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## **Costs and Benefits of Alarm Communication in Aphids**

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## Table of Content

Summary .....	ii
Zusammenfassung.....	v
Introduction.....	1
Alarm Communication.....	2
Aphid-Predator Interactions.....	3
Ecology of Aphid Alarm Pheromone .....	4
Aims and Questions .....	9
General Materials and Methods .....	11
Plants and Animals .....	11
zNose <sup>TM</sup> Settings and Calibration .....	12
Experimental Setups and Procedures.....	16
Overview of Manuscripts .....	25
Discussion.....	30
Alarm pheromone Emission Dynamics.....	30
Kairomone Effect of EBF .....	39
Conclusion .....	52
List of References .....	54
Acknowledgments .....	66
Curriculum Vitae.....	67

List of Publications.....	69
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## Summary

In many prey organisms, elaborate alarm communication is used to reduce an imminent risk of predation. To study costs and benefits of such alarm signals it is mandatory to understand the emission dynamics and link alarm signaling to prey and predator behavior. This has repeatedly been done for alarm calls in mammals and birds by analyzing live vocal recordings and combine them with behavioral analysis. In insects, alarm communication is mainly chemical, but for chemical alarm signaling such analysis has so far been hindered by the availability of real-time analysis techniques.

The pea aphid, *Acyrthosiphon pisum* Harris, serves as the model organism in this thesis. As most aphids, the pea aphid secretes droplets from a pair of tube-shaped structures on their abdomen upon attack. In addition to having direct defensive function by gluing the predator's mouthparts together, these droplets can contain an alarm pheromone which only component is the sesquiterpene (*E*)- $\beta$ -farnesene (EBF). When perceived by colony conspecifics EBF triggers various escape behaviors such as withdrawal of the stylets, dropping off the host plant, kicking or simply walking away.

The aim of this thesis is to investigate the aphid alarm pheromone emission dynamics in more detail and to elucidate the role of aphid alarm signaling in predator-prey interaction in a combination of laboratory and field experiments. Moreover, the ecological costs of the alarm signal by functioning as a kairomone in the field is evaluated.

The first study investigates the EBF emission of single aphids under attack from lacewing or ladybird larvae, by measuring the EBF emission of pea aphids under attack, using a zNose™ - a handheld rapid gas chromatograph. The results indicate that aphid alarm signaling is affected by the predator species attacking. While all aphid bodies always contain EBF, many secreted droplets do not. Alarm signaling in insects can be variable, and both the attacker as well as the attacked may affect alarm signal variation.

While the first study focused on single aphid observations, the second study presents the first long-term monitoring (24 h) of EBF emission in aphid colonies with and without predation using real-time monitoring. EBF emission in colonies under attack is always clearly restricted to defined periods of time and no EBF emission is observed in the absence of predation. There is further no circadian rhythm in alarm pheromone emission and EBF emission patterns within an aphid colony are most likely driven by the feeding behavior of the predator.

The third study investigates the underlying mechanisms of alarm signal variation in aphid-predator interactions in more detail. Therefore real-time alarm signal emission dynamics after predation, measured by the zNose™, are compared with live predator-prey behavioral observations.

Results show that EBF alarm signaling almost exclusively occurs when an aphid is lethally attacked. Further, colony escape response is induced by increasing EBF stimuli rather than total pheromone amounts and stops due to EBF habituation.

Pheromones are intended to provide intraspecific information, but these signals are sometimes utilized by species other than the intended receiver. While the first part of this thesis focuses on the emission dynamics, the second part focuses on the costs of EBF by being used as a host or prey finding cue by aphid enemies. Thus, the fourth study observes the searching behavior of lacewing and ladybird larvae on a plant where an aphid was fixated in the presence and absence of EBF and under field and laboratory conditions. EBF has no effect on the predator's short-range searching behavior or the residence times nor does EBF increase the foraging success. Hence, EBF is not used as an arrestant cue for these aphid predators nor does it have a short-range attractant function.

The fifth study tries to answer the question if EBF is utilized as a kairomone under field conditions, since several aphid natural enemies are believed to use EBF as a host/prey finding cue. Observing predator dynamics under natural conditions using long-term camera monitoring and different concentrations of alarm pheromone suggests that aphid natural enemies do not use the aphid alarm pheromone (*E*)- $\beta$ -farnesene as a kairomone for host/prey localization in the field under natural conditions, but it has the potential to attract additional predators when applied in exaggerated concentrations that do not reflect natural emission amounts.

On the example of aphids, this thesis contributes to the understanding of alarm communication in insects. It reveals the complex nature of EBF emission dynamics and sheds more light on the costs and benefits associated with alarm signaling in aphids. The new knowledge gained from the studies, combining live behavioral observations with real-time headspace measurements, provides a promising approach to fathom the driving factors of EBF emission variation in future research.

## Zusammenfassung

Viele Beutetiere verwenden eine durchdachte Alarmkommunikation um die drohende Gefahr durch Prädation zu reduzieren. Um Kosten und Nutzen solcher Alarmsignale zu studieren, ist es zwingend erforderlich die Emissionsdynamik zu verstehen und die Alarmkommunikation mit dem Verhalten von Räuber und Beute zu verknüpfen. Dies wurde wiederholt für Alarmrufe bei Säugetieren und Vögeln durchgeführt, indem man zuerst Stimm- bzw. Gesangsaufnahmen analysiert und sie anschließend mit Verhaltensanalysen kombiniert hat. Die Alarmkommunikation bei Insekten ist hauptsächlich chemischer Natur und für chemische Signale wurden solche Analysen bisher jedoch durch das Fehlen von Echtzeit-Analysetechniken verhindert.

Die Erbsenlaus, *Acyrtosiphon pisum* Harris, dient in der vorliegenden Arbeit als Modellorganismus. Wie die meisten Blattläuse sondert auch die Erbsenlaus nach einem Angriff kleine Tropfen aus einem Paar röhrenförmiger Strukturen ab, die auf ihren Abdomen sitzen. Zusätzlich zu einer direkten Abwehrfunktion durch ein Zusammenkleben der Mundwerkzeuge des Angreifers, können diese Tropfen ein Alarmpheromon enthalten, dessen oft einzige Komponente das Sesquiterpen (*E*)- $\beta$ -Farnesene (EBF) ist. Wenn Artgenossen der Kolonie EBF wahrnehmen, löst dies verschiedene Fluchtverhalten aus, wie zum Beispiel Herausziehen des Saugrüssels, Herunterfallen von der Wirtspflanze, Treten oder einfaches Weglaufen.

Ziel dieser Dissertation ist es die Dynamik der Alarmpheromone-Abgabe zu untersuchen und die Rolle der Alarmkommunikation in Räuber-Beute Interaktionen durch Labor- sowie Feldexperimente detaillierter zu beschreiben. Ferner werden die ökologischen Kosten des Alarmpheromons als potenzielles Kairomone im Feld untersucht und bewertet.

Die erste Studie untersucht die EBF Emission von einzelnen Blattläusen, die von Florfliegen- oder Marienkäferlarven angegriffen wurden, anhand von EBF-Emissionsmessungen angegriffener Läuse mit Hilfe einer zNose™ - einem portablen, schnellen Gaschromatographen. Die Ergebnisse zeigen, dass das Alarmsignal der Blattläuse abhängig vom angreifenden Räuber ist. Während alle Blattläuse EBF enthalten, enthalten nicht immer alle abgesonderten Tropfen EBF. Alarmsignale bei Insekten können variabel sein; sowohl der Angreifer als auch der Angegriffene kann potentiell die Variabilität der Alarmsignale beeinflussen.

Während sich die erste Studie auf Beobachtungen einzelner Läuse konzentriert, präsentiert die zweite Studie die erste Langzeit-Beobachtung (24 h) von EBF-Emissionen in Blattlauskolonien mit und ohne Räuber in Echtzeit. In angegriffenen Kolonien ist die EBF-Emission immer auf eindeutig definierte Zeiträume beschränkt; EBF-Emission in Abwesenheit von Räubern tritt nicht auf. Die Alarmpheromon-Emission unterliegt keinem Tag-Nacht-Rhythmus und die EBF-Emissionsmuster

innerhalb einer Blattlauskolonie sind wahrscheinlich durch das Fressverhalten der Räuber gesteuert.

Die dritte Studie untersucht eingehender die zugrundeliegenden Mechanismen der Variationen von Alarmsignalen in Blattlaus-Räuber-Interaktionen. Alarmsignal-Emissionsdynamiken nach einem Angriff wurden mit der zNose<sup>TM</sup> in Echtzeit gemessen und mit synchronen Verhaltensbeobachtungen zwischen Räuber und Beute verglichen. Die Ergebnisse zeigen, dass eine Alarmsignal-Abgabe fast ausschließlich auftritt, wenn eine Laus tödlich angegriffen wurde. Des Weiteren wird die Fluchtreaktion der Kolonie wird eher durch Erhöhung des EBF Reizes ausgelöst, als durch die Gesamtmenge an Alarmpheromon und sie stoppt aufgrund EBF Habituation.

