Searching behavior of aphid natural enemies and its implications for host-race maintenance in the pea aphid complex

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von

Diplom-Biologin Ilka Vosteen

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Introduction

Enemy-free space facilitates ecological speciation via host-shifting

The majority of speciation processes are driven by adaptations towards contrasting ecological conditions. If selection under different ecological regimes results in the formation of gene flow barriers between subpopulations, this process is known as 'ecological speciation', in contrast to other forms of speciation that are driven by sexual selection or by chance events like genetic drift and bottleneck effects (Schluter 2000; Schluter 2001; Rundle & Nosil 2005). Host plant-associated diversification of herbivorous insects is a well-studied example of ecological speciation (Matsubayashi, Ohshima & Nosil 2010) and it is assumed that this process generated most of the great diversity of herbivorous insects (Nyman 2010).

Colonization of novel host plants by herbivorous insects may occur for different reasons: (1) A population decline of the original host plant may force a herbivore to colonize novel hosts. (2) Plants that newly invade the habitat of the herbivore or increase in frequency may be colonized (e.g. Carroll 2007; Keeler & Chew 2008; Peccoud *et al.* 2009b) and (3) mutations in the sensory system of the herbivore may increase the attractiveness of plants that were not attractive before (Caillaud & Via 2000). However, speciation via host-shifting will only happen if herbivores have a reduced performance on the novel host plants. Otherwise, the novel host plant would just be incorporated into the existing diet and no host associated differentiation of insect herbivores would occur (Nyman 2010). However, this performance reduction on novel host plants makes it less likely that herbivores settle long enough on novel host plants so that adaptations can evolve. Therefore, the novel host plant should provide some benefits for the herbivore that counterbalance the fitness reduction due to lower performance.

Insect herbivores colonizing novel hosts may benefit from competition release (Feder *et al.* 1995; Berlocher & Feder 2002) or a reduced risk of natural enemy attacks (e.g. Price *et al.* 1980; Jeffries & Lawton 1984; Bernays & Graham 1988), a situation referred to as 'enemy-free space'. These benefits may offset any performance disadvantages on novel hosts and would enable herbivores to colonize nutritionally inferior plants.

The existence of an enemy-free space has been shown to explain the oviposition preference of insects for certain host plants (e.g. Damman 1987; Ballabeni, Wlodarczyk & Rahier 2001; Moon & Stiling 2006; Sadek, Hansson & Anderson 2010), indicating that enemy-free space may indeed be important in structuring plant-herbivore interactions. Moreover, it has been proven repeatedly that strong natural enemy pressure

on ancestral host plants can lead to a host range expansions resulting in the inclusion of nutritionally inferior plants (Mira & Bernays 2002; Mulatu, Applebaum & Coll 2004). If host plant preferences lead to assortative mating, the existence of enemy-free space may lead to host race formation, as known for the goldenrod ball gallmaker *Eurosta solidaginis*, the apple maggot fly *Rhagoletis pomonella* and the Alaskan swallowtail butterfly *Papilio machaon aliaska* (Brown *et al.* 1995; Feder 1995; Murphy 2004; Heard *et al.* 2006) or to speciation as shown for moths of the genus *Heliothis* and *Ostrinia* (Oppenheim & Gould 2002; Pelissie *et al.* 2010). Despite these few examples, it is still unclear how important the existence of enemy-free space is in the process of host race formation.

Multiple causes for enemy free space

To characterize the impact of enemy-free space on host-race formation and speciation, it is important to understand the mechanisms that can generate this phenomenon. These mechanisms might be different depending on the type of natural enemy and its foraging behavior. Thus a host plant that provides an enemy-free space against one predator may be highly preferred by a second predator or parasitoid. In such a case, the existence of an overall enemy-free space depends on the composition of the enemy community and the relative impact of each natural enemy on the herbivore population.

There are several reasons why certain host plants could provide an enemy-free space. Enemies are known to avoid less suitable prey and the nutritional quality of herbivores as prey organisms, including their toxin content, is often influenced by the chemical composition of the host plant (Ode 2006 and cited literature). Denno, Larsson and Olmstead (1990), for example, showed that larvae of the willow leaf beetle *Phratora vitellinae* find an enemy-free space on the salicinoid-rich *Salix fragilis* compare to the salicinoid-poor *Salix viminalis*, because they sequester salicinoids for their defense.

Herbivores colonizing a novel host plant may further escape from their natural enemy in space and time. The apple host race of *Rhagoletis pomonella* finds an enemy-free space on apple trees, its derived host since parasitoids cannot attack larvae that live in apple fruits due to the bigger fruit size and earlier fruiting phenology of apple trees compared to hawthorne, the ancestral host of *R. pomonella* (Feder 1995). Moreover, the abundance of natural enemies increases with habitat complexity and with the complexity of plant architecture (Langellotto & Denno 2004). Thus, plants that have a less complex architecture or grow in less complex habitats may provide an enemy-free space. Habitat quality influences the presence of natural enemies in other ways. Patches where other natural enemies are present are

avoided to prevent intraguild competition and predation (Janssen *et al.* 1995; Taylor, Müller & Godfray 1998; Ruzicka 2001; Nakashima *et al.* 2004), and predators are known to prefer plants where they find alternative food sources like extrafloral nectar or food bodies (Heil 2008 and cited literature). Additionally, herbivores may find an enemy-free space on certain host plants, because it is difficult for their enemies to detect them on these hosts. Natural enemies are known to use a variety of cues to detect their prey/host, including herbivore-induced plant volatiles (e.g. Vet & Dicke 1992; Turlings *et al.* 1995; Takabayashi & Dicke 1996). Thus, differences between plant species in the induction of volatiles may strongly influence natural enemy abundance. Additionally, the searching efficiency of a natural enemy on a plant is influenced by plant architecture (e.g. Andow & Prokrym 1990; Grevstad & Klepetka 1992; Lukianchuk & Smith 1997; Clark & Messina 1998; Gontijo *et al.* 2010) and by plant surface structures like wax layers or trichomes (e.g. Shah 1982; Kennedy 2003; Gentry & Barbosa 2006; Riddick & Simmons 2014 and cited literature). Larvae of the potato tuber moth *Phthorimaea operculella*, for example, are protected from parasitoids on the novel host tomato by trichomes that hamper parasitoid movement, but this benefit is countered by a lower survival rate on this host plant in the absence of natural enemies (Mulatu, Applebaum & Coll 2004; Mulatu, Applebaum & Coll 2006).

Prey and host detection by natural enemies of herbivorous insects

Enemy-free space often results from the inability of the natural enemy to locate the prey. Thus, it is important to have information about the foraging cues and behavior of natural enemies. Predatory insects like ladybirds spend much of their total life foraging (Hassell & Southwood 1978), because they consume prey items that are usually sparsely dispersed. Insect species with predatory larvae and parasitoids similarly spent most of their adult life searching for suitable oviposition places to ensure the successful development of their offspring. Thus, foraging behavior determines where the natural enemy occurs and may strongly influence its population dynamics and impact on prey/host populations.

Foraging insects use different cues and strategies depending on the spacial scale, and insect foraging is generally divided into three hierarchical steps: habitat location, patch location and prey or host location (Hassell & Southwood 1978) (Fig. 1). A habitat is defined as "the environment within which an organism is normally found, and is characterized by the physical characteristics of the environment and/or the dominant vegetation or other stable biotic characteristics" (Lawrence 2005). Thus, the spacial scale of a habitat is not defined and both a forest and a tree could be defined as habitats. Here we use the term

habitat on a landscape scale and a habitat can be seen as an area where prey patches are more likely to be encountered than outside the habitat (e.g. different ecosystems or vegetation communities). Within a habitat, food items are usually not randomly distributed but occur in patches, like herbivores on a plant. A patch is defined as an area containing a stimulus or stimuli at the proper intensity to elicit a characteristic foraging activity in a responsive forager (Hassell & Southwood 1978). Within the patch natural enemies then have to detect and recognize the individual prey or host items.

Habitat location

Due to its large spacial scale, habitat location is difficult to study in insects and thus, little is known about this process. Visual orientation towards prominent landscape features most likely is important in habitat location. The ladybird *Chilocorus nigritus* preys on scales and migrates between citrus orchards and stands of giant bamboo. Hattingh and Samways (1995) showed that it moves towards silhouettes of a horizon with a tree line and towards

1. Habitat location
- optical cues: landscape features, e.g. trees
- olfactory cues: repellent non-host volatiles
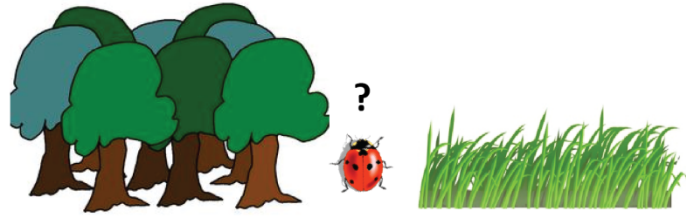
2. Patch location
- optical cues: plant color, shape
- olfactory cues: herbivore induced plant volatiles, sex pheromones
- acoustic cues: courtship calls

3. Prey/host location
- arresting cues: feeding damage, insect frass, honeydew, mandibular gland secretion, herbivore induced plant volatiles
- visual cues: prey/host size, shape, color
- olfactory cues: prey/host odor
- acoustic and vibration cues generated by prey movement and feeding
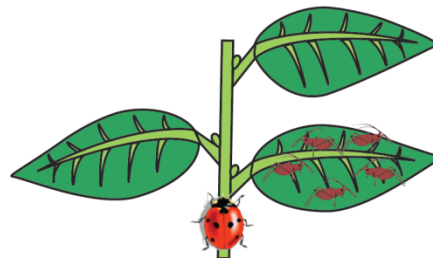
Figure 1: Different cues for prey/host detection are used by natural enemies at different spacial scales

individual trees. Moreover, it prefers vertical over horizontal stripes and is equally attracted by vertical stripes and a tree silhouette. Interestingly, searching beetles habituate faster to the tree silhouette than to the vertical stripes. The authors interpret this to mean that in the case of unsuccessful foraging, it is probably beneficial to habituate faster to a short range cue like the tree silhouette than to longer range
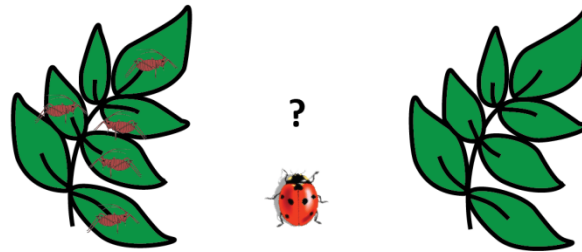
cues like the vertical stripes. Nothing is known about the influence of volatile cues on the habitat location of natural enemies. For insect herbivores it was proposed that they may be repelled by dominant non-host volatiles to avoid habitats where their host plants are unlikely to occur (Andersson 2007). It is likely that natural enemies may also use such volatiles to avoid searching areas where their prey does not occur.

Patch location

Natural enemies that forage within a habitat to find suitable prey/host patches have to face the reliability vs. detectability problem (Vet & Dicke 1992). Cues derived from the herbivore itself (e.g. pheromones) are most reliable, but due to the low biomass of herbivores they are often hard to detect. Plant-derived cues (e. g. plant volatiles) in contrast are easy to detect, but less reliable, because the plant may be herbivore-infested or not. One possible solution to the reliability vs. detectability problem is to use herbivore-induced plant volatiles. These are emitted in higher quantities than volatile cues from herbivores and are highly reliable indicators of herbivore presence.

Thus, herbivore induced plant volatiles are suitable cues to locate a patch (herbivore infested plant or vegetation stand) within the habitat. Attraction of natural enemies towards herbivore-induced plant volatiles has been shown for 26 predatory insect species from 5 taxa and for 49 parasitoid species (Mumm & Dicke 2010; Dicke 2015). Natural enemies can react to an overall increase of volatile emission after herbivory, to certain key induced compounds of the volatile blend or use subtle changes of the volatile ratio within the blend to distinguish between infested vs. non-infested plants. Differences in the volatile ratio may further inform predatory or parasitoid natural enemies about the plant species the insect is feeding on and help to distinguish between prey/host and non-prey/non-host species feeding on the plant. Plant volatile emission, however, is not only altered by herbivory, but also by the plant genotype, abiotic conditions, neighboring plants and additional herbivore species feeding on the plant, so that natural enemies may have to deal with enormous complexity within the spectrum of herbivore-induced plant volatiles (e.g. Dicke 1999; Dicke, van Poecke & de Boer 2003; Dicke, Van Loon & Soler 2009; Mumm & Dicke 2010; Hare 2011; de Rijk, Dicke & Poelman 2013).

Moreover, herbivore-induced plant volatiles often have to be detected against a complex odor background. If background volatiles are detected, continuous exposure to these compounds may lead to habituation and so help the insect to filter the relevant cues from the background. Similarly, sensitisation to volatiles indicating the presence of a prey or host might facilitate recognition of these odors against the background. An insect that encounters an attractive odor will start to fly upwind towards the odor source

(surge behavior). When it loses the odor plume, it will start to fly crosswind until it encounters the odor plume again (cast behavior). The odor plume consists of odor strands with a high odor concentration, interspaced by air strands free of the target odor, which consist only of the background odor. The degree of mixing of plume filaments from different sources depends on the distance from the odor sources and on the distances between the different sources. Pure odor strands even exist at considerable distance from the odor source. While following the odor plume, the insect will pass several target odor strands and background odor strands. Thus, volatiles from spatially separated odor sources will reach the insect antennae at different times, enabling the insect to differentiate the relevant odor from the background odor and to follow the relevant odor towards the source (Beyaert & Hilker 2014 and cited references).

To locate prey/host patches or individuals within a patch, natural enemies may further exploit sexual signals like courtship calls or sex pheromones (e.g. Cade, Ciceran & Murray 1996; Zuk & Kolluru 1998) and also visual cues like plant color and shape may be used for patch location. Hattingh and Samways (1995) and Bahlai *et al.* (2008) for example showed that ladybirds are attracted towards leaf-shaped images. Light quality is an additional cue that may be use in patch and/or habitat detection (Romeis & Zebitz 1997).

Prey/host location with a patch

After a patch has been encountered, contact with a prey/host individual or different prey/host related cues typically induces an area-restricted searching behavior characterized by an increased turning frequency and turning angle which arrests the enemy within the patch (e.g. Laing 1937; Banks 1957; Chandler 1969; Bell 1990). Typical cues that arrest natural enemies are feeding damage (Wang & Keller 2002), insect frass (e.g. Mattiacci *et al.* 2001; Tanaka, Kainoh & Honda 2001; Wang & Keller 2002), honeydew (Budenberg 1990; McEwen *et al.* 1993; Romeis & Zebitz 1997), mandibular gland secretion (Waage 1978), herbivore-induced plant volatiles (Sabelis & Afman 1994) and wing scales (Colazza *et al.* 2014 and cited references). Some parasitoids do not search a patch randomly but follow herbivore tracks on the plant until they encounter the herbivore (Colazza *et al.* 2014 and cited literature). Natural enemies may further mark already searched areas to avoid searching them again (Bernstein & Driessen 1996; Nakashima, Teshiba & Hirose 2002; Meisner & Ives 2013).

Natural enemies often detect a prey or host item before they touch it physically using visual cues like color, shape and size (e.g. Battaglia *et al.* 1995; Harmon, Losey & Ives 1998; Powell *et al.* 1998; Kral, Vernik & Devetak 2000; Morehead & Feener Jr. 2000) as well as olfactory cues like prey odor (Hemptinne *et al.*

2000). Moreover, enemies may use sound and vibrations generated by prey movement and feeding to detect their prey/host from a distance (Lawrence 1981; Sugimonto *et al.* 1988; Pfannenstiel, Hunt & Yeargan 1995).

Plasticity of foraging behavior

Foraging behavior of natural enemies is highly plastic and is influenced by environmental conditions and the physiological state of the insect. Adverse weather condition like wind and rain strongly reduce foraging activity and efficiency (Fink & Völkl 1995; Weisser, Volkl & Hassell 1997; Schwörer & Völkl 2001) and the presence of intraguild predators reduces the attractiveness of a patch (e.g. Ruzicka 1998; Taylor, Müller & Godfray 1998). It was further shown that egg load and hunger level strongly influence foraging activity and foraging strategy (e.g. Minkenberg, Tatar & Rosenheim 1992; Takasu & Lewis 1993; Sadeghi & Gilbert 2000b; Nakashima, Teshiba & Hirose 2002). Moreover, natural enemies are able to learn cues that they perceive during their development and cues that were associated with previous prey/host encounters (Turlings *et al.* 1993; Steidle & Van Loon 2003).

Aphid natural enemies

The searching behavior of aphid natural enemies is well studied (reviewed by Evans 2003; Hatano *et al.* 2008; Almohamad, Verheggen & Haubruge 2009) and may serve as a model to understand the general foraging behavior of natural enemies at different spacial scales. It might also reveal the impact of natural enemies on host race formation and speciation processes. Aphids are highly variable food sources with colony sizes ranging from just a few to several thousand individuals infesting the same plant, and the occurrence of aphid colonies is often ephemeral and unpredictable in space and time (e.g. Kan 1988a; Kan 1988b; Osawa 2000). Most aphid predators are generalists and prey on a variety of different aphid species, even though not all species can be used as 'essential' prey that allows larval development and egg production (e.g. Hodek, Honek & Hodek 1996; Gilbert 2005). Thus, predators may feed on most aphid colonies that they encounter to a certain extent, but leave patches earlier when the prey is of low quality. During foraging, they should mainly react to general cues that signal aphid presence while highly specific cues that differ between different aphid species, like aphid-induced plant volatiles, should be less important. Parasitoids on the other hand are more specialized and thus should also react to cues that are specific for certain aphid species.

Aphid natural enemies can be divided into three groups with different feeding and foraging strategies: parasitoids, highly mobile adult predators like ladybirds, ants and spiders, and less mobile predatory larvae like ladybird and hoverfly larvae. For species with predatory larvae, it is thus crucial that ovipositing females select aphid colonies that ensure successful larval development. Here; I will briefly summarize the searching behavior of (1) ladybirds, as an example of a species with mobile predatory adults and less mobile predatory larvae, of (2) hoverflies, as an example of a species with non-predatory adults that need to find aphid colonies as oviposition sites, and of (3) parasitoids.

Ladybirds

Adult ladybirds are known to move frequently between sites and habitats (Evans 2003 and cited literature), but little is known about how they detect habitats that are likely to contain aphids. It was reported that leaving hibernation sites happens as non-directional dispersal, while flights towards hibernation sites are considered to be directional migration flights that are under partial control of the ladybirds themselves (Hagen 1962 and cited literature). The scale feeding ladybird *Chilocorus nigritus* uses optical cues for habitat detection (Hattingh & Samways 1995), and it is likely that aphidophagous ladybirds can also use optical cues to detect habitats where the occurrence of aphids is likely.

Evans (2003) proposed that the accumulation of ladybirds in aphid-containing patches, which was observed in several studies, is mainly a passive process, with hungry ladybirds searching the upper plant canopy in a random way until they encounter aphids or aphid cues that induce an area-restricted search. However, it may also be possible that ladybirds use volatiles to detect prey patches from a distance. It was shown that methyl salicylate, which is induced by aphid infested plants attracts *C. septempunctata* to field traps (Zhu & Park 2005). Similarly, several olfactometer experiments showed that ladybirds are attracted to aphid induced plant volatiles (Ninkovic, Al Abassi & Pettersson 2001; Han & Chen 2002; Bahlai *et al.* 2008; Tapia, Morales & Grez 2010) and aphid sex-pheromones (Leroy *et al.* 2012b). However, due to the artificial nature of these experiments, it is unclear if aphid-induced plant volatiles function to attract flying ladybirds towards an aphid infested plant or if they work merely as arrestant cues that concentrate the searching activity of the ladybird on aphid infested plants. It is often stated that the aphid alarm pheromone (*E*)-β-farnesene can be used as a kairomone to detect aphid-infested plants, but since the amounts tested were much higher than the range of amounts normally emitted by aphids (Al Abassi *et al.* 2000; Hemptinne *et al.* 2000; Acar *et al.* 2001; Francis, Lognay & Haubruge 2004; Leroy *et al.* 2012a), it is likely that EBF emitted by attacked aphids may not be used as a long range cue by foraging ladybirds and other aphid natural enemies (for details see chapter IV & V).

Cues that arrest a searching ladybird within a prey patch include honeydew and honeydew volatiles (Carter & Dixon 1984; Han & Chen 2002; Leroy *et al.* 2012a; Purandare & Tenhumberg 2012), as well as the odor of aphids, aphid infested leaves and uninfested leaves (Obata 1986). After encountering aphids or aphid cues, ladybirds switch to an area-restricted search pattern with an increase in turning frequency and turning angle on flat surfaces (Carter & Dixon 1982; Nakamuta 1982; Murakami & Tsubaki 1984; Nakamuta 1985). However, ladybirds usually do not search flat surfaces, but three-dimensional plant structures. While searching on plants they usually follow structures like stems, leaf edges and leaf veins and they often leave a leaf without searching most of the leaf area. Observations show that they spent about 80% of the time searching the leaves and only a little time on the stem (Banks 1957). *Propylea quatuordecempunctata* larvae that search a stand of plants often switch between plants when they touch leaves of a neighboring plant, spending a lot of time on leaves that were already visited. This non-systematic search pattern drastically reduces the chance of finding an isolated aphid colony (Banks 1957), and it was shown that *Harmonia axyridis* larvae mark the areas which they already searched to avoid searching them again (Meisner & Ives 2013). Even when coming close to an aphid colony, ladybird larvae often turn around before reaching the colony, indicating that they are not able to detect an aphid from a distance of more than a few millimeters (Banks 1957). Stubbs (1980) showed that crushed aphid prey is detected from a distance of 7 mm and Hemptinne *et al.* (2000) showed that 1st instar larvae are attracted by the aphid alarm pheromone that is emitted when conspecifics feed on an aphid. Moreover, Jamal and Brown (2001) showed that ladybird larvae are attracted by the odor of aphids, indicating that aphid volatiles may play an important role for the prey detection of ladybird larvae. For adult ladybirds, vision seems to be of greater importance to detect aphids from a distance (Harmon, Losey & Ives 1998). Aphids and dummy prey are detected at a distance of 7 to 10 mm (Stubbs 1980; Nakamuta 1984), and aphid color seems to play an important role in prey detection (Harmon, Losey & Ives 1998).

Hoverflies

In contrast to ladybirds, where both larvae and adults feed on aphids, in hoverflies only the larvae consume aphids, while adults feed on nectar and pollen (e.g. Schneider 1969; Gilbert 1981). It is generally assumed that hoverfly larvae have a low dispersal capacity (but see chapter III for hoverfly larval migration) and that female oviposition choice is crucial for successful larval development (e.g. Sadeghi & Gilbert 2000a; Almohamad, Verheggen & Haubruge 2009). According to their oviposition preferences, hoverfly species with aphidophagous larvae are divided into two groups: aphidozetic species like *Episyrphus balteatus*, that oviposit close to aphid colonies and use mainly aphid derived oviposition cues,

and phytozetic species like *Melanostoma mellinum* that also oviposit on uninfested plants and use mainly plant-derived oviposition cues (Chandler 1968; Almohamad, Verheggen & Haubruge 2009).

Figure 2: Hoverfly (*Episyrphus balteatus*), photo: I. Vosteen

Several aphidophagous hoverfly species are known to migrate between their summer habitats in North and Middle Europe where they oviposit, and their overwintering habitats in the Mediterranean (Gatter & Schmid 1990). Still little is known about the cues hoverflies may use to identify suitable habitats for oviposition. The presence of flowers with easily accessible nectar has been shown to increase the abundance of adult hoverflies as well as egg numbers in a habitat (e.g. van Rijn, Kooijman & Wackers 2006; van Rijn & Wäckers 2010; Gillespie *et al.* 2011; Hogg *et al.* 2011), indicating that the presence of adult food sources is an important criterion for habitat selection. Optical cues may also be important in detecting potential oviposition habitats. It was shown that *Syrphus corolla* prefers dark background colors while it searches for oviposition sites (Sanders 1982). The author interprets this as a preference for dense vegetation, where most eggs of this species are found (Peschken 1964). It was further shown that *S. corolla* preferentially searches complex structures that provide vertical and horizontal optical cues and structures that resemble the appearance of a plant (Sanders 1983).

For detecting aphid infested plants from a distance, honeydew volatiles (Leroy *et al.* 2012a) and aphid induced plant volatiles may play a role. Verheggen *et al.* (2008) showed that several common plant volatiles are detected by hoverflies and that (*Z*)-3-hexenol applied to uninfested *V. faba* plants increases searching and landing of *E. balteatus*, but it is not known if hoverflies are attracted by naturally emitted volatile blends containing these compounds. Before hoverflies land on a plant, they usually hover close to the plant, probably inspecting the plant for aphids (Scholz & Poehling 2000). Kan and Sasakawa (1986) showed that optical cues may play an important role in detecting suitable aphid infested plants: in their experiment hoverflies preferred non-winged aphid models (black beads) over winged aphid models. After hoverflies land on a plant, aphid presence and honeydew contact are important oviposition stimuli (Budenberg & Powell 1992; Scholz & Poehling 2000; Leroy *et al.* 2011; Leroy *et al.* 2014).

The decision to lay eggs, however does not just depend on the presence of aphids on a plant. Gravid females were shown to have an innate rank order of preferences for different aphid species and their

decision to accept or reject a given aphid species depends on the preference rank of this species and on the egg load and age of the female (Almohamad, Verheggen & Haubruge 2009 and cited literature). It was further shown that the reproduction potential of an aphid colony influences *E. balteatus* oviposition behavior: hoverflies preferred to oviposit in small aphid colonies consisting mainly of young aphids, while they avoided oviposition in larger colonies with several winged adults. This strategy ensures that hoverfly larvae will find plenty of aphids as food when they hatch and reduces the risk that the aphid colony disperses or goes extinct before hoverfly larvae have finished their development (Kan & Sasakawa 1986; Kan 1989).

However, due to the ephemeral nature of aphid colonies, it is still likely that a colony may collapse before the hoverfly larvae finished their development. Under these circumstances it would be beneficial if hoverfly larvae had the ability to move to other aphid colonies and there is clear evidence from the literature that hoverfly larvae migrate between aphid colonies. Chandler (1969) reported that "unfed first instar larvae were able to travel considerable distances, certainly well in excess of 1 m" and Banks (1968) found several hoverfly larvae in field experiments that must have migrated to the experimental aphid colonies. Similarly Kan (1988a) and Kan (1988b) observed older hoverfly larvae that migrated between aphid colonies in the field. Nevertheless, it is generally assumed that hoverfly larvae are not able to travel long distances (e.g. Sadeghi & Gilbert 2000a; Almohamad, Verheggen & Haubruge 2009), and nothing is known about the migration behavior of hoverfly larvae.

Parasitoids

Aphid parasitoids are often attracted by volatiles emitted from undamaged plants that can be potential food plants for their aphid hosts (Hatano *et al.* 2008 and cited literature). Thus, volatiles from undamaged plants may be used to identify habitats where aphid hosts are likely to be present. To find aphid infested plants within the habitat, aphid parasitoids may use aphid-induced plant volatiles (e.g. Grasswitz & Paine 1993a; Du, Poppy & Powell 1996; Du *et al.* 1998; Guerrieri *et al.* 1999; Han & Chen 2002; Pareja *et al.* 2009) or honeydew volatiles (Bouchard & Cloutier 1985). In autumn when aphids reproduce sexually, parasitoids may further use aphid sex pheromones to detect aphid infested plants (e.g. Hardie *et al.* 1991; Gabryś *et al.* 1997; Glinwood, Du & Powell 1999). Aphid presence and contact with honeydew arrests parasitoids on aphid infested plants (e.g. Budenberg 1990; Budenberg, Powell & Clark 1992; Grasswitz & Paine 1993b) and optical cues like color and shape are important to recognize aphids on the infested plants (Battaglia *et al.* 1995; Battaglia *et al.* 2000). *Aphidius ervi* prefers to attack green over red or brown

aphid clones (Michaud & Mackauer 1994; Libbrecht, Gwynn & Fellowes 2007) and *Praon pequodorum* rarely attacks aphids that are not moving (Michaud & Mackauer 1995). It was further shown that contact chemicals from the host cuticle and aphid cornicle secretion, which is secreted by the aphid during an attack (Goff & Nault 1974), stimulate parasitoid attacks (Battaglia *et al.* 1993; Battaglia *et al.* 1995; Battaglia *et al.* 2000; Weinbrenner & Völkl 2002; Muratori *et al.* 2006).

The response of *A. ervi* towards aphid-induced changes of plant volatile profiles has been intensively studied and it was shown that *A. ervi* can use differences in plant volatile emission to distinguish whether the plant is infested by host or non-host aphids (Du, Poppy & Powell 1996; Guerrieri *et al.* 1999). It was further shown that aphid infestation does not induce a stronger volatile emission of *Vicia faba* and *Medicago sativa*, but that *A. ervi* is able to use minor changes of the volatile ratio to distinguish aphid-infested from non-infested plants (Pareja *et al.* 2009). Moreover, the response of *A. ervi* towards plant volatiles is highly plastic. Volatile exposure during larval development and during adult emergence from the mummy shapes the parasitoid's reaction towards aphid-induced plant volatiles (Takemoto *et al.* 2012). Further exposure to aphid-damaged plants with or without aphids after mating increases the responsiveness of parasitoids towards volatiles from aphid damaged and undamaged plants in wind tunnel assays, indicating that the presence of host-associated cues like honeydew act as reinforcement stimuli in the learning process (Du *et al.* 1997; Powell *et al.* 1998). Additionally, the presence of intraguild predators and their chemical traces decreases oviposition by parasitoids since parasitoid larvae developing in aphids are often accidentally consumed by aphid predators (Taylor, Müller & Godfray 1998; Nakashima *et al.* 2004; Almohamad & Hance 2013).

Impact on aphid colonies

Aphid natural enemies have a strong impact on aphid colony development (Chambers *et al.* 1983; e.g. Chambers & Aikman 1988; Fox *et al.* 2004) and their presence strongly increases the extinction rate of small aphid colonies (Morris 1992). This is not just due to the high voracity of some predator species (e.g. Hindayana 2001; Mishra *et al.* 2011), but also due to strong non-consumptive effects of natural enemy foraging in aphid colonies (Nelson, Matthews & Rosenheim 2004; Nelson 2007; Fievet, Lhomme & Outreman 2008; Fill, Long & Finke 2012). Attacked aphids often manage to escape the predator (Minoretti & Weisser 2000; Nelson & Rosenheim 2006), but this escape behavior results in non-feeding periods that reduce the body weight and reproduction rate of aphids (Nelson 2007). Additionally, attacked aphids often drop off their host-plants. This strategy is highly effective in avoiding predation by the attacking predator (Francke *et al.* 2008), but makes the aphid vulnerable to ground predators, starvation and

desiccation (Losey & Denno 1998b; Losey & Denno 1998a). These non-consumptive effects of foraging predators are further increased by aphid alarm pheromone emission that causes neighboring aphids to disperse and drop off their host plant (e.g. Kislow & Edwards 1972; Minoretti & Weisser 2000), and disturbed aphid colonies produce more winged offspring that leave the host plant (e.g. Kunert & Weisser 2003; Kunert & Weisser 2007). Because of these strong effects of natural enemies on aphid colony dynamics it is likely that the existence of an enemy free space on certain aphid host plants would reduce the disadvantages of colonizing novel, but less-suitable host plants and would facilitate host race formation in aphids.

Pea aphid complex as a model to study ecological speciation via host race formation

The pea aphid complex (*Acyrthosiphon pisum* HARRIS, Fig. 3) is an important model to study ecological speciation via host plant switches (Peccoud & Simon 2010). It consists of 11 distinct host plant-associated sympatric populations, including 3 possible species and 8 host races (Peccoud *et al.* 2009a). These host-associated populations most likely evolved via host plant switches during the anthropogenic range expansion of legume species, and the burst of diversification was dated back to the post-Pleistocene warming (Peccoud *et al.* 2009a). All host races perform well on the broad bean *Vicia faba*, which is viewed as the 'universal host' (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008) and they are specialized on different legume species (their so called native hosts), where they have a high performance and which they prefer for feeding (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008; Peccoud *et al.* 2009a).
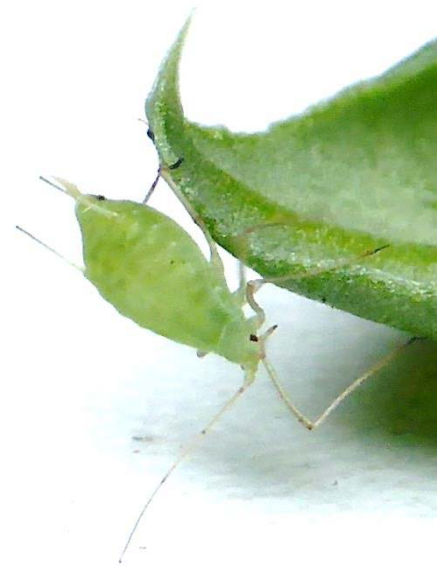
Figure 3: Pea aphid (*Acyrthosiphon pisum),* photo: I. Vosteen

The differences in pea aphid host acceptance have led to assortative mating on their native hosts which reduces the gene flow between different host races and makes specialization on the different host plants possible (Caillaud & Via 2000; Peccoud *et al.* 2009a). When the pea aphid races are placed on other legume species they are not adapted to, they often show a strongly reduced performance or do not

survive at all (Ferrari, Via & Godfray 2008; Schwarzkopf *et al.* 2013). Thus, aphids switching their host plant probably have a strongly reduced performance on their novel host plant and so the novel host plant should provide some benefits that stabilize the aphid populations long enough that adaptations can evolve.

Despite strong evidence that the existence of an enemy-free space may facilitate host race formation and speciation processes of different insect herbivores (Brown *et al.* 1995; Feder 1995; Oppenheim & Gould 2002; Heard *et al.* 2006; Pelissie *et al.* 2010), the influence of aphid natural enemies on host race formation and maintenance within the pea aphid complex has never been studied. Thus, the main aim of this work is to answer the following questions:

- Do some host plants provide an enemy-free space for pea aphids?
- Which mechanisms generate the enemy-free space on certain host plants?
- How does the searching behavior of different aphid natural enemies influence enemy-free space?

Overview of manuscripts

Chapter I: Enemy-free space against multiple natural enemies

Enemy-free space promotes maintenance of host races in an aphid species

Ilka Vosteen, Jonathan Gershenzon, Grit Kunert

Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena, Germany

In the first chapter I show that the native host plants provide an enemy-free space against hoverfly larvae and mobile natural enemies.

Hoverflies preferred to oviposit on *V. faba* and *Pisum sativum* and feeding by hoverfly larvae suppressed aphid population growth on these host plants. Thus, the native host plants *Medicago sativa* and *Trifolium pratense* provided an enemy-free space for pea aphids. Predation by mobile predators was higher on *V. faba* infested with Pisum race aphids than on *P. sativum*, indicating that the enemy-free spaces against different groups of natural enemies may supplement each other and that taken together all three native host plants provide an enemy-free space for pea aphids.

The high predation pressure on the universal host *V. faba* reduces the survival of aphid colonies on the universal host and likely prevents the establishment of mixed colonies. Thus, mating of the different host races on *V. faba* is unlikely and the enemy-free space on native host plants may help to maintain the different host races.

Built on an idea conceived by Ilka Vosteen and Grit Kunert

Designed experiments: Ilka Vosteen (80%) and Grit Kunert

Conducted experiments: Ilka Vosteen (100%)

Performed data analyses: Ilka Vosteen (50%) and Grit Kunert

Wrote the manuscript: Ilka Vosteen (70%), Grit Kunert and Jonathan Gershenzon

Chapter II: Honeydew as oviposition cue for hoverflies

Hoverfly preference for aphids with a high reproduction potential creates an enemy-free space on novel host plants and facilitates host race formation in aphids

Ilka Vosteen, Jonathan Gershenzon, Grit Kunert

Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena, Germany

In this chapter I identified the cause for the observed hoverfly oviposition preferences for plants where aphids have a high reproduction potential. I could show that aphids produced less honeydew on plants where their performance and reproduction rate was low. Field experiments proved that hoverflies use the presence of aphids and honeydew as oviposition cues and that egg number increases with the amount of honeydew on a plant. I could further show that pea aphids find enemy-free space on less suitable host plants, most likely due to reduced honeydew production and reduced aphid size compared to hosts to which the aphids are well adapted. This enemy-free space reduces the performance disadvantages of aphids colonizing a novel host and probably plays an important role in aphid host race formation.

Built on an idea conceived by Ilka Vosteen and Grit Kunert

Designed experiments: Ilka Vosteen (90%) and Grit Kunert

Conducted experiments: Ilka Vosteen (100%)

Performed data analyses: Ilka Vosteen (60%) and Grit Kunert

Wrote the manuscript: Ilka Vosteen (70%), Grit Kunert and Jonathan Gershenzon

Chapter III: Searching behavior of hoverfly larvae

Migrating hoverfly larvae accumulate in large aphid colonies

Ilka Vosteen, Jonathan Gershenzon, Grit Kunert

Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena, Germany

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Field experiments showed that hoverfly larvae often consumed an aphid colony before they completed their development. Thus, they have to leave the plant where they hatched in search of additional food sources. In this chapter I showed that they do not leave a plant before most aphids had been consumed and that they do not distinguish between aphid colonies infesting native and universal host plants. Hoverfly larvae prefer large aphid colonies and this preference explains the distribution of migrating hoverfly larvae in a field experiment. The influence of honeydew and other aphid cues on the searching behavior of hoverfly larvae as well as the potential of hoverfly larvae to use non-aphid food are discussed.

Built on an idea conceived by Ilka Vosteen and Grit Kunert

Designed experiments: Ilka Vosteen (90%) and Grit Kunert

Conducted experiments: Ilka Vosteen (100%)

Performed data analyses: Ilka Vosteen (60%) and Grit Kunert

Wrote the manuscript: Ilka Vosteen (80%), Grit Kunert and Jonathan Gershenzon

Chapter IV: Enemy attraction by aphid alarm pheromone?

Is there any evidence that aphid alarm pheromones work as prey and host finding kairomones for natural enemies?

Ilka Vosteen[1], Wolfgang W. Weisser[2], Grit Kunert[1]

1 Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena, Germany

2 Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85350 Freising-Weihenstephan, Germany

The aphid alarm pheromone (*E*)-β-farnesene (EBF) is generally considered to be used by natural enemies as a prey/host finding kairomone, even though studies show opposing results. Some appear to confirm an attraction of aphid natural enemies by EBF while in others the evidence is unclear. To clarify if aphid natural enemies are attracted by EBF amounts naturally emitted by aphids, we reviewed the existing literature.

Most studies which show an attraction by EBF used much higher amounts of EBF than usually emitted by aphids during a predator attack. Studies with EBF amounts similar to what is emitted by aphids are rare and failed to show an attraction. Moreover, there are only two studies which document an attraction of natural enemies by attacked aphids. Since EBF is emitted in very low amounts, is not very stable, and is only present after an attack, I consider EBF not to be a suitable kairomone for most natural enemy species especially when they are able to use alternative cues. Because EBF is present among herbivore-induced plant volatiles we propose that natural enemies might use it to identify aphid infested plants.

