

Aphid and Plant Secondary Metabolites Induce Oviposition in an Aphidophagous Hoverfly

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Abstract *Episyrphus balteatus* DeGeer (Diptera, Syrphidae) is an abundant and efficient aphid specific predator but usually neglected in integrated pest management programs. Electrophysiological experiments were conducted for the first time on *E. balteatus* in the present work, toward the common aphid alarm pheromone, (E)- β -farnesene (E β F), as well as on a broad range of plant secondary metabolites, including terpenoids (mono- and sesquiterpenes) and green leaf volatiles (C6 and C9 alcohols and aldehydes). Monoterpenes induced significant EAG responses, whereas sesquiterpenes were found to be inactive on EAG, except for the aphid alarm pheromone (E β F). The most pronounced antennal responses were induced by six and nine carbon green alcohols and aldehydes (i.e. (Z)-3-hexenol, (E)-2-hexenol, (E)-2-hexenal and hexanal). To investigate the behavioral activity of the EAG-active plant and aphid secondary metabolites, observations were conducted on *E. balteatus* females exposed to three compounds: R-(+)-limonene (monoterpene), (Z)-3-hexenol (green leaf alcohol) and E β F (sesquiterpene, common aphid alarm pheromone). A single *E. balteatus* gravid female was exposed for 10 min to a *Vicia faba* plant which was co-located with a semiochemical dispenser. Without additional semiochemical, hoverfly females were not attracted toward the plant and no oviposition was observed. The monoterpene R-(+)-limonene had no impact on the foraging behavior of the tested females, whereas (Z)-3-hexenol and E β F increased the time of flight and acceptance of the host plant. Moreover, these two chemicals induced the oviposition of gravid females on aphid-free plants, suggesting that the selection of the oviposition site by predatory hoverflies relies on the perception of chemical blend composed by both prey pheromones and secondary metabolites induced in the host plant.

Keywords *Episyrphus balteatus* • Predator • Plant-insect interaction • Oviposition induction • Green leaf volatiles • Terpenoids • (E)- β -Farnesene • Electroantennography • EAG

Introduction

Episyrphus balteatus DeGeer (Diptera : Syrphidae) is the most frequently-encountered syrphid species at aphid infested sites in temperate regions (Schneider, 1969), and one of the most efficient aphid-specific predators (Entwistle and Dixon, 1989; Tenhumberg and Poehling, 1991). Because syrphid larvae have limited dispersal abilities (Chandler, 1969), oviposition site selection has an important impact on offspring performance. Several studies have already addressed parameters influencing the foraging and oviposition behavior of aphid natural enemies, that include : (1) aphid species and their associated chemicals (Budenberg and Powell, 1992; Bargaen et al., 1998; Sadeghi and Gilbert, 2000a,b; Zhu et al., 2005; Almohamad et al., 2007 ; Verheggen et al., 2007a); (2) host-plant physical and chemical characteristics associated with aphid species (Chandler, 1968; Sanders, 1983; Vanhaelen et al., 2001, 2002; Tumlinson et al., 1992; Zhu et al., 2005; Videla et al., 2006; Harmel et al., 2007; Almohamad et al., 2007) ; (3) aphid colony size and density (Bargaen et al., 1998; Scholz and Poehling, 2000; Sutherland et al., 2001) ; (4) age of the hoverfly female (Sadeghi and Gilbert, 2000c; Frechette et al., 2004) and (5) floral character (Sutherland et al., 1999). Many semiochemicals, either emitted by prey or within their association with host plants are therefore presumed to play an key role in habitat selection, by allowing reduction of searching time and by increasing attack rates on prey (Dicke and Sabelis, 1988; Vet and Dicke, 1992; Harmel et al., 2007).

Many studies on tritrophic interactions that include plant, herbivorous insects and natural enemies demonstrated how effective induced volatiles are against herbivores (Turlings et al., 1990 ; Turlings and Tumlinson, 1992 ; De Moraes et al., 2001). Under herbivore infestation, these plants can qualitatively and/or quantitatively adjust their volatile organic chemical emissions. These emissions usually consist of terpenoids (monoterpenes and

