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Abstract

Predatory ladybirds, as natural enemies of aphids, can influence their prey in various ways, for example by consuming them or making the aphids drop off from their host plants. In this study, I have examined whether aphids can respond to the semiochemicals released from ladybirds. The tritrophic system chosen for my study will be barley plant (*Hordeum vulgare* L) - aphid (*Rhopalosiphum padi* L.) and an aphid predator, the seven-spotted ladybird (*Coccinella septempunctata* L.).

In the laboratory experiments, a significant reduction of aphid host acceptance was observed on plant with previously presented ladybirds in comparison with control plants (without ladybirds). Aphid host acceptance was studied using a no-choice settling test. Aphids could respond to tracks left by ladybirds, showing avoidance responses in the choice test. In the olfactory experiments, aphid avoidance responses to volatiles released from ladybird adults and their track were correlated to the number of ladybirds that had previously been present on the plant. Volatiles released from both ladybird sexes may induce aphid response but aphids responded more intensively to female ladybirds than male.

The results from these experiments indicate that aphids can discover the previous presence of ladybirds. Aphids, like parasitoids or competing female ladybirds, can recognize chemical components in the volatiles exuded by ladybirds and show avoidance responses to the places with former presence of predatory ladybirds. Aphid host plant acceptance and aphid settling on the plants were significantly affected by the previous presence of ladybird adults, both sexes.

From an applied point of view, it would be very interesting to identify semiochemical components from ladybird tracks and potentially utilize them to reduce aphid population development in the field. This might lead to better biological aphid control and a remarkable reduction in the pesticide use.

Introduction

Aphid problem

Aphids are small sap feeding insects; they feed on phloem sap by using their sucking mouthparts called stylets. They are present everywhere in the world and can migrate great distances by passive wind dispersal, or be spread by human-beings. They are one of the most destructive insect pests on cultivated plants, and infestation of aphids can result in a considerable yield loss, in terms of both quality and quantity (Pettersson et al. 2007). Some host-alternating species are especially dangerous to the plants, like *Rhopalosiphum padi* L. (Dixon 1977). In Sweden, *R. padi* hibernates exclusively on Bird Cherry, (*Prunus padus* L.) during the winter. Insecticides can be used to control aphids, but constitute a considerable danger to the environment. Moreover, with long time utilization of insecticides, aphids tend to become resistant, rendering the insecticides less and less effective. However, aphids with their soft body are easily attacked by a wide variety of predatory and parasitoid insects, thus parasites and predators are widely used in bio-control of aphids.

Bio-control of aphids by using parasitoids and predators

Bio-control is an environmentally friendly method to reduce the damage caused by pests, and is a main part of intensive pest management. Because many parasites and predators prey on aphids, they have been utilized to control aphids, and some successful strategies involving parasites have been developed under laboratory conditions. One example of this is the experiments carried out by Hughes et al. (1987), who used an exotic parasite *Trioxys complanatus* to control spotted alfalfa aphid *Therioaphis trifol*. In 1993 Stray et al. introduced bio-control projects with the aim to control some species of cereal aphids with parasitoids. The experiments were fruitful and used as models in Chile. It can be concluded that parasites are capable to reduce aphid population level but that the effectiveness of parasites depends on their ability to adapt to the conditions at hand.

Predators, like ladybirds of the taxon Coccinellidae, are important natural enemies of aphids. They can suppress the number of aphids, thus the predatory ladybird is generally considered the best candidate for bio-control of aphids (Dixon et al. 1995). The aphidophagous ladybird beetles (Coccinellidae) have frequently been used to control aphids (Frazer 1988), but they are rarely effective in reducing aphid abundance when utilized in bio-control (Evans 2000; 2004). One reason for this is that aphid colonies persist for relatively short periods of time (Hemptinne & Dixon 1997) and it’s difficult for ladybirds to synchronize their offspring growth with aphid colony development (Shannag & Obeidat 2008). Other reasons why ladybirds fail to be an effective bio-control agent for aphids are:

1. Aphids can emit an alarm pheromone when they are under attack by a predator, which will make other aphids jump off the host plant (dropping-off) and move to another plant, even if they can’t sense the predator themselves. Pea aphids (*Acyrthosiphon pisum)* emit alarm pheromone when they are under predation by lacewing larvae (Schwartzberg et al. 2008) and Kunert et al. (2005) showed that the exposure to alarm pheromone induces pea aphids, to give birth to winged dispersal morphs which can

leave their host plants. So aphid evasive maneuvers induced by alarm pheromone are likely to be one reason why bio-control of aphids by ladybirds is not effective.

2. Aphid life-cycles are complex and polymorphism is an important characteristic of aphids. During the asexual phase, aphids produce a number of different morphs, which include winged (alate) and unwinged (apterous). The winged morphs will disperse to colonise new hosts if the old host plants get overcrowded, if the quality of host plants deteriorates or in case of a predator attack.

3. Aphids will produce more winged offspring after predatory ladybirds. Weisser et al. (1999) and Poethke et al. (2010) found that the presence of a predator enhanced the proportion of winged morphs among the offspring produced by the aphids. Furthermore, the presence of predator larvae can provoke the morphological shift in aphids. This shift in aphid morphology does not lead to better protection against their natural enemies, but enables them to leave their host plants when risk of predation is high. Minorreti & Weisser (2000) found the presence of individual *Coccinella septempunctata*, (Coleoptera, Coccinellidae) can induce pea aphid (*Acyrthosiphon pisum*) to produce winged offspring as an adaptive strategy to increase offspring survival. Schwartzberg et al. (2008) tested the wing induction response of pea aphid of four clones when attacked by a predatory lacewing larva, and found that three out of four clones increased the proportion of winged offspring under predator attack. The same effect can be found when aphids are exposed to the searching track of ladybirds. Mondor et al. (2004) found that cotton aphids produced a greater proportion of winged offspring when exposed to search tracks from larval or adult ladybird beetles *Hippodamia convergens*. These findings indicated that exposure to adults or larvae of predatory lady beetle induces morphological changes in aphid offspring and that the driving force is the evasion of predation.