Pheromone sind bestimmt für intra-spezifische Kommunikation; diese Signale werden jedoch manchmal von anderen als den vorgesehenen Empfängern verwendet. Während sich der erste Teil dieser Doktorarbeit auf die Emissionsdynamik konzentriert, liegen im zweiten Teil die Kosten von EBF im Fokus, indem es von Blattlausfeinden zur Wirts- oder Beutefindung eingesetzt wird. Somit beobachtet die vierte Studie das Suchverhalten von Florfliegen- und Marienkäferlarven auf einer Pflanze, an der eine Blattlaus fixiert wurde, in An- und Abwesenheit von EBF und unter Feld- und Laborbedingungen. EBF hat keine Auswirkungen auf das Kurzstrecken-Suchverhalten der Räuber, der Verweildauer und EBF erhöht auch nicht den Erfolg der Nahrungssuche. Daher wird EBF weder als Arrestant noch als Kurzstrecken-Hinweis von diesen Blattlausräubern verwendet

Die fünfte Studie versucht die Frage zu beantworten, ob EBF unter Feldbedingungen als Kairomon genutzt wird, da mehrere natürlichen Blattlausfeinde EBF vermutlich als Hinweis zur Wirts- bzw. Beutefindung verwenden. Beobachtungen der Räuberdynamik in einem natürlichen Umfeld durch Langzeit-Kameraaufnahmen in Anwesenheit verschiedener EBF-Konzentrationen legt nahe, dass natürlichen Feinde das Blattlaus-Alarmpheromon (*E*)- $\beta$ -Farnesene nicht als Kairomon unter natürlichen Bedingungen im Feld nutzen, es aber das Potenzial besitzt, Räuber anzulocken, wenn es in übertriebenen, nicht natürlich vorkommenden Konzentrationen ausgebracht wird.

Am Beispiel von Blattläusen, trägt diese Arbeit zum Verständnis der Alarmkommunikation von Insekten bei. Es zeigt die komplexe Natur der Emissionsdynamik von EBF und gibt mehr Aufschluss über die Kosten und Nutzen, die mit der Alarmkommunikation von Blattläusen verbunden sind. Diese neuen Erkenntnisse, die Verknüpfung von Live-Verhaltensbeobachtungen mit Echtzeit-Headspace-Messungen, bietet einen vielversprechenden Ansatz, um die Faktoren der Variabilität der EBF-Emission in zukünftigen Forschungsprojekten zu ergründen.



## Introduction

Communication is the interaction between two individuals, one acting as a sender by providing information and another, the receiver, who uses this information for making decisions (Théry and Heeb 2008). When this information modifies the behavior of the receiver to benefit the sending organism, these detectable facts are called *signal*, when they, however, alter the behavior of receivers in a way that is not beneficial for the sender, they are generally called *cues* (Bradbury and Vehrencamp 1998; Seeley 1989; Théry and Heeb 2008). Bystanders, i.e. individuals not involved in the interaction, can eavesdrop on such signals or cues, although they are not directly involved in the interaction. True or honest communication benefits both, the sender and receiver. When eavesdropping, however, provides an advantage to the bystander at a cost of the actors of the interaction, it is called exploitation, while eavesdropping in general does not imply these costs (Théry and Heeb 2008).

Communication, i.e. signaling, plays an important role in many animal interactions (Bradbury and Vehrencamp 1998): It is essential in sexual interaction to find or attract sexual mates and coordinate reproduction. Communication is necessary for conflict resolution, by providing information of the likely intentions and the fighting ability, or to establish, maintain and defend a territory. Further, many signals are used for social integration, i.e. coordinating task within a group, and providing environmental information by indicating possible food sources or showing the presence of predators.

To convey information to the intended receiver, different signal modalities can be utilized (Bradbury and Vehrencamp 1998; Kost 2008; McGregor 2008). Frequently used are (1) visual signals, such as bright plumage coloration in bird, (2) acoustic and vibrational signals, conveying information from a short-range, e.g. organizing labor in honeybee colonies through vibration signals (Schneider and Lewis 2004), through mid-range, as in bird or frog calls, to long-distances, e.g. infrasonic communication in whales (Payne and Webb 1971), (3) electronic signals, as in social communication of some fishes, e.g. several catfish species, electric skates or gymnotiform and mormyridiform fishes (Hopkins 2009), (4) chemical and olfactory signals, that in contrast to the others signal modalities, have the ability to be still present, although the sending organism is already gone (e.g. scent-marking).

Chemical signaling occurs at different biological levels (Bradbury and Vehrencamp 1998). Chemical compounds facilitating communication inside an organism are called *hormones*, chemical compounds allowing communication within or between species *semiochemicals* (Bradbury and Vehrencamp 1998; Nordlund and Lewis 1976; Théry and Heeb 2008). Semiochemicals encompass *pheromones*, i.e. compounds mediating intraspecific interactions between individuals of the emitting

species, and *allelochemicals*, i.e. compounds mediating interspecific interactions between the sender and individuals other than the sending species (Fellows et al. 2005; Nordlund and Lewis 1976). Allelochemicals can also be further subdivided based on the cost-benefit framework (Kost 2008): While *synomones* are beneficial for both, the sender and the receiver, the only beneficiary of *allomones* is the sender and of *kairomones* the receiver. A chemical compound, however, can serve various purposes and its classification thus depends on the studied ecological context. Pheromones released by herbivore insects, such as alarm pheromones, perceived by natural enemies and utilized for prey localization are hence classified as kairomones in this specific interaction (Hatano et al. 2008b; Kost 2008). The detection of pheromones and allomones can be done using two different reception methods: Olfaction, where the waterborne or airborne chemical is detected from a distance, or taste, where the receiver detects the chemical compound upon direct contact (Bradbury and Vehrencamp 1998; Théry and Heeb 2008).

### Alarm Communication

Alarm communication in the presence of predators is a crucial adaptation for the reduction of predation risk in prey and occurs in a wide range of various animal taxa such as mammals (Zuberbühler 2009), birds (Hollen and Radford 2009), amphibians (Fraker et al. 2009; Rajchard 2006), fishes (Mathis et al. 1995; Smith 1992), gastropods (Snyder and Snyder 1971) and insects (Verheggen et al. 2010). Alarm signaling generally appears in the moment of impending danger – in the presence of a predator or other factors causing a rapid adverse change in the surrounding environment (Verheggen et al. 2010), hence causing immediate direct fitness costs. To deal with these costs, alarm signals can evoke two different types of action: 1) Direct benefits for the signaler, by attracting additional predators which may disrupt the current predation event and help the signaler to escape (Chivers et al. 1996; Mathis et al. 1995), by directly deterring the predator (Ruxton et al. 2004; Zuberbühler et al. 1999) or by eliciting mutualistic anti-predation behavior in con- or heterospecifics that decreases the vulnerability of the signaler such as mobbing calls (Curio et al. 1978; Zuberbühler 2009). 2) Delayed or Indirect benefit for the signaler by alerting, hence saving lives of highly related conspecifics that can reproduce later, mates or group members that respond with escape, hiding, apparent death or attack (Hatano 2010). So alarm signaling can not only be beneficial for the receiver, but also for the sender.

While alarm signals are often visual or auditory, especially in mammals and birds, chemical alarm signals are also widespread (Verheggen et al. 2010; Wyatt 2003). It is believed that most compounds serving as alarm pheromones may have derived from substances involved in predator defense or released during injuries (Verheggen et al. 2010; Wyatt 2003). They are exclusively

released when the sender is in peril and perceived by conspecifics that initiate behavioral reactions close to those when directly exposed to the threat (Wyatt 2003). In insects the chemical composition of alarm pheromones differs greatly between species. In some species, such as the honey bee *Apis mellifera* Linnaeus, the alarm pheromone consists of a complicated chemical blend, whose activity is determined not only by its specific composition, but also the quantitative ratio of its compounds and the stereoisomerism of the dominating substances (Pankiw 2004; Verheggen et al. 2010). In other species, such as the pea aphid *Acyrtosiphon pisum* Harris, it is a single molecule (Francis et al. 2005a).

Aphids are an ideal model organism for studies on alarm communication in insects. Not only are these gregarious insects highly dependent on alarm pheromone emission for colony defense and/or escape behavior, but also is the aphid alarm communication intensively studied since its description in the early 1970<sup>th</sup> (Bowers et al. 1972). Additionally all offspring are produced by parthenogenesis and hence are clones of their mothers (Blackman 1987). So, genetic variation can be excluded when studying the influence of external factors such as predation events. However, aphids show great variation in morphological, physiological and behavioral response to alarm pheromone perception, because this may vary among aphid species, clones, morphs and individuals (see below).

## **Aphid-Predator Interactions**

Aphids (Hemiptera: Sternorrhyncha) are small (1 – 10 mm), soft-bodied, phytophagous insects (Dixon 1998). Most of them feed on phloem sap of plants using a piercing-sucking stylet within the proboscis located behind and between the fore coxae (Dixon 1998). Characteristic morphological features comprise a pair of tube-shaped secretory organs on their fifth abdominal segment called siphunculi, segmented antennae with two short basal segments and a thinner, segmented flagellum, two-segmented tarsi and a cauda, for distribution of honeydew from the anus (Blackman and Eastop 2007).

Due to their great abundance, aphids are used as a food or oviposition source by a wide range of natural enemies. These enemies do not only include highly specialized parasitoids like e.g. the braconid subfamily of aphid wasps (Hymenoptera: Aphidiinae) but also generalist and specialist predators such as beetles (e.g. ladybirds and ground beetles), hoverflies, lacewings, wasps and ants, bugs (e.g. anthrocorid bugs), spiders, mites, opiliones and even birds (Frazer 1987; Völkl et al. 2007). In some families of predators both, the adult and larvae feed on aphids (e.g. ladybirds), while in others families (e.g. lacewings) only the larvae is predaceous (Völkl et al. 2007). Although microbes

are often barely mentioned or completely forgotten in multi-trophic predator-prey interactions, pathogens like entomopathogenic fungi are also important natural enemies of aphids (Hatano 2010; Roy and Cottrell 2008).

