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FACULTE UNIVERSITAIRE DES SCIENCES AGRONOMIQUES DE GEMBLOUX

Production of alarm pheromone in aphids and perception by ants and natural enemies

François VERHEGGEN

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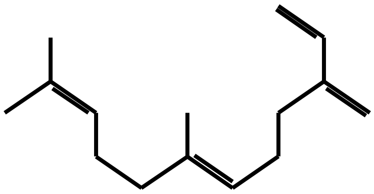

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Verheggen François. (2008). Production of alarm pheromone in aphids and perception by ants and natural enemies (Thèse de doctorat). Gembloux, Belgium, Gembloux Agricultural University, 197p., 9 tabl., 32 fig.

Abstract: Most Aphidinae species produce and use (*E*)-ß-farnesene (Eßf) as an alarm pheromone. This sesquiterpene is released by individuals under attack by a predator, and nearby aphids exhibit a variety of alarm behaviours. This PhD thesis aims to better understand how aphids manage their production and emission of alarm pheromone (Chapter IV). We also wanted, in a second step, to improve our knowledge on the roles that Eßf could play in the relationships that aphids have with their predators (Chapter V) and tending ants (Chapter VI), in order to better pinpoint the problem in this very tough context. The aphid predators have indeed a real advantage to be able to use the odorant cues emitted by their prey, to locate them and to select an adequate oviposition site. Ants establish with certain aphid species mutualistic relationships, which occurrence could be facilitated by the use of aphids' odours. In Chapter IV, we have highlighted that aphid colonies non subjected to attack by predators release constantly small quantities of Eßf in their headspace, which means that this molecule could have additional roles than just acting as an alarm pheromone. In a second study, we demonstrated that the release of Eßf was not contagious, and therefore that a non stressed aphid receiving the alarm signal does not release additional Eßf. Since the production of alarm pheromone is likely to entail physiological cost, we tested and validated the hypothesis that aphids regulate their Eßf production according to their social environment. In Chapter V, we studied the ability of the hoverfly predator *Episyrphus balteatus* (Diptera, Syrphidae) to be used as biological control agent against aphids infesting tomato plants (*Lycopersicon esculentum*). After identifying the odours emitted by aphid infested plants, we have demonstrated that although this Diptera is able to perceive all the odours released by the system, it mainly uses Eßf to select its oviposition site. However, the *E. balteatus* larvae are not adapted to the architecture of tomato plants. We also showed that the Asian ladybeetle *Harmonia axyridis* (Coleoptera, Coccinellidae) olfaction was adapted to the perception of Eßf and that this beetle is also attracted by this sesquiterpene. Finally, in Chapter VI, we characterized the benefits accruing to aphid populations that have established mutualistic relationships with *Lasius niger* (Hymenoptera, Formicidae), and have demonstrated the role of Eßf and honeydew, respectively in locating aphid colonies and in the persistence of the mutualism.

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Résumé: La plupart des espèces de pucerons appartenant à la sous-famille des Aphidinae produisent et utilisent le (E)-ß-farnésène (Eßf) comme phéromone d'alarme. Ce sesquiterpène est relargué par les individus stressés par l'attaque d'un prédateur et cause chez les individus qui le perçoivent un comportement d'alerte. La présente thèse de doctorat a pour objectif de comprendre comment les pucerons gèrent la production et émission de phéromones d'alarme (Chapitre IV). Nous voulions aussi, dans une seconde étape, améliorer les connaissances sur les rôles potentiels que cet Eßf peut jouer au sein des relations que les pucerons entretiennent avec leurs prédateurs (Chapitre V) et avec les fourmis (Chapitre VI) Les prédateurs de pucerons retirent en effet un réel avantage à pouvoir s'aider des odeurs émises par leurs proies pour les localiser et pour sélectionner un site d'oviposition adéquat. Les fourmis, quant à elles, établissent des relations de mutualisme avec certaines espèces de pucerons. Les rencontres entre fourmis et pucerons pourraient être facilitées par l'utilisation des odeurs de pucerons. Les résultats obtenus peuvent être résumés de la manière suivante: Dans le chapitre IV, nous avons mis en évidence que les colonies de pucerons non soumises à l'attaque de prédateurs relarguent constamment de faibles quantités d'Eßf, ce qui permet d'assumer que cette molécule puisse avoir d'autres fonctions que celle de phéromone d'alarme. Dans une deuxième étude, nous avons démontré que l'émission du Eßf n'était pas contagieuse, et donc qu'un puceron non stressé percevant le signal d'alarme n'émet pas à son tour de le Eßf. Puisque la production de phéromone d'alarme a inévitablement un coût physiologique, nous avons testé et validé l'hypothèse selon laquelle les pucerons régulent leur production de Eßf en fonction de leur environnement social. Dans le chapitre V, nous avons étudié la possibilité d'utiliser le syrphe prédateur *Episyrphus balteatus* (Diptera, Syrphidae) en lutte biologique contre les pucerons infestant les plants de tomate (*Lycopersicon esculentum*). Après avoir identifié les odeurs émises par les plants infestés, nous avons démontré que, si ce Diptère est capable de percevoir l'ensemble des odeurs émises par ce système tritrophique, il utilise principalement l'Eßf pour sélectionner son site d'oviposition. Cependant, les larves d'*E. balteatus* ne sont pas adaptées à l'architecture des plants de tomate. Nous avons également montré que la coccinelle asiatique *Harmonia axyridis* (Coleoptera, Coccinellidae) possède le matériel olfactif nécessaire à la perception du Eßf et qu'elle est aussi attirée par ce sesquiterpène. Enfin, dans le chapitre VI, nous avons caractérisé les bénéfices retirés par les populations de pucerons ayant établi des relations de mutualisme avec *Lasius niger* (Hymenoptera, Formicidae), et avons démontré le rôle du Eßf et du miellat, respectivement dans la localisation des colonies de pucerons et dans la persistance du mutualisme.

This PhD thesis is the results of a very exciting collaborative work between scientific and non scientific actors that should find themselves acknowledged here:

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« Des Phéromones.

Des hormones, en fait, qui arrivent à sortir de leurs corps.

On pourrait visualiser chacune de ces molécules

comme un bocal où chaque poisson serait un mot. »

Bernard Werber, *Les fourmis*

Chapter I

GENERAL INTRODUCTION

Aphids (Homoptera, Aphididae) are major pests of arable and horticultural crops, particularly in temperate regions. These range from grain crops and brassicas to potato, cotton, vegetable and fruit crops. They cause damages to their host plant either directly by feeding on their phloem sap, or indirectly by transmitting viruses. In the Aphididae family, large differences are observed on the feeding behaviour, from very polyphagous to monophagous specialist species. Type of life cycle also varies in an important way: certain aphids generally remain on the same host plant species throughout the year (autoecious), while others may alternate between different species of host plant during the annual life cycle (heteroecious). Alternations between primary host, which is often a tree or a shrub, and a secondary herbaceous host are generally observed. Aphids usually have several parthenogenetic generations during summer, a single sexual generation in autumn, and overwinter as eggs. In addition, some aphid families present a high polymorphism, including both alate individuals specialized in reproduction and colonization of new host plants, and apterous individuals. All these observations may result in an exponential reproduction and rapid colonization, making these insects pests of first importance worldwide.

Chemical treatments represent so far the main solution to prevent heavy aphid infestations, but are likely to entail resistance and environmental pollutions. It is therefore no surprise that scientists try to couple the use of biological control agents, such as aphid natural enemies, with conventional treatments to control aphid populations through adequate integrated pest management strategies.

The ecology of aphids is, like that of most insects, highly dependent upon chemical signals. Signals from host and non-host plants convey information that is vital for selecting feeding, larviposition and mating sites. Signals from aphids themselves are important in warning of threats, avoiding competition, attracting a mate, and maybe aggregating with conspecifics. Chemical signals, often called semiochemicals, are volatile and therefore easy to disperse in the environment, relatively specific and, not the least, easy to detect. Aphids make therefore extensive use of semiochemicals, both in gathering information from their environment and in signaling to each other. They are indeed soft-bodied insects and are especially prone to attack by predators and parasitoids, which would have clear advantage to have evolved responses to some of these semiochemicals.

Whereas aphids have means of protection against natural enemies through physical adaptations such as wax protection and sclerotization among soldier forms of social aphids, their primary protection from predators and parasitoids consists of escape responses mediated by the use of alarm pheromone signaling. The asexual forms, and most often the wingless females, of many aphid species release an alarm pheromone when disturbed, that consists, in most Aphidinae species, in a single chemical named (*E*)-ß-farnesene. Nearby aphids exhibit a variety of behaviours, ranging from removal of mouthparts from the host plant and moving away, to running, dropping off the plant and even attack the predator. Previous field studies have already tried to use the aphid alarm pheromone within integrated pest management strategies but concluded that it is necessary to completely understand aphids ecology as well as the way their natural enemies interact with them, before establishing efficient biological control methods.

According to these lacks of knowledge, the overall objective of this PhD was to complete our understanding of aphid chemical ecology, and more specially the production, emission and ecological roles that the aphid alarm pheromone, (*E*)-ß-farnesene, may play in aphids.

Chapter II

APHID CHEMICAL ECOLOGY: AN OVERVIEW

1. Prelude

The main objective of this second chapter is to give the readers who are unfamiliar with aphid chemical ecology the basic knowledge on aphid olfaction as well as an overview of the semiochemicals that are commonly used by aphids. After a brief introduction, the aphid olfactory system will be described, and the most important discoveries on aphid alarm and sexual pheromones will be presented. Finally, the perception of aphid semiochemicals by natural enemies will be described.

2. Introduction to aphid chemical ecology

Aphids represent one of the world's major insect pests, causing serious economic damage to a range of temperate and tropical crops (Remodière and Remodière, 1997). Considering the increasing amounts of insecticides that are needed to counteract the resistance that aphids develop, it is important to consider their semiochemicals as a means of providing new control methods. The understanding of the chemical ecology of aphids is a key factor to know how these insects can locate their host amongst the plant diversity. If semiochemicals from plants are important to explain the aphid distribution, intraspecific infochemicals are also of first importance in the aphid migration in the field. Aphids can indeed select individual of a few closely related host plant species from a wide range of non host plants. This selection relies upon the detection of secondary metabolites released from plants, as well as primary metabolites associated with the physiological condition of the host plant. But recent advances in the field have also pointed out the role of aphid pheromones in the populations dynamic. Sexual and alarm pheromones might indeed be involved in the way aphid constitute colonies, disperse, and select adequate host plants.

Aphid chemical ecology has been previously reviewed in 1992 (Pickett et al., 1992) and more recently by Pickett and Glinwood (2007). In the last thirty years, many studies have investigated the volatile chemical composition of the headspace of many aphid species. Since 1992, the perception of these semiochemicals by predators and parasitoids as well as their resulting behaviour has been studied, demonstrating the kairomonal role of aphids semiochemicals. The major breakthroughs are listed in the following sections of this review.

3. Aphid olfaction

Since the apparition of electrophysiological methods such as electroantennography (EAG) (Schneider, 1957 and 1962 ; Roelofs, 1984), single-cell recording (SCR) (Dawson et al., 1990), or the coupling of these techniques with a gas chromatography system (respectively GC-EAD and GC-SCR) (Arn et al., 1975 ; Struble and Arn, 1984 ; Campbell et al., 1990), studies on aphid olfactory system have been undertaken, aiming the identification of olfactory active compounds of plant or insect origin and the characterization of antennal olfactory receptor neurons.

The use of an insect antenna as a biological detector (EAG), linked with gas chromatography (GC-EAD), has been widely used for identifying olfactory-active compounds from natural odour blends. The application of this technique has been restricted in aphid studies, mostly due to the small, rapidly declining responses and the short usable lifespan of the EAG preparations (Park and Hardie, 1998), especially when excised antennae are used (Wohlers and Tjallingi, 1983). Signal-to-noise ratio of electroantennogram responses were however improved by the use of multiple connected antennae (Park and Baker, 2002). Because of these limitations, GC has been linked to single cell recording (GC-SCR) in aphids and this approach has proved very successful although it lacks the broad spectrum response (Wadhams, 1990; Campbell et al., 1990).

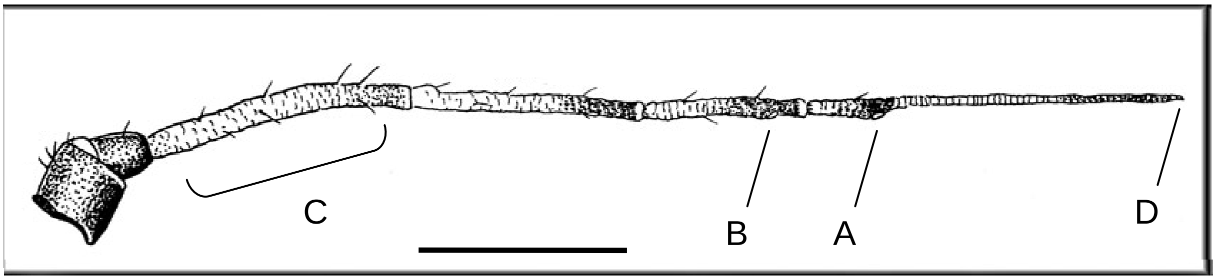

Figure 1. Diagram of an aphid antenna showing olfactory sensilla. (A) Distal primary rhinarium; (B) proximal primary rhinarium; (C) secondary rhinarium; (D) contact chemoreceptors (bar = 0.25 mm).

Nault and coauthors (1973) first demonstrated that the perception of the alarm pheromone is reduced by 66% if the sixth segment of the antenna is removed. Later, three types of olfactory sensilla were identified in adult aphids: proximal primary rhinaria, distal primary rhinaria and secondary rhinaria (Bromley et al., 1979; Park and Hardie, 2004). Whereas the main function of the distal primary rhinaria is in alarm pheromone detection (Wohlers and Tjallingii, 1983; Dawson et al., 1987b; Pickett et al., 1992; Pope et al., 2004), the secondary rhinaria would be a sex pheromone detector (Eisenbach and Miller, 1980; Dawson et al., 1990; Park et al., 2000; Pope et al., 2004). The proximal primary rhinaria of the fifth and sixth antennal segments would be specialized in plant odour detection (Bromley and Anderson, 1982; VanGiessen et al., 1994). Aphid antennae indeed respond to green alcohol and aldehydes (Vangiessen et al., 1994 ; Pope et al., 2004), mono- and sesquiterpenes (Pope et al., 2004), isothiocyanates (Pope et al., 2004) or benzaldehyde (Park et al., 2000 ; Pope et al., 2004). Several studies demonstrated the specificity of these olfactory receptors in term of age, sex, species and morph (Park et al., 2000; Park and Hardie 2002, 2003).

4. The aphid alarm pheromone

Chemicals that affect the behaviour of aphids have been studied for decades, but a major breakthrough was achieved in the 70s, with the identification of an aphid alarm pheromone. Since then, lots of studies have been conducted to understand more completely the modes of production, secretion and action of this very important molecule (Table 1).

Using two cornicles situated on the upper surface of the abdomen near the tail, aphids produce droplets that emit a repellent odour for conspecifics (Kislow and Edwards, 1972). That odour induces alate and apterous *Myzus persicae* to stop feeding and moving away or dropping from the host plant, while waving their antennae before and during movement. Variation in response to alarm pheromone is seen both intra- and inter-specifically and relates to the relative risk of predation and costs of escape.

The droplets secreted by the cornicles were found to contain mainly a sesquiterpene ($C_{15}H_{24}$) named (E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene, or also (*E*)-ß-farnesene or trans-ß-farnesene, symbolized further by the acronym Eßf (Bowers et al., 1972). Its structure is presented in Figure 2. This compound has been identified later in many other aphid species including the green peach aphid *Myzus persicae* Sulzer (Edwards et al., 1973; Wientjens et al., 1973; Pickett and Griffiths, 1980; Francis et al., 2005) and the pea aphid *Acyrthosiphon pisum* Harris (Wohlers, 1981; Mostafavi et al., 1996; Du et al., 1998; Francis et al., 2005).

Germacrene A (Figure 2), a biogenetic precursor of many sesquiterpenes, has later been isolated from the alfalfa aphid and identified as a new intrageneric aphid alarm pheromone (Bowers et al., 1977a). Germacrene A was however found to not act as an alarm pheromone outside the genus Therioaphis. Pickett and Griffiths (1980) showed also *Megoura viciae* to synthesize additional monoterpenes, including α-pinene, ß-pinene and limonene (Figure 2) with (-)-α-pinene having the most important alarm activity. (Z,E)-α-farnesene and (E,E)-α-farnesene were also present in several aphid species (Pickett and Griffiths, 1980 ; Gut and Van Oosten, 1985) but did not show any biological activity (Bowers et al., 1977b).

Recently, Francis and coauthors (2005) undertook the study of the volatiles emitted by 23 aphid species and found that 16 of them were emitting Eßf as their only or major volatile chemical. Eßf was only a minor component of the volatile molecule pattern of five other species. Moreover, two species, *Euceraphis punctipennis* Zetterstedt and *Drepanosiphum platanoides* Schrank, did not release any Eßf, even though other terpenes were isolated. Table 2 summarizes the different components of the aphid alarm pheromone for the previously studied species. During the present PhD work, the volatiles of six additional aphid species of agricultural interest were studied, and were all shown to use Eßf as their only volatile chemical (Verheggen, unpublished data). In *Myzus persicae*, the quantity and mode of action of the alarm pheromone was found to vary with morph and age of aphids (Gut and Van Oosten, 1985). The quantities of Eßf in aphids also increase in relation to increasing body weight (Byers, 2005), but its concentration declines exponentially with increasing body weight.

(*E*)-ß-farnesene is also an ubiquitous plant component. It is found as a constituent of various essential oils from several plants family such as Asteraceae (Reichling and Becker, 1978). Eßf could also be continuously released in the plant volatiles (Agelopoulos et al., 2000; Weissbecker et al., 2000; Bruce et al., 2005) or being punctually emitted by a plant under infestation of herbivores (Turlings et al., 1991, 1998; Rose et al., 1996; Rose and Tumlinson 2004, 2005; Turlings and Ton, 2006) or mechanically damaged (Agelopoulos et al., 1999). Plants would therefore try to get aphids habituated to their own alarm pheromone by emitting important amount of Eßf in order to reduce herbivory by increasing predator and parasitoid efficiency (Petrescu et al., 2001). This hypothesis was however infirmed, as aphids do not seem to get habituated to huge amount of Eßf released by plants. More interestingly, Eßf is contained in trichomes of wild potato that, under aphid infestation, is released causing the aphid dispersion (Gibson and Pickett, 1983). Unfortunately, the alarm activity of Eßf seems to be inhibited by the presence of other sesquiterpenes like (-)-ß-caryophyllene, making aphids able to distinguish between the pure Eßf emitted by conspecifics from the Eßf of plant origin (Dawson et al., 1984). Other terpenes like α-pinene or isothiocyanates seem on the other hand to enhance its dispersal activity (Pickett and Griffiths, 1980; Dawson et al., 1987b).

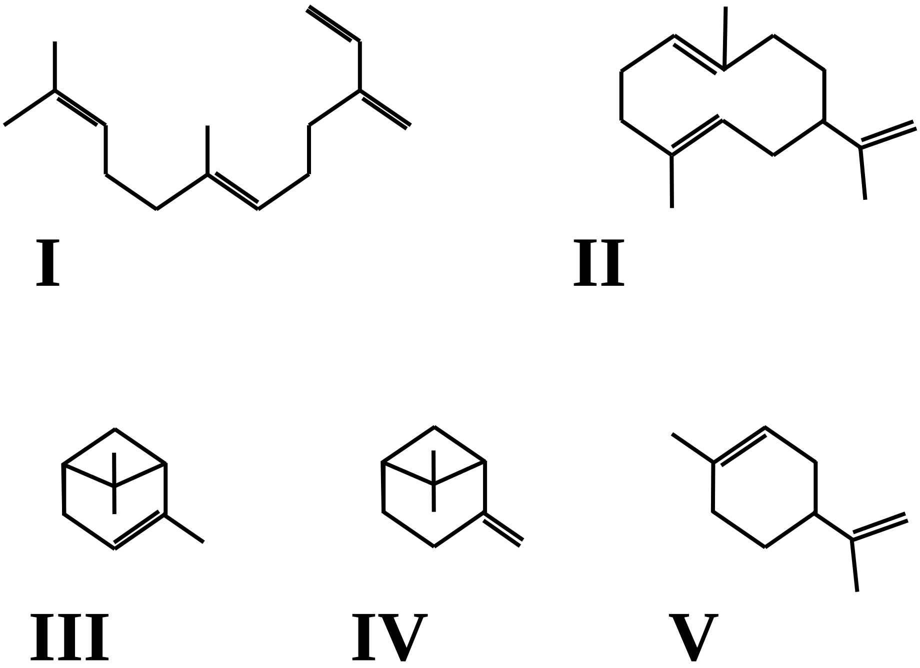

Figure 2. Chemical structures of aphid alarm pheromone components.
I, (*E*)-ß-farnesene. II, Germacrene A. III, α-pinene. IV, ß-pinene. V, limonene

The behavioural effect of alarm pheromone in aphids has been widely studied and may vary with the released amount and the aphid species, from removing the styles from the host plant and stop feeding, jump, fall or walk away from the alarm pheromone source (Edwards et al., 1973 ; Wientjen et al., 1973 ; Phelan et al., 1976 ; Montgomery and Nault, 1977a, 1977b and 1978 ; Roitberg and Myers, 1978 ; Wohlers, 1980 and 1981 ; Clegg and Barlow, 1982 ; Chau and Mackauer, 1997 ; Losey and Denno, 1998 ; Shah et al., 1999 ; Braendle and Weisser, 2001). In the sugar-cane woolly aphid, *Ceratovacuna lanigera* Zehntner (Homoptera, Pemphigidae), the alarm pheromone caused an attacking behaviour from conspecifics (Arakaki, 1989). Eßf has also a repellent effect on the landing behaviour of alate aphids, making them choose another host plant (Lambers and Schepers, 1978; Phelan and Miller, 1982; Wohlers, 1982). Field experiments did confirm the effective dispersal behaviour of aphids subjected to their alarm pheromone in 41 species (Xiangyu et al., 2002). When the Eßf concentration in the aphid environment decreases, aphids re-infest their host plant (Calabrese and Sorensen, 1978). Finally, Kunert et al. (2005) found that Eßf exposure increased winged individual production.

Table 1. (*E*)-ß-farnesene literature time table. This table presents the important discoveries made on aphid alarm pheromone from its first identification to 2006.

1967	(E)-ß-farnesene (Eßf) was synthesized for the first time (Brieger, 1967 ; Cazes et al., 1979)
1972	*Myzus persicae* individuals are repelled by the odour of droplets released from the cornicles and squashed aphids of the same species (Kislow and Edwards, 1972)
	The droplets secreted by aphid cornicles contain Eßf, which serve as alarm pheromone that inform nearby aphids of impending danger. When exposed to Eßf, aphids fall, jump, remove their stylets or walk away to escape (Bowers et al., 1972; Edwards et al., 1973; Wientjen et al., 1973; Montgomery and Nault, 1977a; Roitberg and Myers, 1978; Wohlers, 1981a; Chau and Mackauer, 1997; Losey and Denno, 1998; Shah et al., 1999; Braendle and Weisser, 2001).
1973	Circumstances of Eßf secretion and reception were studied (Nault et al., 1973)
1976	The behaviour of aphids disturbed from their host plant by alarm pheromone has been characterized on several aphid species (Phelan et al., 1976 ; Clegg and Barlow, 1982), as well as the recolonization behaviour (Calabrese and Sorensen, 1978)
	The alarm pheromone of aphids is used to alert tending ants which dispose of attacking predators (Nault et al., 1976)
1977	Germacrene A has been identified as a new aphid alarm pheromone in another aphid family : the Drepanisiphinae (Bowers et al., 1977)
	The structural components essential for activity of the aphid alarm pheromone, Eßf, were determined through synthesis of related analogs (Bowers and Nishino, 1977)
	The responses of various aphid species to their alarm pheromone were compared (Montgomery and Nault, 1977b).
1978	Eßf is found in *Matricaria chamomilla* (Reichling and Becker, 1978 ; Povh et al., 2001) and in hops (Banthorpe et al., 1989)
	Age and wing polymorphism affect the alarm pheromone sensitivity (Montgomery and Nault, 1978)
	Eßf is repellent for landing alate aphids (Lambers and Schepers, 1978 ; Phelan and Miller, 1982)
	The use of Eßf alone in the field is not sufficient to control aphid population (Calabrese and Sorensen, 1978)
1980	Other molecules are identified in the volatiles the vetch aphid (*M. viciae*) such as α-pinene, that can synergize the Eßf activity (Pickett and Griffiths, 1980).
1981	Aphids avoid plants contaminated with Eßf (Wohlers, 1981b)
1982	The flying behaviour of aphids toward Eßf is studied (Wohlers, 1982)
1983	Wild potato repels aphids by releasing aphid alarm pheromone contained in trichomes (Gibson and Pickett, 1983)
	Electrophysiological responses were obtained from of aphids exposed to alarm and sexual pheromones (Wohlers and Tjallingii, 1983 ; Vangiessen et al., 1994 ; Park and Hardie, 1998 ; Zhu et al., 1999 ; Park and Hardie, 2002 ; Pope et al., 2004)
	Insecticide susceptible aphid strains produce more alarm pheromone and respond more quickly than insecticide-resistant strains (Dawson et al., 1983).
1984	The effect of the aphid alarm pheromone is inhibited in presence of ß-caryophyllene. Aphids are able to distinguish the pure Eßf produced by conspecifics and Eßf of plant origin (Dawson et al., 1984).
1985	The quantity and composition of the alarm pheromone varied with morph and age (Gut and van Oosten, 1985)
1987	The turnip aphid, *Lipaphis erysimi*, responds weakly to Eßf, its alarm pheromone, but the response is increased by incorporating plant-derived isothiocyanates (Dawson et al., 1987)
1989	The alarm pheromone in the sugar-cane woolly aphid was found to elicit an attack and a dispersal behaviour under attack by predator (Arakaki, 1989)
1990	The effect of different Eßf isomers application on the morphs and forms of aphids is studied

	(Vanoosten, 1990)
1991	Eßf emission is induced from corn seedlings on which beet armyworm larvae were feeding (Turlings et al., 1991)
	Eßf affect behaviours of predators such as ladybirds (Nakamuta, 1991; Zhu et al., 1999; Mondor and Roitberg, 2000; Hemptinne et al., 2000; Acar et al., 2001; Francis et al., 2004), syrphids (Francis et al., 2005b), beetles (Kielty et al., 1996) or lacewing (Boo, 1998; Zhu et al., 1999).
1992	Eßf increases the effect of an insecticide because of the increased movement that it induces (Elagamy and Haynes, 1992 ; Roditakis et al., 2000)
	Eßf acts as a feeding stimulant for the sand fly (Tesh et al., 1992)
1996	Cotton leaves emit volatiles including Eßf under caterpillar infestation (Rose et al., 1996) that attract parasitoid behaviour (Rose et al., 1998)
1997	A sesquiterpene synthase cDNA clone from peppermint that produces Eßf was isolated and expressed in bacteria (Crock et al., 1997)
1998	Maize plants emit Eßf when mechanically damaged or under infestation of caterpillars but not under aphid infestation (Turlings et al., 1998; Bernasconi et al., 1998; Turlings and Ton, 2006). Potato also emit Eßf when mechanically damaged (Agelopoulos et al., 1999). Cotton plants emit Eßf under herbivore infestation (Rose and Tumlinson, 2004, 2005). Aphids are repelled by the mechanically damaged plant (Bernasconi et al., 1998).
	Parasitoids show an oriented behaviour toward aphid infested plants (Du et al., 1998; Guerrieri et al., 1999; Lo Pinto et al., 2004; Mumm and Hilker, 2005).
1999	Pea aphids *Acyrthosiphon pisum* infected by a fungal pathogen are less sensitive to alarm pheromone (Roy et al., 1999).
2000	Some plants naturally emit or contain Eßf, like potato (Agelopoulos et al., 2000 ; Weissbecker et al., 2000) or *Hemizygia petiolata* (Bruce et al., 2005)
	A quantitative ontogeny study of the Eßf production is conducted on *A. pisum* (Mondor et al., 2000)
	The seven-spotted ladybird possesses two kind of olfactory cells that codes for Eßf and ß-caryophyllene (Eßf inhibitor). Single cell recording was applied (Al Abassi et al., 2000)
2001	Plants do not emit Eßf to disrupt aphid alarm communication (Petrescu et al., 2001)
2002	A terpene synthase gene that encodes for the production of sesquiterpenes including Eßf was isolated from maize (Schnee et al., 2002).
	Aphid behaviour in response to their alarm pheromone is studied in the field (Xiangyu et al., 2002)
	Aphids previously washed with water, are not recognize as host by the parasitoid *Aphidius ervi* (Weinbrenner and Volkl, 2002)
2005	Eßf is found to occur in most Aphidinae species. Some aphid species produce other terpenes (Francis et al., 2005a)
	Eßf concentration in aphid body decreases with body weight (Byers 2005)
	Alarm pheromone induces production of higher proportion of wing morph (Podjasek et al., 2005). Eßf is not the only compound emitted by an aphid colony under attack.
	Aphids react more to the frequency of the Eßf release than to its concentration (Kunert et al., 2005)
2006	The parasitoid *Diaeretiella rapae* is attracted by Arabidopsis infested by *Myzus persicae* but not by Aphids alone or by mechanically damaged plant (Girling et al., 2006). When aphids are removed, the plant is still attractive for the parasitoid.
	Expression of an Eßf synthase gene in transgenic *Arabidopsis thaliana* to produce aphid alarm pheromone that affect aphids natural enemies’ behaviour (Schnee et al., 2006 ; Beale et al., 2006)

Table 2. List of the organic volatile chemicals found in the headspace of various aphid species

Aphid species	Main volatile compound	Other volatile compounds	References
Acyrthosiphon pisum Harris	(E)-β-Farnesene	(Z,E)-α-Farnesene ; (E,E)-α-Farnesene	(1) ; (6) ; (7)
Aphis fabae Scopoli	(E)-β-Farnesene	(Z,E)-α-Farnesene ; (E,E)-α-Farnesene	(6) ; (7)
Aphis idaei Van der Goot	β-pinene	α-Terpinene ; β-Phellandrene ; Myrcene ; α-Thujene ; p-Cymene ; α-Pinene ; (E)-β-Farnesene	(7)
Aphis glycines (Matsumura)	(E)-β-Farnesene		Present work
Aphis gossypii Glover	(E)-β-Farnesene		(1)
Aphis sambuci Linnaeus	(E)-β-Farnesene		(7)
Aphis spiraecola (Pagenstecher)	(E)-β-Farnesene		Present work
Aphis urticata Gmelin	(E)-β-Farnesene		(7)
Aulacorthum solani Kaltenbach	(E)-β-Farnesene	ST204(*) ; β-Pinene	(7)
Brachycaudus cardui Linnaeus	(E)-β-Farnesene		(7)
Brachycaudus persicae (Passerini)	(E)-β-Farnesene		Present work
Brachycaudus schwartzi Börner	(E)-β-Farnesene		(7)
Brevicoryne brassicae Linnaeus	Benzyl-isothiocyanate	α-Gurgunene ; Limonene ; (E)-β-Farnesene ; β-Pinene ; ST204(*)	(7)
Capitophorus elaeagni del Guercio	(E)-β-Farnesene	ST204(*) ; β-Bergamotene	(7)
Chaitophorus populeti Panzer	α-Pinene	β-Pinene ; Isobornyl acetate ; Camphene ; (E)-β-Farnesene ; Limonene	(7)
Dysaphis plantaginea Passerini	ST204(*)	(E)-β-Farnesene	(7)
Drepanosiphum platanoides Schrank	β-pinene	Limonene ; α-Pinene ; β-Phellandrene ; α-Thujene ; Camphene ; Isobornyl acetate ; Terpinolene	(7)
Euceraphis punctipennis Zetterstedt	Germacrene D	β-Bourbonene ; α-Copaene ; ST204(*)	(7)
Hyalopterus pruni Geoffroy	(E)-β-Farnesene		(7)
Hyperomyzus lactucae Linnaeus	(E)-β-Farnesene		(7)
Macrosiphoniella abrotani Walker	(E)-β-Farnesene		(7)
Macrosiphum rosae Linnaeus	(E)-β-Farnesene		(1)
Megoura viciae Buckton	β-pinene	α-Pinene ; (E)-β-farnesene ; Limonene ; (Z,E)-α-Farnesene ; (E,E)-α-Farnesene	(6) ; (7)
Metopolophium dirhodum Walker	(E)-β-Farnesene		(3) ; (7)
Myzus cerasi Fabricius	(E)-β-Farnesene		(7)
Myzus lythri Schrank	(E)-β-Farnesene	ST204(*)	(7)
Myzus persicae Sulzer	(E)-β-Farnesene	(Z,E)-α-Farnesene ; (E,E)-α-Farnesene	(2) ; (3) ; (6) ; (7)
Pherodon humuli Schrank	(E)-β-Farnesene	(Z,E)-α-Farnesene ; (E,E)-α-Farnesene	(6)
Rhopalosiphum padi Linnaeus	(E)-β-Farnesene		(3) ; Present work
Rhopalosiphum maidis Fitch	(E)-β-Farnesene		Present work
Schizaphis graminum (Rondoni)	(E)-β-Farnesene		(1) ; Present work
Sitobion avenae Fabricius	(E)-β-Farnesene	(Z,E)-α-Farnesene ; (E,E)-α-Farnesene	(3) ; (6) ; (7)
Therioaphis maculata Buckton	Germacrene A		(4) ; (5)
Therioaphis riehmi Börner	Germacrene A		(4)

(*) ST204 = Unidentified sesquiterpene

(1) Bowers et al., 1972 ; (2) Edwards et al., 1973 ; (3) Wientjens et al., 1973 ; (4) Bowers et al., 1977 ; (5) Nishino et al., 1977 ; (6) Pickett and Griffiths, 1980 ; (7) Francis et al., 2005

5. The aphid sex pheromone

Although aphids reproduce asexually on their host plants during the summer, many species migrate to a winter host, usually a woody species, where sexual reproduction occurs (Dawson et al., 1987a). As autumn approaches, the asexually reproducing aphids on the summer host respond to the reduced daylight hours by producing winged sexual female precursors, named *gynoparae*, which migrate to the winter host, facilitated by volatile semiochemicals released by these plants (i.e. mainly terpenes). Wingless female aphids, named *oviparae*, are then produced on the winter host and release sex pheromones from their hind legs. The pheromone is detected by male aphids using the secondary rhinaria, which are organs on the third segment of the six-segmented antenna.