There is variability in sensitivity of aphids to the presence of predatory larvae. Responses of two species of aphids *Aphis fabae* and *Megoura viciae* to the presence of predator larvae were tested by Kunert et al. (2008), and they found that the presence of a predator did not induce winged morphs among offspring of these two aphid species, so they suggest that aphid species differ in their susceptibility to natural enemy attack, as well as their sensitivity. Conditions like aphid density, predator consumption rates and aphid species-specific sensitivity to the presence of predators can play important roles in the induction of winged morphs by natural enemies. In spite of the existence of variability of sensitivity to the track left by ladybirds, production of winged morphs can be another reason why ladybirds are not effective as a bio-control agent for aphids.

The existence of avoidance response between predatory ladybirds and parasitoids

Taylor et al. (1998) demonstrated that the aphid parasitoid *Aphidius haliday* (Hymenoptera, Aphidiidae) avoids places where the intraguild predator seven-spotted ladybird (*C. septempunctata*) is present or was present recently. They suggest that chemical trails left by *C. septempunctata* induce avoidance responses in parasitoids. Nakashima & Senoo (2003) carried out a series of experiments by utilizing an aphid parasitoid, *Aphidius ervi,* and an aphid predator, *C. septempunctata*, and they demonstrated that parasitoids try to avoid areas that contain or recently have contained ladybirds and that they react to olfactory traces left by the ladybirds on the plant surface. Moreover, they found that parasitoids without oviposition experience were not sensitive to the intraguild predators' trails. Although avoidance response exists between parasitoids and predators, different parasitoids show different sensitivity to the former appearance of intraguild predators. Nakashima et al. (2006) found that there is an interspecific variability in sensitivity to semiochemical trails of ladybird predators. Different species of parasitoids show different degrees of sensitivity to the trails of *C. septempunctata*. They identified three main chemical components of the trails of *C. septempunctata*: n-tricosane ($C_{23}H_{48}$), n-pentacosane ($C_{25}H_{52}$) and n-heptacosane ($C_{27}H_{56}$) respectively. The avoidance responses of different species of parasitoids to these three main chemical components were also evaluated. Unsurprisingly, the results indicate that different species of parasitoids show different degrees of avoidance responses to these chemical components as well.

The existence of avoidance response between predatory ladybirds and other competing ladybirds or larvae

Avoidance behaviours also take place between conspecific and heterospecific ladybirds when female ladybirds choose their oviposition site. Adult ladybirds are likely to encounter various species of prey when they choose oviposition site, and the optimal oviposition site should be the one most suitable for their offspring development, which often means the one where food is most abundant. To achieve this, they try to avoid laying eggs in patches with former presence of conspecific or heterospecific ladybirds or larvae. Dixon et al. (1977) demonstrated that female ladybirds (*Adalia bipunctata*) are extremely reluctant to lay eggs and try to leave the area when conspecific larvae are present even though prey is abundant. Oliver et al. (2008) found that ladybirds respond to many different cues when selecting patches that will maximize their own and their offspring's fitness. Oviposition cues for coccinellids include prey species and abundance, presence of – and chemicals from – competing females and conspecific larvae. Some evidence confirms that certain heterospecific larval tracks may also inhibit oviposition. Sarmento et al. (2007) found that female predatory ladybird of the species *Cycloneda sanguinea* can use volatile cues to assess patch profitability and avoid patches with heterospecific competitors or intraguild predators. Ruzicka (2006) showed the oviposition-deterring effects of conspecific and heterospecific larval tracks on *Cheilomenes sexmaculata*. These statements indicate that competing females and conspecific larvae definitely leave some chemical cues that the female coccinellids can recognize and undertake evasive behaviour accordingly. This is a good strategy to decrease the risk of cannibalism and enhance the survival rate of offspring (Kajita et al., 2006). However, few eggs will be laid by female ladybirds if they intrude into patches where other competing females have been present. This avoidance response may restrict the reproduction of predatory ladybirds.

The existence of avoidance response between predatory ladybirds and feces of conspecific or heterospecific ladybirds

Fecal cues of ladybird predators were found to contribute to their assessment of cannibalism risk from conspecific and heterospecific competitors in common habitats. Agarwala et al. (2003) indicated that female ladybirds show avoidance responses to the feces produced by conspecific or heterospecific female ladybirds. They investigated the responses of two ladybird species to the fecal cues of conspecific and heterospecific ladybirds, and found that female ladybirds of the species *H. axyridis* respond to conspecific feces, but not to heterospecific feces. Female *P. japonica* ladybirds respond to the feces of both conspecific and heterospecific competitors. Their results demonstrated that feces of ladybirds emit an odour that has the potential to deter other ladybirds of the same species as well as heterospecifics from feeding and oviposition activities.

The study and hypothesis

According to the literature mentioned above, parasitoids show avoidance responses to the former presence of predatory ladybirds. Predatory ladybirds also try to avoid patches with former presence of conspecific or heterospecific female ladybirds or larvae. The odour of ladybirds' track is an important cue for parasitoids and competing female ladybirds. Nelson et al. (2004) conducted an experiment where they compared the effectiveness regarding induction of evasive maneuvers of in aphids of damsel bugs (*Nabis* spp.) which had had their mouth parts removed to the effectiveness of damsel bugs with undamaged mouth parts. The greatest reduction in aphid population growth was caused by damsel bugs that were able to both consume and disturb aphids. However, aphid population growth was also strongly reduced by non-consumptive, disturbance-only damsel bugs. Thus one hypothesis is that the previous presence of ladybirds or tracks left by ladybirds may affect aphid preference. All the experiments conducted in this study will try to prove this.

Materials and Methods

Aphids for laboratory experiments

Multi-clonal cultures of bird cherry-oat aphid (*Rhopalosiphum padi* L.) were reared on barley (cv. Golf) in a glasshouse. The culture was kept in a controlled environment chamber at 18-22°C, 16:8h L:D, and no efforts were made to control humidity.

