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A Differential Role of Volatiles from Conspecific and Heterospecific Competitors in the Selection of Oviposition Sites by the Aphidophagous Hoverfly *Sphaerophoria rueppellii*

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Abstract The selection of oviposition sites by syrphids and other aphidophagous insects is influenced by the presence of con- and heterospecific competitors. Chemical cues play a role in this selection process, some of them being volatile semiochemicals. Yet, little is known about the identity and specificity of chemical signals that are involved in the searching behavior of these predators. In this study, we used olfactometer bioassays to explore the olfactory responses of gravid females and larvae of the syrphid *Sphaerophoria rueppellii*, focussing on volatiles from conspecific immature stages, as well as odors from immature stages of the competing coccinellid *Adalia bipunctata*. In addition, a multiple-choice oviposition experiment was conducted to study if females respond differently when they can also sense their competitors through visual or tactile cues. Results showed that volatiles from plants and aphids did not affect the behavior of second-instars, whereas adult females strongly preferred odors from aphid colonies without competitors. Odors from conspecific immature stages had a repellent effect on *S. rueppellii* adult females, whereas their choices were not affected by volatiles coming from immature heterospecific *A. bipunctata*. The results imply that the syrphid uses odors to

avoid sites that are already occupied by conspecifics. They did not avoid the odor of the heterospecific competitor, although in close vicinity they were found to avoid laying eggs on leaves that had traces of the coccinellid. Apparently adult syrphids do not rely greatly on volatile semiochemicals to detect the coccinellid, but rather use other stimuli at close range (e. g., visual or non-volatile compounds) to avoid this competitor.

Keywords Syrphidae · *Adalia bipunctata* · Olfactometer · Semiochemicals · Oviposition behavior · Intraguild interactions

Introduction

Predatory syrphid larvae are natural enemies of aphids (Brewer and Elliott 2004; Freier et al. 2007; Haenke et al. 2009; Rojo et al. 2003; Tenhumberg and Poehling 1995). Together with generalist coccinellids, they represent the most important predators that reduce the population densities of aphids in agricultural ecosystems (Coderre 1999; Freier et al. 2007; Latham and Mills 2009; Smith et al. 2008; Winder et al. 1994).

Ovipositional preferences by syrphid females have profound effects on the performance of their offspring. Decisions where to oviposit determine not only whether newly hatched larvae will find food (Sadegui and Gilbert 2000), but also the prospects of cannibalism and intraguild predation (Branquart et al. 1997; Frechette et al. 2007; Hindayana et al. 2001). Therefore, gravid syrphid flies ought to select high quality aphid patches, with the ‘quality’ depending not only on the presence and density of aphids, but also on the presence of inter- and intraspecific competitors (Almohamad et al. 2010b; Pineda et al. 2007). Oviposition site selection may rely greatly on the perception of prey-derived volatiles and herbivore-induced plant volatiles (synomones) (De Moraes et al. 2001;

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Dicke and Sabelis 1988; Harmel et al. 2007; Turlings and Tumlinson 1992; Turlings et al. 1990; Verheggen et al. 2008; Vet and Dicke 1992). Such volatiles provide syrphid females with information about host plant characteristics, aphid species and even the presence of con- and heterospecific competitors (Almohamad et al. 2007, 2008b, 2010a; Chandler 1968; Sadeghi and Gilbert 2000; Verheggen et al. 2008).

Because larvae of aphidophagous hoverflies and ladybird beetles occur frequently on the same aphid infested plants, these predators may interact strongly with each other, leading to intraguild predation, especially when prey become scarce (Agarwala and Yasuda 2001). Previous studies have assessed the oviposition-avoidance behavior from syrphid females to chemical cues from con- and heterospecific (e. g., coccinellid) competitors (Almohamad et al. 2010a, b; Hemptinne and Dixon 2000; Laubertie et al. 2006; Verheggen et al. 2008). However, these past studies did not distinguish between the role of volatile compounds and gustatory and visual stimuli. These latter factors also are important for aphid colony selection by syrphid females (Chandler 1966). The present work aimed to study the specific role of volatile compounds on the interaction between syrphids and con- and heterospecific competitors.