The great variety of enemies likewise comprises a great variety on different foraging and feeding behaviors, thus, being gregarious and sedentary, aphids possess a range of defense behaviors with varying degrees of success: When disturbed or attacked by a predator an aphid may remain motionless to avoid detection, twitch or kick, simply walk away, attack when approached and drop or fall from the host plant (Arakaki 1989; Dixon 1958; Dixon 1998; Losey and Denno 1998b; Roitberg et al. 1979). Although dropping is the most common response after being directly attacked by a predator, defense behaviors are moderated or altered by an array of factors including plant quality, aphid density, predator size and physiological factors (Losey and Denno 1998a). However, after an attack aphids may also elicit a waxy droplet from their tube-shaped abdominal cornicles, containing a mixture of fatty acid radicals often accompanied by alarm pheromone (Dixon 1958; Greenway and Griffith 1973).

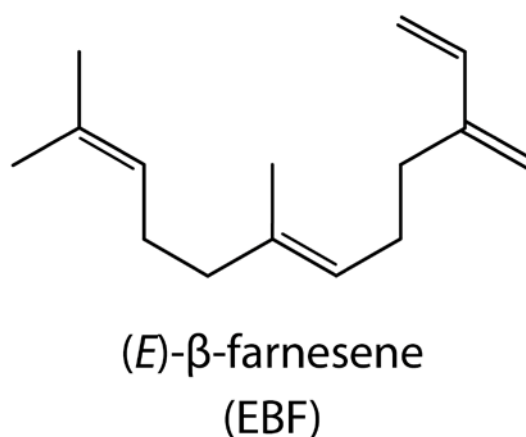
Cornicle droplets are produced by modified oenocytes; several cornicle secretory cells are stored in the cornicle secretory cell sac within the cornicle stalk and the haemocoel below (Chen and Edwards 1972). After an attack, secretory cells (forming a droplet) are released via turgor by retracting a cornicle muscle that is attached to the top and origins at the base of the siphunculi, opening a valve-like flap at the top of the cornicle (Edwards 1966). The emission is regarded as a holocrine secretion, since the secretory cells are disrupted at the time of discharge (Chen and Edwards 1972). The fatty acid radicals hexanoyl, sorboyl, myristoyl and palmitoyl rapidly crystallize on contact with the surface of a predator or parasitoid, eventually halting the attack (depending on the body part the droplet was daubed on), creating an opportunity for the aphid to escape (Dixon 1958; Edwards 1966). The alarm pheromone volatilizes upon emission of the droplets and can then be perceived by neighboring conspecifics, informing about the presence of an enemy in close vicinity (Kislow and Edwards 1972; Nault et al. 1973).

## Ecology of Aphid Alarm Pheromone

Chemical alarm signaling in aphids has been studied intensively since its description in the early 1970s (Bowers et al. 1972) and has been reviewed recently by Pickett and Glinwood (2007). The first characterization of aphid alarm pheromone was done by Bowers et al. (1972), identifying the sesquiterpene (E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene,  $C_{15}H_{24}$  or (E)- $\beta$ -farnesene (Fig. 1) as the primary component for *Macrosiphum rosae* (L.), *Acyrtosiphon pisum* Harris, *Schizaphis*

*graminum* (Rondani) and *Aphis gossypii* Glover. Subsequent studies supported the finding and identified (*E*)- $\beta$ -farnesene also in other aphid species (Edwards et al. 1973; Pickett and Griffiths 1980). Eventually Francis et al. (2005a) characterized the volatile emissions of 23 aphid species, showing that (*E*)- $\beta$ -farnesene is the main or only component in 16, including *A. pisum*, and a minor component in five of them. Two of the 23 aphid species did not release any (*E*)- $\beta$ -farnesene in their pheromone blend, but other terpenes. In addition to Francis et al. (2005a) four more species have been identified to use (*E*)- $\beta$ -farnesene as the only alarm pheromone substance (Verheggen et al. 2010). Other alarm pheromone components include monoterpenes like  $\alpha$ -pinene,  $\beta$ -pinene or limonene and additional sesquiterpenes such as germacrene A and germacrene D, (*Z,E*)-a-farnesene or (*E,E*)-a-farnesene (Francis et al. 2005a; Gut and Vanoosten 1985; Pickett and Griffiths 1980), whereas the latter two molecules show no biological activity (Bowers et al. 1977).

Fig. 1 - Structural formula of (*E*)- $\beta$ -farnesene.

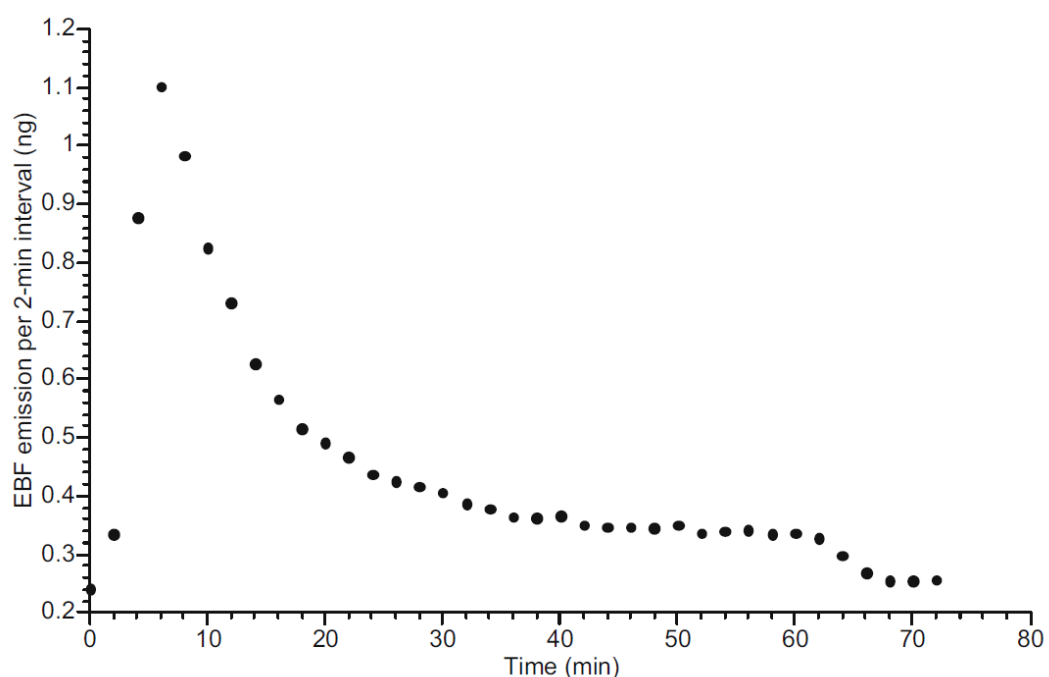


Besides its importance as an aphid alarm pheromone, (*E*)- $\beta$ -farnesene appears in a wide range of both, plants and animals (Crock et al. 1997). In plants, it is not only present in essential oils of various gymno- and angiosperms (Crock et al. 1997; Heuskin et al. 2009), but also in plant volatile emissions induced after herbivore feeding or mechanical damage or in constitutive volatile blends (Agelopoulos et al. 2000; Agelopoulos et al. 1999; Turlings and Ton 2006). In animals, it is found in mammals (Novotny et al. 1990), but used most extensively in insects (Crock et al. 1997): The semiochemical is used as a defense allomone in two andrenid bees (Fernandes et al. 1981), a trail pheromone in several ant species (Ali et al. 1987; Jackson et al. 1990), a feeding stimulant for sand fly *Lutzomyia longipalpis* (Lutz & Neiva) (Tesh et al. 1992), or when released by corn as a synomone for

parasitic wasps *Cotesia marginiventris* (Cresson) to locate their lepidopteran host (Turlings et al. 1991), to name but a few.

The biosynthetic pathway for production of terpenes similar to (*E*)- $\beta$ -farnesene is well understood for plants, the exact biosynthesis of (*E*)- $\beta$ -farnesene in aphids is, however, still unknown (Vandermoten et al. 2012). (*E*)- $\beta$ -farnesene is believed to be produced de novo, probably in anticipation of an attack, and speculated to be involved in an indirect feedback loop with the juvenile hormone (Gut et al. 1987; Gut and Vanoosten 1985; Mondor et al. 2000). It has been shown, that there is a great variation in (*E*)- $\beta$ -farnesene quantities in aphid morphs and age (Gut and Vanoosten 1985). The quantities in aphids increase in relation to increasing body weight, whereas the pheromone concentration declines (Byers 2005). The (*E*)- $\beta$ -farnesene amount is highest in pre-reproductive stages and significantly decreases in adults, indicating that based on their more clustered colony structure, pre-reproductive stages are selected to produce more (*E*)- $\beta$ -farnesene as they face an increased predation risk (Mondor et al. 2000). Schwartzberg et al. (2008) were the first to describe the alarm pheromone headspace emission patterns of a single aphids after attacks of single predator species and reported differences among instars and great variation within instars. The general shape of an (*E*)- $\beta$ -farnesene emission curve after an attack is characterized by a fast increase of pheromone amounts in the headspace, followed by an exponential decline over time (Fig. 2).

Fig. 2 - General shape of an (*E*)- $\beta$ -farnesene emission curve emitted by a pea aphid after successful lacewing larva attack (Schwartzberg et al. 2008, modified).



Aphids can perceive (*E*)- $\beta$ -farnesene by the two primary rhinaria, specialized structures located on the 5<sup>th</sup> and 6<sup>th</sup> antennal segment (Wohlers and Tjallingii 1983), as confirmed for several aphid species (Pickett et al. 1992). When perceiving the pheromone signal, aphids respond by stop feeding, withdrawing their stylet from the plant tissue, waving their antennae, wagging, walking away or dropping from the leaf or host plant (Clegg and Barlow 1982; Kislow and Edwards 1972; Nault et al. 1973; Nault et al. 1976; Roitberg and Myers 1978; Wohlers 1980). As a long-term effect it also has been shown, that maternal detection of (*E*)- $\beta$ -farnesene induces the production of winged offspring (Kunert et al. 2005; Podjasek et al. 2005). (*E*)- $\beta$ -farnesene is, however, only emitted after a direct predator attack and, functioning as an alarm pheromone in aphids, hence warns conspecific aphid receivers of the immediate presence and threat of an enemy. The aphid alarm pheromone has further a repellent effect on alate aphids when landing (Phelan and Miller 1982). For aphid species that produce soldiers it has been demonstrated that after perceiving (*E*)- $\beta$ -farnesene, colony conspecifics become aggressive towards the threat and attack it using their frontal horns (Arakaki 1989).