At the very beginning, the role of the sex pheromone was considered to be no more than a close range aphrodisiac by various entomologists. In-depth behavioural studies conducted in the following years both in the laboratory and in the field have later demonstrated the relatively long range attraction by synthetic sex pheromone. In the early 70s, Pettersson (1970) first suggested the existence of an aphid sex pheromone in the *Schizaphis* genus, released by females and attracting males. This pheromone was first chemically characterized and shown to be released by glandular cells underlying porous plaques on the hind tibiae of females' individuals of the vetch aphid *Megoura viciae* (Dawson et al., 1987a). Later, the sex pheromones of several aphid species were also identified, including the pea aphid *Acyrthosiphon pisum*, the greenbug *Schizaphis graminum*, the green peach aphid *Myzus persicae*, the soybean aphid *Aphis glycines*, the potato aphid *Macrosiphum euphorbiae*, the peach aphid *Tuberocephalus momonis* or the black bean aphid *Aphis fabae* (Marsh, 1972; Eisenbach and Miller, 1980; 1990; Boo et al., 2000; Goldansaz, 2004; Zhu et al., 2006).

The characterization of the sex pheromone was facilitated by the development of electrophysiological recording techniques so that individual olfactory nerve cells within the secondary rhinaria of the male insects could be located and nerves impulses recorded by the insertion of tungsten electrodes. The biological active components of the pheromone, obtained from hind legs of the females, were located on capillary GC column by coupling the effluent to the electrophysiological preparation and, simultaneously, to a flame ionization detector (Dawson et al., 1987a). This identification of the pheromone was confirmed by GC-MS (and later by ^{1}H and ^{13}C nuclear magnetic resonance, NMR) as a mixture of the monoterpenoids (+)-(4aS,7S,7aR)-nepetalactone and (-)-(1R,4aS,7S,7aR)-nepetalactol (Figure 2). The ratio of these two compounds in the leg extract was also found to be different from the ration in the headspace of the emitting females, where (+)-(4aS,7S,7aR)-nepetalactone was found in higher proportion (Dawson et al., 1990). The ratio of the two components varies with the aphid species (Table 3), but also with the age, the optimal released amount in *Myzus persicae* being reached on the sixth day of adulthood (Marsh, 1972; Hardie et al., 1990).

Table 3. Ratios of nepetalactol to nepetalactone found in entrainments collected from oviparae of different aphid species

Species name	Ratio (ol:one)	Reference
Brevicoryne brassicae	0:1	(Gabry et al., 1997)
Sitobion fragariae	0:1	(Hardie et al., 1992)
Sitobion avenae	(trace) 0:1	(Lilley et al., 1995)
Aphis spiraecola	1:2	(Jeon et al., 2003)
Tuberocephalus momonis	1:4	(Boo et al., 2000)
Megoura viciae	1:5 - 1:12 (age effect)	(Hardie et al., 1990)
	1:6	(Dawson et al., 1990)
Aphis spiraecola	1:6 - 1:8 (age effect)	(Jeon et al., 2003)
Aphis fabae	1:29	(Dawson et al., 1990)
Acyrthosiphon pisum	1:1	(Dawson et al., 1990)
Myzus persicae	3:2	(Dawson et al., 1990)
Dysaphis plantaginae	3.3:1 - 3.7:1 (age effect)	(Stewart-Jones et al., 2007)
Macrosiphum euphorbiae	4.1 - 2.1 (age effect)	(Goldansaz et al., 2004)
Schizaphis graminum	8:1	(Dawson et al., 1988)
Cryptomyzus spp.	30:1	(Guldemond et al., 1993)
Rhopalosiphum padi	1:0	(Hardie et al., 1994)
Phorodon humuli	1:0	(Campbell et al., 1990)

Although neither compound was attractive to males when presented individually in an olfactometer, a mixture of the two components produced a behavioural response equal to that elicited by the female leg extract (Bowers et al., 1987a; Goldansaz, 2004). However, there seem to have lack of pheromone specificity when looking at the attraction of male aphids of various species to ratios that are different from the one their females produce. If nepetalactol and nepetaloctone are ubiquitous aphid sex pheromone components, as it has been suggested (Dawson et al., 1990), ratio combinations are limited and additional mechanisms of species isolation are likely to exist. Numerous factors have been proposed, e.g. the use of different blends of diastereoisomers or enantiomers of nepetalactol and nepetaloctone (Hardie et al., 1997). Lactol and lactone were also identified in the emitted volatiles of calling female aphids of several species, the latter showing some inhibitory activity (Eisenbach and Miller, 1980; Dawson et al., 1990). The role of these compounds is however not yet understood (Dawson et al., 1990).

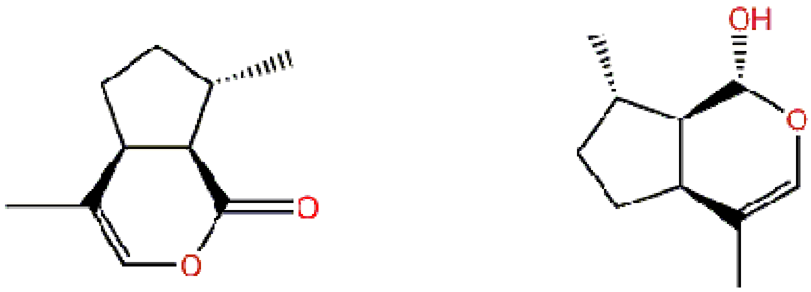

***Figure 3*. Structure of (+)-(4aS,7S,7aR)-nepetalactone and (-)-(1R,4aS,7S,7aR)-nepetalactol, the two compound of the aphid sex pheromone**

The promise of the aphid sex pheromones for alternative methods of pest control not exclusively reliant upon conventional aphicides, but also incorporating the ability to attract beneficial parasitic wasps and predators by means of sex pheromones (Zhang et al., 2004 ; Zhu et al., 2005), has created a need for additional work on this topic, which is discussed in section 7 of this chapter.

6. Perception of plant volatiles

During the late fifties, it was believed that plant chemical cues were detected by winged aphids only after landings on plants. However, because of the accumulation of experimental evidences, the idea that olfactory cues released from the host plant play no role in host plant selection is now completely forgotten. Alikhan (1960) in the early sixties was one of the first to show the role of plant volatiles in the selection of the host plant by the black bean aphid, *Aphis fabae* Scop. Pettersson (1970) quickly showed in a laboratory study that the gynoparae and males of the bird cherry-oat aphid, *Rhopalosiphum padi*, were attracted toward the winter host, *Prunus padus*, but not to the nonhosts *Prunus cerasus* and *Rubus idaei*. Visser and Taanman (1987) later demonstrated that apterous *Cryptomyzus korschelti* individuals also use plant odours to walk toward their summer host, *Stachys sylvatica*. In 1991, Nottingham et al. used a linear track olfactometer to show significant responses of apterous and alate *A. fabae* to summer host *Vicia faba*. Since then, many aphid species have been studied for their perception of host plant volatiles, and identification of several chemicals involved in host plant location by aphids have been made. The use of chemicals by return migrants searching for their hosts in autumn has been reviewed (Powell and Hardie, 2001), providing evidence of semiochemical use by several aphid species, including *A. fabae, P. humuli, R. padi* and *S. fragariae* among many others.

A substantial part of the existing knowledge on the semiochemicals of the secondary host in host alternating aphid species was extensively reviewed by Pickett et al. (1992). While the ability of aphids to perceive chemical cues and the importance of these cues in plant finding is undoubted, eventual host acceptance is expected to depend upon the interaction of chemical, visual and nutritional cues available to the aphids. Therefore, it makes no sense to believe that semiochemicals are the only factors regulating host alternation in aphids. However, Pettersson et al. (1994) demonstrated that the primary host becomes a non-host in the spring because of the associated repellency was perceived by specific olfactory cells, again in the primary rhinaria, that respond to non-host semiochemicals.

7. Interactions with natural enemies

The use of semiochemicals might sounds like a great innovation and a powerfull way to communicate, aggregate, encounter mates and find food resources. However, the use of highly volatile chemicals in intraspecific communication has led the releasing insects to become more vulnerable. An individual may be colored to mimic its host plant color or architecture, when it starts releasing volatiles in its environment, it reveals its presence to any natural enemy that has evolved the ability to detect those signals. Because aphids are largely present in the environment, it is no surprise that numerous examples of natural enemies that have developed the faculty to perceive aphid semiochemicals exist.

Predators suffer of their bad reputation. They have generally been considered as less sophisticated users of host kairomones than parasitic wasps (Pickett and Glinwood, 2007). However, the location of aphid colonies by adult hoverflies, coccinellids and lacewings is also very important for the survival of their offspring. Aphid predators have therefore developed the faculty to locate their prey by orienting their fly or crawling movements using mainly the aphid alarm pheromone. Previous studies have demonstrated this phenomenon in ladybirds (Nakamuta, 1991; Zhu et al., 1999; Mondor and Roitberg, 2000a; Hemptinne et al., 2000; Acar et al., 2001; Francis et al., 2004), syrphids (Francis et al., 2005b), ground beetles (Kielty et al., 1996) and lacewings (Boo, 1998; Zhu et al., 1999). Both adults and larvae were shown to use aphid volatile cues to forage for prey. The most commonly used methods for demonstrating the attractiveness of predators toward Eßf were the linear, the Y or the four-arm olfactometers. These methods were somethings conducted after conventionnal electrophysiological studies that provided electroantennograms from predator's antenna in response to at least one of the semiochemical released by the aphid species under investigation.

Electrophysiological studies have reported olfactory responses of the twelve-spotted lady beetle *Coleomegilla maculata* and the green lacewing *Chrysoperla carnea,* to the nepetalactone and nepetalactol sex pheromone components (Zhu et al., 1999). However, the behavioural responses of ladybeetles to aphid sex pheromone have either not been studied, of have not been reported due to negative outcomes. On the other hand, lacewings (*Chrysopa cognata*) showed positive behavioural responses in an olfactometer to aphid sex pheromone (Boo et al., 1998). Behavioral responses from hoverflies to aphid semiochemicals were notably absent from the litterature until 2007 (Pickett and Glinwood, 2007).

Parasitoids were the first aphid natural enemies to be studied for their perception of aphid semiochemicals. Since honeydew is relatively apparent and likely to "smell", it is no surprise that initial studies on the use of kairomones as host location cues by aphid parasitoids focused on aphid honeydew (Gardner and Dixon, 1985). Hardie et al. (1991) first demonstrated that parasitoids were attracted to aphid sex pheromones through field trials where male flying aphids were attracted by synthetic sex pheromone. The parasitoid *Aphidius uzbekistanicus* was attracted to the aphid alarm pheromone Eßf in a simple Y-tube olfactometer bioassay (Micha and Wyss, 1996).

The future may therefore see greater use of aphid semiochemicals within integrated pest management strategies using aphid natural enemies.

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Chapter III

OBJECTIVES

Beside their negative impact on agricultural crops, aphids represent an awesome model for the study of alarm signaling, mainly because their alarm signal is usually released and perceived by clonemates, but also because the aphid alarm pheromone often consists in a single volatile which is, in most Aphidinae species, the sesquiterpene (*E*)-ß-farnesene (Eßf). This chemical is released in response to predation and other stresses and typically causes nearby aphids to cease feeding, drop from their host plant, and disperse. However, Eßf also serves as a foraging cue for aphid natural enemies, suggesting that the adaptive benefits of signaling via Eßf must be weighed against the ecological costs of increasing apparency to predators and parasitoids.

The overall objective of the present thesis is therefore to have a better understanding on how aphids manage their production and emission of alarm pheromone, as well as to improve our knowledge on the roles that Eßf could play in the relationships that aphids have with their predators and tending ants, in order to better pinpoint the problem in this very tough context.

Preliminary field observations led us to the hypothesis that aphids could release volatile cues even in absence of predators. In the first chapter of this thesis, we therefore wanted to identify and quantify the volatile organic chemicals released from an aphid colony in absence of predators. Secondly, we wanted to test the possibility that aphids perceiving the alarm signal (Eßf), release additional Eßf. Indeed, the amount of alarm pheromone released by a single individual under attack by a predator is likely to be very small and not enough to alert all the surrounding conspecifics. Such a contagious phenomenon could be adaptive if there are benefits to disseminating the alarm farther than would be achieved by the release of Eßf by a single individual. Thirdly, we wanted to explore the possibility for aphids to regulate their alarm pheromone production. Because alarm signaling confers apparent fitness benefits on recipients while the production and release of Eßf likely entails costs for the emitting individuals, it could be adaptive for aphids to regulate their Eßf production in response to variation in the social environment (i.e. presence or absence of conspecifics).

In the second chapter of this thesis, we have hypothesized that aphidophagous insects would have a clear advantage to have evolved in such a way that they would be able to locate their prey and to choose their oviposition sites using aphid volatile cues. The behaviour of an aphidophagous hoverfly, *Episyrphus balteatus* De Geer (Diptera: Syrphidae), was first observed when in presence of aphid infested tomato plants. We wanted to quantify and identify all the volatile chemicals released from this natural system and identify those that were perceived and used by the hoverfly females to locate their oviposition site. We then explored the performance of hoverfly females to reduce aphid infestations on several aphid host plants. We finally tested several volatile organic chemicals, including Eßf, as potential attractant and oviposition stimulant for the hoverfly *E. balteatus*. The attractiveness of the aphid alarm pheromone was also investigated on the Asian ladybeetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), a new invasive coccinellid species.

In the third chapter of this thesis, we studied the mutualistic relationships that occur between some aphid and ant species. Both insect species have clear advantage to meet each other, as aphids represent an easy access to sugars and amino-acids, essential for the fitness of an ant colony, that provide in exchange protection against natural enemies. We tested Eßf as a potential attractant for ants and have tried to understand what could make the aphid-ant mutualistic relationship occur and persist.

The last part of this PhD thesis discussed globally the results obtained and formulate perspectives for additional trials that could complete the understanding of the roles that Eßf could have for aphids.

Chapter IV

(E)-ß-FARNESENE WITHIN AN APHID COLONY

GENERAL INTRODUCTION TO CHAPTER IV

In the previous chapter, the importance of alarm pheromone for aphids was demonstrated. On a first hand it causes aphids to cease feeding and disperse, and on the other hand it may act as a kairomone, attracting various natural enemies that use this sesquiterpene to locate their prey. (E)-ß-farnesene is therefore likely to be a good candidate to be integrated in pest management strategies. Using (E)-ß-farnesene dispensers in a crop field could indeed disperse the pests while keeping a high pressure of predation. However, every pheromone-based pest managments are preceeded by a deep understanding of the ecology and chemical ecology of the pest.

Before including (E)-ß-farnesene in an integrated aphid management program, we need a good knowledge of the modes of production, emission and action of the latter compound in aphids. What are the exact behavioral effects of (E)-ß-farnesene on aphids? Would they simply run away or would they increase the alarm signal by releasing additonnal alarm pheromone? Are they releasing this pheromone under predation only? Does the social environment of aphids have any effect on (E)-ß-farnesene production?

The present chapter aims therefore to understand the circumstances of the production and emission of alarm pheromone in aphids. Two aphid species were mainly used in the following experiments: *Myzus persicae* and *Acyrthosiphon pisum*. Both aphid species belong to the Aphidinae sub-family and are important pest species. In addition, both species use (E)-ß-farnesene as only volatile component of their alarm pheromone.

IV.1

Emission of alarm pheromone by non-preyed aphid colonies

Raki Almohamad[1], François J. Verheggen[1], Frédéric Francis[1], Georges Lognay[2],
Eric Haubruge[1]

[1]*Department of functional and evolutionary Entomology,* [2]*Department of Analytical Chemistry,*
Gembloux Agricultural University, Passage des Déportés 2, B-5030 Gembloux (Belgium)

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Abstract – The sesquiterpene (*E*)-ß-farnesene (Eßf) is the primary component of the alarm pheromone of most aphid species. It is released in response to physical stress including attack by natural enemies and causes aphids to cease feeding and disperse. Eßf also acts as a kairomonal cue for aphid natural enemies. In the present study, we collected the headspace volatiles released by aphid colonies of different sizes. GC-MS analysis demonstrated the presence of Eßf in absence of predator attack. A quadratic relationship was found between the released (*E*)-ß-farnesene amounts and aphid colony size. Behavioural impact of aphid alarm pheromone toward *E. balteatus* female oviposition behaviour was also demonstrated in this work. These results highlight the primary role of the small but continuous release of aphid alarm pheromone in mechanisms of decision-making by aphid predators during their foraging and egg-laying behaviour.

Key words: Semiochemicals, *Episyrphus balteatus*, *Myzus persicae*, aphid alarm pheromone, (*E*)-ß-farnesene

Introduction

(*E*)-ß-Farnesene (Eßf), the main component of the aphid alarm pheromone was identified in 16 aphid species, alone or associated with other molecules (Francis et al. 2005). Previous behavioural studies have demonstrated the kairomonal role of Eßf in various aphid natural enemies, including ladybeetles, hoverflies and parasitic hymenoptera (Du et al. 1998; Al Abassi et al. 2000; Harmel et al. 2007; Verheggen et al. 2007, 2008).

In the hoverfly *E. balteatus,* there is a good evidence from laboratory and field studies for the existence of a positive density-dependent response to aphid colony size in term of oviposition (Dixon 1959; Chandler 1968; Itô and Iwao 1977; Bargen et al. 1998; Scholz and Poehling 2000; Sutherland et al. 2001; Almohamad et al. 2006). However, there is only little work on the role of odour cues in predatory hoverflies attraction (Laubertie et al. 2006; Almohamad et al. 2007; 2008; Verheggen et al. 2008).

The present study aims to understand the role of chemical cues released from non-preyed aphid colonies on decision–making processes that lead to oviposition in female *E. balteatus*. In order to evaluate the olfactory signal released by the tested colonies, we quantified the volatile organic compounds released in their headspaces. The oviposition behaviour of *E. balteatus* was subsequently investigated with respect to M. persicae colonies of different sizes.

Materials and Methods

Plants and insects - Broad bean plants (*Vicia faba* L.) were grown in 30 x 20 x 5 cm plastic trays filled with a mix of perlite and vermiculite (1/1) and maintained in controlled environment growth rooms (16:8 Light: Dark ; 20 ± 1°C). Two aphid species, namely *M. persicae* and *Megoura viciae* Buckton were taken from stock rearing on *V. faba*, in separate air-conditioned rooms under the same conditions as above. Adult *E. balteatus* were reared in 75 x 60 x 90 cm cages and were provided with bee-collected pollen, sugar and water. Broad beans infested with *M. viciae* were introduced into the cages for 3 h every two days to allow oviposition. Hoverfly larvae were mass-reared in aerated plastic boxes (110 x 140 x 40 mm) and were fed daily *ad libitum* with *M. viciae* as standard diet.

Leaf disc system – The leaf disc-system consisted of (1) a circular piece of *V. faba* leaf, (2) aphids and (3) 7 ml of an agar diet (agar 1% solution w:w), placed in a 25 mm diameter Petri dish, to reduce desiccation. Leaves were infested with different quantities of *M. persicae* and were kept for 24h in a controlled conditions incubator (16:8 Light: Dark; 20 ± 1°C) before testing.

Influence of aphid colony size on syrphid oviposition rate – In no-choice experiments, a single *E. balteatus* female was introduced in a net cage and allowed to lay eggs for 3h on the leaf disc supporting a *M. persicae* colony made of 25, 75 or 125 individuals. The leaf disc system was placed on a Plexiglass holder at a height of 20 cm. This setup was previously shown as an efficient method to evaluate the oviposition behaviour of the hoverfly *E. balteatus* in response to aphid-infested plants (Almohamad *et al*. 2006). The number of eggs laid (oviposition rate) on the leaf disc was counted. This experiment was replicated 10 times for each aphid colony size.

Collection and analysis of volatile chemical emissions

Volatile collection system – Volatile chemicals were collected using a push/pull volatile collection system consisting of a glass air-collection chamber (Schott®, 12 cm base-diameter, 35 cm high) placed inside an incubator set at 21 ± 1°C, and previously washed with hot water and *n*-hexane. The leaf disc system was placed on a Plexiglass holder similar to those used in the above-mentioned bio-assays. Incoming air was pushed through an in-line activated charcoal filter before entering the glass chamber at a flow of 200 ml/min. The volatile-enriched air was then pulled through an adsorption trap containing 40 mg SuperQ® (Alltech, Deerfield, IL, USA). Six replicates were conducted for each aphid colony size and four replicates for the control (i.e. an aphid-free leaf disc system). Volatiles were collected during 3h. Filters were eluted with 150 µl of *n*-hexane and nonyl acetate (400 ng) was added to each sample as internal standard. The extracted samples were directly stored in a freezer at − 80 °C until GC analysis. Identification and quantification were performed respectively by GC-MS and GC-FID.

Statistical analyses – Regression analysis was used to correlate aphid density with number of eggs laid by female *E. balteatus* and amounts of released Eßf. The regression analysis was performed using Minitab® software (14.2 version, Minitab Inc, State College, PA, USA).

Results and Discussion

EßF emission from non-preyed *M. persicae* was demonstrated in the present study by volatile collection and subsequent GC-MS analyses. Regression analysis revealed that there was a significant quadratic relationship between the amount of released EßF (Y) and *M. persicae* colony sizes (X) ($F_{2,23}$ = 14.89; P<0.001; r^2 = 0.9997). This relationship can be represented as $Y= 0.0143X^2 - 0.2265X$ (Fig. 1). Other chemical compounds were also identified, such as hexanal, 3-methyl-2-pentanone, ß-terpinene, 6-methyl-5-hepten-2-one and limonene. These latter compounds were found to be released from leaf-disc system and they were not specifically induced by the presence of *M. persicae* colony on leaf disc. Geranyl acetone was also identified and its released amount was slightly decreasing with aphid colony size.

EßF constant emission in absence of predators can have both positive and negative impacts. Aphids would have advantage to release small amounts of alarm pheromone in the case of crowded colonies to cause dispersion of the individuals and to help preserving the host plant. This effect would complete the already well-known effect of winged-morph induction described by Kunert et al (2005). However, a constant emission of alarm pheromone increases the risk of the releasing colony to be located by a natural enemy.

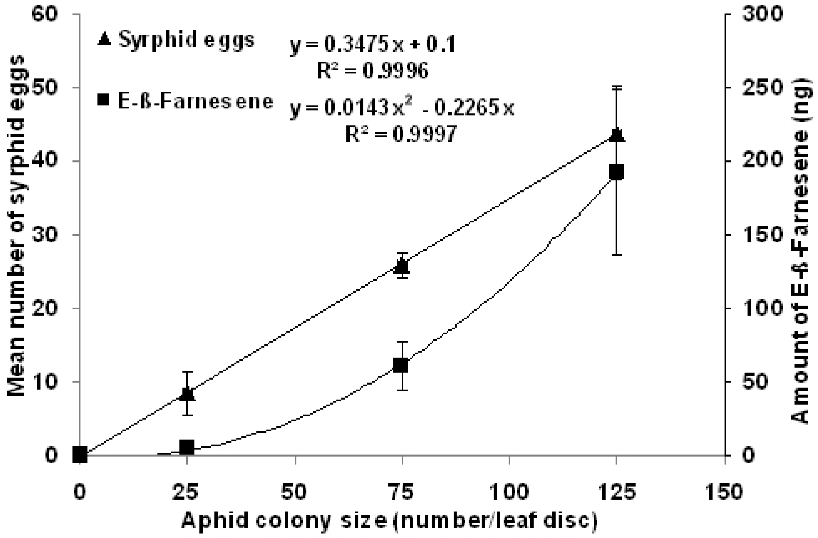

Figure 1. Amount of (*E*)-ß-farnesene and mean number of *E. balteatus* eggs laid in response to increasing M. persicae colony size on broad bean leaf disc. Bars indicate standard errors of the means.

According to previous observations, nearly no egg was laid in absence of aphids on the leaf disc (Fig. 1). The number of eggs laid (Y) increased significantly and linearly with the size of the aphid colony (X) ($F_{1,39} = 94.12$; $P < 0.001$; $r^2 = 0.9996$), according to $Y = 0.3475X + 0.1$. Several studies already suggested that the oviposition rate of syrphid females was a function of aphid densities (Chandler 1968; Itô and Iwao 1977; Bargen et al. 1998; Scholz and Poehling 2000; Sutherland et al. 2001; Almohamad et al. 2006). Our regression analysis suggests an adaptive oviposition behaviour leading the emerging larvae to locate immediately sufficient food resources. According to Bargen et al. (1998), the number of eggs laid by female hoverflies does not only depend on aphid quantity on the plant, but also on the presence and quantity of oviposition-eliciting substances emitted from the prey and the infested plant. Previous observations have indeed shown that predatory hoverflies oviposit in response to volatile compounds emitted from aphids and their liquid secretions such as honeydew (Dixon 1959; Bombosch and Volk 1966; Budenberg and Powel 1992; Shonouda 1998; Verheggen et al. 2008).

Along with the previous results of Verheggen et al. (2008) on the role of oviposition stimulant of synthetic Eßf, these results demonstrate the strong implication of aphid alarm pheromone in aphidophagous syrphid oviposition behaviour.

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IV.2

Emission of alarm pheromone in aphids: a non-contagious phenomenon

F.J. Verheggen[1], M.C. Mescher[2], E. Haubruge[1], C.M. De Moraes[2] and E.G. Schwartzberg[2]

[1]*Department of Functional and Evolutionary Entomology, Gembloux Agricultural University, Passage des Déportés 2, 5030 Gembloux, Belgium.*
[2]*Department of Entomology, The Pennsylvania State University, University Park, PA, 16801, USA*

Reference – Verheggen FJ, Mescher MC, Haubruge E, De Moraes CM, Schwartzberg E (2008). Emission of alarm pheromone in aphids: A non-contagious phenomenon. Journal of Chemical Ecology 34:1146-1148.

Abstract – In response to attack by natural enemies, most aphid species release an alarm pheromone that causes nearby conspecifics to cease feeding and disperse. The primary component of the alarm pheromone of most studied aphid species is (*E*)-ß-farnesene. We recently demonstrated that the production and accumulation of (*E*)-ß-farnesene during development by juvenile aphids is stimulated by exposure to odour cues, most likely (*E*)-ß-farnesene itself, emitted by other colony members. Here we examined whether the release of (*E*)-ß-farnesene can be triggered by exposure to the alarm pheromone of other individuals and thereby amplify the signal. Such contagious emission might be adaptive under some conditions because the amount of (*E*)-ß-farnesene released by a single aphid may not be sufficient to alert an appropriate number of individuals of the colony to the presence of a potential threat. Using a push-pull headspace collection system, we quantified the (*E*)-ß-farnesene released from aphids exposed to conspecific alarm signals. Typical avoidance behaviour was observed with exposure to (*E*)-ß-farnesene (i.e., they ceased feeding and dropped from host-plant); however, no additional alarm pheromone was detected, suggesting that contagious release of (*E*)-ß-farnesene does not occur.

Key Words – Aphid alarm pheromone production, *Acyrthosiphon pisum*, (*E*)-ß-farnesene, headspace collection system.

Introduction

As a result of parthenogenetic reproduction, aphids typically have a clonal colony structure and are surrounded by other genetically identical individuals. This social environment favors communal defense mechanisms, and in most aphid species, individuals respond to attack by natural enemies by releasing an alarm pheromone (Bowers et al., 1972) which induces perceiving individuals to stop feeding, disperse locally, and often drop from the host plant (Braendle and Weisser, 2001).

Like most insect species, aphids are highly dependent upon chemical signals (Pickett and Glinwood, 2007). Whereas alarm pheromones in other insects and mites usually consist of a mixture of chemicals (e.g. Verheggen et al., 2007a), the aphid alarm pheromone appears to contain a single chemical in most Aphidinae species (Bowers et al., 1972 ; Francis et al., 2005): the sesquiterpene (*E*)-ß-farnesene (Eßf). Eßf has been identified as a unique volatile compound in 13 aphid species, including the pea aphid, *Acyrthosiphon pisum* Harris (Francis et al., 2005). Eßf also acts as a kairomone used by predators and parasitoids to locate their aphid prey (Pickett and Glinwood, 2007; Verheggen et al., 2007b; Verheggen et al., 2008). These recent findings highlight the possibility of direct negative effects of alarm pheromone production in the form of increased apparency to natural enemies. Beale et al. (2006) effectively exploited these properties by adding an Eßf synthase gene to *Arabidopsis thaliana* plants, increasing their attraction of aphid parasitoids.

In a recent study, we found that juvenile aphids reared in social isolation on artificial diet release less Eßf than those reared in colony or those reared in isolation but exposed to colony odours (Verheggen et al., submitted). We suggested that aphid, plant or aphid-induced plant volatiles may stimulate the production of additional Eßf in downstream aphid signal recipients. In this study we examined whether exposure to Eßf stimulates the release of Eßf by receiving individuals by measuring the pheromonal response of individuals exposed to Eßf from conspecifics. Such a contagious phenomenon could be adaptive if there are benefits to disseminating the alarm farther than would be achieved by the release of Eßf by a single individual.

Materials and Methods

Insects and Plants. Pea aphids were reared on broad beans *Vicia faba* in an environmentally controlled greenhouse (L16:D8, RH 35 ± 5 %, 25 ± 2 °C) for several months prior to the experiment. Plants were grown in square 9 x 9cm plastic pots filled with a peat-based, general-purpose potting soil (Metro Mix 200 Series, SunGrow Agriculture Distribution Inc., Bellevue, WA, USA).

Push-pull Headspace Collection System. The push-pull headspace collection system consisted of two cylindrical chambers (12 cm diameter x 30 cm) made of glass and Teflon® (Figure 1). Chambers were sealed on both ends and connected to one another with Teflon® tubing. To maintain ambient humidity and normal atmospheric pressure within the chambers, activated-carbon-filtered air was pumped into the system at the same rate that air was removed via air entrainment filters, in a manner consistent with push-pull headspace collection setups described elsewhere (e.g., Tholl et al. 2006).

To generate natural Eßf emissions, we crushed 50 3rd instar aphids inside our volatile collection chambers using a glass pestle left inside the chamber after use. To quantify Eßf produced by the crushed (lead) and undisturbed (downstream) aphids, an adsorbent filter containing 40 mg of SuperQ® (Alltech, Deerfield, IL, USA) was connected to each chamber. Clean air was pushed into the system at a rate of 1.5 L/min and sampled air was pulled through the filters from both the lead and downstream chambers at a rate of 0.75 L/min per chamber. Five experiments were conducted for 1 hr each with 9 replicates (Table 1). The first experiment (crushed – empty) was a positive control designed to document the Eßf distribution in our system. The second experiment (empty – infested) measured the amount of Eßf released by a colony of 50 *A. pisum* under our laboratory conditions. The third (empty – non infested) and fifth (crushed – non infested) experiments are controls, respectively devoted to the evaluation of the potential amount of Eßf that could be released from an uninfested broad bean unexposed or exposed to Eßf. The fourth experiment (crushed – infested) was conducted to show whether “Downstream” aphids emit additional alarm signal at the time they are exposed to an alarm signal from conspecifics.

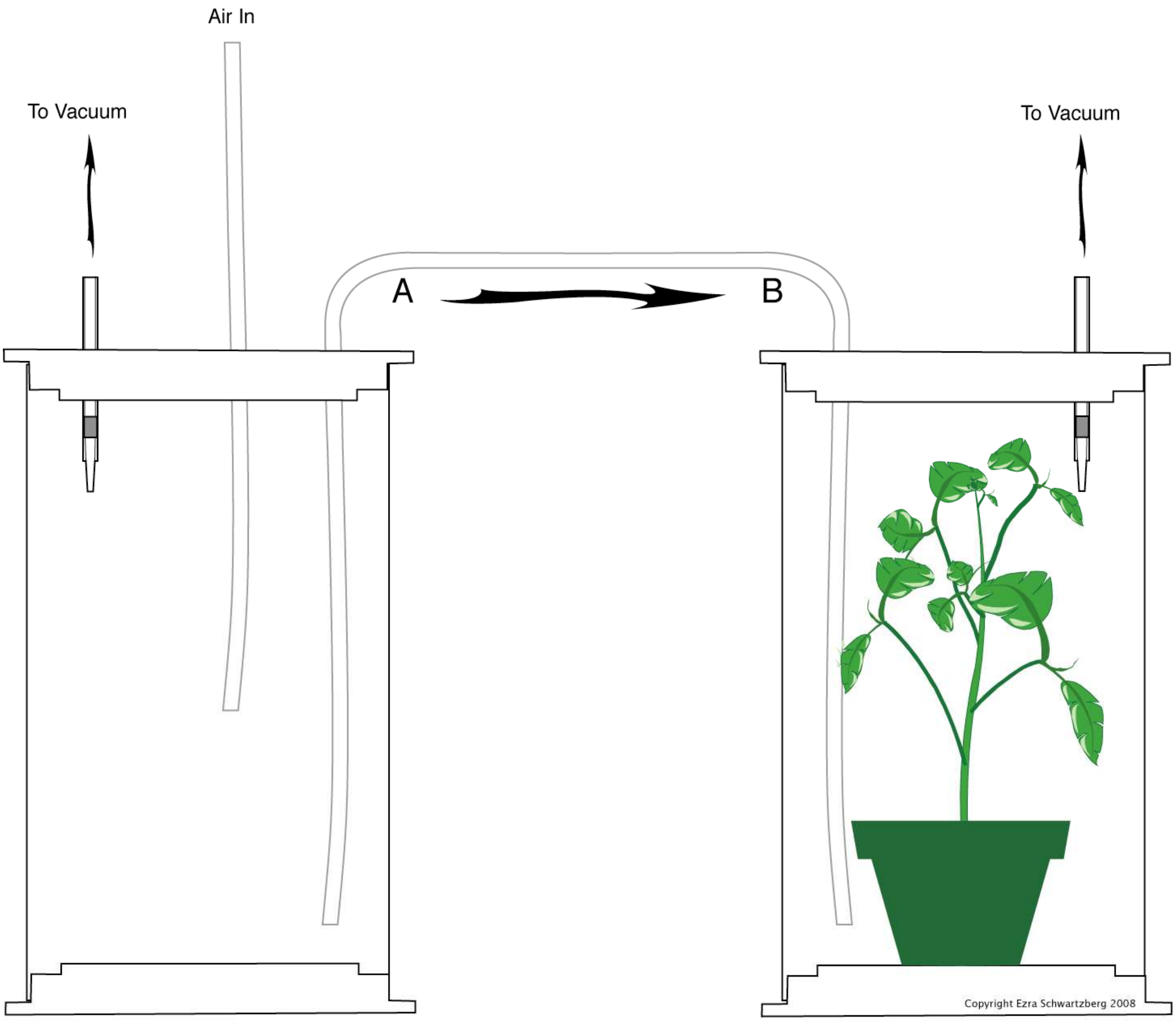

Figure 1. Push-pulled headspace collection set-up. Pumps are used to push and pull air through this system, maintaining normal atmospheric pressure in both chambers while allowing air to pass from the lead chamber (A) to the downstream chamber (B).