Built on an idea conceived by Ilka Vosteen and Wolfgang W. Weisser

Performed literature review: Ilka Vosteen (100%)

Wrote the manuscript: Ilka Vosteen (80%), Grit Kunert and Wolfgang W. Weisser

Chapter V: No effect of EBF on predators searching on a plant

The aphid alarm pheromone (*E*)-β-farnesene does not act as a cue for predators searching on a plant

Christoph Joachim[1], Ilka Vosteen[2] , Wolfgang W. Weisser[1]

1 Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85350 Freising-Weihenstephan, Germany

2 Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena, Germany

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To test if the aphid alarm pheromone works as a short range attractant or arrestant, the searching behavior of lacewing and hoverfly larvae on a plant was observed in the presence and absence of the aphid alarm pheromone (*E*)-β-farnesene (EBF). Neither the application of natural (50 ng) nor unnaturally high (1 μg) amounts of EBF induced longer patch residence times or increased the foraging success of the predator compared to solvent application. Thus, EBF does not serve as an attractant or arrestant cue for predators searching on a plant.

Built on an idea conceived by Ilka Vosteen, Christoph Joachim and Wolfgang Weisser

Designed experiments: Christoph Joachim, Ilka Vosteen (40%) and Wolfgang Weisser

Conducted experiments: Christoph Joachim and Ilka Vosteen (20%)

Performed data analyses: Christoph Joachim and Ilka Vosteen (10%)

Wrote the manuscript: Christoph Joachim, Ilka Vosteen (10%) and Wolfgang Weisser

Chapter I: Enemy-free space against multiple natural enemies

Enemy-free space promotes maintenance of host races in an aphid species

Ilka Vosteen*, Jonathan Gershenzon, Grit Kunert

Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena

Abstract

The enormous biodiversity of herbivorous insects may arise from ecological speciation via continuous host plant switches. Whether host plant switches can be successful depends on the trade-off between different selection pressures which act on herbivores. Decreased herbivore performance due to suboptimal nutrition might be compensated by a reduced natural enemy pressure. As a consequence an 'enemy-free space' on a certain plant might facilitate host plant switches and maintain biotypes. To test this hypothesis, we used the pea aphid (*Acyrthosiphon pisum*) complex which consists of at least 11 genetically distinct host races that are native to specific legume host plants, but can all develop on the universal host plant *Vicia faba.* Three *A. pisum* host races native to *Trifolium pratense*, *Pisum sativum* and *Medicago sativa* living on the universal host plant *V. faba* and on their respective host plant were investigated.

We found that hoverflies preferred to oviposit on *P. sativum* and the universal host *V. faba*. Since feeding by hoverfly larvae suppressed aphid population growth on these host plants, the native host plants *M. sativa* and *T. pratense* provided an enemy-free space for the respective *A. pisum* races. Mobile predators, such as ants and ladybirds preferred Pisum race aphids on *V. faba* over *P. sativum*. Thus all three native host plants studied supply an enemy free space for *A. pisum* compared to the universal host *V. faba*. Reduction of encounters between aphid races on *V. faba* would reduce gene flow among them and contributes to maintain the host races.

Keywords: enemy-free space, host race, speciation, aphid, hoverfly, ladybird beetle, parasitoid, multitrophic interactions, extrafloral nectaries

Introduction

With approximately 5 million species, insects are by far the most diverse group of eukaryotic organisms inhabiting our world (e.g. Stork 1993; Odegaard 2000; Novotny *et al.* 2002). Taxa of plant feeding insects contain more species than their non-herbivorous sister groups (Mitter, Farrell & Wiegmann 1988), and so it is assumed that phytophagous insects together with parasitiods (Smith *et al.* 2008) constitute the vast majority of species. In fact, estimates of global biodiversity are often based on estimated species numbers of phytophagous insects (e.g. Erwin 1982; Odegaard 2000; Novotny *et al.* 2002). Comparisons of phylogenies of plants and their insect herbivores indicate that most of insect biodiversity has been generated via continuous host-shifting during insect evolution, and that 'cospeciation' and 'escape and radiate'-scenarios are less important (Nyman 2010). The high number of cryptic insect species and host races (as defined by Dres & Mallet 2002) specialized on different plant species (e.g. Diehl & Bush 1984; Dres & Mallet 2002; Stireman, Nason & Heard 2005; Bickford *et al.* 2007) further supports the contribution of host plant switches to insect speciation.

Speciation via host-shifting can only happen if herbivores have a reduced performance on novel host plants. Otherwise the novel host plant would just be incorporated into the existing diet and no host associated differentiation would occur (Nyman 2010). However, this performance reduction on novel host plants makes it less likely that herbivores will settle long enough on novel host plants so that adaptations can evolve. Therefore, novel host plants should provide some benefits for insect herbivores that counterbalance the fitness reduction due to their lower performance. These benefits may come from competition release (Feder *et al.* 1995; Berlocher & Feder 2002) or a reduced risk of natural enemy attacks on novel host plants (e.g. Price *et al.* 1980; Jeffries & Lawton 1984; Bernays & Graham 1988), a situation referred to as 'enemy-free space'. Reduction in enemies may offset any performance disadvantages on a novel host plant and could enable herbivores to colonize and adapt to nutritional inferior plants.

The existence of an enemy-free space has been shown to explain the oviposition preference of insects in a number of cases (e.g. Damman 1987; Ballabeni, Wlodarczyk & Rahier 2001; Moon & Stiling 2006; Sadek, Hansson & Anderson 2010), indicating that enemy-free space may indeed be important in structuring plant-herbivore interactions. Moreover, it has been demonstrated repeatedly that strong natural enemy pressure on ancestral host plants can lead to host range expansions resulting in the inclusion of nutritional inferior plants (Mira & Bernays 2002; Mulatu, Applebaum & Coll 2004; Murphy 2004). If host plant preferences lead to assortative mating, the existence of enemy-free space may lead to host race formation (Brown *et al.* 1995; Feder 1995; Heard *et al.* 2006) and speciation (Oppenheim & Gould 2002; Heard *et al.* 2006; Pelissie *et al.* 2010). Feder (1995) showed for example, that the apple host race of *Rhagoletis pomonella* experiences an enemy-free space on its derived host. This is due to the earlier fruiting phenology and bigger fruit size of apple trees compared to hawthorn,

the ancestral host of *R. pomonella*. Despite these examples it is still unclear how important the existence of enemy-free space is in the process of host race formation.

In natural communities, insect herbivores are usually attacked by a wide range of enemies with diverse life histories and behaviors that differ in their impact on herbivore populations. However, most of the literature on enemy-free space focuses on the effect of only one or two natural enemy species/genera (e.g. Feder 1995; Oppenheim & Gould 2002; Moon & Stiling 2006; Sadek, Hansson & Anderson 2010) or on the effect of the entire natural enemy or parasitoid community (e.g. Damman 1987; Gratton & Welter 1999; Ballabeni, Wlodarczyk & Rahier 2001; Heard *et al.* 2006). There are only few studies that try to disentangle the effects of different natural enemy species or groups (Brown *et al.* 1995; Keese 1997; Mira & Bernays 2002; Mulatu, Applebaum & Coll 2004; Murphy 2004; Pelissie *et al.* 2010).

The *Acyrthosiphon pisum* (pea aphid, HARRIS) complex is an important model for studying ecological speciation via host shifting, but studies about enemy-free space are lacking. Within the *A. pisum* complex, population genetic studies have detected 11 distinct host plant associated sympatric populations, including three possible species and eight host races (Peccoud *et al.* 2009a). The establishment of these subspecific divisions has been dated back to the post-Pleistocene warming and coincides with the anthropogenic range expansion of legume species (Peccoud *et al.* 2009a). Host-associated *A. pisum* populations are specialized on different legume species (their so called native hosts), where they have a high performance and which they prefer for feeding (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008; Peccoud *et al.* 2009a). These differences in host acceptance lead to assortative mating of races on their native hosts which reduces the gene flow between them and makes specialization on the different host plants possible (Caillaud & Via 2000; Peccoud *et al.* 2009a). When *A. pisum* races are placed on legume species they are not adapted to, they often show a strongly reduced performance or do not survive at all (Ferrari, Via & Godfray 2008; Schwarzkopf *et al.* 2013) because, as shown by studies using the electrical penetration graph (EPG) technique, they have difficulties to establish feeding (Caillaud & Via 2000; Schwarzkopf *et al.* 2013).

All host races perform well on the broad been *Vicia faba* (L.), which is viewed as a 'universal host' (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008). By accommodating different *A. pisum* host races at the same time in mixed colonies, *V. faba* plants provide a "conduit" for gene flow among the different host races, and hybrid individuals are known in nature (Peccoud *et al.* 2009a). The persistence of the *A. pisum* host races indicates that mechanisms must exist to minimize gene flow and to maintain the differences between them. Previous studies on *A. pisum* host races have focused on the ongoing divergence and on physiological adaptations of the different host races (Caillaud & Via 2000; Del Campo, Via & Caillaud 2003; Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008; Frantz *et al.* 2009; Schwarzkopf *et al.* 2013) with only scattered attention to mechanisms that reduce the gene flow

between host races (Via, Bouck & Skillman 2000; Hawthorne & Via 2001). One mechanism might be the existence of an enemy-free space. If a higher predation pressure on (potentially mixed) aphid colonies living on the universal host plant *V. faba* would negate the potential advantages of a better nutrition on the universal host plant, aphids living on their native host plant might have a better survival and chance to reproduce. Thus the enemy-free space may have significant impact on gene flow between races and thus facilitate host race formation and maintenance.

Here we tested whether enemy-free space plays a role in host race persistence in the *A. pisum* complex by comparing natural enemy pressure on the native vs. universal host plants of three aphid host races, the Trifolium race, the Pisum race and the Medicago race. Like most herbivores, aphids are attacked by a wide range of natural enemies, including low mobility predatory larvae, such as hoverfly larvae, ladybird beetle and lacewing larvae and higher mobility predatory adults, such as ladybird beetles, ants and spiders and different parasitoid species, which all can have a strong impact on aphid population dynamics (Stary 1995; Müller & Godfray 1999; Schmidt *et al.* 2003; Brown 2004). We assessed the effects of these enemies by measuring individual and colony survival in the presence and absence of enemies as a whole and by censusing specific enemies, such as hoverflies (eggs) and parasitoids (mummies). We also investigated the influence of factors like the existence of extrafloral nectar and duration of aphid infestation on the existence of enemy free space.

We found that all three native host plants studied supply an enemy free space for *A. pisum* compared to the universal host *V. faba*. Native host plants *M. sativa* and *T. pratense* provide an enemy free space since hoverflies preferred to oviposit on *P. sativum* and the universal host *V. faba*, and feeding by hoverfly larvae suppressed aphid population growth on these host plants. *P. sativum* provide an enemy free space since mobile predators preferred Pisum race aphids feeding on *V. faba* over aphids feeding on *P. sativum*.

Material and Methods

Organisms

Three different host races of the *Acyrthosiphon pisum* complex were used for this study. In order to avoid variation due to different colour morphs only green aphid clones were used: the Trifolium race (clone T3-8V1, here called T), the Pisum race (clones P136 and Colmar; called P1 and P2) and the Medicago race (clones L1-22 and L84; called M1 and M2). They were originally collected from their native host plants *Trifolium pratense* (L.), *Pisum sativum* (L.)and *Medicago sativa* (L.), respectively and genotypically assigned to the respective host race (for detailed information see Table S1 in Peccoud *et al.* (2009a)). Stock cultures were maintained for several generations in the climate chamber on their native host and on the universal host plant *Vicia faba*. Plants used in the experiments and for aphid rearing were 3 to 4 weeks old and were cultivated in soil (7:20 mixture of Klasmann Tonsubstrat and

Klasmann Kultursubstrat TS1) in climate chambers (20 °C, 16:8 L:D, 70% relative humidity). *P. sativum* and *V. faba* were grown individually in pots (10 cm in diameter), while *T.pratense* and *M. sativa* were grown in groups of 3 to 7 plants to get a similar plant biomass in each pot. All plants hosting aphids were covered with air permeable cellophane bags (18.8 x 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland) to prevent the escape of aphids.

Aphid population development on native vs. universal host in the absence of natural enemies

To test if and how the host plant influences the aphid population development in the absence of natural enemies, survival and reproduction rate of the three host races on native and universal host plants were assessed in a climate chamber (20 °C, 16:8 L:D, 70% relative humidity). One adult aphid from each clone was placed in a clip cage on its native or universal host plant. After 24 h the adult aphid and all offspring except one were removed from the cage. Survival of this one offspring was monitored every day until reproduction started. Afterwards survival and reproduction was checked every second day for a total of 17 (clones P2, M2) or 18 days (clones T, P1, M1) and offspring were removed after each counting. Each of the six clone-plant combinations (treatments) was replicated ten times. Due to time and space limitations clones T, P1 and M1 were tested in a separate experiment from clones P2 and M2.

General set-up of field experiments

All field experiments, which are described in detail below, were carried out in small meadows next to the building of the MPI for Chemical Ecology, Jena, Germany. These were mostly dominated by grasses and were mowed once every one to two months. The legumes *Vicia sepium*, *Lotus corniculatus*, *Trifolium repens*, *Trifolium pratense, Medicago lupulina* and *Medicago sativa* grew in these meadows providing potential hosts for wild *A. pisum* colonies and hence prey for potential enemies.

The general setup of all five field experiments was similar. Aphid clones were kept on their native host plant or the universal host *V. faba*, and a randomized block design was used. One block consisted of all aphid clone - host plant combinations (treatments) used in the respective experiment. Inside one block, treatments were arranged randomly in two rows, with 35 cm between the treatments. Blocks were separated by 3 m. Several measures were employed to prevent the escape of aphids. Plants were tied to sticks to maintain an upright position and not trail over the edge of the pot. Then, the pots (10 cm in diameter) containing the aphid infested plants were placed in bigger pots (19 cm in diameter) which were half filled with soil so that aphids dropping off the plant would fall into the larger pot. In addition, the fluoropolymer resin Fluon (SIGMA-ALDRICH Chemie GmbH) was used to cover the inner and the outer side of the big pots hindering aphids from leaving the pot. Thus aphids trying to escape were caged in the pot and could only stay there or climb back to the plant. If not

stated otherwise aphid infested plants were placed in the meadows at 10 am (start of experiment) and exposed to natural predation for 30 h. After this exposure time, all plants were covered again with cellophane bags and transferred to the laboratory for further analysis. In these experiments, aphids were all in the juvenile stage (L3 or L4), and did not reproduce.

Hoverfly oviposition on universal vs. native host (Field experiments conducted in July & August 2011, August 2013)

The aim of this experiment was to assess whether native host plants can provide an enemy-free space against hoverflies. The clones T (Trifolium race), P1 (Pisum race) and M1 (Medicago race) were tested on their native and universal host plants, resulting in 6 different treatments. To include some temporal variability in hoverfly oviposition the experiment was done for periods in two different months in summer 2011 (25th to 29th of July and 22nd to 26th of August). Each month 20 replicates (blocks) were tested, and were split into two groups of 10 replicates, started with a three day time-lag.

To rear the "experimental aphids", one adult aphid from each aphid clone (from stock culture) was placed on each experimental plant (20 native and 20 universal host plants per aphid clone) six days before the start of the experiment. The aphid was allowed to reproduce for two days and was then removed. After four days, all offspring were collected from the plants, and aphids from the different plants belonging to one treatment were pooled to avoid bias due to potential maternal effects. From this pool, 15 aphids each were randomly chosen and transferred back to each of the experimental plants of the respective treatment. Two days later, the plants with the 15 juvenile aphids (L3/L4) were placed in the field. After 30 h in the field, hoverfly eggs and remaining aphids on each plant were counted in the laboratory. Some hoverfly larvae that hatched from the eggs were reared until adulthood to determine the species.

To test if enemy-free space on native host plants exists also for other clones of the Pisum and Medicago host races, another set of clones (P2 and M2) was tested on its native and universal host plants in the field in August 2013 (20th to the 21th). Each treatment was replicated 20 times. The experimental plants were induced for 6 days by 15 "induction aphids", which were then replaced one day before the start of the experiment by 15 five-day-old "experimental aphids" that were reared on another set of plants. Plants were placed for 30 h in the field and afterwards hoverfly eggs were counted.

Aphid survival on universal vs. native hosts with natural enemies (Field experiments conducted in July 2013, 2014)

The aim of the 2013 experiment was to test whether (1) native host plants also provide enemy-free space against aphid parasitoids and predators other than hoverflies, (2) the duration of aphid infestation and (3) the availability of extrafloral nectar influence the presence of the enemy free space. This led to the following factorial design: the 3 aphid races (clones: T, P1, M1) were placed on their native host plant, on the universal host plant *V. faba* with its typical extra-floral nectaries, and on *V. faba* with extrafloral nectaries covered with wax to make them inaccessible. The plants were aphid infested one (short infestation) or six days (long infestation) before the start of the experiment, resulting in 18 different treatments in total (Table 1). The experiment was split temporally into two parts (16th to 17th and 22nd to 23rd of July) with 10 replicates in each part.

Table 1: Treatments for the aphid survival experiment

treatment number	pea aphid race (clone)	host plant	extrafloral nectaries	aphid infestation (days)
1	Pisum race (P1)	universal: *Vicia faba*	open	6
2				1
3			closed	6
4				1
5		native: *Pisum sativum*	none	6
6				1
7	Trifolium race (T)	universal: *Vicia faba*	open	6
8				1
9			closed	6
10				1
11		native: *Trifolium pratense*	none	6
12				1
13	Medicago race (M1)	universal: *Vicia faba*	open	6
14				1
15			closed	6
16				1
17		native: *Medicago sativa*	none	6
18				1

To cover the extrafloral nectaries of *V. faba*, the nectar droplet was first removed with a paper towel, and melted wax (paraffin) was applied with a small brush to the extrafloral nectaries. For the plants with nectaries left open, a paraffin droplet was also added but next to the nectaries. As an additional control, 10 wax droplets (5 x 2) were placed on leaves of native host plants even though these do not have extrafloral nectaries.

Aphids used in this experiment were reared on an additional set of plants. Therefore, 6 days before the start of the experiment adult aphids (4 aphids / plant) were placed on plants and were allowed to reproduce for 2 days and then removed. Their offspring ("experimental aphids") were placed on the "experimental plants" (15 aphids / plant) one day before the start of the experiment. For the short infestation treatment, fresh uninfested plants were used as experimental plants. For the long infestation treatment, plants were first infested with 15 two-day-old "induction aphids" of the respective clone. After five days, these aphids were replaced by 15 "experimental aphids".

After 30 h in the field, hoverfly eggs and surviving aphids were counted. Aphids were placed on a fresh set of plants and kept for 10 days to allow mummification of parasitized aphids. The number of plants per treatment containing mummies was recorded.

To test if the observed pattern of aphid survival is stable over different years, this experiment was repeated in 2014 excluding the treatment where the extrafloral nectaries were covered with wax and without keeping the aphids after the experiment to allow parasitoid development. It was again divided into two parts (15th to 17th and 22nd to 24th of July) with 10 replicates each.

Aphid colony development on native vs. universal host in the presence of natural enemies (Field experiment conducted in June, 2014)

This experiment was done in order to test if differences in predation pressure on the different host plants have an influence on aphid population development in the field. One clone of each host race (T, P1 and M1) was tested on its native and universal host plant, resulting in six different treatments which were replicated 15 times. Plants were infested with 10 two-to-three-day-old aphids which had been reared in low densities on another set of plants of the respective species. Eight days later, on the 13th of June, plants with the now adult aphids were placed in the field. After three, five, seven, ten and thirteen days in the field, plants were taken into the laboratory to count aphids, hoverfly eggs and hoverfly larvae. After each counting, all insects were placed back on their plants and plants were covered for 2 h with cellophane bags to allow the insects to settle before they were taken back to the field.

Statistics

In order to test whether the number of offspring produced in the absence of natural enemies differed between the aphid clones on the different plant species, Poisson generalized linear models (glm) were used. In cases of overdispersion, standard errors were corrected using quasi-glm models. P-values for explanatory variables were obtained by deleting explanatory variables one after another and subsequent comparison of the more complex model with the simpler model (Zuur *et al.* 2009). Survival data were analysed with Cox proportional hazard models. In case of significant influences of the aphid clone – plant combinations on the number of offspring or on the aphid survival, a factor level reduction was used to find out which aphid clone – plant combinations were different from each other (Crawley 2013).

All field data were analysed with generalized linear mixed models (glmm with the lmer function of the lme4 package (Bates *et al.* 2014) to account for the randomized block design. Experimental blocks were treated as random effects (random intercept), and treatments as fixed effects. P-values for explanatory variables were obtained by deleting explanatory variables one after another and

comparison of the likelihood of the more complex model with that of the simpler model (Zuur *et al.* 2009). Factor level reductions were used to reveal differences between levels of a treatment.

The dependencies of the number of hoverfly eggs on the aphid clone – plant combination were analysed by glmm with a Poisson error distribution. The dependencies of the number of surviving aphids on the aphid clone – plant combination and the duration of aphid infestation were analysed by glmm with a negative binomial error distribution. During some field experiments, a low number of hoverfly eggs were laid, few aphids were parasitized, and few aphids survived. In those cases presence / absence data were analysed with Bernoulli glmms.

The influence of induction of a plant by aphids on the number of plants with hoverfly eggs was only analysed for the second experimental week of 2014, since this was the only time hoverfly eggs were laid.

In order to test the influence of extrafloral nectaries on the parasitization probability of a certain aphid colony or on the number of surviving aphids, only aphid colonies on *Vicia faba* plants (with open and closed nectaries) were analysed. Four out of 20 experimental blocks where none of the plants contained parasitized aphids were excluded from the analysis. Since extrafloral nectaries did not play a significant role, treatments with closed extrafloral nectaries (the artificial situation) were excluded from the analyses of the influence of aphid clone – host plant combination and aphid induction.

All data were analysed with R version 3.1.1 R (Core Team 2014).

Results

Aphid population development on universal vs. native host plants without natural enemies

Clones of each of the three *Acyrthosiphon pisum* races, the Trifolium, Pisum and Medicago race, were reared on their native host plants and the universal host plant *Vicia faba* in a climate chamber. Reproduction and survival differed between the different host race - plant combinations (aphid numbers: aphid clones T, P1, M1: $F = 15.337$, $p < 0.001$; aphid clones P2, M2: $F = 8.160$, $p < 0.001$; survival: aphid clones T, P1, M1: likelihood ratio = 23.600, $p < 0.001$; aphid clones P2, M2: likelihood ratio = 19.820, $p < 0.001$), and was influenced in some cases by the host plant species (Fig. 1). In general, Medicago race aphids produced less offspring than the other races independent of host plant. Negative effects of the native host plant could be seen for both Trifolium and Medicago race aphids. The Trifolium race produced about 50 % less offspring on *Trifolium pratense* than on the universal host plant *V. faba,* while the survival did not differ between host plants. For the Medicago race we found the opposite pattern. Offspring production of both clones did not differ between host plants, but the M1 clone of the Medicago race had a lower survival on the native host *Medicago sativa* than on the universal host, whereas survival of the M2 clone did not differ between the two host plants. For Pisum race aphids, no negative effect of the native host plant (*Pisum sativum*) could be observed.

Reproduction rate did not differ between aphids on native and universal host plants for both clones. For the clone P2, aphid survival higher on the native host plant compared to the universal host plant, whereas no difference in aphid survival between native and universal hosts could be found for P1.
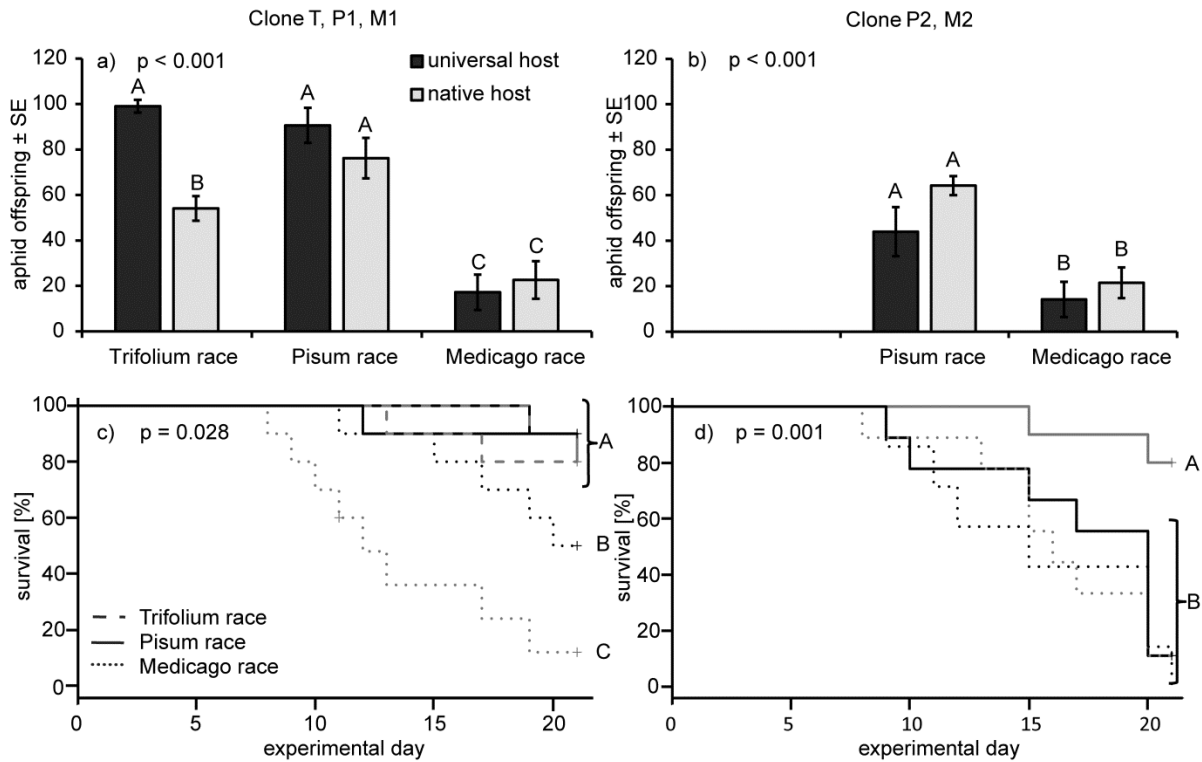
Figure 1: Mean aphid offspring production (a, b) and survival (c, d) of the Trifolium clone T, the Pisum clone P1 and the Medicago clone M1 (a, c) and the Pisum clone P2 and the Medicago clone M2 (b, d). Dark color represents aphids on universal host *V. faba,* and grey color represents aphid on their native host plants. Dashed lines represent the Trifolium race, solid lines the Pisum race, and dotted lines the Medicago race. Bars represent means ± standard error of cumulative offspring number after 18 (a) and 17 (b) days. Different letters indicate significant differences (p ≤ 0.05) between treatments.
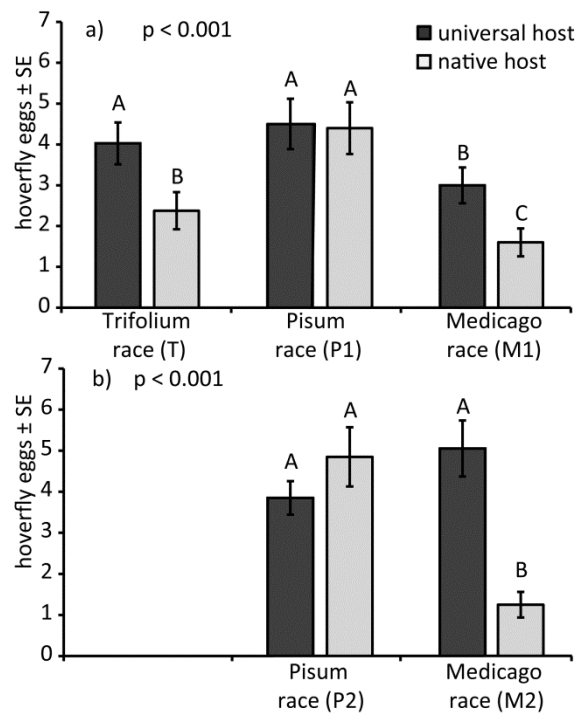
Figure 2: Number of hoverfly eggs laid on universal and native host plants infested with different pea aphid host races. a) Field experiment in July and August 2011, b) Field experiment August 2013. Bars represent means ± standard error; different letters indicate significant differences (p ≤ 0.05) between treatments.

Hoverfly oviposition on universal vs. native host plants

Plants infested with each of the *A. pisum* host races, on either their native host or the universal host, were placed in the field and hoverfly oviposition was monitored. The different aphid clone – plant combinations differed in the number of hoverfly eggs (clones T, P1, M1: χ^2 = 90.818, p < 0.001, clones P2, M2: χ^2 = 58.702, p < 0.001). Hoverflies preferred to oviposit on the universal host plant *V. faba* compared to the native host plants when the plants were infested with Trifolium and Medicago race aphids (Fig. 2). No such preference could be observed for plants infested with Pisum race aphids. These patterns were stable over two years and for different clones of the same race. All hoverfly larvae that were kept until adulthood were identified as *Episyrphus balteatus* (DE GEER).

Aphid survival on universal vs. native hosts with natural enemies

Another series of experiments was carried out to investigate the pressure from both hoverflies and additional enemies. Plants of each of the aphid clone-host plant combinations were placed in the field and monitored for hoverfly oviposition, aphid parasitism and aphid survival. In these experiments, no hoverfly eggs were found on plants during the 2013 trial and the first half of the 2014 trial due to periods of bad weather prior to the experiments. Sampling of adult hoverflies showed that females were carrying no or few eggs during the experimental periods. In the second week of the 2014 trial, about half of all experimental plants carried hoverfly eggs. Egg distribution followed the general oviposition pattern we had found in the previous series of experiments, with less oviposition on the native hosts than the universal host *V. faba* (Fig. 2). However, due to the low egg number, these differences were only significant for the Medicago race aphids (χ^2 = 14.296, p = 0.014, Fig. S1). Duration of aphid infestation did not influence the presence of hoverfly eggs (χ^2 = 0.160, p = 0.689). Parasitism as measured by the frequency of plants with mummified aphids did not differ between the different aphid race – host plant combinations (χ^2 = 3.645, p = 0.602, Fig. 3a, Table S2). The proportion

of plants with parasitized aphids was not influenced by the accessibility of extrafloral nectaries (χ^2 = 0.451, p = 0.502, Table S1), nor by the duration of aphid infestation (χ^2 = 0.025, p = 0.875, Table S2).
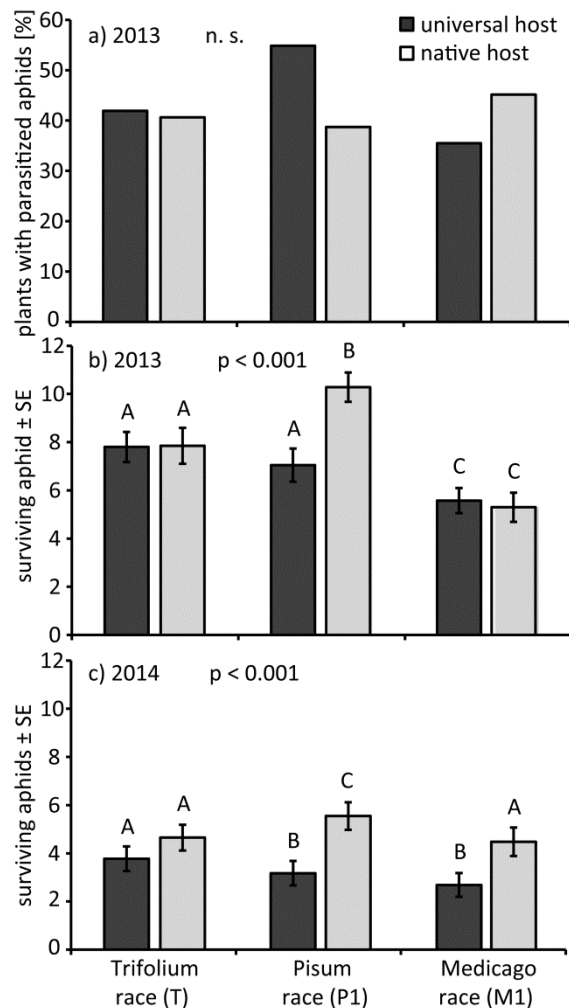
Mobile predators, such as ants, spider and ladybird beetles were also observed on the experimental plants placed in the field. To assess the collective effects of these mobile enemies the number of surviving aphids was measured on the different aphid clone – plant combinations. Strong differences in survival were observed in both years (2013: χ^2 = 54.729, p < 0.001; 2014: χ^2 = 33.538 , p < 0.001, Fig. 3b, c, Table S4, S5) with more surviving aphids of the Pisum clone (P1) on its native host plant *P. sativum* than on the universal host *V. faba* in both years. This was also true for the Medicago clone (M1) in 2014 with more surviving aphids on its native host plant *M. sativa* compared to *V. faba*. However, the number of surviving aphids of the Trifolium clone in both years and of the Medicago clone (M1) in 2013 did not differ between native and universal host plants.

The duration of aphid infestation on plants before being set out in the field had only had a

Figure 3: Proportion of plants with parasitized aphids (a) and number of aphids surviving in field (b, c) on native and universal host plants infested with different pea aphid host races b,c). a, b) Field experiment July 2014, c) Field experiment July 2014. Bars represent means ± standard error; different letters indicate significant differences (p ≤ 0.05) between treatments.

marginally significant effect on aphid survival in 2013 (χ^2 = 6.044, p = 0.014, Table S4): On average just one aphid more survived on plants infested for six days than on plants infested for three days. In 2014 this effect could not be detected (χ^2 = 2.838, p = 0.092, Table S5). The accessibility of extrafloral nectaries did not influence aphid survival (χ^2 = 0.021, p = 0.886, Table S3).

Aphid colony development on universal vs. native hosts in the presence of natural enemies

In another experiment, we monitored the changes in aphid populations in the field to see how they reflected differences in predation pressure on various host plants. Colonies of all *A. pisum* races decreased strongly in size during 13 days of natural enemy exposure in the field on both native hosts and the universal host (Fig. 4a). There was a much stronger decrease in population size of those aphid

race - host plant combinations that started out with a high aphid number on day 3 than of those with a low number at day 3. Although most aphid colonies went extinct during the 13 days in the field, there was a significant difference in the number of surviving aphid colonies depending on the aphid clone – plant combination (χ^2 = 26.357, p < 0.001). Survival of Trifolium and Medicago race colonies was higher on native than universal host plants, but for the Pisum race colony survival did not differ between native and universal host plants (Fig. 4b).

The number of hoverfly eggs on the plants was also assessed in this experiment and was dependent on the aphid clone – plant combination (χ^2 = 60.868, p < 0.001). The oviposition pattern was similar to the one observed after 30 h (Fig. 2) with more eggs on the universal host *V. faba* compared to native host plants when plants were infested with Trifolium and Medicago race aphids (Fig. 4c).
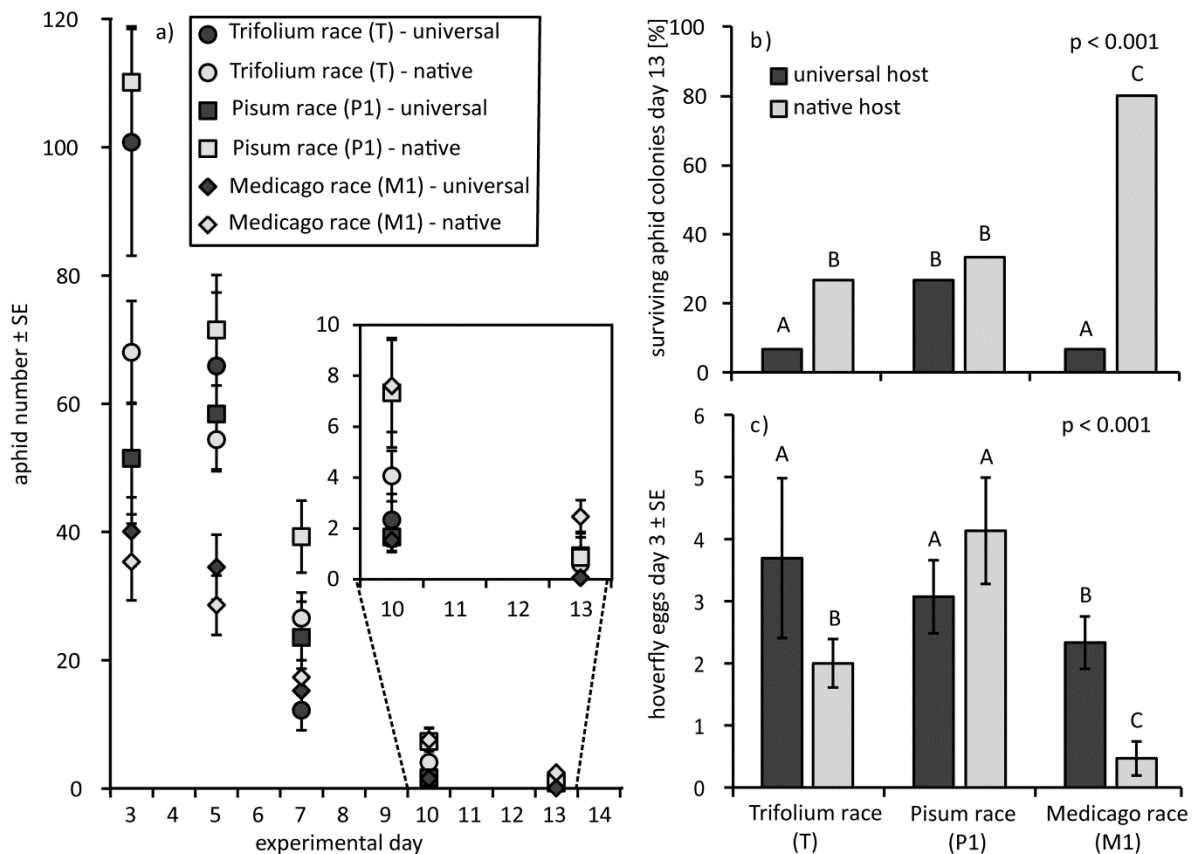
Figure 4: Size (a) and survival rate (b) of pea aphid colonies of different races on native and universal host plants in the field. The number of hoverfly eggs was counted on day 3 (c). a, c) Bars and symbols represent means ± standard errors ; different letters indicate significant differences (p ≤ 0.05).

Discussion

Natural enemy pressure maintains Acyrthosiphon pisum *host races*

Within the *A. pisum* complex, many host races are specialized on different legumes, but readily feed on the broad bean *Vicia faba*, known as a universal host of pea aphid races (Ferrari et al, 2006, 2008; Peccoud et al., 2009b). In this study, most of the clones of pea aphid races investigated had a better performance on the universal host (clones T and M1) in the absence of natural enemies or performed equally well on *V. faba* and native hosts (clones P1 and M2), corroborated by patterns in earlier work (Ferrari et al., 2008; Schwarzkopf et al., 2013). Only clone P2 of the Pisum race showed a better performance on its native host *P. sativum*. These data suggest that different races could frequently settle and survive on *V. faba* in the field. In fact, Peccoud et al 2009 could find aphids from various host races on *V. faba* and closely related plant species. In such cases mixed-race colonies might occur and lead to inter-racial mating if they persist until aphids reproduce sexually. For all but three host races, hybrids can be found in the field (Peccoud et al 2009).