We used a system consisting of sweet-pepper [*Capsicum annuum* L. (Solanaceae)], a horticultural species largely extended in the Mediterranean area, and the aphid *Myzus persicae* (Sulzer) (Hemiptera:Aphididae) a species that causes severe economic losses due to its role as a vector of many plant viruses (Blackman and Eastop 2000; Minks and Harrewijn 1989). We studied the olfactory responses of *Sphaerophoria rueppellii* (Wiedemann) the main predatory syrphid in Mediterranean greenhouse crops and a promising aphid control agent in such agricultural systems (Amorós-Jiménez et al. 2012; Pineda and Marcos-García 2008). We focussed specifically on the possible avoidance of the odor of conspecifics, as well as the odor of *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), a coccinellid that is frequently commercialized for its use as a biocontrol agent in greenhouses. In olfactometer tests, we studied the choices made by gravid *S. rueppellii* females and second-instars when offered the volatile compounds emitted from unoccupied aphid-infested plants and plants carrying con- or heterospecific individuals or only their tracks. We also studied the acceptance of oviposition sites by gravid *S. rueppellii* females that also could see and contact con- and heterospecific competitors to compare and contrast the results from the olfactometer trials.

Methods and Materials

Olfactometers

The attraction of female syrphid flies to different odor sources was tested in a six-arm olfactometer (Turlings et al. 2004), which allows up to six odor sources to be tested simultaneously

for their relative attractiveness. The attraction of *S. rueppellii*'s larvae towards different odors was studied in a 4-arm olfactometer (D'Alessandro and Turlings 2006).

Plant Material, Aphids and Syrphids

Sweet-pepper plants (*Capsicum annuum* var. California Wonder) were grown from seed in a climate room ($T=25\text{ }^{\circ}\text{C}\pm 1\text{ }^{\circ}\text{C}$, $RH=60\text{ }\%\pm 10\text{ }\%$, Photoperiod=12 L:12D) in plastic pots ($5\times 5\times 6\text{ cm}$). A base colony of the aphid *M. persicae* was maintained on sweet pepper plants in mesh-netting insect-proof cages ($30\times 30\times 30\text{ cm}$; Megaview Science, Taiwan). Adults of syrphid *S. rueppellii* originally came from a colony kept at the CIBIO research institute (Alicante, Spain). Adults were reared in cages similar to those explained above, containing multi-flower bee pollen, sucrose, water, and an aphid-infested pepper plant as oviposition stimulus. Syrphid larvae were maintained on *M. persicae* colonies on sweet pepper plants in similar cages. Larvae of the coccinellid *A. bipunctata* were obtained from Koppert Biological Systems (The Netherlands) and maintained until adults on sweet pepper plants infested with *M. persicae*, in similar cages as for the syrphids.

Odor Sources

All plants used in experiments were 9 weeks of age (6–7 extended leaves). When aphid-infested plants were required, sweet pepper plants were infested with 2–3 adults of *M. persicae* 2 weeks before the start of the experiment, in order to get a colony of approximately 400 individuals per plant at the start of the trials. Aphid-infested plants containing con- or heterospecific immature stages also were used as odor sources in some tests. To obtain plants with predator eggs, sweet-pepper plants were placed in a rearing cage containing mature adults of one of the two predators for enough time to obtain 50 eggs per plant, and after that time plants were removed from the cage and kept without predators in separate glass vessels inside a climatic chamber. To obtain plants carrying larvae, five larvae of either *S. rueppellii* or *A. bipunctata* (of second and third instar, respectively) were placed carefully on the infested plant with a soft paintbrush. As a final odor source, we used aphid-infested plants with larval tracks from syrphids or coccinellids. To obtain larval tracks, five larvae of either *S. rueppellii* or *A. bipunctata* were placed on a circular piece of parafilm (8 cm diam), inside a petri dish. The Petri dish was sealed with parafilm and kept in a rearing chamber for 24 h. After this time, the larvae and all their observed by-products were removed carefully, and the parafilm disc was attached to an infested plant. All odor sources were prepared on the evening before the start of an experiment, and after that plants were transplanted into a glass pot with a ground-glass male connector that was inserted into an odor source vessel (see Turlings et al. 2004).

Olfactometer Bioassays

Bioassays were carried out with both larvae and female adults of *S. rueppellii*. Only those insects that made a choice were included in the analysis of the results (this represented 45 % of the individuals tested).