Ladybirds for laboratory experiments

A laboratory culture of seven-spotted ladybird *Coccinella septempunctata* L. was already established and kept in cages (40 x 40 x 80 cm) where reproduction takes place. The cereal aphids *R. padi* L. on barley (cv. Golf), flowering *Brassica napus* L. plants, and Pea aphid (*Acyrthosiphon pisum)* on bean plants (*Vicia. faba)* were used as food sources for ladybirds. The culture was kept in a controlled environment chamber at 18-22°C, 16:8h L:D, and 80% relative humidity. The sex of the adults was determined according to Baungaard (1980).

Plant materials

For the aphid settling tests, barley (*Hordeum vulgare* L) plants of cultivar Scandium were grown in plastic pots (8.5 cm wide × 7 cm high) in potting soil (Special Hasselfors Garden, Hasselfors, Sweden). Plants were grown in a separate greenhouse chamber under the same conditions as *R. padi*.

Aphid preference

No-choice aphid settling test on plants

A no-choice settling test (Ninkovic et al. 2002) was used to investigate aphid behavioural responses to plants on which ladybirds had previously been present. The youngest leaf of a barley plant in the two-leaf stage was placed in a 50 ml polystyrene tube. The upper end of the tube was covered with a net and the lower end with a plastic foam plug with a slit for the leaf. To avoid mechanical damage to the test plant, a stick was used to support the tube. Ladybird adults of the same sex were placed in the tubes with the plants. Three treatments; one ladybird, three ladybirds and an empty tube as a control, were tested in fourteen replicates. After 24 hours, ladybirds were removed from the plants and the tubes were replaced with new ones to avoid any residues left by ladybirds on the tube wall. Ten aphids were then placed in each tube. After two hours the number of aphids feeding/settled was counted for each tube.

Choice test- Petri dish bioassay

The effect of ladybird residues on aphid behaviour was further studied in an arena choice experiment. This test method covers responses to both contact stimuli and volatile components and was performed with separate tests to look at aphid’s responses to tracks from the two sexes and different numbers of ladybirds. A Petri dish (diameter 12 cm) was divided into two parts using a plastic partition whose position was marked in as a line on the bottom of the dish. Randomly chosen ladybirds of the species *C. septempunctata* were placed in one half of the Petri dish while the other half was left empty. The Petri dishes were sealed with perforated Parafilm (Bemis, USA) to provide ladybirds with air. After 24 hours the ladybirds and the partition were removed and the Petri dish was placed on a piece of white paper with precautions taken to avoid the influence of light on the arena. When larvae were tested, they were removed from the Petri dish after 6 hours, rather than 24 hours to avoid cannibalism (Ruzicka 2006). The size of larvae that were put into each Petri dish was similar. The different sexes and developmental stages of ladybirds were tested separately.

Ten randomly selected aphids were placed in the middle of the dish and allowed 10 minutes to acclimatize, after which the number of aphids in each half of the Petri dish was recorded every three minutes for 1 h. Three minutes was chosen as an interval because it was long enough to permit an aphid to move from one side of the arena to the other. The position of treatments, side with ladybird residues and side without residues in divided Petri dishes, was alternated randomly between the left and right side in each separate test to account for any positional bias. Fifteen replicates were made for each experiment. As a control, a blank test was done in a Petri dish without any coccinellid residues using the same protocol as for the other treatments.

To test the hypothesis that semiochemical tracks of adult *C. septempunctata* alone can affect individuals of *R. padi*, a series of choice experiments was conducted. To obtain ladybird tracks without ladybird feces we used unfed ladybird adults and changed the Petri dish every 24 hours. After the 3rd replacement Petri dish tracks were obtained without any visible feces and this dish was then used to test aphid's preferences in the same way as described above.

Five different arrangements were utilized in choice test:

1. One side of the petri dish had previously been occupied by 1, 3 or 5 male ladybird adults and therefore contained both tracks and feces. The other side of the petri dish was empty.
2. One side of the petri dish had previously been occupied by 1, 3 or 5 female ladybird adults and therefore contained both tracks and feces. The other side of the petri dish was empty.
3. One side of the petri dish had previously been occupied by 1, 3 or 5 ladybird larvae, and therefore contained both tracks and feces. The other side of the petri dish was empty.
4. One side of the petri dish had previously been occupied by 1, 3 or 5 male, unfed ladybird adults and contained only tracks. The other side of the petri dish was empty.
5. One side of the petri dish had previously been occupied by 1, 3 or 5 female, unfed ladybird adults and contained only tracks. The other side of the petri dish was empty.

Aphid olfactory bioassay

Aphid behavioural responses to volatiles released from *C. septempunctata* were tested using two-arm olfactometers as described by Ninkovic et al. (2009). The odour from Petri dishes was introduced through two extended zones/arms of the olfactometers into a central zone/arena (2.5 x 2.5 cm). Each Petri dish contained holes in the middle of the bottom to achieve equal distribution of air and was turned upside down and connected directly by Teflon tubing to the olfactometer arm inlet. The position of the treatments was switched between the left and right arms in of the olfactometers to account for any positional bias. Airflow through the system was set to 250 ml min^{-1}, measured and controlled by a flow meter.

Four treatment arrangements with ladybird adults were used in olfactometry experiments:

1. One olfactometer arm was connected to a petri dish with a female ladybird, while the second arm was connected to an empty petri dish.

2. One olfactometer arm was connected to a petri dish with a male ladybird, while the second arm was connected to an empty petri dish.
3. A petri dish which had previously been occupied by a female ladybird was connected to one olfactometer arm while the second arm was connected to an empty petri dish.
4. A petri dish which had previously been occupied by a male ladybird was connected to one olfactometer arm while the second arm was connected to an empty petri dish.