The existence of *A. pisum* host races implies certain barriers to gene flow among them. Here we demonstrated that all three host races studied experience reduced enemy pressure on their native hosts compared to *V. faba*, which may minimize co-occurrence on this universal host. Reduced enemy pressure on native hosts was shown directly in cases where natural enemies oviposited less on the native than the universal host plant, or indirectly in cases where the number of surviving aphids or aphid colonies was higher on native host plants than on the universal host plant. Increased performance on native hosts decreases the probability that mixed colonies of different host races survive on *V. faba* and sexually reproduce. The enemy-free space we found on the native host plants may not only be crucial for maintaining *A. pisum* host races at present, but may have also contributed to host race formation by counterbalancing any performance reduction *A. pisum* may have faced when they first started to colonize novel host plants 6500 to 9500 years ago (Peccoud *et al.* 2009b).

More than one group of enemies is involved

Different groups of natural enemies may contribute to the enemy-free space on the native host plants vs. the universal host. In both experimental years, we found a strong hoverfly oviposition preference for the universal host *V. faba* when the plants were infested with Trifolium and Medicago race aphids. Hoverfly larvae are assumed to be among the most important aphid predators in central Europe (Chambers & Adams 1986; Tenhumberg & Poehling 1995). Thus the higher number of hoverfly eggs on *V. faba* would have resulted in a much higher predation pressure on *V. faba*, since 3rd instar hoverfly larvae consume up to 33 mg aphids per day (approx. 22 4th instar *A. pisum*) (Hindayana *et al.* 2001). However, even when hoverfly larvae were rare or absent, differences in aphid survival or population size could be detected for some host races. For example, in 2013 we observed significantly more

surviving aphids on native host plants for Pisum race aphids than the universal host *V. faba*, while in 2014 both the Pisum and the Medicago race aphids had a higher survival on their respective native hosts vs. *V. faba*. Thus the native host plants seem to provide reduced pressure from enemies other than hoverflies though this is more variable than the enemy-free space from hoverflies. The other enemies could include mobile predators, such as ladybird beetles, spiders and ants which were seen in the experimental plots but whose presence was not systematically recorded. Parasitoids could theoretically also contribute to survival disparities between aphids on native vs. universal host plants, but in this study the degree of parasitism did not differ between the types of host plant.

Comparing host races over different experimental years, individual or colony survival was not always greater on native vs. the universal host plant due to changes in enemy pressure and the nature of the natural enemy community. Sometimes no significant differences were observed, but we never observed the opposite situation, that *A. pisum* races survived better on the universal host than on their respective native host plant. This suggests that the effects of different natural enemy groups complemented each other leading to an overall enemy-free space on the native host plants for all three host races. However, in the experiment on aphid population development in the presence of hoverfly larvae this resulted only in the case of Trifolium and Medicago race aphids to a higher colony survival on native host plants.

The complementary effects of different natural enemies in contributing to greater survival on one host plant than another were noted in previous work on lepidopteran herbivores (Mira & Bernays 2002; Mulatu, Applebaum & Coll 2004). However, other studies found counteracting effects of natural enemies on different host plants. In such cases, the overall survival on different hosts depends on the relative abundance and effectiveness of the different enemy species associated with the hosts. The overall survival of two lepidopteran and one dipteran species was higher on the novel vs. the original host when natural enemies were present (Brown *et al.* 1995; Murphy 2004; Pelissie *et al.* 2010), but one study (Keese 1997) found a higher overall survival of a chrysomelid beetle on its original host.

Association of enemies with specific host plants

The differential association of herbivore enemies with specific hosts may be explained by several different factors. First, enemies may prefer hosts with higher nutrient content in their tissues and reduced defensive toxins because they offer more nutritious and less toxic prey. Among aphid enemies, the predator *E. balteatus* and the parasitoid *Diaeretiella rapae* (which does not parasitize *A. pisum*) prefer to oviposit on plants where aphids have the highest nutritional quality for their offspring (Almohamad *et al.* 2007; Kos *et al.* 2012). *A. pisum* were shown to have a 1.17-1.3 fold higher caloric content when reared on *M. sativa* compared to *V. faba*, and ladybird beetles and lacewing larvae have an increased performance when they are reared on *A. pisum* from *M. sativa* (Giles *et al.* 2000; Giles *et*

al. 2001; Giles *et al.* 2002). Thus if nutritional factors had a major effect on predation pressure in our study, we might expect that Medicago race aphids on *M. sativa* would suffer a higher predation rate than on *V. faba*. Since this is not the case, nutritional factors may not be of central importance.

Another factor we hypothesized would lead to the preference of herbivore enemies for specific plant species is the presence of extrafloral nectaries. These structures, which are found on the universal host *V. faba* but not on the native hosts in this study, are known to attract natural enemies and to serve the plant as indirect defences (Heil 2008). Ladybird beetles (Pemberton & Vandenberg 1993), ants (Engel *et al.* 2001) and the aphid parasitoid *D. rapae* (Jamont, Crépellière & Jaloux 2013) are reported to feed on the extrafloral nectaries of *V. faba*, and a removal of extrafloral nectaries reduces the number of ants that visit this species (Katayama & Suzuki 2004). Moreover, aphid infestation leads to increased extrafloral nectar production by *V. faba* (Jaber & Vidal 2009). These results suggest that extrafloral nectaries could be important in attracting aphid enemies. Surprisingly, we could not detect any influence of extrafloral nectaries on predation and parasitization rate between aphids on *V. faba* plants with open or closed (wax covered) extrafloral nectaries. Similarly, in previous work the parasitism rate of the caterpillar parasitoid *Microplitis mediator* did not increase in the vicinity of extrafloral nectaries, and this species was not attracted by volatiles emitted from extrafloral nectar (Géneau *et al.* 2013), even though the availability of extrafloral nectar increases parasitoid longevity. Another enemy, ladybird beetles were mainly observed to feed on extrafloral nectaries in the absence of aphids, suggesting that extrafloral nectaries have no direct effect on ladybird predation rate (Pemberton & Vandenberg 1993). Taken together, these results indicate that in our system extrafloral nectaries may not directly increase parasitization and predation rates on individual plants, but could act on community scales and over longer time spans by maintaining natural enemies in the absence of prey and by increasing their longevity.

Another factor affecting the distribution of natural enemies might be their attraction to different aphid-infested species as mediated by volatiles. It is well known that plants react to herbivore attack with an increased emission of volatile compounds that can attract herbivore enemies (e.g. Dudareva *et al.* 2006; Unsicker, Kunert & Gershenzon 2009; Hare 2011). Attraction by aphid-induced plant volatiles has been documented for the ladybird beetle *Coccinella septempunctata* (Ninkovic, Al Abassi & Pettersson 2001; Zhu *et al.* 2005) and for several aphid parasitoid species (e.g. Guerrieri, Pennacchio & Tremblay 1993; Du, Poppy & Powell 1996; Hatano *et al.* 2008; Takemoto, Kainoh & Takabayashi 2011) in laboratory experiments. Moreover, it was shown that the hoverfly *E. balteatus* perceives several common plant volatiles (Verheggen *et al.* 2008). However, we could not detect differences in predation and parasitization rates between plants infested by aphids for one or six days. We had expected that longer infestation times would alter volatile blends and lead to greater enemy predation or parasitism. Different explanations are possible for the lack of increased enemy pressure. Some enemies, like the ladybird *Adalia bipunctata* are not attracted by the volatiles emitted by *V. faba*

plants infested with *A. pisum* (Francis, Lognay & Haubruge 2004). Interestingly, Schwartzberg, Boroczky and Tumlinson (2011) and Pareja *et al.* (2009) showed that pea aphid infestation not only does not induce the volatile emission of *V. faba* and *M. sativa*, but that *A. pisum* actually suppresses the emission caused by chewing herbivores. Pareja *et al.* (2009) further showed that although the parasitoid *Aphidius ervi* is able use minor changes in the ratio of certain plant volatiles to distinguish between infested and uninfested *V. faba*, it is not attracted by aphid-damaged *M. sativa.* This was probably because all parasitoids used in this study were reared on *V. faba*, but it also highlights the complexity of the aphid – natural enemy interactions. Another explanation is that previous studies on natural enemy attraction to aphid damaged plants were done under controlled laboratory conditions, in contrast to our experiment which was carried out in the field. The complex odor background in the field may decrease the detectability of minor changes in volatile blends to aphid enemies. Additionally, different parasitoid and predator species may react in a different manner in the field, especially since it was shown that experience can strongly influence parasitoid behavior (e.g. Daza-Bustamante *et al.* 2002; Takemoto, Kainoh & Takabayashi 2011). Furthermore, since plant derived volatiles are sometimes considered to function as long range cues (Hatano *et al.* 2008), aphid induced volatiles may have guided natural enemies to our experimental plots, where they then searched for aphids on all available plants, independent on their volatile profile. In addition, changes in volatile emission may happen directly after aphid infestation such that volatile blends do not differ between plants that were infested for one and six days. Finally, we only used 15 aphids per plant in our experiments which may not have been enough to increase the attractiveness of the volatile blend for enemies (Guerrieri *et al.* 1999).

If the differential attraction of aphid enemies to various infested plants is not due to an increased nutritional quality of aphids, differences in volatile emission, or the presence of extrafloral nectaries, it may result from the differences in accumulation of honeydew on aphid infested plants. The presence of this sticky, sugar-rich secretion is known to serve as an attractant and arrestant for hoverflies, ladybirds and parasitoids (e.g. Budenberg 1990; Budenberg & Powell 1992; Leroy *et al.* 2012). In this study, hoverfly oviposition preferences for plants in the field (Fig. 2a) closely corresponded to the reproductive rate of aphids in those plants in the laboratory (Fig. 1a). If the reproductive rate of aphids on a plant correlates with honeydew production, honeydew may be a good measure of future plant suitability for hoverfly offspring when laying eggs.

Conclusions

Although *A. pisum* races may perform better on the universal host *V. faba*, we showed that they suffered less enemy pressure on their native host plants. For the Trifolium and Medicago race aphids, this enemy-free space was shown to counterbalance the reduced survival and reproduction rate on

native host plants, leading to a higher colony survival on the native hosts. For the Pisum race there were no performance drawbacks on native compared to universal host plants, so even the slight reduction in enemy pressure on *P. sativum* vs. *V. faba* may make this native host more favorable than the universal host. Thus enemies may play an important role in maintaining races on their native hosts. While hoverflies were the major enemies in this study, other enemy species also contributed to the patterns of aphid individual and colony survival. Neither the presence of extrafloral nectaries of *V. faba*, the potential difference in volatile production between the different plants, nor the nutritional value of aphids from different plants explained the differences in enemy pressure we found in the field, so further research is needed to determine the mechanisms that generate enemy free space for *A. pisum*.

Acknowledgements

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Chapter II: Honeydew as oviposition cue for hoverflies

Hoverfly preference for aphids with a high reproduction potential creates an enemy free space on novel host plants and facilitates host race formation in aphids

Ilka Vosteen*, Jonathan Gershenzon, Grit Kunert

Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena

Abstract

The existence of an enemy-free space can play an important role in host race formation processes, but little is known about the mechanisms that generate an area of low predation pressure on particular host plants. In this paper we identify a mechanism generating an enemy-free space that promotes the maintenance of the different host races of the pea aphid (*Acyrthosiphon pisum*) complex, a well-studied model for ecological speciation. This model species consists of at least 11 genetically distinct host races which are native to specific host plants of the legume family, but can all develop on the universal host plant *Vicia faba*. The experiments described in the previous chapter demonstrated that hoverfly (*Episyrphus balteatus*) oviposition preferences contribute to the enemy-free space that helps to maintain the different pea aphid host races. Hoverflies were found to oviposit preferentially on plants colonized by aphids with a strong reproductive potential. In these experiments, the actual number of aphids could not have been a cue for oviposition since it was kept constant. Thus we hypothesized that honeydew is produced in higher amounts by aphids with a higher reproductive potential, and that higher amounts of honeydew are more attractive to ovipositing hoverflies. Here we could show that aphids with a higher reproductive rate do indeed have a higher rate of honeydew production, and that honeydew is an important quantitative oviposition cue for hoverflies under field conditions. We could further show that pea aphids suffer less predation on less suitable plants, most likely due to reduced honeydew production and a reduced aphid size on those plants. This enemy-free space can reduce the performance

disadvantages of aphids colonizing a novel plant, and probably plays an important role in aphid host race formation.

Introduction

Insect herbivores are the most diverse group of eukaryotic organisms inhabiting our world (e.g. Stork 1993; Ødegaard 2000; Novotny *et al.* 2002), and most of their diversity presumably arose via continuous host-shifting during insect evolution (Nyman 2010). However, speciation via host-shifting can only happen if herbivores have a reduced performance on novel host plants. Otherwise the novel host plant would just be incorporated into the existing diet and no host-associated differentiation of insect herbivores would occur (Nyman 2010). However, this performance reduction on novel host plants makes it less likely that herbivores will remain long enough on novel host plants so that adaptations can evolve. Therefore, novel host plants should provide some benefits for insects to counterbalance a reduction in performance. These benefits may come from competition release (Feder *et al.* 1995; Berlocher & Feder 2002) or a reduced risk of natural enemy attacks on novel host plants (e.g. Price *et al.* 1980; Jeffries & Lawton 1984; Bernays & Graham 1988). A reduction in enemy pressure often referred to as enemy-free space may offset performance disadvantages on the novel host plant and thus enable herbivores to colonize nutritionally inferior plants.

The existence of enemy free space has been reported to explain oviposition preferences of insects for certain host plants (e.g. Damman 1987; Ballabeni, Wlodarczyk & Rahier 2001; Moon & Stiling 2006; Sadek, Hansson & Anderson 2010), which can in turn structure plant-herbivore interactions. Moreover, it has been proven repeatedly that strong natural enemy pressure on ancestral host plants can lead to host range expansions resulting in the inclusion of nutritionally inferior plants (Mira & Bernays 2002; Mulatu, Applebaum & Coll 2004; Murphy 2004). If host plant preferences also lead to assortative mating, the existence of enemy-free space may lead to host race formation (Brown *et al.* 1995; Feder 1995; Heard *et al.* 2006) and speciation (Oppenheim & Gould 2002; Heard *et al.* 2006; Pelissie *et al.* 2010).

While there are many indications of enemy-free space, only a few studies have tried to identify the causes for an enemy-free space on a certain host plant. One possibility is that insect herbivores colonizing a novel host plant may escape their natural enemies in time if the periods of natural enemy activity and herbivore vulnerability no longer match (Feder 1995). Herbivores might also escape their natural enemies in space. Host plants have been shown to provide a structural refuge for herbivores (Feder 1995; Oppenheim & Gould 2002) and the presence of trichomes may generate an enemy-free space by reducing the mobility and searching efficiency of natural enemies (Mulatu, Applebaum & Coll 2004; Mulatu, Applebaum & Coll 2006). Another cause of enemy-free space may be the sequestration of host-specific plant toxins by

herbivores (Nishida 2002; Opitz & Müller 2009), since natural enemies often avoid chemically defended prey (e.g. Schaffner *et al.* 1994; Camara 1997; Narberhaus, Zintgraf & Dobler 2005; Kos *et al.* 2012). In addition, the volatiles typically emitted by plants after herbivore attack, which attract natural enemies of herbivores (e.g. Vet & Dicke 1992; Turlings *et al.* 1995; Takabayashi & Dicke 1996), might not be released if a plant does not recognize a novel herbivore after a host plant switch. Or, the new host plant may emit volatiles that are not recognized by the natural enemy. In both cases, the novel host plant could provide an enemy-free space. These studies show that the mechanisms that generate an enemy-free space on a certain host plant often depend on certain chemical or physical properties of the plant. But, interactions with other organisms can also result in enemy free space. For example, ant-tended herbivores can find an enemy-free space on plants where ant number is high. The mistletoe butterfly *Ogyris amaryllis* does not oviposit on plants in the absence of ants even if the plants are nutritionally acceptable (Atsatt 1981). The attraction of ants to herbivores is also influenced by host plant species and plant genotype (Cushman 1991; Mooney & Agrawal 2008), indicating that the level of ant protection can be influenced by the host plant.

Enemy-free space is likely to be involved in the ecological speciation that has occurred via host-shifting in the pea aphid complex (*Acyrthosiphon pisum* HARRIS) (chapter I). This model for ecological speciation consists of at least 11 distinct host plant-associated sympatric populations, including eight host races and three possible species (Peccoud *et al.* 2009). These populations are specialized on different legume species (referred to as native hosts), on which they perform well and prefer for feeding (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008; Peccoud *et al.* 2009). When pea aphid races are placed on other legume species, they usually show a strongly reduced performance (Ferrari, Via & Godfray 2008; Schwarzkopf *et al.* 2013). These differences in aphid ability to use a certain plant as host and the fact that the aphids mate where they feed, lead to assortative mating on their native hosts. This reduces the gene flow between different host races and makes adaptation to different host plants possible (Caillaud & Via 2000; Peccoud *et al.* 2009). However, despite their specialization on different host plants, all host races perform well on the broad bean *Vicia faba*, which is viewed as the 'universal host' (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008). This universal host can be colonized by different pea aphid host races at the same time and may provide a "window" for gene flow between the different host races, resulting in hybrid formation (Peccoud *et al.* 2009). But, if the gene flow is too high, it would counteract any further differentiation of the host races and may even reduce existing differences between the host races. Thus the continued existence of pea aphid races is evidence for a lack of high gene flow among them. One mechanism for reducing gene flow could be the existence of enemy-free space. It was already shown that pea aphids of the Trifolium race, the Pisum race and the Medicago race find an enemy-free space against

different aphid predators on their native host plants compared to the universal host *V. faba* (chapter I). The higher predation pressure on *V. faba* reduces the probability of mixed colonies and therefore of gene exchange via the universal host. For the Trifolium and Medicago races, this enemy free space on the native hosts may be especially important for host race maintenance, because under laboratory conditions without natural enemies these races have a reduced performance on their native hosts compared to the universal host *V. faba* (Schwarzkopf *et al.* 2013, chapter I). In the field however, high predation pressure strongly reduces the probability of colony survival on *V. faba* leading to a higher survival on the native hosts *Trifolium pratense* and *Medicago sativa*.

Our previous work showed that the oviposition preference of hoverflies (*Episyrphus balteatus*) plays a very important role in the existence of the overall enemy-free space, because the release from predation pressure is stable in time (chapter I) and hoverfly larvae consume a large amount of aphids during their development (Hindayany et al. 2001). When hoverflies were allowed to choose between different host plant - aphid race combinations, they preferred to oviposit on the universal host *V. faba* compared to the native hosts, when plants were infested with Trifolium and Medicago race aphids, while plants infested with Pisum race aphids were highly attractive independent of the plant species (chapter I, see also Fig. 1 a, b). These patterns cannot be explained by the presence of extrafloral nectaries on *V. faba* (chapter I). Also differences in prey quality are not likely to be the reason. Thus the mechanisms responsible for this oviposition pattern remain unclear to date. Strikingly, the hoverfly oviposition pattern on different plant species in field experiments with constant numbers of aphids reflects aphid reproduction rate on those plants under laboratory conditions. This indicates that hoverflies may make an optimal oviposition choice by placing most eggs on plants were their larvae will find a lot of food. But how do hoverflies judge aphid reproductive potential if aphids are still in the juvenile phase and not reproducing? Since honeydew is an important oviposition cue for hoverflies (Budenberg & Powell 1992; Scholz & Poehling 2000; Leroy *et al.* 2011; Leroy *et al.* 2014) and since honeydew production increases with increasing aphid performance (Auclair 1959), hoverflies may use differences in honeydew production as a cue to distinguish between aphids with different reproduction potential. To test this hypothesis, we collected honeydew from different host plant - aphid race combinations to see if it reflects the oviposition pattern. We further tested if hoverflies use honeydew as an oviposition cue under field conditions and if qualitative or quantitative differences of aphid honeydew explain the oviposition pattern. Moreover, we investigated whether aphids that colonize a novel host plant on which their performance is reduced, experience an enemy-free space against hoverflies on these plants due to their reduced honeydew production.

Material & Methods

Organisms

Three different host races of the pea aphid complex were used for this study: the Triflolium race (clone T3-8V1, called T), the Pisum race (clones P136 and Colmar; called P1 and P2) and the Medicago race (clones L1-22 and L84; called M1 and M2). They were originally collected from their native host plants *Trifolium pratense*, *Pisum sativum* and *Medicago sativa* (for detailed information see table S1 in Peccoud *et al.* (2009)). The stock cultures have been maintained for several generations in a climate chamber on their native hosts and on the universal host plant *Vicia faba.* Plants used in the experiments and for aphid rearing were 3 to 4 weeks old and were cultivated in soil (7:20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1) in climate chambers (20 °C, 16:8 L:D, 70% relative humidity). *P. sativum* cv. 'Baccara' and *V. faba* cv. 'The Sutton' were grown individually in pots (10 cm in diameter), while *T. pratense* cv. 'Dajana' and *M. sativa* cv. 'Giulia' were grown in groups of 3 to 7 plants to obtain a similar plant biomass in each pot. All plants hosting aphids were covered with air permeable cellophane bags (18.8 x 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland) to prevent the escape of aphids.

Honeydew collection and application

To collect the honeydew for subsequent field experiments, plants with many aphids were surrounded by parafilm cones and were left for 24 to 48 h in the climate chamber. Afterwards the parafilm was removed and the honeydew droplets that had accumulated on the parafilm were dissolved in 200 µl of distilled water that had been heated up to 50 °C to increase the solubility of dried honeydew droplets. The solution was than filtered through a hydrophilic cellulose filter (pore size 0.45 µm, 13 mm, WICOM Germany GmbH) and was stored at – 80°C until freeze drying (Christ, Alpha 1-4 LD Plus, 48 h, 0.001 mbar, -76 °C). Freeze-dried samples were weighed (Metter Toledo, XP26), resuspended in a defined amount of distilled water, and stored at – 80°C until they were used in the experiments.

Small droplets of 1 or 2 µl honeydew solution (depending on the experiment) were applied with a micropipette to the experimental plants one day before the start of the experiment. Three droplets were always applied together on the upper side of one leaf. On *V. faba* and *P. sativum*, the application started on one randomly selected leaf of the lowest leaf pair and was continued along the leaves until the youngest expanded leaf was reached. The second leaf of each leaf pair was left without honeydew. If the plant did not contain enough expanded leaf pairs to apply all droplets, application started again at the lowest leaf pair on the honeydew free leaf. On *T. pratense* and *M. sativa* plants, leaves for honeydew application were selected randomly.

Quantitative honeydew collection

In order to estimate the amount of honeydew that is produced by Trifolium, Pisum and Medicago race aphids feeding on their native or universal hosts, 50 adult aphids were placed on each plant. The aphids had been reared on the same host plant species as used in the experiments. After approximately 24 h, the plant was surrounded by parafilm cones which were removed 24 h later. Honeydew collection was done according to the method described above. This procedure was replicated 6 to 10 times for each host plant - aphid race combination.

General set-up of field experiments

Several field experiments, which are described in detail below, were conducted to investigate hoverfly oviposition preferences. These experiments were done on small extensively managed meadows next to the building of the MPI for Chemical Ecology, Jena, Germany. The experimental site was covered mostly by grasses and was mowed every one to two months. The Leguminosae *Vicia sepium*, *Lotus corniculatus*, *Trifolium repens*, *Trifolium pratense, Medicago lupulina* and *Medicago sativa* grew on the meadow and may have provided habitats for wild pea aphid colonies and their natural enemies.

A randomized block design was used in all field experiments. One block consisted of all treatments that were used in the respective experiment. Inside one block, treatments were arranged randomly in two rows, with 35 cm between the treatments. Blocks were separated by 3 m. To prevent the escape of aphids the plants were tied to sticks to ensure an upright position. The pots (10 cm in diameter) containing the experimental plants were placed in bigger pots (18 cm in diameter) which were filled half with soil. Thus all aphids dropping off the plant would fall into the big pot. Fluon (Sigma-Aldrich) covering the inner and the outer side of the big pots hindered the aphids from leaving the pot. Plants infested with aphids were placed in the field at 10 am (start of experiment) and were exposed to natural predation. After this exposure time, all plants were covered again with cellophane bags and transferred the laboratory to count the number of hoverfly eggs.

Oviposition cues experiment

To test which oviposition cues (aphid presence, aphid – induced plant cues, honeydew) are used by hoverflies, 6 different treatments of the universal host plant *V. faba* and the native host plant *T. pratense* were tested simultaneously in a field experiment. We used three kinds of plant treatments: plants that were never before infested by aphids and therefore did not contain any aphid cues or plant cues induced by aphids (Fig. 1: treatment 1 & 2); plants that were never aphid infested before, but treated with honeydew (Fig. 1: treatment 3 & 4); and plants that were infested with 15 one to two day-old Trifolium race aphids 6 days before the start of the experiment and contain therefore aphid cues like honeydew as

well as any plant cues induced by aphids (Fig. 1: treatment 5 & 6). These aphids were removed one day before the plants were used for the experiment. On half of the plants from each of the three groups, 15 five to six day-old Trifolium race aphids were placed one day before plants were brought out to the field.

Honeydew added in this experiment had been collected from Trifolium race aphids feeding on *T. pratense* and *V. faba.* It was used at a concentration of 1 µg of freeze - dried honeydew dissolved in 8 µl H_2O. 24 droplets (2 µl) were applied on *V. faba* and 15 droplets (2 µl) on *T. pratense.* The experiment was replicated 10 times for each plant species and was run from the 15th to the 16th of August 2013.

no prior infestation   no prior infestation   prior infestation
                         + honeydew
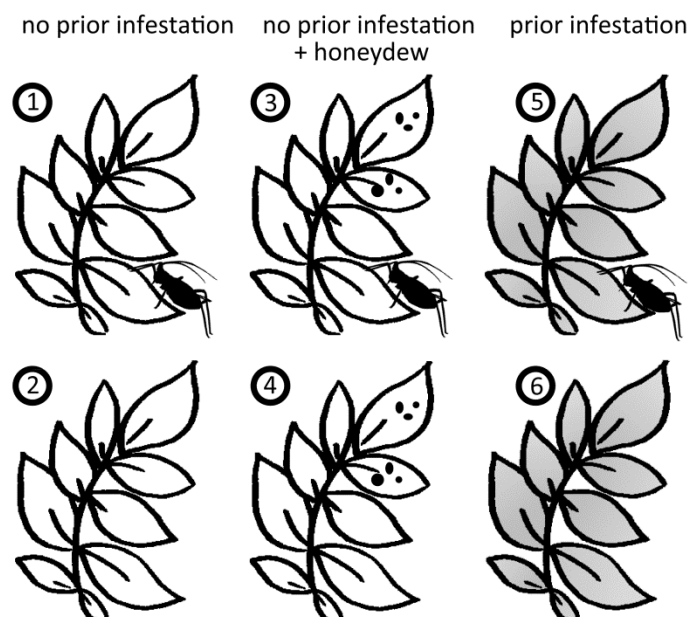
Figure 1: Treatments used in the honeydew experiment. 1) and 2) Non induced plants, 3) and 4) Non induced plants with honeydew, 5) and 6) Plants that had been infested with 15 induction aphids six days before start of field the experiment. Induction aphids were removed one day before start of the field experiment. 15 experimental aphids were placed on treatment 1), 3), and 5) one day before start of the field experiment.

Qualtitative effects of honeydew on hoverfly oviposition

We tested if qualitative differences between the honeydew types that were produced by aphids feeding on their respective native and universal host plants explain the observed hoverfly oviposition pattern. Therefore, honeydew was collected from Pisum race aphids (clone P1), Trifolium race aphids (clone T) and Medicago race aphids (clone M1) feeding on their native host plants and on the universal host *V. faba*. 24 one µl droplets of each honeydew type (1 µg freeze - dried honeydew dissolved in 10 µl H_2O) were applied to different uninfested *V. faba,* resulting in 6 different treatments that were replicated 14 times. The plants were placed for 48 h in the field (24th to 26th of August 2013).

Quantitative effects of honeydew on hoverfly oviposition

For this experiment, honeydew that was collected from Pisum race aphids (clone P1) feeding on the universal host *V. faba*. 6, 12 or 18 droplets (1 µg freeze - dried honeydew dissolved in 10 µl H_2O) were applied to uninfested *V. faba.* 10 replicates were placed in the field from the 11th to the 12th of August, 2014.

Enemy free space on less suitable host plants

Since hoverflies lay less eggs on plants with less honeydew and since honeydew production is influenced by the quality of aphid hosts plants (Auclair 1959), aphids may find an enemy free space on less suitable plants and may therefore benefit when they colonize novel (less suitable) plants. This hypothesis was tested using *T. pratense* as native host for Trifolium clone T and novel plant for Pisum clone P1, and *P. sativum* as native host for Pisum clone P1 and novel plant for Trifolium clone T.

Trifolium and Pisum race aphids were reared both on *T. pratense* and *P. sativum*, so that each race was reared on its native host and a less suitable plant (Schwarzkopf *et al.* 2013). Six days before the start of the experiment experimental plants were infested with 15 one to two day-old Trifolium or Pisum race aphids to induce the plants. Two days before the start of the experiment, these induction aphids were replaced by four to five day-old experimental aphids of the same race. Plants were placed in the field on the 1st of June, 2014. After two, six, nine and fourteen days in the field, plants were taken into the laboratory to count aphids, hoverfly eggs and hoverfly larvae. Additionally, three randomly selected aphids from each plant were weighed on day two. After each counting, all insects were placed back on their plants and plants were covered for 2 h with cellophane bags to allow the insects to settle before they were taken back to the field. Treatments were replicated 15 times.

To test if temporal differences in hoverfly abundance and egg load affect the hoverfly-aphid interactions on non-host plants, this experiment was repeated in August (starting day 5th of August, 2014). Number of hoverfly eggs, hoverfly larvae and aphids were only checked on day two and six, because several aphid colonies already were extinct by day 6.

Statistics

The amount of honeydew produced by aphids feeding on different host plants was analyzed with a One-Way-ANOVA. In cases of non-homogenous variances, data were log-transformed. The Tukey Honest Significant Difference method was used for post-hoc comparison.

To account for the randomized block design of the field experiments, all data collected during the field experiments were analyzed with mixed effects models using blocks as random effects (random intercept) and treatments as fixed effects. P-values for explanatory variables were obtained by deleting explanatory variables one after another and comparison of the likelihood of the more complex model with that of the simpler model (Zuur *et al.* 2009). Factor level reductions were used to reveal differences between levels of a treatment. Number of hoverfly eggs and larvae were analyzed with generalized linear mixed models with either a Poisson error distribution, or a negative binomial error distribution (glmm with the lmer function of the lme4 package (Bates *et al.* 2014) whichever fitted best the data of a certain experiment. In

cases were only a few hoverfly eggs or larvae were present and several plants without hoverfly eggs or larvae occurred, presence / absence data were used and analyzed with Bernoulli glmms. Which error structure was applied for analyzing a certain data set can be found in the supplemental material (Tab. S1). The occurrence of aphid colonies on plants was also analyzed with Bernoulli glmms. In order to investigate whether aphid weight differed depending on the plant the aphids were feeding on a linear mixed model was used (lme function of the nlme package (Pinheiro *et al.* 2013). All data were analyzed with R version 3.1.1 R (Core Team 2014).

Results

Quantitative honeydew production

The honeydew production of the different aphid races infesting the different host plants differed significantly (Clone T, P1, M1: ANOVA, F = 6.984, p < 0.001, Fig 1e, Clone P2, M2: F = 7.165, p < 0.001, Fig. 2 f) and reflected the offspring production, and the hoverfly oviposition pattern on those host plants which was described earlier (chapter I, Fig. 2 a – d). The highest amount of honeydew was produced by Pisum race aphids infesting their native and universal hosts and by Trifolium race aphids infesting the universal host. These were also the plants where the aphid offspring production was highest and where most hoverfly eggs were laid. Less honeydew was produced by Trifolium race aphids infesting their native hosts and by the Medicago race infesting both the native and the universal host plant, which again mirrored the offspring production on those plants. The hoverfly ovipostion pattern, however, was not exactly reflected by this pattern. Despite the low honeydew and offspring production of Medicago race aphids infesting the universal host, the number of hoverfly eggs on the plants was intermediate (clone M1) or high (clone M2).
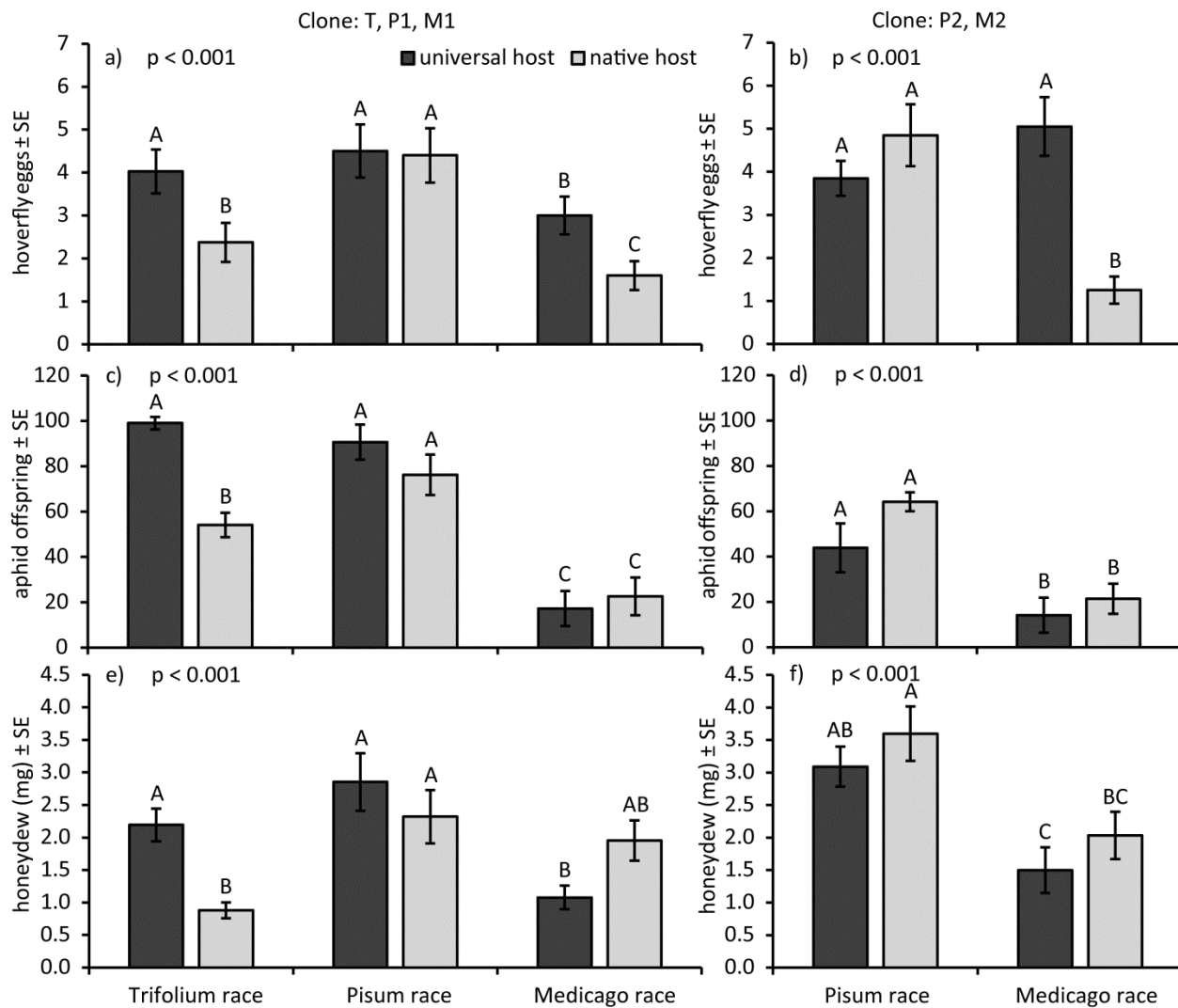
Figure 2: Influence of host plant and aphid race on hoverfly oviposition (a, b), cumulative aphid offspring production after 18 (c) or 17 days (d) and honeydew production of 50 adult aphids (weight of freeze-dried honeydew) (e, f). Bars represent means with standard errors; different letters indicate significant differences (p ≤ 0.05). Panels a – d modified from chapter I.

Oviposition cues experiment

In order to investigate which cues are important oviposition stimuli for hoverflies, *V. faba* and *T. pratense* plants containing different cues or combinations of cues were placed in the field. *E. balteatus* hoverflies laid more eggs on *V. faba* than on *Trifolium pratense* (χ^2 = 8.0123, p = 0.005, Fig. 3, Tab. S2) and more eggs were laid in the presence of aphids than without aphids (χ^2 = 16.379, p < 0.001). Egg number was further influenced by plant treatments (χ^2 = 18.683, p < 0.001) with honeydew-containing plants receiving the most eggs. However, there was a significant interaction between plant treatment and aphid presence (χ^2 =

12.787, p = 0.002). The addition of aphids to plants without prior infestation or with just honeydew addition made the plants more attractive for hoverflies, whereas it did not change the attractiveness of previously infested plants. Hoverflies rarely oviposited on plants in the absence of aphid stimuli (lack of prior infestation, lack of current aphid presence). Aphid presence and honeydew acted together in an additive way – both stimuli presented together doubled the amount of *E. balteatus* eggs per plant.

Figure 3: Influence of aphid-induction, honeydew and aphid presence on hoverfly oviposition. Bars represent means with standard errors; different letters indicate significant differences between treatments (p < 0.05). Number of hoverfly eggs differed significantly between *Vicia faba* (a) and *Trifolium pratense* (b) as indicated by upper and lower case letters (p = 0.005).

Qualitative and quantitative effects of honeydew on hoverfly oviposition

V. faba plants with honeydew originating from different aphid race-host plant combinations or with different amounts of honeydew were placed in the field to reveal the importance of qualitative and quantitative differences for hoverfly oviposition. Honeydew collected from different aphid race-host plant combinations differed in its attractiveness to ovipositing hoverflies (χ^2 = 19.546, p = 0.002; Fig. 4 a). However, in most cases hoverflies did not differentiate between plants containing qualitatively different honeydew, and only honeydew collected from Trifolium race aphids infesting *V. faba* was significantly less attractive than honeydew from the other aphid race-host plant combinations.

The quantity of honeydew on *V. faba* plants was important for the number of hoverfly eggs laid on the plants (χ^2 = 8.9439, p = 0.003; Fig. 4 b). More honeydew droplets led to a higher attractiveness.

Figure 4: Qualitative and quantitative effects of honeydew on hoverfly oviposition. a) Number of hoverfly eggs on *V. faba* plants with honeydew droplets from different aphid race-host plant combinations, Different letters indicate significant differences (p ≤ 0.05). b) The number of hoverfly eggs was dependent on the number of honeydew droplets present on *V. faba* plants. The solid line is the fitted Poisson glmm curve based on the estimated intercept and slope of the statistical model. Bars and dots represent means with standard errors.