Larvae: 4 – 5 day-old larvae (second-instar) were removed from their cage with a soft paintbrush and carefully placed at the central choice chamber of the olfactometer. Because syrphid larvae are commonly nocturnal (Hopper et al. 2011) and illumination induces escape rather than foraging responses (Amorós-Jiménez, pers. observ.), all larvae were tested during the scotophase. In the experiments that included plants as odor sources, the olfactometer was illuminated during 30 min prior to releasing the larvae in order to ensure volatile emission by plants (Gouinguene and Turlings 2002). Each replicate consisted of three groups of six larvae. Larvae within each group were tested simultaneously.

Adults: Mated 6-7-day-old females were used, and all test insects were naïve in that they had never encountered a prey or plant as an adult. They were removed from their cage with an aspirator and placed directly in a vial attached to a glass frit at the bottom of the central choice chamber of the olfactometer.

When attracted by an odor, the insect would crawl or fly into an olfactometer arm through which the odor entered the central chamber. At the end of the arms, the insects' passage was blocked by a stainless steel screen, and eventually they would walk up into a trapping bulb, where they could be counted and removed (Turlings et al. 2004). All insects were given 30 min to make a choice, after which they were removed and a new group was released. Each replicate consisted of three groups of either four or six individuals (see sections below). Insects within each group were tested simultaneously. On a given day, all insects were tested with the same odor sources, which remained in the same position. The positions were kept this way because odors can adsorb to the glass surface, and arms could remain attractive even after removal of the odor sources (Turlings et al. 2004). For this reason, after each experiment all parts of the olfactometers (glass and Teflon) were cleaned thoroughly with distilled water and then with acetone and pentane at the end of each day. After solvents had evaporated, the glass parts were placed overnight in an oven at 250 °C.

Attraction to Aphid-infested Plants

The attractiveness of aphid-infested plants to syrphid larvae and adult females was first assessed in order to test if the olfactometer was suited for such assays and to determine if syrphids are able to distinguish aphid-

infested plants from uninfested plants, which would be essential for the next experiments.

Larvae: An aphid-infested sweet pepper plant was placed into one of the arms of a four-arm olfactometer, while a non-infested plant was positioned in a different arm. The two remaining arms were left empty.

Adults: Two infested and two non-infested sweet pepper plants were placed in different arms of the six-arm olfactometer, leaving the remaining two empty.

Each day, three groups of six larvae or three groups of four female adults were released into the choice chamber, and their choices were recorded. This was repeated six times, with the odor sources being introduced through different arms each time.

Although observations in these two preliminary experiments showed that most larvae crawled into one of the four arms, they kept coming back to the central chamber and exploring new arms, showing no clear preference for a particular odor source. Figure 1a illustrates the final choice of larvae after they stopped searching and shows that they do not discriminate between plants and infested plants. Based on the results of these experiments we decided to not test the response of syrphid larvae to volatiles from con- or heterospecific cues.

Responses to Con- and Heterospecific Volatile Cues

To assess the effect of conspecific volatile compounds on *S. rueppellii* female choices, two arms with only clean air were alternated with arms containing an aphid-infested plant with eggs, larvae, or larval tracks from the same syrphid species. The remaining arm consisted of an infested plant without syrphids. Three times, six naïve female adults were released per replicate with a total of six replicates, and their choices were determined 30 min after release. For each replicate, the odor sources were placed in a different position.

To test the effect of heterospecific volatiles on host plant selection of *S. rueppellii* females, we followed a procedure similar to that of the previous experiment, but in this case aphid-infested plants with eggs, larvae, or larval tracks of *A. bipunctata* were used as odor sources.

Oviposition Site Preferences

To study the effect of potential competitors on acceptance of oviposition sites by *S. rueppellii* females, the following multiple-choice experiment was carried out. An arena consisting of an insect-proof cage (30×30×60 cm) was placed inside a climatic chamber under the same conditions explained above. Four aphid-infested sweet-pepper plants were placed