Although abiotic factors such as degradation (Kourtchev et al. 2009; Pinto et al. 2007), habitat structure or wind may have a big effect on (*E*)- $\beta$ -farnesene evaporation and transmission over distance, the alarm signal is not amplified by colony conspecifics (Hatano et al. 2008a; Verheggen et al. 2008b).

Although (*E*)- $\beta$ -farnesene emission is apparently beneficial by revealing the presence of an enemy in close vicinity, releasing this semiochemical comes along with both, physiological and ecological costs (Vandermoten et al. 2012). Electroantennogram (EAG) recordings and behavioral studies have demonstrated that several natural enemies are adapted to perceive (*E*)- $\beta$ -farnesene and are able to exploit it. Nault et al. (1976) was the first to describe interspecific communication involving (*E*)- $\beta$ -farnesene, by observing recruitment behavior for aphid protection in the aphid-tending ant *Formica subsericea* Say. Parasitoids may use host plants odors as long-range olfactory cues for host finding (Vandermoten et al. 2012), but studies indicate short-range response to the aphid alarm pheromone (Du et al. 1998; Foster et al. 2005; Micha and Wyss 1996). Although predators are considered as less sophisticated users of host kairomones than parasitoids (Pickett and Glinwood 2007) both, larvae and adults of several species seem to use the aphid alarm pheromone as a foraging cue, such as ladybirds (Francis et al. 2004; Nakamuta 1991; Verheggen et al. 2007), hoverflies (Almohamad et al. 2009; Francis et al. 2005b; Verheggen et al. 2008a), lacewings (Boo et al. 1998; Zhu et al. 2005; Zhu et al. 1999) and ground beetles (Kielty et al. 1996). However, (*E*)- $\beta$ -farnesene does not only pose indirect costs by attracting natural enemies, but also direct fitness costs for the signaler and conspecific receiver. When the receivers walk away from the emission

source they abandon good feeding sites, facing the risks of starvation, desiccation or predation of ground-foraging predators (Dill et al. 1990; Losey and Denno 1998a; Outreman et al. 2010). Additional physiological costs are, among others, delayed offspring production in third to fourth instars of *A. pisum* when disturbed to release cornicle secretion compared to non-secreting individuals (Mondor and Roitberg 2003); lower fecundity and lighter weight of *Aphid gossypii* Glover adults when exposed to aphid alarm pheromone and prolonged developmental times in first and third instars (Su et al. 2006).

Since aphids face manifold costs when producing or receiving (*E*)- $\beta$ -farnesene, the behavioral response to alarm pheromone perception shows intra- and interspecific variations and differences on species (Losey and Denno 1998b), clone (Braendle and Weisser 2001) or colony level, strongly according to their environmental framework (Dill et al. 1990). Xiangyu et al. (2002), for example, showed that the response of several different aphid species to (*E*)- $\beta$ -farnesene was related to host-plant species, whereas strong response was found in aphids residing on their specific host plant, without ant-attendance. Within one species there can be considerable differences among instars in the defense and escape behavior. Early instars nymphs of *A. pisum* (Roitberg and Myers 1978), *Myzus persicae* (Sulzer) (Montgomery and Nault 1978) and *Diuraphis noxia* (Mordvilko) (Shah et al. 1999) are less sensitive to alarm pheromone than late instars or adults, since young nymphs possess an increased risk of starvation when dropping from the host plant. Also different aphid morphs display variation in their escape tactics. Alate morphs of *M. persicae* are more sensitive to (*E*)- $\beta$ -farnesene than apterous, based on EAG response (Visser and Piron 1997), as are soldier morphs (Arakaki 1989; Rhoden and Foster 2002). Braendle and Weisser (2001) could demonstrate clone type differences in escape response, i.e. dropping, by exposing red and green clones of *A. pisum* to an artificial predator or letting the host plant drop on a wooden surface. Additionally host races, i.e. host-adapted populations of *A. pisum* feeding from four different host plants also differ in their defense behavior after (*E*)- $\beta$ -farnesene perception, suggesting that host shifts can lead to ecological specialization (Kunert et al. 2010). These differences in alarm response may arise from biotic and abiotic variations in diverse habitats where aphid species, morphs and clones can be under different selection pressures favoring specific escape response. Even within one aggregation, individual aphids can behave differently. So, aphids may adaptively modify their escape tactics with changes in prevailing environmental conditions. Factors as food quality, temperatures, humidity and predator pressure may alter especially dropping behavior (Dill et al. 1990).



## Aims and Questions

While alarm signaling in aphids is thought to be adaptive, hence beneficial, little is known about EBF emission dynamics and its implication in actual aphid- aphid or aphid-predator interactions.

To study costs and benefits of alarm signals it is mandatory to know and understand the emission dynamics and link alarm signaling to prey and predator behavior. As stated above, this has repeatedly been done for alarm calls in mammals and birds by analyzing live vocal recordings and combining them with behavioral analysis (Seyfarth et al. 1980; Templeton et al. 2005; Zuberbühler 2001). For chemical alarm signals, however, such an analysis of costs and benefits has so far been hindered due to elaborate sampling techniques: To be detected, chemical signals need to be trapped in the headspace of the signaling organism. Generally, pre-concentration techniques are used, because the number of molecules emitted is often low. Here, headspace volatiles are first absorbed and pre-concentrated for a certain time on an organic polymers, desorbed by rapid heat or eluted in solvents, and subsequently analyzed using gas chromatography (Agelopoulos and Pickett 1998). Sampling the headspace for a certain time implies, however, that any information on the timing of emission is lost. This is a problem in case the alarm signaling prey individual encodes information in the sequence of volatile emission, e.g. altering the emission pattern in response to varying predator-prey interactions in a group of prey under attack by a predator. To face this problem, a zNose™ (Electronic Sensor Technology, Newbury Park, CA, USA), a handheld rapid gas chromatograph capable of repeated quantitative sampling of headspace volatiles (Kunert et al. 2002), is used in this thesis. This “electronic nose” has recently been successfully employed to analyze alarm pheromone emission in aphids in almost real-time (Schwartzberg et al. 2008). Applied to the questions asked in this thesis, this novel device will give new insight in real-time emission dynamics of aphid chemical alarm signaling.

For measuring the costs that are associated with alarm signaling it is further important to understand the ecological implications of (*E*)- $\beta$ -farnesene emission. As stated above, several aphid natural enemies are believed to use EBF as a host/prey finding kairomone, hence bearing a cost for the emitting aphid and the colony conspecifics (Hatano et al. 2008b). While several electroantennogram studies and olfactometer assays demonstrate that a variety of aphid natural enemies are capable of perceiving EBF or show an attractant behavioral response to EBF presence, these studies were mainly conducted under laboratory conditions using exaggerated EBF amounts that were higher than natural occurring amounts (e.g. Francis et al. 2005b; Micha and Wyss 1996; Zhu et al. 1999). Field studies investigating the ecological implications of natural occurring amounts are, however, lacking so far, and are therefore also part of this thesis.

The general aim of this thesis is to increase the knowledge of the role of the aphid alarm pheromone, (*E*)- $\beta$ -farnesene, for aphid fitness. Therefore the studies comprised in this thesis (1) investigate the emission dynamics in single aphids and aphid colonies with predator interaction and link them to behavioral observations, and (2) elucidate the ecological costs of alarm signaling under field conditions.

### Overall question

How are the aphid alarm pheromone emission dynamics shaped in different environmental conditions and which role does the aphid alarm pheromone, (*E*)- $\beta$ -farnesene, play in aphid-aphid and aphid-natural enemy interactions?

### Specific questions

1. Are the dynamics of (*E*)- $\beta$ -farnesene emission pattern affected by the predator species that attacks the aphid?
2. How are (*E*)- $\beta$ -farnesene emission dynamics shaped in aphid colonies with and without predation?
3. How do (*E*)- $\beta$ -farnesene emission dynamics affect aphid-predator interaction?
4. Do aphid natural enemies utilize (*E*)- $\beta$ -farnesene as a cue in the field?

## General Materials and Methods

The general experimental setups and methods used in this thesis are given below. A detailed description of the specific experimental design can be found in each of the corresponding Manuscripts.

In brief, to investigate the first, second and third question, the emission dynamics of single aphids and aphid colonies with and without predation are analyzed using an electronic nose, supported i. a. by live behavioral recordings of predator-prey interactions. Question four is addressed by testing EBF as an arrestant and long range attractant cue under field conditions using observations of foraging behavior and long-term camera monitoring.

## Plants and Animals

### Aphids and Plants

Two different clones of the pea aphid *Acyrtosiphon pisum* Harris were used: The green clone, originally collected in Jena, Germany, and the red clone, originally collected in Bayreuth, Germany. Almost all experiments were conducted with the red clone, except the ones presented in Manuscript 1. Pea aphids were reared on 3-week-old broad bean plants (*Vicia faba* L., variety “The Sutton”; Nickerson-Zwaan, UK) in 10 cm diameter plastic pots. To prevent escape of aphids, plants were covered with air-permeable cellophane bags (Unipack GmbH, Germany). Aphids were kept in a climate chamber under constant environmental conditions (20°C, 75 % relative humidity, photoperiod: 16:8 h L:D).