A single aphid was placed in the central zone of the olfactometer and its position in the arena was recorded 10 times at 3-min intervals after ten minutes adaptation time. Test insects were randomly chosen from the culture and if the test individual did not move between two observations, it was removed, the data discarded and a new series of 10 observations started with a new insect. Each individual was used only once and the total number of visits by an aphid in the zones/arms after 10 observations was regarded as one replicate. Twenty replicates were carried out for each experiment.

In experiments where larvae were used as odour source, 1, 3 or 5 individuals were kept in a Petri dish for only 6 hours prior to being connected to the olfactometer to avoid cannibalism (for more detailed description see Petri dish bioassay). The way of observation and recording of data was the same.

Two treatment arrangements with ladybird larvae were used in this olfactometry experiments:

1. One olfactometer arm was connected to a petri dish with ladybird larvae, while the second arm was connected to an empty petri dish without larvae.
2. A petri dish, from which larvae were removed, was connected to one olfactometer arm while the second arm was connected to an empty petri dish.

Tracks as odour source

To test whether volatile semiochemicals from tracks may play a role in the aphid avoidance behaviour observed in the previous experiments, tracks without visible ladybird feces produced in the same way as described earlier were used as test odour sources in the olfactometer.

Two treatment arrangements with tracks left by 1, 5 or 10 ladybird adults were used in this olfactometry experiments:

1. One olfactometer arm was connected to a petri dish which had previously been occupied by female ladybirds, and thus contained ladybird tracks, while the second arm was connected to an empty petri dish without ladybird tracks.
2. One olfactometer arm was connected to a petri dish which had previously been occupied by male ladybirds and thus contained ladybird tracks, while the second arm was connected to an empty petri dish.

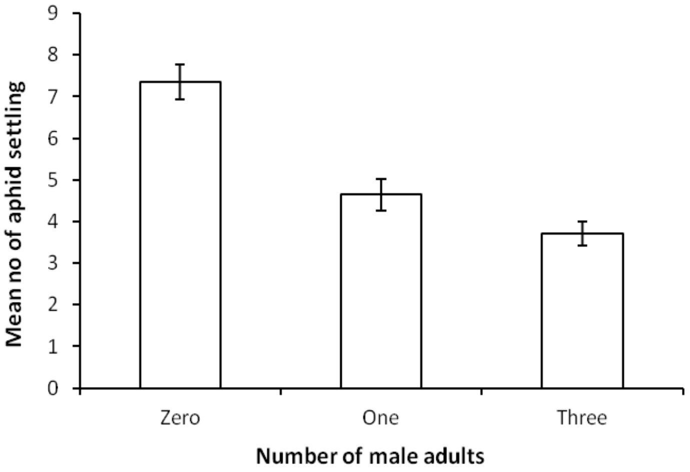

Figure 1. Mean aphid settling (± standard error) on the barley plants which had previously been occupied by 0, 1 or 3 male ladybirds.

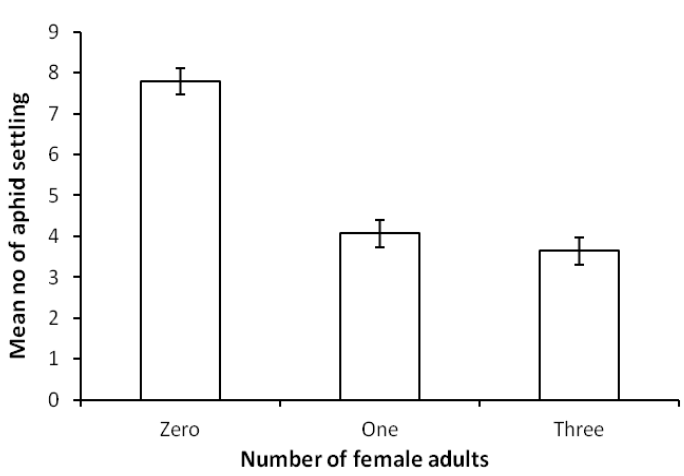

Figure 2. Mean aphid settling (± standard error) on the barley plants which had previously been occupied by 0, 1 or 3 male ladybirds.

Statistical analysis

To compare different levels of treatment with the control, the statistical analysis related to the choice test and aphid olfactory responses was done with Wilcoxon Matched Pairs Test. Results showing p-values at the 5% level were considered to be significant. All the statistical analysis related to no-choice tests was done with ANOVA followed by Tukey test (HSD: honestly significance difference test). All statistical analyses were performed with the Statistica software (Statsoft Inc. 2011). The results showing p-values at the 5% level were considered to be significant here as well.

Results

Aphid preference

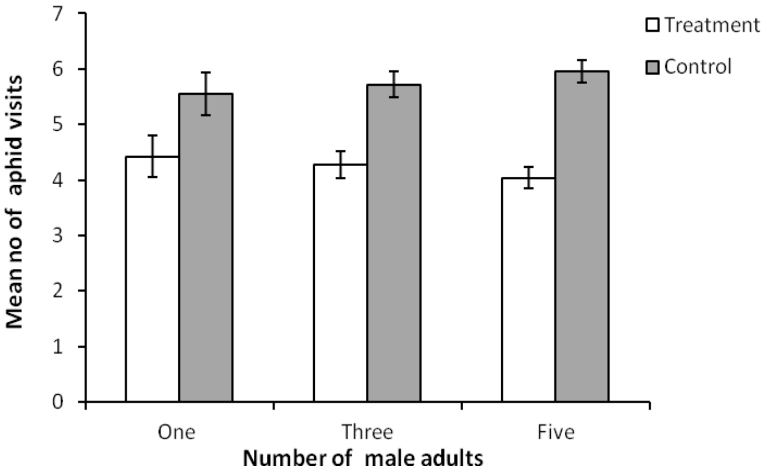

Figure 3. Mean (± standard error) distribution of aphids over the two sides of the Petri dish after the previous presence of 1, 3 or 5 male ladybird adults.