Volatile Analysis. Filters were eluted using 150 µl of dichloromethane. Nonyl acetate (320 ng) was added to each sample as an internal standard. Extracts were analyzed by GC-FID using a Hewlett-Packard 6890 series gas chromatograph. Aliquots of 1 µL were injected with a splitless injector held at 260°C. The column (Equity-1, Supelco, Bellefonte, PA, USA, 30 m x 0.25 mm i.d.) was maintained at 40°C for 1 min before being heated to 260°C at a constant rate of 15°C/min. This final temperature was maintained for 10 min. Quantifications of compounds were obtained by comparing individual peak areas to the internal standard. Identification of Eßf was made by comparison of its retention time with that of synthetic Eßf (Bedoukian Research, Inc., Danbury, CT, USA) and confirmed by GC-MS.

Results and Discussion

Eßf was the only detectable volatile released by *A. pisum* in our experiments, which is consistent with previous findings (Francis et al., 2005). In experiment one (crushed – empty), an average of 48.52 ng of Eßf per 3rd instar *A. pisum* larva was found. The higher Eßf levels observed in our study compared to those found by Mondor et al. (2000) and Schwartzberg et al. (2008) may be explained by differences in Eßf elicitation techniques (crushing versus probing or natural attack). These Eßf doses are larger than what we would expect to see in a natural condition; however we feel that these doses would be better to show the effects of a response by receiving aphids. Within a colony, signaling and receiving aphids are much closer to each other and if we had lower emission from signaling aphids in our experiments we may have underexposed aphids as compared to a natural setting.

The ratio of downstream aphid to lead aphid emission would be equal to 1.0 if no additional Eßf was produced from the downstream chamber. Any increases in the amount of Eßf collected from the downstream chamber therefore reflect emission of Eßf from aphid/host plant complexes subjected to the alarm signal. Amounts are listed in Table 1 as downstream and lead aphid emissions and downstream/lead aphid emission ratios.

Table 1. (E)-ß-farnesene emissions by unstressed aphids exposed to Eßf from crushed conspecifics. Experiments 1, 2, 3 and 5 are controls.

n°	Lead chamber	Downstream chamber	Average EßF amounts (± SE) [d]		Average Downstream/Lead EßF ratios (± SE) [d]
			Lead chamber	Downstream chamber	
1	Crushed aphids [a]	Empty	1295.74 ± 261.43	1130.25 ± 148.87	1.056 ± 0.190
2	Empty	Infested plant [b]	/	/	/
3	Empty	Non infested plant [c]	/	/	/
4	Crushed aphids	Infested plant	1585.06 ± 288.37	957.69 ± 153.83	0.769 ± 0.094
5	Crushed aphids	Non infested plant	1384.22 ± 275.00	1048.26 ± 133.65	0.859 ± 0.113

[a] 50 crushed 3rd Instar larvae *A. pisum*
[b] Single 20 cm high *V. faba* infested with 50 3rd Instar larvae *A. pisum*
[c] Single 20 cm high non infested *V. faba*
[d] Nine replicates were performed for each experimentation

No Eßf was emitted from downstream plant and plant/aphid complexes in experiments with empty lead chambers (Table 1, Experiment 2 (empty – infested) and 3 (empty – non infested)). These observations confirm that *V. faba* do not emit Eßf and demonstrate that undisturbed aphids under the conditions of this experiment do not produce a detectable alarm signal.

Eßf was detected in experiments 1 (crushed – empty), 4 (crushed – infested) and 5 (crushed – non infested). Analysis of variance demonstrated the equivalence of the EßF ratios obtained in these three experiments (ANOVA, $F_{2,24}$=1.12, P=0.342). The downstream/lead ratio found in experiment 1 was close to 1.0 as predicted. This ratio was not significantly different from the ratio obtained with a non-infested *V. faba* plant in the downstream chamber (*Tukey*, α=0.05). The very small reduction in the EßF ratio is likely due to the presence of the plant, which may act as an absorbent surface for airborne compounds to adhere to. In the fourth experiment (crushed – infested) aphids were present in the downstream chamber, yet there was no significant difference in the EßF ratio compared to that observed in experiment 5 (crushed – non infested) (*Tukey*, α=0.05). The downstream aphids did appear to perceive the EßF coming from the lead chamber, as the number of aphids in the downstream chamber that dropped from their host plant increased from 0 to 14% (Figure 2). These results indicate that amplification of the EßF alarm signal does not occur. This result is consistent with further observations that the amount of EßF released by a single aphid under attack is similar to the average amount of alarm pheromone released per consumed aphid in a colony (Schwartzberg et al., in press).

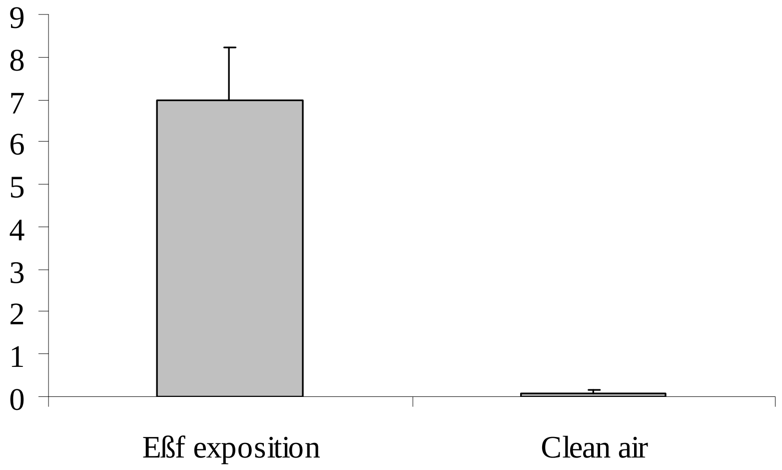

Figure 2. Number of aphids that dropped from the host plant after 1 hour of Eßf exposition (n=12)

An understanding of how alarm pheromone is emitted in a natural setting, or at least an intact aphid colony subject to environmental cues, may be important when studying the effects of alarm signaling among aphids and their predators. We have seen that a single, environmentally ubiquitous alarm signal can influence aphid ecology in the form of both inter- and intra-specific signaling. The way that such signals convey information in an aphid colony may be important in both the effectiveness of alarm signals within a colony as well as in reducing the costs of signal production in an environment where signal eavesdropping by prey can add a fitness cost to signal production.

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IV.3

Production of alarm pheromone by developing aphids varies in response to their social environment

Verheggen FJ[1], Haubruge E[1], De Moraes CM[2], Mescher MC[2]

[1]*Department of Functional and Evolutionary Entomology, Gembloux Agricultural University, Passage des Déportés 2, 5030 Gembloux, Belgium.*

[2]*Department of Entomology, The Pennsylvania State University, University Park, PA, 16801, USA*

Reference - Verheggen FJ, Haubruge E, De Moraes CM, Mescher MC (2008). Production of alarm pheromone by developing aphids varies in response to their social environment. Accepted for publication in Behavioural Ecology.

Abstract - In most aphid species, the volatile sesquiterpene (*E*)-β-farnesene (Eβf) is released as an alarm pheromone. It is also emitted continuously at low levels and may have a number of signaling functions. Aphid predators also use Eβf as a foraging cue, suggesting that the benefits to aphids of signaling to conspecifics via Eβf must be weighed against the cost of increasing apparency to natural enemies. To determine whether aphids vary Eβf production in response to features of their social environment, we compared the production of Eβf by *Acyrthosiphon pisum* (Harris) individuals reared in isolation to that of individuals reared among conspecifics or among individuals of a different aphid species, *Myzus persicae*. Production of EβF by *A. pisum* reared in isolation was significantly lower than that of aphids reared among conspecifics, among *M. persicae* individuals, or among conspecifics of another aphid clone. *A. pisum* individuals also produced less EβF when reared among *M. persicae* than among conspecifics, though this difference fell just short of statistical significance. In a second experiment, we reared *A. pisum* individuals in isolation but exposed them to the odor of conspecifics. Under these conditions, EβF production was similar to that of aphids reared among conspecifics, suggesting that aphids use volatile cues to assess their social environment and regulate their production of alarm pheromone accordingly. Finally, we examined the attraction of a predatory hoverfly to groups of aphids reared in isolation or in a colony and found that groups comprising individuals reared in isolation were significantly less attractive to the predator.

Keywords: Aphids, Alarm pheromone, (E)-beta-farnesene, *Acyrthosiphon pisum*, *Myzus persicae*, social environment, pheromone production

Introduction

Most aphid species reproduce parthogenetically between periods of sexual reproduction. As a result, individual aphids are often surrounded by other individuals that are genetically identical to themselves. This atypical social environment may explain the evolution in aphids of cooperative behavioral strategies similar to those encountered in eusocial insects such as termites, ants and honeybees. Many aphid species exhibit high degrees of polymorphism and cooperative behavior (Dixon *et al*. 1998; Foster 2002). For example, some gall-forming aphid species include sterile morphs that engage in defense and maintenance of the colony (Aoki 1977; Stern & Foster 1997; Kurosu *et al.* 2003).

Communal defense against predators is a commonly invoked benefit of social cooperation (e.g. Alexander *et al*. 1991; Bourke 1997), and a widespread form of coordinated defense in aphids involves the production and release of alarm pheromone, which occurs in most species (Francis *et al*. 2005). In response to attack by natural enemies (Pickett *et al*. 1992), individual aphids release droplets from two cornicles on the upper surface of the abdomen that emit an odor repellent to conspecifics (Kislow & Edwards 1972). In most Aphidinae species, the active component of this alarm pheromone is the sesquiterpene (*E*)-β-farnesene (EβF) (Bowers *et al*. 1972; Francis *et al*. 2005), which is also released as a volatile from various plant families including Solanaceae (Agelopoulos *et al*. 1999), Poaceae (Turlings *et al*. 1998), and Malvaceae (Rose & Tumlinson 2004). In addition to being actively released from the cornicles, EβF is emitted as a result of physical disruption of the aphid body during predation events.

Other aphids perceiving the pheromone typically remove their stylet from the host plant and fall, jump, or walk away to escape potential danger (Edwards *et al*. 1973; Wientjen *et al*. 1973; Montgomery & Nault 1977; Roitberg & Myers 1978; Wohlers 1981; Pickett *et al*. 1992; Chau & Mackauer, 1997; Losey & Denno, 1998; Shah *et al*. 1999; Braendle & Weisser 2001) but do not themselves release additional alarm pheromone (Verheggen *et al*. 2008; Hatano et al., 2008) . In the aphid *Ceratovacuna lanigera*, the alarm pheromone induces conspecific soldiers to attack predators or parasitoids (Arakaki 1989). As a consequence of its alarm function, Kunert *et al*. (2005) suggested that Eβf increases the production of alate morphs by increasing aphid movement and thus the frequency of physical contact among aphids.

Apart from serving as a pheromone for conspecifics, EβF serves as an attractant for aphid-tending ants (Nault *et al*. 1976 ; Mondor and Addicott 2007) , which may provide aphids with protection from natural enemies. However, EβF is also exploited by aphid predators and parasitoids that have developed the faculty to locate their prey by perceiving and responding to this compound, including ladybirds and other beetles (Nakamuta 1991; Zhu *et al*. 1999; Acar *et al*. 2001; Francis *et al*. 2004; Verheggen *et al*. 2007a; Kielty *et al*. 1996); syrphid flies (Almohamad *et al*. 2007 ; Verheggen *et al.*, 2008); and lacewings (Boo 1998; Zhu *et al*. 1999). Thus, the fitness benefits of signaling to conspecifics or mutualists via EβF presumably must be weighed against the ecological costs of increasing apparency to natural enemies (Almohamad et al., 2008), as well as any energetic or other physiological costs (Mondor and Roitberg, 2003), associated with the production and release of EβF.

Because the benefits of alarm signaling appear to fall primarily on individuals receiving the signal, while the costs are borne to an equal or greater extent by individual emitting the signal, we hypothesized that it might be adaptive for individual aphids to regulate their Eβf production in response to variation in the social environment. That is, aphids might be expected to produce more Eβf when other individuals are present to receive the signal and when a high proportion of potential recipients are clone-mates or close relatives. In some vertebrate systems the frequency of alarm calls can be correlated to features of population structure. For example, the propensity of female Belding's ground squirrels to produce alarm calls more frequently than males appears to be explained by the fact that males commonly disperse and, thus, are less likely than females to be in social environments including a high proportion of close genetic relatives (Sherman 1977, 1981).

Previous studies demonstrated that the frequency of the emission of alarm pheromone droplets varies with features of the social environment (Robertson et al., 1995). To investigate whether the production of aphid alarm pheromone might be sensitive to features of the social environment, we explored the production of Eβf by young *Acyrthosiphon pisum* (Harris) aphids under different social conditions. This aphid species has been extensively studied and its production and emission of alarm pheromone, as well as the response of individuals receiving the signal are well characterized (e.g., Kunert *et al*., 2005 ; Verheggen *et al*. 2008 ; Schwartzberg *et al.* 2008). We examined Eβf production by *A. pisum* individuals reared in isolation, among clone mates, among unrelated conspecifics, or among individuals of another aphid species, *Myzus persicae*. We also reared individual aphids in isolation but exposed them to odors from aphid colonies, to see whether volatile cues might be involved in regulating Eβf production. Finally, to explore the potential ecological relevance of socially mediated variation in Eβf production, we examined the behavioral response of a predatory hoverfly to groups of aphids comprising individuals reared in different social environments.

Material and Methods

***Plants and insects* –** Pea aphids, *A. pisum*, and green peach aphids, *M. persicae* (Sulzer), were reared in incubators (16-hr photophase, 25°C, 60% RH) for several years before the experiments. *Acyrthosiphon pisum* colonies were fed on *Vicia faba* (L.) and *M. persicae* were fed on turnips (*Brassica rapa* L.). Both plants were grown in square 9 x 9 x 8 cm high plastic pots filled with a peat-based general-purpose soil under the same conditions used for aphid rearing. Two *A. pisum* clones, D26 and LSR1, were provided by Dr David Stern (Department of Ecology and Evolutionary Biology, Princeton University, USA). Adult *E. balteatus* hoverflies were collected in Gembloux (Belgium) in June 2007, reared in 75 x 60 x 90 cm cages and fed pollen, sugar, and water *ad libitum*. Hoverfly oviposition was induced by placing aphid infested 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 birth.

***Experiment 1: isolation treatments* –** *A. pisum* individuals were reared in four different social environments, and their Eβf content was measured when they reached the adult stage. In each treatment, new-born *A. pisum* D26 were transferred from *Vicia faba* to 5cm-diameter Petri dishes enclosed by two layers of Parafilm® between which 400 µl of the chemically defined diet was placed. Artificial diet was prepared according to the methods of Dadd & Mittler (1966). Sachets were changed three times a week. The following treatments were employed: (1) Individual new-born *A. pisum* D26 were placed in empty Petri dishes; (2) Groups of 30 new-born *A. pisum* D26 were placed in empty Petri dishes; (3) Individual new-born *A. pisum* D26 were transferred to Petri dishes each containing a group of 30 *Myzus persicae* adults; (4)

Individual new-born *A. pisum* D26 were transferred to Petri dishes each containing a group of 30 *A. pisum* clone LSR1 individuals.

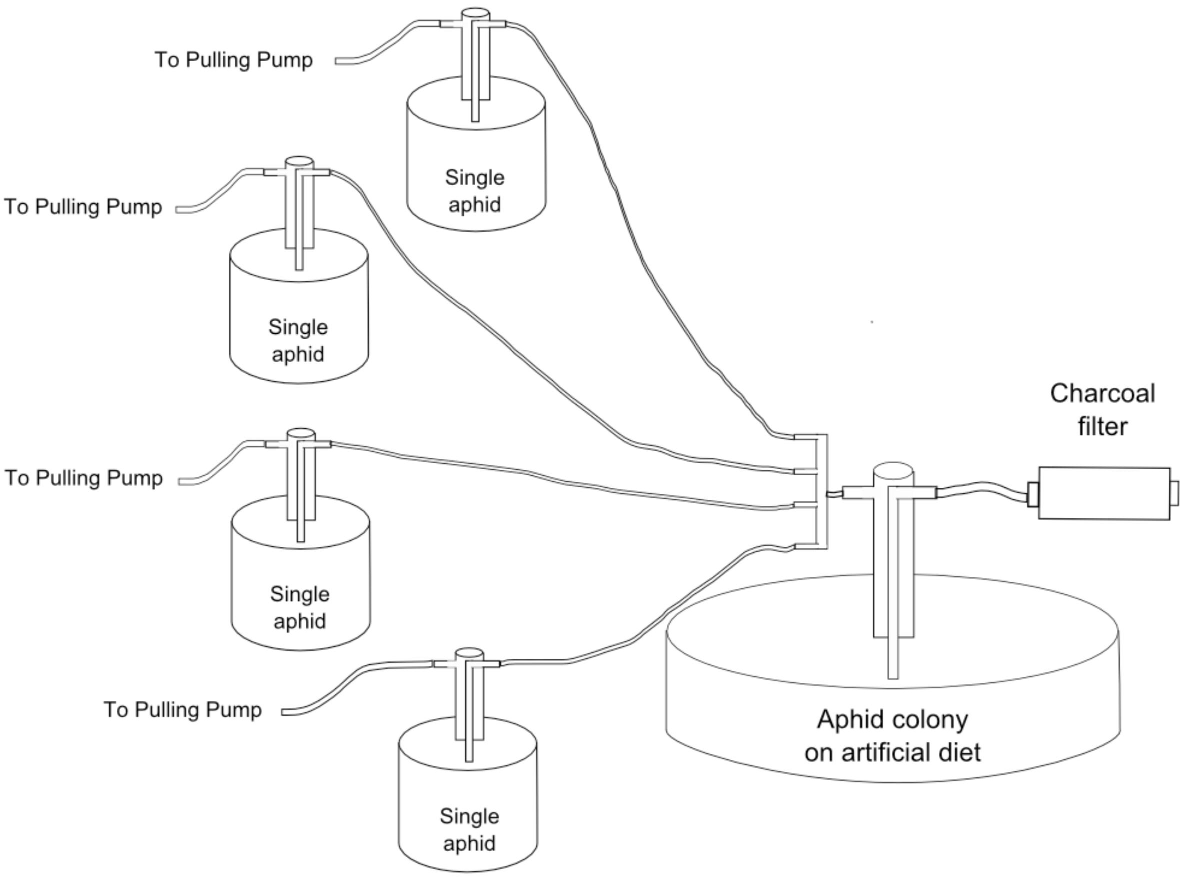

Figure 1. Push-pull system used to expose individual aphids to odour produced in the headspace of a conspecific colony.

Experiment 2 : exposure to conspecific odors – To assess the effects of conspecific odors on individual aphids, we employed a 15cm diameter, 0.5L glass chamber connected to four smaller glass chambers (200ml, 5cm diameter) using Teflon tubing and glass flow dividers (Fig. 1). Filtered air was pulled into the main chamber at 0.4 l/min using four pulling pumps (0.1 l/min each) connected to the four small chambers. A 10cm diameter glass Petri dish was placed in the main chamber and covered with two layers of Parafilm® between which was placed 1000 µl of the artificial diet described above, and 100 mg of *A. pisum* individuals were allowed to feed and reproduce throughout the experiment. In each of the four small chambers a single *A. pisum* first-instar larvae was reared from birth to the adult stage on a 3cm diameter glass dish covered with a sachet of artificial diet. All sachets were changed three times a week. The Eβf content of the individually reared aphids was measured when they reached the adult stage (see methods below). As controls, Eβf content was also measured from individual aphids taken from the central colony as well as from aphids reared individually in the small glass chambers not connected to the central colony.

Experiment 3: Ecological relevance of Eβf production – We previously demonstrated that the predatory hoverfly *Episyrphus balteatus* (Diptera: Syrphidae) uses aphid alarm pheromone to locate aphid colonies (Verheggen et al., 2008). To test hoverfly attraction to aphids reared in different social environments, we released individual gravid hoverfly females into a net cage (60x60x60cm) containing two 20 cm-high *Vicia faba* plants, each containing 20 adult *A. pisum* that had either been reared in social isolation or among conspecifics (30 individuals reared together from the first instar). The cage was placed in an air-conditioned room under uniform light at 25°C, 60% RH. All aphids were reared on artificial diet, using the methods described above. Hoverfly behavior was recorded for 10 minutes, using the software The Observer5.0® (Noldus information Technology, version 5.0, Wageningen - The Netherlands), which allows hoverfly behavior to be analyzed (Harmel et al., 2007). Five behavioral categories, similar to those described in Verheggen *et al*. (2008), were employed to assess behavior relative to each target plant. Briefly, these included (1) Immobile: no movement of the hoverfly is observed; (2) Searching: the hoverfly flies in the cage; (3) Selecting: the hoverfly flies near one of the two plants; (4) Accepting: the hoverfly lands on one of the two plants; and (5) Ovipositing: the hoverfly actively moves its ovipositor and/or lays eggs. Behavioral trials were conducted in a climate-controlled room at 24 ± 1°C. The *E. balteatus* females were approximately 15-30 days old and no aphid-infested plant was offered for 24h prior the experiment, to avoid oviposition induction. A total of 16 hoverfly females were tested one after the other, leaving 5 minutes between each assay. In cases where a hoverfly landed and/or oviposited on one of the two plants, the plant was replaced.

Analysis of Eβf content in aphids – In each experiment, once aphids reached the adult stage, their *Eβf* content was analyzed using a push/pull volatile collection system employing chambers made from modified funnels that were 7.5 cm diameter and 7.5 cm high. These funnel-chambers were placed upside-down on a glass plate. Both the chambers and plates were previously washed with *n*-hexane. Clean air was pumped into the chambers (0.55 l/min) via Teflon® tubing and pulled out (0.5 l/min) through traps containing 40 mg SuperQ®

(Alltech, Deerfield, IL, USA). A single aphid was crushed on the glass plate, using a small glass pestle that was left inside the chamber. Volatiles were collected over the next 30 min.

Traps were eluted using 150 µl of dichloromethane; *n*-Octane (80 ng) and nonyl acetate (400 ng) were added to each sample as internal standards. Extracts were then analyzed by gas chromatography coupled with flame ionization detection (GC-FID) using a Hewlett-Packard model 6890 series gas chromatograph. Aliquots of 1 µl were injected with a splitless injector held at 220°C. The column (15 m x 0.25 mm i.d.) was maintained at 35°C for 0.5 minute and then heated to 180°C at a constant rate of 12°C/min. Quantification of Eβf was realized by comparing the EβF GC area with those of internal standards, using Chemstation software (Agilent Technologies, Palo Alto, CA, USA). Identifications were made by comparing retention times with those of known standards and confirmed by mass spectrometry.

***Statistical analysis* –** Comparisons between averages of (E)-β-farnesene emissions were realized using 1-way ANOVA followed by a Tukey's HSD means comparison test. Student's t-tests were used to evaluate differences between the durations of behavioral categories observed in experiment 3. All tests were conducted with Minitab15.2® software.

Results

***Experiment 1: isolation treatments* –** Eβf was the only compound isolated from individual *A. pisum* D26 headspace. The Eβf content in individual D26 *A. pisum* was found to vary as a function of the presence of other aphids (ANOVA, $F_{3,59}$=2.92, P=0.041) (Fig. 2). When reared individually from the first instar, individual adult *A. pisum* contained significantly less alarm pheromone upon reaching the adult stage than *A. pisum* D26 individuals reared in a group of 30 D26 individuals or in a group of 30 LSR1 individuals (Tukey's test, P<0.05). Eβf content was not significantly different between *A. pisum* D26 individuals reared among other *A. pisum* D26 individuals and those reared among clone LSR1 individuals (Tuckey's test, P>0.05). When reared among individuals of another aphid species, *M. persicae*, *A. pisum* D26 individuals contained significantly more Eβf on reaching the adult stage than those reared individually (Tuckey's test, P<0.05), significantly less than those reared among LSR1 individuals (Tuckey's test, P<0.05) and less (but not significantly so) than those reared among other *A. pisum* D26 individuals (Tuckey's test, P>0.05).

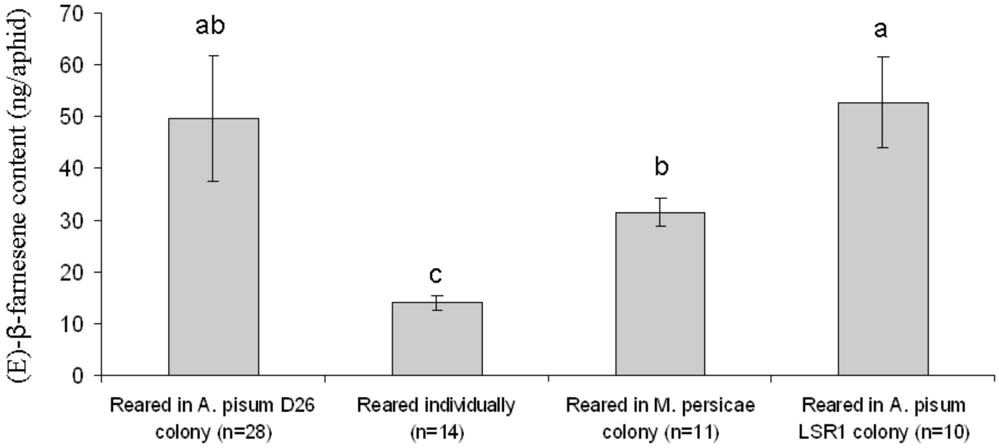

Figure 2. (*E*)-ß-farnesene content of individual A. pisum green clone according to different social environment. Averages with letters in common are not significantly different (ANOVA, P>0.05).

***Experiment 2: exposure to conspecific odors* –** Aphids reared in complete isolation exhibited lower Eβf levels when reaching the adult stage than aphids reared in a colony of conspecifics (ANOVA, $F_{2,48}$=7.43, P=0.002 ; Tukey's test, P<0.05) (Fig. 3). Aphids reared in physical isolation but exposed to conspecific odors produced similar amounts of Eβf to those reared in the colony (Tukey's test, P>0.05) and significantly more than aphids reared in complete isolation (Tukey's test, P<0.05).

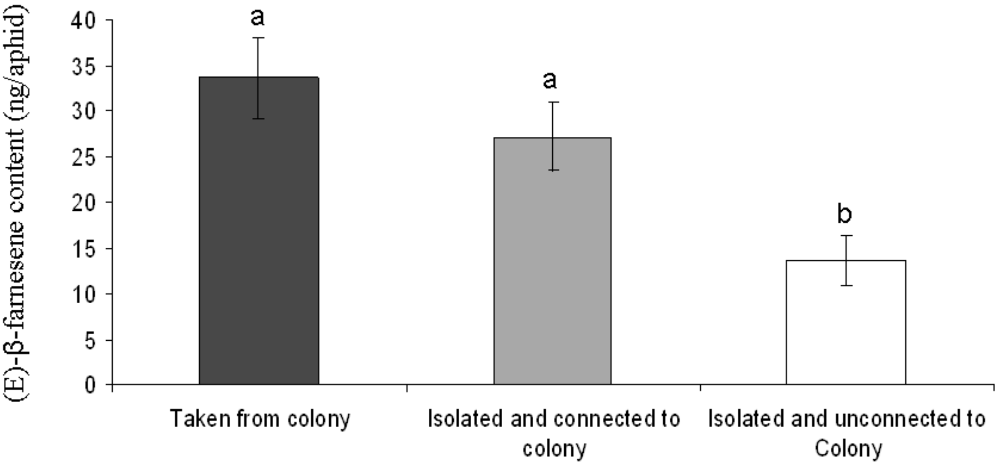

Figure 3. (*E*)-ß-farnesene content of individual *A. pisum* green clone. Three environmental conditions were tested: *A. pisum* reared in colony of conspecifics (N=17), *A. pisum* reared individually (N=17) and *A. pisum* reared individually with air connection to a conspecific colony (N=17). Averages with letters in common are not significantly different (ANOVA, P>0.05)

Experiment 3: Ecological relevance of Eβf production – Plants inhabited by aphids reared in isolation (low-Eβf aphids) were less attractive to the hoverfly predator *E. balteatus* than those inhabited by aphids reared in a colony (Figure 4). Time spent on the plant (Behavioral class 4: Acceptance) was significantly lower for plants inhabited by aphids reared in isolation (Student t-test, t_{obs}= 2.15, P=0.046). Time spent flying near the plant (Behavioral class 3: Selection) was also higher for these plants, although this difference fell just short of statistical significance. Almost no ovipositions (Behavioral class 5) occurred during the short times allowed for these experiments and no meaningful pattern was observed.

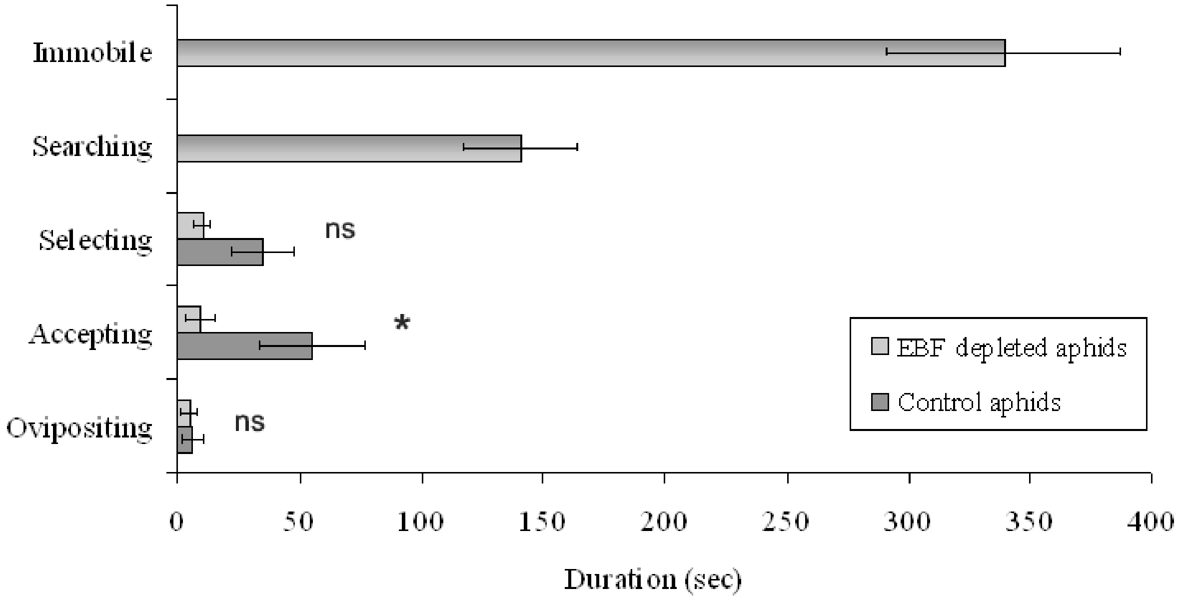

Figure 4. Durations of the five observed behavioural sequences of the foraging behaviour of *Episyrphus balteatus* females (N=16, Mean durations +/- SE) subjected to the dual choice between two plants infested respectively by an Eßf-depleted aphid colony and a control aphid colony. The star indicates that both means were significantly different from each other (Student t-test, *P*<0.05).

Discussion

Some social insects have previously been shown to use pheromonal cues to assess and respond to the social environment. For example, pheromones produced by queens and brood suppress ovary activation in honeybees (Slessor *et a*l. 1988). Moreover, some insects regulate their own pheromone release in response to social cues; for example, the confused flour beetle, *Tribolium confusum* DuVal, regulates the release of both aggregation and epideictic pheromones according to food availability and the local abundance of conspecifics (Verheggen et al. 2007b). Our results indicate that pheromonal or other volatile cues regulate the production of alarm pheromone by aphids. Individual *A. pisum* reared in isolation produced significantly less Eβf than those reared among conspecifics or those reared in isolation but exposed to conspecific odors. Thus it appears that some volatile cue, perhaps Eβf itself, acts to up-regulate the production of Eβf during development.

Although we found that Eβf production was stimulated by the presence of conspecifics, we found no evidence that Eβf production varies in an adaptive way in response to the genetic relatedness of neighboring individuals. There appeared to be no difference in Eβf production between individuals reared amongst clone-mates and those reared in a different *A. pisum* clone. *A. pisum* individuals reared in a *M. persicae* colony did produce less Eβf than those reared in conspecific colonies, but this difference was statistically significant for only one of the two aphid clones.

In our second experiment, Eβf was the only volatile found in the headspace of the central *A. pisum* colony, which is in accordance with findings from previous studies (Bowers *et al*. 1972; Mondor *et al*. 2000; Francis *et al*. 2005; Verheggen et al., 2008). Thus, it is very likely that Eβf itself is the volatile signal that induces increased production of Eβf by developing aphids. If so, Eβf may potentially function not only to signal the presence of other individuals that are potential recipients of alarm signals but also to provide information about the frequency with which alarm signals are being released under local conditions. Previous work has suggested that EβF may also regulate the production of alate aphids under crowded conditions (Kunert *et al*. 2005; Podjasek *et a*l. 2005) and increased production in response to high levels of juvenile exposure would seem compatible with such a mechanism.

Individual pea aphids typically release large amounts (16.33±1.54 ng) of their alarm signal when attacked by predators (Mondor et al., 2000 ; Schwartzberg et al., 2008). However, aphid colonies also continuously release smaller amounts of EβF (on the order of 1ng/aphid; Almohamad et al., 2008). As noted above, EβF is known to serve as foraging cue for aphid enemies. We have previously demonstrated that female *E. balteatus* hoverflies are attracted to broad bean plants from which EβF is experimentally released. To test the potential ecological relevance of the variation in EβF production among aphids reared in different social environments observed in the current study, we examined the attraction of gravid *E. balteatus* females to plants inhabited by groups of (20) aphids that were reared either in isolation or in a colony. In choice tests, female hoverflies spent significantly more time on plants inhabited by aphids reared socially than on plants inhabited by aphids reared in isolation, and also spent more time flying near these plants. These results indicate that the diminution in EβF production associated with social isolation during development has potentially significant ecological implications.

Because EβF production appears to entail a significant ecological cost by increasing apparency to natural enemies, it is plausible that the calibration of EβF production to EβF exposure during development might be adaptive. Presumably, such calibration would be favored by selection if the ratio of benefits to costs of EβF production is positively correlated with greater EβF exposure (which will increase with increasing aphid numbers or when EβF emissions per individual are higher). As noted above, EβF exposure may signal the presence of nearby individuals toward which future signals might be directed and might provide information about the frequency or intensity of EβF signals under local conditions (e.g., the intensity of predation). Moreover, if signaling via EβF plays a role in promoting social cohesion or dispersal (e.g., Kunert *et al*. 2005; Podjasek *et a*l. 2005) these functions may be more significant in larger, more crowded, colonies. It is also possible that the costs of EβF production, if they stem largely from increased apparency to predators, may be higher for small colonies. Kan (1988) found that hoverfly females preferred to lay their eggs near newly established aphid colonies lacking winged individuals and containing a higher proportion of nymphs which are more vulnerable to attack by hoverfly larvae. It also seems reasonable to assume that avoiding detection through stealth is a more plausible strategy for small aphid colonies than for larger colonies. For example, natural enemies may be less likely to find newly established aphid colonies by exploiting non-EβF odor cues such as those associated with honeydew or plant volatiles induced by herbivory (e.g., Ide et al. 2007; Pickett and Glinwood 2007; Verheggen et al. 2008) as the production of these cues will increase with aphid numbers and over time.