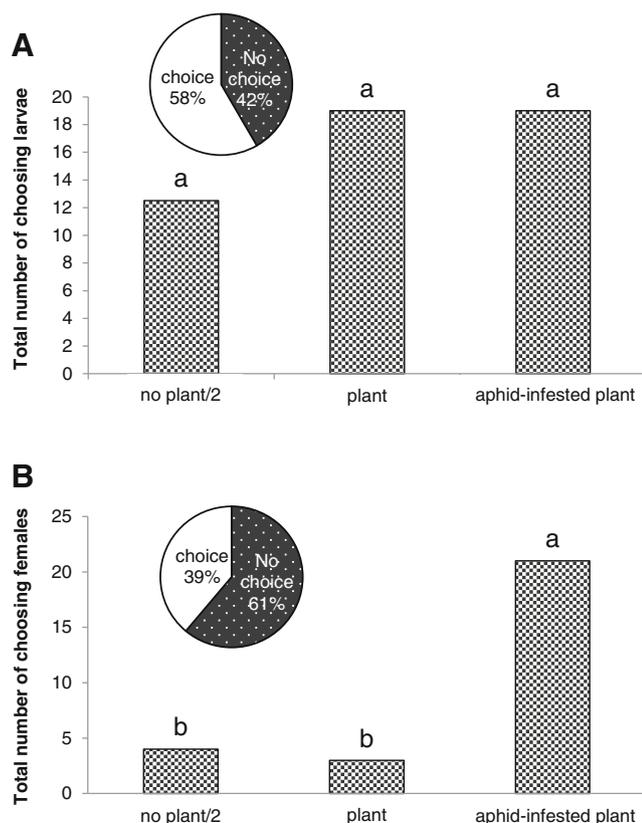


Fig. 1 Attraction of *Sphaerophoria rueppellii* to odors from sweet-pepper plants infested with the aphid *Myzus persicae* ($N=6$). The pie chart shows the percentage of insects that entered an olfactometer arm. Choices for the two control arms were summed and divided by two. Different letters indicate significant differences between the treatments ($P<0.01$). **a** Choices made by second-instar larvae **b** Choices made by female adults

in different corners of the cage to test the oviposition preference in the presence of conspecific predators, and their relative position was randomised for every new replicate: 1) a predator-free plant as positive control; 2) a plant with syrphid eggs; 3) a plant with syrphid larvae; and 4) a plant with larval tracks of *S. rueppellii*. To evaluate the influence of heterospecific competitors, a similar procedure was followed.

A 6–7 day-old mature syrphid female was placed in the center of the cage for 24 h. After this, plants were checked by visual inspection, and the number of eggs laid on each plant was counted. Each fly was used only once, and they had never experienced the test conditions before. Fifteen replicates were performed for each predator.

Statistical Analysis

Olfactometer choices were analysed using a log linear model that takes into account overdispersion (including effects of insects influencing each other's choices) as described elsewhere (D'Alessandro and Turlings 2006). Data from the oviposition behavior experiment (proportion of eggs laid) were

square-root-transformed to achieve homocedasticity prior to running a Kolmogorov-Smirnov normality test. A repeated measures ANOVA test then was performed to study the ovipositional preference of syrphid females, with "oviposition site" defined as within-subject factor, followed by a Bonferroni *post-hoc* test for multiple pair-wise comparisons. All data were analysed with the statistical packages R (<http://stat.ethz.ch/CRAN/>) and SPSS V20.0. (IBM Co., NY, USA).

Results

Olfactometer Bioassays

Attraction to Aphid-infested Plants Larvae and adult females of *S. rueppellii* responded differently to test stimuli. While the larvae that entered an arm (58 %) distributed uniformly over all four arms ($df=2$, 69; deviance ratio=2.70; $P=0.39$) (Fig. 1a), the great majority of choosing females (39 %) selected arms with infested plants over non-infested plants or empty arms ($df=2$, 105; deviance ratio=20.47; $P<0001$) (Fig. 1b).

Con- and Heterospecific Volatile Cues In the first experiment, the response of naïve *S. rueppellii* females to infested plants with different conspecific cues as odor sources was tested. While 37 % of the flies made a choice, 63 % remained in the central chamber of the olfactometer. Most females that responded chose the arm associated with odor cues from aphid-infested plants without predators and preferred this odor to odors from aphid-infested plants with predatory syrphid larvae ($df=4$, 103; deviance ratio=11.25; $P<0.05$; Tukey test: $P<0.01$). There was no evidence of differences in behavior among replicates ($df=1$, 102; deviance ratio=1.35; $P=0.22$) (Fig. 2a).