Enemy-free space on less suitable host plants

In order to test whether aphids benefit from enemy free space when colonizing new plants, *T. pratense* and *P. sativum* plants were each infested with either Trifolium or Pisum race aphids, resulting in two treatments where aphids were on their native plants and two treatments with aphids on less suitable plants, which mimic the colonization of a new plant. The fate of the aphid colonies and the attractiveness for hoverflies were investigated. Aphids feeding on a plant they were not adapted to where were much lighter than aphids feeding on their native host plant (F = 78.005, p < 0.001, Fig. 5 a). The strength of reduction, however, was different for the two aphid host races (F = 21.183, p < 0.001). When Trifolium race aphids were reared on *P. sativum*, their weight was reduced by about 50% compared to aphids reared on the native host *T. pratense*. Pisum race aphids grown on *T. pratense* showed an even stronger reduction in body weight of approximately 70%. In general, the two aphid races did not differ in their weight (F = 1.947, p = 0.163). After two days where aphid-infested plants were exposed to natural enemies, only about 20 % of all plants contained hoverfly eggs. Only one less suitable plant was carrying hoverfly eggs, while several native host-plants with hoverfly eggs were recorded (χ^2 = 11.850, p < 0.001; Fig. 5 b). More plants infested with Pisum race aphids contained hoverfly eggs than plants infested with Trifolium race aphids (χ^2 = 4.716, p = 0.030). This resulted in fewer less suitable plants with hoverfly larvae
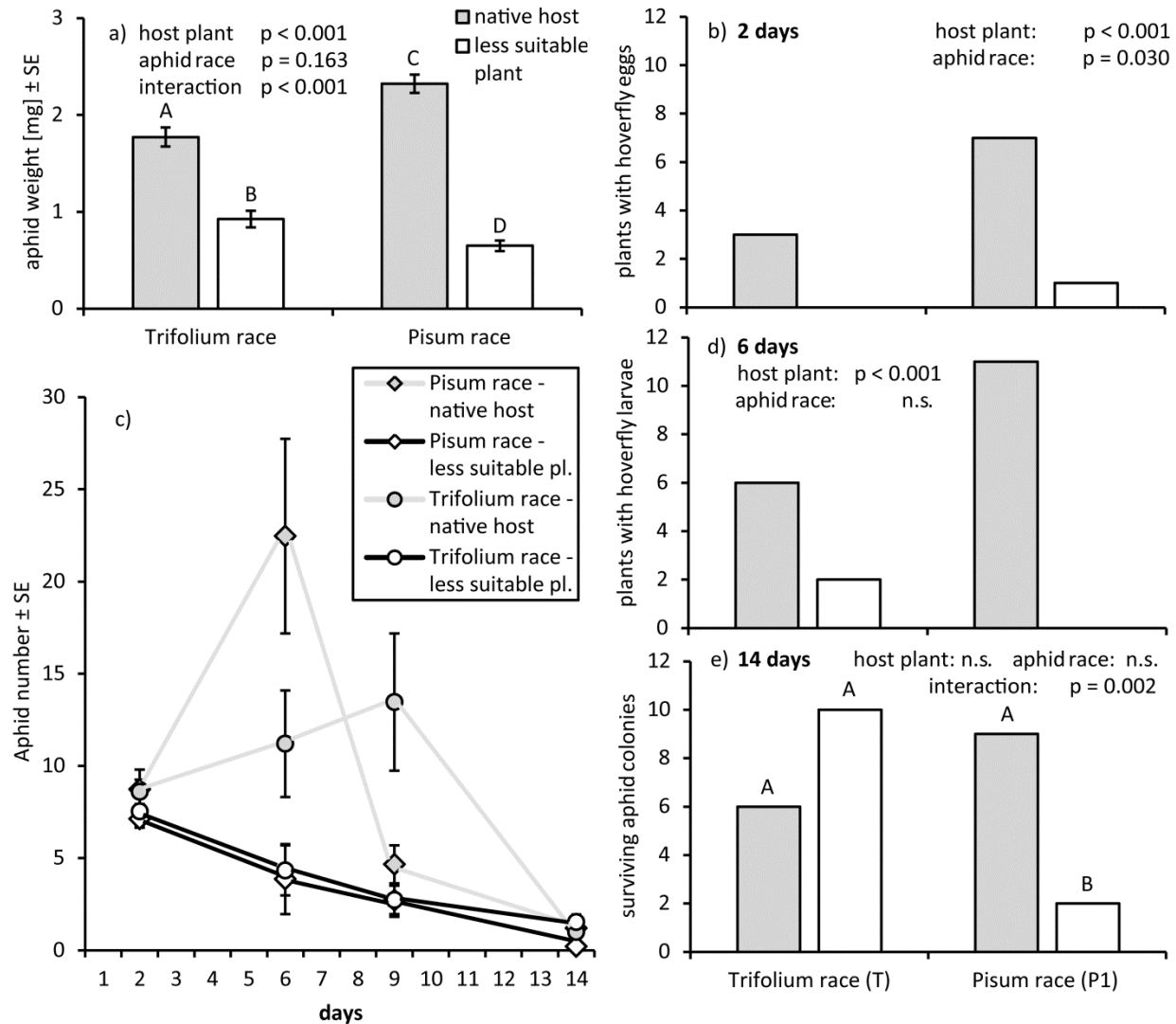
Figure 5: Influence of native and less suitable plants on interactions between hoverflies and pea aphids (Trifolium race clone T, Pisum race clone P1) in the field (July 2014). a) Aphid weight, b) number of plants with hoverfly eggs on day 2, c) aphid population development, d) plants with hoverfly larvae on day 6, e) number of aphid colonies which survived until day 14. Bars and symbols represent means with standard errors (a, c), and total plant number with hoverfly eggs (b), larvae (d) and surviving aphid colonies (e); different letters indicate significant differences of host plant-aphid race interaction (p ≤ 0.05).

than native host plants after 6 days (χ^2 = 19.170, p < 0.001; Fig. 5d). Aphid populations on the native host plant increased during the first 6 to 9 days of the experiment, but decreased strongly towards the end of the experiment, while aphid populations decreased steadily on non-host plants (Fig. 5 c). This led to a similar number of surviving Trifolium race populations on native and non-host plants, whereas the number of Pisum race populations surviving 14 days was much higher on the native host *P. sativum* compared to the non-host *T. pratense* (influence of host race: χ^2 = 1.511, p = 0.219; influence of plant: χ^2 = 0.453, p = 0.501; influence of interaction: χ^2 = 9.209, p = 0.002; Fig. 5e).
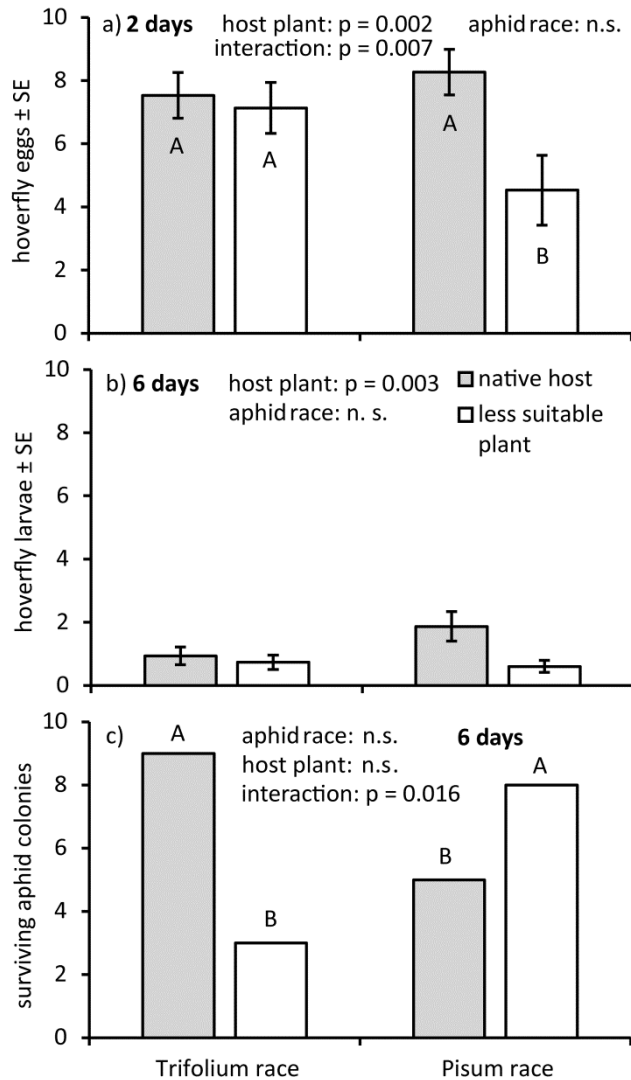
Figure 6: Influence of native and less suitable plants on interactions between hoverflies and pea aphids (Trifolium race clone T, Pisum race clone P1) in the field (August 2014). a) Number of hoverfly eggs on day 2, b) number of hoverfly larvae on day 6, c) number of aphid colonies which survived until day 6. Bars represent means with standard error (a, b), and total number of surviving aphid colonies (c).

This experiment was repeated one month later (in August) to see if temporal differences in hoverfly number and egg load influenced the outcome of this interaction. In August, all plants contained hoverfly eggs and the number of hoverfly eggs per plant was high (mean over all treatments = 6.9 eggs per plant). Hoverfly egg number after 2 days was influenced by plant suitability (χ^2 = 9.366, p = 0.002; Fig. 6 a, Tab. S3), with more eggs being laid on native hosts compared to less-suitable plants. The difference in egg number laid on native hosts vs. less suitable plants, however, was only significant for Pisum race aphids (χ^2 = 7.371, p = 0.007). Although the number of hoverfly larvae on day 6 was much lower than egg number on day 2, the pattern of the distribution of hoverfly larvae was similar to the distribution pattern of hoverfly eggs (Fig. 6 b) with more larvae on native host plants compared to less suitable plants (χ^2 = 8.594, p = 0.003, Tab. S4). Aphid colony survival was higher on less-suitable plants compared to native hosts for Pisum race aphids, while it was lower on less suitable plants compared to native hosts for the Trifolium race (χ^2 = 5.793, p = 0.016, Tab. S5).

Discussion

Hoverfly oviposition cues

Our previous work showed that hoverfly oviposition on native and universal hosts infested with Trifolium race, Pisum race and Medicago race aphids reflected the aphid reproduction potential on those hosts (chapter I). These results suggest that hoverflies make an optimal oviposition choice by laying the highest number of eggs on those plants where their offspring will find the highest amount of food. Here we investigated the basis by which hoverflies decide where to lay eggs and make such an optimal choice.

Many cues important for hoverfly oviposition are already known (summarized in Almohamad et al. 2009). Present and future prey availability, as reflected by aphid colony size and age, are obviously very important. For instance, Kan and Sasakawa (1986) and Kan (1989) showed that hoverflies preferred to oviposit in small aphid colonies consisting mainly of young aphids, while they avoided oviposition in older colonies with several winged adults. This strategy ensures that hoverfly larvae will find plenty of aphids as food when they hatch and reduces the risk that the aphid colony will collapse before hoverfly larvae are old enough to migrate. However, neither colony size nor colony age nor the presence of winged aphids can explain the oviposition pattern in our previous experiments, since an equal number of aphids was used, and the aphids were all of the same age and still in the juvenile stage.

Another possibility is that the aphids themselves differ depending on the host plants they are feeding on, and differences between host plants may influence hoverfly searching and oviposition behavior. It is known that several common plant volatiles can be detected by hoverflies and that (*Z*)-3-hexenol applied to uninfested *V. faba* plants increases searching and landing of *E. balteatus* (Verheggen *et al.* (2008). However, we do not attribute the oviposition pattern we observed to differences in aphid-induced plant volatiles, because aphid induced plants did not contain more hoverfly eggs than non-infested plants (Fig. 3). In addition, pea aphid infestation does not induce volatile emission from *V. faba* and *M. sativa* and aphids actually suppress the volatile induction by chewing herbivores in *V. faba* (Pareja *et al.* 2009; Schwartzberg, Boroczky & Tumlinson 2011).

A physical plant characteristic potentially important for hoverfly oviposition choice is the presence of trichomes which has been shown to negatively influence search efficiency of aphid predators (Belcher & Thurston 1982; Simmons & Gurr 2004; Verheggen *et al.* 2009). Whereas *V. faba* and *P. sativum* are trichome free, *M. sativa* and *T. pratense* surfaces contain trichomes which might make these two plants less attractive for hoverflies. However, at least the difference in attractiveness between *V. faba* and *T. pratense* in our experiments was quite small, indicating that the presence of trichomes seems to have little influence in comparison to other potential cues tested in our experiment (Fig. 3). Plant architecture could also alter the search efficiency of aphid predators (Kareiva & Sahakian 1990; Clark & Messina 1998b;

Clark & Messina 1998a; Reynolds & Cuddington 2012). This plant feature also seemed to be of minor importance since although *V. faba* and *P. sativum* plants differ in their architecture, *P. sativum* for example has many tendrils, while *V. faba* does not have any, they did not differ in their attractiveness for ovipositing hoverflies.

Since none of the potential oviposition cues mentioned above could convincingly explain the oviposition pattern we found in our experiments, we tested whether the cues used by hoverflies come from the aphids themselves. Aphid presence was found to make plants more attractive (Fig. 3), probably due to visual cues provided by the aphids, since it is known that vision is important for aphid identification by hoverflies. Kan and Sasakawa (1986) documented that hoverflies prefer non-winged over winged aphid models (black beads). Aphid presence may also be detected by odor. One important aphid-originated volatile is the aphid alarm pheromone (*E*)-β-farnesene, emitted when aphids are attacked by natural enemies. However, (*E*)-β-farnesene seems not to be important since it was shown that undisturbed pea aphids do not emit any volatiles cues (Joachim *et al.* 2013; Joachim & Weisser 2013), and that highly mobile adult predators do not react towards aphid alarm pheromone released at natural concentrations (chapter IV). We therefore deduced that visual cues from aphids elicit oviposition by hoverflies. Aphid honeydew is also known to be an important cue for hoverfly oviposition (Budenberg & Powell 1992; Bargen, Saudhof & Poehling 1998; Scholz & Poehling 2000; Leroy *et al.* 2014). Since we demonstrated that hoverfly oviposition on individual plants reflects aphid reproductive potential (chapter I), and since Auclair (1959) showed that performance and honeydew production of pea aphids are closely linked, we hypothesized that the reproductive rate of the different host races on their native and universal host plants might be reflected by honeydew production. And, we further surmised that these differences in honeydew production might be employed by hoverflies to distinguish between aphids with high and low reproductive potential.

Our results show that honeydew production is well reflected by aphid performance and reproductive potential (Fig. 2). The lower honeydew production on plants where aphid performance was low was most likely due to a lower frequency of droplet production, which may have resulted from prolonged non-feeding periods and increased changes of feeding sites when aphids faced difficulties in establishing feeding (Auclair 1959).

We also showed for the first time that honeydew applied on uninfested plants elicits hoverfly oviposition behavior under field conditions. This is in line with the results of several laboratory experiments which showed that honeydew is an important oviposition cue for *E. balteatus* (Budenberg & Powell 1992; Bargen, Saudhof & Poehling 1998; Scholz & Poehling 2000; Leroy *et al.* 2014) and that volatiles produced by honeydew degrading microorganisms are attractive for hoverflies (Leroy *et al.* 2011). The

attractiveness of honeydew can also explain why plants infested with aphids for 6 days stayed attractive even when aphids were removed. Interestingly, honeydew presence and aphid presence seemed to be the most important cues. Each caused a similar number of hoverfly eggs to be laid on the experimental plants, and these cues acted in an additive way (Fig. 3). Therefore, we conclude that visual cues from aphid presence and gustatory cues von honeydew are the most important cues for aphid location by hoverflies in the field.

The effectiveness of honeydew as a cue could differ depending on aphid host plant species. It was previously reported that honeydew composition differs between different aphid species and host plants (Hendrix, Wei & Leggett 1992; Fischer, Volkl & Hoffmann 2005). However, our results show that honeydew collected from the different pea aphid host races feeding on native and universal host plants does not differ in its attractiveness for hoverflies in most cases, and that only honeydew collected from Trifolium race aphids feeding on *V. faba* is less attractive than all other honeydew types (Fig. 4 a). Thus, qualitative differences in honeydew composition do not explain the general hoverfly oviposition pattern in this study. Instead, we showed that an increase in number of honeydew droplets that were applied to *V. faba* increased the number of eggs laid on these plants (Fig. 4 b). Thus the quantitative difference in honeydew production, which reflects the reproductive potential of the aphids, is the main factor that explains the observed oviposition pattern. However, there are probably additional factors that make the universal host *V. faba* attractive, since hoverfly ovipostion was much higher on this species infested with the Medicago race than we would have expected from aphid reproduction rate and honeydew production. Possibly the large leaves of *V. faba* may collect more honeydew droplets than the smaller leaves of the native hosts.

Implications for early steps of host race formation

The close relationship we have demonstrated between pea aphid performance on a host plant, the resulting honeydew production and the incidence of hoverfly oviposition may contribute to host race formation. Aphids that colonize a novel host can be assumed to suffer reduced performance and smaller size due to their lack of adaptation. But, small aphid size and low amounts of honeydew may lead to less hoverfly oviposition and thus to an enemy-free space. Hence colonization of novel host plants may lead to a similar aphid survival compared to typical hosts despite the lower performance. When we tested these predictions in the field in July, 2014, Trifolium and Pisum race aphids were indeed shown to be much smaller when they were reared on non-host plants and hoverflies laid eggs less often on less suitable plants compared to the native hosts *T. pratense* and *P. sativum* (Fig. 5). This resulted in fewer less suitable plants with hoverfly larvae. Meanwhile, aphid populations on non-host plants declined steadily during the

entire experiment. In contrast, populations showed sharp increases on native host plants, but this was followed by equally sharp declines, leading to similar population levels as on less suitable plants. The decline coincides with the time that hoverfly larvae started to consume considerable amounts of aphids. It was already shown that feeding by hoverfly larvae has a strong impact on aphid population dynamics (chapter I). In this experiment it led to a similar aphid colony survival on host and non-host plants for the Trifolium race, indicating that the reduced performance on the non-host *P. sativum* is balanced by the high predation pressure by hoverfly larvae on the native host. For the Pisum race however, colony survival was still lower on the non-host, even though predation by hoverfly larvae led to a drastic population decline on the native host *P. sativum*. The performance difference between native and non-host plants is especially strong for Pisum race aphids indicating that this enemy-free space is not strong enough to balance the strong performance reduction Pisum race aphids face when feeding on *T. pratense*.

When we repeated the field experiment one month later (Fig. 6), the pattern of results was different. Hoverflies laid very high numbers of eggs on native and non-host plants infested with Trifolium race aphids and on the native host *P. sativum* infested with Pisum race aphids, while they laid an intermediate number on the non-host *T. pratense* infested with Pisum race aphids. The high total number of eggs as well as the reduced differentiation between suitable and non-suitable hosts suggests that hoverflies had a very high egg load in this second experiment (Courtney, Chen & Gardner 1989; Sadeghi & Gilbert 2000b). Yet, despite the high egg number, the number of hoverfly larvae was very low after 6 days. This is probably due to the fact that several aphid colonies were already extinct on day 6, so that hoverfly larvae had already left these plants. However, there were still significantly more hoverfly larvae on the native host *P. sativum* infested with Pisum race aphids than on the non-host *T. pratense* leading to a higher colony survival for this race on less suitable plants than on native hosts. For Trifolium race aphids, however, the pattern was opposite with colony survival much lower on less suitable plants than on native hosts. The pattern for this host race was also reversed from that seen in the July, 2014 experiment.

The differences between the experiments conducted in July and August, 2014 show that the existence of an enemy-free space on non-host plants may depend on the egg load of hoverflies or the influence of other antagonists. However, in both experiments, we found a similar colony survival on native vs. less suitable plants for one out of two tested aphid races, indicating that enemy-free space on novel host plants has the potential to stabilize aphid populations inhabiting novel hosts at least temporarily and so facilitate early steps of host race formation. In contrast to other studies that identified the mechanisms which generate enemy-free space (Denno, Larsson & Olmstead 1990; Feder 1995; Oppenheim & Gould 2002; Mulatu, Applebaum & Coll 2004; Mulatu, Applebaum & Coll 2006), reduced predation in our system is not directly due to the chemical or structural properties of the novel host plant, but due to ecological

interactions between host plants and the herbivore. For pea aphids, the low performance on the novel host plant indirectly generates enemy-free space on these plants, because aphids with low performance are smaller and produce less honeydew than high performing aphids, and are thus less conspicuous towards searching hoverflies.

Host plant switches probably played an important role in aphid evolution (Peccoud *et al.* 2010; Jousselin *et al.* 2013). Host plant-associated differentiations are known or suspected for at least 16 aphid species or genera (Müller 1985a; Müller 1985b; Guldemond 1990; Sunnucks *et al.* 1997a; Sunnucks *et al.* 1997b; Shufran *et al.* 2000; Miller, Kift & Tatchell 2005; Lozier, Roderick & Mills 2007; Margaritopoulos *et al.* 2007; Carletto *et al.* 2009; Peccoud *et al.* 2009; Mezghani-Khemakhem *et al.* 2012) indicating that host race formation might be a common process in aphids. Since several hoverfly species are generalists that prey on multiple aphid species (Sadeghi & Gilbert 2000a; Gilbert 2005), enemy-free space due to lower aphid performance and honeydew production probably plays a role in host race formation of aphid species besides the pea aphid if they also suffer performance reductions on novel host plants.

Conclusion

Hoverflies use differences in honeydew production to select aphid populations with high reproductive potential for oviposition. This differential oviposition results in lower hoverfly predation on novel host plants where aphids have a low performance. The resulting enemy-free space has the potential to compensate for the performance reduction aphids suffer on novel host plants, and may result in similar colony survival on novel and native host plants. This pattern could improve the prospects for survival of aphid colonies inhabiting novel host plants, and may facilitate the early steps of host race formation.

Acknowledgements

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Chapter III: Searching behavior of hoverfly larvae

Migrating hoverfly larvae accumulate in large aphid colonies

Ilka Vosteen*, Jonathan Gershenzon, Grit Kunert

Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena

Abstract

Predatory larvae like aphidiphagous hoverfly larvae often have to face food shortages during their development due to the ephemeral occurrence of aphid colonies. Thus, it would be an advantage if developing larvae would be able to disperse and find new feeding sites. But, little is known about the dispersal capacity of predatory larvae and the cues they may use to find new feeding sites. In this study, we estimated the dispersal capacity of hoverfly (*Episyrphus balteatus*) larvae and investigated other aspects of their foraging behavior.

We showed that hoverfly larvae do not leave a plant as long as there are aphids available, and that migrating larvae are able to find other aphid colonies in the field. Migrating hoverfly larvae accumulated on large aphid colonies, most likely because these are easier to detect and because hoverfly larvae search an area more thoroughly after they encounter aphids or aphid cues like honeydew. Searching hoverfly larvae did not distinguish between different pea aphid - legume species combinations, and the performance of hoverfly larvae fed with pea aphids reared on different host plant species was not influenced by the rearing plant. Thus, hoverflies should forage on any of these host plants if they encounter them in the field, and indeed hoverfly larvae were found on all host plants provided in a field experiment.

Introduction

Survival of insects depends on several crucial events like finding the right food or avoiding predation. Most of the decisions an insect makes directly influence the individual itself, but ovipositing females make

decisions that influence their offspring. In most insect species, females oviposit on or close to larval food sources and often select oviposition sites that maximize larval survival (Thompson & Pellmyr 1991; Ohsaki & Sato 1994; Singer *et al.* 2004; Gripenberg *et al.* 2010). But, unpredictability of food sources or trade-offs between female foraging and offspring performance (Thompson 1988; Scheirs & De Bruyn 2002) might lead the egg-laying female to make suboptimal decisions. In these cases it would be an advantage if developing larvae would be able to disperse and find new feeding sites. Whereas dispersal of insect larvae has been reported (Doak 2000), little is known about their actual dispersal capacities and how they orient if they switch feeding sites (Chew 1977; Bernays & Chapman 1994; Berdegué, Reitz & Trumble 1998; Soler *et al.* 2012). Whilst herbivorous larvae are often attracted by volatiles from their host plants (e.g. Visser 1986; Dickens 2002; Castrejon, Virgen & Rojas 2006; Becher & Guerin 2009; Soler *et al.* 2012), studies considering the orientation of predatory larvae are rare (Branco *et al.* 2006), even though predatory larvae are more likely to encounter food shortages than herbivorous larvae. This is especially true if predatory larvae are specialized on a certain prey with an unpredictable distribution. Aphids, for example, are a highly unpredictable food source. Depending on abiotic and biotic conditions, colonies were found to survive up to 50 days, but often they survive only a few weeks or even days in the field (e.g. Weisser 2000; Weisser & Härri 2005; Outreman *et al.* 2010, chapter I & II). However, the developmental time of the larvae of most aphid predators takes about two weeks at temperatures above 20 °C (Hart, Bale & Fenlon 1997; Lanzoni *et al.* 2004) and is even longer under adverse conditions (Hart, Bale & Fenlon 1997). Larval development may therefore take much longer than the availability of a single aphid colony, and consequently, larval development in most cases cannot be completed with one aphid colony as food source. In the search for additional food to complete their development, it is known that aphidophagous ladybird larvae use pollen, extrafloral nectar and foliage as alternative food sources and are often able to complete their development with these alternative food sources (Lundgren 2009). On the other hand, aphidophagous hoverfly larvae are thought to feed solely on aphids and to have a limited dispersal capacity (e.g. Sadeghi & Gilbert 2000; Almohamad, Verheggen & Haubruge 2009). Field experiments showed, however, that hoverfly larvae left plants if aphid colonies went extinct (chapter I & II). Since nothing is known about the migration behavior of hoverfly larvae, we aimed to find out whether hoverfly larvae migration is a general behavior and under which circumstances they leave a plant.

If hoverfly larvae leave plants, they also have to find new aphid infested plants. The decision to climb a certain plant might not only depend on the availability of aphids but also on the plant species itself, possibly due to factors like plant architecture or surface structures. For instance, it was shown by Verheggen *et al.* (2009) that trichomes hamper the movement of hoverfly larvae. To test whether plant – aphid combinations differ in their attractiveness for hoverfly larvae, we used different pea aphid - legume

species combinations. The pea aphid (*Acyrthosiphon pisum* HARRIS) is actually a species complex consisting of at least 11 genetically distinct host races which are native to particular legume species, but can all develop very well on the universal host plant *Vicia faba* (Ferrari *et al.* 2006; Ferrari, Via & Godfray 2008; Peccoud *et al.* 2009a; Schwarzkopf *et al.* 2013). It is assumed that natural enemies contribute to the maintenance of the different host races by preferring aphids living on the general host plant *V. faba*, and therefore minimizing the occurrence of mixed colonies (chapter I). It was shown that adult hoverflies oviposit especially on the universal host plant (chapter I & II), but it is unknown if hoverfly larvae also have a certain preference.

To get a better understanding of the role of hoverfly larvae on aphid colony development in the field, we investigated the migration behavior of hoverfly larvae and focused on the following questions: Do hoverfly larvae leave a plant only after most aphids were consumed, and is this migration a general phenomenon? If hoverfly larval migration is a common behavior, the larvae not only have to decide when to leave a plant, but also have to find a new aphid-infested plant. We tested if hoverfly larvae are able to find aphid-infested plants and whether aphid colony size changed the attractiveness of a plant. We additionally investigated if different plant species − aphid biotype combinations were differentially attractive, and tested if larval performance is influenced by the host plant-aphid race combination.

Material & Methods

Organisms

Three different host races of the pea aphid complex were used for this study: the Trifolium race (clone T3-8V1), the Pisum race (clone P136) and the Medicago race (clone L1-22). They were originally collected from their native host plants *Trifolium pratense*, *Pisum sativum* and *Medicago sativa* and genotypically assigned to the respective host race (for detailed information see table S1 in Peccoud *et al.* (2009b)). The stock cultures have been maintained for several generations in the climate chamber (20 °C, 16:8 L:D, 70% relative humidity) on their native hosts and on the universal host plant *V. faba.* Plants used in the experiments and for aphid rearing were 3 to 4 weeks old and were cultivated in soil (7:20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1) in climate chambers (20 °C, 16:8 L:D, 70% relative humidity). *P. sativum* cv. 'Baccara'and *V. faba* cv. 'The Sutton' were grown individually in pots (10 cm in diameter), while *T.pratense* cv. 'Dajana' and *M. sativa* cv. 'Giulia' were grown in groups of 3 to 7 plants to get a similar plant biomass in each pot. All plants hosting aphids were covered with air-permeable cellophane bags (18.8 x 39 cm, Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland) to prevent the escape of aphids.

Hoverfly eggs (*Episyrphus balteatus*) were obtained from a commercial supplier (Katz Biotech AG, Baruth, Germany) and fed with aphids until they were used in the experiments (for rearing details see description of experiments). Rearing of insects and all laboratory experiments were done in climate chambers (20 °C, 16:8 L:D, 70% relative humidity). The leaving rate and performance experiments were performed in insect rearing tents (60x60x60 cm, Bugdorm, MegaView Science Co., Ltd., Taiwan).

Leaving rate experiment

To prepare the aphid-infested plants used in this experiment, *V. faba* plants were infested with 40 adult Pisum race aphids one day before the start of the experiment. Before the start of the experiment, two uninfested *V. faba* were placed 10 cm apart from each other in an insect rearing tent. Two of the infested *V. faba* plants were arranged on both sides of the non-infested plants, with 10 cm distance to the non-infested plants so that the four plants formed a trapezoid. One hoverfly larvae was placed on one of the aphid-infested plants and the position of the larva was noted 24 h later. Due to logistic reasons the ten replicates were done on two consecutive days. Hoverfly larvae were kept on *V. faba* plants that were strongly infested with Pisum race aphids until they were 5 to 6 days old and were then used in the experiment.

Larval preference – effect of aphid number

Experimental *V. faba* plants were infested with 0, 10, 20, 40 and 60 adult Pisum race aphids one day before they were used in the experiment. Plants were arranged randomly around a petri dish with an even distance towards each other and 10 cm distance towards the petri dish in the insect rearing tents. Eight larvae were placed in the petri dish and were allowed to forage for 24 h in the tent. Afterwards their position and number of the surviving aphids were recorded. Due to spatial limitations, the 25 replicates were done on five consecutive days. Hoverfly larvae were kept on *V. faba* plants that were strongly infested with Pisum race aphids until they were 5 to 9 days old and then were used in the experiment. Every day the biggest larvae from the cohort were selected for the experiments.

Larval preference – effect of plant species

To test if hoverfly larvae prefer certain host plant - aphid race combinations, 20 adult aphids of the three pea aphid host races were placed either on their native or on their universal host plant one day before the start of the experiment. Plants were again arranged in a random order in the insect rearing tents with 10 cm distance around a petri dish which contained 10 hoverfly larvae. Larvae were allowed to forage for 21 h and their position and the number of surviving aphids was recorded at the end of the experiment. Due

to aging of hoverfly larvae, the experiment was done with two larval cohorts. The first cohort was reared in a large plastic box (19 x 25 x 39 cm, covered with gauze) that contained 15 to 20 *Raphanus sativus* var. *sativus* infested with *Myzus persicae* aphids to avoid habituation to cues from the plant species used in the experiments. Due to the low body mass and slow growth of *Myzus persicae*, larvae had to be fed additionally with a mixture of pea aphids that contained all three host races, reared either on their native or the universal host plant. The second cohort was reared without plants in a plastic box (16 x 12 x 6 cm, covered with air-permeable cellophane to prevent escape of larvae), and was fed daily with a mixture of pea aphids that contained the same amounts of the three host races, reared either on their native or the universal host plant. Due to spatial limitations only five replicates could be done simultaneously. With the first larval cohort 25 replicates were done on five consecutive days, while with the second larval cohort 20 replicates were done on four consecutive days. Larvae used in this experiment were 3 to 9 days old and every day the biggest larvae from a cohort were selected for the experiments.

Hoverfly larval distribution in the field

To test how hoverfly larvae are distributed in the field, native and universal host plants were infested with 10 three day-old aphids of the Trifolium, Pisum and Medicago races. After eight days of aphid population growth, plants were placed in the field on the 14th of June, 2013. Plants were distributed randomly in 3 double rows that were 3 m apart from each other, and each treatment was replicated twelve to seventeen times. Plants within one double row were placed 35 cm apart. To prevent the escape of aphids, the plants were tied to sticks to ensure an upright position. The pots (10 cm in diameter) containing the aphid-infested plants were placed in bigger pots (19 cm in diameter) which were filled half with soil, thus all aphids dropping off the plant would fall into the big pot. Fluon (SIGMA-ALDRICH Chemie GmbH) covering the inner and the outer side of the big pots hindered the aphids from leaving the pot. After 7 days in the field, plants were brought into the laboratory and the number of aphids, hoverfly larvae, and hoverfly eggs on the different host plants was counted. The conditions of the hoverfly eggs were examined in order to estimate the age of the eggs and evaluate whether and how many hoverfly larvae hatched on the plants. Older eggs or eggs where larvae hatched can be distinguished from newly laid hoverfly eggs by their texture. When a larva hatches or when an egg dies, it gets dry. Therefore, the number of dry eggs is a measure of the maximal number of hoverfly larvae that could have hatched on a plant and reflects hoverfly oviposition at the beginning of the experiment when aphid number was mainly influenced by aphid reproduction rate under laboratory conditions and less by predation.

Hoverfly larval performance

To test if larval performance differs depending on their food, freshly hatched larvae were placed individually in small Petri dishes (5.5 cm in diameter) that contained a moist piece of paper towel (2 x 2 cm) to prevent desiccation of larvae. Larvae were fed daily with 15 or 40 mg juvenile aphids of the three host races that were reared either on their native or on the universal host plant. Larval survival and development stage were checked daily. Afterwards larvae were transferred to clean Petri dishes containing moist pieces of paper towels and the aphid prey. At day seven, larval weights were also recorded. Hoverfly development was followed until adults hatched from the pupae. Adults were sexed and their head width, as a robust measurement of the body size, was measured under a stereo microscope. Due to logistic reasons, the experiment was split into two parts. In part A, larvae were fed with Pisum and Medicago race aphids reared on their native and universal host plants, and in part B larvae were fed with Trifolium race aphids reared on the native and universal host. To check if larval performance differed between part A and B, larvae fed with Pisum race aphids reared on the universal host were again included in part B. Each experimental part was repeated four times with each time starting with a new set of larvae. Each treatment was replicated 32 times. Larval weight was only recorded in the experimental part A.

Statistical analyses

For all larval preference tests presence / absence data were analyzed with generalized linear mixed models (glmm with the glmer function of the lme4 package (Bates *et al.* 2014) to account for the block design. Experimental tents (blocks) were treated as random effects (random intercept), and treatments as fixed effects. P-values for explanatory variables were obtained by deleting explanatory variables one after another and comparison of the likelihood of the more complex model with that of the simpler model (Zuur et al., 2009).

Field data were analyzed with generalized linear models (glm). Whether the presence of dry hoverfly eggs on a plant was dependent on the host plant - aphid race combination was analyzed using a glm with the Bernoulli error structure. For the analysis of the number of hoverfly larvae and aphids on the plants, a glm with the Poisson error structure was used. And, a negative binomial error structure was applied for the analysis of the number of aphids.

Survival data were analyzed with Cox proportional hazard models using the amount of food and the plant species - aphid race combination as explanatory variables.

The influence of the amount of food, the kind of food (host plant - aphid race combination) and the sex of the hoverfly on hoverfly larval weight, larval and total developmental time and head width were analyzed

using linear mixed effects models (lme function of the nlme package (Pinheiro *et al.* 2015), to account for the different larval batches used. Larval batches were treated as random effects, and food amount, the kind of food and gender as fixed effects. Since only adult hoverflies can be sexed, data were only used from larvae that successfully developed into adult hoverflies.

For all analyses p-values for explanatory variables were obtained by deleting explanatory variables one after another and comparison of the likelihood of the more complex model with that of the simpler model (Zuur *et al.* 2009). In cases of significant differences, factor level reductions were used to reveal differences between levels of a treatment.

All data were analyzed with R version 3.1.1 R (Core Team 2014).

Results

Leaving rate

Nine out of ten hoverfly larvae survived until the end of the experiment. Of the nine surviving larvae, none left the aphid infested plant within 24 h. They consumed on average of 17 ± 1.5 aphids of the 40 adult aphids placed on the plant before the start of the experiment.

Larval preference

The number of plants with either hoverfly larvae or showing evidence of previous hoverfly presence (feces, leftovers from aphid feeding) significantly increased with the number of aphids infesting the plants (χ^2 = 14.000, p < 0.001, Fig. 1a). The number of plants where larvae were present at the end of the experiment also increased as well with the number of aphids infesting the plant (χ^2 = 13.357, p < 0.001, Fig. 1b). The number of plants with either
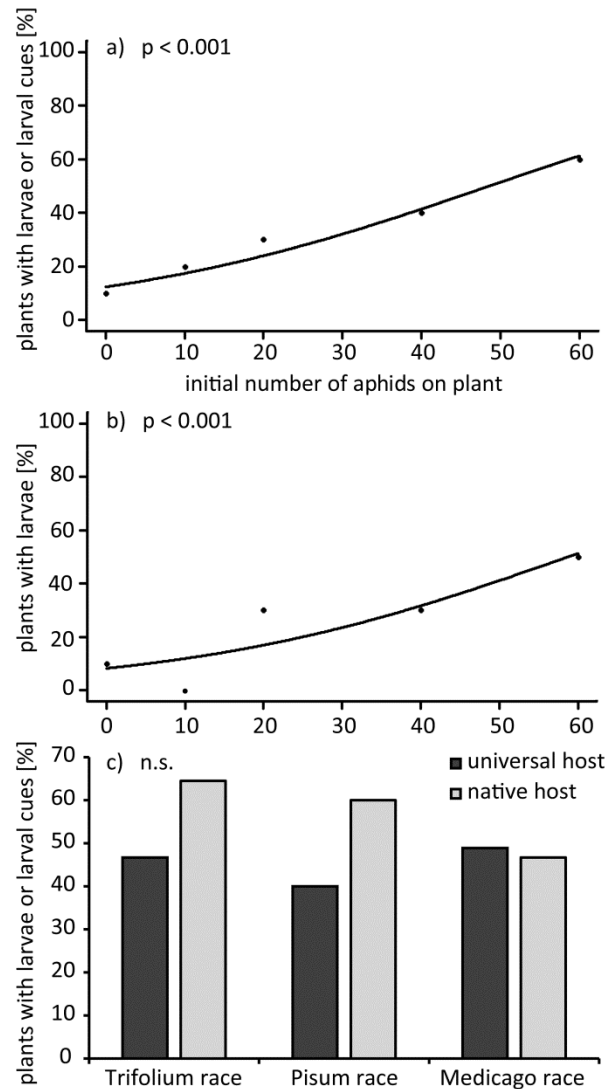
Figure 1: Effect of aphid infestation size on plant selection by larval hoverflies. Graphs depict (a) the proportion of plants with hints of hoverfly larvae presence, and (b) the proportion of plants with larvae actually present after 24 h. The curve presents the regression line obtained from the generalized linear mixed effects model. (c) The effect of various host plant - aphid race combinations on the proportion of plants with evidence of hoverfly larvae presence.

larvae or larval cues present at the end of the experiment was not influenced by the various host plant-aphid race combinations tested (χ^2 = 8.907, p = 0.113; Fig. 1c).

Hoverfly larvae distribution in the field

The distribution of hoverfly eggs that were laid at the beginning of the experiment, when the aphid population size was not altered by predation, was influenced by the host plant – aphid race combination (Binomial glm: likelihood ratio = 19.003, p = 0.002). Dry eggs, which had been laid during the first days of the experiment, were more often present on the universal host plant *V. faba* compared to the native host plants (Fig. 2a).