### Aphid Lines

For all experiments a split-brood design was employed to control for any effect of previous rearing conditions on aphid alarm pheromone emission or predator attraction. By distributing individuals from one aphid line equally among treatments, any variation due to rearing conditions is distributed equally over all experimental treatments, since one line consists of genetically identical aphids (Kunert et al. 2005). To initiate aphid lines the corresponding number of adult foundress aphids ( $F_0$  generation), randomly collected from a single population consisting of the same clone, were placed individually on broad bean plants where they were allowed to reproduce for 24 h. Subsequently they were removed from the plants. So, to initiate e.g. ten lines, ten adult foundress aphids collected from a single colony were placed singly on ten plants. After 8 to 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 used for the experiments as soon as they reached the needed developmental stage (e.g. third instar or adult). A split-brood design was achieved by using aphids from one line once for all treatments, i.e. for each line the needed amount of aphid individuals was chosen once for each treatment.

## Predators

Two predator species were used in the experiments, chosen to show differences in foraging and feeding strategies: 1) Larvae of the common green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera - Chrysopidae), piercing-sucking predators, slowly consuming their prey (Bänsch 1964); 2) larvae and adults of the seven-spot ladybird, *Coccinella septempunctata* L. (Coleoptera – Coccinellidae), chewing predators that consume their prey quickly (Hodek and Honěk 1996).

Predators were obtained from a commercial supplier (Katz Biotech AG, Baruth, Germany), arriving as 1<sup>st</sup> instar larvae (*C. carnea*) and as eggs (*C. septempunctata*). Both predator species were generally reared on broad bean plants infested with pea aphids until they reached the desired developmental stage, e.g. 3<sup>rd</sup> instar or adulthood. Infested plants and predators were either covered with air-permeable cellophane bags or placed in 6 L fauna boxes (Savic NV, Heule, Belgium) covered with a mesh to avoid escape. When ladybird larva were used in the experiments, hatching ladybird larvae were collected from the fauna boxes, after reaching 1<sup>st</sup> to 2<sup>nd</sup> instar, and reared individually in Petri dishes with sufficient pea aphid supply.

Predators were generally starved 24 h before each experiment to enhance their incentive to forage. Preliminary experiments and personal behavioral observation showed that longer starvation times can lead to a decreased foraging likeliness, since the predators seem to be in a weak condition. This phenomenon has also been described by Bänsch (1964) for lacewing larvae. Shorter starvation times are also not chosen for, since predators, especially lacewings, tend to rest for a few hours after successful predation and before continue foraging. Predators were reared at 20°C, 75% humidity and a photoperiod of 16:8/ L:D.

## zNose™ Settings and Calibration

To study alarm signaling and the associated costs and benefits, it is mandatory to know and understand the dynamics of the pheromone emission dynamics. Recently the zNose™ (Electronic Sensor Technology, USA), a handheld rapid gas chromatograph capable of repeated quantitative

sampling of headspace volatiles (Kunert et al. 2002), has been successfully employed to analyze alarm pheromone emission in aphids in almost real-time (Schwartzberg et al. 2008). Its short measurement intervals allow to test chemical alarm signaling for patterns in emission dynamics - a mandatory necessity, since it has been shown for mammals and birds, that slight variation in alarm signaling can deliver information about the predator size or type of predator attacking (Templeton et al. 2005; Zuberbühler 2001).

To monitor EBF emissions a zNose™ model 4100 was used in the first study (Manuscript 1), conducted by co-author C. Linse, and for all following studies (Manuscript 2 – Manuscript 5) a zNose™ model 4300 was used.

A single zNose™ sample includes three phases: 1) sampling and trapping of volatiles, 2) rapid discharge of the trapped compounds onto the column followed by a specifically designed temperature-programmed elution with following compound detection, and 3) recovery phase of the surface acoustic wave (SAW) quartz microbalance detector (Kunert et al. 2002). Such a sample is defined by a method carefully designed for each experimental setup, defining the steps the instrument requires for analyzing a sample by containing information on the sampling duration, temperature of the different components and temperature gradients for the column (EST 2008). An example of such a method is shown in Tab. 1. In general, the zNose™ was programmed to sample volatiles at a flow-rate of 30 ml min<sup>-1</sup> in experiment-depending time intervals (e.g. every 2 minutes) for a pre-set duration (e.g. 10 sec. sampling time). After pre-concentration of the volatile compounds on a Tenax® trap, the compounds were transported to a DB5 column (1 m, film thickness 0.25 µm, ID 0.25 mm) for separation. After fast elution under a programmed temperature gradient (generally from 40°C to 175°C at 5°C s<sup>-1</sup>), compounds were monitored and quantified by the SAW detector, giving one EBF peak, i.e. amount of EBF in the headspace sample, for every specific time interval, when EBF was present. The temperature of the SAW detector was adjusted to 40°C to obtain optimum sensitivity for quantification of EBF. The threshold below which the identification of EBF was not considered to be reliable was set to 100 Hz, as recommended by the manufacturer (EST 2008). Carrier gas (Linde, Helium 6.0 T 10, ultra high purity) was supplied by a large gas cylinder.

*Tab. 1 - Example of a zNose™ method as used in Manuscript 3. The method was repeated every 90 sec., i.e. a new analysis started automatically every 90 seconds, allowing a continuous sampling of headspace volatiles.*

Event	Duration [s]	Description
Sample	0.5	Rotor inside the valve is placed in the “sample” position
Pump	20	Pump runs and pulls air through the inlet, i.e. sampling the headspace
Inject	0.5	Rotor inside the valve is placed in the “inject” position
Wait	2.0	Method pauses
Focus	-	Trap fires for 15 ms at 250°C
Wait	1.0	Method pauses
Ramp	-	Column ramps, i.e. is heated, from 40°C to 200°C at a rate of 5°C s <sup>-1</sup>
Data	40.0	Detector collects data.
Bake	20.0	SAW detector bakes automatically at 150°C
End	-	Sampling cycle ends
Total	84.0	Total method time

### Calibration of zNose™

While the SAW detector of the zNose™ always oscillates at a detector-specific frequency, as determined by the manufacturer, each analyte that deposits on the detector during a sampling cycle causes a frequency shift in this oscillation. This frequency shift is characteristically depending on the amount of material deposited on the detector and hence allows quantization of the analyte (EST 2008). The zNose™ is, however, not capable to link the frequency shifts automatically to the corresponding amount, but has to be calibrated to known standards of the analyte of interest before starting an analysis.

To quantify EBF amounts detected by the zNose™, response factors were calculated by comparison to synthetic EBF (Bedoukian Research Inc., USA). The calibration of the zNose™ was run under the same method and the same settings as the actual corresponding experiment but with two differences: 1) the pump time, i.e. the time volatiles were collected was always set to 10 sec. and 2) the repeat function was disabled. While these differences had, however, no influence on EBF perception or retention times, they allowed a faster calibration. A dilution series was created by dissolving synthetic EBF in methanol (Carl Roth Germany, 99.8%), generally at concentrations of 0.25, 0.5, 1.0, 2.0, 3, 4, 6.0 and 10.0 µg EBF ml<sup>-1</sup>. An aliquot (0.5 µL) of each diluted sample was either

directly injected into the LUER inlet of the zNose<sup>TM</sup> or into a heated desorber tube (3100 Vapor Calibrator, Electronic Sensor Technology, Newbury Park, CA, USA) attached to the LUER-inlet and set to 190°C while the zNose<sup>TM</sup> was sampling for 10 s, using a 1 µL syringe. This allowed trapping of total pheromone amounts of the injected solution. The volatilized samples were eluted under programmed conditions. Each concentration was tested at least five times. So, (*E*)-β-farnesene was identified by comparison to a synthetic standard. Regression analysis showed that the response of the SAW detector to EBF changed in a linear fashion in all performed experiments. The calibration curve was described by  $y(x) = a \cdot x$ , whereas  $y$  is the response of the SAW detector [Hz];  $x$  is the amount of EBF [ng] and  $a$  is the constant describing the slope or gradient of the line.

*Brief differences between Manuscripts:* The calibration for Manuscript 1 was performed by co-author C. Linse using a zNose<sup>TM</sup> 4100 and a heated desorber tube. The calibrations for the other Manuscripts were performed by me using a zNose<sup>TM</sup> 4300, whereas a heated desorber tube was only used in Manuscript 2, and not in Manuscript 3 or Manuscript 5. In Manuscript 3 and Manuscript 5 the aliquot was directly injected into the LUER inlet.

### Calibration of the Experimental Vessels

To measure the emission dynamics of the aphid alarm pheromone (*E*)-β-farnesene, it is mandatory to ensure that the amounts detected by the zNose<sup>TM</sup> resemble the amounts actually emitted by the aphid. While this cannot be guaranteed when sampling the headspace of aphids in an open experimental setup, where EBF might get lost due to volatilization, experimental vessels were used, creating a closed, controlled environment. When EBF is not sampled in a small vial where the total amount of air can be extracted from within one pump and sample cycle by the zNose<sup>TM</sup>, e.g. 4 ml glass vials, a calibration of the experimental container is essential.