In the second experiment, we tested the response of naïve adult females to infested plants with odors from immature coccinellid stages. In this case, 42 % of the released females made a choice, while 58 % of them remained in the central choice chamber. There were no statistical differences ($df=4$, 103; deviance ratio=7.59; $P=0.11$) that indicated discrimination between infested plants with or without heterospecific competitors (Fig. 2b).

Oviposition Preferences

The proportion of eggs laid by syrphid females was significantly influenced by the presence of conspecific immature stages ($df=2.299$, 22.985; $F=3.487$; $P=0.042$). Females preferably laid their eggs on aphid-infested plants without syrphid larvae than on those with syrphid larvae (Bonferroni test, $P<0.05$), whereas there was no significant difference compared to plants with syrphid eggs or larval tracks (Fig. 3a).

In contrast, female adults faced with a choice between plants with or without coccinellid eggs or larval tracks laid fewer eggs

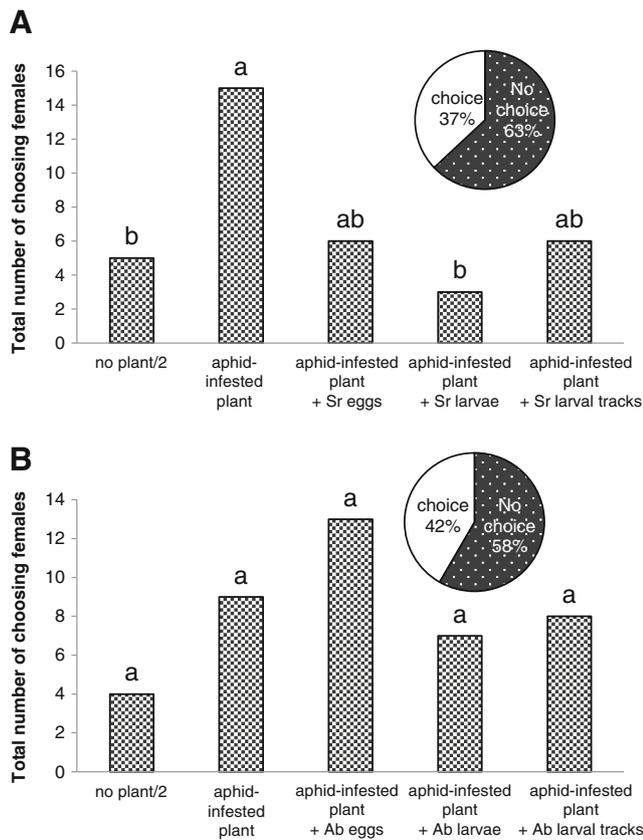


Fig. 2 Choices made by *Sphaerophoria rueppellii* adult females in the presence and absence of con- and heterospecific cues ($N=6$). The pie chart shows the percentage of syrphid females that entered an olfactometer arm. Choices for the two control arms were summed and divided by two. Different letters indicate significant differences between the treatments ($P<0.01$). **a** With odors of *S. rueppellii*. **b** with odors of *Adalia bipunctata*

on such plants than on aphid-infested plants without traces of the competing predator ($df=2.624, 36.738$; $F=3.608$; $P=0.027$; Bonferroni test, $P<0.05$). There were no statistical differences in the proportion of eggs laid on predator-free infested plants and those laid on infested plants with coccinellid larvae (Fig. 3b).

Discussion

Olfactometer bioassays were conducted to explore whether volatile compounds are important for syrphid larvae and adults to detect and orient towards food sources and oviposition sites, respectively. Similar experiments were carried out to study if the odor cues of intra- and interspecific competitors affect the egg-laying preferences of *S. rueppellii* females. Females were found to effectively discriminate between aphid-infested plants and non-infested ones, clearly responding to olfactory stimuli. Choice of oviposition sites in aphidophagous hoverflies is a complex process that involves visual, auditory, olfactory, and gustatory cues (Almohamad