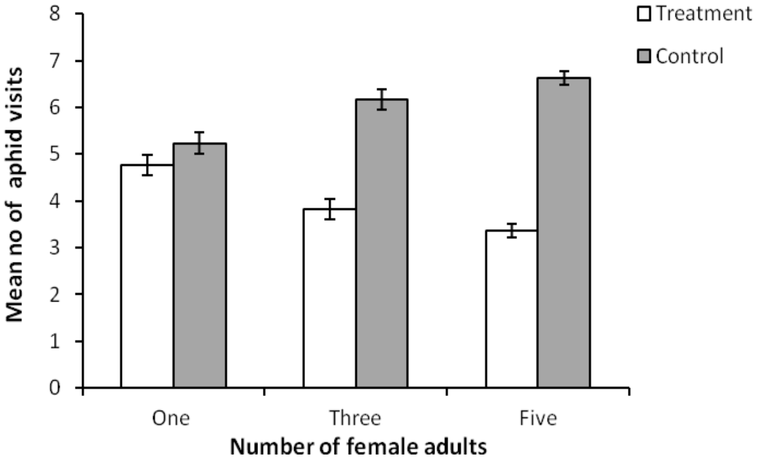

Figure 4. Mean (± standard error) distribution of aphids over the two sides of the Petri dish after the previous presence of 1, 3 or 5 female ladybird adults.

No choice aphid settling test on plants

In this series of tests, all the aphids will eventually have to accept the test plants, and the willingness of the aphids to settle on the plants indicates how the former presence of ladybirds affects aphids' choice of host plant. The fewer aphids that were found on the plants, the more intensively aphids respond to the former presence of ladybirds. The results indicated that the treatment with either a single adult male or a single female adult ladybird can elicit a significant effect on aphid settling (*P* = 0.0002 and *P* = 0.0001, respectively), compared to the control plants which had not been occupied by ladybirds. There is no significant effect of sex in this aphid settling test. A similar reduction of aphid settling was also observed on the leaves on which three ladybird adults were previously placed compared to non-exposed for both males (*P* = 0.0001) and females (*P* = 0.0001).

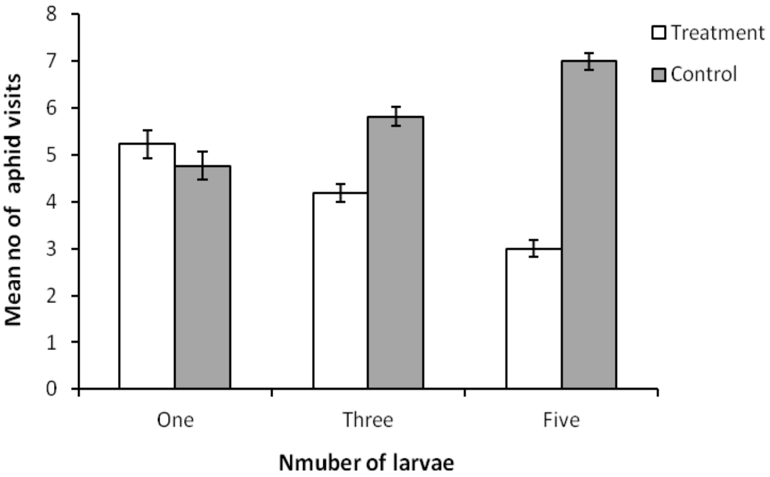

Figure 5. Mean (± standard error) distribution of aphids over the two sides of the Petri dish after previous presence of 1, 3 and 5 ladybird larvae.

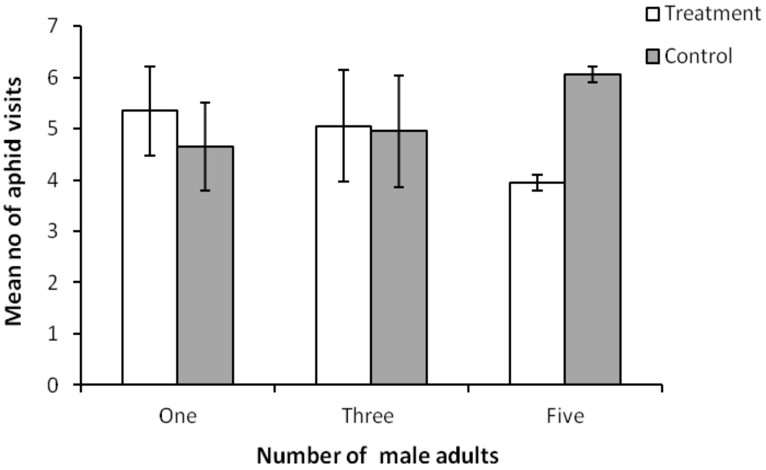

Figure 6. Mean (± standard error) aphid distribution over the two sides of the Petri dish in response to tracks left by 1, 3 and 5 male ladybird adults.

Choice test- Petri dish bioassay

Results of a blank test done in a Petri dish without any coccinellid residues did not show any patchy distribution of aphids in the dish arena (*P* = 0.78).

Aphid responses depended on how many ladybirds had been present in the petri dish. This was true for both male and female ladybirds. In all experiments, aphids were recorded more frequently in the control side of the Petri dish compared to the side exposed to ladybird adults (Figure 3 and 4). These differences were significant when 3 (*P* = 0.012) and 5 males (*P* = 0.002) were used (Figure 3).

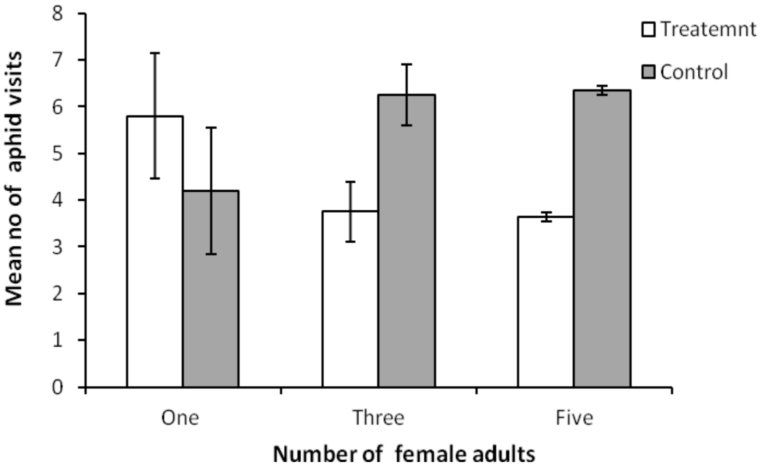

Fig7. Mean (± standard error) aphid distribution over the two sides of the Petri dish in response to tracks left by different 1, 3 and 5 female ladybird adults.