For calculating the total amount of aphid-emitted EBF in a vessel with airflow, the discrepancy between EBF perception of the zNose<sup>TM</sup> and the amount of EBF evaporated due to constant air flow was determined (see Manuscript 2 and Manuscript 3). Therefore the vessel was also calibrated by creating a dilution series of EBF in methanol, generally at concentrations of 5, 10, 15 and 20 µg mL<sup>-1</sup>, respectively. In contrast to the calibration of the zNose<sup>TM</sup> to EBF, the calibration of the experimental vessel was run under the exact same method and same setup as the actual corresponding experiment. An aliquot (1.0 µL) of each diluted sample was applied with a 1 µL syringe on a piece of filter paper (1 cm x 1 cm) attached to a wooden stick in between the plant leaves, at the exact same conditions as the actual experiment. Each sample was tested at least four times and the

amount of the volatilized samples measured by the zNose<sup>TM</sup> was compared to the applied amount. The resonance frequency [Hz] of the SAW detector changed in a linear fashion with increasing EBF amount. The calibration curve was described by  $y(x) = a \cdot x$ , whereas  $y$  is the EBF amount detected by the zNose<sup>TM</sup> [ng];  $x$  is the EBF amount applied to filter paper within the vessel [ng] and  $a$  is the constant describing the slope or gradient of the line. The lag time, i.e., the time after EBF application into the vessel and the first EBF detection by the zNose<sup>TM</sup>, was also accounted for.

*Brief differences between Manuscripts:* While in Manuscript 2 a three liter vessel was used, in Manuscript 3 a smaller 250 ml vessel and hence also different experimental parameters (e.g. decreased airflow) were used in the setup of the experiment.

## Experimental Setups and Procedures

### Setup for Volatile Collection using a zNose<sup>TM</sup>

Investigating the emission dynamics in single aphids and aphid colonies with and without predator interaction is crucial to increase the knowledge of the role of (*E*)- $\beta$ -farnesene for aphid fitness. To get an insight into various aspects of EBF emission, e.g. general emission pattern (shape of emission), duration of emission, total pheromone amount, etc., it is mandatory to ensure that when measuring EBF emission dynamics 1) the time intervals for each measurement are small enough to identify the pattern of the signal and that 2) the detected amounts resemble the emitted amounts. While the high resolution, i.e. short time intervals, is ensured by using the zNose<sup>TM</sup>, the correct detection of EBF is ensured by using a setup, specifically designed for volatile collection.

In laboratory experiments, where EBF emission is measured, recorded and/or monitored, two different setups for volatile collection were used: 1) Small experimental vials, where more than the total volume of headspace air within the vial was collected by the zNose<sup>TM</sup> within one sampling cycle, as in Manuscript 1 and 5; 2) large experimental vessels, where the total volume of the vessel exceeded the amount of air pulled in during one zNose<sup>TM</sup> sampling cycle, as in Manuscript 2 and 3.

For volatile collection in small vials, an air-collection chamber was constructed from a 4 ml glass vial (Macherey & Nagel, Düren, Germany). The vial with its lid and septum was connected to the zNose<sup>TM</sup> by inserting its stainless steel needle (Hamilton, 50 mm) through the septum (CS Chromatographie Service, Langerwehe, Germany). Additionally, a hypodermic needle (0.9×40 mm; B. Braun Melsungen AG, Melsungen, Germany) was inserted in the septum to allow influx of air to the



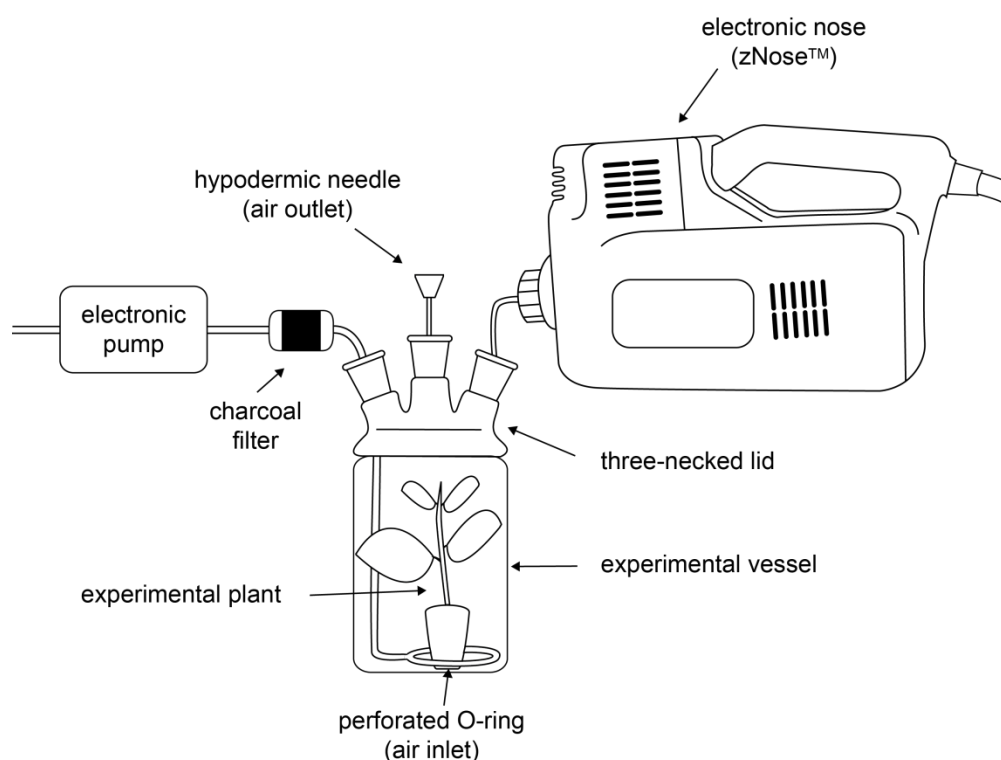
system during the collection. The method of the zNose™ was set to extract 5 ml air per measurement from the 4 ml chamber, hence pulling in more than the total volume of headspace air within one sampling cycle to minimize accumulation of EBF in the chamber. While for the studies in Manuscript 5 an EBF slow releaser bead was placed in an intact 4 ml vial and the observation started, for the studies in Manuscript 1 (performed by co-author C. Linse) a vial with its bottom cut-open was used. This bottom-less chamber attached to the zNose™ was then placed over the predator/prey, sitting on the lower surface of an excised broad bean leaf embedded in 1.5 % agar in a Petri dish (diameter: 90 mm, height: 20 mm), using the open bottom of the vial. The system was sealed with a ring of damp cotton wool surrounding the open bottom of the vial upon the leaf. The volatile collection started immediately after positioning of the predator/prey pair inside the collection system. A new collection chamber was used for every replicate.

For volatile collection in larger vessels, e.g. volatile collection of aphid colony with and without predators on plants, an airstream was generated to achieve an even air distribution in the vessel, minimizing accumulation of EBF. Therefore flange vessels with three-necked lids (Schott, Germany) were used in the experiments. The setup of these experiments is of the same design (Fig. 3), only the size of the vessel and for some components the manufacturers differed. By connecting an electronic pump (Thomas Memmingen, Germany or Schego, Germany) with a flowmeter to the first neck of the 3 L or 250 ml vessel, respectively for Manuscript 2 and Manuscript 3, air was blown through a charcoal filter (custom made at the MPI for Chemical Ecology, Jena, Germany, or Carl Roth, Germany) into the vessel at a rate specifically adjusted to the size of the vessel used in the experiment, replacing the complete air every ten or 15 minutes, respectively. A tube reached to the bottom of the vessel, forming a perforated O-ring as an air inlet. An air outlet was created at the second neck by either using a hole-plug with two air-outlet holes (custom made at MPI for Chemical Ecology, Jena, Germany) or by piercing a hypodermic needle (B. Braun Medical AG, Switzerland) through a plug that sealed the neck. The third neck was covered with a septum plug (Gebr. Rettberg GmbH, Göttingen, Germany) and the stainless steel inlet needle of the zNose™ was inserted through a small hole in the septum of this plug.

For the experiments, plants (potted in odorless glass beakers) with aphid colonies were placed inside the vessel and aphids were allowed to settle down prior to the start of an experiment and before a predator was introduced through the second neck of the vessel. In the experiments presented in Manuscript 2 the EBF emission from aphid colonies without a predator was monitored for the minimum of one hour before introducing the predator. In all experiments the vessel was placed on a desk and sterilized with boiling water before each replicate. Aphids were counted after each experiment.

*Brief differences between Manuscripts:* While in Manuscript 1 and Manuscript 5 small 4 ml vials were used as volatile collection chambers, in Manuscript 2 and Manuscript 3 larger vessels were used to collect the headspace from aphid colonies. In addition to the volatile collection, also the behavior of predators and aphids was recorded in Manuscript 3.

*Fig. 3 - General experimental setup for volatile collection in larger vessels using a zNose™*



### Setup for Behavioral Observations of Predators without Volatile Collection

To analyze costs associated with EBF emission is important to check for chemically induced behavior in foraging predators. If the presence of EBF leads to more intense foraging behavior or increased residence times in aphid predators, its emission would possess a high risk for the colony fitness.