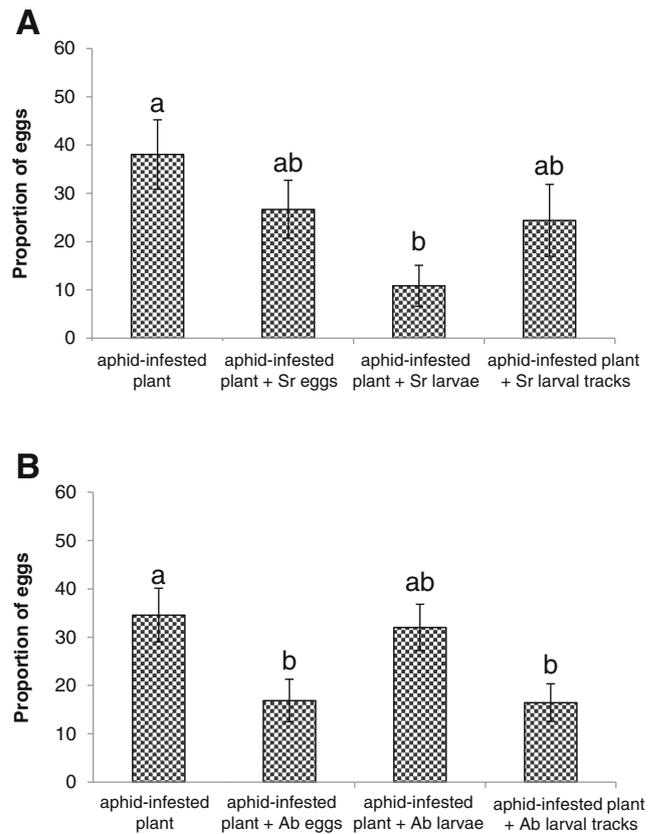


Fig. 3 Proportion of eggs (Mean±SE) laid by *Sphaerophoria rueppellii* females in the presence and absence of conspecific (**a**) and heterospecific (*Adalia bipunctata*) competitors (**b**) ($N=15$). Different letters indicate significant differences among treatments (repeated measures ANOVA and Bonferroni test, $P<0.05$)

et al. 2009). Yet, volatile compounds emitted from aphids and host plants can be sufficient to stimulate egg-laying in females even in absence of the other cues (Almohamad et al. 2008a; Verheggen et al. 2008). Indeed, most females in our olfactometer trials showed abdominal protraction and oviposition after entering an arm with an infested plant, despite the fact that they did not sense anything other than olfactory stimuli (data not shown). These observations highlight the key role that volatile semiochemicals play in choice-making behavior of hoverflies. Green leaf volatiles (GLVs) and the aphid-alarm pheromone [(*E*)- β -farnesene] have been recognized as important semiochemicals for the location and acceptance of oviposition sites for syrphids and other aphid predators (Dicke 1994; Francis et al. 2004; Verheggen et al. 2008).

In contrast to these positive results, plant and aphid volatiles did not attract *S. rueppellii*'s larvae, which distributed themselves equally over the olfactometer arms, including both infested and non-infested plants, but also the arms carrying clean air (Fig. 1a). Literature on the role of semiochemicals in the foraging behavior of syrphid larvae is scarce, and results more or less support the notion that larvae do not use volatiles to locate aphids, or that they have limited importance for first

instars at short distances (Bargen et al. 1998; Francis et al. 2005). Instead, non-volatile (gustatory) compounds from, for instance, honeydew triggers specific larval locomotory behaviors (slowing, turning, and 'casting') and seem to have a greater role in their prey-locating success (Bargen et al. 1998; Chandler 1969). In our experiments, only second instars were used, as first instars are hard to manipulate without causing extensive mortality and require very high humidity to remain active (Amorós-Jiménez et al. 2012). Whether first instars of *S. rueppellii* use infochemicals to forage for aphids and to what extent they are important is a question that this study cannot answer. Females of the syrphid fly *Episyrphus balteatus* are known to regularly lay their eggs on uninfested plants (Sadeghi and Gilbert 2000), forcing the hatching larvae of *E. balteatus* to forage for themselves. Indeed, larvae of this species are able to move long distances and survive long periods without encountering aphids (Rojo et al. 1996). In contrast to this strategy, *S. rueppellii*'s larval stages have a more limited movement capacity due to their lower tolerance to starvation periods, and females mostly lay their eggs close to or even inside aphid colonies (Amorós-Jiménez, pers. observ.). We, therefore, hypothesise that for *S. rueppellii* larvae to encounter sufficient prey, they rely on their mothers to select suitable oviposition sites.