In sum, our findings demonstrate that production of an aphid pheromone regulating important social behaviors—including coordinated defense and possibly dispersal—varies in response to features of the social environment experienced during aphid development. We further demonstrate that this variation is mediated by odor cues--most likely exposure to EβF itself which may provide information about the presence and abundance of nearby conspecifics as well as the frequency of alarm signaling under local conditions. Finally, we show that this variation in EβF is sufficient to influence the apparency of aphids to foraging natural enemies, suggesting that the observed effects likely have ecological relevance and that socially mediated variation in EβF production may be adaptive.

Acknowledgements

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Chapter V

(E)-ß-farnesene in aphids-aphidophagous predators interactions

GENERAL INTRODUCTION TO CHAPTER V

Aphids are subjected to predation by various arthropod insects including ladybeetles, hoverflies, parasitoids and lacewings that limit the expension of their populations, and subsequent yield losses of crops. These beneficials use odors associated with the presence of their prey to locate adequate oviposition sites or hosts. These odors might be released from the prey themselves or from the infested host plant. Most asexual aphids belonging to the Aphidinae sub-family release a single semiochemical named (E)-ß-farnesene that acts as an alarm pheromone. This pheromone is one of the only means of protection aphids have developed against natural enemies. This molecule was already known to act as a kairomone for some aphid natural enemies. However, previous field studies that have tried to use (E)-ß-farnesene within integrated pest management strategies failed to increase predation, and the aphid populations remained uncontrolled. These authors concluded that it is first necessary to completely understand aphids ecology as well as the way their natural enemies interact with them.

We therefore conducted a series of experiments in order to shed light on the behavioral impact the aphid alarm pheromone may have on two aphid predators: the hoverfly *Episyrphus balteatus* De Geer (Diptera: Syrphidae) and the Asian lady beetle *Harmonia axyridis* (Coleoptera: Coccinellidae). *Episyrphus balteatus* is often used as a model species of generalist hoverfly, and several studies have addressed the potential factors affecting its foraging and oviposition behavior. While adults feed on pollen, females have to forage for suitable oviposition sites (i.e. plants infested with aphids) where their larvae will find prey to feed on, directly after emergence. It would therefore make sense for the hoverfly females to have evolved their olfactory systems for the perception and interpretation of aphid odor blends. The Asian ladybeetle is an exotic species of ladybirds imported in Europe for aphid control. Since its introduction, *Harmonia axyridis* spread out in the whole continent. Through direct and indirect competition with indigenous coccinellids, they cause disappearance of the latter species. Similarly with hoverfly females, ladybeetles would have clear advantage to locate aphid colonies by detecting their alarm pheromone.

V.1

Tomato-aphid-hoverfly: A tritrophic interaction incompatible for pest managment

François J. Verheggen[1], Quentin Capella[1], Ezra G. Schwartzberg[2] and Eric Haubruge[1]

[1] *Department of Functional and Evolutionary Entomology. Gembloux Agricultural University, 2 Passage des Déportés, 5030 Gembloux, Belgium*

[2] *Department of Entomology, The Pennsylvania State University, University Park, Pennsylvania, USA*

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Abstract – Trichome-based tomato resistance offers the potential to reduce pesticide use, but its compatibility with biological control remains poorly understood. We evaluated *Episyrphus balteatus* De Geer (Diptera, Syrphidae), an efficient aphidophagous predator, as a potential biological control agent of *Myzus persicae* Sulzer (Homoptera, Aphididae) on trichome-bearing tomato varieties. *Episyrphus balteatus*' foraging and oviposition behavior, as well as larval mobility and aphid accessibility, were compared between two tomato varieties (*Lycopersicon esculentum* Mill. 'Moneymaker' and 'Roma') and two other crop plants; broad bean (*Vicia faba* L.) and potato (*Solanum tuberosum* L.). Hoverfly adults landed and laid more eggs on broad beans than on three species of Solanaceae. Hoverfly larval movement was drastically reduced on tomato, and a high proportion of hoverfly larvae fell from the plant before reaching aphid prey. After quantifying trichome abundance on each of these four plants, we suggest that plant architecture, specifically trichomes, are a key factor contributing to reduced efficacy of *E. balteatus* as a biological agent for aphid control on tomatoes.

Keywords *– Episyrphus balteatus*; *Lycopersicon esculentum*; *Myzus persicae*; Trichome-based resistance

Introduction

Aphids are major agricultural and horticultural pests throughout the world. While they can result in direct damage to crops through feeding on phloem tissue, they can also contribute to severe indirect damage by acting as primary vectors of many plant viruses. Aphids reproduce rapidly and have been shown to adapt quickly to host-plant phenology and ecology, as well as plant physiology and biochemistry (e.g. Pettersson et al. 2007).

While chemical insecticides can provide adequate control of aphid populations, increased resistance among aphid populations to chemical products highlights the need for alternative control methods, including the use of natural enemies (Cook et al. 2007). In order to increase the effectiveness of such alternative control techniques we need a better understanding of the ecology and behavior within these tri-trophic interactions.

Aphid communities are subject to predation by a broad range of specialist and generalist arthropod predators and parasitoids. Aphid natural enemies such as hoverflies (Gilbert 1986, 2005), coccinellid beetles (Hodek and Honek 1996), lacewings (Principi and Canard 1984), cecidomyiid midges (Nijveldt 1988), spiders (Sunderland et al. 1986) and parasitoids (Stáry 1970), are major components of the predatory guild associated with aphid colonies. Hoverflies are efficient aphidophagous predators (Alhmedi et al. 2008). Our study organism, *Episyrphus balteatus* De Geer (Diptera, Syrphidae), is an economically important syrphid and accepts a broad range of aphid species in the field (e.g. Völkl et al. 2007). The larvae of this species are voracious predators of aphids and are important biological control agents (e.g. Ankersmit et al. 1986; Chambers and Adams 1986).

Most predators have been shown to have specific food resource preferences (e.g. Hodek 1993; Schoonhoven et al. 1998; Almohamad et al. 2007; Almohamad et al. 2008a), and these preferences need to be considered when exploiting them as biological control agents. These preferences are especially important for syrphids because syrphid larvae have limited dispersal abilities (Chandler 1969). Therefore oviposition site discrimination has a strong impact on offspring performance (Scholz and Poehling 2000). The oviposition preference of female syrphids has recently been correlated with offspring performance on preferred host plants (Almohamad et al. 2007). The behavioural impact of various plant and prey secondary metabolites, including volatile and non-volatile chemicals, in the localization and selection of an oviposition site in *E. balteatus* has recently been demonstrated (Chandler 1968; Kan 1988; Sadeghi and Gilbert 2000a, 2000b; Harmel et al. 2007; Almohamad et al. 2008b; Verheggen et al. 2008). Among these chemicals, the main component of the aphid alarm pheromone, the sesquiterpene (E)-ß-farnesene (Francis et al. 2005), acts as an attractant and an oviposition stimulant for *E. balteatus* female (Almohamad et al., 2007, 2008b; Verheggen et al., 2008).

While studying induced volatile emissions of aphid-infested tomato plants, and the subsequent effect on *E. balteatus* attraction, we observed their apparent failure in aphid control on tomato plants. We therefore estimated in this study the ability of hoverfly females to orient toward and forage on tomato plants infested by *M. persicae*. Herein we test the hypothesis that trichome architecture plays an important role in predator success on tomato plants. Two tomato varieties (Moneymaker and Roma) as well as two additional host plants of *M. persicae* Sulzer (Homoptera, Aphididae), namely broad bean (*Vicia faba* L., Fabaceae, variety “Grosse ordinaire”) and potato (*Solanum tuberosum* L., Solanaceae, variety “Bintje”) were selected to test larval movement and success in reaching aphid colonies. *Vicia faba* and *S. tuberosum* were chosen because they were shown in previous work to be easily exploited as host plants by *E. balteatus* (Almohamad et al. 2007; Harmel et al. 2007). In this work, we tested *E. balteatus* as a possible biological agent against aphids infesting four host plants, and explain how plant architecture may play a role in predator efficacy within this system.

Materials and Methods

Plants and insects - Broad beans (*V. faba*) were grown in 30 x 20 x 5 cm plastic trays filled with a mix of perlite and vermiculite (1:1). Potatoes (*S. tuberosum*) and tomatoes (*L. esculentum* ´Moneymaker´ and *L. esculentum* ´Roma´) were grown in 8 x 8 x 10 cm plastic pots filled with a mix of compost, perlite, and vermiculite (1:1:1). All plants were grown in controlled environment growth rooms (16:8 h Light:Dark; 20 ± 2 °C; RH: 70 ± 5%). The peach aphid, *M. persicae*, was reared on the four previously mentioned plants in separate controlled environment growth rooms set at the same conditions as described above. Adult specimens of *E. balteatus* were reared in a separated room, in 75 x 60 x 90 cm cages and were fed with bee pollen, sugar and water. Broad beans infested with *M. persicae* were introduced into the cages for 3 hours every 2 days to allow oviposition by hoverfly adults. Hatched hoverfly larvae were mass-reared in aerated plastic boxes (110 x 140 x 40 mm) and were fed daily *ad libitum* with *M. persicae* as a standard diet. All the hoverfly adults tested in the following experiments were 2 to 4 weeks old, which corresponds to sexual maturity and high fecundity (Sadeghi and Gilbert, 2000c), and had not previously been exposed to aphid-infested plants.

Host plant preference - In no-choice experiments, hoverfly females were individually placed in screened cages (30 x 30 x 60 cm) with one of the four tested plant species infested with 100 adult *M. persicae* 24 h prior to observations. Plants, roughly 20 cm tall, having four fully expanded leaves were presented in a plastic pot filled with the same soil composition as presented above.The soil was c overed with aluminum foil to avoid any volatile chemicals released by the compost to affect the hoverfly behavior. The hoverfly foraging behavior was visually observed and recorded for 10 min using the Observer$^{®}$ software (Noldus information Technology, version 5.0, Wageningen, The Netherlands). Descriptions of the five observed behavioral subdivisions (Immobility, Fly, Searching, Acceptance, Oviposition) are presented in Table 1. In another series of similar no-choice experiments, single *E. balteatus* females were allowed to lay eggs for 3 h and the number of eggs laid on each aphid infested plant was counted. The experiments were conducted at a controlled room temperature (20 ± 2 °C) and relative humidity 70 ± 5%). *E. balteatus* females were approximately 21-28 days old. One replicate was limited to each plant. There were a total of 20 replicates for each of the two aforementioned experiments.

Larval movement observation – In above mentioned net cages, 20 cm tall plants were infested by placing 20 adult *M. persicae* on the adaxial surface of two fully expanded leaves with. All other lower leaves were removed from the plant. Aphids were allowed to feed 4 h before starting the experiment. After this initial aphid settlement period a 3rd instar larva of *E. balteatus*, starved 5 h prior to the experiment, was placed in the middle of the plant on the stem. Twenty hoverfly larvae were observed on the four plants. Three types of reactions were observed ocularly and recorded: (1) When a larva moved and reached the top of the plant within 15 minutes of placement on the plant, (2) when a larva fell from the plant within 15 minutes of placement on the plant, and (3) when a larva remained stationary or did not reach the top of the plant within the 15 minutes of observation. Each plant and insect was tested once. The number or replicates tested on each of the four plants, *V. faba*, *S. tuberosum*, *L. esculentum* Roma and Moneymaker were: 11, 10, 4 and 5 respectively. Larval velocity (mm*sec^{-1}) was calculated as the distance traveled divided by the time needed to reach the top of the plant or by 15 minutes if the larva did not reach the top of the plant.

Table 1. Description of the behavioural events recorded for aphidophagous hoverfly *Episyrphus balteatus* exposed to different aphid infested host plants

Observed behavior	Description
Immobility	Predator immobilized on the cage
Fly	Predator flying in the cage
Searching	Predator flying near the plant (<5cm)
	Predator touching sporadically the plant
Acceptance	Predator landing and walking on the plant
	Predator extending its proboscis and identifying the stimulatory substrate to accept the host.
Oviposition	Predator laying eggs on plant

Trichome density evaluation – To evaluate the impact that trichome density has on the movement of *E. balteatus* adults and larvae, we quaantified one parameter of trichome density by counting the number of trichomes per square centimeter of stem surface under binoculars (Olympus®, SZ40). A 1cm long piece of the stem was cut with a razor blade from the middle of a 20 cm tall plant with four fully expanded leaves. Ten plants of each of the four tested species and varieties were cut this way. According to Luckwill (1943) and Simmons and Gurr (2005), there up to 5 types of trichomes are found on a *L. esculentum* stem. Here, only types I and III were counted as they represent the longest trichomes. Indeed, according to our observations, no contact between the tested insects and the smallest trichomes occurred.

Statistical analyses – One-way ANOVA followed by *Tukey's* post-hoc test (pairwise comparisons) were used in the host plant preference experiment. A general linear model followed by *Tukey's* test (pairwise comparisons) was used to compare the average larval speed observed on the four tested host plants.

Results

Host plant preference – In no-choice experiments, the durations of the five observed behavioral subdivisions (Immobility, Flying, Searching, Acceptance, Oviposition) were recorded. Immobility and searching behavior were similar for the four plants tested (ANOVA, Tukey post-hoc test, $P > 0.05$). However, hoverfly females spent more time (recorded as acceptance) on *V. faba* than on *S. tuberosum* and *L. esculentum* ´Moneymaker´ (ANOVA, Tukey post-hoc test, $P < 0.05$) (Fig. 1). In addition, the time spent ovipositing on broad bean plants was greater than on the three species of Solanaceae (ANOVA, Tukey post-hoc test, $P < 0.05$). These observations are in accordance with the number of eggs laid on each of the four tested plants. An average of 28.65 ± 5.48 eggs where laid on *V. faba*. Significantly fewer eggs were laid on *S. tuberosum* and *L. esculentum* ´Roma´ and ´Moneymaker´, with 8.95 ± 2.69, 11.80 ± 2.60 and 10.75 ± 3.69 eggs respectively (ANOVA, Tukey post-hoc test, $P < 0.05$).

Larval foraging efficiency – To evaluate the ability of *E. balteatus* larvae to reach their aphid prey, their velocity on each of the four plants was recorded (Fig. 2). Hoverfly larvae had an average speed of 2.69 ± 0.37 $mm*sec^{-1}$ on *V. faba*, significantly higher than the speed observed on both tomato varieties: 0.75 ± 0.33 mm*sec^{-1} and 0.70 ± 0.23 mm*sec^{-1}, for *L. esculentum* ´Roma´ and ´Moneymaker´ respectively (Tukey post-hoc test, $P < 0.05$). The average larval speed on *S. tuberosum* (1.44 ± 0.47 mm*sec^{-1}) was similar to the three other aphid host plants tested (Tukey post-hoc test, $P > 0.05$). In addition, the number of larvae that fell from the plant was counted. No larvae fell from *V. faba* and one larva fell from *S. tuberosum*. On tomatoes, 10 larvae out of 20 fell while walking on *L. esculentum* ´Roma´ and 5 larvae out of 20 fell from a *L. esculentum* ´Moneymaker´.

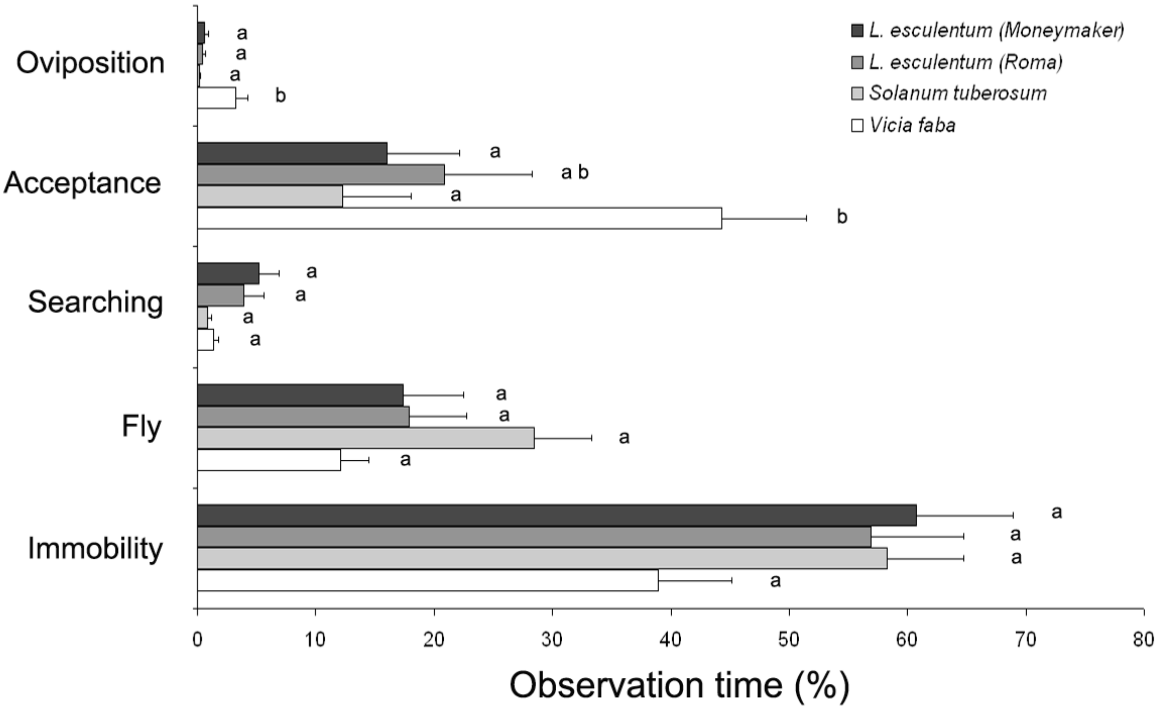

Figure 1. Behavioural observations (% of observation time + SE) of *Episyrphus balteatus* females on aphid host plants inno-choice experiments. Means with similar letters are not significantly different (n=20, Tukey post-hoc test, P<0.05).

Trichome density evaluation – While no trichomes were found on the plant surface of *V. faba*, an average (± SE) of 42.10 ± 5.21 trichomes were present per cm of *S. tuberosum* stem. *L. esculentum* ´Moneymaker´ and ´Roma´ had 170.80 ± 13.28 and 180.80 ± 13.66 trichomes (types I and III pooled together) per cm of stem, respectively (Fig. 3). Trichome densities are significantly different between *S. tuberosum* and both *L. esculentum* varieties but no significant difference was observed between the two tomato varieties (Tukey post-hoc test, $P < 0.05$).

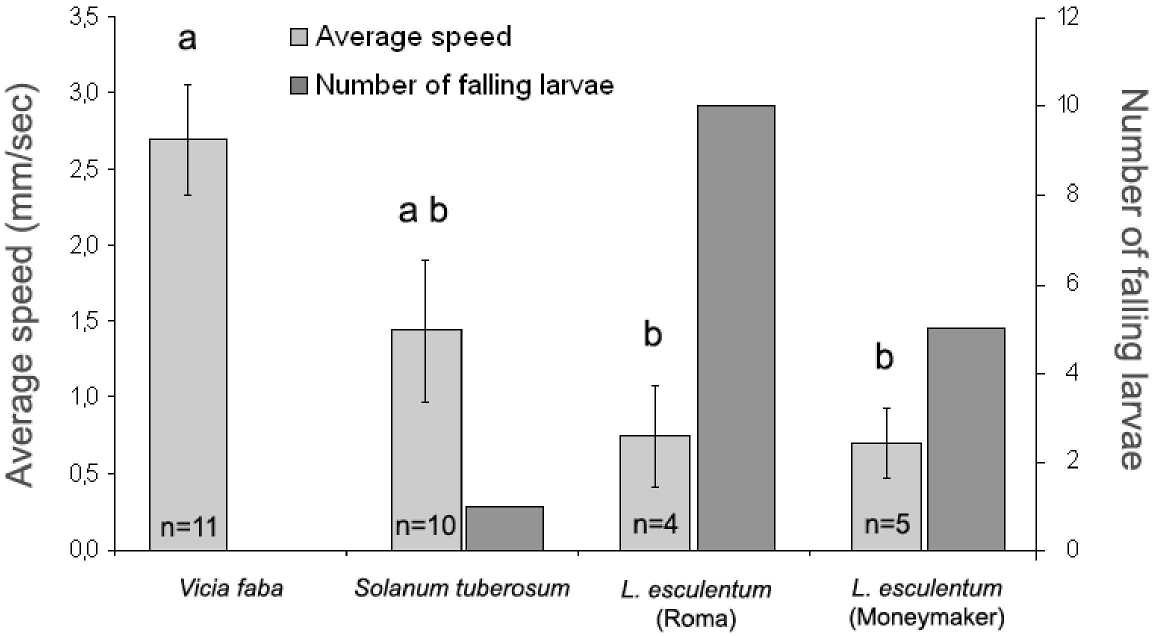

Figure 2. Effects of aphid host plants on *Episyrphus balteatus* larval velocity (mean velocity ± SE) and on the number of larvae falling off of the stem. Means with similar letter are not significantly different (General linear model, n=20, Tukey post-hoc test, P<0.05).

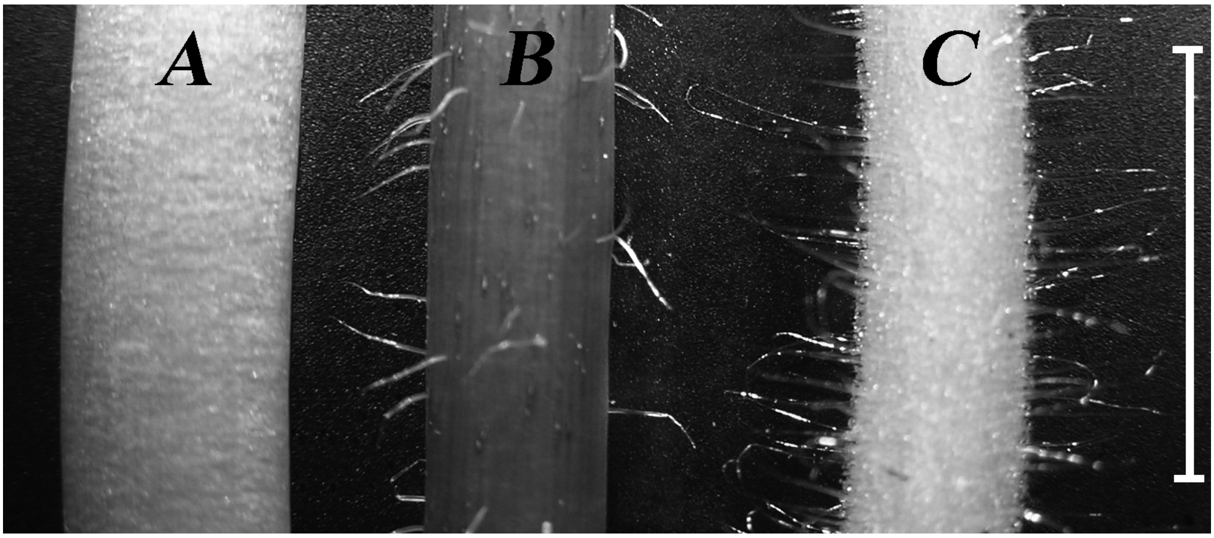

Figure 3. Comparison of the architecture of the three tested crop plant species (A) *Vicia faba*, (B) *Solanum tuberosum*, (C) *Lycopericon esculentum* (Roma). The white bar represents 1cm of stem.

Discussion

Insect-plant interactions involving the genus *Lycopersicon* have been previously studied and reviewed by Kennedy (2003), and interactions with hoverfly predators have received little attention. In our behavioral experiments, the negative effect of trichomes on the accessibility of hoverfly adults was visually observed. We showed that *E. balteatus* females preferred to land and oviposit on *V. faba* rather than on the three representative species of Solanaceae studied, which is likely to lead to a more efficient foraging of aphid preys. In addition to being guided by odorant cues, hoverflies use tactile foraging before laying eggs. Although hoverfly females were flying close to tomato plants (see “searching behavior” in Fig. 1), they had clear difficulties landing, presumably due to the presence of long Type I and III trichomes.

Trichome density on the stem surface of tomatoes also has an influence on the foraging speed of hoverfly larvae. Many larvae fell while trying to reach the top aphid-infested leaves. These results contrast with those obtained in studies of coccinellid (Seagraves and Yeargan 2006) and fire ant (Styrsky et al. 2006) predators. This may be due in part to different morphological features not shared with syrphid larvae, namely the presence of articulating legs. The effects of trichomes may be more apparent on syrphid larvae than on coccinellid larvae because of this.

Trichome-based tomato resistance against pest insects offers the potential to reduce pesticide use in tomato production, but its compatibility with biological control agents remains unclear. Trichome-mediated plant defenses have been demonstrated as being implicated in the third trophic level, either by direct contact of predators and parasitoids with trichomes, or indirectly by negatively affecting the host plant or positively affecting the prey insect (Simmons et al. 2003). The behavior of two aphid predators, *Macrolophus pygmaeus* Rambur (Heteroptera, Miridae) and *Orius niger* Wolff (Heteroptera, Anthocoridae), were influenced by the trichome density of tomato plants (Economou et al. 2006). The ability of lacewings to be used as biological control agents against aphids on pubescent plants has been questioned in previous work for *Mallada signata* (Simmons and Gurr 2004). Increased trichome density has been shown to reduce aphid accessibility on tomatoes, however they can also negatively affect predator efficacy through increase predator cannibalism and predator mortality due to trichome-related entrapment. Advantages to herbivorous insects have also been demonstrated on trichome-rich plants (Oku et al. 2006) where trichomes provide refuge and reduce predators' accessibility. *Coleomegilla maculate* (Coleoptera: Coccinellidae), a frequent predator of *Helicoverpa zea* eggs, prefers to oviposit on plants that have glandular trichomes, presumably as a means to provide its offspring from larval/egg cannibalism as well as from intra-specific predation (Seagraves and Yeargan 2006).

Movement of the hoverfly predator *Episyrphus balteatus* therefore seems compromised on trichome-rich solanaceous plants due to decreased mobility and subsequent aphid accessibility.

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V.2

What makes *Episyrphus balteatus* (Diptera : Syrphidae) oviposit on aphid infested tomato plants ?

FJ Verheggen[1], Q Capella[1], J-P Wathelet[2], E Haubruge[1]

[1] Gembloux Agricultural University, Dept. Functional and Evolutionary Entomology
[2] Gembloux Agricultural University, Dept. General Chemistry
2, Passage des Déportés, B-5030 Gembloux, Belgium

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Abstract - Under attack by insect pests, many plant species change their volatile chemical emissions to attract natural enemies. Most of the tomato (*Lycopersicon* sp., Solanaceae) varieties are subjected to infestation by molluscs and insects, including the generalist aphid *Myzus persicae* Sulzer (Homoptera, Aphididae). *Episyrphus balteatus* De Geer (Diptera: Syrphidae) is a generalist aphid predator that was here observed to lay eggs on *M. persicae* infested tomato but not on non-infested plants. In order to identify the volatile chemicals that guide the foraging and oviposition behaviour of *E. balteatus*, we collected and identified volatiles released in the headspace of both aphid infested and uninfested tomato plants by SPME-GC-MS. The identified chemicals were subsequently tested by electroantennography (EAG) on *E. balteatus*. Monoterpenes and sesquiterpenes were identified, the main volatile chemicals being ß-phellandrene, 2-carene, α-phellandrene, 3-carene and α-pinene. Electrical depolarizations were observed for each tested monoterpene, with optimal responses ranging from -0.2 to -0.8 mV. *Episyrphus balteatus* antennae showed dose-response relationships towards all the active chemicals. (*E*)-ß-farnesene, the main component of the aphid alarm pheromone, was the only active sesquiterpene, and is presumed to act as an ovipositing stimulus for *E. balteatus*.

Key words: *Lycopersicon esculentum*; *Myzus persicae*; Electroantennography; Volatile collection.

Introduction

Aphids represent major agricultural pests in temperate regions, damaging plants directly through feeding and indirectly by acting as important vectors of plant viruses. Increased resistance among aphid populations to crop protection products highlights the need for alternative control methods including the use of natural enemies (Cook et al., 2007). Attempts to develop such alternative control techniques will benefit from a more complete understanding of predators and parasitoids ecology. Aphid communities are indeed subjected to predation by a broad range of specialist and generalist predators or parasitoids arthropods whose distributions vary according to host plant species and phenology, season and weather conditions. Aphid natural enemies such as hoverflies (Gilbert, 1986), coccinellids (Hodek and Honek, 1996), lacewings (Principi and Canard, 1984), gall-midges (Nijveldt, 1988), spiders (Sunderland et al., 1986) and parasitoids (Stary, 1970), are major components of predatory guild associated with aphid colonies. The larvae of about one third of the species, classified in the subfamily Syrphinae, are efficient aphid predators. They are voracious feeders on aphids and are important biological control agents (Ankersmit et al., 1986; Chambers and Adams, 1986). However, many of the recent studies were focused on coccinellids (e.g. Ferran and Dixon, 1993; Sengonça and Liu, 1994 ; Verheggen et al., 2007a).

Plants respond to insect feeding damages by releasing a variety of volatile chemicals from the damaged and the undamaged sites, and the profile of the emitted volatiles is markedly different from those of undamaged plants (Paré and Tumlinson, 1999; D'Alessandro & Turlings, 2006). Two types of induced plant responses might be cited : (1) The plant responds to herbivory with the production of novel volatile chemicals and/or (2) the plant responds to herbivory with the production of the same compounds as when undamaged or damaged mechanically, but in larger quantities and over a longer time. Like aphid semiochemicals (Pickett & Glinwood, 2007), these induced plant volatiles serve as important foraging cues for natural enemies such as hoverflies, ladybeetles or parasitoids, to locate their prey, adapt their foraging behaviour and orientate towards sites appropriate for offspring fitness (Guerrieri et al., 1999; Scholz and Poehling, 2000; Turlings & Wäckers, 2004; Harmel et al., 2007; Verheggen et al., 2008; Almohamad et al., in press). *Episyrphus balteatus* DeGeer has been poorly studied although it is one of the most economically important syrphid, as it accepts a broad range of aphid species (Völkl et al., 2007). This species discriminates between aphid host plants, aphid colony sizes and aphid species, to select the most suitable oviposition site for larval fitness (Sadeghi & Gilbert, 2000; Sutherland et al., 2001; Almohamad et al., 2007a). *Episyrphus balteatus* also discriminates parasitized aphids from healthy ones, adapting its searching and oviposition behaviour accordingly, suggesting the perception of aphid semiochemicals (Almohamad et al., 2007b).

The tomato-induced defences have been studied in previous works (Dicke et al., 1998; Ryan, 2000; Vercammen et al., 2001; Kennedy, 2003; Kant et al., 2004), demonstrating that under herbivore infestation, 20 defence-related proteins are activated leading to changes in volatile emission profile. Whereas the attraction of ladybeetle towards aphid-infested tomato plants has been clearly demonstrated (Rodriguez-Saona & Thaler, 2005), tomato-aphid-hoverfly tritrophic interactions have received little attention. In this study, we evaluated the ability of *E. balteatus* males and females to perceive and orientate towards the various volatile organic chemicals (VOCs) released from tomato plants infested by *Myzus persicae*, a significant pest of tomatoes (Yardim and Edwards, 1998). We collected and identified the VOCs released in the headspace of healthy and aphid-infested tomato plants by SPME-GC-MS. This technique has indeed been widely used in tomato volatile analysis (e.g. Markovic et al., 2007). The identified mono- and sesquiterpenes were subsequently tested by electroantennography (EAG) to highlight their antennal perception by hoverfly antennae.

Materials and methods

Plants and insects

Broad beans (*Vicia faba* L.) were grown in 30 × 20 × 5 cm plastic trays filled with a mix of perlite and vermiculite (1:1). Tomatoes (*Lycopersicon esculentum* cultivar Roma) were grown in 8 × 8 × 10 cm plastic pots filled with a mix of compost, perlite, and vermiculite (1:1:1). Both plant species were grown in climate chambers (L16:D8 ; 20 ± 2 °C ; RH : 70 ± 5%). The peach aphid, *M. persicae*, was mass-reared on broad beans in separate climate chambers set at the same conditions as described above. Adult *E. balteatus* were reared in 75 ×60 × 90 cm cages and were fed with bee-collected pollen, sugar and water. Broad beans infested with *M. persicae* were introduced into the cages for 3 hrs every 2 days to allow oviposition. Hoverfly larvae were mass-reared in aerated plastic boxes (110 × 140 × 40 mm) and were daily fed ad libitum with *M. persicae* as a standard diet. All the hoverfly adults tested in the following experiments were 2 to 4 wks old.

Oviposition assays

Tomato plants were infested with 100 *M. persicae* 24 hrs prior to the experiment. In no-choice experiments, single *E. balteatus* females were allowed to lay eggs for 3 hrs on a 20 cm-high non-infested or infested tomato plant. The experiments were conducted in a controlled temperature room at 21 ± 2 °C. E. balteatus females were approximately 21-28 days old and no induction of oviposition had been realized for 24 hrs prior to the experimentation. There were 20 replicates for each of the aforementioned experiments.

Volatile collection

Potted 20 cm-high tomato plants were infested by 100 *M. persicae* and placed in the volatile collection chamber 24hrs prior to volatile analyses. Volatiles were collected from both uninfested and infested plants using solid-phase microextraction (SPME, Supelco®, Pennsylvania, USA). While the quantitative precision of SPME may not be as reliable as that achievable by other methods, the sensitivity, simplicity, speed, low cost, and gentle treatment of compounds outweigh this disadvantage for the purposes of the present study (Tholl et al., 2006). The adsorbent material covering the SPME fiber consisted of PDMS/CAR/DVB (polydimethylsiloxan / carboxen / divinylbenzen : 50/30µm). The plastic pot was covered with aluminum foil and introduced in a glass volatile collection chamber (Schott®, 12 cm base-diameter, 35 cm high), previously washed with acetone and n-hexane. The SPME fiber was cleaned in a GC Split/Splitless injector at 250°C for 1 hr before being exposed in the chamber for 1 hr. The adsorbed chemicals were analyzed by gas chromatography (Hewlett-Packard model 6890 series) coupled with mass spectrometer (Agilent Technologies 5973N), using a splitless injector held at 250°C. The column (30 m x 0.25 mm i.d.) was maintained at 40°C for 3 min before heated to 180°C at a constant rate of 10°C/min. The oven was then heated to 280°C at a constant rate of 20°C/min and maintained for 3 min. Identifications were made by comparing retention times with those of known standards and confirmed by mass spectrometry.