Hoverfly larvae were identified as *E. balteatus* and as *Scaeva pyrastri*. After seven days in the field, hoverfly larvae were present on an average of 70 % of all experimental plants (Fig. 2b), whereas hoverfly eggs, which were laid at the beginning of the experiment, were present on an average of only 25 % of experimental plants, indicating that most hoverfly larvae migrated to the experimental plants. The number of hoverfly larvae found on the plants after 7 days was not dependent on the number of hoverfly eggs laid at the beginning of the experiment (Poisson GLM: likelihood ratio = 1.292, p = 0.256), but dependent on the host plant – aphid race combination (Poisson GLM: likelihood ratio = 119.339, p < 0.001, Tab. S1). The highest number of hoverfly larvae was found on *P. sativum*, while intermediate numbers of hoverfly larvae were

Figure 2: Proportion of plants with (a) hoverfly eggs that were laid at the beginning of the experiment and (b) with hoverfly larvae after 7 days. Number of (c) hoverfly larvae and (d) aphids found on native and universal host plants after seven days in the field (July 2013). c, d): Bars represent means with standard error.

recorded on the universal host, *V. faba*, infested with Trifolium and Pisum race aphids. The lowest number of hoverfly larvae was found on *T. pratense*, on *M. sativa* and on *V. faba* infested with Medicago race aphids (Fig. 2c). The number of aphids that were present at the end of the experiment had an additional

influence on the number on hoverfly larvae (Poisson GLM: likelihood ratio = 8.593, p = 0.003, Tab. S1) with more larvae on plants with high aphid numbers.

The number of aphids after 7 days in the field was mainly influenced by the plant species – aphid race combination (Negative binomial glm: likelihood ratio = 15.998, p = 0.007; Fig. 2d, Tab. S2). Fewer aphids were found on *V. faba* infested with Pisum and Medicago race aphids compared to all other plants. Neither the number of hoverfly eggs at the beginning of the experiment (Negative binomial glm: likelihood ratio = 3.555, p = 0.059), nor the number of hoverfly larvae at the end (Negative binomial glm: likelihood ratio = 3.115, p = 0.078) significantly influenced the number of aphids on the plants (Tab. S2).

Hoverfly larval performance and survival

The amount of aphids as food strongly influenced all tested hoverfly parameters. More aphids resulted in heavier larvae, shorter larval development times and total developmental times, and wider heads of adult hoverflies. The influence of the aphid amount was also significant for the total development time in experiment B (Pisum race from universal host, Trifolium race from universal and native host) (Tab. 1). Whilst males and females differed in size (as larvae and as adults) with males being the bigger individuals, they needed similar times for their development (Tab. 1).

The influence of the kind of aphid food (host plant – aphid race combination) was different for the two experimental parts. It did not have an influence on any of the measured parameters (larval weight, larval and total development time, head width) in experiment A (Pisum race and Medicago race from native and universal hosts), while it influenced larval development time and adult head width in experiment B (Pisum race from universal host, Trifolium race from native and universal host (Tab. 1)).
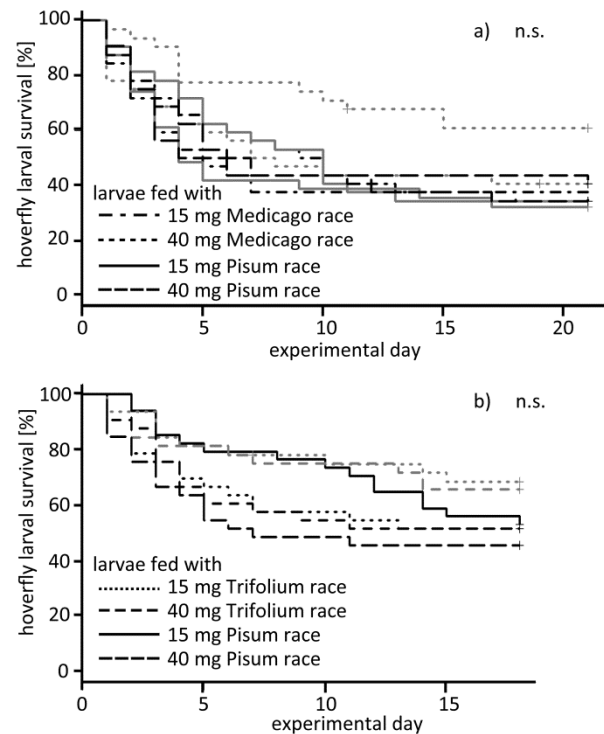
Figure 3: Kaplan-Meier survival curves of hoverfly larvae fed with different amounts and types of aphids as food. Larvae were fed with (a) different amounts of Medicago and Pisum race aphids reared on their native and universal host plants, or (b) with different amounts of Trifolium race aphids reared on their native and universal hosts and with Pisum race aphids reared on the universal host. Dark color represent survival curves of hoverfly larvae fed with aphids originating from the universal host *V. faba*, grey color represent survival curves of hoverfly larvae fed with aphids reared on their native host plants.

Larvae fed with Trifolium race aphids had a shorter development time than larvae fed with Pisum race aphids. The resulting adult hoverflies had also wider heads when fed with Trifolium race aphids (Tab. 1).

The survival of the hoverfly larval was neither influenced by the amount nor by the kind of food (host plant – aphid race combination) offered (experiment A - Medicago & Pisum race: kind of food: likelihood ratio = 5.159, p = 0.161; amount of food: likelihood ratio = 0.352, p = 0.553; interaction: likelihood ratio = 4.070, p = 0.254; experiment B - Trifolium & Pisum race: kind of food: likelihood ratio = 4.965, p = 0.084; amount of food: likelihood ratio = 0.811, p = 0.368; interaction: likelihood ratio = 0.667, p = 0.716; Fig. 3).

Table 1: Influence of food amount (pea aphids), gender of hoverflies and type of food (host plant – aphid race combination) on hoverfly larval weight, larval development time, total development time and adult head width. Data is presented as means ± standard error, and the likelihood ratio (LR) and p-values of linear mixed effects models are given.

Experiment A	larval weight [mg]	larval development time [days]	total development time [days]	adult headwidth [mm]
food amount	**LR = 57.773 p < 0.001**	**LR = 13.875 p < 0.001**	**LR = 8.711 p = 0.003**	**LR = 18.870 p < 0.001**
15 mg/day	22.12 ± 0.78	10.05 ± 0.25	17.63 ± 0.23	4.481 ± 0.047
40 mg/day	34.18 ± 1.48	9.02 ± 0.30	16.83 ± 0.31	4.761 ± 0.040
hoverfly gender	**LR = 7.629 p = 0.006**	LR < 0.001 p = 1.000	LR = 0.124 p = 0.724	**LR = 24.941 p < 0.001**
female	26.12 ± 1.19	9.59 ± 0.24	17.29 ± 0.24	4.513 ± 0.033
male	31.38 ± 1.99	9.41 ± 0.37	17.07 ± 0.35	4.821 ± 0.060
type of food	LR = 5.882 p = 0.118	LR = 1.306 p = 0.728	LR = 0.357 p = 0.949	LR = 5.545 p = 0.136
Pisum race from *V. faba*	26.15 ± 2.28	10.06 ± 0.42	17.53 ± 0.27	4.723 ± 0.101
Pisum race from *P. sativum*	24.93 ± 1.96	9.55 ± 0.47	17.20 ± 0.52	4.553 ± 0.051
Medicago race from *V. faba*	30.15 ± 2.24	9.21 ± 0.31	17.05 ± 0.33	4.635 ± 0.064
Medicago race from *M. sativa*	30.49 ± 1.95	9.35 ± 0.41	17.13 ± 0.41	4.601 ± 0.059
interactions				
type of food x hoverfly gender	LR = 2.241 p = 0.524	LR = 1.591 p = 0.661	LR = 2.964 p = 0.397	LR = 1.524 p = 0.677
food amount x type of food	LR = 1.507 p = 0.681	LR = 3.167 p = 0.367	LR = 3.847 p = 0.279	LR = 3.124 p = 0.373
food amount x hoverfly gender	LR = 0.047 p = 0.828	LR = 0.328 p = 0.567	LR = 0.674 p = 0.412	LR = 0.733 p = 0.392
Experiment B		larval development time [days]	total development time [days]	adult headwidth [mm]
food amount		**LR = 4.026 p = 0.045**	LR = 3.459 p = 0.063	**LR = 22.420 p < 0.001**
15 mg/day		9.67 ± 0.25	17.70 ± 0.31	2.616 ± 0.030
40 mg/day		8.93 ± 0.22	16.79 ± 0.26	2.800 ± 0.027
gender		LR = 0.382 p = 0.537	LR = 2.107 p = 0.147	**LR = 9.749 p = 0.002**
female		9.19 ± 0.18	16.89 ± 0.25	2.658 ± 0.032
male		9.4 ± 0.32	17.17 ± 0.35	2.795 ± 0.032
type of food		**LR = 7.124 p = 0.028**	LR = 4.522 p = 0.104	**LR = 6.205 p = 0.045**
Pisum race from *V. faba*		9.89 ± 0.29 A	17.77 ± 0.42	2.623 ± 0.043 A
Trifolium race from *V.faba*		9.04 ± 0.26 B	17.07 ± 0.34	2.744 ± 0.031 B
Trifolium race from *T. pratense*		8.94 ± 0.30 B	16.875 ± 0.31	2.767 ± 0.036 B
interactions				
type of food x hoverfly gender		LR = 0.013 p = 0.994	LR = 0.201 p = 0.904	LR = 0.608 p = 0.738
food amount x type of food		LR = 0.073 p = 0.964	LR = 0.214 p = 0.900	LR = 0.130 p = 0.937
food amount x hoverfly gender		LR = 0.364 p = 0.546	LR = 0.039 p = 0.844	LR = 0.341 p = 0.559

Discussion

Even though not much is known about hoverfly larval migration, there are clear hints from the literature that hoverfly larvae migrate between aphid colonies. Banks (1968) found several hoverfly larvae in field experiments that must have migrated to the experimental aphid colonies. Similarly Kan (1988a) and Kan (1988b) observed older hoverfly larvae that migrated between aphid colonies in the field, and Chandler (1969) reported that that even "unfed first instar larvae were able to travel considerable distances, certainly well in excess of 1 m". Nevertheless, it is generally assumed that hoverfly larvae are sedentary and that therefore, hoverfly oviposition choice determines the fate of the developing larvae (e.g. Sadeghi & Gilbert 2000; Almohamad, Verheggen & Haubruge 2009). In fact, oviposition choice was detected in adult hoverflies: they prefer certain aphid species and host races and distinguish between aphids feeding on different plant species. It was shown that they generally prefer aphids that result in a high performance of their offspring and plants where aphids have a high reproductive rate (Almohamad *et al.* 2007; Almohamad, Verheggen & Haubruge 2009, chapter I & II). And indeed, our experiment showed that *E. balteatus* larvae do not leave a plant as long as there are aphids available as food. However, when most aphids on a plant are consumed, larvae have to leave the plant to search for additional food to complete their development (chapter I & II). This was found in our field experiment where we discovered new hoverfly larvae in aphid colonies. More than half of the observed hoverfly larvae must have migrated to the experimental plants, because the number of dry hoverfly eggs (equivalent to the maximum number of larvae that could have hatched on the plant) was often much lower than the number of larvae actually present. Most hoverfly larvae were found on those host plant - aphid race combinations where the aphids have high reproductive rates like *V. faba* infested with Trifolium and Pisum race aphids and *P. sativum* infested with Pisum race aphids, but differences between host plants seem to be less important (chapter I). But how do hoverfly larvae find aphid colonies?

We showed that the probability that a migrating larva will visit a certain plant increases with increasing aphid number. Since it is known that hoverfly larvae intensify their search activity in areas where honeydew is present (Leroy *et al.* 2014), high amounts of honeydew that accumulate on aphid infested plants and in the vicinity might be an important cue for the hoverfly larvae. Larger aphid colonies produce more honeydew which increases the probability that larvae will climb those plants that contain high numbers of aphids. Once a hoverfly larva has encountered an aphid, it will increase its turning rate and search the surrounding area more thoroughly (Chandler 1969). This would again increase the probability of encountering more aphids in large colonies and explains the observed arrestance of hoverfly larvae in such colonies.

As already pointed out, honeydew is most likely the cue that informs hoverfly larvae that they are close to an aphid colony. However, it is not known if larvae are able to perceive aphid colonies over greater distances. Honeydew and aphid volatiles can only be perceived over a few centimeters and *E. balteatus* larvae are not attracted by volatiles from pea aphid - *V. faba* complexes (Bargen, Saudhof & Poehling 1998). This suggests that hoverfly larvae do not perceive aphid colonies over larger distances and that they randomly search the vegetation canopy until they encounter honeydew or aphids. They probably use other cues as well as is known for ladybird larvae which mark the areas which they have already searched to avoid examining them again (Meisner & Ives 2013).

Assuming that hoverfly larvae forage for aphids randomly within the vegetation canopy, it may take them several days to find another aphid colony. The ability of hoverfly larvae to survive starvation periods increases with larval age: 3 day old *E. balteatus* are able to survive 3 days of starvation, while 7 day old larvae survive 6 days without food at 19 to 21 °C (Rojo, Hopper & Marcos-Garcia 1996). But still, it would be beneficial for hoverfly larvae to use alternative food sources while searching for aphids. However, it is not known if aphidophagous hoverflies are able to use plant-derived food like nectar and pollen, as is known for other aphid predators (e.g. Banks 1957; Lundgren 2009), or other insects besides aphids. The latter is not unlikely, since hoverfly larvae have already been observed to act as intraguild predators (Hindayana 2001; Fréchette *et al.* 2007). Whether such alternative food just prolongs the time a hoverfly larva can live without aphids or whether it is sufficient to complete their development is not known. Hints that alternative food might at least influence the performance of the hoverflies come from studies where different aphid species were used as food. Hoverfly performance was dependent on the aphid species the hoverfly larvae were feeding on, and some aphid species were reported to be toxic for hoverfly larvae (e.g. Ruzika 1975; Sadeghi & Gilbert 2000; Almohamad *et al.* 2007). The suitability of aphids for larval development may depend on which plant species or cultivar they were feeding (Giles *et al.* 2000e.g. ; Vanhaelen, Gaspar & Francis 2002; Almohamad *et al.* 2007; Kos *et al.* 2011). The mortality of hoverfly larvae fed with the cabbage specialist *Brevicoryne brassicae* for instance increases drastically when aphids had been reared on a glucosinolate-rich host plant compared to hosts that contain less glucosinolates (Vanhaelen, Gaspar & Francis 2002; Chaplin-Kramer *et al.* 2011). Pea aphids reared on *M. sativa* were shown to have a higher caloric content than pea aphids reared on *V. faba* which results in better survival and/or faster development of lacewings and ladybirds (Giles *et al.* 2000; Giles *et al.* 2001; Giles *et al.* 2002). However, our experiments showed that aphids of all three pea aphid host races tested allow successful development of hoverfly larvae, and that survival was not influenced by the host plant species the aphid was feeding on. This fits well with the observation that migrating hoverfly larvae do not prefer

any host plant - aphid race combination in the laboratory and that larvae were found on all combinations in the field experiment.

Now that we know that hoverfly larvae migrate and that they prefer large aphid colonies over small colonies, most likely due to the greater amount of honeydew, we can consider the consequences for aphid populations. An accumulation of hoverfly larvae in large aphid colonies will reduce the survival of aphids with a high performance and reproductive rate. This was also observed during a previous field experiment. There the number of Trifolium race aphids on *V. faba* and the number of Pisum race aphids on both host plants was much lower than would be expected from their high reproductive rates (chapter I). This was most likely due to the feeding of several hoverfly larvae that were found on those plants. In turn, aphids with a low performance and reproduction rate will be less apparent to migrating hoverfly larvae. This can have consequences especially for aphids which colonize novel host plants. On novel hosts, they will most likely have a reduced performance and hence, produce little honeydew, but therefore also might benefit from reduced predation (chapter II). Thus the reduced performance might be compensated by an enemy-free space. This mechanism was already shown for hoverfly oviposition preference. Plants where aphid reproduction was low created an enemy-free space. Because this balances performance reductions that aphids have to face when they colonize novel host plants, it may facilitate host race formation and may help to maintain existing host races (chapter I & II).

Conclusion

One aphid colony is often not sufficient to enable hoverfly larval development and so older hoverfly larvae migrate in search of other aphid colonies. We showed that hoverfly larvae accumulate in large aphid colonies, probably because these are easier to detect and the act of encountering aphids or honeydew arrests larvae on plants. Hoverfly larvae are likely not able to use long range cues to detect an aphid colony over larger distances, but search the vegetation canopy randomly until they encounter honeydew.

Acknowledgments

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Chapter IV: Enemy attraction by aphid alarm pheromone?

Is there any evidence that aphid alarm pheromones work as prey and host finding kairomones for natural enemies?

Ilka Vosteen*[1], Wolfgang Weisser[2], Grit Kunert[1]

1 Max-Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07749 Jena

2 Department of Ecology and Ecosystemmanagement, Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85350 Freising-Weihenstephan

Abstract

The aphid alarm pheromone (E)-β-farnesene (EBF) is often considered to be used by natural enemies as a prey/host finding kairomone. However, studies show opposing results, some appear to confirm an attraction of aphid natural enemies by EBF while others do not provide any evidence for the kairomone function of EBF.

To clarify if aphid natural enemies are attracted by amounts of EBF naturally emitted by aphids, we reviewed the existing literature about EBF attractiveness to aphid natural enemies with consideration of the amounts of EBF used in the studies.

We found 31 publications that investigated the ability of EBF, aphid cornicle secretion and attacked aphids, to attract aphid natural enemies. Several studies showed an attraction by EBF, but these used much higher amounts of EBF than usually emitted by aphids during a predator attack. Studies investigating EBF amounts similar to what is emitted by aphids are rare, and failed to show an attraction. Moreover, there are only two studies which document an attraction of natural enemies by attacked aphids.

Since EBF is emitted in very low amounts, and since it is not very stable, and only present after an attack, we consider aphid derived EBF not to be a suitable kairomone for most natural enemy species, especially when they are able to use alternative cues. However, since EBF, amongst other volatiles, is also emitted by herbivore-induced plants we propose that natural enemies might use plant derived EBF as a synomone to identify aphid infested plants via an altered plant volatile bouquet.

In order to confirm that aphid alarm pheromone is used as prey / host finding kairomone more experiments are needed which use EBF amounts naturally emitted by aphids.

Keywords: aphid, alarm pheromone, (*E*)-β-farnesene, hoverflies, kairomone, ladybirds, lacewings, natural enemies, parasitoids, volatiles

Introduction

Natural enemies employ several different cues for prey and host detection. These cues can be optical such as color, shape, and size (e.g. Caldwell 1986; Endler 1991; Harmon, Losey & Ives 1998), acoustic such as courtship calls, nestling begging calls, and sounds generated by prey movement (e.g. Payne 1971; Tuttle & Ryan 1981; Ryan & Tuttle 1987; Haskell 1994), mechanical like movement vibration (e.g. Horridge & Boulton 1967; Brownell & Farley 1979), electrical like bioelectric fields of aquatic organisms (e.g. Kalmijn 1982; Freund *et al.* 2002) and chemical such as prey odor, pheromones and herbivore induced plant volatiles (Wiskerke, Dicke & Vet 1993 and cited literature; Hare 2011). These cues are either linked to prey movement or feeding, or they might play a role in the intraspecific communication of the prey. Additionally, natural enemies can use cues from herbivore-associated organisms such as food plants or associated microorganisms (Vet & Dicke 1992).

If cues created inevitably attract enemies, natural selection acts on the prey or host to minimize these cues and to become as inconspicuous as possible (Vet & Dicke 1992). If natural enemies eavesdrop on the communication of their prey or hosts, a trade-off between the necessity to avoid predation and the need for communication arises. Especially when communication is vital for the survival and reproduction of the prey and host organisms, natural enemies have many opportunities to exploit the signal (Wertheim, Vet & Dicke 2003). This is the case for many sexual signals like the conspicuous coloring of males, courtship calls and sex pheromones (Zuk & Kolluru 1998 and cited literature). A special case is the exploitation of alarm signals such as distress calls or alarm pheromones, because these aim to prevent predation. If instead these alarm signals attract more natural enemies this would decrease their benefits drastically.

Alarm signals are known from a wide variety of taxa, including insects (Blum 1969; Verheggen, Haubruge & Mescher 2010), gastropods (e.g. Snyder & Snyder 1971; Atema & Stenzler 1977), amphibians (Rajchard 2006), fishes (Smith 1992), birds and mammals (Hollen & Radford 2009). They might benefit the signaling individual directly if the anti-predation behavior of alerted con- and heterospecifics reduces the probability of successful predation (e.g. Charnov & Krebs 1975; Perrone 1980; Sherman 1985) or if additional predators disrupt the predation event (Perrone 1980; Hogstedt 1983; Koenig *et al.* 1991; Chivers, Brown & Smith 1996). Alarm signals may also have delayed benefits for the signaler, for example by saving the lives of individuals who will reciprocate in the future

(Trivers 1971), of potential mates (Witkin & Ficken 1979) or other group members in circumstances where group living is beneficial (Smith 1986). Warning calls may prevent predators from specializing on the caller's species and locality (Trivers 1971). Alarm signals which do not benefit the signaler itself should only evolve if the surrounding conspecifics are closely related kin of the signaler (Hamilton 1964; Maynard Smith 1965; Sherman 1977). An example for this is the alarm signaling behavior of aphids. Most often the aphid attacked by a natural enemy emits an alarm pheromone to warn closely related conspecifics, but the attacked aphid itself only very rarely manages to escape and survive. Alarm signaling may also have negative effects for the signaler and surrounding conspecifics, if it attracts additional enemies. But can aphid natural enemies really use the emitted alarm pheromone to find their prey?

In this article we review the literature in order to judge if the aphid alarm pheromone is used by natural enemies as a prey/host finding cue. First, we briefly summarize the knowledge on aphid detection by natural enemies and the alarm pheromone emission in aphids. We then review the evidence for natural enemy attraction to EBF. We contrast the results of studies that used EBF in amounts similar to the amounts naturally emitted by attacked aphids with studies that use higher EBF amounts. Finally, we discuss the suitability of EBF as a kairomone for natural enemies and why the reaction of natural enemies may be different towards different dosages of EBF.

Aphid detection by natural enemies

For insect natural enemies such as aphid predators and parasitoids, the search for prey or hosts can be divided into three steps during which different cues are used: 1) locating a plant with aphids, 2) locating aphids on the plant and 3) accepting the aphid as suitable host/prey (Vinson 1976; Hatano *et al.* 2008b). Prey/host cues can act in two different ways; attractants are perceived from a distance and guide the natural enemy towards its prey/host organism, whereas arrestance cues keep the natural enemy in the area where the cue was perceived (Fellowes, van Alphen & Jervis 2005). While searching for aphids, natural enemies face the reliability-detectability problem (Vet & Dicke 1992). Cues derived from aphids are most reliable, but due to the low biomass of aphids they are often hard to detect. In contrast, plant-derived cues (e. g. plant volatiles) are easy to detect, but less reliable, because the plant may be aphid-infested or not. Most predators and parasitoids solve the reliability-detectability problem by using plant volatiles that are induced during an aphid attack (Vet & Dicke 1992 ; Hatano *et al.* 2008b and cited literature). These cues are present in higher amounts and are very specific. Aphid parasitoids are even able to discriminate between plants that were previously infested by host and by non-host aphids (Du, Poppy & Powell 1996; Du *et al.* 1998; Guerrieri *et al.* 1999; Powell, Tosh & Hardie 2006).

For detecting aphids on the plant, natural enemies may use optical or short range chemical cues. Optical cues can be aphid color and shape (Nakamuta 1984; Kan & Sasakawa 1986; Battaglia *et al.* 1995; Harmon, Losey & Ives 1998). Chemical cues that are emitted from the aphid itself and are used by natural enemies for aphid detection are honeydew (e.g. Budenberg 1990; Budenberg & Powell 1992; Budenberg, Powell & Clark 1992; Evans & Richards 1997; Han & Chen 2002; Ide, Suzuki & Katayama 2007; Leroy *et al.* 2011), and sex pheromones (Hardie *et al.* 1991; Glinwood, Du & Powell 1999; Zhu *et al.* 2005). However, the signal most often suspected to work as a host/prey finding cue is the aphid alarm pheromone (*E*)-β-farnesene (EBF).

Aphid alarm pheromone emission

Aphids respond to attacks of natural enemies by secreting droplets from their cornicles (paired tubes arising from the 5th or 6th tergite) (Nault, Edwards & Styer 1973; Heie, Minks & Harrewijn 1987). These sticky droplets are composed mainly of triglycerides (Strong 1967) which are reported to impede the attack, giving the aphid the chance to escape (Edwards 1966; Dixon 1975). The cornicle droplets also contain alarm pheromone that alerts nearby conspecifics which may escape predation by walking away and dropping off the host plant (e.g. Kislow & Edwards 1972; Wientjens, Lakwijk & Van Der Marel 1973; Nault & Bowers 1974; Montgomery & Nault 1977; Minoretti & Weisser 2000). Although the attacked aphid often dies during predator attack the alarm signaling is thought to be beneficial for the attacked aphid since it saves the live of colony members which are mostly genetically identical clones of one colony-founding female (McAllister & Roitberg 1987; Kunert *et al.* 2010).

The alarm pheromone of several aphid species is (*E*)-β-farnesene (EBF) either alone or in combination with other components (Bowers *et al.* 1972; Edwards *et al.* 1973; Wientjens, Lakwijk & Van Der Marel 1973; Nault & Bowers 1974; Pickett *et al.* 1992; Francis *et al.* 2005a).

Single *Acyrthosiphon pisum*-aphids (De Geer) emit on average 8 to 16 ng EBF and always less than 50 ng when they are attacked by the lacewing *Chrysoperla carnea*, while they emit even smaller amounts during a ladybird attack (Schwartzberg *et al.* 2008; Joachim *et al.* 2013; Joachim & Weisser 2013). Cornicle droplets of *A. pisum* contain on average less than 15 ng EBF and some droplets contain no EBF at all (Mondor & Roitberg 2000; Joachim *et al.* 2013; Joachim & Weisser 2013). Micha and Wyss (1996) reported even lower EBF amounts (0.7 ng on average) for cornicle droplets of *Sitobion avenae.* Since only single or few aphids in a colony are attacked at the same time and the signal is not amplified by emission of neighboring aphids (Hatano *et al.* 2008a) EBF amounts naturally emitted by aphids can be considered to be always lower than 200 ng (corresponding to about four adult attacked pea aphids).

Importance of a carful estimation of emission rates

While comparing the reactions of natural enemies towards high and low amounts of EBF one has to keep in mind that the EBF release rates most likely differ depending on the dispersers used in the experiment and that applying certain amounts of EBF in rubber septum or on filter paper does not necessarily means that the same amount evaporates. Most studies use synthetic EBF dissolved in organic solvents that was applied to filter paper (e.g. Nakamuta 1991; Du *et al.* 1998; Acar *et al.* 2001; Francis *et al.* 2005b; Ameixa & Kindlmann 2012; Joachim, Vosteen & Weisser 2014) or to a variety of different dispersers (e.g. Micha & Wyss 1996; Zhu *et al.* 1999; Verheggen 2008), without quantifying the release rate. Thus, we can only speculate which EBF amounts mimic natural EBF emission rates. But since the total amounts of EBF emitted from attacked aphids are always lower that 50 ng, it is likely that even in the case that only a fraction of applied EBF amount really evaporates, EBF amounts higher than 200 ng lead to unnaturally high EBF emission rates. Even in cases when the emission rate of a disperser has been quantified (Francis *et al.* 2005b; Heuskin *et al.* 2011; Cui *et al.* 2012; Heuskin *et al.* 2012; Joachim & Weisser 2015), the EBF amount in the headspace most likely varies, because of depletion of the EBF source and differences in the environmental conditions (Heuskin *et al.* 2012). Some studies only give the total release rate during several hours or days (Cui *et al.* 2012; Heuskin *et al.* 2012). In these cases we calculated the average emission rate per hour to have a comparable estimate for the EBF amount. So far, there is only one study that measured the release rate of the EBF dispersers and loaded the dispersers accordingly to get a certain EBF amount in the headspace (Joachim & Weisser 2015).

Similarly, in studies with disturbed or crushed aphids and cornicle secretion, most authors did not quantify the actual release rate (e.g. Grasswitz & Paine 1992; Hemptinne *et al.* 2000; Francis *et al.* 2005b). EBF content of aphids depends on the aphid species (Francis *et al.* 2005a), and EBF emission rates further depend on the aphid's reproductive stage (Mondor *et al.* 2000; Schwartzberg, Tumlinson & Jones 2014), as well as on the natural enemy species that attacks the aphid (Joachim *et al.* 2013). Additionally, some cornicle droplets do not contain EBF at all (Joachim *et al.* 2013). Thus, one cannot simply infer the EBF amount in the headspace from the amount of aphids or cornicle secretion used. Also crushing several aphids leads to unnatural high emission rates since cornicle droplets only contain 11 to 17 % of the EBF that is stored in the aphid body (Joachim *et al.* 2013). However, the closer the number of disturbed aphids used in an experiment is to the actual number of aphids that would be disturbed during a predator attack, the more realistic the experimental scenario is.

Reactions of natural enemies towards EBF

Experiments that use EBF amounts close to natural amounts are rare and hardly provide any evidence that aphid emitted EBF works as a kairomone (Fig. 1). Joachim and Weisser (2015) showed in a field

experiment that different groups of natural enemies are neither attracted nor arrested by EBF amounts close to natural emission rate (around 100 ng/h).
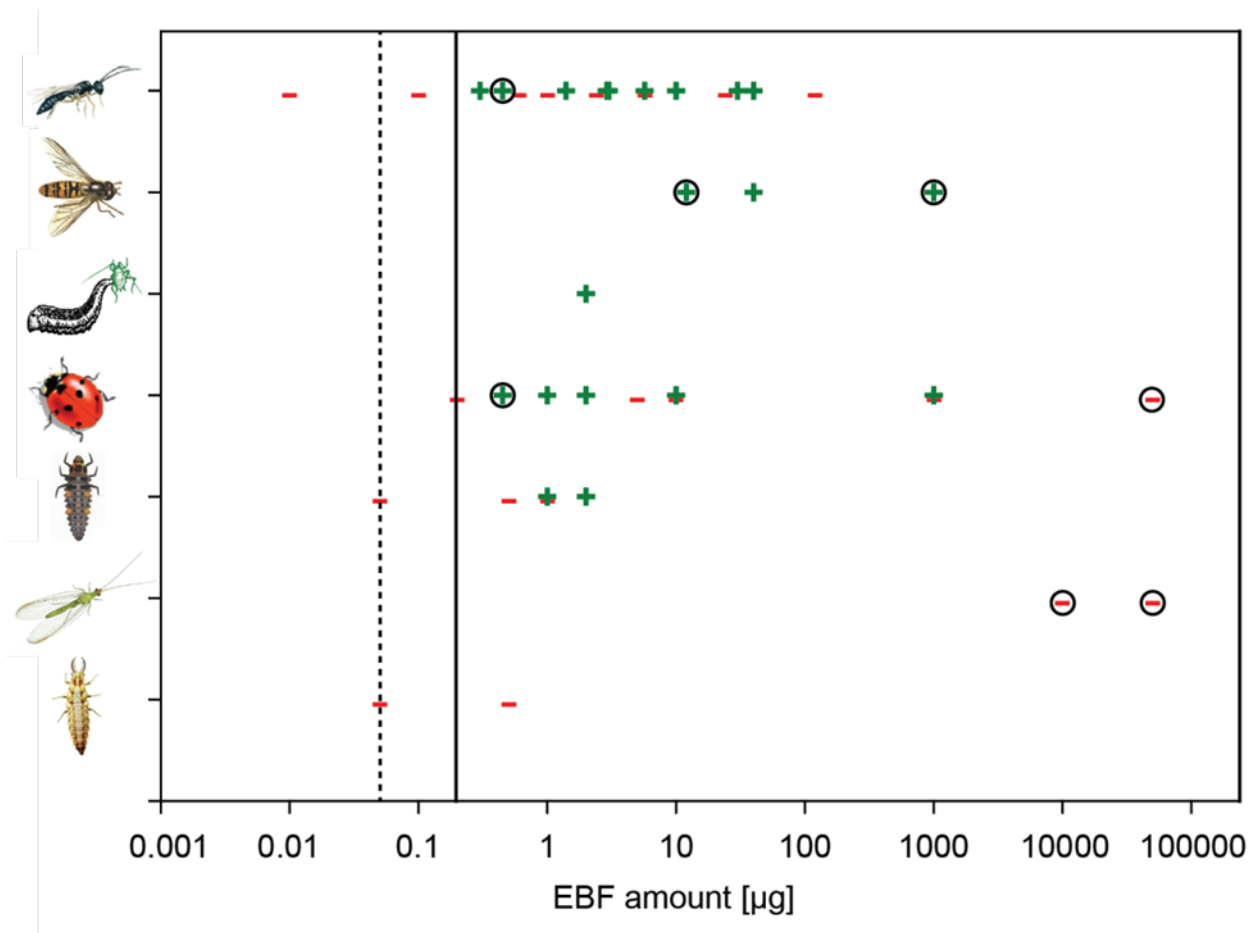
Figure 1: Reaction of different aphid natural enemies to different amounts of artificially applied EBF. + positive reaction towards EBF, - no reaction to applied EBF; natural enemies from top to bottom: parasitoids, hoverflies, hoverfly larvae, ladybirds, ladybird larvae, lacewing larvae. The dotted line indicates the maximal amount of EBF (50 ng) emitted from one pea aphid (Schwartzberg et al., 2008), the solid line marks the maximal amount of EBF approximately occurring naturally in an aphid colony (200ng, corresponding to about 4 crushed pea aphids), circles indicate field trapping experiments.

Parasitoids (Hymenoptera: Aphidiidae)

Under laboratory conditions, two generalist parasitoid species (*Aphidius colemani* and *Lysiphlebus testaceipes*) were tested with natural amounts of EBF (2.4 ng to 240 ng). None of them showed a reaction, even when EBF amounts were increased to 1 µg or higher (Micha & Wyss 1996; Ameixa & Kindlmann 2012), indicating that these species do not respond to EBF at all. Other tested parasitoids like the generalists *Aphidius ervi* and *Praon volucre* as well as the specialists *Aphidius uzbekistanicus* and *Diaeretiella rapae* were however, attracted by high EBF amounts in wind tunnel, olfactometer and search arena experiments (Micha & Wyss 1996; Du *et al.* 1998; Heuskin *et al.* 2011). Thus at least the response towards high amounts of EBF is regardless of the degree of host specialization but species specific.

In field experiments Cui *et al.* (2012) documented an increased number of aphid parasitoids in EBF releasing yellow traps with a mean emission rate of approximately 450 ng EBF/h (measured at 20 °C, 65% relative humidity, air flow 0.5 l/min). The number of mummified aphids on plants however, did not differ between EBF treated and control plots. Since it is not documented how many dispensers per plot were used, and since the EBF used was not very pure (83.8 ± 0.3%, extracted from essential oil of *Matricaria chamomilla*) it is difficult to judge whether EBF was used as a host finding volatile.

Since it is known, that parasitoids are able to learn, their experience might influence the reaction towards EBF. This was found for instance for *A. uzbekistanicus*. Experience decreased its response threshold from 5.7 μg to 1.4 μg EBF, but 600 ng were still not attractive (Micha & Wyss 1996), indicating that even experienced parasitoids do not respond to EBF amounts close to natural emission range. Also for the specialist *D. rapae* an experience effect was detected. Whilst experienced *D. rapae* females were attracted by the odor of secreting and non-secreting *Brevicoryne brassicae* aphids, naïve females were not attracted. The experienced females also preferred secreting aphids over non-secreting ones, while naïve females were not attracted by aphid volatiles at all. This indicats that *D. rapae* females were able to learn the odor of disturbed and undisturbed aphids and that learning is probably more important than volatiles of the cornicle secretion (Moayeri, Rasekh & Enkegaard 2014).

A few experiments were done with aphid cornicle secretions as EBF source. In these cases results must be interpreted very carefully, since cornicle secretions not only contain the volatile alarm pheromone but also other non-volatile components which might be used by parasitoids. Thus naïve *A. ervi* and *A. uzbekistanicus* did not react to volatiles emitted from cornicle secretion, but both species showed an oviposition response after physical contact with cornicle secretion (Battaglia *et al.* 1993; Battaglia *et al.* 1995; Micha & Wyss 1996; Battaglia *et al.* 2000). Similarly, *L. testaceipes* showed an increased oviposition and examination behavior towards glass beads coated with cornicle secretion and body fluids of its host *Rhopalosiphum padi*-aphids, but not of the non-host *Aphis nerii*. Application of cornicle secretion of *R. padi* to *A. nerii* increased examination and oviposition by *L. testaceipes* (Grasswitz & Paine 1992). This indicates that natural EBF amounts emitted from cornicle secretion most likely do not influence oviposition behavior of aphid parasitoids, but that non-volatile substances from cornicle secretion help to distinguish between host and non-host aphids.

Thus, even though some parasitoid species reacted to high amounts of EBF and showed oviposition responses after physical contact with cornicle secretion, EBF most likely does not play a role in attraction of aphid parasitoids towards aphid colonies.

Adult ladybirds (Coleoptera: Coccinellidae)

Adults of three tested ladybird species (*Coccinella septempunctata, Harmonia axyridis, Coleomegilla maculata*) showed neurological responses to EBF in single cell recording and EAG experiments (Al Abassi *et al.* 2000; Zhu *et al.* 2005; Verheggen *et al.* 2007). This however, not necessarily means that

the ladybirds also react to EBF, but all tested species were attracted to high EBF amounts in olfactometer experiments (Al Abassi *et al.* 2000; Hemptinne *et al.* 2000; Acar *et al.* 2001; Francis, Lognay & Haubruge 2004; Leroy *et al.* 2012a). *Adalia bipunctata* and *H. axyridis* were further attracted by high amounts of crushed *A. pisum* and *Myzus persicae* (Verheggen *et al.* 2007). *H. axyridis* is also attracted by extremely high amounts on EBF (1 mg in rubber septum) under laboratory conditions (Leroy *et al.* 2012b), while high amounts (5 µg, 10 µg) were not attractive anymore (Alhmedi, Haubruge & Francis 2010).

Experiments with natural amounts of EBF or with high amounts, but under field conditions, could however, not confirm these results. Lower amounts of EBF like the 200 ng used in the experiment of Nakamuta (1991) did not induce locally restricted search in *C. septempunctata* and Mondor and Roitberg (2000) did not find an attraction of *H. axyridis* adults to fresh cornicle secretion collected from five *A. pisum*. *H. axyridis* was also not attracted by extreme high EBF amounts (1 mg) under field conditions (Alhmedi, Haubruge & Francis 2010). Also (Zhu *et al.* 1999) could not find any attraction of ladybirds to traps baited with 50 mg of EBF indicating that even though ladybeetles in general are attracted by high amounts of EBF the effect of EBF under field conditions may vary with environmental conditions and species composition. Only Cui et al. (2012) documented an increased the number of ladybirds (*H. axyridis*, *C. septempunctata*) in EBF treated plots compared to control plots under field conditions. The EBF used in this experiment was extracted from essential oil of *M. chamomilla* and was not very pure (83.8 ± 0.3%,). Thus it cannot be excluded that other volatile compounds besides EBF were responsible for the attractiveness of the EBF-containing dispensers.