Table 1 Aphid olfactory response to odour from a Petri dish with different numbers of male ladybird adults.

Number of coccinellids	Olfactometer arm (mean ± SE)		*Z*	*P*
	Treatment	Control		
(a) male ladybird adult(remained in PD)				
One ladybird	3.00 ± 0.37	3.65 ± 0.35	1.14	0.256
Five ladybirds	2.95 ± 0.25	3.45 ± 0.35	0.75	0.453
Ten ladybirds	2.50 ± 0.27	4.20 ± 0.32	2.70	0.007
(b) male ladybird adult (previously presented)				
One ladybird	3.15 ± 0.37	3.35 ± 0.25	0.44	0.658
Five ladybirds	3.15 ± 0.28	3.50 ± 0.26	0.80	0.423
Ten ladybirds	2.65 ± 0.34	3.80 ± 0.34	1.79	0.074

Treatment represents the side where the male ladybird adults were placed in the Petri dish. Control represents the control side which was connected to an empty Petri dish. *P* and *Z* values are from Wilcoxon matched pairs test, *Mean* is the average number of aphid visits with per olfactory arm, ± SE is standard error.

Differences in aphid distribution were larger when females were used (3e females: *P* = 0.0006; 5 females: *P* = 0.0006) (Figure 4). The same aphid avoidance response to the side of the Petri dish that was previously exposed to ladybirds was observed with ladybird larvae (Figure 5). The significant reduction of aphid visits to the treated side was observed when 3 (*P* = 0.002) and 5 (*P* = 0.0006) larvae were used to test aphid preference.

Table 2 Aphid olfactory response to odour from a Petri dish with different numbers of female ladybird adults.

Number of coccinellids	Olfactometer arm (mean ± SE)		*Z*	*P*
	Treatment	Control		

(a) female ladybird adult (remained in PD)				
One ladybird	3.25 ± 0,34	2.70 ± 0.33	0.79	0.426
Five ladybirds	2.95 ± 0,22	3.70 ± 0.30	1.51	0.132
Ten ladybirds	2.00 ± 0,16	3.70 ± 0.16	3.64	0.0003
(a) female ladybird adult (previously presented)				
One ladybird	3.15 ± 0.34	2.95 ± 0.38	0.35	0.729
Five ladybirds	3.20 ± 0.20	3.55 ± 0.29	1.05	0.293
Ten ladybirds	2.25 ± 0.23	3.65 ± 0.18	2.98	0.003

Treatment represents the side where the male ladybird adults were placed in the Petri dish. Control represents the control side which was connected to an empty Petri dish. *P* and *Z* values are from Wilcoxon matched pairs test, *Mean* is the average number of aphid visits with per olfactory arm, ± SE is standard error.

Table 3 Aphid olfactory response to odour from Petri dish with different numbers of larvae

Number of coccinellids	Olfactometer arm (mean ± SE)		*Z*	*P*
	Treatment	Control		
(a) larvae (remained in PD)				
One larva	4.00 ± 0.28	2.25 ± 0.29	2.86	0.004
Three larvae	3.20 ± 0.41	3.40 ± 0.29	0.28	0.777
five larvae	2.95 ± 0.26	3.95 ± 0.15	2.45	0.014
(b) larvae (previously presented)				
One larva	4.30 ± 0.40	2.30 ± 0.32	2.72	0.0006
Three larvae	3.40 ± 0.37	3.45 ± 0.25	0.12	0.906
five larvae	2.80 ± 0.27	3.65 ± 0.34	1.50	0.132

Treatment represents the side where the male ladybird adults were placed in the Petri dish. Control represents the control side which was connected to an empty Petri dish. *P* and *Z* values are from Wilcoxon matched pairs test, *Mean* is the average number of aphid visits with per olfactory arm, ± SE is standard error.

When the influence of feces was avoided, a similar pattern was observed in the distribution of aphids as in the previous choice tests (Fig 6 and 7). For instance, tracks left by 5 female ladybirds ($p = 0.0002$) is a significantly better aphid deterrent than that of 5 males ($p = 0.0003$). Moreover, if we compare tracks left by 3 male ladybirds ($p = 0.851$) with tracks left by 3 females ($p = 0.0005$), we find that the aphids responded much more intensively to the tracks left by 3 females. However, single ladybirds regardless of sex were generally not enough to repel aphids (Fig 6 and 7).

So, from our aphid performance tests we can deduce two things. The first is a positive relationship between aphid responses and the number of ladybirds we put in the petri dish. The second one is that female ladybirds affect aphid performance more intensely than male. The results of aphid performance tests prove our hypothesis that aphids exhibit this sort of avoidance responses, decreasing their activities in places with previous presence of ladybirds, especially the female ladybirds.

Table 4 Aphid olfactory responses to odour from Petri dish with tracks from different number of adults.

Number of ladybird adults' track in PD	Olfactometer arm (mean ± SE)		*Z*	*P*
	Treatment	Control		
(a) female ladybird adult				
One ladybird	4.7 ± 0.38	1.7 ± 0.35	2.67	0.008
Five ladybirds	3.1 ± 0.17	3.6 ± 0.21	1.35	0.176
Ten ladybirds	2.6 ± 0.15	3.8 ± 0.13	2.52	0.012
(b) male ladybird adult				
One ladybird	4.2 ±0.37	2.3 ±0.25	2.34	0.019
Five ladybirds	3.3 ±0.20	3.5 ±0.21	0.53	0.594
Ten ladybirds	3.0 ±0.20	3.7 ±0.14	2.02	0.043

Treatment represents the side where the male ladybird adults were placed in the Petri dish. Control represents the control side which was connected to an empty Petri dish. *P* and *Z* values are from Wilcoxon matched pairs test, *Mean* is the average number of aphid visits per olfactory arm, ± SE is standard error.