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., 2007a, 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 second antenna. A 0.5-cm2 piece of filter paper that was impregnated with 10 µl 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 air stream. Paraffin oil was used to make chemical solutions with concentrations ranging from 10ng/µl to 105ng/µl (by 10x increments). Electroantennograms were collected using Autospike 3.0 (Syntech®, Hilversum, The Netherlands). Stimulations with paraffin oil were executed as negative controls before and after the stimulations with the five doses cited above. Stimulations were induced thirty seconds from each other, from the lowest to the highest dose. Previous results indicated that this length of time was adequate to allow the insect to recover its full reactivity to stimuli (Verheggen et al., 2008).

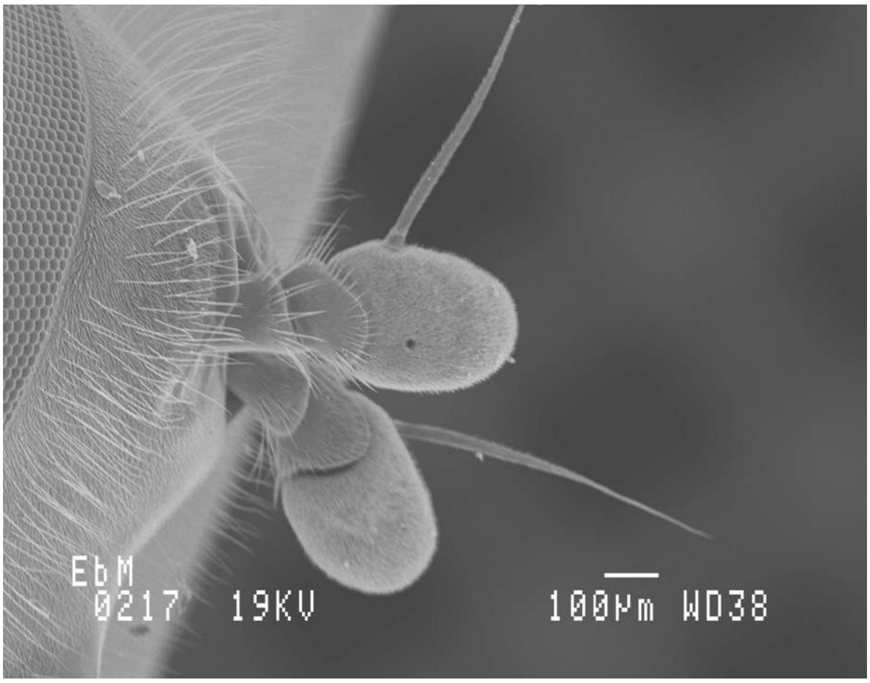

Figure 1. Electron miscroscopy picture of male E. balteatus antennae

Results

Oviposition assays

In no-choice experiments, the hoverfly gravid females laid 0.45 ± 0.33 egg on non-infested tomato after 3 hrs (N=20), whereas 11.80 ± 2.60 eggs were laid on a tomato plant infested by 100 *M. persicae* (N=20) (t_{obs} = 4.33 , P<0.001) (Fig. 1).

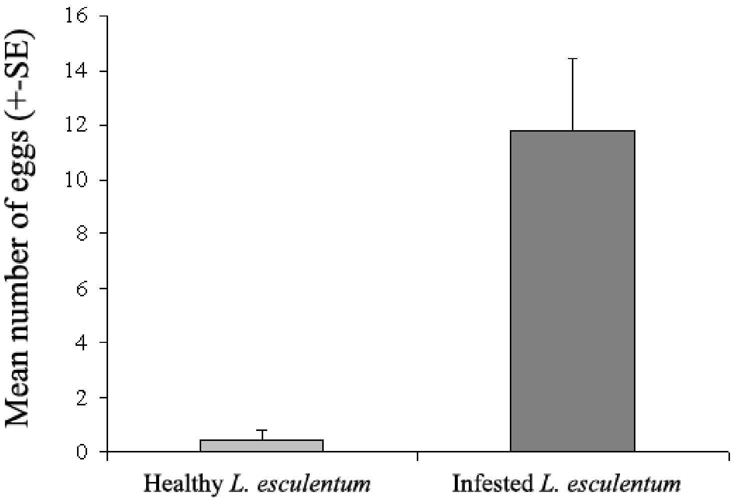

Figure 2. Mean number of eggs laid by *Episyrphus balteatus* gravid females on non infested and infested tomato (Lycopersicon esculentum) plants in no-choice bio-assays.

Volatile collection

Gas chromatography analysis of the volatile blend released by aphid infested tomatoes (L. esculentum Roma) (n=4) revealed the presence of several volatile compounds that are listed in Table 1. A typical chromatogram from a *M. persicae* infested tomato is presented on Fig. 2. Ten monoterpenes were identified while an 11th one could not be identified due to its too small concentration. Three sesquiterpenes were also identified, including (*E*)-ß-farnesene, the aphid alarm pheromone. The only chemical to be emitted in significant different amount with aphid presence is (*E*)-ß-farnesene (1-sample t-test, P<0.05). The major chemicals found in the headspace of aphid-infested tomato were subsequently tested for their perception by E. balteatus antennae using electroantennography.

Table 1. Relative amounts of the identified mono- and sesquiterpenes found in the headspace of healthy and *Myzus persicae*-infested *Lycopersicon esculentum* Roma (n=4). The letters "s" and "m" significantly mean sesquiterpenes and monoterpenes. Compounds (1) to (11) are monoterpenes. Compounds (12) to (14) are sesquiterpenes.

n°	Volatile Chemicals	Relative amounts (% of identified chemicals)			
		Uninfested plants		Aphid-infested plants	
		Averages	(min_{obs} - max_{obs})	Averages	(min_{obs} - max_{obs})
1	α-Pinene	4.88	(2.96 - 6.95)	3.06	(1.67 - 5.60)
2	ß-Pinene	1.17	(0.26 - 1.98)	2.15	(0.50 - 3.76)
3	2-Carene	23.46	(17.34 - 32.81)	22.54	(19.76 - 26.70)
4	α-Phellandrene	11.51	(6.02 - 19.82)	9.18	(4.98 - 12.80)
5	3-Carene	5.99	(2.98 - 10.71)	4.73	(1.90 - 6.93)
6	α-Terpinene	3.29	(1.28 - 5.34)	1.61	(0.15 - 4.83)
7	Cymene	1.48	(nd - 2.90)	2.22	(nd - 4.77)
8	ß-Phellandrene	38.44	(31.88 - 46.91)	42.65	(36.90 - 49.81)
9	γ-Terpinene	1.40	(nd - 3.83)	0.92	(nd - 1.91)
10	Terpinolene	1.17	(0.71 - 1.80)	0.72	(nd - 1.20)
11	Non identified Monoterpene	0.39	(nd - 1.11)	0.34	(nd - 0.90)
12	ß-Caryophyllene	4.89	(nd - 13.32)	3.12	(1.90 - 4.17)
13	α-Humulene	1.94	(nd - 6.23)	1.12	(0.13 - 2.94)
14	(*E*)-ß-Farnesene	0.00	/	5.66	(2.18 - 9.24)

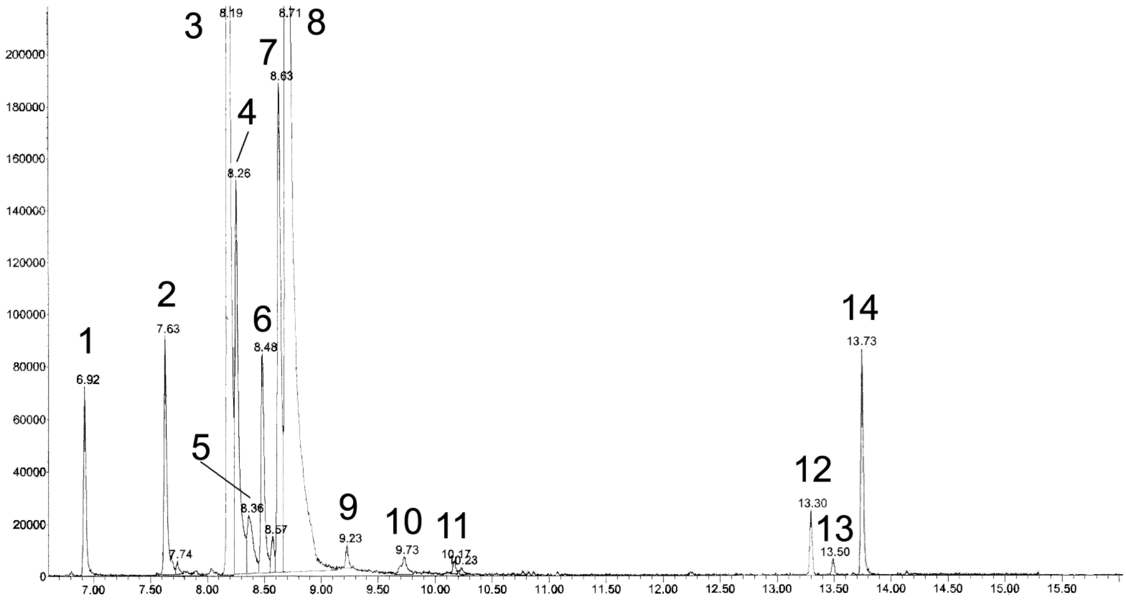

Figure 3. Chromatogram of volatile compounds emitted by tomatoes submitted to feeding by 100 *Myzus persicae*. Major compounds are labelled as follows: (1) α-Pinene ; (2) ß-Pinene; (3) 2-Carene ; (4) α-Phellandrene ; (5) 3-Carene ; (6) α-Terpinene ; (7) Cymene ; (8) ß-Phellandrene ; (9) γ-Terpinene ; (10) Terpinolene ; (11) Non identified monoterpene ; (12) ß-Caryophyllene ; (13) α-Humulene ; (14) (*E*)-ß-Farnesene. Compounds (1) to (11) are monoterpenes. Compounds (12) to (14) are sesquiterpenes.

Electroantennography

Olfactory responses towards 8 of the major volatile chemicals found in the headspace of aphid-infested tomatoes are presented in Fig 3. All the tested monoterpenes induced antennal activities, while only one of the three tested sesquiterpenes (i.e., (*E*)-ß-farnesene) induced antennal responses. All the EAG active chemicals induced similar responses in males and females, except the aphid alarm pheromone, (*E*)-ß-farnesene, that induced higher EAG responses in females (432 ± 65 µV, N=5) than in males (208 ± 29 µV, N=5) at the highest tested dose only (t_{obs}=3.17, P=0.025). Due to its isomeric instability, ß-phellandrene could not be tested using EAG.

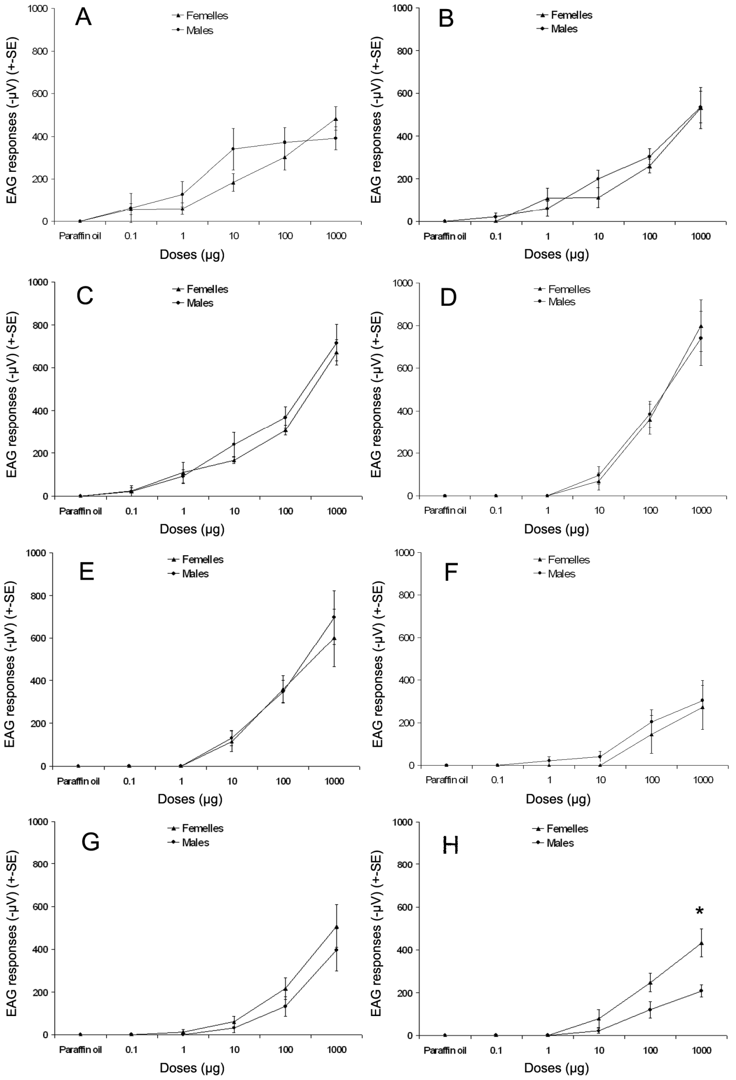

Figure 4. Effect of tomato released mono- and sesquiterpenes ((A) α-Pinene ; (B) ß-Pinene ; (C) 2-Carene ; (D) 3-Carene ; (E) α-Phellandrene ; (F) Cymene ; (G) α-Terpinene ; (H) (*E*)-ß-Farnesene) on the antennal responses (±SE) of female and male *Episyrphus balteatus* (n=5). * indicates significant difference between male and female average EAG responses (Student t-test, P<0.05).

Discussion

Tritrophic interactions between infested plants, herbivorous arthropods and their natural enemies are complex because of the large number of semiochemicals that are typically involved. Several tritrophic systems were investigated in the past (e.g. De Moraes et al., 1998; De Boer et al., 2004; Almohamad et al., 2007) demonstrating that plants are able to manipulate their odours to interact (selectively) with natural enemies of the infesting herbivorous arthropods. Thus, plants that attract parasitoids and predators that respond to herbivore-induced plant volatiles will experience selective advantages. The role of plant secondary metabolites is indeed important for natural enemies of pests as they contribute to their orientation towards appropriate oviposition site (Bargen et al., 1998), rich in food for either adults and/or emerging larvae. Insect-plant interactions involving the genus Lycopersicon have been reviewed by Kennedy (2003). Several tomato species show good morphological and chemical adaptations to pest infestation, involving more than 20 defence-related proteins (Ryan, 2000). Although most research on tritrophic effects involving Lycopersicon has focused on parasitoids, effects involving predaceous arthropods have been documented as well (Kennedy, 2003). Our results complement the available studies on hoverfly-tomato interactions data.

Some aphidophagous syrphids are attracted and lay their eggs on some plant species in absence of aphid, suggesting the role of particular plant secondary metabolites on the oviposition site selection (Chandler, 1968). Several species respond with oviposition to aphid-produced honeydew (or other aphid remains), whereas in others, the aphid prey themselves are needed for oviposition to occur (Steidle and van Loon, 2002). Electroantennogram (EAG) study revealed antennae of female *Eupeodes corollae* (Diptera: Syrphidae) to be sensitive to some green plant volatile substances (6-carbon alcohols) (Hood Henderson and Wellington, 1982). Shonouda et al. (1998a,b) demonstrated an aphid-synomone, consisting of a group of long chain hydrocarbons, to stimulate the oviposition of *E. corollae* Fabricius females. Almohamad et al. (2007) explained the preference of *E. balteatus* for *Solanum tuberosum* over *S. niger* as oviposition site by the absence of the main component of the aphid alarm pheromone in the volatile blend of the latter plant species. Our results demonstrated the poor specificity of *E. balteatus* antennae, which are able to perceive the majority of the secondary metabolites produced by tomato plants.

These volatile chemicals consist mainly of monoterpenes and sesquiterpenes, which is in accordance with previous studies (Vercammen et al., 2001; Kant et al., 2004) that also demonstrated ß-phellandrene, 2-carene and α-phellandrene as the major tomato-released volatile chemicals. The same authors found linalool and indole to be released by *L. esculentum* specifically under cotton leafworm (*Spodoptera littoralis* B.) feeding. We did not isolate these two alcohols under aphid infestation. The composition of herbivore-induced plant volatiles depends on plant species and plant cultivar. But many plant species are also known to respond differently to different herbivore infestations (Gatehouse, 2002), especially when comparing different types of wounds such as those of mites, aphids or caterpillars (Dicke et al., 1998; De Boer et al., 2004). In our case, (*E*)-ß-farnesene only was emitted in case of aphid infestation. Because *M. persicae* is likely to emit (*E*)-ß-farnesene when disturbed, we can assume that this sesquiterpene is being released from the aphids. However, the infestation time (24hr) might be too short and could explain the absence of induced volatile emissions. Indeed, under spider mite infestation, *L. esculentum* plants change their volatile emissions after four days (Kant et al. 2004). However, E. balteatus was here shown to discriminate between healthy and infested tomato plants, which suggest (*E*)-ß-farnesene as a potential ovipositing stimulus.

The perception of (*E*)-ß-farnesene, the main component of the aphid alarm pheromone (Francis et al., 2005), differs between both sexes of adult *E. balteatus*. The fact that female antennae produce higher electrical depolarization exclusively to (*E*)-ß-farnesene demonstrates their richness in specialized olfactory sensillae and in corresponding receptors in the Syrphidae family (Hood Henderson, 1982). As females are likely to search for suitable oviposition site, this discrimination in the perception of (*E*)-ß-farnesene sounds justified. The aphid alarm pheromone seems therefore to be a key compound in prey-seeking behaviour in aphidophagous hoverflies. The two other identified sesquiterpenes, namely ß-caryophyllene and α-humulene, showed no antennal activities on *E. balteatus*. They are both common plant volatiles and ß-caryophyllene is also used by another aphid predator, the multicolored Asian Lady beetle *Harmonia axyridis*, as a potential component of its aggregation pheromone (Verheggen et al., 2007a).

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V.3

Aphid and Plant Secondary Metabolites Induce Oviposition in an Aphidophagous Hoverfly

Francois J. Verheggen[1]• Ludovic Arnaud[1] • Stefan Bartram[2] • Marie Gohy[3] • Eric Haubruge[1]

[1]*Department of Functional and Evolutionary Entomology*
Gembloux Agricultural University (Belgium)
[2]*Department of Bioorganic chemistry*
Max Planck Institute for Chemical Ecology (Germany)
[3] *Unité Technique COVs Qualité de l'air*
Institut scientifique de service public (Belgium)

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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*)-ß-farnesene (Eßf), 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 (Eßf). 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 behavioural 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 Eßf (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 behaviour of the tested females, whereas (Z)-3-hexenol and Eßf 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)-ß-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 behaviour of aphid natural enemies, that include : (1) aphid species and their associated chemicals (Budenberg and Powell, 1992; Bargen 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 (Bargen 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)-ß-farnesene (Eßf), 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 ß-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 behavioural activity of various plant and aphid volatile organic chemicals in order to highlight those that may act on the prey-seeking behaviour of *E. balteatus*, and how they could be included in modern integrated pest management methods.

Methods and Materials

Chemicals All chemicals, except Eßf, were purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany) and had chemical purity >97 % (determined by GC). Eßf 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±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, *Acyrthosiphon 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; $CaCl_2$: 0.21g/l; KCl : 0.35g/l; $NaHCO_3$: 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-cm^2 piece of filter paper that was impregnated with 10 µl 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}ng/µl to 10^{5}ng/µl (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.

Table 1. Description of the behavioural 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

Behavioural 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 *Acyrthosiphon 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 behavioural activity of three chosen semiochemicals: *(R)*-(+)-limonene (monoterpene), *(Z)*-3-hexenol (green leaf volatile), and Eßf (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 behaviour was then recorded for 10 min using the software The Observer5.0® (Noldus information Technology, version 5.0, Wageningen - The Netherlands) which allows hoverfly behaviour to be easily observed, subdivided and recorded (Harmel et al., 2007). Descriptions of the four observed behavioural 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 ± 1°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 *Dunnett's* test (comparison with a control) was used to compare the behavioural 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 behavioural 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).

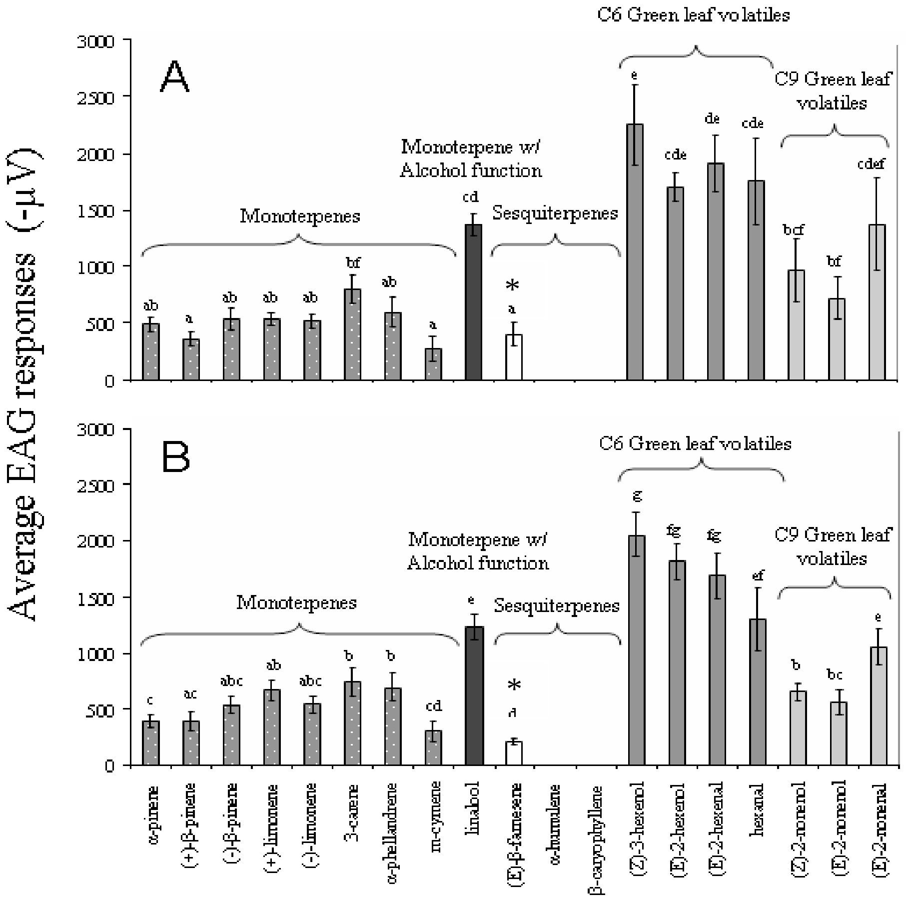

Figure 1 : EAG activity of female (A) and male (B) *Episyrphus balteatus* antennae to aphid and plant secondary metabolites (100µg/µl). Means (+/-SE) with no letter in common are significantly different (ANOVA followed by *Tukey's test, P*<0.05). An asterisk indicates significant difference in EAG activity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

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). Eßf 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 ß-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 -1750µV to -2250µV for the six-carbon chain GLV and from -600µV to -1400µV for the nine-carbons chain GLV.

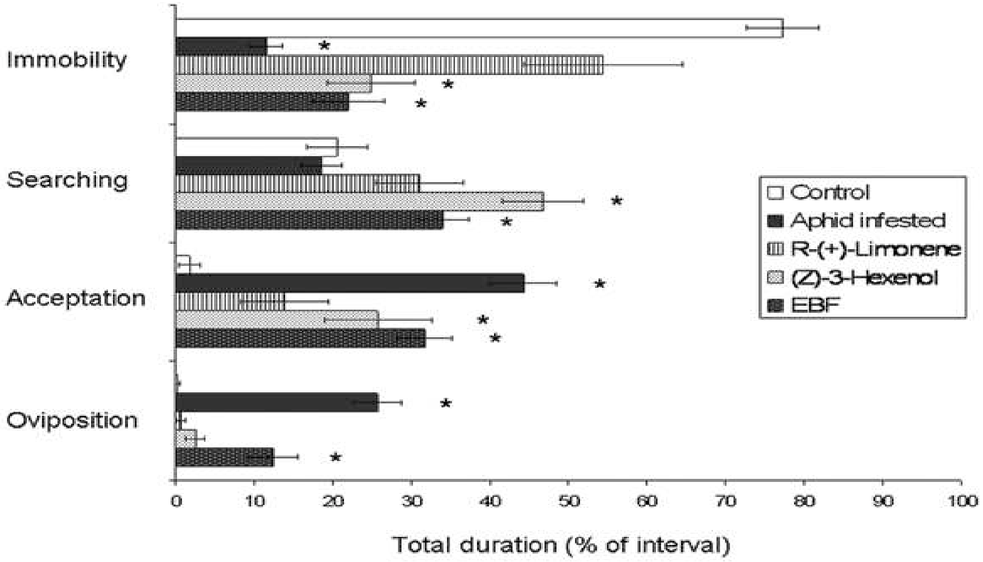

Figure 2: Effect of plant and aphid secondary metabolites on the foraging behaviour of *Episyrphus balteatus* females (Mean duration in % of interval, +/- SE). Stars indicate means that are significantly different from the non-infested semiochemical-free plant (ANOVA followed by *Dunnett's test, P*<0.05).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

Behavioural 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 behaviour 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 (Eßf) significantly increased hoverfly mobility, searching duration, acceptance of the host plant and oviposition behaviour. 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).

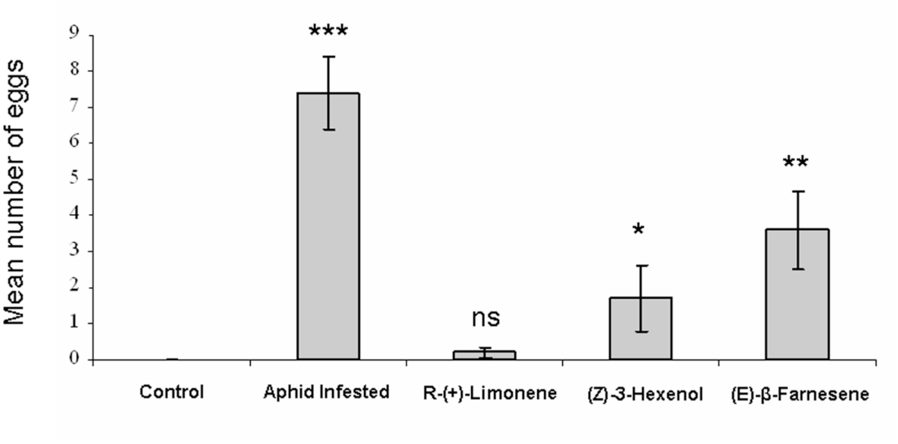

Figure 3: Effect of plant and aphid semiochemicals on the oviposition of *Episyrphus balteatus* females (Mean number of eggs +/- SE). The signs “ns”, “*”, “” and “***” indicate no significant and significant differences from control at P<0.05, P<0.01 and P<0.001 respectively (*1-sample Student t-test*).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

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 (Nevroptera, 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 behaviour 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 behaviour. 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 odours. 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 behaviour, 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 behavioural effect on a gravid hoverfly female.

Green leaf volatiles were previously thought to play a role in prey finding behaviour, 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.

Eßf, 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, Eßf 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 Eßf in hoverfly foraging behaviour, as females are looking for suitable oviposition site. Eßf antennal activity is also underlined when compared to the lack of response to two other tested sesquiterpenes (i.e. α-humulene and ß-caryophyllene) that are commonly released by plants or insects as semiochemicals (e.g. De Moraes et al., 2001; Brown et al., 2006). Whereas ß-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, Eßf is as volatile as ß-caryophyllene and α-humulene, but antennal activity was recorded. Eßf significantly increased hoverfly mobility, acceptance of the host plant, the oviposition behaviour, and the number of eggs laid (3.6 eggs/females). The aphid alarm pheromone is therefore a key compound in prey-seeking behaviour 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. Behavioural results obtained with a Eßf-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 EßF, but also secondary metabolites related to plant damages such as green leaf volatiles.

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V.4

Electrophysiological and Behavioural Responses of the Multicolored Asian Lady Beetle, *Harmonia axyridis* Pallas, to Sesquiterpene Semiochemicals

Francois J. Verheggen[1]• Quentin Fagel[1] • Stéphanie Heuskin[2] • Georges Lognay[2] • Frédéric Francis[1] • Eric Haubruge[1]

[1] Department of Functional and Evolutionary Entomology
[2]Department of Analytical Chemistry
Gembloux Agricultural University, Belgium

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Abstract – The role of two volatile sesquiterpenes, (*E*)-ß-farnesene and (–)-ß-caryophyllene, in the chemical ecology of the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, was investigated by using both electrophysiological and behavioural techniques. (*E*)-ß-Farnesene is the major component of the alarm pheromone of most aphid species, which are preyed on by *H. axyridis*. (–)-ß-Caryophyllene was previously isolated from the headspace volatiles above overwintering and aggregated *H. axyridis* females. These sesquiterpenes elicited significant EAG activity from both *H. axyridis* male and female antennae. In a four-arm olfactometer, male and female *H. axyridis* were highly attracted toward (*E*)-ß-farnesene, whereas only males were significantly attracted to (–)-ß-caryophyllene. In a bioassay technique that used a passively ventilated plastic box, both male and female *H. axyridis* aggregated in the (–)-ß-caryophyllene-treated side of the box. These results support the potential usefulness of (*E*)-ß-farnesene and (–)-ß-caryophyllene in push-pull strategies that use *H. axyridis* as a biological control agent in aphid-infested sites, or to control this new urban pest in residential structures.

Keywords – Aggregation pheromone • Behavioural assays • (–)-ß-Caryophyllene • Electroantennography • (*E*)-ß-Farnesene • Four-arm olfactometer • *Harmonia axyridis*

Introduction

Several insect species release aggregation pheromones to attract conspecifics for mating, optimizing resource use, or to aggregate in an environmentally favorable spot to overwinter. Aggregation pheromones, as well as sex pheromones, are essential components of integrated pest management methods, used in push-pull strategies to repel the target pests from a protected host, while luring them toward an attractive trap (Cook et al., 2007). Although the multicolored Asian lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), was introduced in Europe and North America as an effective natural enemy of various pests including aphids and mites, adverse effects of *H. axyridis* on nontarget insects, humans, and crops have been identified. Further, *H. axyridis* negatively impacts native coccinellids, as *H. axyridis* is the top predator in the aphidophagous guild. It occupies the ecological niches of endemic lady beetles and other aphid-specific predators such as the two-spotted lady beetle, *Adalia bipunctata* L., or the seven-spotted lady beetle, *Coccinella septempunctata* L. (Koch, 2003; Sato et al., 2005). *Harmonia axyridis* is also considered as a nuisance pest because it migrates from the field into houses and other structures when temperatures decline, and forms mass overwintering aggregations in elevated, dark, and concealed portions of structures (Huelsman et al., 2002). In addition to its invasive behaviour, *H. axyridis* when disturbed secretes hemolymph that imparts an unpleasant odour to any surfaces (walls, furnishings, etc.) where the beetles aggregate (Huelsman et al., 2002; Magnan et al., 2002). The secretions and the unpleasant odour can lead to allergic reactions in human occupants.

To develop efficient push-pull strategies, a better knowledge is required of the potential semiochemicals involved in the establishment and persistence of overwintering aggregations of *H. axyridis*. While observing *H. axyridis* aggregation sites, Nalepa et al. (2000) concluded that there was little evidence for volatile aggregation pheromones, and that contact chemoreception with conspecifics probably mediates the aggregation behaviour. However, Brown et al. (2006) reported the identification of (–)-ß-caryophyllene from the headspace of *H. axyridis* females under conditions that simulated natural overwintering conditions. (–)-ß-Caryophyllene is also commonly released from various plant families, including the Brassicaceae (Rohloff and Bones, 2005), Solanaceae (Farag and Paré, 2002) and Poaceae (Dean and De Moraes, 2006).

In order to demonstrate the potential role of (–)-ß-caryophyllene in the aggregation behaviour of *H. axyridis*, and the impact of (*E*)-ß-farnesene (major component of the aphid alarm pheromone) on the foraging behaviour of this aphid predator, we conducted both electrophysiological and behavioural experiments. These two methodological approaches have been used widely in the identification of lady beetle-related semiochemicals (Zhu et al., 1999; Al Abassi et al., 2000; Acar et al., 2001; Francis et al., 2001, 2004; Ninkovic et al., 2001).

Methods and Materials

Insects Larvae of *H. axyridis* were collected in May 2007 on the edges of a bean field and placed in aerated plastic boxes (up to 25 individuals per container). The larvae and the resulting adults were provisioned daily *ad libitum* with aphids, *Acyrthosiphon pisum* Harris, reared on beans. Sugar, water-impregnated cotton, and multi-flower pollen were also provided. The boxes were placed in controlled environment incubators (16 hr light photoperiod; 25±2°C; 70% RH). Males and females were separated at least 1 wk prior to electrophysiological and behavioural bioassays.

Electrophysiology (EAG) The *H. axyridis* antenna was carefully excised from the head, so that all the segments and the basal nerve were still attached. The antenna was mounted between two glass Ag-AgCl electrodes (Harvard Apparatus, Holliston, MA, USA; 1.5 mm o.d. x 1.17 mm i.d.) filled with saline solution (NaCl: 7.5 g/l; $CaCl_2$: 0.21g/l; KCl: 0.35 g/l; $NaHCO_3$: 0.2 g/l) and in contact with a silver wire. The base of the antenna was first inserted into the ground glass electrode. The tip of the recording electrode was bowl-shaped, and half of the last distal antennal segment was immersed into the saline solution. This setup was previously shown to produce elegant results while studying the olfactory responses of the confused flour beetle, *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae), to its aggregation pheromone (Verheggen et al., 2007). The DC potential was recorded on a computer (Auto Spike v. 3.0) by using an amplifier (IDAC-4, Syntech®, Hilversum, The Netherlands) with 100-fold amplification. A 0.5-cm^2 piece of filter paper that was impregnated with 10 µl of the chemical under examination was placed in a Pasteur pipette and used to puff an air sample in a constant 1.5 l/min airstream. As a negative control, the antennae were first stimulated with semiochemical-free filter paper (=mechanical stimulus). Later, the antennae were stimulated with (–)-ß-caryophyllene or (*E*)-ß-farnesene (Eßf). (–)-ß-Caryophyllene was purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany) and had a chemical purity of >97% (determined by GC). Eßf was synthesized from farnesol (Tanaka et al., 1975) and had a chemical purity of 98% (also determined by GC). Ten antennae were tested for each sex.