These studies show that EBF can be perceived and that high amounts are attractive for adult ladybirds, while lower amounts do not lead to an attraction. Thus, it is unlikely that adult ladybirds use EBF that is emitted by an aphid attack to detect an aphid colony.

Ladybird larvae

In contrast to adult ladybirds, larvae are less mobile and may focus more on short range cues like EBF during their search for aphids. 4th instar *C. septempunctata* larvae were, for example, shown to walk towards the smell of a crushed aphid at a distance of 0.69 cm (Stubbs 1980). This ability to smell aphid prey over short distances, however, did not increase the ability of 3rd and 4th instar larvae of *C. septempunctata* to find a glued aphid on a plant when 50, 500 or 1000 ng of EBF were applied compared to solvent application and neither attraction nor arrestance by EBF could be observed in this experiment (Joachim, Vosteen & Weisser 2014).

However, 1st instar larvae of *A. bipunctata* were attracted and arrested by aphids that were preyed on by 10 1st instar ladybird larvae (Hemptinne *et al.* 2000). Since the capture of the first aphid prey is

critical for survival of 1st instar ladybird larvae (Dixon 1959), they might have adapted to detect the small amounts of EBF emitted by aphids which are attacked by their siblings (Hemptinne *et al.* 2000). Even a short detection distance of a few mm as calculated for *C. septempunctata* by Stubbs (1980) will increase the perceptive field of the larvae drastically. Since female ladybirds lay their eggs in clusters close to aphid colonies, EBF might in this case be a useful kairomone for short range detection of aphids (Fig. 2). Since 1st instar larvae only consume few aphids, several young larvae could prey on the same aphid colony without competing with one another. In the case when aphids are rare the cannibalistic larvae may prey on each other. However, this result should not be extrapolated to other natural enemy species without further testing.
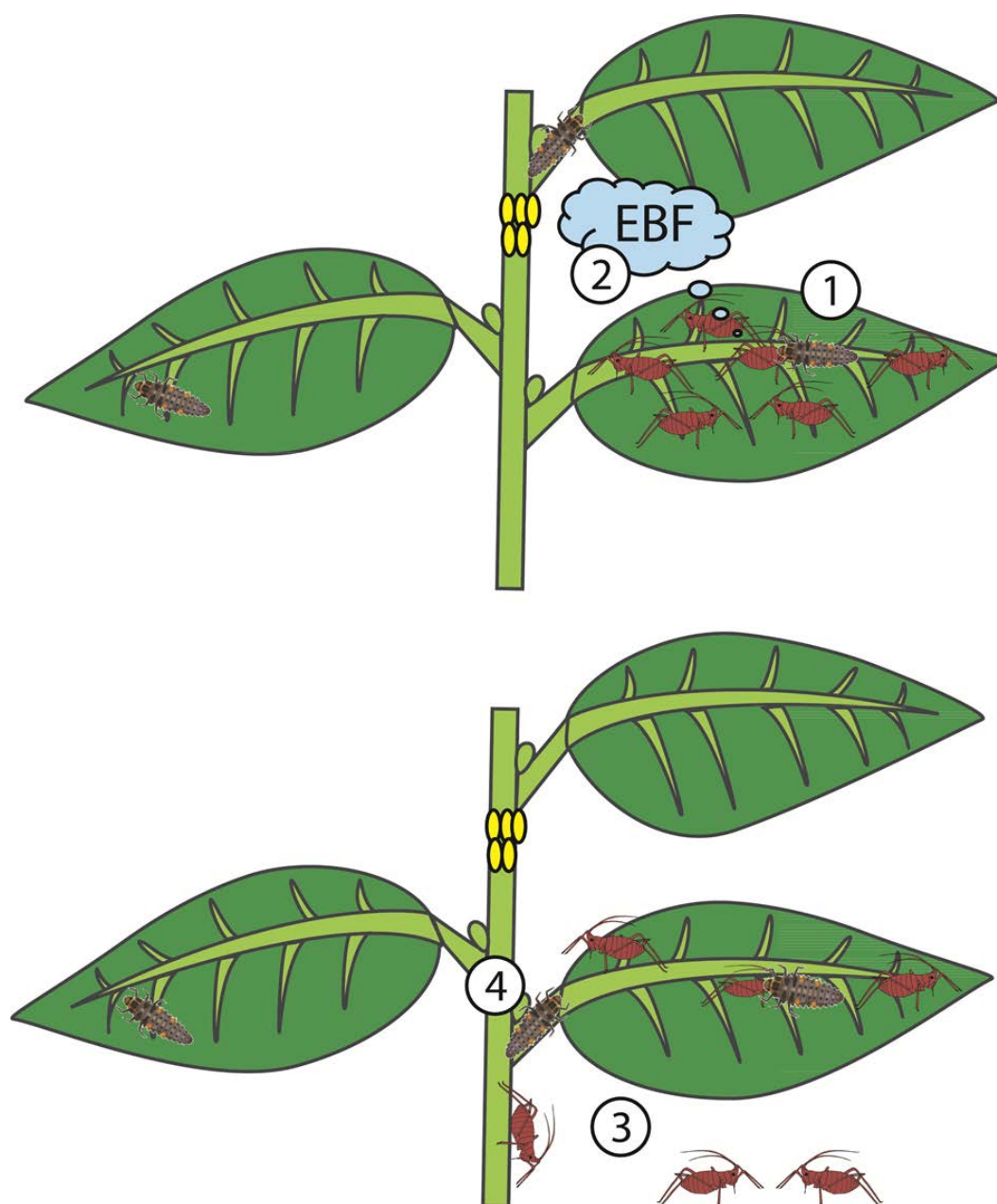
Figure 2: Function of EBF as a short range kairomone for ladybird larvae. **1** A ladybird larvae attacks an aphid. **2** The EBF emitted by the attacked aphid alerts nearby aphids and is perceived by a close-by newly hatched ladybird larva searching for prey. **3** Alerted aphids disperse. **4** The close-by newly hatched ladybird larva is moving towards the attacked aphid and may encounter aphids from the colony that did not disperse after the EBF emission.

Hoverflies (Diptera: Syrphidae)

Larvae and females of the hoverfly *Episyrphus balteatus* also showed EAG responses to EBF and were attracted in an olfactometer by high amounts (Francis *et al.* 2005b; Verheggen *et al.* 2008; Leroy *et al.* 2010), and adults were attracted in the field to EBF emitting traps (traps filled with 10 mg EBF with unknown release rates and traps with release rates up to 12 µg/h, depending on weather conditions) (Alhmedi, Haubruge & Francis 2010; Heuskin *et al.* 2012). Furthermore, *E. balteatus* larvae react to unstressed and crushed *Aphis fabae*, *A. pisum* and *Megoura viciae* (Francis *et al.* 2005b). Since unstressed aphids do not emit EBF unless they are disturbed by handling (Joachim *et al.* 2013; Joachim & Weisser 2013) it is hard to interpret these results. If the aphids are really unstressed, this would indicate that other cues than EBF would be used by hoverfly larvae to detect aphids. If the aphids were disturbed by the handling procedure, this would suggest that hoverfly larvae like ladybeetle larvae are able to react to natural amounts of EBF. The last possibility is further supported by an observation from Joachim and Weisser (2015) during a field experiment: hoverfly larvae were only found foraging on plants were low or high EBF amounts were applied, but not on control plants where only the solvent was applied.

Lacewings (Neuroptera: Chrysopidae)

Larvae of the lacewing *Chrysoperla carnea* were not attracted or arrested by low or high amounts of EBF and its search efficiency on a plant was not increased (Joachim, Vosteen & Weisser 2014). Even though adult *C. carnea* showed an EAG response to EBF, they were not attracted to field traps with 10 or 50 mg EBF (Zhu *et al.* 1999; Zhu *et al.* 2005). Another lacewing species, *Chrysopa cognata* did not show an EAG response and it was also not attracted to EBF when it was applied in very high amounts (10 mg) (Boo *et al.* 1998). These results show that EBF in high dosages does not play a role for the searching behavior of lacewing larvae and adults.

Ground beetles (Coleoptera: Carabidae)

The ground beetles *Pterostichus melanarius* and *Harpalus rufipes* were responding to different volatile mixtures containing variable concentrations of EBF. However, the reaction towards the two mixtures varied substantially suggesting that other volatile compounds of the samples have repellent or attractive effects (Kielty *et al.* 1996).

To summarize, most studies on EBF detection and attraction showed that the aphid natural enemies are able to detect EBF and react to this odor by attraction or arrestment, but only when it was available in quite high amounts (Tab. 1). This raises the question why all these insects belonging to different taxa (Coleoptera, Diptera Hymenoptera and Neuroptera) react to this compound.

Tab. 1: Attraction of different natural enemy species by (E)-β-farnesene , disturbed and undisturbed aphids

Ref.	Natural enemy species	life stage (gender), experience	EAG or SCR response	high EBF amounts	natural EBF amounts	crushed aphids (no., amount)	disturbed aphids (no.)	cornical secretion (no. aphids)	undisturbed aphids alone (no., amount)	undisturbed aphids on plant (no., amount)
	parasitoids									
1	Aphidius colemani	ad (f), naïve		no (1 µg)	no (100; 10 ng)					
2	Aphidius ervi	ad (f), naïve	yes	yes (10 µg)						
	Aphidius ervi	ad (f), exp		yes (10 µg)						
3	Aphidius ervi	ad (f), naïve		yes (40 - 400 µg/h)						
4	Aphidius umbekistanicus	ad (f), naïve		yes (5.7 µg)	no (600 ng)			no (5 dr)	no (50)	
	Aphidius umbekistanicus	ad (f), exp		yes (5.7; 2.9; 1.4 µg)			yes (50)			
	Lysiphelebus testaceipes	ad (f), naïve		no (5.7 µg)						
	Paron volucre	ad (f), exp		yes (5.7 µg)						
5	Lysiphlebus testaceipes	ad (f)		no (119; 24; 2.4 µg)	no (240; 24; 2.4 ng)	yes (2)		yes (10 - 15)		
6	Diaeretiella rapae	ad (f), naïve		yes (3; 30 µg)	yes (300 ng)					
7	Diaeretiella rapae	ad (f), naïve					no (40)		no (40)	
	Diaeretiella rapae	ad (f), exp					yes (40)		yes (40)	
8	different parasitoid spec	ad (f), exp		yes (7.5 µg/min)						
	hoverfly									
9	Episyrphus balteatus	1st inst		yes (2 µg)		yes (250 mg)			yes (250 mg)	yes (250 mg)
	Episyrphus balteatus	1st inst		yes (2 µg)		yes (250 mg)			yes (250 mg)	yes (250 mg)
	Episyrphus balteatus	1st inst		yes (2 µg)		yes (250 mg)			yes (250 mg)	yes (250 mg)
10	Episyrphus balteatus	ad (f, m)	yes	yes (40 µg *)						
11	Episyrphus balteatus	ad (f)		yes						
12	Episyrphus balteatus	ad (f)		yes (40 µg *)						
13	different hoverfly spec	ad		yes (up to 12 µg/h)						yes (1 g)
	lacewing									
14	Chrysoperla carnea	ad	yes	no (50 mg)						
15	Chrysoperla carnea	ad	yes	no (10 mg)						
16	Chrysopa cognata	ad	no (10 mg)	no (10 mg)						

f = female, m = male, inst = instar, ad = adult, exp = experienced, * in rubber septum, dr = dropletts

References: 1 = Ameixa & Kindlmann 2012, 2 = Du et al. 1998, 3 = Heuskin et al. 2011, 4 = Micha & Wyss 1996, 5 = Grasswitz & Paine 1992, 6 = Foster et al. 2005, 7 = Moayeri et al. 2014, 8 = Cui et al. 2012, 9 = Francis et al. 2005b, 10 = Verheggen et al. 2008, 11 = Leroy et al. 2010, 12 = Almohamad et al. 2008, 13 = Heuskin et al. 2012, 14 = Zhu et al. 1999, 15 = Zhu et al. 2005, 16 = Boo et al. 1998

Ref.	Natural enemy species	life stage (gender), experience	EAG or SCR response	high EBF amounts	natural EBF amounts	crushed aphids (no., amount)	disturbed aphids (no.)	cornical secretion (no. aphids)	undisturbed aphids alone (no., amount)	undisturbed aphids on plant (no., amount)
	ladybirds									
17	*Adalia bipunctata*	1st inst		yes (1; 2 µg)		yes (40 mg)	yes		no (40 mg)	
18	*Adalia bipunctata*	2nd inst, ad		yes (2 µg)		yes (250 mg)				no (250 mg)
	Adalia bipunctata	2nd inst, ad		yes (2 µg)		yes (250 mg)				no (250 mg)
	Adalia bipunctata	2nd inst, ad		yes (2 µg)		yes (250 mg)				no (250 mg)
	Adalia bipunctata	2nd inst, ad		yes (2 µg)		yes (250 mg)				no (250 mg)
	Adalia bipunctata	2nd inst, ad		yes (2 µg)		no (no EBF)				no (250 mg)
	Adalia bipunctata	2nd inst, ad		yes (2 µg)		no (no EBF)				no (250 mg)
19	*Coccinella septempuctata*	ad	yes	yes (10 µg)						
20	*Coccinella septempuctata*	ad			no (200 ng)					
21	*Coccinella septempuctata*	4st inst								
22	*Coleomegilla maculata*	ad	yes	no (50 mg)		yes (1)				
23	*Harmonia axyridis*	ad	yes			yes (20)				
24	*Harmonia axyridis*	ad						no (5)	yes (100)	yes (100)
25	*Harmonia axyridis*	ad		yes (1 mg *)					yes (25 - 30)	yes (50)
26	*Hippodamia convergens*	ad		yes (1 µg)						
8	different ladybird spec	ad			yes (450 ng/h)					
	ground beetle									
27	*Pterostichus melanarius*	ad		yes					yes (500 mg)	
	Harpalus rufipens	ad		yes					no (500 mg)	
	Nebria brevicolis	ad		no						
	spiders									
8	different spider spec			no (7.5 µg/min)						

f = female, m = male, inst = instar, ad = adult, exp = experienced, * in rubber septum, dr = dropletts

References: 17 = Hemptinne et al. 2000, 18 = Francis et al. 2004, 19 = Al Abassi et al. 2000, 20 = Nakamuta 1991, 21 = Stubbs 1980, 22 = Zuh 1999, 23 = Verheggen et al. 2007, 24 = Mondor & Roitberg 2000, 25 = Leroy et al. 2012b, 26 = Acar et al. 2001, 27 = Kielty et al. 1996

Why are natural enemies attracted to high amounts of EBF?

When thinking about the ecological function of EBF detection in natural enemies of aphids, one has to keep in mind that EBF is a widely distributed volatile which is produced by many different organisms (Crock, Wildung & Croteau 1997 and cited literature). Moreover, EBF is often among the herbivore induced plant volatiles, i.e. it is emitted when a plant is attacked by a herbivore, in most cases not an aphid (Gibson & Pickett 1983; Loughrin *et al.* 1994; Paré & Tumlinson 1997; Bernasconi *et al.* 1998; Turlings *et al.* 1998; Kessler & Baldwin 2001; Schnee *et al.* 2002; Martin, Gershenzon & Bohlmann 2003). Plant volatile blends containing EBF have been shown to attract the natural enemies of these herbivores although the role of EBF within these blends is unclear (Röse, Lewis & Tumlinson 1998; Turlings *et al.* 1998; Schnee *et al.* 2006). Natural enemies of aphids also reacted to aphid induced plant volatiles other than EBF (Guerrieri, Pennacchio & Tremblay 1993; Du, Poppy & Powell 1996; Guerrieri *et al.* 1999; Ninkovic, Al Abassi & Pettersson 2001; Han & Chen 2002; Zhu & Park 2005). Du *et al.* (1998) showed that EBF is among the attractive volatiles emitted by aphid-infested *Vicia faba*, but did not test if EBF was emitted by the aphid or the plant. However, there are other studies which document an increase of EBF after aphid infestation (Harmel *et al.* 2007; Gosset *et al.* 2009). That EBF emission by plants can arrest aphid natural enemies was shown by Beale *et al.* (2006) with EBF-producing transgenic *Arabidopsis thaliana*. Thus, the reaction of aphid natural enemies to high dosages of EBF may be explained by their reaction towards aphid induced plant volatiles.

Reaction of natural enemies to other aphid alarm pheromone compounds

Francis *et al.* (2005a) investigated the composition of the alarm pheromone of over 20 aphid species. While some aphid species such as *A. pisum* solely emit EBF as an alarm pheromone, other species like *Aphis idaei* (vd Goot) (Hemiptera: Aphididae) emit a mixture of up to 10 different terpenoids. Some species do not emit EBF at all or like *B. brassicae* only in very little amounts (Francis *et al.* 2005a). Thus, it is not surprising that crushed *B. brassicae* are not attractive for *A. bipunctata*. The attractiveness of most volatile blends has not been tested, but some individual compounds from those blends have been studied. Limonene works in high amounts (5 to 10 µg) as an attractant and arrestant for the ladybird *H. axyridis* and elicits oviposition, while β-pinene does not influence its searching behavior (Alhmedi, Haubruge & Francis 2010; Leroy *et al.* 2012a). α-pinene however, can be perceived by the ladybird *C. maculata* and the lacewing *C. carnea* (Zhu *et al.* 1999) and limonene, α- and β-pinene, β-myrcene and α-phellandrene are perceived by *E. balteatus*-hoverflies (Verheggen *et al.* 2008; Stökl *et al.* 2011). However, limonene alone did not influence hoverfly oviposition behavior (Verheggen *et al.* 2008; Leroy *et al.* 2011), but a blend of α- and β-pinene, β-myrcene and α-phellandrene elicited oviposition of hoverflies. This blend is emitted by flowers of the orchid *Epipactis veratrifolia* which is pollinated by ovipositing hoverflies and believed to mimic an aphid alarm

pheromone (Stökl *et al.* 2011). The floral blend of *E. veratrifolia* is quite similar to the alarm pheromone of *M. viciae*, but the authors did not test if the alarm pheromone blend itself is attractive in low amounts like emitted by the aphid. All these compounds which are part of the alarm pheromone blend are also common plant volatiles, so it is not surprising if some can be perceived by aphid predators and are attractive at high amounts. But it remains to be tested if they also work in low amounts as kairomones for aphid natural enemies.

Are aphid alarm pheromones suitable kairomones for natural enemies?

In order to work successfully as an alarm signal, pheromones should be difficult to detect by natural enemies. If there are some natural enemies which are able to use EBF as a kairomone, the aphid will be under a strong selective pressure to decrease the suitability of EBF as a kairomone. The EBF emission should therefore have evolved to be just high enough for neighboring aphids to detect, but too low to be recognized by predators over longer distances. This seems to have happened in aphids, because most aphid species respond to EBF dosages less than 20 ng (Montgomery & Nault 1977) and secretion was always found to be of similar magnitudes (Micha & Wyss 1996; Mondor & Roitberg 2000; Schwartzberg *et al.* 2008; Joachim *et al.* 2013; Joachim & Weisser 2013). Most natural enemies, however, are attracted to EBF amounts higher than 1 µg, which is more than 50 times higher than the detection threshold for most aphid species (Montgomery & Nault 1977). Besides the EBF amount the operating distance might be important. Aphids only react to EBF emission from cornicle droplets within a range of 1-3 cm (Nault, Edwards & Styer 1973), suggesting that the EBF amount present in the air rapidly decreases with distance from the odor source. Furthermore, EBF is known to be not very stable and to degrade within one hour (Pinto *et al.* 2007; Kourtchev *et al.* 2009). The low amounts of aphid emitted EBF and the short life time of the volatile should limit the possibilities for natural enemies to employ EBF as a kairomone at least over long distances. Aphid enemies might exploit the alarm signals at short distance, but it is doubtful how ecologically worthwhile this is. Since conspecific aphids will leave the site of an attack, newly arriving predators would most likely not find more food but rather other natural enemies with which they would compete for food. Encountering other predators may even be harmful for natural enemies of aphids, because intraguild predation often occurs among them (e.g. Hindayana 2001; Meyhöfer & Klug 2002).

Additionally, EBF is not a very reliable cue: the absence of EBF does not necessarily mean the absence of aphids, but only the absence of another enemy that is attacking the aphid. Although Almohamad *et al.* (2008) and Verheggen *et al.* (2009) reported EBF emission by non-preyed *M. persicae*, *M. viciae* and *A. pisum* colonies, other studies did not find any EBF emission by non-preyed colonies of the same (*M. persicae* and *A. pisum*) and other aphid species (*S. avenea* and *B. brassicae*) (Micha & Wyss 1996; Francis, Haubruge & Gaspar 2000; Joachim & Weisser 2013). EBF emission recorded from non-preyed

aphids might be due to disturbance during handling. This is supported by Joachim and Weisser (2013) which reported EBF emission of "undisturbed" aphid colonies up to 134 min after aphids were placed in the air collection chamber. The authors explain this initial EBF emission in the absence of predators with aphid disturbances during handling procedure.

We therefore conclude that EBF should only be used as a kairomone when other aphid cues cannot be used. This might be the case in ladybird larvae, because they are not able use optical cues (Stubbs 1980) and honeydew only reveals the presence of the aphid on the plant, but not the actual location of the aphid.

Conclusions

There are only few well documented examples were attacked aphids attract natural enemies but all experiments where EBF amounts close to rates emitted by aphids were applied, failed to attract predators and parasitoids. It is therefore quite unlikely that adult natural enemies may use it as a long range cue to detect aphid infested plants, especially because they are able to use several different cues for aphid detection (Hatano *et al.* 2008b). Predator larvae however, may use EBF as a short range cue to detect aphids, as shown by Hemptinne *et al.* (2000) for 1st instar ladybird larvae. For them the first meal is crucial for their survival (Dixon 1959) and since they have limited visual capacities (Stubbs 1980), EBF may be a useful cue to detect aphids at a distance of a few mm. We therefore suggest that more experiments with both adults and larvae of aphid natural enemies using EBF amounts similar to aphid emission should be done before accepting the kairomone effect of EBF as a general occurring mechanism in prey and host location of aphid enemies. In upcoming experiments researchers should carefully quantify the release rates of their disperses and select the applied EBF amounts accordingly to achieve realistic headspace concentrations of EBF.

Acknowledgements

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RESEARCH PAPER

The aphid alarm pheromone (*E*)-β-farnesene does not act as a cue for predators searching on a plant

Christoph Joachim · Ilka Vosteen · Wolfgang W. Weisser

Abstract Insect enemies use several environmental cues for host or prey finding. In insects these cues are often chemical, deriving from the host plant or from the prey itself. The aphid alarm pheromone (*E*)-β-farnesene (EBF) that is emitted by aphids when attacked by a predator is believed to be such a cue, as it has been shown to be perceived by several aphid enemies. It is unclear, however, if EBF is used as an arrestant stimulus or a cue for short-range prey localization, i.e., attractant stimulus, on the plant. We observed the searching behavior of larvae of two aphid predators, lacewing (*Chrysoperla carnea* (Stephens), Neuroptera—Chrysopidae) and ladybird (*Coccinella septempunctata* L., Coleoptera—Coccinellidae), on a plant where an aphid was fixed, in the presence and absence of EBF, and under field and laboratory conditions. EBF had no effect on predator searching behavior, either when natural amounts of 50 ng EBF or unnaturally high amounts of 1,000 ng were used. EBF also did not induce longer predator patch residence times under laboratory (ladybird only: 600.8 ± 35.1 s) and field (ladybird: 644.9 ± 50.7 s, lacewing: 1,108.4 ± 49.5 s) conditions. Predators found the aphid on the plant within the allocated time in only 34.72 and 17.13 % of the cases in the laboratory and field, respectively, but the presence of EBF did not increase the foraging success. We conclude that aphid alarm pheromone is not used as an arrestant cue for these important aphid predators nor does it have a short-range attractant function.

Keywords *Acyrthosiphon pisum* · Prey-associated cue · Foraging behavior

Introduction

Predators and parasitoids utilize various cues to localize their herbivore prey or host (Jervis 2005). These cues can be visual, auditory or mechanical, but in insects they are predominantly chemical (Carde and Bell 1995; Fellows et al. 2005). For insect enemies, potential chemical cues on which they can eavesdrop include prey pheromones and allelochemicals, i.e., compounds mediating interactions between the prey and other species, such as herbivore-induced plant volatiles (Agelopoulos et al. 1999; Fellows et al. 2005).

When searching for a host or prey (henceforth prey), insect enemies follow a hierarchical behavioral pattern that includes two steps before a prey is encountered (Vinson 1976): the first step is the prey habitat localization, i.e., long-range localization of the habitat where prey might be present. In case of insect herbivores that only feed on one plant species, a single plant individual may be the habitat. The second step is prey localization, i.e., short-range localization of the prey itself within the habitat, e.g., on the plant (Hatano et al. 2008b). In addition to the dichotomy of short-range and long-range localization, Fellows et al.

C. Joachim (✉) · W. W. Weisser
Department of Ecology and Ecosystem Management, Terrestrial Ecology Research Group, Center for Food and Life Sciences Weihenstephan, Technische Universität München, 85354 Freising, Germany
e-mail: Christoph.Joachim@tum.de

I. Vosteen
Institute of Ecology, Friedrich-Schiller University, Dornburger Str. 159, 07743 Jena, Germany

Present Address:
I. Vosteen
Department of Biochemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745 Jena, Germany

(2005) described the dichotomy of attractant vs. arrestant stimuli. Attractant stimuli direct the searching forager to areas containing prey, independent of the range over which the forager is attracted. In contrast, the perception of arrestant stimuli results in a reduction in distance or area covered per unit of time, e.g., it may increase the residence time of a forager in the area where the stimulus is detected (Fellows et al. 2005). For example, Noldus et al. (1991) described arrestant effects of the sex pheromones of noctuid hosts for two parasitoid wasp species of the genus *Trichogramma*. In the experiment, natural emission amounts of the host's sex pheromone induced higher patch residence times and prolonged locomotion of wasps compared to a set-up with clean air. As another example, aphid honeydew has been shown to act as both, an attractant for habitat localization (Saad and Bishop 1976) as well as a chemical arrestant stimulus for ladybird larvae that intensify their search in the prey patch in response to honeydew contact (Carter and Dixon 1984).

In many aphid species (Hemiptera: Aphididae), (E)-β-farnesene (EBF) is the only alarm pheromone component (Francis et al. 2005) and although it has the benefit of warning colony members of impending danger, EBF emission has been proposed to carry the cost of attracting aphid enemies (cf. Hatano et al. 2008b; Vandermoten et al. 2012). Several electroantennogram (EAG) studies and olfactometer assays demonstrate that a great variety of aphid enemies are capable of perceiving EBF or display a behavioral response to EBF presence (Hatano et al. 2008b). The evidence that EBF is used by predators or parasitoids as an attractant or arrestant stimulus is, however, weak, for a number of reasons. One problem is the low emission rate of EBF. While recent studies found that the EBF amounts released by individual aphids after an attack can range from less than 1 ng up to 50 ng (Schwartzberg et al. 2008; Joachim et al. 2013) with no further amplification by colony members (Hatano et al. 2008a; Verheggen et al. 2008), older studies on the attractant role of EBF used much higher amounts in their experiments, raising doubts about the biological significance. For example, Zhu et al. (1999) conducted EAG studies and showed that both the ladybird *Coleomegilla maculata* (Degeer) and the lacewing *Chrysoperla carnea* (Stephens) were able to perceive EBF, with increasing sensitivity in the range from 1 to 1,000 μg EBF, with the highest sensitivity at 1,000 μg. Thus, even the lowest concentration used was at least 20 times higher than the maximum amount released by aphids, a very unlikely scenario, since attacked aphids generally do not always emit maximum EBF amounts and the aphid colony would have dispersed after such frequent attacks (Minoretti and Weisser 2000; Joachim et al. 2013). In addition, EAG responses only give information of a predator's potential to use EBF as a host-finding cue, rather than revealing if the

predator actually employs EBF as a chemical signal in prey search. Results from EAG studies on EBF have also been shown to fail in correctly predicting the behavior of aphid enemies in the field (cf. Zhu et al. 1999).

In contrast to EAG studies, olfactometer assays reveal behavioral responses to a chemical signal, but also here the amounts of EBF used are often well above natural emission rates. For example, Micha and Wyss (1996) found that naive females of the parasitoid *Aphidius uzbekistanicus* Luzhetski showed attractant behavior to EBF in an olfactometer, but only at concentrations of 5.7 μg and not for lower concentrations. Further, Francis et al. (2004) found an attractant role of EBF only for amounts above 2 μg for larvae and adults of the ladybird *Adalia bipunctata* L.–concentrations more than 40 times higher than the maximum emitted amounts measured in aphids attacked by a predator. It is unclear whether reactions found for very high doses have any implications for aphid enemy behavior under natural conditions.

Another problem is that studies so far have rarely distinguished between the potential attractant and potential arrestant role of EBF. While EAG studies cannot distinguish between these two possibilities, olfactometers and wind tunnels (Du et al. 1998; Zhu et al. 1999; Al Abassi et al. 2000; Verheggen et al. 2007) focus on the role as an attractant stimulus. It is thus unknown if EBF can act as an arrestant stimulus, thus triggering an aphid enemy to stay and search longer or more intensively within a habitat.

In this paper, we investigate the influence of EBF on the foraging behavior of two different aphid predators on a plant. Here, in contrast to former studies on the kairomone effect of EBF, we will ascertain if EBF concentrations that naturally occur in aphid–predator interactions evoke a behavioral change in aphid predators. Further, we addressed the following questions under both laboratory and field conditions since laboratory studies do not always reflect natural conditions: (1) does EBF act as an arrestant stimulus for aphid predators? (2) Is short-range prey localization in aphid predators faster in the presence of EBF, i.e., does it serve as an attractant stimulus?

Materials and methods

Experimental plants and animals

Identical rearing conditions were established for aphids and predators (20 °C, 75 % humidity, photoperiod: L16:D8). Experiments were conducted with red clones of the pea aphid *Acyrthosiphon pisum* Harris (Hemiptera—Aphididae), originally collected in Bayreuth, Germany. Aphids were reared on two-week-old broad bean plants, *Vicia faba* L. (The Sutton; Nickerson-Zwaan, UK). To avoid aphid escape, plants were covered with air-permeable cellophane

bags (Unipack GmbH, Germany). A split-brood design was employed according to Kunert et al. (2005). By distributing individuals from one line equally among treatments, any variation due to rearing conditions is equally distributed over all treatments. To do so, we established 22 lines for the laboratory experiment (experiment 1) and 36 lines for the field experiment (experiment 2) by placing 22 and 36 adult foundress aphids (F_0 generation), randomly collected from a single population consisting of the same clone, on 22 or 36 bean plants, respectively, where they were allowed to reproduce for 24 h. After 8–9 days, the offspring (F_1 generation) reached the adult stage. For each line, one F_1 individual was selected and transferred to a new plant where it was allowed to reproduce for 24 h. The resulting offspring (F_2 generation) were eventually used for the experiments. A split-brood design was achieved for each experiment by choosing for all lines 1 F_2 individual for each EBF treatment. So, one aphid line was used once for each treatment.

Predators were chosen to show differences in foraging strategies. Lacewing larvae, *Chrysoperla carnea* (Stephens) (Neuroptera—Chrysopidae), are piercing-sucking predators, slowly consuming their prey and ladybird larvae, *Coccinella septempunctata* L.(Coleoptera—Coccinellidae), are chewing predators, quickly consuming their prey. Predators were obtained from a commercial supplier (Katz Biotech AG, Germany). Lacewings were reared on bean plants infested with pea aphids until they reached the 3rd instar, at which point they were used in the experiments. Hatching larvae of the seven-spot ladybird were reared individually in Petri dishes with sufficient pea aphid supply until they reached the 4th instar, at which point they were used in the experiments. All predators were starved 1.5 h (laboratory experiment) and 24 h (field experiment) before each experiment, respectively.

General experimental setup

For the behavioral assay "The Observer XT" Version 10.5 (Noldus Information Technology, Netherlands) was run on a laptop PC. Predator behavior was classified as follows: SEARCH—walking or foraging on the plant, STOP—resting or cleaning on the plant without moving, ENCOUNTER—the predator comes in physical contact with the aphid prey, and WALK OFF—the predator leaves the plant.

Each predator was observed until it encountered the aphid, until it left the plant, or until the maximum time was reached (15 min, hence 900 s, for experiment 1; 25 min, hence 1,500 s, for experiment 2), whatever came first. Longer observation times were chosen for in experiment 2 to account for abiotic factors, such as wind, and hence the second application of alarm pheromone (see below).

The following target, i.e., response, variables were used for analysis: (1) experimental outcome, the frequency of replicates in which the predator either found the prey, left the plant before the end of the experiment (900/1,500 s) or stayed on the plant for the whole time of the experiment—hence three possible outcomes; (2) foraging success, the frequency of replicates in which the predator found the aphid on the plant; (3) patch residence time, the duration of an replicate in seconds before the observation ended. In cases where the predator found the aphid prey, we refer to the patch residence time also as time until prey encounter. In case the predator left the plant without encountering the aphid and before the experimental time had ended we refer to the patch residence time also as time until the predator left the plant. If the predator stayed on the plant for the full 15/25 min without finding the aphid or leaving the plant a patch residence time of 900/1,500 s was noted as patch residence time. (4) Predator search time, the duration in seconds a predator was in motion, hence walking or searching for potential prey, not resting during a replicate; (5) Predator rest time, the duration in seconds a predator was motionless on the plant, not walking during a replicate.

Experiment 1—predator search behavior
in the laboratory

Experiment 1 tested the influence of different EBF concentrations (EBF treatment), as evaporated from a filter paper, on the foraging behavior of ladybird predators under laboratory conditions. One aphid was glued by its hind legs close to the leaf edge of the 2nd leaf pair (as seen from the base) of three- to four-week-old *Vicia faba* plants (three to five leaf pairs) and 1.5 cm away from the leaf stem using glue (UHU Alleskleber 45015 (all purpose adhesive), UHU GmbH & Co. KG, Germany) 1.5 h before a predator was introduced to the plant. Although pea aphids are known to feed on the underside of the leaf, close to the leaf petiole at (c.f. Keiser et al. 2013), due to an enhanced visibility aphids were fixed to the upper side of the leaf, but close the petiole in the experiments. While the glue mainly consists of methyl acetate, and also ethanol and acetone, the delay time before the start of the experiment allowed for evaporation of the solvents and hence solidification of the adhesive to an inert, physiologically indifferent and neutral drop. After they have been glued to the plant, at the start of each replicate, the aphids were all alive, looked healthy (no signs that the glue had an influence on the physiological appearance) and predominantly continued feeding (i.e., the aphid's stylet pierced the plant tissue).

Each plant with an aphid was subjected to one of three levels of the EBF treatment: (1) control (hexane), (2) low EBF amount (50 ng), and (3) high EBF amount (1,000 ng). Treatment three was established to check for possible overdose effects. Two plants, each within their flowerpot and with a fixed aphid, were placed in a no-choice

experiment, on a lab table at a distance of ∼50 cm to each other to prevent any confounding effects from EBF application or aphid presence in the neighboring treatment. A piece of filter paper (0.5×1 cm), attached to a wooden stick, was placed close (∼1–2 cm) to the aphid without touching the plant. Alarm pheromone solutions were created in hexane (Carl Roth Germany, 99.8 %) at an EBF concentration of 12.5 and 250 μg mL^{-1} (Bedoukian Research Inc., USA). For each treatment, 4.0 μL of the respective solution, hence 50 and 1,000 ng EBF, respectively, was applied with a syringe to the filter paper at the start of each replicate. So, EBF was released by the filter paper during the experiment, as demonstrated by Joachim and Weisser (2013). After EBF application, one predator was carefully set free from a Petri dish (5 cm in diameter) at the base of the plant stem. The behavioral monitoring started at the same time as the predator release.

Two replicates of the same treatment were observed simultaneously, to increase the sample size. Each treatment was replicated at least 16 times, resulting in $N = 54$ total replicates.

Experiment 2—predator search behavior in the field

Experiment 2 was set up similar to experiment 1, with some modifications. In this experiment we used two different predator species, lacewings and ladybirds (predator treatment). Because the experiment was carried out under field conditions, some parameters were changed to account for the particular abiotic factors, including wind. One aphid was glued by its hind legs using glue to the base of a leaf of the uppermost leaf-pair of two-week-old *Vicia faba* plants (three leaf pairs), as mentioned above. After they have been glued to the plant, at the start of each replicate, the aphids were all alive, looked healthy (no signs that the glue had an influence on the physiological appearance) and predominantly continued feeding (i.e., the aphid's stylet pierced the plant tissue).

Each plant with an aphid was subjected to one of three levels of the EBF treatment: (1) control (methanol), (2) low EBF amount (2×50 ng EBF), and (3) high EBF amount (2×500 ng). Treatment three was again established to check for possible overdose effects. Further, in contrast to experiment 1, EBF application was done twice to counteract EBF drift and fast EBF evaporation due to a slight but permanent breeze. Thus, in experiment 2, there were three EBF treatments × two predator treatments, i.e., six treatment combinations in a no-choice experiment. Methanol was used in contrast to experiment 1 since preliminary experiments showed that hexane might influence the behavior of additional aphid enemies, such as ants. As experiment 2 was conducted in the field with the possibility of attracting additional predators, a different solvent was chosen to exclude potential hexane effects on other predators.

Six plants, each within their flowerpot and with a fixed aphid, were placed in an experimental field site in Freising, Germany (fallow grassland, geographic coordinates: latitude, longitude: 48.405191, 11.6910). Plants were placed on the ground at a distance of ∼40 cm to each other in an open semicircle.

Thus, to be able to observe them all at the same time, the plants were arranged slightly closer to each other as in experiment 1. Preliminary behavioral observations revealed that this distance seems to be still adequate to prevent any confounding effects from EBF application or aphid presence in the neighboring treatment since EBF is only applied in small amounts, in the presence of wind, and is believed to degrade fast, thus having an impact only at a local scale (Kourtchev et al. 2009). Further, when predators left the plant, observations showed that their search was not instantly directed to plants with (higher) EBF concentration. A piece of filter paper (0.5×1 cm), attached to a wooden stick, was placed near to the aphid in the distance of 1–2 cm without touching the plant. Alarm pheromone solutions were created in methanol (Carl Roth Germany, 99.8 %) at an EBF concentration of 100 and 1,000 μg mL^{-1}, respectively. For each treatment 0.5 μL of the respective solution was applied with a syringe to the filter paper at the start of each replicate and again after 10 min. So, EBF was released by the filter paper during the experiment, as demonstrated by Joachim and Weisser (2013). Subsequently the predators, each kept individually in a Petri dish (5 cm in diameter) were carefully set free at the base of the plant stems. The behavioral monitoring started at the same time as the predator release. All six treatment combinations were observed simultaneously and the block was replicated 36 times, resulting in $N = 216$ total replicates.

Statistical analysis

Data were analyzed using the R software 3.0.1 (www.r-project.org). All data are presented as mean ± standard error (SE).

The count data for frequencies of occurrences, i.e., the variables of experimental outcome and foraging success, were analyzed following Crawley (2007): after fitting saturated generalized linear models (GLM) with a Poisson distribution to account for count data, the interaction of interest was removed and the updated model was compared with the saturated model using analysis of variance (ANOVA) with a Chi-squared test. The interaction of interest was either the influence of the treatment or the influence of the predator species on the experimental outcome or foraging success. For example, in experiment 2, the interaction between predator species, EBF concentration

and foraging success was tested first by removing it from the saturated model, i.e., it was tested if foraging success of the predator depended on the interaction between predator and EBF concentration. In the same way we proceeded to remove further terms where necessary.