Aphid olfactory bioassay

When we kept different numbers of male ladybirds in the petri dish, only 10 male ladybird adults gave us a significant difference (*P* = 0.007) between treatment and control. After ladybirds were removed from the Petri dish, the results we got were similar. There was a trend in the direction that the odour of 10 male ladybirds' residues can have some effect on aphids (*P* = 0.07) but the results were not significant. .

A parallel tendency was found in the olfactometer experiments with female ladybirds. Aphids respond most intensively when 10 female ladybird adults remained in the Petri dish (*P* = 0.0003). Odour of 10 female adults gave results that were most significantly different from the control (*P* = 0.003) among the three different treatments of previously presented female adults (Table 2).

Avoidance behaviour was only observed when 5 larvae were used as odour source (*P* = 0.014). Aphids showed a significant attractant/arresting response when one larva was used in a Petri dish as the odour source (*P* = 0.004) (Table 3).

Significant avoidance responses were observed when Petri dishes with tracks produced by ten adults were used as odour source, female (*P* = 0.012) and male (*P* = 0.043). The volatiles released from female tracks induced much stronger responses than volatiles from male tracks (Table 4). However, significant arresting/attracting effects were observed to volatiles released from the tracks of single adult of both sexes, female (*P* = 0.008) and male (*P* = 0.019).

The conclusions we got from the aphid olfactory responses tests were in line with the choice tests above. It's interesting to observe the attracting/arresting response of aphids to tracks left by single ladybirds or larvae.

Discussion

The experiment results indicate that aphids show different degrees of avoidance responses to different types of odour from adult and larval ladybirds. The degree of avoidance behaviour exhibited by aphids was concentration dependent: the more ladybirds we used, the stronger was the aphid response. Moreover, the degree of aphid avoidance response depends on the sex of the ladybirds involved in the experiment. Former presence of female ladybirds provokes stronger aphid responses than male ladybirds. In summary, the previous appearance of ladybird adult or larvae can affect aphid behaviour.

No-choice test with plants

This no-choice test may be less sensitive than a test in which the aphids are offered more than one feeding site choice, since with this method all aphids will eventually have to accept the test plant (Ninkovic et al., 2002). The aphid plant acceptance decreased profoundly on plants previously occupied by ladybirds. Three previously presented ladybird adults provoke a slightly higher effect on aphid response than one ladybird. This avoidance behaviour may contribute to the aphid's search for optimal feeding sites and enemy-free space for the establishment of new colonies. It would be an obvious advantage for aphids to distinguish plants that have been exposed to ladybird foraging from those that have not. In the no-choice test, aphid host acceptance was significantly affected by the presence of single ladybirds. Glinwood et al. (2004) demonstrated that barley plants which were exposed to volatiles from thistles (*Cirsium* spp.) were less acceptable to *R.padi*. Ninkovic & Åhman (2009) found that aphid acceptance is affected by plant volatile exposure, and aphid host acceptance will change depending on their host status assessment. If volatile exposure is negative to aphid performance, aphids will give up settling on the host. Therefore it can not be ruled out that barley plants might respond to the volatiles from ladybirds, and that the response will make the plant less acceptable to the aphids. Pettersson et al. (2007) reported that aphids have an intimate relationship with their host and they are very sensitive to changes in host plant status. Therefore, the aphids can quickly detect changes in their host and avoid settling, if the previous presence of predatory ladybirds is negative to the aphid performance. This could be an explanation of why only single ladybirds can provoke a significant aphid response in this no-choice test.

Choice test

The former presence of different numbers of ladybirds affects aphid performance differently. The results of the choice tests with adults indicate that aphids show avoidance responses of different degrees. Although the results with only one ladybird were not significantly different from the control, the trend is that they try to avoid going to the places where predatory ladybirds have previously been present. A difference in degree of avoidance between odour sources that contain visible feces and those without visible feces was observed. When tracks (no visible feces) were used as odour source, aphids show more intensive responses to the Petri dish with more ladybirds, because higher number of ladybirds covered larger area with their cues. So concentration is an important factor, and the feces left by ladybirds also.

Recently a similar study has been done by WeiZheng Li *et al*. (2010), who used different experimental materials and different methods, but found results similar to ours. They found that the apterous *Myzus persicae* can detect the presence of the predatory ladybird *Propylaea japonica* by combining visual and olfactory cues. They also used the extract of whole dead *P. japonica* to test the dose-response of *M. persicae*, and found that there was a positive linear relationship between the dose of ladybird extract and the repelling effect on aphids. They conclude that *M. persicae* have the ability to detect the presence of its predator and initiate avoidance responses to reduce the predation risk. The positive relationship between aphid response and number of ladybirds used as test odour source in our experiments was in line with the results reported by them from their experiments with dead body extracts, which can help us explain why the tested *R. padi* respond more intensively to higher numbers of previously present ladybird adults. However, in their experiments, the visual cue is a significant factor and the dose they use was extracted from the whole dead *P. japonica*, which was different from our studies. In our choice tests, we exclude the visual cues and only test the aphid responses to the track left by ladybirds without visible feces. Despite this, the *R.padi* in our experiment still shown avoidance responses to the tracks left by their predators. Our experiments proved that tracks left by ladybirds are an important factor in predation risk assessment and predator detection by aphids, and that positive relationship exists between aphid response and the amounts of present/previously present predators.