Four-arm Olfactometer Assays The four-arm olfactometer was similar to that previously described by Vet et al. (1983). It was constructed entirely of Teflon® and was closed with a removable glass roof, both previously cleaned with *n*-hexane. The walking arena was 40 cm wide (from center to odour source) and 1.5 cm high (from Teflon® walking arena to glass ceiling). Charcoal-filtered air was pushed in each of the four olfactometer arms through Teflon® tubing, and adjusted to 100 ml/min with a digital flowmeter. A pump ventilated the walking arena by removing air from the center at 400 ml/min. A 0.5 l glass chamber was connected to one of the four olfactometer arms, and was used to dispose of the odour source. Three stimuli were tested on both *H. axyridis* males and females: (1) The four arms of the olfactometer were connected to pure air sources, the glass chamber remained empty; (2) Twenty unwinged adult aphids, *Myzus persicae* Sultzer, were rapidly crushed inside of the glass chamber using a small glass pestle left inside the chamber [as a natural source of Eßf: According to previous studies, the volatiles released by crushed *M. persicae* consist exclusively of Eßf (Edwards et al., 1973; Francis et al., 2005)]; (3) A 0.5 cm^2 piece of filter paper was impregnated with 10 µl of (–)-ß-caryophyllene and placed inside the glass chamber. The glass chamber was randomly connected to one of the four arm of the olfactometer. Both the walking arena and the glass ceiling were washed with *n*-hexane after each lady beetle was tested. The olfactometer was divided into one central 10 cm squared area, and four other areas related to the four odour sources. The choice of the lady beetle was determined by (a) the first area it entered, (b) the first area where it stood for 30 consecutive sec, and (c) the area where the lady beetle stood at the end of the 3 min of observation (=last entered area). The behavioural observations were conducted in a laboratory at 22±1°C and under uniform lighting to avoid interference with behaviour of the test insects.

Aggregation Assays Three 5 cm diameter holes were cut into a 30 x 15 x 15 cm plastic box, and the holes were covered with metal screening. One hole was located on each lateral side (30 cm apart) and a third hole was located on the top. A 4 cm^2 piece of filter paper impregnated with 10 µl of (–)-ß-caryophyllene was attached to the screen cover of one of the lateral holes by using a rubber band. Ten males and 10 females *H. axyridis* were introduced in the box for 1 hr, and the side where they stood was recorded after 30 and 60 min. The behavioural observations were conducted under the same conditions as mentioned above.

Statistical Analyses A Student's *t*-test was performed to compare the mean EAG responses to the semiochemical stimuli with the EAG responses to the mechanical stimulant, as well as to compare EAG responses between the sexes. Observed frequencies related to the choice of *H. axyridis* in olfactometer assays (four-arm and aggregation bioassays) were compared to corresponding theoretical frequencies by using a χ^2 goodness-of-fit test. All statistical tests were conducted using Minitab® release 14.2.

Results

Electroantennography To the best of our knowledge, this is the first report of successful EAG recordings from *H. axyridis* antennae. Both pure Eßf and (–)-ß-caryophyllene elicited good activity from antennae of both sexes. Female and male antennal responses to the latter semiochemical were significantly higher than those produced mechanically (Student's *t*-test, $t_{obs}=4.89$, $P<0.001$ and $t_{obs}=5.49$, $P<0.001$, for females and males respectively). Male antennae were more sensitive to (–)-ß-caryophyllene (338±31 µV, $N=10$) than female antennae (178±19 µV, $N=10$) ($t_{obs}=4.41$, $P=0.001$).

The mean EAG responses to Eßf were significantly higher than those to mechanical stimuli ($t_{obs}=2.43$, P=0.027 and $t_{obs}=2.53$, $P=0.022$, for females and males, respectively). The mean EAG responses to Eßf was higher in males (202±17 µV) than in females (120±18 µV) ($t_{obs}=3.30$, $P=0.004$). (–)-ß-Caryophyllene induced higher EAG responses than Eßf ($t_{obs}=2.22$, $P=0.040$ and $t_{obs}=3.84$, $P=0.002$, for females and males, respectively).

Four-arm Olfactometer Assays In the four-arm olfactometer, Eßf (crushed *M. persicae* as a natural source), and (–)-ß-caryophyllene elicited significant behavioural activity from both *H. axyridis* males and females (Table 1). According to the three behavioural criteria that we observed and recorded, males were attracted to (–)-ß-caryophyllene. Females were less attracted than males to (–)-ß-caryophyllene, as significant results were only obtained while observing the last area entered (χ^2 = 7.92, *P*=0.048). Eßf attracted both male and female *H. axyridis* (Table 1).

Table 1. Behavioural responses[a] **of *Harmonia axyridis* to two sesquiterpenes**

Odor source	*H. axyridis* gender	Behavioral observations	Observed frequencies	χ^2	*P*
(-)-β-Caryophyllene	Female	First entered area	0.38	4.56	0.207
		First area entered for 30 sec	0.38	4.56	0.207
		Last entered area	0.42	7.92	0.048
	Male	First entered area	0.44	9.68	0.021
		First area entered for 30 sec	0.46	11.92	0.008
		Last entered area	0.44	9.84	0.020
(*E*)-β-Farnesene[b]	Female	First entered area	0.50	16.72	0.001
		First area entered for 30 sec	0.52	16.60	< 0.001
		Last entered area	0.54	22.80	< 0.001
	Male	First entered area	0.52	19.60	< 0.001
		First area entered for 30 sec	0.54	22.48	< 0.001
		Last entered area	0.58	29.20	< 0.001

[a] Observed frequencies (*N*=50) were compared to expected frequencies assuming random distribution (one odour source and three controls) by using a χ^2 test.

[b] Natural source of Eßf was twenty unwinged adult aphids, *Myzus persicae* Sultzer (crushed in the olfactometer release chamber).

Aggregation Assays After 30 min, both sexes of *H. axyridis* aggregated significantly on the (–)-ß-caryophyllene side of the box (χ^2 = 20.48, *P*<0.001 and χ^2 = 18.00, *P*<0.001, for males and females, respectively) (Fig. 1). Similar results were observed after 1 hr, as both males and females were still aggregated in the side of the box where (–)-ß-caryophyllene was released (χ^2 = 11.52, *P*=0.001 and χ^2 = 13.52, *P*<0.001, respectively).

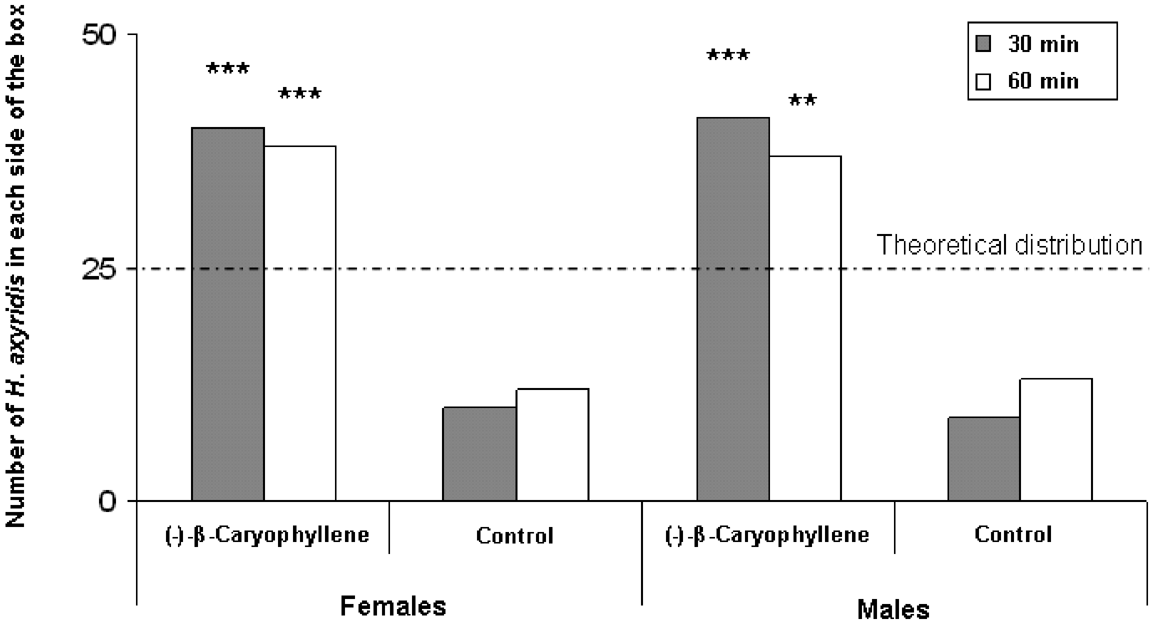

Figure 1 Observed distribution of female and male *Harmonia axyridis* (*N*=50 for each sex) relative to the release site of (–)-ß-caryophyllene in an aggregation bioassay. ** and * indicate significant differences between observed and expected distributions at P<0.01 and P<0.001, respectively (χ^2 goodness-of-fit test).**

Discussion

Comprehension of the chemical ecology of *H. axyridis* is essential before developing push-pull strategies for the efficient use of this lady beetle in aphid control or for the control of this new urban pest in structures.

(–)-ß-Caryophyllene and EßF are ubiquitous plant volatiles, and previous studies have demonstrated the role of plant semiochemicals in the searching behaviour of aphid natural enemies (Tumlinson et al., 1992). In our experiments, (–)-ß-caryophyllene and Eßf elicited antennal activity in both sexes, and significant attraction in a four-arm olfactometer. It has been suggested that lady beetles orientate toward aphid prey by discriminating aphid punctual and important Eßf emission from the continuous plant release of Eßf, usually joined with (–)-ß-caryophyllene emission (Dawson et al., 1984). The latter has also been previously shown previously to counteract the attraction of lady beetles to Eßf, acting therefore as a natural alarm pheromone inhibitor, allowing the beetles to differentiate between Eßf of plant and aphid origin. As *H. axyridis* releases its aggregation pheromone at the end of autumn, when the temperatures get lower and aphids are present in lower densities, no inhibition happens.

Our results demonstrated that (–)-ß-caryophyllene and Eßf act as semiochemicals for *H. axyridis*. Indeed, (1) there are some specific neuronal receptors that allow their perception, and (2) a behavioural attraction/aggregation was noted in the two behavioural assays. Al Abassi et al. (2000) obtained similar electrophysiological results while studying *Coccinella septempunctata* olfactory cell responses toward the same two sesquiterpenes: Cells having high specificity for (–)-ß-caryophyllene and Eßf were identified. In the twelve-spotted lady beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae), the highest EAG responses were obtained while testing Eßf, (–)-ß-caryophyllene, and two other terpenoid alcohols (Zhu et al., 1999). Therefore it is not surprising that *H. axyridis*, which also belongs to the Coccinellidae, possesses similar specific receptors.

(–)-ß-Caryophyllene is specifically released by *H. axyridis* females in overwintering aggregation conditions (Brown et al., 2006). The production of (–)-ß-caryophyllene by females of *H. axyridis* was confirmed in the present study by volatile collection and subsequent GC-MS analysis (data not shown). Whereas cases of aggregation pheromone release by one sex only are common in Coleopterans (e.g., Verheggen et al., 2007), the use of a chemical commonly found in the headspace of various plants as an aggregation pheromone might be inappropriate. One might put forth the assumption that lady beetles have different antennal sensitivity to (–)-ß-caryophyllene according to the season, being more sensitive during winter conditions. (–)-ß-Caryophyllene has an attractive effect on *H. axyridis,* although the responses of the females in the four-arm olfactometer were significant in only one of the three behavioural observations. However, in the second set of behavioural assays, the distribution of both males and females in the (–)-ß-caryophyllene-treated side of the box was significant. These behavioural data, the antennal perception of (–)-ß-caryophyllene, and its specific period of release (i.e., in the early winter) support the hypothesis that it acts as a component of an aggregation pheromone rather than a sex pheromone.

Like other lady beetle species, including *A. bipunctata* (Hemptinne et al., 2000; Francis et al., 2004), *H. convergens* (Acar et al., 2001) and *C. septempunctata* (Nakamuta, 1991; Al Abassi et al., 2000), *H. axyridis* is able to perceive and orientate toward Eßf, the main component of the aphid alarm pheromone. Our electrophysiological and behavioural results support previous research that demonstrated that *H. axyridis* has a high ability to track aphid populations in space and time (Osawa, 2000). Indeed, this aphid predator showed fast and pronounced orientation behaviour toward the Eßf source in the four-arm olfactometer. Having the ability to localize aphids under predation represents an undeniable advantage for a mobile and voracious predator like *H. axyridis*. Although previous studies demonstrated that *H. axyridis* responded to volatiles from aphids and aphid-damaged plants (Han and Chen, 2002), our results do not support previous work that demonstrated that *H. axyridis* was attracted toward a colony of *A. pisum*, but not toward cornicle secretions (Mondor and Roitberg, 2000), which are known to contain Eßf (Francis et al., 2005).

In conclusion, push-pull strategies aiming the use of *H. axyridis* as biological control agent in aphid infested sites, or designed to control this new urban pest in human dwellings, should take into account the potential attractive effect that this newly found aggregative chemical, named (–)-ß-caryophyllene, could have in outdoor conditions. Our data also suggest that in aphid biological control strategies, one should incorporate Eßf as essential chemical attractant for various aphid natural enemies, including *H. axyridis*.

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Chapter VI

(E)-ß-farnesene in myrmecophilous aphids – ants interactions

GENERAL INTRODUCTION TO CHAPTER VI

The mutualistic relationships that occur between myrmecophilous aphids and ants are based on the rich food supply that honeydew represents for ants and on the protection they provide against aphid natural enemies. While aphid predators and parasitoids actively forage for oviposition sites by using aphid semiochemicals, scouts of aphid-tending ant species would also benefit from locating honeydew resources by orienting toward aphid pheromone sources. The aphid alarm pheromone was previously demonstrated to alert tending ant soldiers that dispose of attacking predators, but no study addressed the potential impact (E)-ß-farnesene may have on scouts.

We decided to focus our research on the mutualism between the black bean aphid *Aphis fabae* (Homoptera: Aphididae) and the black garden ant *Lasius niger* (Hymenoptera: Formicidae). Both species occur in Belgium and are largely distributed in Europe. They are also often used as model species for the study of aphid-ant mutualism. In this chapter, we first realized a field study where we quantified the beneficial impact this mutualism may have for *A. fabae*. Secondly, we tried to shed light on the potential behavioral impact that (E)-ß-farnesene, the aphid alarm pheromone, may have on *L. niger* scouts while looking for sugary resources.

VI.1

Mutualisme pucerons-fourmis : étude des bénéfices retirés par les colonies d'*Aphis fabae* en milieu extérieur

François Verheggen(1)*, Lise Diez(1), Claire Detrain(2), Eric Haubruge(1)

[1] *Unité d'Entomologie fonctionnelle et évolutive, Faculté universitaire des Sciences agronomiques de Gembloux, Passage des Déportés 2, B-5030 Gembloux (Belgique)*
[2] *Unité d'Ecologie sociale, Université libre de Bruxelles, CP231, boulevard du Triomphe, 1050 Bruxelles*

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Résumé - La relation de coopération entre pucerons-fourmis est un bel exemple de mutualisme dans le règne animal, les premiers cherchant protection et hygiène, les seconds une source de sucres nécessaires à la survie de la colonie. La présente étude s'est intéressée à recenser les bénéfices retirés par *Aphis fabae* Scopoli (Homoptera, Aphididae) de ses relations de mutualisme avec *Lasius niger* L. (Hymenoptera : Formicidae). Plusieurs paramètres ont été observés en milieu extérieur sur des plants de fèves des marais infestés initialement par 100 individus en présence ou non d'une colonie de *L. niger*. En présence de fourmis, les plantes étaient constamment infestées par un nombre de pucerons plus important, et la proportion d'individus ailés y était également similaire ou plus grande, selon la date d'observation. Un nombre moins important de prédateurs aphidiphages sur les plantes en présence de fourmis a permis d'expliquer en partie ces observations. Les nombres moyens de pucerons parasités ne différaient pas, que les plantes soient explorées ou non par les fourmis. Cela suggére que *L. niger* est peu efficace face aux attaques de parasitoïdes. Par contre, très peu de pucerons appartenant à des espèces différentes d'*A. fabae* ont été observés sur les plants mis en présence des fourmis. Ces observations suggèrent que *L. niger* adopte un comportement de prédation sur les pucerons avec lesquels elle n'entretient aucune relation mutualiste. Les observations menées n'ont pas permis de mettre en évidence un quelconque effet des fourmis sur la vigueur des plantes hôtes des pucerons, bien que sensiblement moins d'exuvies et de taches de miellat étaient présentes sur les plantes dont les colonies de pucerons étaient visitées par *L. niger*. L'ensemble de ces résultats confirme que *L. niger* améliore les conditions de vie des colonies de pucerons d'*Aphis fabae* dont elle exploite le miellat, principalement grâce à la protection qu'elle apporte contre les prédateurs et la réduction de la pression de compétition exercée par les autres espèces non myrmécophiles de pucerons.

Mots-clefs – *Aphis fabae*, *Lasius niger*, mutualisme pucerons – fourmis, balance coûts-bénéfices

Aphid-ant mutualism: an outdoor study of the benefits for *Aphis fabae*

Abstract - Aphid-Ant relationships are common examples of mutualism. Aphids are indeed submitted to predation and therefore require protection, while ants are continuously looking for new sugar sources. The present work aimed to study the benefits that a mutualistic relationship with *Lasius niger* (Hymenoptera : Formicidae) could bring to the black bean aphid *Aphis fabae* (Homoptera, Aphididae). Several parameters were observed in the field, on broad bean plants infested with an initial amount of 100 *A. fabae* and in presence or not of a *L. niger* colony. More aphids were observed on plants being visited by ants as well as a higher proportion of winged individuals. One explanation is that fewer predators were observed on plants being visited by ants, demonstrating their protective role. However, the number of parasitized aphids was not reduced in presence of *L. niger*. On the other hand, fewer different aphid species were present on plants foraged by ants, what suggests that they could exert a predation on unattended aphids. Our observations do not allow to conclude on any impact of *L. niger* on the fitness of the aphid host plant, although fewer exuvia and honeydew spots were observed when they were present. All these results confirm that *L. niger* increase the fitness of *A. fabae* colonies mainly by decreasing the number of predators and by reducing competition from aphid species unattended by ants.

Keywords – *Aphis fabae*, *Lasius niger*, Aphid-ant relationship, Cost-benefit analysis

Introduction

Les interactions entre espèces sont généralement classées par les spécialistes en catégories telles que la compétition, la prédation, le parasitisme et le mutualisme (Cushman et Addicott 1991). Les termes servant à parler de mutualisme sont divers : on parle de symbiose, de commensalisme, de coopération, d'aide mutuelle, de facilitation, d'altruisme réciproque et d'entraide (Boucher et al., 1982). Le mutualisme peut être défini comme une association à bénéfices réciproques pour deux organismes (Boucher et al., 1982). La mise en place d'une telle relation dépend des avantages que chacun peut apporter à l'autre, mais aussi des coûts que le mutualisme entraîne (Way, 1963). Des études théoriques suggèrent que le mutualisme s'applique uniquement à des situations où le coût de maintien de ce système est faible pour chacun des participants et où les bénéfices sont relativement importants (Bristow, 1991).

La coopération entre les groupes des pucerons et des fourmis est un exemple bien connu de mutualisme puisqu'il s'apparente à la relation homme-bétail : un apport nutritionnel est échangé contre protection et entretien (Dixon, 1985 ; Stadler & Dixon, 2005). En Europe, on estime qu'un tiers des espèces d'aphidés ne sont pas soignées par les fourmis, un tiers à une relation de mutualisme facultatif et un tiers à un mutualisme obligatoire (Stadler & Dixon, 1998). La balance entre coûts et bénéfices a fait pencher maintes fois la relation dans l'une ou l'autre direction et la diversité actuelle des espèces dans ces groupes et de leurs relations illustrent bien la multitude des facteurs qui entrent en compte (Stadler & Dixon, 2005).

Alors que les fourmis y trouvent une source de sucres nécessaires à la survie de leur colonie, les pucerons retirent de nombreux bénéfices d'une telle relation et ont ainsi tout intérêt à la faire perdurer (Banks, 1962 ; Dixon, 1985). C'est ainsi qu'en présence de l'autre, chaque espèce manifeste des changement de comportement, voir de morphologie (Buckley, 1987). Certaines espèces de pucerons augmentent la quantité de phloème ingérée et adaptent alors la quantité et la qualité de leur miellat afin de satisfaire les demandes des fourmis (Völkl et al., 1999 ; Fischer & Shingleton, 2001 ; Yao and Akimoto, 2001). Suite à la palpation que les fourmis pratiquent avec leurs antennes sur le corps des pucerons, ceux-ci excrètent des gouttes de miellat qu'ils évitent alors d'éjecter, afin de faciliter leur récolte par les fourmis (Sudd, 1967). En échange, les fourmis change leur comportement initial de prédateurs pour devenir éleveuses de pucerons (Huber 1810) et tendre à améliorer leurs santé et durée de vie, les protégeant contre leurs nombreux prédateurs (Pontin 1959 ; Yao et al 2000) et participant activement à l'hygiène de la colonie (Way 1963).

Les fourmis augmentent généralement la durée de vie des colonies de pucerons qu'elles soignent (Bristow 1984 ; Mahdi and Whittaker 1993 ; Sloggett & Majerus, 2000). Cependant les études dévouées à la mise en évidence des bénéfices retirés par les colonies de pucerons souffrent d'être réalisées en conditions de laboratoire. L'objectif de la présente étude est d'évaluer en milieu extérieur l'impact de la présence de *Lasius niger* L. (Hymenoptera : Formicidae) sur le bien-être de colonies d'*Aphis fabae* Scopoli 1763 (Homoptera, Aphididae), par le biais d'observations de terrain en condition semi-contrôlées.

Matériel et méthodes

Matériel biologique – Les plants de fève des marais (*Vicia faba* L.) proviennent de semences mises à germer dans des pots de 9x9x10 cm à raison de 8 graines par pot. Après germination, les plantes sont repiquées individuellement dans des pots de taille identique. Le substrat est composé de perlite et de vermiculite dans le rapport 1:1, permettant à la fois l'aération du milieu et la conservation de l'humidité apportée par un arrosage pratiqué trois fois par semaine. L'élevage des pucerons (*A. fabae*) est pratiqué par infestation naturelle à partir d'une plante âgée infestée vers des plantes saines disposées dans la même cage. La culture des fèves et l'élevage des pucerons sont réalisés dans des pièces séparées et climatisées, à une température de 23 ± 3°C et une humidité de 70 ± 5%.

La récolte de la fourmilière de *L. niger* a été réalisée sur le campus de la Plaine de l'ULB à Bruxelles le 18 avril 2007 par temps ensoleillé. Une motte de terre de 20 cm de diamètre environ avec herbe et racines est prélevée et désagrégée pour découvrir certaines chambres de la fourmilière. Les fourmis et la terre sont placées dans un bac dont les bords sont fluonés (polytetrafluoroéthylène) afin d'empêcher toute fuite des ouvrières. L'excavation est ensuite examinée afin de localiser les chambres de la fourmilière où est logé le couvain, qui est alors placé dans le même bac. Afin de conserver la fourmilière jusqu'au début de l'expérimentation, des tubes d'eau et de sucre sont ajoutés pour compléter les besoins nutritionnels. L'apport en protéines est constitué d'insectes frais ou congelés : deux fois par semaine, drosophiles, blattes ou de vers de farine sont déposés dans le bac.

Dispositif expérimental – Sur un terrain de 11,4 x 9,7 m fraichement labouré, un traitement herbicide a été pratiqué 15 jours avant le début de l'expérimentation. Seize plants de *V. faba* sont disposés en deux cercles concentriques, à 0,5 et 1,50 m de rayon, à raison de 8 plants sur chaque cercle (Figure 1). Un dispositif de 16 plants est mis en présence d'une fourmilière de *L. niger*, un autre dispositif de 16 plants est placé en l'absence de fourmilière en son centre. Les plantes sont placées dans des pots de 13x13x13 cm contenant 3 à 4 cm de billes d'argex dans le fond et de la terre prise dans la parcelle expérimentale. Chaque pot placé autour de la fourmilière de *L. niger* est disposé dans un bac en plastique. Celui-ci est rempli de cailloux et de billes d'argex afin de permettre le passage facile des fourmis du sol vers les pots. L'infestation par *A. fabae* est réalisée manuellement sur des plantes âgées d'une semaine. Sur chaque plante, 100 adultes aptères ont été introduits au début de l'expérience. Les pots témoins sont placés dans des bacs similaires remplis d'eau, pour éviter le passage de fourmis vers les plantes.

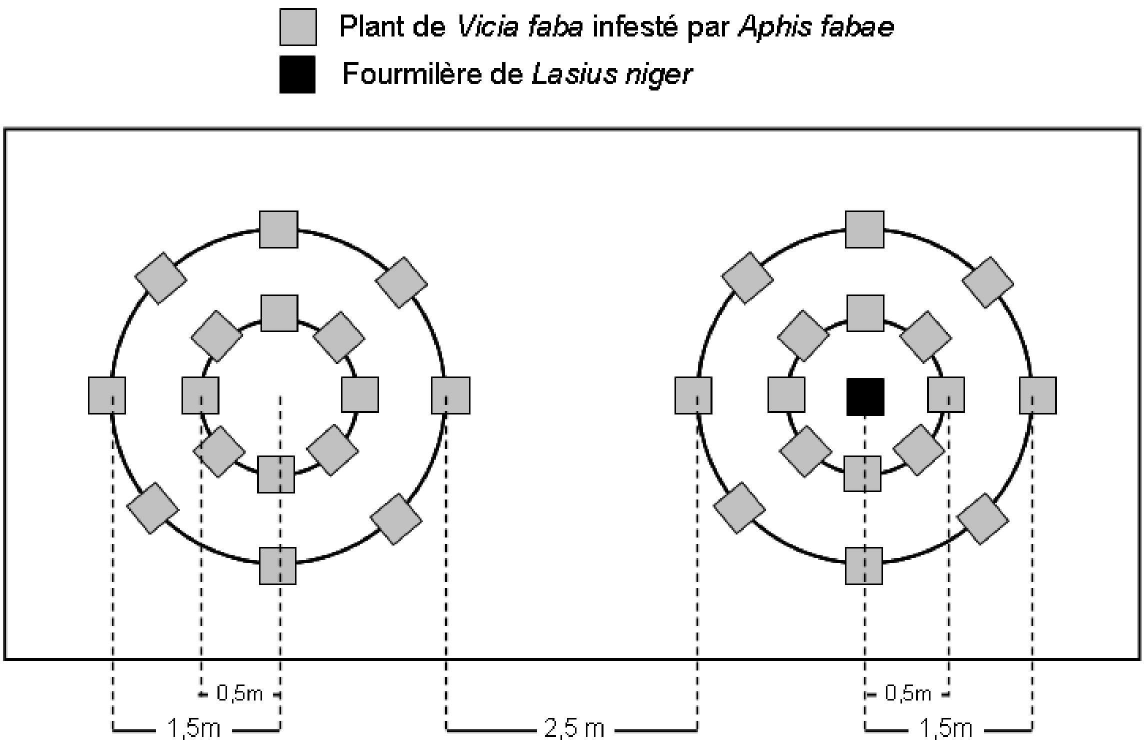

Figure 1. Dimensions de la parcelle expérimentale et disposition des plants de *Vicia faba* infestés par *Aphis fabae*

Observations réalisées – Afin de mettre en évidence l'effet de la présence de fourmis sur la l'hygiène et le développement de colonies d'*A. fabae*, différentes observations (présentées ci-dessous) ont été réalisées sur les acteurs biologiques suivants : (1) *A. fabae*, (2) les espèces d'autres pucerons, (3) les prédateurs et parasitoïdes présents, (4) les plants de *V. faba* et (5) les fourmis elles-mêmes. Les observations ont été réalisées une fois par semaine du 09 mai au 20 juin 2007. Les hauteurs des plants de *V. faba* ont été mesurées et une évaluation de leur vigueur a été établie sur base d'observations visuelles portant sur la présence de rouilles, nécroses, chloroses et autres dégâts dus à la présence de phytophages. Les colonies d'*A. fabae* ont été caractérisées sur base (1) du nombre de pucerons, (2) de la proportion de pucerons ailés, (3) de la proportion de pucerons momifiés et (4) de la présence d'autres espèces d'aphidés. Les prédateurs et parasitoïdes présents sur les plants ont également été comptés et identifiés. Enfin, le nombre de fourmis présentes sur la plante et se nourrissant des nectaires extrafloraux a également été noté.

Analyse statistique – L'analyse des données a été réalisée à l'aide du logiciel MINITAB v.14 par analyse de la variance à un facteur fixe (présence de fourmis), séparément pour chaque date d'observation. Les tests de corrélation de Pearson ont également été réalisés.

Résultats et discussions

3.1. La présence de fourmis influence-t-elle le développement des colonies de pucerons ?

Taille des colonies de pucerons – L'observation de la figure 2 montre l'évolution au cours du temps du nombre moyen d'*A. fabae* présents sur les plants de *V. faba* en fonction de la présence ou l'absence de *L. niger*. Le nombre d'*A. fabae* est supérieur ou égal sur les plantes avec fourmis tout au long des sept semaines d'observation. L'analyse de la variance montre en effet que les pucerons soignés par les fourmis sont plus nombreux le 9 mai (ANOVA, $F_{1,31}$=4,97 ; P=0,030), le 30 mai (ANOVA, $F_{1,31}$=14,25 ; P=0,001) et le 6 juin (ANOVA, $F_{1,31}$=5,15 ; P=0,031). Les fourmis semblent donc favoriser la croissance des colonies de pucerons. Cette conclusion est en accord avec l'étude de El-Ziadi & Kennedy (1956) qui avaient déjà mis en évidence que les colonies d'*A. fabae* étaient de plus grandes tailles en présence de fourmis en conditions naturelles. Par contre, Stadler et al. (2002) ont obtenu des résultats opposés. Cependant, cette dernière étude a été réalisée en laboratoire où les pucerons soignés n'ont donc jamais été attaqués par leurs ennemis naturels. La reproduction d'*A. fabae* est favorisée par la présence de fourmis si les bénéfices qu'elle entraîne sont supérieurs aux coûts engendrés. Dans le cas présent, comme les pucerons qui ont été soignés par les fourmis sont plus nombreux, on peut supposer que les bénéfices retirés par ces dernières ont été supérieurs aux coûts occasionnés par la protection et l'entretien d'une telle colonie de pucerons. Aucune différence de taille de colonie n'a été observée entre les deux cercles concentriques autour de la fourmilière.

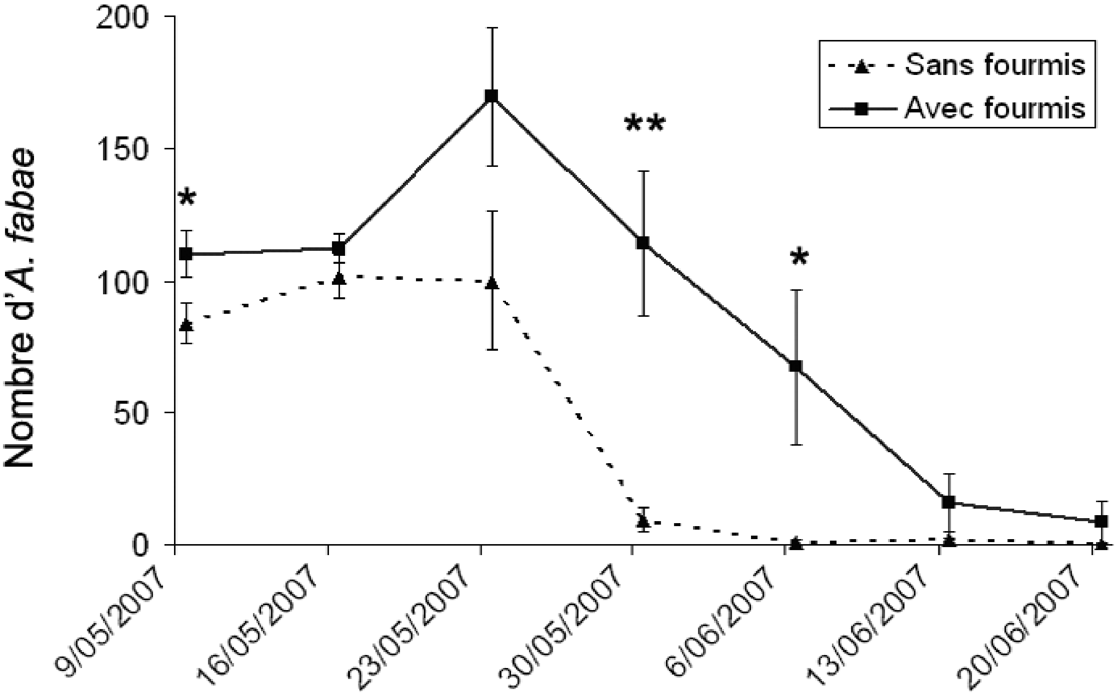

Figure 2 Évolution au cours du temps du nombre moyen d'*Aphis fabae* (Moyenne ± Erreur standard) présents sur les plants de *Vicia faba* en fonction de la présence ou l'absence de *Lasius niger.* Les moyennes surmontées de * et ** sont significativement différentes avec P<0.05 et P<0.01 respectivement

Proportion de pucerons ailés - L'évolution de la proportion de pucerons ailés au cours des semaines d'observation est présentée à la figure 3. La proportion de pucerons ailés est globalement supérieure dans le cas des plantes avec fourmis mais la différence n'est significative qu'à la date du 30 mai (ANOVA, $F_{1,31}$=13,79 ; P=0,001). Selon de nombreuses études antérieures (El-Ziady & Kennedy, 1956 ; Johnson, 1959 ; Hölldobler & Wilson, 1990), la production d'ailés est retardée dans la saison par la présence de fourmis. En effet, on observe sur le graphique un décalage d'une semaine de la proportion maximale de pucerons ailés en présence de fourmis sur les plantes infestées. Les pucerons ailés étant généralement produits en plus grandes proportions en conditions défavorables, comme la présence répétée de prédateurs ou la baisse de la qualité ou de la quantité de nourriture, il aurait été normal de constater également un nombre d'ailés moins important en présence de fourmis. Cependant, la densité des populations d'*A. fabae* étant plus importante en présence de fourmis, et la production de formes ailées étant positivement corrélée à la densité des populations (Kunert et al 2005), il n'est pas anormale de retrouver une proportion d'ailés supérieure en présence de fourmis. Nos résultats confirment ceux obtenus par El-Ziady & Kennedy (1956), qui expliquent la stabilisation de la proportion d'individus ailés comme le résultat engendré par l'ensemble des bénéfices accordés par la présence de fourmis, qui tendent également à augmenter la taille et la vigueur de la colonie de pucerons.