The presence of syrphid larvae-derived semiochemicals in odor blends from aphid-infested plants was apparently detected by *S. rueppellii* females, and rendered such odor sources less attractive, since females significantly preferred the non-contaminated infested plants. Similarly, females preferred to oviposit on aphid-infested plants that carried no conspecific larvae (Fig. 3a). The fact that the deterrent effect of eggs and larval tracks was less intense than that of larvae might be due to lower concentration of freshly deposited oviposition-deterrent substances. Indeed, attractive stimuli associated with plants and aphids may overpower the repellent effects of volatiles from eggs and larval tracks of conspecific competitors (Almohamad et al. 2010b). Although it has already been established that detection of intraspecific competitors involves chemical cues derived from eggs, larvae, or larval tracks (e.g., Almohamad et al. 2010a, b; Scholz and Poehling 2000) our study reveals the specific importance of volatiles in these interactions, and shows that syrphids respond to these cues even in the absence of other stimuli.

Syrphid female choices were not affected by volatile emissions from immature stages of the coccinellid *A. bipunctata*. Although a greater number of females tended to prefer arms containing a plant over an empty vessel (Fig. 2b), contrary to our expectation, infested plants without coccinellids did not attract more females. Yet, the oviposition experiments produced the anticipated result: females reduced their oviposition rate in response to the presence of coccinellid immatures (eggs and larval tracks), but there were not differences between aphid-infested plants with or without coccinellid larvae

(Fig. 3b). That syrphid oviposition was not significantly deterred by the presence of coccinellid larvae may be related to the high aphid-alarm pheromone production they induce. It is known that *A. bipunctata* induces a stronger antipredator behavior in *M. persicae* than do *S. rueppellii* larvae, including an enhanced secretion of exudate from their cornicles, which contains the alarm pheromone (*E*)- β -farnesene (Belliere et al. 2011). This pheromone may enhance the attraction of natural enemies such as gravid syrphid flies (Francis et al. 2004, 2005). When larvae are no longer present (larval tracks treatment) and the production of the alarm pheromone has stopped, this added attractiveness ends, possibly explaining the differences observed in syrphid's oviposition preferences.

Hence, *S. rueppellii* females responded strongly to conspecific volatiles, whereas they did not respond to volatiles from *A. bipunctata*. This contrasts with previous work that has reported an avoidance by syrphid females of aphid colonies with heterospecific competitors (Almohamad et al. 2009, 2010a; Hindayana et al. 2001; Laubertie et al. 2006; Putra et al. 2009) and in the specific case of Coccinellidae it has been shown that chemical cues released by larval stages play a role in this avoidance behavior (Almohamad et al. 2010a). Our results suggest that the sole perception of related volatiles is not deterrent enough for syrphid females. This is similar to what has been found for certain coccinellids, where adult females were not deterred from ovipositing in the presence of heterospecific cues (Almohamad et al. 2010a; Yasuda et al. 2000). Possible explanations for our results are that adult syrphids do not rely expressively on semiochemicals to detect heterospecific competitors, or that heterospecific volatiles elicit a response only when they are combined with other stimuli. For example, Almohamad et al. (2010a) found that *Episyrphus balteatus* females laid fewer eggs on leaf discs contaminated with *Harmonia axyridis* larval tracks, and that this involved a mixture of volatile and gustatory stimuli, as well as visual perception of the plant. In another study (Putra et al. 2009), *E. balteatus* female response to coccinellid larvae varied depending on the instar they encountered, again suggesting that the syrphids' response to heterospecifics may rely largely on visual cues.

The present study reveals an important role for volatile semiochemicals in the foraging behavior of syrphid flies and their selection of oviposition sites. It also shows a differential importance of volatiles in the avoidance of plants that carry con- and heterospecific predators. Information on the identity of such volatiles is scarce. Hemptinne and Dixon (2000) found that coccinellid larvae and larval tracks release a mixture of alkanes and Almohamad et al. (2010b) identified a blend of acids, alcohols, and aldehydes in the emissions from *E. balteatus* larvae and their tracks. Identifying the specific volatile-compounds that are involved in the recognition of conspecifics by *S. rueppellii*, as well as further studies on the possible perception of other heterospecific competitors—

including other syrphid species—will be important to obtain a full understanding of the ability of *S. rueppellii* to detect and avoid intraguild competitors.

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