The sex of the formerly present ladybirds affects aphid performance. Aphids respond more intensively to female tracks than male tracks. One possible explanation for this may be that female ladybirds always mark their own oviposition site when they come into a new area, and thereby release some special chemical substance which male ladybirds can’t produce. Another explanation may be that the chemical cues from male trails is not as strong as female’s, so the aphids show less intensive responses to the male tracks.

Aphid olfactory response

Results from olfactometer trials confirmed the findings from the choice test. The residues from female ladybirds induced a stronger avoidance response than residues from males. When the influence of ladybird feces was excluded, the aphids still showed similar avoidance responses to the tracks left by the ladybirds. Thus we suggest that aphids can use volatile cues from ladybirds to detect their natural enemies and show avoidance response when those volatile cues reach a certain level. The olfactometer results showed that odour from a single larva and its' residues alone provoked an arresting/attracting response in aphids. This effect was also observed when aphids were exposed to volatiles from the tracks of a single adult. This finding may indicate that ladybirds may be deliberately luring aphids through some sort of deception, which could be one of the factors that contribute to the survival of *C. septempunctata*. Further studies of the chemical composition of the residues may reveal whether it is part of a strategy contributing to successful coccinellid foraging.

Avoidance responses

The results we got proved our hypothesis that aphids show avoidance responses to the former presence of predatory ladybirds or the tracks left by predatory ladybirds. As previously mentioned, there is a variability of sensitivity among aphid species to the previous presence of ladybirds, so our results may not be valid for all species of aphids. We can only say there is an avoidance response among *R. padi* to its predator *C. septempunctata*. Jacobsen & Stabell (2004) found that active predatory crabs or crude extract of crabs which feed on *Tegula funebralis* can induce avoidance responses in the marine snail *Tegula funebralis* while crabs actively feeding on another snail species or non-feeding crabs did not induce snail avoidance responses. Thus this kind of avoidance response can be highly specific and needs to be researched deeply if it is to be understood in detail.

Ecological importance

Both parasitoids and competing female ladybirds avoid laying eggs in the same site as other intraguild predators because they want to choose a perfect oviposition site and perfect growth environment for their offspring. Aphids too want to choose a profitable site for their offspring. They want to increase the survival rate of their offspring, and to achieve this they produce an alarm pheromone when they get attacked by their natural enemies. They also increase the proportion of winged dispersal morphs in the following generation, to allow their offspring to escape the potential predation risk. Because compared to aphid colony development, the growth of the ladybird population feeding on it usually lags behind. This decreases the risk of cannibalism among the ladybird larvae but makes ladybirds less effective as a bio-control agent for aphids. Aphids can utilize the olfactory cues of its predators to avoid areas with previous presence of ladybirds. So from aphid’s point of view, the ability of detection, assessment, and taking precautions in response to the presence/previous presence of natural enemies is crucial for the aphid to increase survival rate.

Practical use

The avoidance response to ladybirds in aphids is potentially very useful for controlling aphid problems, although the responses are highly specific for each combination of aphid and ladybird species. Three main components were extracted from the track of seven-spotted ladybirds, *C.septempunctata* L, by Nakashima et al. (2006), but they didn’t use them to test any aphids. Thus we can extract the chemical components from the tracks left by *C. septempunctata*, and utilize the extracted chemical cues to test aphid response in the laboratory. If aphids show similar avoidance response to the extracted chemicals as to real ladybirds, we can use them for bio-control of aphid outbreaks in the field. If it works in the field, maybe we can use these chemicals and predatory ladybird together in the bio-control, to see if this additive effect will be positive or negative. If it is effective, we can use this to replace insecticides, which would be beneficial to our environment.

Conclusion and future research need

To our knowledge, this is the first time that aphid avoidance responses to the previous presence of ladybirds and tracks left by ladybirds has been tested. From our results, we draw two main conclusions: one is that there is a positive relationship between aphid responses and numbers of ladybirds, the other is that female ladybirds affect aphid performance to a larger extent than males. The discovery of this avoidance response can help us solve many problems, and hopefully the chemicals involved can ultimately be incorporated into biological pest control strategies for *R. padi*.

Of course, our experiments have some research limitations. Firstly, as we mentioned, we need to go deeply and test more by using predators fed on other types of food. If the tested aphids still show avoidance responses, then what we got here will be more persuasive. Secondly, in this series of experiments, we use larvae as odour source. During the experiments, larvae were chosen for their size because we wanted to avoid the risk of cannibalism. However, we neglected one factor; the instar of the larvae. As we know, different instars of larvae show different prey consumption rates and the prey consumption rate of larvae might be a factor which is also important to aphid behaviour. So in subsequent studies, we will compare the aphids’ responses to different instars of larvae, to see whether the avoidance responses of aphids will be affected by this factor or not. Thirdly, we will try to extract the chemical components from ladybird tracks, and use them to test aphid responses. We will also test aphid responses to different dose concentrations. If aphids respond more intensively to higher concentrations, then this will prove our first conclusion that there is a positive relationship between aphid response and the number of ladybirds we use. Then we will use this chemical extract in the field to test aphid responses. If plants sprayed with this extract are less acceptable to the aphids, then maybe those chemicals can be used in bio-control. We also want to combine chemical agents from ladybird tracks with active predatory ladybirds or other synthesized chemicals, such as the aphid induced plant stress volatile methyl salycilate (Ninkovic 2003; Zhu & Park 2005) to test whether the additive effect will be positive or negative. Last but not least, from the no-choice settling tests, we deduced that plants themselves can respond to the chemicals released from ladybirds. Former research has demonstrated that certain combinations of genotypes of barley cultivars can attract predatory ladybirds to come to the mixed habitat earlier than aphids (Ninkovic et al., 2011). Thus in the future, we will do more experiments to see whether plants can respond to these chemicals and lower the aphid host acceptance. Then we can create a new type of cropping system and add these two positive effects together to reduce aphid attacks on barley plants. If our plan works, this will be a promising prospect in bio-control, to decrease the frequency of insecticide use in the field.

In summary, the results are very promising and potentially very useful for bio-control, although we still need to do more studies.

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