sesquiterpenes) and green leaf volatiles (alcohols, aldehydes or esters), the latter being specifically released as a result of tissue damage (Paré and Tumlinson, 1997; Farag and Paré, 2002; Tholl et al., 2006). These indirect defenses generally include semiochemicals that can be used as synomones (Nordlund and Lewis, 1976) by natural enemies to locate the infested plant and subsequently their prey (Tumlinson et al., 1992; Vet and Dicke, 1992). Compared to the body of information on parasitoids, much less information is available on those chemical cues that guide predators during location and acceptance of oviposition sites (Steidle and van Loon, 2002). Hoverflies are however subjected to various natural chemical blends when searching for an oviposition site. These blends consist of plant and insect semiochemicals such as (E)- β -farnesene (E β F), the main component of the alarm pheromone of most aphid species (Nault et al., 1973; Francis et al., 2005a). This sesquiterpene was found to act as a kairomone for several efficient aphid predators, including *E. balteatus* larvae, *Harmonia axyridis* larvae and *Adalia bipunctata* larvae and adults (Francis et al., 2004, 2005b ; Verheggen et al., 2007a). More than twenty additional chemicals, including α - and β -pinene, cymene, α -phellandrene or limonene, were found by Francis et al. (2005a) in some aphid species. These aphid secondary metabolites are also commonly found in the headspace of many plant families, such as Solanaceae, Fabaceae or Brassicaceae (Agelopoulos et al., 1999; Farag et Paré, 2002; Verheggen et al., 2005; Harmel et al., 2007). Terpenoids, as well as green leaf volatiles (GLVs), are potential semiochemicals that can be used by aphid predators, such as syrphids, lady beetles or lacewings, to locate their prey (Zhu et al., 1999 ; Steidle and van Loon, 2002 ; Harmel et al., 2007).

In this study, we investigated the olfactory perception and behavioral activity of various plant and aphid volatile organic chemicals in order to highlight those that may act on the prey-seeking behavior of *E. balteatus*, and how they could be included in modern integrated pest management methods.

Methods and Materials

Chemicals All chemicals, except E β F, were purchased from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany) and had chemical purity >97 % (determined by GC). E β F was synthesized from farnesol (Tanaka et al., 1975) and had a chemical purity of 98% (also determined by GC).

Biological Material All plants, aphids and hoverflies used in the present work were reared in climate-controlled rooms (16 hr light photoperiod ; 70% RH ; 20 \pm 2°C). Broad beans (*Vicia faba* L.) were grown in square 9cm x 8cm plastic pots filled with a mixture of vermiculite and perlite (1/1), and were used as host plants for the pea aphid, *Acyrtosiphon pisum* Harris. Adult *E. balteatus* were reared in cages (75 x 60 x 90 cm) and fed pollen, sugar, and water *ad libitum*. Hoverfly oviposition was induced by placing broad beans in the cage for 3 hr. *E. balteatus* larvae were fed *A. pisum* and pupae were placed in aerated plastic boxes (14 x 11 x 4 cm) until hatching. Experiments were carried out using two to four wk old adults.

Electroantennography The hoverfly was immobilized by covering its abdomen and thorax with modeling clay. This setup enabled the recording of electroantennograms for longer time period than if the antenna was excised (Verheggen et al., 2007b). Two glass Ag-AgCl electrodes (Harvard Apparatus; 1,5mm OD x 1,17mm ID) filled with saline solution (NaCl : 7.5g/l; CaCl₂ : 0.21g/l; KCl : 0.35g/l; NaHCO₃ : 0.2g/l) and in contact with a silver wire, were placed on the insect antennae. The ground glass electrode entirely covered one antenna while the recording electrode, linked to an amplifier (IDAC-4, Syntech®, Hilversum, The Netherlands) with a 100 times amplification, was placed on the bottom of the last segment of the other antenna. A 0.5-cm² piece of filter paper that was impregnated with 10 μ l of the chemical under examination was placed in a Pasteur pipette, which was then used to puff an air sample in a constant 1.5 l/min airstream. Paraffin oil was used to make chemical solutions

with concentrations ranging from $10^{-1}\text{ng}/\mu\text{l}$ to $10^5\text{ng}/\mu\text{l}$ (by 10x increments). Electroantennograms were collected using Autospike 3.0 (Syntech, Hilversum, The Netherlands). Stimulation with paraffin oil was executed as a negative control before and after the stimulations with the seven concentrations cited above of the tested chemical. Stimulations were induced thirty seconds from each other. Preliminary results indicate this length of time was adequate to allow the insect recover its full reactivity to stimuli. Five insects from both sexes were tested with each chemical.