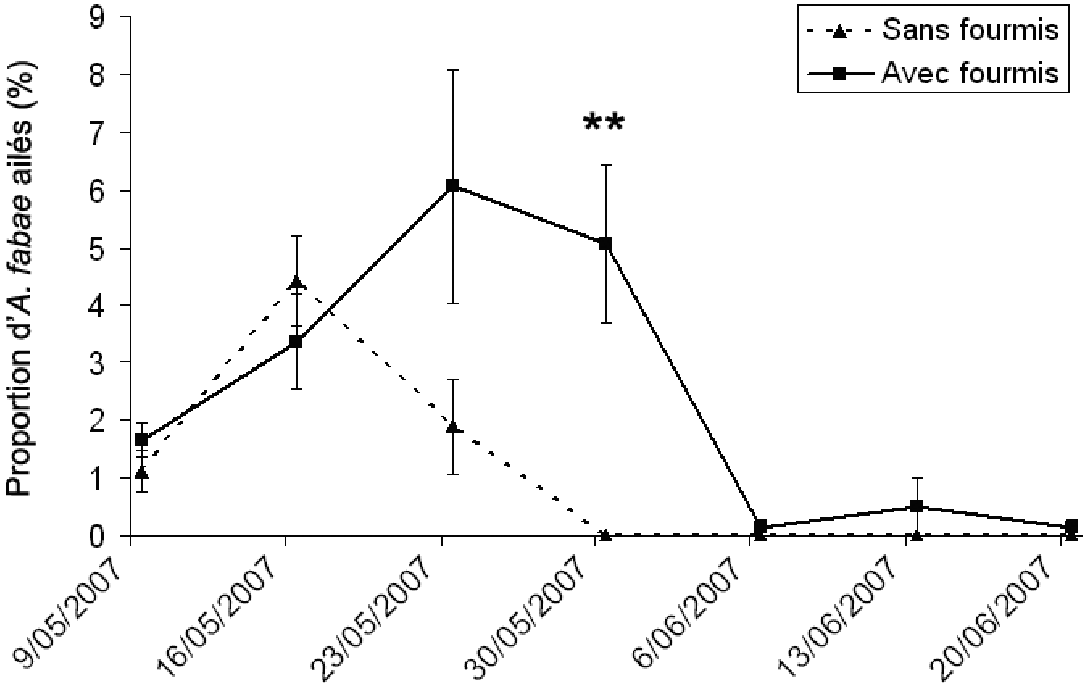

Figure 3 Évolution au cours du temps du nombre moyen d'*Aphis fabae* ailés (Moyenne ± Erreur standard) présents sur les plants de *Vicia faba* en fonction de la présence ou l'absence de *Lasius niger.* Les moyennes surmontées de ** sont significativement différentes avec P<0.01

3.2. Comment les fourmis influencent-t-elles le développement des colonies de pucerons ?

Prédateurs - Les résultats précédents montrent le rôle des fourmis sur le nombre de pucerons composant les colonies et sur la proportion d'individus ailés. Il serait intéressant de mettre en évidence les facteurs sur lesquels les fourmis ont une influence.

Les espèces de prédateurs aphidiphages ont été recensées et dénombrées. Trois espèces de syrphes ont été observées : *Syrphus ribesii* L., *Metasyrphus corollae* Fabricus et *Episyrphus balteatus* De Geer. Les espèces *Propylea quatuordecimpunctata* L., *Calvia decemguttata* L., *Coccinella septempunctata* L. (Coleoptera : Coccinellidae) ont été également observées. Le site expérimental étant placé à proximité (<10m) de l'arboretum de la Faculté des Sciences agronomiques de Gembloux, il n'est pas étonnant de retrouver certaines espèces plus forestières comme *C. decemguttata*.

Le nombre moyen de prédateurs observés sur une plante infestée d'*A. fabae* en présence ou non de fourmis est présenté à la figure 4. On peut observer que le nombre de prédateurs présents sur les plantes visitées par les fourmis est resté, durant toute la durée des observations, égal ou inférieur au nombre de prédateurs sur les plants sans fourmi. La différence entre le nombre de prédateurs observés sur les plantes avec et sans pucerons se révèle significative à la date du 16 mai (ANOVA, $F_{1,31}$=6,97 ; P=0,013). Le nombre étonnamment élevé de prédateurs sur les plantes sans fourmi du 06 juin ne peut être interprété : le nombre de pucerons sur les plantes à cette date étant très bas. Néanmoins, la présence de miellat sur feuilles et tiges pourrait expliquer l'attraction de ces prédateurs aphidiphages.

Ces résultats accordent d'autant plus d'importance au rôle des fourmis que le nombre de pucerons était toujours supérieur sur les plantes visitées par celles-ci. En effet, on s'attendrait à observer un nombre plus important de prédateurs là où le nombre de proies est plus important. Or ce ne fut pas le cas. Ces résultats confirment d'autres études telles que celles de El-Ziadi & Kennedy (1956) et de El-Ziadi (1960) qui concluent que les fourmis ont tendance à faire diminuer le nombre de prédateurs sur les plantes où elles sont présentes.

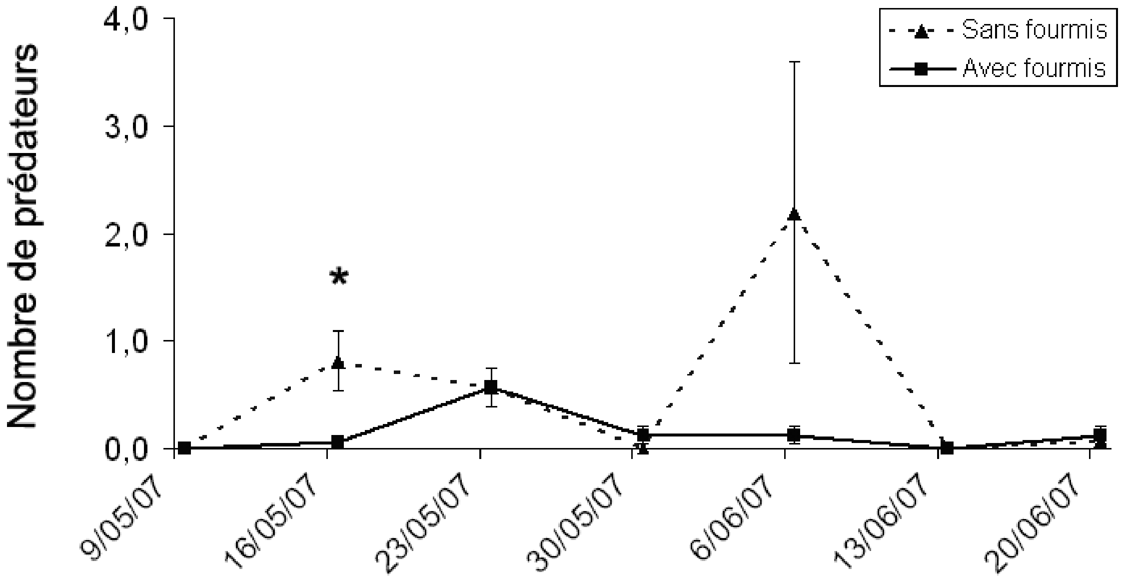

Figure 4 Nombre moyen de prédateurs aphidiphages (Moyenne ± Erreur standard) (tous stades de développement confondus) observés sur un plant de *Vicia faba* infestés d'*Aphis fabae* en fonction de la présence ou l'absence de *Lasius niger*. Les moyennes surmontées de * sont significativement différentes avec P<0.05

Parasitoïdes - Les espèces de parasitoïdes ont été recensées, et la proportion de pucerons parasités a été calculée lors de chaque semaine d'observation (Figure 5). Les espèces de parasitoïdes identifiées sont *Aphidius ervi* Haliday, *Praon volucre* Haliday, *Lysiphlebus fabarum* Marshall, *Lysiphlebus testaceipes* Cresson et *Adialytus ambiguus* Haliday. La proportion de pucerons parasités est restée inférieure à 10% sauf en date du 30 mai où cette même proportion s'est accrue tant au niveau des plantes non-visitées que de celles visitées par les fourmis. L'analyse de la variance ne montre pas de différence significative pour aucune des sept dates. Les fourmis ne procureraient donc pas de protection des pucerons vis-à-vis des parasitoïdes.

Malgré l'absence de différence significative, on constate que le nombre de pucerons momifiés est supérieur en présence de fourmis, à la date du 30 mai. Les parasitoïdes ont en effet développé des comportements de défense qui leur permet de pratiquer l'oviposition malgré tout (Kaneko, 2003). De plus, la même étude a démontré que la présence de fourmis, notamment *L. niger*, favorisait l'émergence de parasitoïdes adultes en repoussant notamment les prédateurs intraguildes, en protégeant les pucerons parasités encore vivants des prédateurs et en empêchant l'oviposition d'hyperparasitoïdes (Kaneko, 2003).

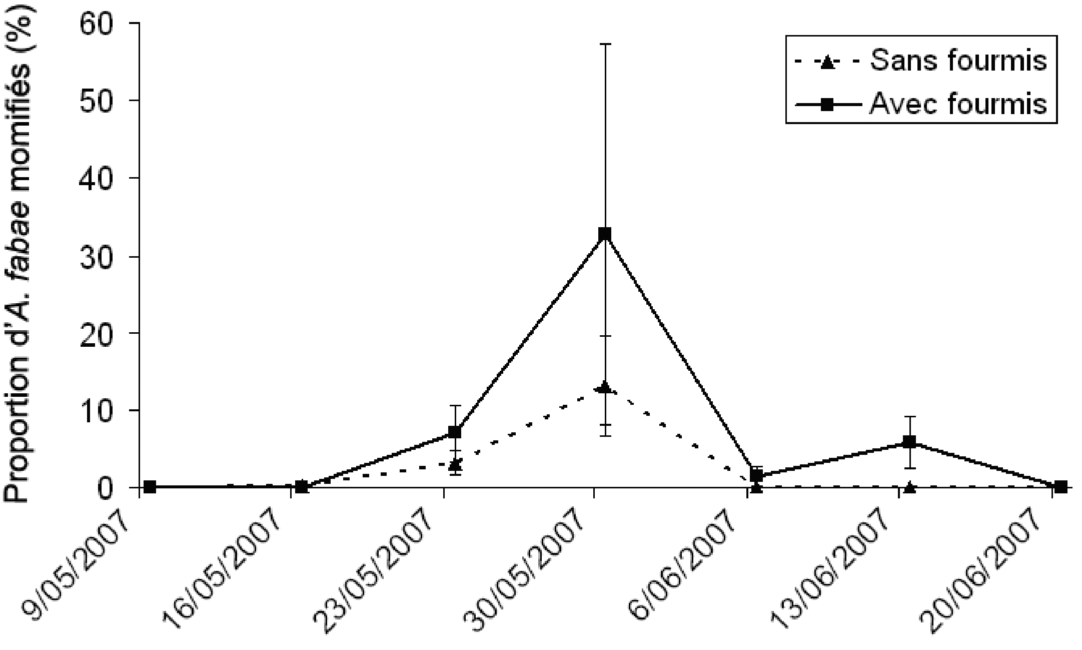

Figure 5. Nombre moyen d'*Aphis fabae* momifiés (Moyenne ± Erreur standard) observés sur un plant de *Vicia faba* en fonction de la présence ou l'absence de *Lasius niger*

Autres espèces de pucerons – D'autres espèces de pucerons ont été dénombrées et déterminées. Quatre espèces supplémentaires ont ainsi été identifiées : *Acyrthosiphon pisum* (Harris), *Megoura viciae* (Buckton), *Macrosiphum euphorbiae* (Thomas) et *Aulacorthum solani* (Kaltenbach). Certains pucerons n'ont pas pu être identifiés, en raison de leur stade de développement précoce au moment des observations.

On remarque à la figure 6 que presque aucun puceron d'espèces différentes d'*A. fabae* n'a été observé sur les plantes visitées par les fourmis. De petites colonies de pucerons d'espèces précitées ont été décelées sur plusieurs plantes sans fourmis alors qu'aucune colonie (à l'exception de deux pucerons ailés isolés) n'a été observée sur les plantes visitées par les fourmis. L'explication qui peut être donnée à ces différences est celle de la prédation des fourmis sur les pucerons d'espèces autres qu' *A. fabae*. Les espèces observées durant l'expérience ne sont pas myrmécophiles (Engel et al, 2001 ; Almehdi, communication personnelle), les fourmis présentes ont donc probablement supprimé ces espèces au profit d' *A. fabae*. Une autre hypothèse consiste à expliquer l'absence de pucerons d'espèces différentes comme le résultat de compétitions interspécifiques entre pucerons, où la prospérité des colonies myrmécophiles prendrait le dessus sur l'installation de nouvelles colonies.

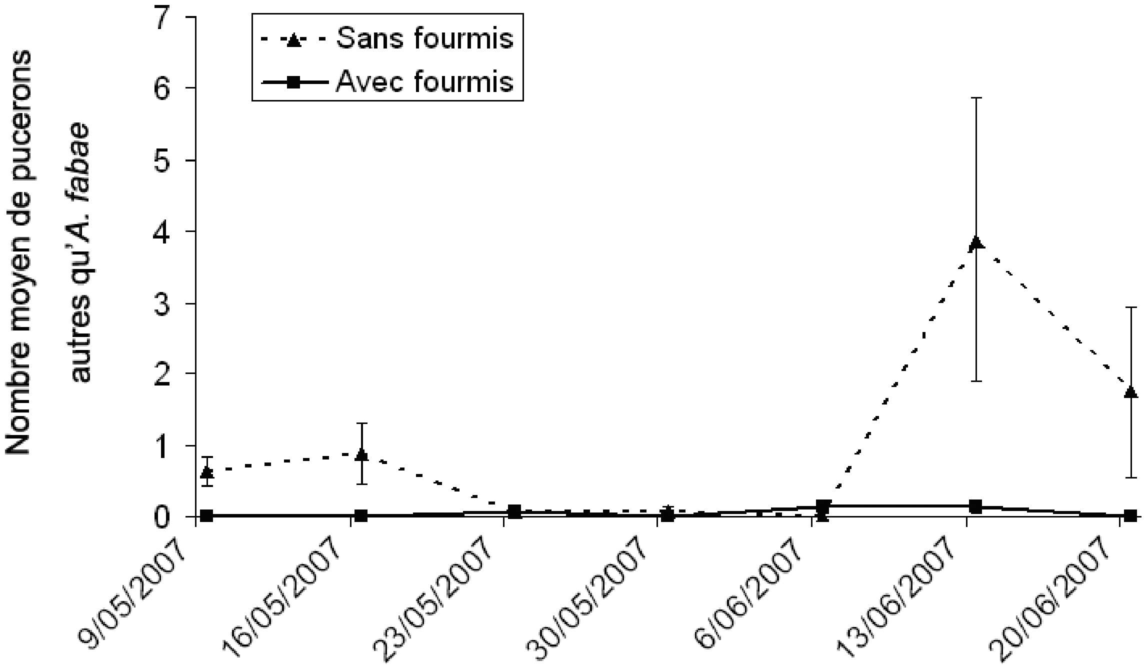

Figure 6. Nombre moyen de pucerons d'espèce différente d'*Aphis fabae* (Moyenne + Erreur standard) observés sur un plant de *Vicia faba* en fonction de la présence ou l'absence de *Lasius niger*

3.3. Les fourmis améliorent-elles la vigueur des plantes infestées ?

Les observations réalisées sur les plantes infestées ont pour objectif de décrire brièvement leur vigueur afin de déterminer si la présence de fourmis est bénéfique pour celles-ci. Les variables concernant la plante qui ont été observées sont la hauteur et les symptômes apparents de maladies. On observe que la hauteur moyenne des plantes est inférieure à 20 cm jusqu'au 30 mai, c'est-à-dire jusqu'à ce que le nombre de pucerons chute. Les plantes ont grandit à partir de l'absence de pucerons : ces deux variables sont en effet négativement corrélées (Pearson = -0,303 ; P<0,001).

Les hauteurs moyennes des plantes visitées et non visitées par fourmis sont restées identiques durant toute la durée de l'observation, à l'exception du 16 mai où les plantes visitées par les fourmis étaient significativement plus grandes (ANOVA, $F_{1,31}$=5,8 ; P=0,022).

L'observation régulière des plantes a conduit à la constatation d'une meilleure « hygiène » des plantes visitées par les fourmis, surtout du point de vue du nombre d'exuvies et de taches de miellat, même si toutes présentaient régulièrement des nécroses, chloroses, rouilles, feuilles boursouflées ou des traces d'attaques de phytophages.

3.4. Comment a évolué la fréquentation des plantes par les fourmis ?

La présence des fourmis sur les plantes du dispositif a été observée durant toute l'expérience, Le nombre total de fourmis sur chaque plante augmente jusqu'au 30 mai puis diminue. Ce nombre est corrélé avec le nombre de pucerons sur les plants de fèves de manière hautement significative (Pearson = 0,291; P=0,002). Par contre, aucune corrélation significative n'a été observée entre la température journalière moyenne (Pearson = 0,068; P=0,477) ou les précipitations (Pearson = -0,116; P=0,222).

Le nombre de fourmis qui se trouvent sur les colonies de pucerons a aussi été observé. Celui-ci évolue parallèlement au nombre total de fourmis sur la plante ; la corrélation entre ces deux variables se révèle très hautement significative (Pearson = 0,916; P<0,001). Cela suggère que les fourmis se déplacent jusqu'aux plantes afin d'établir des relations de mutualisme avec les pucerons.

Le nombre de fourmis présentes sur les nectaires extrafloraux a enfin été compté. Il est nul jusqu'au 23 mai puis augmente légèrement. Cette évolution est due à deux facteurs principaux : (1) la décroissance du nombre de pucerons sur les plantes et (2) la croissance de la taille des plantes et du nombre de nectaires extrafloraux. En effet, si le nombre de pucerons diminue, les fourmis recherchent d'autres sources de sucres dont le nombre augmente alors que la plante grandit.

Construction d'une structure protectrice – De nombreuses espèces de fourmis construisent des structures physiques externes à leur colonie, telles que des ponts, des tunnels, des abris et des avant-postes (Anderson & McShea, 2001). Lorsque ces structures sont destinées à protéger les espèces d'insectes avec lesquelles les fourmis entretiennent des relations de mutualisme, elles peuvent porter les noms que Linné leur avait initialement prêté : « étable » ou « bergerie », mais le terme d'« abris » est plus communément utilisé (Anderson & McShea, 2001). Lors de la présente étude, la colonie de *L. niger* initialement placée au centre du dispositif expérimental a installé un avant-poste dans le pot d'un plant infesté de *V. faba*. En date du 8 mai 2007, une ébauche de construction de terre a été observée à la base de la plante. Cette construction s'est ensuite agrandie pour couvrir l'entièreté de la tige, enfermant les pucerons présents. En date du 24 mai celle-ci mesurait plus de 5 centimètres de haut. De nombreuses fourmis étaient présentes continuellement sur la plante. Ce nombre dépassait fréquemment la vingtaine alors que 2,9 ± 0,6 fourmis en moyenne étaient présentes sur l'ensemble des plantes visitées par les fourmis. Aucun prédateur, parasitoïde ou espèce de pucerons autre qu'*A. fabae* n'a été observé.

Figure 7 Structure de terre bâtie par une colonie de *Lasius niger* autour d'une tige de *Vicia faba* infestée par *Aphis fabae*

Conclusion

L'observation du mutualisme pucerons-fourmis en conditions semi-naturelles a permis d'évaluer les bénéfices retirés par les pucerons. L'évolution différente des colonies de pucerons se nourrissant sur les plantes auxquelles les fourmis avaient accès par rapport à celles dont elles étaient exclues montre que les pucerons retirent un intérêt certain à être soignés par les fourmis. En effet, les pucerons des colonies soignées se sont mieux reproduits que les pucerons des colonies qui ne l'étaient pas. De plus, les fourmis excluent les prédateurs et les pucerons non-myrmécophiles des plantes où *A. fabae* est présent, diminuant la prédation et la compétition interspécifique pour le phloème. Les soins apportés par les fourmis aux colonies et à la plante (notamment le nettoyage du miellat et des exuvies) bénéficient aux pucerons en ralentissant le développement de champignons. Ces bénéfices pour les pucerons rejoignent directement l'intérêt des fourmis : elles maintiennent leur source de nourriture dans les meilleures conditions possibles.

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VI.2

Aphid-ant mutualism: How do aphids focus ant foraging?

François J. Verheggen[1§] · Claire Detrain[2§] · Lise Diez[1] · Bernard Wathelet[3] · Eric Haubruge[1]

[§] Both authors have contributed equally to the present work
[1] Department of Functional and Evolutionary Entomology - Gembloux Agricultural University, Belgium
[2] Department of Social Ecology – University of Brussels, Belgium
[3] Department of Biological Chemistry - Gembloux Agricultural University, Belgium

Reference – Verheggen FJ, Detrain C, Diez L, Wathelet B, Haubruge E (2008). Aphid-ant mutualism: How do aphids focus ant foraging? Insectes sociaux. Submitted.

Abstract – The mutualistic relationships that occur between myrmecophilous aphids and ants are based on the rich food supply that honeydew represents for ants and on the protection they provide against aphid natural enemies. While aphid predators and parasitoids actively forage for oviposition sites by using aphid semiochemicals, scouts of aphid-tending ant species would also benefit from locating honeydew resources by orienting toward aphid pheromone sources. The present study aims to identify the chemical factors that attract ants and that maintain their mutualistic relationships with aphids. The perception and behavioural impact of *Aphis fabae* alarm pheromone, namely (*E*)-ß-farnesene, on *Lasius niger* were firstly investigated using electroantennography and a four-arm olfactometer. *Aphis fabae* honeydew sugar composition was subsequently analyzed while the foraging and recruiting behaviour of *L. niger* scouts towards each of the identified sugars was studied. Clear electrical depolarisations were observed from *L. niger* scout antennae to stimulations of *A. fabae* alarm pheromone. Scouts were significantly attracted toward (*E*)-ß-farnesene in the four-arm olfactometer, suggesting for the first time that the latter compound is a key chemical in the establishment of the mutualism. *Aphis fabae* honeydew consisted of 9 identified mono-, di- and tri-saccharides and 8 hydrocarbons that could not be identified. The main identified sugars were sucrose, fructose, glucose and melezitose. *L. niger* scouts showed the following drinking preferences for the tested sugars: melezitose = sucrose = raffinose > glucose = fructose > maltose = trehalose = melibiose = xylose, with a recruitment launched toward the first three sugars. Therefore, ant scouts may use aphid semiochemicals to locate at distance an aphid colony and subsequently estimate honeydew quality by tasting it before recruiting conspecifics and establishing a mutualistic relationship.

Key words – Aphid alarm pheromone, (*E*)-ß-farnesene, sugar preference, *Lasius niger*, *Aphis fabae*, honeydew.

Introduction

Aphids (Homoptera, Aphidoidea) and ants (Hymenoptera, Formicidae) are the protagonists of one of the most studied model of mutualistic relationships in the animal kingdom : the first ones produce a carbohydrate- and nitrogen-rich excretion named honeydew, which is collected by some ant species (Way 1963) who provide aphids in return with protection and hygiene (Buckley 1987). Honeydew represents an important nutriment source for ants, whose collective exploitation by ant colonies is facilitated by aphids' gregarious lifestyle. It is usually considered, through its quantity and quality, as the key factor conditioning the future relation between both protagonists. This mutualistic relationship is expected to last as long as the benefits linked to nutriment source that honeydew represents are more important that the costs of aphid protection and maintenance (Way 1963 ; Bristow 1991 ; Yao et al 2000).

In presence of each other, both insects show behavioural changes (El-Ziady and Kennedy, 1956). Aphids tend to increase the amount of feeding and, hence, the amount of excreted honeydew (Völkl et al., 1999). The honeydew sugar composition has also been shown to be dependent of ants presence (Yao and Akimoto, 2001 ; Fischer and Shingleton 2001) and even to be adapted to ant sugar preferences (Fischer and Shingleton 2001). Under palpation by ant antennae, aphids extrude their honeydew droplets without ejecting them to facilitate collection by ants (Sudd, 1967). The latter become aphid breeders, providing aphids with protection against natural enemies (Pontin 1959 ; Yao et al 2000) and cleaning the colony from the exuviae and the uncollected honeydew droplets what tend to increase aphid health and colony lifespan (El-Ziady 1960 ; Way 1963).

The communication between both partners was thought to be essentially tactile, as ants palpate aphids' abdomen using alternatively their two antennae to stimulate the ejection of the honeydew droplets. The interactions between ants and aphids are also chemically mediated. Nault et al. (1976) have demonstrated ants' ability to react behaviourally to (*E*)-ß-farnesene (Eßf), the main component of the alarm pheromone of most aphid species (Edwards et al., 1973 ; Francis et al., 2005). When *Formica subsericea* were attending aphids and exposed to the latter sesquiterpene, they extended their antennae and opened their mandibles being prepared for attacking potential aphid enemies. Besides, more recent studies have demonstrated that ants detect specific blend of cuticular hydrocarbons on aphids' body what allows them to discriminate myrmecophilous aphids from potential prey (Lohman et al 2006). However, little is known about the possible chemical detection of aphids by ants at longer distances, the encounter between both insect species being assumed to occur by chance.

In this respect, most aphid natural enemies, including parazitoid hymenoptera (Du et al., 1998), hoverflies (Almohamad et al., 2007; Verheggen et al., 2008), lacewings (Han and Chen, 2002) or ladybeetles (Verheggen et al., 2007a), are able to use Eßf as a kairomonal substance to locate at long distances their host or prey. We therefore studied whether ants are also able to locate aphids from a distance by detecting their alarm pheromone.

Honeydew is commonly thought to be the main factor involved in the maintenance of ant-aphid mutualism through its quality and quantity. Previous work (Vander Meer et al. 1995; Völkl et al.1999: Tinti and Nofre,2001) showed that the ant foragers' flows and/or the amount of ingested food differ depending on the nature of sugar sources. Here we investigated how different sugars present in aphid honeydew may influence, at the individual level, the foraging behaviour as well as the laying of a recruitment trail by scouts. This is a major issue to explain possible differences in the exploitation rate of aphids by ant nests. Indeed, the dynamics of food exploitation as well as the selection of a food source among several ones by the entire colony is greatly determined by the first steps of food exploitation – in other words by the way scouts will forage and recruit nestmates to food (Beckers et al. 1993, Detrain et al. 1999, Portha et al. 2004, Detrain and Deneubourg 2008).

Materials and Methods

Ants and aphids – The common black ant, *Lasius niger* L. is a well-known aphid-tending species widespread in European temperate regions. Colonies were collected in Brussels in April 2007 and placed in plastic containers whose edges were covered with polytetrafluoroethylen (Fluon) to prevent them from escaping. Test tubes covered with a red transparent foil were disposed as laboratory rearing nests. Sucrose solutions (1M) and water filled test tubes were provided. Twice a week, dead insects, including cockroaches or drosophila, were also added as protein sources. The colonies were kept in an environmentally controlled room (L16:D8, humidity 65 ± 5 %, and 23 ± 2 °C). The black bean aphids, *Aphis fabae* Scopoli, were mass reared on broad beans (*Vicia faba* L.) grown in 10cm³ plastic pots filled with a mix of perlite and vermiculite (1:1) and placed in similar conditions as above.

Electroantennography – The *L. niger* antenna was carefully excised from the head. The scape was removed to improve electrical contact and subsequently decrease background noise (Kleineidam et al, 2005). The antenna was mounted between two glass Ag-AgCl electrodes (Harvard Apparatus, Holliston, MA, USA; 1.5 mm o.d. x 1.17 mm i.d.) filled with saline solution (NaCl: 7.5 g/l; $CaCl_2$: 0.21g/l; KCl: 0.35 g/l; $NaHCO_3$: 0.2 g/l) and in contact with a silver wire. The pedicel was first inserted into the ground glass electrode. The tip of the recording electrode was bowl-shaped, and half of the last funicle segment was immersed into the saline solution. This setup has already produced elegant results while studying the olfactory responses from beetle antennae (Verheggen et al., 2007a,b). The DC potential was recorded on a computer (Auto Spike v. 3.0) by using an amplifier (IDAC-4, Syntech®, Hilversum, The Netherlands) with 100-fold amplification. A 0.5-cm^2 piece of filter paper that was impregnated with 10 µl of the chemical under examination was placed in a Pasteur pipette and used to puff an air sample in a constant 1.5 l/min airstream. Paraffin oil was used to make four Eßf solutions with concentrations ranging from 0.1 µg/µl to 100µg/µl (by 10x increments). Stimulation with semiochemical-free paraffin oil was executed as a negative control (=mechanical stimulus) before and after the stimulations with the four Eßf solutions cited above. Stimulations were induced thirty seconds from each other. Preliminary results indicate that this length of time was adequate to allow the insect recover its full reactivity to stimuli. Eßf was synthesized from farnesol (Tanaka et al., 1975) and with a chemical purity of 98% (determined by GC).

Four-arm Olfactometer Assays – The four-arm olfactometer was similar to that previously described by Verheggen et al. (2007a) and was adapted to be connected to a *L. niger* colony. It was constructed entirely of Teflon® and was closed with a removable glass roof, both previously cleaned with *n*-hexane. The walking arena was 40 cm wide (from center to odour source) and 1.5 cm high (from Teflon® walking arena to glass ceiling). Charcoal-filtered air was pushed in each of the four olfactometer arms through Teflon® tubing, and adjusted to 100 ml/min with a digital flowmeter. A pump ventilated the walking arena by removing air from the center at 400 ml/min. A *L. niger* colony was placed under the olfactometer and a plastic tube allowed scouts to climb up to the walking arena. A "T" glass piece allowed the connection of the plastic tube to the olfactometer, and at the same time the aspiration of the outgoing air. This piece also allowed to close the access to the olfactometer and thus controlled the entrance of only one scout per replicate. A 0.5 l glass chamber was connected to one of the four olfactometer arms, and was used to introduce twenty unwinged adult *A. fabae*, that were rapidly crushed inside the glass chamber using a small glass pestle left inside the chamber [as a natural source of Eßf]. According to previous studies, the volatiles released by crushed *A. fabae* consist exclusively of Eßf (Francis et al., 2005). The glass chamber was randomly connected to one of the four arm of the olfactometer. Both the walking arena and the glass ceiling were washed with *n*-hexane after each *L. niger* scout was tested. The olfactometer was divided into one central 10 cm squared area, and four other areas related to the four odour sources. The observations were conducted for 3 min, starting when the scout entered the walking arena. The choice of the tested scout was determined by (a) the first area it entered and (b) the time spent in the four areas. The behavioural observations were conducted in a laboratory at 22±1°C and under uniform lighting to avoid any bias in the orientation behaviour of the tested insects.

Honeydew sugar composition – Using 0.5µl microcapillaries, honeydew was collected from a colony made of around 50 unattended *A. fabae*. The filled capillary was then transferred into a microtube containing 50µl of milli-Q water, where the honeydew was extracted. Samples were kept at -18°C until chromatography analysis. The sugar composition of *A. fabae* honeydew was measured by high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) using a Carbopac^{TM} PA-100 column (4x250mm) and a ED40 amperometric detector (Dionex, Sunnyvale, CA). This setup was previously shown to be adapted for mono- and polysaccharide analyses (Yao and Akimoto, 2001 ; Ronkart et al., 2007). The elution was conducted by mixing a 0.5M NaOH solution with milli-Q water. Equilibration was conducted for 10 minutes with 3% NaOH solution before injection. After injection of 24 µl of sample, the concentration of the NaOH solution was increased from 3 to 58.8% during 25 min. Detection was operated for 25 min starting at the injection time. A control sample comprising a mix of fourteen sugars of known concentration was injected prior to the analysis of the samples, and was used for sugar identifications.

Sugar preferences – The preferences of individual *L. niger* scouts were investigated for the different sugars previously identified in the honeydew of *A. fabae.* We allowed a single scout to reach a foraging arena on which we placed the sugar under investigation. The setup was aluminium-made and consisted in three different parts (Figure 1). (1) The ramp consisted in a 15cm long and 1cm wide piece of aluminium that was placed near the nest entrance at a declination of 45° and that allowed scouts to reach the bridge. (2) The 20cm long bridge connected the ramp to the foraging arena and was covered with filter paper. A 3cm long section of the bridge – located close to the ramp – could be manually removed to allow only a single scout to access the bridge and the foraging area. (3) The 5X5 cm squared foraging arena was entirely covered with a piece of filter paper. Papers covering the bridge and the foraging arena were renewed before each test in order to remove spots of trail pheromone laid by the scout previously tested. In the centre of the arena, 0.4 ml of the 0.5M tested sugar solution was placed using a piece of bowl-shaped aluminium piece. The following sugars were tested: fructose, glucose, maltose, melezitose, melibiose, raffinose, sucrose, trehalose and xylose (Sigma-Aldrich, Chemie Gmbh, Steinheim, Germany). These sugar solutions were made with distilled water, which was therefore used as control. The sugar solutions were tested randomly. For comparison purpose, the response of each colony was quantified for all sugars. All sugars were tested once per experimental day.

The tested colonies were deprived of any food but water during 4 days. Before the observations, the colony was connected to the experimental setup for 1hr and the sugar solutions, stored at -5°C, were placed at ambient temperature. After 1hr, the ants, if any, were removed from the setup and the tested sugar solution was placed in the centre of the foraging arena. Once a scout reached the mid-part of the bridge, the movable section of the bridge was removed. The foraging behaviour of this scout was then recorded using the Observer® software (Noldus information Technology, version 5.0, Wageningen, The Netherlands) until they left the foraging arena and the bridge to go back to their nest. Four behavioural sequences were recorded: (1) the time spent on searching on the bridge and the arena before having drunk the sugar solution, (2) the time spent on touching and drinking the sugar solution, (3) the time spent on searching on the bridge and the arena after having drunk the sugar solution, (4) the time spent on cleaning itself. The number of trail spots laid on the bridge was also assessed by observing how many times a scout bent its gaster downwards and contacted the substrate on its way back to the colony. This trail-laying behaviour was also used as an additional parameter for the evaluation of the ant sugar preference. All the ants having contacted (with mandibles or antennae) the sugar solution at least once were observed until they reached the end of the bridge. Sixteen different scouts from four different colonies were tested for each sugar solution.