Patch residence time, time until prey encounter, time until the predator left the plant, the predator search time, and predator rest time were compared using GLMs with model simplification, i.e., stepwise backward selection of independent variables to obtain the minimal adequate model. Due to the time-bound nature of the data, a Gamma distribution with an 'inverse' link function was used.

Results

Experiment 1—predator search behavior in the laboratory

When only predator foraging success was considered (i.e., aphid found/not found), there was no effect of the EBF

treatment on the frequencies an aphid was found on the plant or not (GLM: $\chi^2 = 2.48$, df = 2, $P = 0.290$).

When all experimental outcomes were considered, i.e., the frequencies of whether a predator found the prey, left the plant before the end of the replicate time or searched unsuccessfully for the whole 900 s of the experiment, there was also no effect of the EBF treatment on the outcome (GLM: $\chi^2 = 3.12$, df = 4, $P = 0.539$, Fig. 1a).

Patch residence time of ladybirds was 612.6 ± 41.6 s and not affected by the EBF treatment (GLM: $F_{2,51} = 0.45$, $P = 0.642$). In trials where the predator found the prey, the time until prey encounter was 415.9 ± 65.7 s and also not affected by the presence of EBF (GLM: $F_{2,13} = 0.09$, $P = 0.913$, Fig. 1b). For replicates where the predator did not encounter the prey item and left the plant before 15 min had elapsed, the time until the predator left the plant was 335.1 ± 49.0 s, also not affected by the EBF treatment ($F_{2,12} = 1.68$, $P = 0.240$, Fig. 1c).

Ladybird search time was 297.6 ± 26.9 s and not affected by the EBF treatment (GLM: $F_{2,51} = 0.92$,
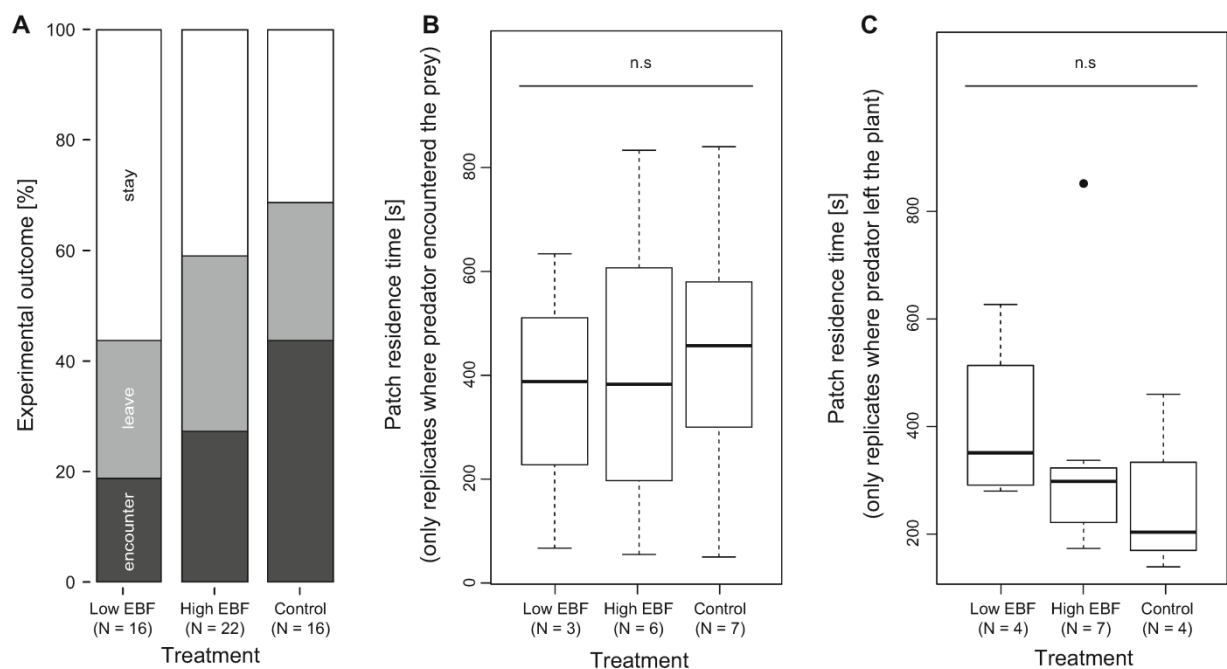
Fig. 1 Behavior of ladybird predators as a function of the presence of aphid alarm pheromone in experiment 1 (laboratory experiment) in which a ladybird larva was placed on plants where an aphid was fixed. EBF treatments: low = 50 ng, high EBF = 1,000 ng, control = 0 ng, $N = 54$. **a** Outcome of the experiment. *Full bars*—successful search (encounter), i.e., the predator found the aphid on the plant. *Gray bars*—the predator left the plant before the experimental run time of 900 s had elapsed without having encountered the aphid (leave). *Open bars*: the predator stayed on the plant for the entire experimental period of 900 s but did not encounter the aphid (stay).

b Patch residence time of replicates in which the ladybird predator encountered the aphid (time until prey encounter, $N = 16$), (**c**) patch residence time of the predator for replicates in which the ladybird predator left the plant before the maximum time (900 s) had elapsed, without finding the aphid (time until the predator left the plant, $N = 15$). *Box plots* show the median value (*solid line*), the 25 and 75th percentile; the *error bars* below and above the *box* indicate the 10 and 90th percentile, respectively. *Black dots* indicate outliers. *ns* not significant

$P = 0.405$). Predator rest time was 314.3 ± 37.3 s and also not influenced by the EBF treatment (GLM: $F_{2,51} = 0.03$, $P = 0.973$).

Experiment 2—predator search behavior in the field

Overall, the outcome of the field experiment was similar to the laboratory experiment.

Under field conditions, the aphid prey was also rarely found by the predator. In only 37 out of 216 replicates (17.13 %), the prey was encountered by the predator, half as often as under laboratory conditions. This foraging success, i.e., whether an aphid was found on the plant or not, did not depend on the interaction between predator species and EBF treatment (GLM: $\chi^2 = 1.58$, df = 2, $P = 0.453$). The main effects, predator treatment (GLM: $\chi^2 = 2.67$, df = 3, $P = 0.102$) and EBF treatment (GLM: $\chi^2 = 0.45$, df = 4, $P = 0.797$), were also not significant.

When all experimental outcomes were considered, i.e., the frequencies of whether a predator found the prey, left the plant before the 1,500 s had elapsed, or stayed on the plant for the entire time, there was a difference between the predator species (GLM: $\chi^2 = 42.71$, df = 6, $P < 0.001$, Fig. 2): lacewings stayed on the plant more often for the entire time period while ladybirds left the plant more often before the experimental time elapsed. This was independent of the EBF treatment (main effect EBF: GLM: $\chi^2 = 2.39$, df = 8, $P = 0.665$, interaction predator treatment $\times$ EBF treatment GLM: $\chi^2 = 1.29$, df = 4, $P = 0.864$).

Patch residence times of ladybirds and lacewings were 644.9 ± 50.7 s and $1,108.4 \pm 49.5$ s, respectively, and differed between the predator species (GLM: $F_{1,212} = 34.88$, $P > 0.001$), but were not affected by the EBF treatment (GLM: $F_{2,213} = 0.13$, $P = 0.882$). The interaction between EBF and predator treatment was also not significant (GLM: $F_{2,210} = 0.19$, $P = 0.828$).

In replicates where the predator found the aphid, the time until prey encounter did not differ between predator species (GLM: $F_{1,33} = 1.54$, $P = 0.224$). There was, however, a slight effect of the EBF treatment on the time until prey encounter, but in the opposite direction as expected (GLM: $F_{2,34} = 4.30$, $P = 0.023$, Fig. 3a): aphids were found after 549.6 ± 91.5 s in the control treatment, 615.7 ± 152.3 s at low EBF concentrations, and 986.5 ± 113.9 s at high EBF concentrations, i.e., the predators searched longer until the aphid was found when more EBF was present in the headspace of the plant. The interaction between predator treatment and EBF treatment was also significant (GLM: $F_{2,31} = 4.30$, $P = 0.023$): lacewings encountered aphids after longer time periods than ladybirds in the presence of EBF, but not in the control.
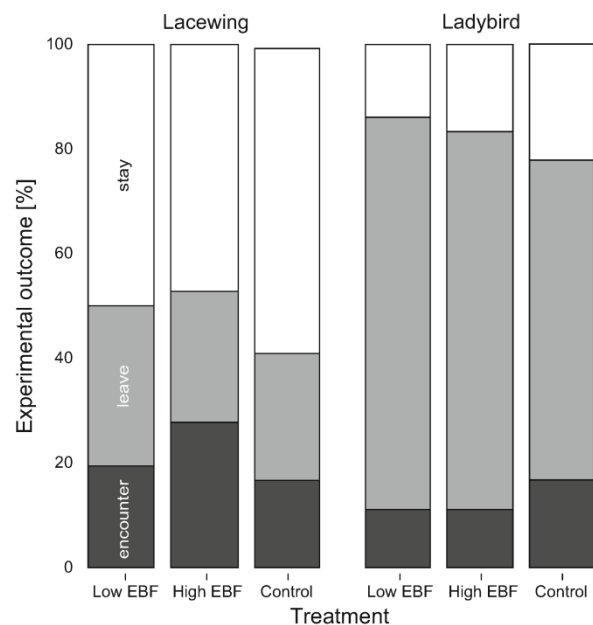
Fig. 2 Behavior of ladybird and lacewing predators as a function of the presence of aphid alarm pheromone in experiment 2 (field experiment) in which a predator larva was placed on plants where an aphid was fixed. EBF treatments: low = 2 × 50 ng = 100 ng, high = 2 × 500 ng = 1,000 ng, control = 2 × 0 ng. $N = 36$ (per predator and treatment). *Full bars*—successful search (encounter), i.e., the predator found the aphid on the plant. *Gray bars*—the predator left the plant before the experimental run time of 900 s had elapsed without having encountered the aphid (leave). *Open bars*: the predator stayed on the plant for the entire experimental period of 1,500 s but did not encounter the aphid (stay)

The time until a predator left the plant (replicates where the predator did not encounter the aphid and left the plant before 25 min had elapsed, Fig. 3b) did not differ between ladybirds (438.2 ± 41.8 s) and lacewings 579.9 ± 79.6 s (GLM: $F_{1,100} = 2.57$, $P = 0.112$). There was also no effect of EBF treatment (GLM: $F_{2,101} = 2.76$, $P = 0.068$) and the interaction was also not significant (GLM: $F_{2,98} = 0.11$, $P = 0.894$).

Predator search time was 343.5 ± 28.5 s and 303.9 ± 19.0 s for ladybirds and lacewings, respectively, and did not differ between predator species (GLM: $F_{1,214} = 1.42$, $P = 0.235$) or between EBF treatments (GLM: $F_{2,212} = 0.61$, $P = 0.543$). The interaction of EBF and predator treatments was also not significant (GLM: $F_{2,210} = 0.82$, $P = 0.442$). Predator rest time was much shorter for ladybirds (313.4 ± 42.8 s) than for lacewings (838.4 ± 44.6 s, GLM: $F_{1,212} = 43.11$, $P < 0.001$). There was no influence of the EBF treatment on predator rest time (GLM: $F_{2,213} = 0.09$, $P = 0.915$) and the interaction between EBF and predator treatment was also not significant (GLM: $F_{2,210} = 0.495$, $P = 0.611$).

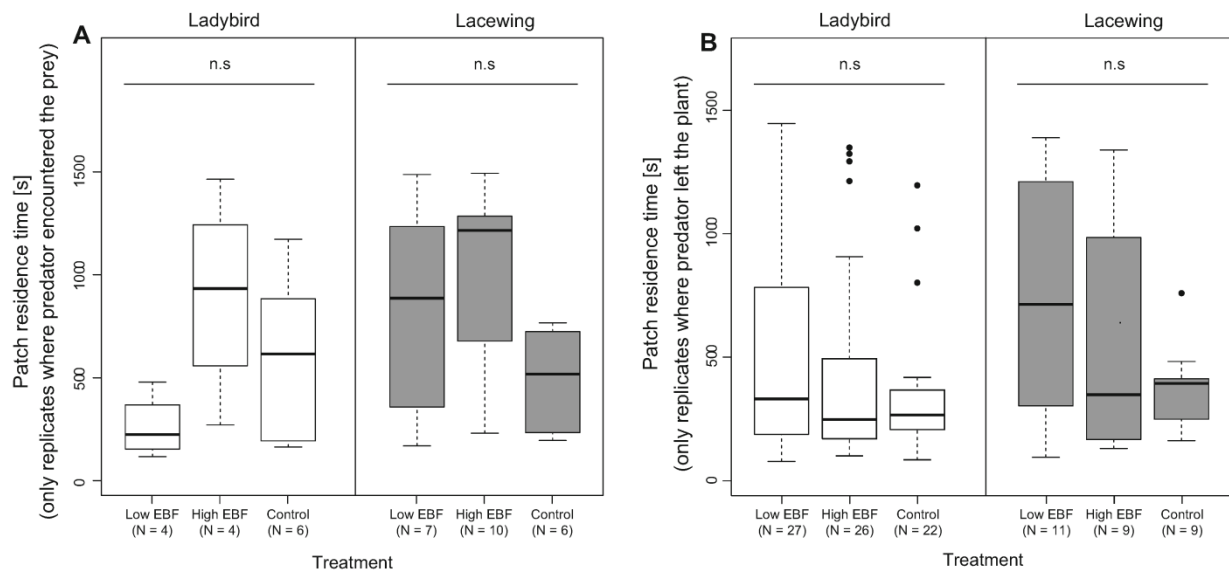The aphid alarm pheromone (*E*)-β-farnesene does not act as a cue

Fig. 3 Behavior of ladybird and lacewing predators as a function of the presence of aphid alarm pheromone in experiment 2 (field experiment) in which a predator larva was placed on plants where an aphid was fixed. EBF treatments: low = 2 × 50 ng = 100 ng, high = 2 × 500 ng = 1,000 ng, control = 2 × 0 ng. **a** Patch residence time of replicates in which the ladybird and lacewing predator encountered the aphid (time until prey encounter, $N = 37$), (**b**) patch residence time of the predator for replicates in which the ladybird and lacewing predator left the plant before the maximum time (1,500 s) had elapsed, without finding the aphid (time until the predator left the plant, $N = 104$). *Box plots* show the median value (*solid line*), the 25 and 75th percentile; the *error bars* below and above the *box* indicate the 10 and 90th percentile, respectively. *Black dots* indicate outliers. *ns* not significant

Discussion

Our study suggests that lacewings and ladybirds do not use the aphid alarm pheromone (*E*)-β-farnesene (EBF) as an environmental foraging cue to increase their foraging efficiency on the plant; in particular, it does not serve as either an arrestant or an attractant stimulus (Fellows et al. 2005; Purandare and Tenhumberg 2012). In both our laboratory and our field experiment, none of the different aspects of predator foraging behavior, in particular patch residence time and the chance of finding the aphid, was affected by the presence of EBF, either when predators were exposed to natural levels of EBF emission, or when they were exposed to exaggerated high amounts of EBF in the headspace.

In the process of prey localization by predators, EBF could play two distinct roles after the predator has arrived on a plant that may host aphid prey: first, it may disclose the presence of aphids to the predator. Consequently predators should search more intensively on the plant, given that the chances of encountering prey are higher than on a plant where no EBF is perceived. EBF would thus act as an arrestant stimulus and one would expect that patch residence times and ultimately also the fraction of replicates where the fixed aphid is found are higher than in the non-EBF treatments, which was not the case. This is consistent with findings of Nakamuta (1991) who showed for

ladybirds that EBF presence had no influence on area-concentrated search behavior, which is typically displayed by coccinellids after having consumed an aphid prey.

The second potential role of EBF for a foraging predator on a plant could be that of a short-range attractant, i.e., it could serve as a guiding signal for the predators. Consistent with a large number of studies that have shown that lacewing and ladybird larvae do not show directed search towards aphids on a plant (e.g., Bänsch 1964; Clark and Messina 1998; Minoretti and Weisser 2000), our study also found no evidence that the presence of EBF changed the efficiency of ladybird or lacewing search. As EBF in our study was placed close to or behind the aphid (from the position of the predator), both the probability of finding the aphid and also the time needed to find it should have been shorter in the presence of EBF, which was not the case.

There are at least two reasons why it may not be adaptive for aphid predators to use EBF as a stimulus for within-plant foraging behavior. First, the presence of EBF in the headspace of an aphid colony indicates a predation event which implies that the aphid colony is disturbed, i.e., that aphids may be walking, may have dropped off the plant or are otherwise scattered over the plant (e.g., Kislow and Edwards 1972; Wohlers 1981; Minoretti and Weisser 2000). Thus, particularly when the aphid colony was initially small, there may in fact be no aphids available for consumption. Second, the presence of EBF in the

headspace indicates the presence of a competitor or even intraguild predator on the plant. Thus, foraging success may in fact be very low on the plant and there is an additional mortality risk for the foraging predator. In addition to these disadvantages, there may also be physiological constraints: EBF is only emitted in very low quantities and it is not amplified by the colony (Verheggen et al. 2008; Hatano et al. 2008a; Joachim et al. 2013), yet EBF degenerates when in contact to air (Kourtchev et al. 2009). However, very little is known about how EBF disperses within the plant and how far it can be detected, not only by the predator but also by conspecifics. More information is needed on the movement of EBF molecules when emitted by an attacked aphid, and the rate of decay with distance. For EBF to be a short-range attractant, a gradient needs to build up.

The only significant effect of EBF presence was when unnaturally high amounts (1,000 ng) of EBF were offered in the field experiment. Interestingly, this led to longer but not shorter times until the aphid was found, compared to the control, in replicates where the foraging was successful. It is, however, unclear why this effect is observed. It has, however, been observed that unnaturally high concentrations of a semiochemical can lead to unusual behaviors in the receiver, which are not displayed, when natural amounts of that semiochemical are present, as e.g., in males of different moth species after perceiving different ratios of their sex pheromone (e.g., Roelofs 1978).

In contrast to alarm pheromones, which trigger prey dispersal, other chemical signals, such as products of prey feeding or prey body odor, may better disclose the presence of prey on a plant, and are potentially more suitable as arrestant or short-range attractant cues. For example, honeydew that is released by aphids when feeding is such a cue and is indeed known to acts as a contact kairomone in ladybirds (Carter and Dixon 1984; Buitenhuis et al. 2004; Ide et al. 2007). Ladybird larvae show a difference in foraging behavior only after they have been in close (physical) contact with honeydew. A number of other aphid enemies also induce intensive area-restricted foraging behavior or increase their rate of oviposition upon direct contact with honeydew or, for syrphid flies (Diptera—Syrphidae), after perceiving honeydew volatiles (Budenberg 1990; Budenberg and Powell 1992).

To summarize, our study provides no evidence for a role of aphid alarm pheromone in the foraging behavior of predators on the host plant. While we present evidence that (E)-β-farnesene is not utilized as an arrestant or short-range attractant cue for two important aphid predators, a more detailed knowledge of its role as a kairomone is desirable for a better understanding of the costs associated with chemical alarm signaling.

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Discussion

Host plants provide enemy free space

Assortative mating on native host plants is an important mechanism that helps to maintain the different host races of the pea aphid complex (Caillaud & Via 2000; Peccoud *et al.* 2009a), but hybridization may occur if aphids from different host races mate on the universal host *V. faba*. As shown in chapter I, in the absence of natural enemies most of the pea aphid clones of the Trifolium, Pisum and Medicago races tested performed better on the universal host *V. faba* (aphid clones T and M2) or equally well on both universal and native hosts (aphid clones P1 and M2). Only the clone P2 of the Pisum race showed an increased performance on its native host *P. sativum*. This suggests that pea aphids, at least from the investigated aphid races, may settle on *V. faba* in the field. And, in fact Peccoud et al 2009 could find aphids from other host races on *V. faba* and closely related plant species. In such cases, mixed colonies might occur and if they persist until aphids reproduce sexually, aphids from different host races may mate with each other and produce hybrids. In all but three host races, hybrids can be found in the field (Peccoud et al 2009). Since hybrids usually show a reduced performance on both host plants of their parents, there is strong post zygotic selection against hybrids in the field (Müller 1971; Via, Bouck & Skillman 2000; Peccoud *et al.* 2014). But hybrids perform well on the universal host *V. faba* and some wild vetches (V*icia. sativa*, *Vicia hirsuta*) (Peccoud *et al.* 2014), and these universal hosts may not only promote hybridization by hosting different pea aphid host races at the same time, but they may further offer an environment where hybrids can survive. If this gene flow via hybrids would be too high, it would counteract any further differentiation of the pea aphid host races and may even reduce existing differences between host races. Since this is not the case, mechanisms besides assortative mating and hybrid unfitness on parental native hosts must exist which prevent high hybridization rates. One important factor might be the existence of enemy-free space.

For all three host races I found an enemy free space on their native host plants (chapter I). This was either shown directly in cases where natural enemies preferred to oviposit on the universal host plant compared to the native host plant, or indirectly in cases where the number of surviving aphids or aphid colonies was higher on native host plants than on the universal host. Predators strongly affected aphid populations that have a high reproduction rate (e.g. Trifolum race on *V. faba* and Pisum race of both hosts), while there was less predation pressure on populations with a low reproduction rate (e.g. Medicago race on both plants), resulting in a decreased aphid colony survival on the universal hosts for the Trifolium and

Medicago race. This would reduce the probability that mixed colonies of different host races survive on *V. faba* until sexual reproduction and may prevent mating among different races. Thus, the enemy free space I found on the native host plants may be crucial for structuring the pea aphid complex.

Different groups of natural enemies contributed to the enemy free space on native host plants

It is very likely that different groups of natural enemies contributed to the enemy-free space on the native host plants. We found for both experimental years a strong hoverfly oviposition preference for the universal host *V. faba* compared to native hosts of the respective host races when the plants were infested with Trifolium and Medicago race aphids (chapter I). Hoverfly larvae are assumed to be among the most important aphid predators in central Europe (Chambers and Adams 1986, Tenhumberg and Poehling 1995) that can consume up to 33 mg aphids per day (approx. 22 4st instar *A. pisum*) (Hindayany et al. 2001). Thus the higher number of hoverfly eggs on *V. faba* would have resulted in a much higher predation pressure on aphids feeding on *V. faba*. However, even in times when hoverfly larvae were rare or absent, differences in aphid survival or number could be detected. Thus other natural enemies must have caused these differences. In 2013 we observed significantly more surviving aphids on native host plants for Pisum race aphids, while in 2014 both the Pisum and the Medicago race aphids had a higher survival on their respective native host plants. Thus the native host plants seem to provide an enemy-free space against mobile aphid predators which is more variable and temporally less stable than the enemy free space against hoverflies. This high variability was probably due to changes in the mobile predator community, which can consist of ladybirds, spiders, ground beetles and ants (Sunderland *et al.* 1987; Mohamed, Lester & Holtzer 2000; Ximenez-Embun, Zaviezo & Grez 2014) or due to changes in the activity of these predators. Since these species are very mobile, actual predation events were rarely seen. However, aphid parasitization did not differ between aphid colonies on native and universal host plants.

Although I cannot attribute the reduced aphid survival on the universal host plant *V. faba* in the absence of hoverflies to a certain aphid natural enemy species, and found the effect of natural enemies other than hoverflies was not stable over time, I never observed that aphids on the universal host plants survived better than on their respective native host plant. This suggests that the activities of different natural enemy groups complemented each other and led at least temporarily to an overall enemy-free space on the native host plants for all three host races. However, only the enemy-free space against hoverfly larvae that was found on *T. pratense* and *M. sativa* resulted in higher aphid colony survival on native than on universal hosts.

Cues important for aphid detection by natural enemies

To understand the mechanisms that generate enemy-free space against different groups of natural enemies and to estimate the impact of different natural enemies on aphid population dynamics, it is important to identify the cues natural enemies use for aphid detection under field conditions.

Aphid induced plant volatiles

Volatiles are often considered to be important for the long rage attraction of aphid natural enemies to prey/host patches (e.g. Hatano *et al.* 2008; Almohamad, Verheggen & Haubruge 2009). Under laboratory conditions, attraction by aphid-induced plant volatiles could be documented for the ladybird *Coccinella septempunctata* (Ninkovic, Al Abassi & Pettersson 2001; Zhu *et al.* 2005) and for several aphid parasitoid species (e.g. Hatano *et al.* 2008; Takemoto, Kainoh & Takabayashi 2011), and it was shown that the hoverfly *E. balteatus* reacted to several common plant volatiles (Verheggen *et al.* 2008). Thus, I would have expected that plants infested with aphids for six days to be more attractive for parasitoids and predators than plants infested for just one day, but both plant types were equally attractive in field experiments (chapter I). Several reasons may explain these unexpected findings. First, if changes in the volatile blend caused by aphid infestation occur in less than 24 h, there would be no difference between one- and six-day infested plants. Additionally, the situation in the field is much more complex than in laboratory experiments. The complex background odor in the field may have decreased the detectability of minor changes in volatile blends. Moreover, different parasitoid and predator species may have reacted in a different ways to changes of the volatile blends, masking the responses of individual species in field experiments. Since plant derived volatiles are considered to function as long range cues (Hatano *et al.* 2008), aphid induced volatiles may have guided natural enemies to my experimental plots, where they searched for aphids on all available plants, independent of their volatile profile. However, I did not measure volatile emission during my experiments. Thus, aphid infestation may not have caused changes in the volatile blends of the plants that I used in my field experiments. Guerrieri *et al.* (1999) showed that more than 20 aphids per plant are needed to increase the attractiveness of the volatile blend for aphid parasitoids. Thus, the 15 aphids which were used in my experiments to induce the plants may not have been enough to induce changes in the volatile blends. It was further shown that aphid infestation does not induce increased volatile emission of *V. faba* and *M. sativa*, and that pea aphid infestation actually suppressed the volatile induction caused by chewing herbivores in *V. faba* (Pareja *et al.* 2009; Schwartzberg, Boroczky & Tumlinson 2011). Thus, aphid infestation does not induce volatile emission in

two of the four host plants used in my experiments, and so the other host plants, which belong to the same family, may react in a similar way. However, the aphid parasitoid *A. ervi* is able to use minor changes in the ratio of certain plant volatiles to distinguish between infested and uninfested *V. faba,* but is not attracted by aphid-infested *M. sativa* under laboratory conditions. This was probably because all parasitoids used in this study were reared on *V. faba* (Pareja *et al.* 2009). Thus, the lack of increased parasitoid attraction to the six day- infested plants may be due to a lack of experience of natural enemies with these host plants.

My results show that the attraction of aphid natural enemies by volatile blends altered by aphid infestation, as previously shown in laboratory experiments, does not necessarily mean that these cues are also used by foraging aphid enemies in the field. In my system, aphid-induced plant volatiles apparently play at most only a minor role for detecting pea aphids under field conditions.

Aphid alarm pheromone (E)-β-farnesene

Aphid odor is another cue that may be used by natural enemies of aphids while searching for prey, but undisturbed aphids usually do not emit any measurable amounts of volatiles (Micha & Wyss 1996; Francis, Lognay & Haubruge 2004; Kunert *et al.* 2005). The aphid alarm pheromone (*E*)-β-farnesene (EBF) is emitted during a natural enemy attack and is often considered as an important cue for aphid detection by natural enemies (Micha & Wyss 1996; Al Abassi *et al.* 2000; Hemptinne *et al.* 2000; Acar *et al.* 2001; Francis, Lognay & Haubruge 2004; Francis *et al.* 2005; Almohamad *et al.* 2007; Verheggen *et al.* 2007; Hatano *et al.* 2008; Verheggen *et al.* 2008). However, most of the evidence for the kairomone function of EBF comes from studies that use unnaturally high amounts of EBF, while studies that use EBF in amounts naturally emitted by an attacked aphid have never showed any effect (chapter IV, Fig. 1). Thus, EBF does not seem to work as a long distance cue that attracts natural enemies towards a patch containing aphids and there are several reasons that make EBF a less suitable cue for long range attraction (chapter IV). However, there is some evidence that predatory larvae may use it to detect aphids from a short distance. According to Stubbs (1980) 4th instar larvae of the ladybird *C. septempunctata* are able to smell crushed aphid prey at a distance of 0.69 mm. This ability to smell aphid prey over short distances did, however, not increase the ability of 3rd and 4th instar larvae of *C. septempunctata* to find a glued aphid on a plant when 50, 500 or 1000 ng of EBF were applied, and neither attraction nor arrestance by EBF could be observed (Joachim, Vosteen & Weisser 2014 (chapter V)). This result might be due to the searching behavior of ladybird larvae. Searching larvae usually move up and down the stem of a plant and search leaves when they are encountered (Banks 1957). Thus, whether or not larvae will cross the EBF odor

plume probably depends on the plant architecture and chance proximity to aphids under attack. Since we do not know how close the larvae got to the odor source, and because too few larvae may have come close enough to the aphid to detect the EBF plume, any short-range attraction towards EBF may have been overlooked in our experiment (Joachim, Vosteen & Weisser 2014 (chapter V)). The situation might be different for 1st instar larvae. Since the capture of the first aphid prey is critical for survival of 1st instar ladybird larvae (Dixon 1959), these insects might have adapted to detect the small amounts of EBF emitted by aphids when they are attacked by their siblings (Hemptinne *et al.* 2000). Even a short detection distance of a few millimeters as calculated for *C. septempunctata* by Stubbs (1980), will increase the perceptive field of the larvae drastically. Since female ladybirds lay their eggs in clusters close to aphid colonies, EBF might in this case be a useful kairomone for short range detection of aphids (chapter V, Fig. 2). Because first instar larvae consume only a few aphids each, several young larvae could prey on the same aphid colony without competing with each other, and in the case that aphids are rare the cannibalistic larvae may prey on each other. This is supported by an olfactometer experiment, where 1st instar larvae of *Adalia bipunctata* were attracted and arrested by aphids that were preyed on by 10 1st instar ladybird larvae (Hemptinne *et al.* 2000).

Visual cues

It was shown that several aphid natural enemies use optical cues like color and shape to recognize aphids on infested plants and to evaluate the quality of their prey (e.g. Stubbs 1980; Kan & Sasakawa 1986; Battaglia *et al.* 1995; Battaglia *et al.* 2000). Ladybirds and parasitoids for example distinguish between aphids of different color (Michaud & Mackauer 1994; Harmon, Losey & Ives 1998; Libbrecht, Gwynn & Fellowes 2007), and ladybirds detect aphids and dummy prey at a distance of 7 to 10 mm (Stubbs 1980; Nakamuta 1984). Kan and Sasakawa (1986) showed that optical cues like the presence of winged aphids are used by hoverflies to evaluate the quality of an aphid colony. In my experiments, I showed that aphid presence makes non-induced plants attractive for hoverfly oviposition, indicating that visual cues are used by hoverflies to identify aphid infested plants. As reported by Scholz and Poehling (2000), I often observed *E. balteatus* hovering close to the experimental plant during my field experiments, probably looking for aphids.

Honeydew

Visual and volatile cues are not the only ones which come along with aphid presence. Since aphids excrete honeydew this could also be used as an indication of aphid presence. In several laboratory experiments, it was shown that honeydew is an important arrestment cue and oviposition stimulus for parasitoids and mobile aphid predators like ladybirds and hoverflies (e.g. Carter & Dixon 1984; Budenberg 1990; Budenberg & Powell 1992; Budenberg, Powell & Clark 1992; Grasswitz & Paine 1993b; Han & Chen 2002; Leroy *et al.* 2012a), but field studies that evaluate the response of freely-moving aphid enemies are lacking. In cage experiments, mobile insects are restricted to small searching arenas with a limited amount of choices. They do not have the possibility to leave the arena to search for more suitable oviposition or feeding sites, and may accept plants which they would have never accepted under field conditions. Host-deprived hoverflies, for example, even start to oviposit on green plastic lids if they were kept in cages without an aphid infested plant (personal observation). Thus, feeding and oviposition cues that where identified under laboratory conditions should also be tested under field conditions. I showed that honeydew applied on uninfested plants elicited oviposition behavior of *E. balteatus* hoverflies under field conditions and the egg number increased with an increasing amount of honeydew (chapter II). This validated the results from several laboratory studies (Budenberg & Powell 1992; Bargen, Saudhof & Poehling 1998; Scholz & Poehling 2000; Leroy *et al.* 2014). Foraging hoverflies usually land several times on the plant, testing the plant surface with their ovipositors before they decide to lay an egg or to leave the plant. Interestingly, *E. balteatus* was observed to hover close by a plant, to land and to test the plant surface independently of the presence of aphids or honeydew (personal observation). This indicates that hoverflies search all potential host plants for honeydew and visual aphid cues before deciding to lay eggs or to leave, and that volatile cues are of minor importance at most in detecting aphid infested plants. Thus, to identify the mechanism that generates the enemy-free space against hoverflies, we focused mainly on honeydew and visual cues.

Cues used by migrating hoverfly larvae

Hoverfly oviposition choice has a strong influences on aphid population survival (chapter I and II) and on hoverfly larval development (e.g. Almohamad, Verheggen & Haubruge 2009), because hoverfly larvae will first consume the aphids on the plant where they hatched (chapter III). Single aphid colonies, however, often do not provide enough food for larvae to complete their development, they have to leave the plant in search of other aphid colonies (chapter I, II & III). Because the migration capacity of hoverfly larvae was often ignored in the past (e.g. Sadeghi & Gilbert 2000a; Almohamad, Verheggen & Haubruge 2009) little is

known about the cues they may use to find aphid colonies. In principle they could use all the available cues discussed above such as volatiles like the aphid alarm pheromone EBF, visual cues, or honeydew (Leroy *et al.* 2014).

I showed in a laboratory experiment that the probability that a migrating larva will visit a certain plant increased with increasing aphid number and that larvae accumulate in large aphid colonies in a field experiment. This is in line with other studies that showed that aphid predators accumulate in large aphid colonies (e.g. Turchin & Kareiva 1989; Osawa 2000), which was probably due to high amounts of honeydew that accumulated in the vicinity of large colonies. Since the search activity of hoverfly larvae increases in areas where honeydew is present (Leroy *et al.* 2014), and thus the probability that a larvae will climb an aphid infested plant increases with increasing honeydew amount and correlatively aphid colony size. If migrating hoverfly larvae are more likely to find plants where aphid performance is high and colonies are big, this contributes to the enemy-free space for aphids on plants where aphid performance is low.

After encountering an aphid, hoverfly larvae increase their turning rates and search the surrounding area more thoroughly (Chandler 1969). This increases the probability of encountering more aphids in big colonies and could explain the observed arrestance of hoverfly larvae in big colonies. If there are other predators on the plant, hoverfly larvae may use the aphid alarm pheromone EBF as short distance cue to detect aphids, as was shown by Hemptinne *et al.* (2000) for 1st instar ladybird larvae. Similarly, *E. balteatus* larvae were attracted by crushed aphids and by aphids that probably were disturbed by the handling procedure and may have emitted EBF (Francis et al., 2005b). In another field experiment hoverfly larvae were observed foraging on plants were EBF was applied, but not on control plants (Joachim & Weisser 2015).

Aphid volatiles and honeydew most likely can only be perceived over a few centimeters and *E. balteatus* larvae are not attracted by volatiles from pea aphid - *V. faba* complexes (Bargen, Saudhof & Poehling 1998). This suggests that hoverfly larvae do not perceive aphid colonies over large distances and that they randomly search the vegetation canopy until they encounter honeydew or aphids. In light of such random searching, it may take migrating larvae several days to find another aphid colony. Even though the ability of hoverfly larvae to survive starvation periods increases with larval age (Rojo, Hopper & Marcos-Garcia 1996), it would be highly beneficial for them to use alternative food sources. Thus hoverfly larvae should accept any non-toxic aphid species as prey that increase their survival (chapter III). Thus, the detectability

of an aphid colony (which depends on colony size and honeydew production) would determine in most cases if a migrating larvae will prey on the colony.

Mechanisms that generate the enemy-free space

In the first chapter, I showed that hoverfly oviposition preferences create an enemy-free space on the native hosts *T. pratense* and *M. sativa*. These hoverfly oviposition preferences were shown in chapter II to reflect the aphid reproductive potential on different host plants. Hoverflies are able to distinguish between aphid colonies of different sizes. Under laboratory conditions hoverfly larvae prefer bigger colonies (Scholz & Poehling 2000; Almohamad *et al.* 2008), indicating that an increase in the intensity of oviposition cues like honeydew and aphid presence leads to an increase in egg number. During field experiments with naturally infested plants, however, hoverflies prefer young aphid colonies that are very small over older and bigger colonies (Kan 1988a; Kan 1988b; Kan 1989). Thus, during my experiments with reproducing aphids, hoverflies may have been directly evaluating aphid reproduction potential via aphid colony size (chapter I, Fig. 4). In these field experiments, the decline of aphid numbers was strongest on plants that had high aphid numbers (resulting from high reproduction rates during aphid rearing in the laboratory). The high number of hoverfly larvae hatching from the many eggs laid on plants where aphids had a high reproductive rate led to an increased rate of predation and to the strong decline in aphid numbers. Predation by other mobile predators may have also played a role, such as ladybirds which are known to accumulate in large aphid colonies (e.g. Turchin & Kareiva 1989; Osawa 2000), probably because big colonies are easier to detect from a distance or because of the high density of aphid related cues that arrest predators in big colonies. Besides the colony detection rate, the foraging efficiency of aphid predators within a colony also increases with the increasing aphid density in larger colonies, because of an increased chance of prey encounter (Yasuda & Ishikawa 1999).

In most of my experiments (chapter I & II) however, the aphid population size was kept stable and hoverflies could not have directly used the number of aphid offspring on a plant to assess aphid reproductive potential. Hence, hoverflies must have used other cues to evaluate reproductive potential indirectly. Aphid size differs between host plant species and the different pea aphid races grew to the largest size on plants were they had a high performance and reproduction rate (Schwarzkopf *et al.* 2013, chapter I). Since optical cues are very important oviposition cues for hoverflies (Kan & Sasakawa 1986, chapter II), and since aphid size is a good predictor for their reproduction rate (e. g. Dixon & Wratten 1971; Dixon 1976; Kempton, Lowe & Bintcliffe 1980; Traicevski & Ward 2002), differences in aphid size

may be used as an cue to identify aphid colonies with high reproduction rates. Similarly, hoverflies are known to use visual cues from winged aphids to avoid older aphid colonies that are likely to collapse soon (Kan & Sasakawa 1986).

I also showed that aphid honeydew production reflects aphid performance and reproduction potential quite well (Schwarzkopf *et al.* 2013, chapter I & II), and for *E. balteatus*, the dominant hoverfly in our study, aphid honeydew was an important oviposition cue under laboratory and field conditions (Budenberg & Powell 1992; Bargen, Saudhof & Poehling 1998; Scholz & Poehling 2000; Leroy *et al.* 2014, chapter II). Thus, hoverflies may use either qualitative differences in honeydew produced by different aphid races feeding on certain plants, or quantitative differences in the honeydew production to distinguish between aphids with different reproduction potential. Qualitative differences in the honeydew composition from aphids infesting the different host plants did not explain the general oviposition patter, but an increased number of honeydew droplets on *V. faba* enhanced the hoverfly egg number that was laid on these plants. Thus, quantitative differences in aphid honeydew production most likely contribute to the enemy-free space on the native host plants *T. pratense* and *M. sativa*.