When the influence of different EBF concentrations was tested on the foraging behavior of ladybird and lacewing larvae under laboratory and field conditions with simultaneous behavioral recordings as in Manuscript 4 one aphid was glued to the leaf edge of the 2<sup>nd</sup> leaf pair (as seen from the base) of three to four-week old or to the uppermost leaf-pair of two-week old *Vicia faba* L. plants and 1.5 cm away from the leaf stem using adhesive, respectively for the laboratory and field trials, as

described below. Plants, each within their flowerpot and with a fixated aphid, were placed on a lab table or on the ground in an experimental field site in Freising, Germany (fallow grassland, geographic coordinates: latitude, longitude: 48.405191, 11.6910) at a distance of approx. 50 cm and 40 cm to each other, respectively. The distance ensured to prevent any confounding effects from aphid presence or EBF application in the neighboring treatment, since EBF is only emitted in low amounts, degrades fast and is believed to have only an impact on a local scale (Manuscript 1, Kourtchev et al. 2009; Pinto et al. 2007). A piece of filter paper (0.5 cm x 1 cm), attached to a wooden stick, was arranged close (~1-2 cm) to each aphid without touching the plant. Alarm pheromone solutions were created by dissolving EBF (Bedoukian Research Inc., USA) either in hexane (laboratory trials, Carl Roth Germany, 99.8%) or methanol (field trials, Carl Roth Germany, 99.8 %) at two different concentrations: For the laboratory trials EBF concentrations were 12.5  $\mu\text{g mL}^{-1}$  and 250  $\mu\text{g mL}^{-1}$  and for the field 100  $\mu\text{g mL}^{-1}$  and 1000  $\mu\text{g mL}^{-1}$ . The influence of EBF on the foraging behavior was always tested against a control, where only the solvent was used. For each laboratory treatment 4.0  $\mu\text{L}$  of the respective solution was applied with a syringe to the filter paper at the start of each replicate. For each field treatment 0.5  $\mu\text{L}$  of the respective solution was applied with a syringe to the filter paper at the start of each replicate and again after 10 minutes to account for longer observation times and the presence of wind. Subsequently predators, each kept individually in a Petri dish (5cm in diameter) were carefully set free at the base of the plant stem.

The behavior of the predators was recorded using the software “The Observer XT” (Noldus Information Technology, Netherlands), as described below. Each predator was observed until it encountered the aphid on the plant, until it left the plant, or until the maximum time was reached (15 min for the laboratory experiment, 25 minutes for field experiment), whatever came first.

### **Setup for Camera Monitoring in the Field**

Observing predator-prey interactions under field conditions in the presence and absence of alarm pheromone can reveal the ecological importance of EBF in a natural environmental setting. Attracting additional aphid natural enemies can have an overall impact on the colony fitness and while several aphid enemies are known to be able to perceive EBF or show attractant behavior towards EBF, based on electroantennogram or olfactometer assays (c.f. Hatano et al. 2008b), its utilization as a kairomone in a natural environmental setting, i.e. with natural emission amounts in the field, is still unknown. Utilization of cameras provides both, undisturbed monitoring of an environmental setup and the capability of long-term observations.

When camera observations were conducted to monitor predator-prey dynamics on plants in presence and absence of EBF in the field (Manuscript 5) SONY® CUT-HR-SHAD-day/night auto-iris-cameras, capable of night recordings due to infrared lights, were used. Each camera was mounted individually on wooden boards (20 cm x 20 cm) for stabilization. Power supply was ensured by 12 V batteries (Banner Energy Bull, 956 01, K5/60Ah K20/80Ah K100/90Ah). Lens distance to the object was 25 cm. One camera observed one object/plant.

To monitor aphid colonies in the field, a small artificial colony of pea aphids was established on three-week-old *V. faba* plants. Therefore four aphids were fixated, as described below, close to the mid-vein of the underside of a leaf from the bottom leaf pair by their hind legs using glue (UHU All Purpose Adhesive, UHU GmbH & Co. KG, Germany) directly before each experimental replicate. To stay in focus of the camera, the leaf was held upright and hence stabilized by two small wooden sticks and thin pliable wire. Since preliminary experiments revealed the possibility of plant destruction by mice (*Myodes glareolus*, *Microtus arvalis*), the plant pots were placed into plastic water containers (ø 25 cm), dug plain into the ground. To include ground foraging predators, such as ants, carabid beetles or spiders, six wooden sticks connected the plant pot with the surrounding environment. Alarm pheromone slow releaser beads (production described below) were placed, hence glued, next to the fixated aphids in concentrations according to the treatment. In case of the control treatment an alginate bead containing no EBF was used. Observation spots were arranged randomly over the field site and positions changed after each replicate to avoid possible aggregation of predators to a certain area.

Observation started between 7 am and 10 am and lasted for 24 hours. The numbers of aphids left on the plants were counted after each replicate.

## **Specific Procedures used in the Experiments**

### ***Gas Chromatograph Analysis***

To rule out any failures in detecting EBF with the zNose™, co-authors E. Hatano and C. Linse conducted additional gas chromatographic (GC) analysis in Manuscript 1 to check for EBF presence in aphid bodies or aphid cornicle droplets (Manuscript 1). Therefore 2 µl of the corresponding sample, i.e. either cornicle droplets dissolved in hexane or whole aphids immersed in hexane both containing β-caryophyllene (Sigma Aldrich, St. Louis, MO, USA) as an internal standard, was injecting in a GC-MS with a Hewlett-Packard 6890 gas chromatograph equipped with a Hewlett-Packard 7683 auto sampler and a Hewlett-Packard 5973 quadrupole-type mass-selective detector operated in electron impact mode (Agilent Technologies, Santa Clara, CA, USA). The mass detector had a transfer line

temperature of 230 °C, a source temperature of 230 °C, a quadrupole temperature of 150 °C, electron energy of 70 eV, and a scan range of 50–400 amu. Helium was used as a carrier gas at a linear flow rate of 1 ml min<sup>-1</sup>. All samples were analyzed on a DB-5MS (J & W, Agilent Technologies, Inc., Santa Clara, CA, USA) column. After sample injection, the column oven was kept at 60 °C for 4 min, increased to 150 °C at a rate of 5 °C min<sup>-1</sup>, and then increased further at 60 °C min<sup>-1</sup> until 300 °C and kept for 2 min. Mass spectra of EBF and  $\beta$ -caryophyllene were compared to those in the National Institute of Standards and Technology and the Wiley libraries for identification of peaks.

### ***Behavioral Recordings***

Behavioral observations in the presence or absence of a signal are important to investigate the influence and significance of a signal on a species. In this thesis, behavioral observations were performed in EBF presence / absence and also directly linked to emission dynamics. These studies are essential to gather information of the importance of different aspects of a chemical signal, such as intensity, duration or signal frequency, on chemically induced changes in the receiver's behavior to increase the knowledge of the ecological significance of EBF as an alarm pheromone and as a kairomone in short-range prey localization.

When behavioral assays were recorded, as for the studies presented in Manuscript 3 and Manuscript 4, the software "The Observer XT" Version 10.5 (Noldus Information Technology, Netherlands) was used.

Generally, predators could display one of the following behaviors: SEARCH – the predator moves on the plant, searching for prey; STOP – the predator rests on the plant, not moving; ENCOUNTER- physical contact to an aphid, ATTACK – predator bites, successfully or unsuccessfully, into an aphid; FEED – predator clearly feeds on an aphid. In Manuscript 4 only SEARCH, STOP and ENCOUNTER, were recorded and further the additional behavior WALK OFF, whereby: WALK OFF – the predator leaves the plant. This additional behavior was noted for, since the experimental design allowed the predator to leave the plant, hence terminating the observation.

In case of Manuscript 2 also the behavior of the pea aphids was accounted for. The behavior of all aphid colony members was noted, i.e. initially 10 individuals. While it was not possible to track all aphids individually, the number of aphids displaying a particular behavior was noted down, using colony modifiers (1-10). The classified behaviors were: FEED – feeding, hence sucking on plant tissue; WALK – walking; STOP – an aphid that was previously walking now stops; DROP – dropping from the host plant/leaf; WAVE – moving the antennae. Whenever aphids showed multiple behaviors, e.g. walking away and moving the antennae, the most agitated behavior was recorded, in this case

walking away. Initially, when the predator was introduced to a plant, all 10 aphids were in the state FEED. If one aphid was encountered or attacked by the predator, additional behaviors of this particular individual were recorded, i.e., KICK - aphid kicks and/or shakes its body; DROPLET – the aphid secreted at least one droplet from the cornicles; CONSUMED – attacked aphid has been consumed by the predator, and is now dead.

When alarm pheromone emission was monitored in addition to the recording of behavioral interactions as in the studies of Manuscript 3, both, the zNose<sup>TM</sup> and “The Observer XT” were started simultaneously.

*Brief differences between Manuscripts:* In Manuscript 3 the behavior of the predator, the aphid colony and the attacked aphid was accounted for, while in Manuscript 4 only the general behavior of the predator was observed. Further there were less and slightly different variables recorded for the predator in Manuscript 4, as described above.

### ***Fixating Aphids on Plants***

For some studies it is important to present real aphid prey to foraging aphid natural enemies in the field to create more realistic foraging conditions. In such cases the aphids' appearance and movements may act as a visual cue and the odor may also have an important function (Hatano et al. 2008b) , e.g. to measure foraging success or frequencies of predator-prey interactions. Since aphids show various escape behaviors after a predator encounter, such as walking away or dropping off the host plant (Dixon 1958), but it was crucial that the prey does not disperse, aphids were fixated using adhesive.

Single or small colonies of pea aphids were fixated to a plant in both, Manuscript 4 and Manuscript 5. To do so UHU All Purpose Adhesive (UHU GmbH & Co. KG, Germany) was filled into a syringe (Braun, Omnifix, 10 ml Leur Lock Solo) equipped with a fine disposable hypodermic needle (Braun, Sterican Gr. 1, 0.90 x 40 mm) for a more precise handling of the adhesive. A drop of glue was then placed with the syringe onto the plant used in the experiment (position on the plant according experimental design). In a next step an aphid was carefully taken from its host plant by a moist, not wet brush and transferred to the drop of glue on the experimental plant, by dipping the hind legs into the slightly dried glue.

Aphids were fixated directly, but at least 15 min before each experimental replicate, to allow evaporation of solvents and hence solidification of the adhesive. Aphids used in the experiments were all alive and predominantly continued feeding at the start of the experiment.

*Brief differences between Manuscripts:* While the aphids for the laboratory experiment in Manuscript 4 were fixated to the plants by co-author I. Vosteen, the aphids of the field experiments in Manuscript 4 and all aphids in Manuscript 5 were fixated by me. There were no differences in the method of fixating the aphids between the Manuscript 4 and Manuscript 5.