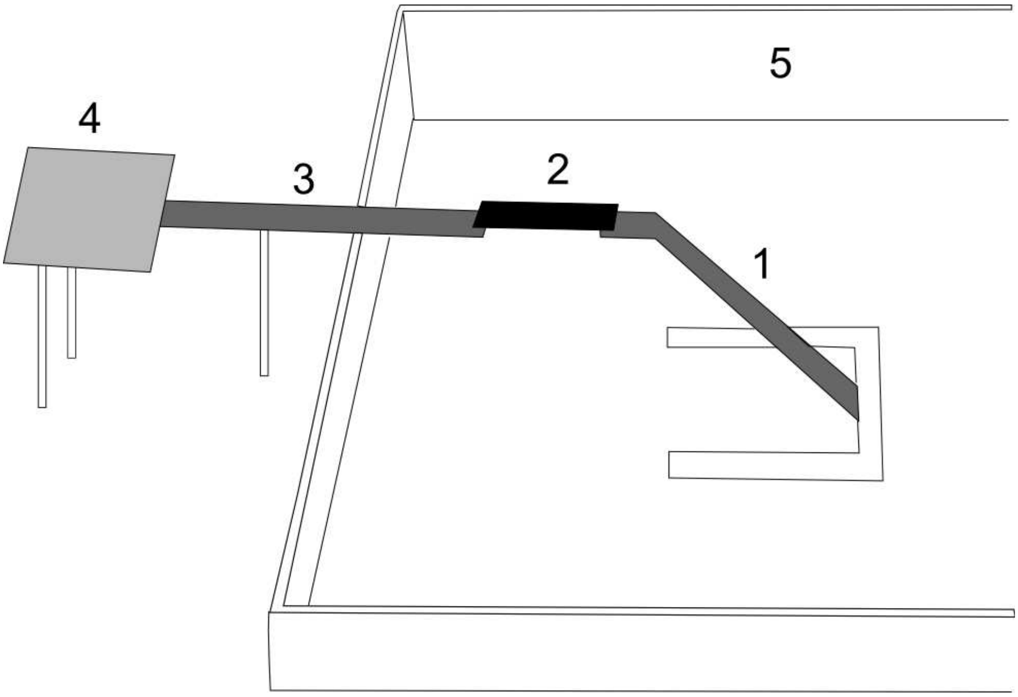

Figure 1. Schematic representation of the aluminium bioassay used to evaluate sugar preferences of individual scouts. (1) 15cm-long ramp, (2) 3cm-long movable bridge, (3) 20cm-long bridge, (4) 5X5cm squared foraging arena in the middle of which the sugar solution was placed, (5) plastic box containing the colony.

Results

Electroantennography – A positive dose–response relationship in EAG to (*E*)-ß-farnesene was observed ($F_{4,24}$=9.46, P<0.001). The highest tested dose elicited EAG responses of -0.692±0.197mV (average ± SE) statistically higher than the paraffin oil control (Dunnett’s comparison with control, P<0.05) (Figure 2).

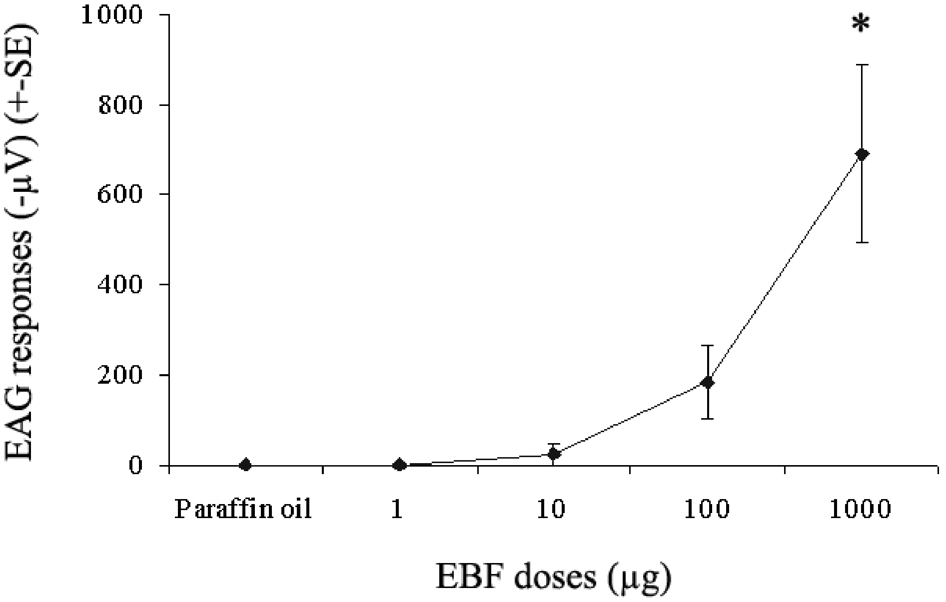

Figure 2. Effect of (*E*)-ß-Farnesene on the antennal responses (±SE) of *Lasius niger* scouts (n=5). * indicates significant EAG responses (Dunnett's test, P<0.05).

Four-arm Olfactometer Assays – In the four-arm olfactometer, Eßf (crushed *A. fabae* as a natural source) elicited significant behavioural activity from the tested *L. niger* scouts. According to the first area visited, 53.3% of the scouts were first attracted to the Eßf source (χ^2 = 9.63, *P*=0.003, n=30). They also spent most of their time in the arena connected to the Eßf source, as the tested scouts spent 42.7±6.2% of the observation time (time spent in the neutral area deducted) in the Eßf arm of the olfactometer ($F_{3,116}$=3.02, P=0.033, n=30).

Honeydew sugar composition – Nine sugars were identified in aphid honeydew by comparison of their retention times with those of known standards: trehalose, glucose, xylose, fructose, melibiose, sucrose, melezitose, raffinose and maltose (Table 1). Eight additional peaks were also detected but could not be identified. The main sugars were sucrose (15.778g/l), fructose (9.039g/l) and glucose (4.661g/l).

Table 1 : Average (±SE) sugar proportions and concentrations in *A. fabae* honeydew. nq = not quantified.

Sugars	Average proportions (%)	S.E.	Average concentrations (g/l)	S.E.
Sucrose	27.34	3.41	15.778	0.938
Glucose	26.78	4.72	4.661	1.406
Fructose	21.94	1.96	9.039	1.981
X1	7.72	0.97	nq	
Melibiose	4.14	0.62	0.677	0.136
X4	3.53	1.82	nq	
X8	1.81	0.30	nq	
X3	1.26	0.33	nq	
Xylose	1.19	0.28	0.386	0.092
X5	1.14	0.70	nq	
X7	1.08	0.18	nq	
Raffinose	0.90	0.63	0.645	0.356
Melezitose	0.75	0.37	0.601	0.323
Trehalose	0.73	0.32	0.315	0.112
X2	0.49	0.22	nq	
X6	0.10	0.06	nq	
Maltose	0.04	0.03	0.059	0.041

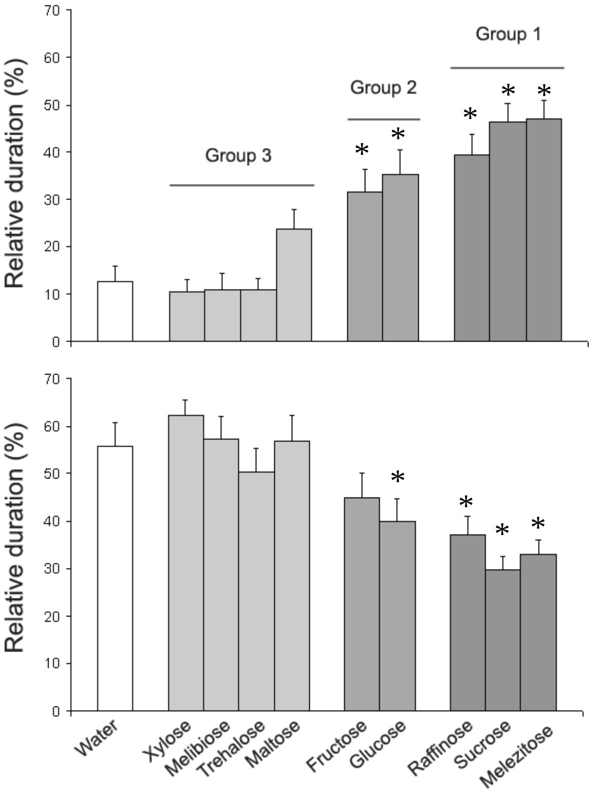

Figure 3. Behavioural observations (relative durations + SE) on the feeding behavior (above) and on the searching behavior that occurred after eating (below) of *Lasius niger* scouts in relation with sugar source in no-choice experiment; * indicates significant differences with water control (Dunnett's test, P<0.05).

Sugar preferences – Whatever the sugar source presented, the relative time spent by ants in cleaning themselves was too short (<1%) to be presented in Figure 3 and this behaviour was performed by only one or two scouts for all tested sugars. The only exception is found for workers feeding on maltose since half of the tested ants spent at least 0.5 sec to clean their legs and/or antennae. For every tested sugar sources, the time spent to explore the foraging area before reaching the sugar source was not statistically different from the water control (Dunnett's comparison with control, P>0.05). Compared to water, five sugar solutions induced a significantly longer lasting feeding behaviour: fructose, glucose, melezitose, raffinose and sucrose (Dunnett's comparison with control, P<0.05). Time spent to explore and leave the foraging area after having drunk at the sugar solution were significantly lower than that with water for all the above-mentioned sugars, excepted fructose (Dunnett's comparison with control, P<0.05). This suggests that scouts were quite satisfied and did not search for other sugary resources after having found sucrose, raffinose or melezitose sources. Scouts deposited a significant higher number of trail spots on their way back to the colony after having tasted melezitose, sucrose or raffinose (Figure 4). This behaviour was also observed in case of fructose and glucose without statistical significance when compared to the water control. These results allow us to discriminate the tested sugars into three groups. Firstly, sucrose, raffinose and melezitose are sugars that trigger along-lasting drinking behaviour, a short-lasting searching duration after feeding and a pronounced trail-laying behaviour. Glucose and fructose both belong to the second group of sugars that were significantly longer ingested than water, which induced short food-searching after their consumption but that did not induce significant trail-laying behaviour. Finally, the third group consists of those sugars that were not appreciated and poorly ingested by scouts –i.e. melibiose, xylose, trehalose and maltose.

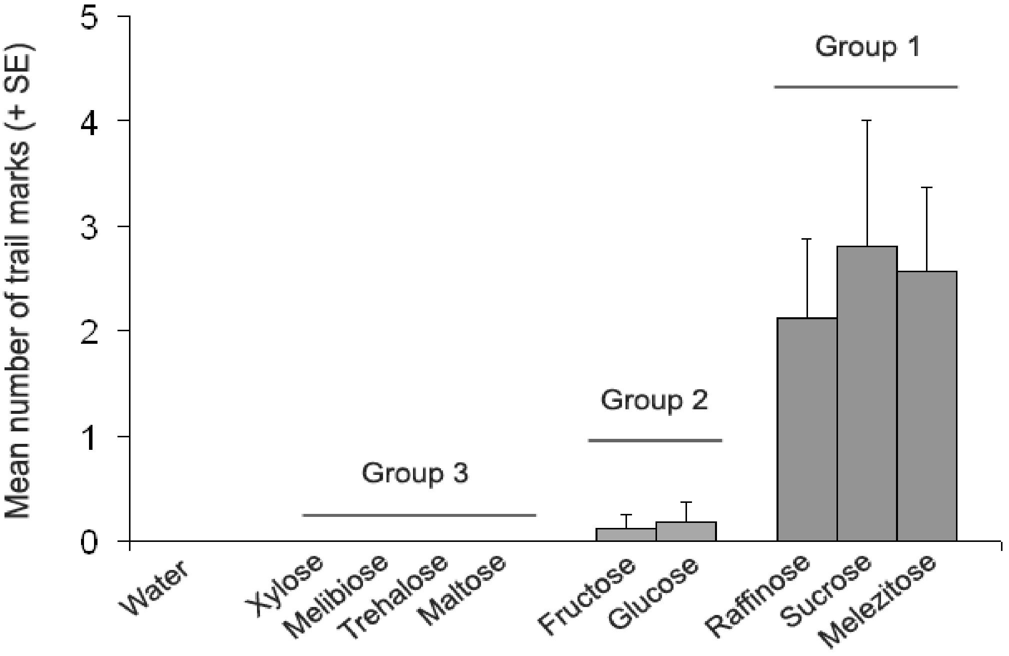

Figure 4. Mean number of trail marks (+ SE) made by *Lasius niger* scouts having tasted a sugar solution (Student t-test, ** and * representing significant difference with P<0.01 and P<0.001 respectively).**

Discussion

Most aphid natural enemies, including lady beetles, hoverflies or lacewings, have evolved in order to adapt their olfactory system to the perception of aphid-released volatile chemicals and subsequently locate their prey (e.g. Han and Chen, 2002; Harmel et al., 2007; Verheggen et al., 2007a, 2008). Ants would therefore have a clear advantage to perceive aphid odourant cues, which would increase their chance to establish a mutualistic relationship. Our results demonstrate that *L. niger* have olfactory receptors perceiving *A. fabae* alarm pheromone, (*E*)-ß-farnesene. Moreover, while using Eßf at the same dose, and with similar equipment and method, Verheggen et al. (2007a ; 2008) obtained EAG responses twice lower with the predatory hoverfly *Episyrphus balteatus* (Diptera: Syrphidae), and three times lower with the Asian lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). The olfactory system of foraging ant workers seems therefore well-adapted for the perception of small amounts of aphid alarm pheromone, and subsequent for a high sensitivity to aphid presence. This is in accordance with previous findings of Nault et al. (1976) who found that *Formica subsericea* respond to Eßf by raising and extending their antennae and opening their mandibles. Presentation of a filter paper impregnated with Eßf also induced typical attacking behaviour.

That aphid alarm pheromone could be used by ants to locate aphid colonies has never been demonstrated earlier. Single ant foragers were clearly attracted by Eßf in the four-arm olfactometer. In addition to being their first orientation choice, the Eßf arena was also the most explored. The fact that Eßf has an attractive effect on ant scouts has important ecological implications. Alarm pheromone is emitted either in case of attacks by natural enemies (Edwards et al. 1973) but is also released from non-attacked crowded colonies (Verheggen, unpublished data). In the latter case, the released amount is usually low. Many myrmecophilous ant species, including *L. niger*, are known to switch continuously from a “breeder” to a “predator” behaviour according to aphid colony size: the increased aphid density per ant led to an increase in the rate of predation (Sakata, 1994, 1995), but no information was available on the potential chemical signals that inform ants on the number of available aphids. As Eßf was here shown to be well perceived by ants, the latter chemical could induce predation when its released amount reach high level, i.e. when the aphid colony get crowded. As Eßf emissions induce aphids to drop or run away from each other, this hypothesis is in accordance with the work of Sakata (1995) who correlated ant predation with aphid movement. Ants are also likely to perceive the high Eßf levels released from attacked aphids. Secondly, Eßf is used as unique component of the alarm pheromone in most aphid species, including unattended ones (Francis et al, 2005). If Eßf leads mostly to aphid colonies, and sounds like reliable semiochemical for aphid presence, one could consider its perception by ants as an indicator of mutualism opportunity. Regarding aphids, they would have strong advantage to emit low amounts of semiochemicals to attract ants at for the first steps of this mutual relationship. Later on, other factors such as the laying of a recruitment trail and/ or a memorization of the foraging path allow ants to return to already discovered aphid colonies (Hölldobler and Wilson 1990). Within aphid-ant mutualism, the main roles of aphid alarm pheromone would be the attraction of ant scouts, as well as to alert ants about a predator attack.

Once located, an aphid colony will trigger and maintain a, mutualistic relationship with ants only if aphids supply ants with honeydew of adequate quality and quantity (Way, 1963). Honeydew composition vary among aphid species (Völkl et al., 1999), aphid host plants (Fischer et al., 2005), between age classes (Fischer et al., 2002), and even in response to interactions with ants (Fischer & Shingleton, 2001; Yao & Akimoto, 2001). Ants are expected to focus their honeydew collection on aphid colonies which are the most profitable , either in terms of high volume of honeydew (see e.g. Mailleux et al. 2000, 2003) , or in terms of sugar content (Beckers et al. 1993). In addition to xylose, fructose, glucose, sucrose, maltose trehalose, melezitose and maltose, already identified in the honeydew of *Aphis fabae* feeding on *Vicia faba* (Völkl et al., 1999; Fischer at al., 2005), we found traces of melibiose. Here, sucrose, glucose and fructose were the most abundant sugars in *A. fabae* honeydew. Conversely to Fischer et al. (2005) study on honeydew composition from ant-attended *A. fabae*, we found out that melezitose was present only in minor amounts (<1g/l) and represented less than 1% of the total amount of sugar. The absence of ants tending aphids may explain the poor melezitose content of their honeydew since some aphid species are known to increase their melezitose production in presence of *L. niger* ants (Fischer & Shingleton, 2001). Melezitose seems to play a key role in aphid-ant mutualistic relationships. Indeed, *L. niger* workers are able to detect melezitose at very low concentrations (Schmidt 1938) and respond quite intensively to honeydew or water solution containing the latter trisaccharide (Kiss, 1981, Völkl et al 1999). Woodring et al. (2004) even demonstrated that a combination of honeydew abundance, sugar richness and melezitose content accounts for the rate of ant-attendance. Likewise, in the present study, ant scouts spent a long time drinking at melezitose solution. When fed, they explore very shortly the foraging area and head on back to the nest what strongly suggests that they are quite satisfied by the ingestion of this trisaccharide. Raffinose and sucrose gave similar results in terms of preference: tested ants show long drinking times, spent less time to search over the area and reached quickly their colony after food discovery. Moreover, these three sugars (melezitose, raffinose, sucrose) similarly elicited high frequencies of trail-laying behaviour, an additional evidence of their primary role in aphid-ant mutualism. Indeed, a sugar source of interest for ant colonies (i.e. an aphid colony with adequate honeydew production) should trigger an intense trail-laying behaviour by scouts in order to ensure the quick recruitment of nestmates as well as the monopolisation of this food resource by the ant colony. Fructose and glucose belong to a second group of sugars: they are appreciated by ants that spend a long time drinking and when fed, that did not search long over the foraging arena. Nevertheless, they do not induce a trail-laying behaviour. Future studies should investigate whether trail-laying may arise over successive trips or encounters with these two sugars. Most probably, these two sugars are appreciated because these monosaccharides provide energy that can be quickly metabolized. For instance, glucose can be converted in trehalose, abundantly present in insect's hemolymph (Turunen, 1985). Finally, the third group of sugars- i.e. xylose, trehalose and maltose seems of poor interest to the ants that did not stay drinking and that spent most of their time searching for additional resources over the foraging area. Ant's sugar preferences are surely connected to metabolic needs (Boeve and Wackers, 2003), e.g. sugars being less digestible or providing less gains regarding to the energy needed to digest them could be less preferred. According to Percheron et al., (1981), xylose is hardly metabolized by animal's cells. Melibiose is hydrolyzed by a ß-galactosidase, usually absent from ant's digestive tract (Boeve and Wackers, 2003). This explanation could however not be applied in the cases of trehalose and maltose, whose enzymes are present in insects digestive tract (Percheron et al. 1981). Finally, we did not find any correlation between the molecular structure of the tested sugars and their preference by the tested ants.

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Chapter VII

Conclusions, Discussions & Perspectives

In Europe, aphids represent a recurrent problem in agriculture. Without the use of adapted chemical treatments, these pests cause severe damages to the cultivated crops. The European parliament decided recently to encourage the reduction of insecticide, to reach a 50% of reduction in 2010. Because we will not be able to control aphids with reduced amounts of chemicals, there is a real need to switch to integrated strategies. Several biological methods, including resistant cultivars or natural enemies of the pests, have already showed their efficiency. However, before being able to control living organisms, it is necessary to fully understand the tritrophic interactions that occur between a pest, its host plant, and its natural enemies. This PhD thesis provides additional elements that help to understand aphids' ecology as well as the way ants and aphid natural enemies interact with them.

For the first time, we showed that aphids do not produce Eßf exclusively in case of attack by natural enemies. The release of the alarm pheromone by unstressed individuals highlights the potential roles that this sesquiterpene could play in aphids. This molecule could play indeed several roles in the population dynamic of aphids. Previous studies have already demonstrated that Eßf can promote the production of winged individuals, and therefore regulate the population density. It is well known in some other insect species that a single chemical can attract or disperse conspecifics, depending whether the released amount is low or high, respectively. Eßf could then act at very low concentrations as a semiochemical carrying the information that a particular host plant is already colonized but that this plant fits the aphids' needs, and that it can provide sufficient food for additional conspecifics. One should not neglect to potential side-roles of Eßf, including the possible dual function as alarm and aggregation pheromone. A very simple experiment to start testing that hypothesis could be to place a piece of filter paper impregnated with a very low dose of Eßf on a plant. A group of aphids could be placed between two plants and the distribution of the aphids on the two plants would be observed. Behavioural assays using conventional olfactometer could also help in studying the potential attraction induced by very low Eßf amounts.

Because the production of an alarm signal is likely to entail ecological, physiological and even sometimes morphological costs for aphids, it was not surprising that these pests reduce their production of Eßf in case of isolation. They save the energy needed for the production of that sesquiterpene, and in the same time become less visible to predators. We concluded from our experiments that Eßf was carrying the information about the presence of conspecifics, because it was the only chemical to be identified in the headspace of pea aphids. However, to complete and confirm the study presented in chapter IV.3, one could expose developing aphids with pure Eßf and check their production of alarm pheromone. This would confirm that Eßf is actually the chemical carrying the information about conspecifics' presence. The methods developed in chapter IV.3 provides the researchers with particularly interesting biological material: Eßf-depressed aphids. Indeed, aphids having a reduced Eßf production are useful to study the ecological roles that this sesquiterpene may play in an aphid's life. These Eßf-depressed aphids could by example be used to study aphids' aggregation or aphids' relationships with natural enemies or tending ants.

More surprisingly, we found that aphids do not amplify the alarm signal released by a conspecific under attack, and simply run away from the emitter. This result was confirmed by a similar study conducted and published simultaneously, but independently, by a German team. Using deuterated Eßf, they were able to differentiate between applied Eßf and Eßf released by treated aphids. Aphid colonies were treated with deuterated Eßf and headspace volatiles were collected but no aphid-derived Eßf was detected, also suggesting that amplification of the alarm signal does not occur. The emission of alarm pheromone by aphids that perceive the initial signal but are not directly attacked would have helped to amplify the warning to ensure that most individuals of a colony are alerted to the presence of a natural enemy. The amounts of Eßf emitted by individual pea aphids are small and the compound might be subject to chemical oxidation or its concentration diluted rapidly by air movement. Thus aphids feeding far away from the site of initial attack will not interrupt their feeding given the low quantities emitted. If a predator continues feeding in a colony, the frequency of emissions will increase and a large proportion of individuals will respond by walking or dropping from the plant. On the other hand, disadvantage of signal reinforcement is that it could make aphids unable to locate the feeding site of a predator. Colony members situated far away from the site where an aphid is initially attacked might be alerted and start walking; they could end up closer to the danger than they were previously. Additionally, some natural enemies are known to use Eßf to detect their prey. Under these circumstances, signal amplification is clearly not adaptive. We can conclude that the response of aphids at the colony level to the alarm pheromone is adjusted to the number of single emissions and their proximity.

Although the aphid alarm pheromone was found to act as a foraging cue for various aphid predators, it is surely not the only volatile organic chemical that is specifically released from an aphid infested plant. Indeed, many plant species react to herbivore infestation changing their volatile emission profile, e.g. by releasing specific chemicals in their headspace or by changing the relative amounts of each continuously released volatile. In addition to the semiochemicals that have been tested on the foraging and oviposition behaviour of the hoverfly predator *Episyrphus balteatus*, one should focus on the potential attractiveness of the whole blend of odour released from an aphid infested plant, and compare the resulting behaviour to that showed with the whole odour blend of a non infested plant. In our tomato trials, although Eßf was the only volatile which amount was found to vary after 24 hours of aphid infestation, it did not induce pronounced oviposition by itself. Additional chemicals could therefore be involved and mixtures should be constituted according to the absolute and relative amounts released naturally, to determine the volatile chemicals that guide hoverfly predators to oviposition. Other factors may also have an effect on the hoverfly foraging and oviposition behaviour, such as vision. Placing on a plant both artificial aphids (similar in size and color to real ones) and a semiochemical diffuser could be an interesting experiment and could show the potential synergetic action of vision and chemical cues.

One could therefore expect different chemicals to be involved in either the foraging or the oviposition behaviour (or both) of predatory hoverflies, and more generally in aphid natural enemies. Eßf could act as an attractant, providing information about the presence of prey, but could be much less informative about the fitness of the prey colony, which has been shown to be evaluated by hoverfly females when looking for a suitable oviposition site. Hoverfly females that have landed on a plant usually use their proboscis before laying eggs, which is another indication for a potential existence of an oviposition inductor, probably less volatile. Aphids produce a secretion named honeydew that consists of a mixture of amino-acids and sugars. The chemical composition of honeydew, often excreted in large amounts on aphid host plants, could be a good indication of the fitness of the colony. Some component of this aphid secretion could therefore act as oviposition stimulants. One should study the honeydew chemical composition according to the age of the aphid colony and its apparent fitness. The presence of sensillae on the proboscis of hoverfly females and their reaction to certain of the chemicals present in the honeydew would be a good indication for the use of honeydew by hoverflies in their search for suitable oviposition site. Moreover, honeydew might release additional volatile organic chemicals, which we have not isolated on our trials, probably because our aphids were feeding on the tested host plant for a too short period. The volatile composition of aphid honeydew should therefore also be studied. Because the amount of honeydew can be very large on an aphid infested plant, aphid natural enemies would have clear advantage to be sensitive to these volatiles, and to use them in their search for prey. A similar approach as those followed in the present thesis for the study of the olfactory perception of volatile molecules could be conducted: (1) the volatile collection in headspace of a plant infested with aphids for a long period ; (2) the collection of large amounts of honeydew directly from the aphid anus using microcapillaries ; (3) the GC-MS analysis of diluted honeydew solutions; (4) a volatile collection from the headspace of a concentrated solution of honeydew ; (5) the olfactory study of the identified chemicals on *E. balteatus* antennae using electroantennography (EAG) or the coupled gas chromatography - electroantennography (GC-EAD) ; (6) the behavioural assays using the single active molecules or the volatile blends made of the different active chemicals in absolute and relative amounts similar to that present in the headspace of an aphid infested plant.

The aphid alarm pheromone was found to attract the Asian ladybeetles, *Harmonia axyridis*, in our bioassays. ß-Caryophyllene was also showed to attract the beetles and cause aggregation in a ventilated plastic box. However it is likely that these beetles use additional chemical of physical cues to aggregate in winter. The main reason is that a similar aggregation site can be colonized each year, where other sites in the same building or in the neighbourhood are never colonized. Moreover, no trace of ß-caryophyllene was detected in the headspace of overwintering ladybeetles (unpublished data). Among the other possible factors leading these beetles to aggregation: light, temperature, thigmotactism, magnetism, cuticular hydrocarbons, vision, alkaloids… One should therefore investigate the impact of these factors on *H. axyridis* aggregation behaviour. These lady beetles could also perceive the two tested semiochemicals (and others) differently according to their environment or to the seasons. If they use an aggregation pheromone to aggregate in the (early?) winter, they would have clear advantage to produce and react to it at a certain period of the year only, because its production is likely to entail cost and because ß-caryophyllene, a possible component of this aggregation pheromone, is an ubiquitous plant volatile chemical. One should therefore collect the volatile released by *H. axyridis* individuals during a whole year and should check its olfactory perception at the same time. Their content in pheromone should also be studied in parallel to the volatile collections.

Because both ants and aphids would take full advantage to find each other, we tested the potential attractiveness of Eßf for ant scouts. It was already known that tending ants react to aphid alarm pheromone by running toward the emitter and by attacking the aphid enemy. It was therefore no surprise to observe in our assays attraction toward aphid alarm pheromone. This attraction suggests that ants, that have never been exposed to aphid odours and that are likely searching for food resources, may use this volatile chemical to orientate toward aphid colonies. However, as previously suggested in this discussion, the aphid alarm pheromone might not be the only aphid-specific odorant cue to be used by ant scouts. Honeydew and the volatile organic chemicals it may release might complete the attractiveness of Eßf. Testing a mixture of Eßf and the volatile components of aphid honeydew on ant scouts, in a two-choice bioassay, could provide interesting information on the signals that guide an ant to an aphid colony.

The close relationships that aphids have with their natural enemies or their host plants have resulted in the evolution of fascinating chemical ecological interactions, involving complex chemical molecules, that are rich in information for all the living actors of this system. The aphid alarm pheromone, and more specially the sesquiterpene Eßf, appears in various roles in this work. It should be borne in mind that, beside the suspected but underestimate importance of this molecule for further pest management strategies, there are still areas where new chemicals of interest will be identified, for example those involved in the chemical ecology of aphid parasitoids and predators.

Beside the modes of production, olfactory perception or behavioral impacts of aphid semiochemicals, the identification of the aphid genome seems to be of prime importance to improve our understanding of aphid chemical ecology. Additional elements on the genes and resulting enzymes involved in volatile chemical production and perception would lead to more perspectives for aphid control strategies. The discovery of the genes in plants that are involved in the production of semiochemicals used by aphids can also lead to biological-resistant crop plants that would produce repulsive substances or substances disrupting aphid communication. *Arabidopsis thaliana*, the model plant for this field of studies, has already led to promising results. Finally, these studies will be greatly enhanced by a deeper understanding of the molecular biology of aphid chemosensory receptors, particularly those pathway induced by external signals.

Chapter VIII

List of Publications, Oral Presentations and Posters

The results previously described were taken from the publications listed below. Most of them were presented orally or through the use of posters.

1. Publications

1. **Verheggen F,** Arnaud L, Capella Q, Francis F and Haubruge E (2005). Perception of aphid infested tomato plant volatiles by the predator *Episyrphus balteatus*. *Comparative biochemistry and physiology* **141**A, 3
2. **Verheggen FJ**, Fagel Q, Heuskin S, Lognay G, Francis F & Haubruge E (2007). Electrophysiological and behavioural responses of the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, to sesquiterpene semiochemicals. *Journal of Chemical Ecology* **33**: 2148-2155
3. Harmel N, Almohamad R, Fauconnier M-L, Du Jardin P,**Verheggen F**, Marlier M, Haubruge E and Francis F (2007). Role of terpenes from aphid-infested potato on searching and oviposition behaviour of *Episyrphus balteatus*. *Insect Science* **14**, 57-63.
4. **Verheggen FJ**, Mescher MC, Haubruge E, De Moraes CM, Schwartzberg E (2008). Emission of alarm pheromone in aphids: A non-contagious phenomenon. *Journal of Chemical Ecology* **34**:1146-1148.
5. **Verheggen FJ** , Arnaud L, Bartram S, Gohy M and Haubruge E (2008). Aphid and plant secondary metabolites induce oviposition in an aphidophagous hoverfly. *Journal of Chemical Ecology* **34**(3): 301-307
6. Almohamad R, **Verheggen FJ**, Francis F, Lognay G & Haubruge E (2008). Emission of alarm pheromone by non-preyed aphid colonies. *Journal of Applied Entomology*. **132**(8):601-604.
7. **Verheggen FJ**, Haubruge E (2008). Que faire lors d'invasions de Coccinelles asiatiques? *Ligue des Amis du Kauwberg*, **66**: 14-16
8. **Verheggen FJ**, Diez L, Detrain C, Haubruge E (2008). Mutualisme pucerons-fourmis : étude des bénéfices retirés par les colonies d'*Aphis fabae* en milieu extérieur. *Accepted for publication in B.A.S.E.*

9. **Verheggen FJ**, Capella Q, Wathelet JP, Haubruge E (2008). What makes *Episyrphus balteatus* (Diptera: Syrphidae) oviposit on aphid infested tomato plants? *Accepted for publication in Communications in Agricultural and Applied Biological Sciences*.
10. **Verheggen FJ,** Haubruge E, De Moraes CM, Mescher MC (2008). Production of alarm pheromone by developing aphids varies in response to their social environment. *Accepted with minor revisions in Behavioural Ecology*.
11. **Verheggen FJ**, Capella Q, Schwartzberg E, Haubruge E (2008). Tomato-aphid-hoverfly: An incompatible tritrophic interaction for pest management. *Accepted with major revisions in Arthropod-Plant Interactions.*
12. **Verheggen FJ**, Detrain C, Diez L, Wathelet B, Haubruge E (2008). Aphid-ant mutualism: How do aphids focus ant foraging? *Insectes sociaux*. Submitted.

2. Oral Presentations

1. **Verheggen F,** Arnaud L, Haubruge E (2005). Isolation of tomato plant volatiles and their perception by the predator *Episyrphus balteatus* De Geer. Syrphidae Symposium Leiden (Hollande), 2-4 Septembre 2005.
2. **Verheggen, F,** Arnaud, L, & Haubruge, E (2005). Isolation of tomato plant volatiles and their perception by the predator *Episyrphus balteatus* De Geer. Symposium "Entomology in Belgium", Brussels, 2nd December.
3. **Verheggen FJ**, Schwartzberg E, Haubruge E and Tumlinson J (2007). Emission of alarm pheromone in aphids : a contagious phenomenon ? 59th International Symposium on Crop Protection, Gent (Belgium).
4. **Verheggen FJ**, Mescher MC, Haubruge E, De Moraes CM (2008). Production of alarm pheromone by developing aphids varies in response to their social environment. 24th Meeting of the International Society of Chemical Ecology. August 17-22de, State College, PA, USA.
5. **Verheggen FJ**, Capella Q, Wathelet J-P, Haubruge E (2008). What makes *Episyrphus balteatus* (Diptera: syrphidae) oviposit on aphid infested tomato plants ? 60th International Symposium of Crop Protection, Gent, 20 mai: 35

6. **Verheggen FJ**, Detrain C, Diez L, Wathelet B, Haubruge E (2008). Aphid-ant mutualism: How do aphids focus ant foraging? International congress of Entomology, Belgian Crongress of Zoology, Liège, Belgium, October 30-31.
7. **Verheggen FJ**, Diez L, Detrain C, Haubruge E (2008). Mutualisme pucerons-fourmis : étude des bénéfices retirés par les colonies d'*Aphis fabae* en milieu extérieur. Belgian Crongress of Zoology, Liège, Belgium, October 30-31.

3. Posters

1. **Verheggen FJ,** Mescher M-C, Haubruge E, De Moraes C-M (2007). Aphids adapt their alarm pheromone production to presence or absence of conspecifics. 13 th Symposium on insect – plant relationships, 29 – 2 august, Uppsala, Sweden : 116
2. **Verheggen FJ**, Capella Q, Wathelet JP, Haubruge E (2008). What makes *Episyrphus balteatus* (Diptera : syrphidae) oviposit on aphid infested tomato plants? International Symposium of Plant Physiology, Merida (Mexico), June 26th
3. **Verheggen FJ**, Detrain C, Diez L, Wathelet B, Haubruge E (2008). Aphid-ant mutualism: How do aphids focus ant foraging? International congress of Entomology, Durban, South Africa, July 6-12
4. **Verheggen FJ**, Mescher MC, Francis F, Haubruge E, De Moraes CM, Schwartzberg E (2008). Emission of alarm pheromone in aphids: A non-contagious phenomenon. International congress of Entomology, Durban, South Africa, July 6-12
5. **Verheggen FJ,** Haubruge E, De Moraes CM, Mescher MC (2008). Production of alarm pheromone by developing aphids varies in response to their social environment. Belgian Crongress of Zoology, Liège, Belgium, October 30-31.