Behavioral Observations A single female hoverfly was placed in a cage (30x30x60 cm) with a 20 cm tall *Vicia faba* plant. As a positive control, the female hoverflies were offered a *Vicia faba* plant which was infested with 1 g of *Acyrtosiphon pisum* 24 h before the experiment. A non-infested *Vicia faba* was offered to the hoverfly as a negative control. A rubber septum was used to test the behavioral activity of three chosen semiochemicals: (*R*)-(+)-limonene (monoterpene), (*Z*)-3-hexenol (green leaf volatile), and E β F (sesquiterpene). The diffuser was placed on the first pair of true leaves and contained a 100 μl paraffin oil solution (400ng/ μl) of the chemical to be tested, and the solution was changed after each replication. Paraffin oil was chosen for its chemical inertness and ability to continuously release chemicals that are diluted within it. The female hoverfly foraging behavior was then recorded for 10 min using the software The Observer5.0® (Noldus information Technology, version 5.0, Wageningen - The Netherlands) which allows hoverfly behavior to be easily observed, subdivided and recorded (Harmel et al., 2007). Descriptions of the four observed behavioral subdivisions are presented in Table 1. The number of eggs laid by each female was counted at the end of each observation. Experiments were conducted in a climate-controlled room at $22 \pm 1^\circ\text{C}$. The *E. balteatus* females were approximately 15-30 days old and no aphid-infested plant was offered for 24h prior the experiment. Ten replications were performed for each tested chemical.

Statistical Analyses One-way ANOVA followed by *Tukey's* test (pairwise comparisons) were used with the EAG results. Two sample *t-test* was used to compare EAG responses from males and females. One-way ANOVA followed by *Dunnnett's* test (comparison with a control) was used to compare the behavioral data observed for the four treatments compared to our control. The one sample *t-test* was applied to compare the mean number of eggs laid in behavioral assays to the "0" value observed with the control. All statistical tests were conducted using Minitab v.14 for Windows®.

Results

Electroantennography Antennal activity increased significantly in both sexes with the concentration of the tested compound (that ranged from 0.1ng/μl to 0.1mg/μl). No saturation of the antenna was observed for any tested chemical. The three lowest concentrations (0.1ng/μl, 1ng/μl and 10ng/μl) did not elicit antennal response, regardless of the tested compound. Because we aimed to compare EAG data to results previously obtained when similar chemicals were tested on other aphid predators, we did not correct our EAG responses by taking into account their differences of volatility, as stated by Brockerhoff and Grant (1999).

EAG responses were significantly different between the five tested chemical families ($F_{4,152} = 117.82$, $P < 0.001$), namely monoterpenes, monoterpenes with alcohol function, sesquiterpenes, C6 and C9 green leaf chemicals (Figure 1). Male and female antennal activity over the range of chemical compounds tested were not significantly different from each other ($F_{1,152} = 2.85$, $P = 0.094$). The eight tested monoterpenes elicited electrical depolarization that ranged from -400μV to -800μV and they were all equally perceived by both sexes. Linalool induced an average depolarization of -1300μV, and was similarly perceived by males and females ($t_{obs} = 0.95$, $P = 0.372$). EβF was the only chemical to be perceived differently by males and females ($t_{obs} = 2.62$, $P = 0.031$). The two other tested sesquiterpenes (α-humulene and β-

caryophyllene) did not elicit electrical depolarization in either sex. The green leaf alcohols and aldehydes tested in this study elicited high EAG responses, statistically equal in both males and females, ranging from $-1750\mu\text{V}$ to $-2250\mu\text{V}$ for the six-carbon chain GLV and from $-600\mu\text{V}$ to $-1400\mu\text{V}$ for the nine-carbons chain GLV.

Behavioral Observations Hoverfly gravid females showed no interest for a non-infested *V. faba*, staying immobile during 80% of the observation time (Figure 2). However, while presenting an aphid-infested plant, the immobility duration is significantly reduced and the time spent on the plant (acceptance) is increased, as well as the oviposition behavior and the number of laid eggs (7.4 eggs/female; $t_{obs}=7.38$, $P<0.001$) (Figure 3). (*R*)-(+)-Limonene did not significantly attract the tested predatory hoverflies toward the non-infested plant and did not increase the number of eggs laid by females, comparing with a non-infested semiochemical-free plant (0.2 egg/female; $t_{obs}=1.50$, $P=0.084$). In addition to the high sensibility of hoverfly antennae for the green leaf volatiles, (*Z*)-3-hexenol, significantly increased the mobility of females and plant acceptance, and induced the oviposition of a significant number of eggs (1.7 eggs/female; $t_{obs}=1.85$, $P=0.049$). The main compound of the aphid alarm pheromone (E β F) significantly increased hoverfly mobility, searching duration, acceptance of the host plant and oviposition behavior. The mean number of laid eggs was 3.6 per female, which was significantly higher than the control ($t_{obs}=3.31$, $P=0.005$) and lower than the number of eggs laid when testing an aphid-infested *V. faba* ($F_{1,18}=6.60$, $P=0.019$).