I identified differences in honeydew production and aphid body size as important factors that explain the observed hoverfly oviposition pattern. However, other factors may also contribute to this pattern. It was for example shown that movement of hoverfly larvae is hampered by trichomes (Verheggen *et al.* 2009). Thus, hoverflies may lay fewer eggs on *T. pratense* and *M. sativa*, because these plants contain trichomes. Moreover, as I have shown in chapter III, hoverflies may leave the plant where they hatched and migrate to another aphid-infested plant. Hoverfly larvae only leave a plant after most aphids had been consumed and they are more likely to find big aphid colonies and do not show a preference for native or universal host plants infested with the same aphid number during migration (chapter III). Thus hoverfly migration would not counteract the enemy-free spaces created by hoverfly oviposition preferences.

Since honeydew was shown to be an important arrestment cue for parasitoids and mobile aphid predators like ladybirds in laboratory experiments (e.g. Carter & Dixon 1984; Budenberg 1990; Budenberg, Powell & Clark 1992; Grasswitz & Paine 1993b; Han & Chen 2002; Leroy *et al.* 2012a), one may also expect a correlation between direct predation and parasitation rate, honeydew production and reproduction potential of aphids on the different host plants. However, such correlations could not be found. Different parasitoid and mobile predator species most likely react differently towards honeydew and other aphid cues. This might have masked the effect that honeydew may have on individual enemy species.

Additionally, the effect of honeydew may be less pronounced under field condition than under laboratory conditions.

The way that natural enemies detect aphids – by using visual cues like aphid size or colony size, and honeydew – has consequences for the predator pressure that acts on an aphid colony. Aphids that colonize plants where their performance is low escape from their predators because of their small size, the decreased honeydew production and the small population size resulting from a decreased reproduction rate. The simultaneous action of these factors leads to a reduction of performance differences between aphids on different host plants. This phenomenon could be observed during the field experiment where Medicago race aphids exhibited a low performance on *M. sativa* without natural enemies, but suffered least from hoverfly oviposition on their native host, and consequently had the highest colony survival compared to other aphid – plant combinations (chapter I, Fig. 4).

Consequences of enemy-free space for the colonization of novel host plants

Aphids that colonize novel host plants escape their predators due to their low performance
The fact that aphids find enemy-free space on plants were they have a low performance might have strong consequences for the colonization of novel host plants. It can be hypothesized that aphids that settle on novel host plants on which they have a low performance will most likely not suffer strong overall fitness disadvantages, because the low performance will be alleviated by the formation of an enemy-free space.

To test this hypothesis, we reared the Trifolium and Pisum race aphids both on *T. pratense* and *P. sativum*, so that both races were reared on their native hosts and on a less suitable plant which would represent a novel host plant. As expected, aphids on the less suitable plants were much smaller than aphids reared on their native hosts, indicating that their performance was lower (chapter II, Fig. 5a). Consequently, hoverflies laid fewer eggs in the field on less suitable plants vs. native host plants, when they were infested with the same aphid race (chapter II, Fig. 5b, 6a). This resulted in fewer less suitable plants than native host plants with hoverfly larvae (chapter II, Fig. 5d, 6b). The higher number of hoverfly larvae reduced the number of aphids on the native host plants so that the aphid numbers on native and less suitable plants were similarly low at the end of the experiment (chapter II, Fig. 5c). Despite a much higher predation rate of aphids on their native hosts than on the less suitable plant, colony survival was not consistently changed. All three possible scenarios were detected during the two field experiments

conducted: higher as well as a lower colony survival on the native host, and also similar colony survival on both plant species. These results indicate that under certain circumstances aphids can find an enemy-free space on novel host plants, and the lower predation pressure could stabilize aphid populations on novel hosts and facilitate early steps of host race formation. The fact that enemy-free space due to reduced hoverfly oviposition did not always lead to improved aphid colony survival on novel host plants can be attributed to various reasons. Colony survival might depend not only on the mean number of hoverfly eggs laid on a certain plant, but also on the general predation pressure and the community of natural enemies at a certain time. For instance, intraguild interactions for instance might reduce the effectiveness of natural enemies (e.g. Hågvar 1972; Ferguson & Stiling 1996; Frago & Godfray 2013). How effective natural enemies are in terms of aphid eradication or reduction also depends on the actual reproduction of aphids on a certain plant and host plant properties like architecture or the presence of trichomes (e.g. Clark & Messina 1998; Simmons & Gurr 2004; Verheggen *et al.* 2009; Reynolds & Cuddington 2012).

Hypothetic host race formation process in aphids

The escape from predators due to a reduced performance on novel host plants may also facilitate host race formation of other aphid species. Host plant switches seemed to be important in aphid evolution (Peccoud *et al.* 2010; Jousselin *et al.* 2013), and host plant-associated differentiations are known or suspected for at least 16 aphid species or genera (Müller 1985; Guldemond 1990; Sunnucks *et al.* 1997a; Sunnucks *et al.* 1997b; Shufran *et al.* 2000; Miller, Kift & Tatchell 2005; Lozier, Roderick & Mills 2007; Margaritopoulos *et al.* 2007; Carletto *et al.* 2009; Peccoud *et al.* 2009a; Mezghani-Khemakhem *et al.* 2012). This indicates that host race formation is a quite common process in aphids, and the influence of natural enemies might be a contributing factor.

The host race formation of aphids could start with winged females that leave host plants, apterous females that leave a crowded plant, or juveniles that drop off their host plants to escape from predators (Braendle & Weisser 2001). Despite their high host fidelity (Via 1999; Caillaud & Via 2000; Ferrari *et al.* 2006), only a very low percentage of these aphids will find a suitable host plant (Ward *et al.* 1998). Most of the aphids will die because they landed on unsuitable plants, but some might land on plants were they can establish feeding and survive, although with a much lower performance (Via 1999; Peccoud *et al.* 2009a). Reduced performance on less suitable plants can be caused by reduced feeding (Schwarzkopf *et al.* 2013) due to the lack of feeding stimulants (Caillaud & Via 2000; Del Campo, Via & Caillaud 2003), or the presence of toxins (Goggin 2007), or the reduced ability to manipulate a plant to establish feeding

(Walling 2008). However, despite reduced performance, small colonies may become established on less suitable novel hosts.

While a high percentage of aphid colonies are eradicated by predators, the colonies on a novel host may escape this fat due to their small size and low honeydew production (chapter II). If the colony persists long enough, beneficial mutations may occur that increase the performance. Due to asexual reproduction during the summer, these beneficial mutations are likely to persist and to increase in frequency if they increase the reproduction rate of the individual carrying the mutation. Since aphids mate in autumn on their host plant, beneficial mutations might be transferred to individuals in the following season.

Since only a few winged females will find the 'correct' host plant during dispersal (Ward *et al.* 1998), to promote host race formation colonies with beneficial mutations for feeding on a novel host must produce enough winged females that some of them will find another specimen of the novel host plant during dispersal. A high abundance of the novel host would facilitate this process. After winged females landed on the novel host, they need to establish feeding. However, it was shown that aphids may reject a plant after brief probing of the plant tissue with their stylets due to the absence of feeding stimuli (Caillaud & Via 2000; Del Campo, Via & Caillaud 2003). Thus, migrating females may reject plants on which they would able to establish feeding because they do not recognize them. However, it has been shown that the rearing plant influences preference and acceptance of different host plants by *A. pisum* and *Aphis fabae* (Gorur, Lomonaco & Mackenzie 2007; McLean, Ferrari & Godfray 2009). Thus winged females that disperse from a novel host may be more likely to accept this novel host species than aphids dispersing from the original host. Evolution of the ability to recognize substances from the novel host and having these stimulate feeding would then be a further step towards host race formation. In pea aphids, the genes for host acceptance and performance are closely linked. This may either be caused by close physical linkage on the corresponding chromosome or by pleiotrophy – acceptance and performance on a host plant are influenced by the same genes (Hawthorne & Via 2001), in which case reduced performance on a novel host plant may indeed be due to reduced acceptance of the plant because of the absence of feeding stimuli (Caillaud & Via 2000; Del Campo, Via & Caillaud 2003).

After recognition of the novel host has evolved, the aphids may migrate between different individuals of the novel host while assortative mating on the novel host enables further genetic differentiation of a new host race (e.g. Via 1999; Peccoud & Simon 2010). Selection against hybrids as found in the pea aphid complex would reduce the gene flow between the host races (Via, Bouck & Skillman 2000).

Population genetic studies of the pea aphid complex have shown that the different host races show a high degree of differentiation in non-coding regions of the genome close to genes that encode for salivary proteins and odorant and gustatory receptors (Jaquiéry *et al.* 2012; Nouhaud *et al.* 2014), indicating that the evolution of the ability to manipulate a novel host to establish feeding and to recognize the novel host plant are indeed crucial steps in the host race formation process.

It may seem hard to imagine that aphid colonies on novel host plants persist long enough that adaptation to the novel host plant can evolve, even with the reduced natural enemy pressure I found on novel host plants (chapter II). However, even if this process is quite unlikely, repeated colonization of less-suitable plants may lead to repeated establishment and extinction of colonies on less suitable plants, while few colonies exist long enough so that adaptations can evolve. Additionally, some ecological scenarios could increase the likelihood of aphid host race formation. A population decline of the original host plant may increase the probability that a less suitable plant will be colonized. An increase in abundance and geographic range of a less suitable plant species would also increase the probability that migrants from a colony on a novel host, which already developed some adaptations to this host, but so far no recognition, will again leave offspring on this novel host plant species. This hypothesis is supported by the fact that the burst of diversification in the pea aphid complex, which was dated back to the post-Pleistocene warming, may have been initiated by the beginning of the domestication of legumes and their anthropogenic range expansion (Peccoud et al. 2009a).

Conclusion & Outlook

Enemy foraging for aphids is a highly complex process that can affect aphid population dynamics in different ways. Different cues that indicate aphid presence are used at different special scales and by different natural enemy species. However, a general pattern was observed in this study: aphid predation is generally higher in larger than in smaller aphid colonies, probably because large colonies are easier to detect and repeated encounters with aphids or aphid cues arrest predators in large colonies.

Predation by hoverfly larvae strongly influences aphid colony survival since larvae stay on the plant until most aphids are consumed. Therefore hoverfly oviposition behavior is crucial for the fate of an aphid colony. Aphid size and the amount of honeydew on a plant influence the number of hoverfly eggs that are laid on a plant, leading to a low number of hoverfly eggs laid on plants where aphids have a low performance.

Due to the preference of aphid predators for aphids that produce a lot of honeydew and form big colonies, the low performance of aphids that colonize a novel (less suitable) host plant creates the enemy-free space that reduces the performance disadvantages these aphids have to face on a less suitable host. This may help to establish a stable colony on the novel host and increase the possibility that aphids would adapt to it. In addition to facilitating aphid host race formation, enemy-free space probably also helps to maintain the established pea aphid host races by reducing the gene flow via the universal host *V. faba*.

This thesis provides information which leads to a better understanding of the interaction between pea aphids and their natural enemies. It sheds light on the role of pea aphid host plants in providing an enemy free space for the aphids and discusses the possible consequences for the maintenance of the different pea aphid host races. However, it also raises new questions:

- The existence of host plant specific biotypes is known or suspected also for other aphid species. It would be interesting whether in these systems certain host plants also provide an enemy free space and whether it would support host race or biotype maintenance.

- It is now known that hoverfly larvae are able to migrate to aphid infested plants in the field. In order to understand under which conditions aphid colonies are visible or hidden to hoverfly larvae, the cues relevant to locate aphid colonies need to be investigated.

- Even if the common aphid alarm pheromone (E)-β-farnesene is suspected to work as a host finding cue for aphid natural enemies, a closer examination reveals that most studies were done

with unnatural high concentrations. Thus to finally answer the question if aphid emitted EBF works as a host finding kairomone it is important to quantify the release rate of EBF in experiments and use concentration in the headspace that is similar to the ones released during an aphid attack.

- Some of the short range cues used by natural enemies to find aphids are known, but we lack information about long range cues used to identify the habitat where aphids are likely to occur. In order to estimate the foraging efficiency of natural enemies it is important to know over which distances certain cues are attractive and how other environmental cues interfere the host finding process of natural enemies.

Summary

Herbivorous insects are the most diverse group of multicellular organisms inhabiting our world. One process that may have contributed to this enormous biodiversity is ecological speciation via continuous host plant switches, but the process of host associated differentiation is not well understood. Differentiation of herbivorous insects on a novel host plant requires reduced performance or the new host would simply be included in the host range of the original species. But, this makes the establishment of a stable population on the novel host plant less likely. Thus, mechanisms should exist to provide benefits on the novel host plant and stabilize herbivore populations that colonize novel hosts. Novel host plants have been previously shown to provide enemy-free space for herbivores which may stabilize the population that colonizes the novel host so that host-associated differentiation can occur. However, little is known about the impact of different natural enemy groups on the overall enemy-free space and about the mechanisms that generate an enemy-free space on novel host plants.

The pea aphid complex (*Acyrthosiphon pisum* HARRIS) is an important model to study ecological speciation via host plant switches and consist of at least 11 genetically distinct host races. These are native to specific host plants of the Legume family, but can all develop on the universal host plant *Vicia faba.* Assortative mating on native host plants is an important mechanism that helps to maintain the different host races of the pea aphid complex, but hybridization may occur if aphids from different host races mate on the universal host *V. faba*. I could show that in the absence of natural enemies, pea aphids of the Trifolium, Pisum and Medicago race have in most cases similar or better performance on the universal host *V. faba* compared to the respective native host plants. This suggests that pea aphids at least from the investigated aphid races may settle on *V. faba* in the field, and mating within mixed colonies may lead to hybrid formation. If this gene flow via hybrids would be too high, it would counteract any further differentiation of the pea aphid host races and may even reduce existing differences between host races. Since this is not the case, mechanisms must exist which prevent high hybridization rates. Natural enemies may prefer certain plants for prey searching or oviposition and the non-preferred plants may provide an enemy-free space for aphids and help to maintain the different pea aphid host races. I used three pea aphid host races adapted to *Trifolium pratense*, *Pisum sativum* and *Medicago sativa*, respectively, to test this assumption.

I showed that hoverflies (*Episyrphus balteatus*, De Geer) prefer to oviposit on *V. faba* and *P. sativum* and that feeding by hoverfly larvae suppresses aphid population growth on these host plants. Thus, the native host plants *M. sativa* and *T. pratense* provide an enemy-free space for pea aphids. Mobile predators prefer *V. faba* infested with Pisum race aphids over *P. sativum*, while parasitoid pressure was not

influenced by the aphid's host plant. This indicates that the enemy-free spaces from different groups of natural enemies complement each other such that all three native host plants provide an enemy-free space for pea aphids.

The oviposition pattern of hoverflies reflects aphid performance on native and universal host plants, and differences in honeydew production between the different host races was identified as the mechanism that generates the enemy-free space against hoverflies on *T. pratense* and *M. sativa*. I further showed that pea aphids find an enemy-free space on less suitable plants, most likely due to reduced honeydew production and a reduced aphid size compared to more suitable hosts. This enemy-free space reduces the performance disadvantages of aphids colonizing a novel host and probably facilitates the aphid host race formation processes.

Hoverfly larvae were shown to be quite mobile and to leave the plants on which they hatched in order to search for other aphid colonies after they consumed most of the aphids. I showed that migrating hoverfly larvae show no preference for native or universal host plants and that they are more likely to climb plants with large than small aphid colonies, probably due to the higher number of honeydew droplets near the large aphid colonies. In a field experiment, migrating hoverfly larvae accumulated on *V. faba* and *P. sativum* plants, most likely due to the high aphid number on these plants.

The mechanism that generates an enemy-free space against mobile predators others than hoverflies, however, could not be identified. The extrafloral nectaries of *V. faba* did not influence aphid survival and number of surviving aphids did not differ between plants infested by aphids for one or six days, indicating that aphid-induced plant volatiles and honeydew amount are not involved in creating the enemy-free space from predators other than hoverflies. However, the cues that can be used by different natural enemies to detect aphids are highly diverse and their foraging behavior is not well understood especially at higher spatial scales. For example, it is often stated that mobile predators and parasitoids use the aphid alarm pheromone (*E*)-β-farnesene (EBF) to find their aphid prey, even though this is mainly based on studies that use much higher EBF amounts than those typically emitted by attacked aphids. Thus, to understand which mechanisms lead to the enemy-free space against mobile adult predators, it is important to identify the dominant predator species preying on pea aphid populations inhabiting different host plants and to study their foraging behavior in more detail.

When aphid population development was followed in the field, the overall predation rate was generally higher in large than small aphid colonies, probably because large colonies are easier to detect and repeated encounters with aphids or aphid cues arrested predators in large colonies. Thus, the low

performance of aphids that colonize a novel (less suitable) host plant creates the enemy-free space that reduces the performance disadvantages these aphids suffer on a less suitable host. This may help to establish a stable colony on a less suitable host and would increase the possibility that aphids would adapt to this novel host. Thus, enemy-free space may facilitate aphid host race formation and probably helps to maintain the different pea aphids host races by reducing the gene flow via the universal host *V. faba*.

Zusammenfassung

Herbivore Insekten sind eine enorm diverse Artengruppe und tragen erheblich zur globalen Artenvielfalt bei. Es wird vermutet, dass ein Großteil dieser Diversität durch wiederholten Wechsel der Wirtspflanze und anschließender ökologischer Artbildung entstanden ist. Wie dieser Prozess genau abläuft, ist jedoch noch weitgehend unerforscht. Damit eine wirtspflanzenabhängige Differenzierung stattfinden kann, muss die Performance der herbivoren Insekten auf den neuen Wirtspflanzen niedriger sein als auf den ursprünglichen Wirtspflanzen. Das führt jedoch dazu, dass die Etablierung stabiler Population auf neuen Wirtspflanzen relativ unwahrscheinlich ist. Daher müssen Mechanismen existieren, die den Herbivoren Vorteile auf den neuen Wirtspflanzen verschaffen und dadurch die Populationen auf den neuen Wirtspflanzen stabilisieren. Es wurde bereits mehrfach gezeigt, dass Herbivore auf neuen Wirtspflanzen einen ‚Feind-freien Raum' finden, welcher die Populationen stabilisiert und eine wirtspflanzenabhängige Differenzierung ermöglicht. Es ist bisher aber noch weitgehend unbekannt, wie die verschiedenen Gruppen der natürlichen Feine den gesamten ‚Feind-freien Raum' beeinflussen und welche Mechanismen zur Bildung eines ‚Feind-freien Raumes' führen.

Der Erbsenblattlauskomplex (*Acyrthosiphon pisum* HARRIS) ist ein wichtiges Modelsystem für die Untersuchung der ökologischen Artbildung durch Wirtspflanzenwechsel. Er besteht aus mindestens 11 genetisch differenzierten Wirtsrassen, wovon jede an eine oder einige wenige Wirtspflanzen aus der Familie der Leguminosen angepasst sind. Alle Wirtsrassen können sich jedoch auf dem universellen Wirt *Vicia faba* entwickeln. Weil die Paarung der Blattläuse auf ihren Wirtspflanzen stattfindet, kommen Paarungen deutlich häufiger innerhalb derselben Wirtsrasse als zwischen den verschiedenen Rassen zustande, was zur Aufrechterhaltung der unterschiedlichen Wirtsrassen beiträgt. Trotzdem kann es zu Hybridisierungen kommen, da sich Individuen verschiedener Rassen auf *V. faba* paaren können. In der Abwesenheit von Feinden haben die Blattläuse der Trifolium-, Pisum- und Medicago-Rasse in den meisten Fällen auf dem universellen Wirt *V. faba* eine ähnliche oder bessere Performance als auf ihren spezifischen Wirten. Dies lässt annehmen, dass sich zumindest die Blattläuse der untersuchten Wirtsrassen im Freiland auf *V. faba* ansiedeln können und dass Paarungen innerhalb von gemischten Kolonien zur Hybridisierung zwischen diesen Rassen führen. Wäre der Genfluss durch Hybridisierung auf *V. faba* zu stark, dann würde dies einer weiteren Differenzierung der Wirtsrassen entgegenwirken und bestehende Unterschiede zwischen den Rassen würden reduziert werden. Da dies nicht der Fall ist, muss es Mechanismen geben, die einer zu starken Hybridisierung entgegenwirken. Wenn natürliche Feinde bestimmte Pflanzen für die Beutesuche oder Eiablage bevorzugen, dann könnten die weniger präferierten Pflanzen einen ‚Feind-freien Raum' bieten, welcher zur Aufrechterhaltung der Wirtsrassen beitragen

könnte. Zur Überprüfen dieser Annahmen habe ich Feldexperimente mit drei Erbsenblattlausrassen, die an *Trifolium pratense*, *Pisum sativum* und *Medicago sativa* angepasst sind, durchgeführt.

Ich konnte zeigen, dass Schwebfliegen (*Episyrphus balteatus*, De Geer) ihre Eier bevorzugt auf *V. faba* und *P. sativum* ablegen, und dass der Fraß der Schwebfliegenlarven das Wachstum der Blattlauskolonien auf diesen Wirtspflanzen unterdrückt. Die spezifischen Wirtspflanzen *M. sativa* und *T. pratense* stellen daher einen ‚Feind-freien Raum' dar. Mobile Prädatoren bevorzugen mit der Pisum-Rasse infizierte *V. faba*-Pflanzen gegenüber *P. sativum*-Pflanzen. Die Parasitoideneiablage wurde jedoch nicht von den Wirtspflanzen beeinflusst. Dies zeigt, dass sich die ‚Feind-freien Räume' gegen verschiedene Feinde ergänzen und dass alle drei spezifischen Wirtspflanzen einen ‚Feind-freien Raum' darstellen.

Das Eiablagemuster der Schwebfliegen spiegelt die Blattlausperformance auf den verschiedenen Wirtspflanzen wider. Unterschiede in der Honigtauproduktion der verschiedenen Blattlausrassen auf den verschiedenen Pflanzenarten führten zu dem ‚Feind-freien Raum' auf *T. pratense* und *M. sativa*. Ich konnte außerdem zeigen, dass Erbsenblattläuse einen ‚Feind-freien Raum' auf jeweils suboptimalen Pflanzen finden, vermutlich weil sie sich dort kleiner sind und weniger Honigtau produzieren als auf Pflanzen, an die sie angepasst sind. Dieser ‚Feind-freien Raum' reduziert die Performance-Nachteile von Blattläusen, wenn diese eine neue Wirtspflanze kolonisieren und unterstützt so vermutlich den Rassenbildungsprozess.

Es hat sich gezeigt, dass Schwebfliegenlarven relativ mobil sind. Sie verlassen die Pflanzen, auf denen sie geschlüpft sind, nachdem sie dort einen Großteil der Blattläuse gefressen haben, und suchen nach anderen Blattlauskolonien. Migrierende Schwebfiegenlarven haben jedoch keine Präferenzen für bestimmte Blattlaus-Pflanzen Kombinationen, sondern orientieren sich an der Größe der Blattlauskolonien. Sie lassen sich mit einer höheren Wahrscheinlichkeit auf Pflanzen mit großen Blattlauskolonien finden. So konnte in einem Feldexperiment gezeigt werden, dass migrierende Schwebfliegenlarven auf blattlausbefallenen *V. faba* und *P. sativum* akkumulieren, beides Pflanzen, auf denen Blattläuse sich stark reproduzieren und auf denen die Blattlauskolonien groß waren. Die Akkumulation der Schwebfliegenlarven lag vermutlich an dem verstärkten Vorhandensein von Honigtautropfen in der Umgebung von großen Blattlauskolonien.

Der Mechanismus, der zu der Bildung des ‚Feind-freien Raumes' gegen andere mobile Prädatoren führt, konnte nicht identifiziert werden. Die Zugänglichkeit der extrafloralen Nektarien von *V. faba* hatte keinen Einfluss auf das Blattlausüberleben, und die Anzahl der überlebenden Blattläuse unterschied sich nicht zwischen Pflanzen, die einen oder sechs Tage infiziert waren. Dies zeigt, dass blattlausinduzierte

Pflanzenduftstoffe und Honigtau höchstwahrscheinlich keinen Einfluss auf die Existenz des 'Feind-freien Raumes' gegen andere Prädatoren als Schwebfliegen haben. Blattlausfeinde sind in der Lage, sehr unterschiedliche Hinweise auf Blattlauspräsenz zu nutzen, und gerade auf größeren räumlichen Skalen weiß man wenig über ihr Suchverhalten. Es wird zum Beispiel oft angenommen, dass mobile Prädatoren und Parasitoide das Blattlausalarmpheromone (E)-β-farnesene (EBF) nutzen, um Blattläuse zu finden. Dieses Ergebnis stützt sich jedoch hauptsächlich auf Studien, die deutlich größere EBF-Mengen einsetzen als von einer angegriffenen Blattlaus abgegeben werden. Um zu verstehen, welcher Mechanismus zu dem 'Feind-freien Raum' gegen mobile Prädatoren führt, ist es wichtig, die dominanten Prädatorenarten zu identifizieren, die die Blattlauskolonien auf den verschieden Wirtspflanzen attackieren und ihr Suchverhalten zu erforschen.

Untersuchungen der Blattlauskolonieentwicklungen im Freiland haben gezeigt, dass die Gesamtprädationsrate in großen Kolonien deutlich höher ist als in kleinen Kolonien. Dies ist vermutlich darauf zurück zu führen, dass große Kolonien einfacher zu finden sind und dass häufige Begegnungen von Prädatoren mit Blattläusen oder mit Hinweisen auf Blattlauspräsenz zu einem längeren Verweilen der Prädatoren in großen Blattlauskolonien führen. Daher bildet die niedrige Performance der Blattläuse, die neue (weniger geeignete) Wirtspflanzen kolonisieren, direkt den 'Feind-freien Raum', der die Performancenachteile der Blattläuse auf den neuen Pflanzen ausgleicht. Dies könnte die Etablierung stabiler Populationen auf weniger geeigneten Wirtspflanzen unterstützen und die Wahrscheinlichkeit erhöhen, dass sich Blattläuse an neue Wirtspflanzen anpassen. Die Wirtsrassenbildung von Blattläusen könnte so erleichtert werden. Dieser Mechanismus könnte außerdem dazu beitragen, die verschiedenen Wirtsrassen des Erbsenblattlauskomplexes zu erhalten, da so der Genfluss durch Hybridbildung auf dem universellen Wirt *V. faba* reduziert wird.

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Eigenständigkeitserklärung

Hiermit erkläre ich, entsprechend der zur Zeit gültigen Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena, dass ich die vorliegende Promotionsarbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind am Beginn der Arbeit ("Overview of the manuscripts") und am Ende jedes Manuskriptes angegeben. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen, noch haben Dritte geldwerte Leistungen für Arbeiten im Zusammenhang mit der vorliegenden Dissertation erhalten. Zu keinem früheren Zeitpunkt wurde die vorgelegte Dissertationsarbeit, eine in wesentlichen Teilen ähnliche Arbeit oder eine andere Arbeit an der Friedrich-Schiller-Universität Jena oder einer anderen Hochschule eingereicht.

Jena, den 25.06.15

Curriculum vitae

Ilka Vosteen, PhD-candidate
MPI for Chemical Ecology
Hans-Knöll-Str. 8
07749 Jena
Germany
03641/571314
ivosteen@ice.mpg.de

Date of Birth: September 14th, 1984

Education

since 01/2011	**PhD thesis** at the **MPI for Chemical Ecology**, Jena thesis: **The role of enemy free space for the maintenance of pea aphid races** supervisors: Dr Grit Kunert, Professor Jonathan Gershenzon
10/2004 - 07/2010	Studies of biology at the University of Bremen Major subject: marine biology, minor subjects: ecology and zoology **Diploma degree, grade: 1.1**
08/2009 – 03/2010	**Diploma thesis** at the Biologischen Anstalt Helgoland supervisor: Dr Markus Molis thesis: Feeding defense of macroalgae and chemical mediated tritrophic interactions
01/2009 – 06/2009	Semester abroad in Sweden at the University of Gothenburg
1997 – 2004	Gymnasium Sulingen **Abitur, grade: 1.6**

Committee work

10/2011 – 09/2013	Elected member of the "Rat der Graduierten Akademie"
05/2012 – 09/2013	PhD-committee that initiated the formation of a PhD-council at the FSU Jena
10/2012 – 09/2013	Organization committee of the "Visions in Science"-Conference, an interdisciplinary conference for junior scientists
10/2012 – 03/2015	PhD-representative of the Biochemistry Department at the MPI for Chemical Ecology
10/2013 – 09/2014	Elected member of the "DR.FSU" (PhD-council at the FSU Jena)

Teaching experience

07/2013 and 07/2014	Supervision of student groups within the field ecology course at the FSU Jena, including course preparation, lectures and supervision of field and laboratory work
summer 2013/2014	Supervision of internship students

Jena, den 24.06.2015

Overview of scientific publications

Publications

Joachim, C., Vosteen, I., Weisser, W. W. (2014). **The aphid alarm pheromone (*E*)-beta-farnesene does not act as a cue for predators searching on a plant.** Chemoecology.

Oral Presentations

<u>Vosteen I.</u> **Science Slam: NSA – Natural Spy Affairs**. Sommerfest der Graduiertenakademie, Friedrich-Schiller-Universität Jena, Jena, Germany, May 2015

<u>Vosteen I.</u> **Science Slam: NSA – Natural Spy Affairs**. Visions in Science, Max Planck Institute for Infection Biology, Berlin, Germany, Sep 2014

<u>Vosteen I.</u> **Role of hoverfly – plant species interaction in maintenance of pea aphid host races** ICE Symposium, MPI for Chemical Ecology, Jena, Germany, Sep 2014

<u>Vosteen I.</u> **Role of hoverfly – plant species interaction in maintenance of pea aphid host races**. 15th International Symposium on Insect-Plant Relationships, University of Neuchâtel, Nêuchatel, Switzerland, August 2014

<u>Vosteen I.</u> **Potential role of hoverfly – plant species interaction in sympatric speciation of the pea aphid complex**. 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, Feb 2014

<u>Vosteen I.</u> **Potential role of hoverfly-plant species interaction in sympatric speciation of the pea aphid complex.** 19th European Meeting of PhD students in Evolutionary Biology, University of Exeter, Falmouth, Great Britain, Sep 2013

<u>Vosteen I.</u> **Role of hoverfly - plant species interaction in maintenance of pea aphid host races.** XXIV. International Congress of Entomology - "New Era in Entomology", The Entomological Society of South Korea, Daegu, South Korea, August 2012

<u>Vosteen I.</u> **The role of enemy free space in sympatric speciation of the pea aphid *Acyrthosiphon pisum*.** Insect Chemical Ecology Course - ICE 11, Swedish University of Agriculture Science, Alnarp, Sweden, Jun 2011

Poster Presentations

Vosteen I., Gershenzon J., Kunert G. **Enemy-free space: Could hoverfly oviposition preference create niches of low predation pressure for pea aphid host races?** ICE Symposium, MPI for Chemical Ecology, Jena, Germany, Sep 2013

Vosteen I. **Potential role of hoverfly–plant species interaction in sympatric speciation of the pea aphid complex.** 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany, Apr 2013

Vosteen I., Gershenzon J., Kunert G. **Where to lay the eggs? - Influence of plant species on hoverfly reproduction.** Workshop "Multitrophic Interactions", Gesellschaft für Ökologie, University Göttingen, Göttingen, Germany, Mar 2012

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Supplementary

Chapter I: Enemy free space against multiple natural enemies

Tab. S1: Statistical values for the analysis of proportion of *V. faba*-plants with parasitized aphids in 2013: glmm with binomial error structure. Plants were aphid infested for one or six days and had either open or wax-covered extrafloral nectaries.

factor	χ^2	p-value
aphid race	4.212	0.122
extrafloral nectaries	0.451	0.502
infestation time	0.043	0.836
aphid race x extrafloral nectaries	1.143	0.565
infestation time x extrafloral nectaries	0.220	0.639
aphid race x infestation time	0.514	0.774

Tab. S2: Statistical values for the analysis of proportion of plants with parasitized aphids in 2013: glmm with binomial error structure. Trifolium, Pisum and Medicago race aphids had infested their native and universal host plants for one or six days.

factor	χ^2	p-value
aphid race - plant species combination	3.645	0.602
infestation time	0.025	0.875
aphid race - plant species combination x infestation time	2.615	0.759

Tab. S3: Statistical values for the analysis of aphid survival on *V. faba*-plants in 2013: glmm with negative binomial error structure. Plants were aphid infested for one or six days and had either open or wax-covered extrafloral nectaries.

factor	χ^2	p-value
aphid race	12.266	0.002
infestation time	3.700	0.054
extrafloral nectaries	0.021	0.886
infestation time x extrafloral nectaries	1.834	0.176
aphid race x infestation time	0.260	0.878
aphid race x extrafloral nectaries	0.982	0.612

Tab. S4: Statistical values for the analysis of aphid survival in 2013: glmm with negative binomial error structure. Trifolium, Pisum and Medicago race aphids had infested their native and universal host plants for one or six days.

| factor	χ^2	p-value
aphid race - plant species combination	54.729	< 0.001
infestation time	6.044	0.014
aphid race - plant species combination x infestation time	7.603	0.180

Tab. S5: Statistical values for the analysis of aphid survival in 2014: glmm with negative binomial error structure. Trifolium, Pisum and Medicago race aphids had infested their native and universal host plants for one or six days.

factor	χ^2	p-value
aphid race - plant species combination	33.538	< 0.001
infestation time	2.838	0.092
aphid race - plant species combination x infestation time	0.340	0.997
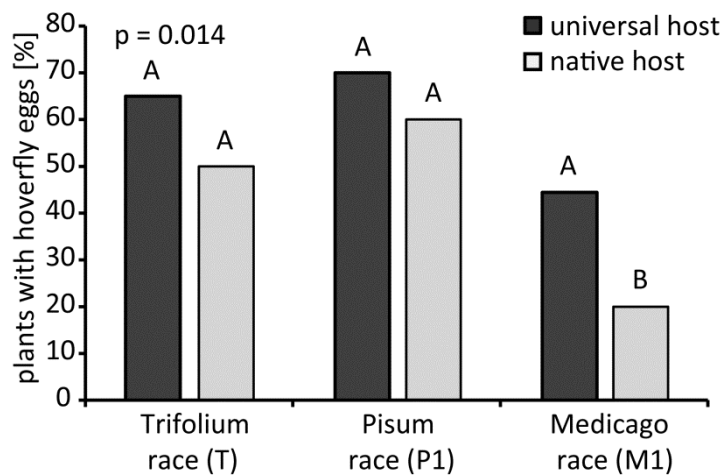
Fig. S1: Proportion of host plants infested with different pea aphid races that carried hoverfly eggs during a field experiments in July 2014. The native hosts are Trifolium race- *T. pratense*, Pisum race- *P. sativum*, and Medicago race- *M. sativa*, and the universal host is *V. faba*. Bars represent means ± standard error; different letters indicate significant differences (p ≤ 0.05).

Chapter II: Honeydew as oviposition cue for hoverflies

Tab. S1: Overview of statistical tests, error structure and data transformation.

experiment - variable	figure	test	error structure	transformation
quantitative honeydew production; clone T, P1, M1 - honeydew amount	2 e	ANOVA	normal	log
quantitative honeydew production; clone P2, M2 - honeydew amount	2 f	ANOVA	normal	-
oviposition cues experiment - number of hoverfly eggs	3	glmm	negative binomial	-
qualitative effects of honeydew - number of hoverfly eggs	4 a	glmm	poisson	-
quantitative effects of honeydew - number of hoverfly eggs	4 b	glmm	poisson	-
less suitable host plant experiment; July 2014 - aphid weight	5 a	glmm	normal	-
less suitable host plant experiment; July 2014 - presence of hoverfly eggs	5 c	glmm	binomial	-
less suitable host plant experiment; July 2014 - presence of hoverfly larvae	5 d	glmm	binomial	-
less suitable host plant experiment; July 2014 - surviving aphid colonies	5 e	glmm	binomial	-
less suitable host plant experiment; August 2014 - number of hoverfly eggs	6 a	glmm	poisson	-
less suitable host plant experiment; August 2014 - number of hoverfly larvae	6 b	glmm	poisson	-
less suitable host plant experiment; August 2014 - surviving aphid colonies	6 c	glmm	binomial	-

Tab. S2: Statistical values of the analysis of hoverfly egg number laid on *Vicia faba* and *Trifolium pratense* (factor: plant species), with and without aphids (factor: aphid presence), treated with and without honeydew and previous aphid infestation (factor: treatment): glmm with negative binomial error structure.

factor	χ^2	p-value
treatment	18.683	< 0.001
host plant species	8.0123	0.005
aphid presence	16.379	< 0.001
treatment x host plant species	5.098	0.078
treatment x aphid presence	12.787	0.002
host plant species x aphid presence	0.552	0.457
treatment x host plant species x aphid presence	0.995	0.6081

Tab. S3: Statistical values of the analysis of hoverfly egg number laid on native hosts and less-suitable plants (factor: plant suitability) infested with Trifolium and Pisum race aphids (factor: aphid race)in August 2014: glmm with poisson error structure.

factor	χ^2	p-value
plant suitability	9.366	0.002
aphid race	1.904	0.168
plant suitability x aphid race	7.371	0.007

Tab. S4: Statistical values of the analysis of number of hoverfly larvae on native hosts and less-suitable plants (factor: plant suitability) infested with Trifolium and Pisum race aphids (factor: aphid race) in August 2014: glmm with poisson error structure.

factor	χ^2	p-value
plant suitability	8.594	0.003
aphid race	2.702	0.100
plant suitability x aphid race	2.843	0.092

Tab. S5: Statistical values of the analysis of surviving aphid colonies on native hosts and less-suitable plants (factor: plant suitability) infested with Trifolium and Pisum race aphids (factor: aphid race) in August 2014: glmm with binomial error structure.

factor	χ^2	p-value
aphid race	0.069	0.792
plant suitability	0.618	0.432
plant suitability x aphid race	5.793	< 0.001

Chapter III: Searching behavior of hoverfly larvae

Tab. S1: Statistical values for the analysis of hoverfly larval number after 7 days in the field: glm with poisson error structure. Fixed effects: Trifolium, Pisum and Medicago race aphids on their native and universal host plants (aphid race-plant species combination), number of hoverfly eggs that were laid at the beginning of the experiment and aphid number after 7 days in the field.

factor	likelihood ratio	p-value
aphid race - plant species combination	119,339	< 0.001
aphid number	8,568	0,003
egg number	1,292	0,256
aphid race - plant species combination x aphid number	5,855	0,321
egg number x aphid race - plant species combination	3,102	0,541
egg number x aphid race - plant species combination x aphid number	7,340	0,119

Tab. S2: Statistical values for the analysis of aphid number after 7 days in the field: glm with negative binomial error structure. Fixed effects: Trifolium, Pisum and Medicago race aphids on their native and universal host plants (aphid race-plant species combination), number of hoverfly eggs that were laid at the beginning of the experiment and number of hoverfly larvae after 7 days in the field.

factor	likelihood ratio	p-value
aphid race - plant species combination	15,998	0,007
egg number	3,555	0,059
larval number	3,115	0,078
egg number x larvae number	0,017	0,897
egg number x aphid race - plant species combination	3,840	0,428
aphid race - plant species combination x larvae number	6,131	0,294
egg number x aphid race - plant species combination x larvae number	3,745	0,442