Discussion

Tritrophic interactions between infested plants, herbivorous arthropods and their natural enemies are complex because of the many semiochemicals that are typically involved. In addition to the semiochemicals that are emitted by the herbivorous insects, most plant species respond to insect infestation by synthesizing and releasing complex blends of volatile

compounds, which can be used by predators and parasitoids as foraging cues, thereby enhancing the plants' defense ability (Dicke et al., 1990; Dicke, 1994; Turlings et al., 1995). Previous studies have provided electroantennogram recordings from aphid natural enemies, including lady beetles (Coleoptera, Coccinellidae) (Zhu et al., 1999; Al Abassi et al., 2000; Verheggen et al., 2007a) and lacewings (Neuroptera, Chrysopidae) (Zhu et al., 1999; 2005), to semiochemicals released from prey and host plants. Some of the tested chemicals attracted the tested predators but no information was available about their impact on predators foraging behavior and oviposition (Zhu et al., 1999, 2005). However, several species responded with oviposition to aphid-produced honeydew alone, whereas in others, the aphid prey themselves are needed for oviposition to occur (Steidle and van Loon, 2002).

Syrphid larvae do not use semiochemicals to locate aphids, or exclusively at very short distance (Bargen et al., 1998; Francis et al., 2005b). Because of their limited dispersal abilities (Chandler, 1969), the choice of the oviposition site by adult females has an important impact on the offspring performance, and volatile organic compounds are therefore presumed to guide their foraging behavior. To the best of our knowledge, this is the first published report of successful EAG recordings from *Episyrphus balteatus* antennae. Various VOCs that are usually released by plants and insects have been here tested using EAG and we found that hoverflies are able to sense their environment by odors. Generalists need to invest less time in searching particular host and prey species than specialists, therefore the use of infochemicals in order to reduce searching time should be less important (Vet and Dicke, 1992). However, previous studies indicated that the use of infochemicals for foraging is an adaptive strategy regardless of dietary specialization and that physiological constraints on sensory processing in generalists might be less severe than supposed (Steidle and van Loon, 2003). Our EAG results confirm that generalist natural enemies do not focus on some volatile chemicals to locate an appropriate oviposition site, as their sensory perception is not specialized. In addition, we

demonstrated that some active compounds play a key role in their foraging behavior, by attracting natural enemies toward potential prey and by inducing oviposition, even in absence of aphids.

α -Pinene, β -pinene, α -phellandrene, and limonene are common plant volatiles (Farag and Paré, 2002; Tholl et al., 2006) but are also emitted by some aphid species such as *Megoura viciae* Buckton or *Drepanosiphum platanoides* Schrank (Francis et al., 2005a). Therefore, it is not surprising that these compounds, like other monoterpenes, elicited EAG responses in both male and female *E. balteatus*. Additionally, we confirmed that *E. balteatus* females do not lay eggs on a non-infested plant (Scholz and Poehling, 2000). (*R*)-(+)-Limonene did not attract the tested predatory hoverflies and the number of eggs laid by females exposed to (*R*)-(+)-limonene was not significantly different from a non-infested semiochemical-free plant. This monoterpene is commonly found in various plant headspace (Agelopoulos et al., 1999; Farag and Paré, 2002; Verheggen et al., 2005), and does not provide any information about prey presence on the stressed plant. This might explain why presence of this chemical does not provoke a direct behavioral effect on a gravid hoverfly female.

Green leaf volatiles were previously thought to play a role in prey finding behavior, due to their emission by damaged plants (De Moraes et al., 2001; Farag and Paré, 2002; Tholl et al., 2006). While the tested monoterpenes induced small EAG responses, the green leaf volatiles showed EAG responses that were 3-4 times higher for the 6-carbon chain GLVs, and 2-3 times higher for the 9-carbon chain GLVs. *E. balteatus*, in opposition to other aphid predators (Zhu et al., 1999), reacts differently to different chemical families and volatile organic compounds. In addition, our results showed that short-chain alcohols like (*Z*)-3-hexenol significantly increased the female's mobility and plant acceptance, in addition to inducing the oviposition of the hoverfly females in absence of prey.

E β F, the common aphid alarm pheromone (Francis et al., 2005a) which acts as a kairomonal substance for several aphid predators (Francis et al., 2004, 2005b ; Verheggen et al., 2007a) is detected by both male and female *E. balteatus* adults. As opposed to lacewings and lady beetles, E β F is perceived differently by male and female hoverflies (Zhu et al., 1999; Verheggen et al., 2007a). This difference in antennal activity between sexes accentuates the importance of E β F in hoverfly foraging behavior, as females are looking for suitable oviposition site. E β F antennal activity is also underlined when compared to the lack of response to two other tested sesquiterpenes (i.e. α -humulene and β -caryophyllene) that are commonly released by plants or insects as semiochemicals (e.g. De Moraes et al., 2001; Brown et al., 2006). Whereas β -caryophyllene induced antennal activity in both lady beetles and lacewings, α -humulene was not tested on these two aphidophagous predators (Zhu et al., 1999 ; Verheggen et al., 2007a). The lack of electrical response to these two sesquiterpenes as well as the lower responses observed to the C9-GLV compared to the C6-GLV, can also be due to their lower volatility. Brockerhoff and Grant (1999) indeed stated that EAG responses should be corrected by taking into account the volatility of the tested chemicals. However, E β F is as volatile as β -caryophyllene and α -humulene, but antennal activity was recorded. E β F significantly increased hoverfly mobility, acceptance of the host plant, the oviposition behavior, and the number of eggs laid (3.6 eggs/females). The aphid alarm pheromone is therefore a key compound in prey-seeking behavior in aphidophagous hoverflies. Previous results demonstrated its kairomonal role for *E. balteatus* larvae, which were attracted in a four-arm olfactometer (Francis et al., 2005b). In this study, we confirmed that female hoverflies are able to perceive this sesquiterpene and use it to select an oviposition site to lay their eggs. Behavioral results obtained with a E β F-treated plant and an aphid-infested plant were different. Therefore, this research suggests that predatory hoverfly oviposition site

selection is influenced by a blend of chemicals rather than by a single chemical, including not only E β F, but also secondary metabolites related to plant damages such as green leaf volatiles.

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Table 1. Description of the behavioral sequences recorded for aphidophagous hoverfly *Episyrphus balteatus* exposed to *Vicia faba*.

Observed behavioral sequences		Descriptions
Immobility		Predator immobilized on the cage
Searching	Fly/cage	Predator flies in the cage
	Fly/plant	Predator flies near the plant
Acceptance	Immobile/plant	Predator lands on the plant
	Walking/plant	Predator moves on the plant
	Proboscis/plant	Predator extends its proboscis and identifies the stimulatory substrate to accept the host
Oviposition	Immobile abdomen/plant	Predator exhibits an abdominal protraction
	Walking abdomen/plant	
	Egg laying	Oviposition

Figure legends

Fig. 1 : EAG activity of female (A) and male (B) *Episyrphus balteatus* antennae to aphid and plant secondary metabolites (100 μ g/ μ l). Means (+/-SE) with no letter in common are significantly different (ANOVA followed by *Tukey's test*, $P<0.05$). An asterisk indicates significant difference in EAG activity between male and female antennae (2-sample *Student t-test*, $P<0.05$). N=5 for both sexes and each chemical.

Fig. 2 : Effect of plant and aphid secondary metabolites on the foraging behavior of *Episyrphus balteatus* females (Mean duration in % of interval, +/- SE). Stars indicate means that are significantly different from the non-infested semiochemical-free plant (ANOVA followed by *Dunnnett's test*, $P<0.05$). N=10 for each treatment.

Fig. 3 : Effect of plant and aphid semiochemicals on the oviposition of *Episyrphus balteatus* females (Mean number of eggs +/- SE). The signs "ns", "*", "**" and "***" indicate no significant and significant differences from control at $P<0.05$, $P<0.01$ and $P<0.001$ respectively (*1-sample Student t-test*). N=10 for each treatment.

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