### ***Production of EBF Slow Releaser***

EBF is believed to be unstable and degenerate fast (Kourtchev et al. 2009; Pinto et al. 2007). When checking for a kairomone effect of EBF in the field, it is therefore important to use pheromone releasers that protect the pheromone from degrading and are capable of a controlled release. Most frequently used slow releasers are reservoir dispensers, consisting of a reservoir and a diffusion area, and solid matrix dispensers, such as polyethylene tubes (Heuskin et al. 2011). Heuskin et al. (2012) were the first to present an efficient EBF slow releaser, optimized for integrated pest management in the field: By encapsulating EBF in an alginate bead, their dispenser allows to hold but also slowly release the pheromone. Thus, the alginate beads are slow releasers.

Since EBF is emitted only in low quantities, not amplified by the colony (Manuscript 1, Hatano et al. 2008a; Verheggen et al. 2008b), and the concentration of semiochemicals released by matrix slow-release systems is believed to decrease in distance to the dispenser (Heuskin et al. 2011), hence resembling a real emission event within an aphid colony on a plant, alginate EBF slow releaser were established according to Heuskin et al. (2012) with slight modifications. The following recipe was used to establish three treatments (1. no EBF (control), 2. natural EBF amounts, 3. exaggerated EBF amounts): 4 mL sodium alginate solution (1.5 % w/v), 0.9 mL sunflower oil, 0.075 mg  $\alpha$ -tocopherol (Sigma Aldrich, Schnellendorf, Germany) and 0.00 g / 0.05 g / 0.02 g (*E*)- $\beta$ -farnesene, respectively. The compounds were mixed (Ultra Turrax IKA T18 basic) at 24.000 rpm for 30 sec. The homogenous emulsion was then extruded by a syringe (Braun, Omnifix, 10 ml Leur Lock Solo) with a fine disposable hypodermic needle (Braun, Sterican Gr. 1, 0.90 x 40 mm) in a calcium solution (0.5 M) stirring at 200 rpm due to a magnetic stir bar. The distance between the needle and the calcium solution was fixed to 20 cm. So formed spherical EBF alginate beads were maturing in the solution for 48 h and dried on filter paper thereafter. The slow releasers were produced shortly before the experiment and stored in 25 ml Falcon Tubes in the fridge at 4-6°C.

The EBF release rate of the alginate beads was calculated by measuring the real-time evaporation of EBF using a zNose<sup>TM</sup> as described above for small vial sizes.

### ***Statistical Analysis***

Data was generally analyzed using the R software 2.13.1 or 3.0.1 ([www.r-project.org](http://www.r-project.org)). All data are presented as mean  $\pm$  standard error (SE).



## Overview of Manuscripts

This thesis contains five manuscripts, of which a brief summary, the publication status and the contribution of the authors is given below.

### Manuscript I

Modulation of Aphid Alarm Pheromone Emission of Pea Aphid Prey by Predators (2013)

**Joachim, C.**, Hatano, E., David, A., Kunert, G., Linse, C., Weisser, W. W.

*Status: published in Journal of Chemical Ecology 29 (6): 773-782.*

This manuscript addresses the first question by studying the shape of the (*E*)- $\beta$ -farnesene emission pattern after predator attack. Recent studies on animal alarm signaling have shown that alarm calls are not generally uniform, but may vary depending on the type and intensity of threat. While alarm call variability has been studied intensively in birds and mammals, little is known about such variation in insects. We investigate the variability in alarm signaling in aphids, using pea aphids as a model. A handheld gas chromatograph (zNose<sup>TM</sup>), which allows real-time volatile analysis, was used to measure EBF emission by pea aphids under attack from lacewing or ladybird larvae.

We demonstrate that aphid alarm signaling is strongly affected by the predator species attacking. Ladybirds generally elicited smaller EBF emission peaks and consumed aphids more quickly, resulting in lower total EBF emission compared to lacewing attacks. In 52 % of the replicates with lacewings and 23 % with ladybirds, no EBF was detectable in the headspace although aphids secreted cornicle droplets after attack. Examining EBF amounts contained in these droplets and the aphid body revealed that while all aphid bodies always contained EBF, many secreted droplets did not. We show that alarm signaling in insects can be variable and both the attacker as well as the attacked may affect alarm signal variation. While underlying mechanisms of such variation in aphid-predator interactions need to be investigated in more detail, we argue that at least part of this variation may be adaptive for the predator and the aphid. It is revealed that there are significant predator-dependent differences in EBF release in aphids, showing that not only in mammals or birds, but also in insects there is the potential for variation in the intensity of prey alarm signaling.

CL, EH and WWW conceived and designed the experiment. CL carried out the experiment with the help of EH. MK and AD assisted in conducting the zNose<sup>TM</sup> experiment. CJ analyzed the data. CJ wrote the manuscript that was edited by CL and WWW. All authors approved the manuscript.

## Manuscript II

Real-time Monitoring of (*E*)- $\beta$ -Farnesene Emission in Colonies of the Pea Aphid, *Acyrtosiphon pisum*, Under Lacewing and Ladybird Predation

Joachim, C., Weisser, W. W.

*Status: published in Journal of Chemical Ecology 39 (10):1254–1262.*

This manuscript addresses the second question by long-term observation (24 h) of EBF emission in aphid colonies using real-time monitoring. Aphids are constantly under attack by a variety of predators and parasitoids. Upon attack, most aphids release droplets from their cornicles that may contain aphid alarm pheromone that induces escape behavior in other aphid colony members such as dropping off the host plant. While it has previously been shown that EBF emission is predator dependent, but highly variable between single aphid observations, and it was lately proposed that EBF is constantly emitted and used as a social cue, the emission dynamics of aphid colonies under predation over a long period of time are still unknown.

In this manuscript we present the first long-term observation of a preyed and unpreyed aphid colony with real-time alarm pheromone monitoring using a handheld gas chromatograph (zNose<sup>TM</sup>). We analyzed the headspace of pea aphid colonies under lacewing larvae and ladybird adult predation for 26 hours and show that there is no emission of EBF in the absence of predation. Placing ladybird adults or lacewing larvae in aphid colonies resulted in a series of emission peaks that correlated well with the number of predation events that were assessed at the end of the experiment. There was also no circadian rhythm in alarm pheromone emission and both predators were active during the night and the day. The results show that the alarm pheromone emission pattern within an aphid colony is affected by the feeding behavior of a predator. There was no evidence of a constant release of aphid alarm pheromone in an unpreyed aphid colony, suggesting that the proposed constant release could be a handling effect rather than a true constant release. This, however, needs further investigation.

CJ and WWW conceived and designed the experiment. CJ performed the experiment. CJ analyzed the data. CJ wrote the manuscript that was edited by WWW.

**Manuscript III**

Linking real-time headspace analysis to behavioural observations reveals the complex nature of alarm signalling in an insect predator-prey interaction

**Joachim, C.**, Weisser, W. W.

*Status: submitted to Proceedings of the Royal Society of London Series B*

This manuscript addresses the third question and compares real-time alarm signal emission dynamics after predation with simultaneous live observations of predator-prey behavior. In many prey, elaborate alarm communication is used to reduce an imminent risk of predation. To study costs and benefits of such alarm signals it is mandatory to link alarm signaling to prey and predator behavior. This has repeatedly been done for alarm calls in mammals and birds by combining live vocal recordings with behavioral analysis. For chemical alarm signaling such analysis has been hindered by the availability of real-time analysis techniques.

Using aphids as a model organism, we directly compare in a novel approach real-time alarm signal emission dynamics, measured by rapid gas-chromatography, with predator-prey behavioral observations. When attacked, the pea aphid emits cornicle droplets containing the aphid alarm pheromone (*E*)- $\beta$ -farnesene that causes escape reactions in conspecifics. Here we therefore analyze the headspace of small pea aphid colonies, using a zNose<sup>TM</sup>, under lacewing larvae and adult ladybird predation and record the behavior of all, the predator and aphid colony members and attacked aphids. We demonstrate that cornicle droplet production is no reliable information source for aphid colony escape response, since not only can EBF be absent in droplet presence, but also present in droplet absence. The colony escape response is induced by changes in EBF headspace amounts rather than total amount itself and stops due to EBF habituation rather than the end of EBF emission. We conclude that combining live behavioral observations with real-time headspace measurements is a promising approach to fathom chemical alarm communication and underlying chemically-mediated predator-prey interactions.

CJ and WWW conceived and designed the experiment. CJ performed the experiment. CJ analyzed the data. CJ wrote the manuscript that was edited by WWW.

## Manuscript IV

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

**Joachim, C.**, Vosteen, I., Weisser, W. W.

*Status: submitted to Chemoecology*

This manuscript addresses the fourth question by testing EBF as an arrestant cue in the field. While pheromones are intended to provide intraspecific information, these signals are sometimes utilized by species other than the intended receiver, since natural insect enemies use several environmental cues for host or prey finding. EBF is believed to be such a cue, as it has been shown to be perceived by several aphid natural enemies. It is unclear, however, if EBF is used as an arrestant stimulus or a cue for short-range prey localization on the plant.

We observed the searching behavior of larvae of the common green lacewing *Chrysoperla carnea* (Stephens) and the seven-spot ladybird *Coccinella septempunctata* L. on a plant where an aphid was fixated, in the presence and absence of EBF, and under field and laboratory conditions. EBF had no effect on predators' searching behavior, neither when natural occurring amounts of 50 ng EBF and unnaturally high amounts of 1000 ng were used. EBF did also not induce longer predator patch resistance times under laboratory (ladybird only: 600.8 $\pm$ 35.1 sec.) and field (ladybird: 644.9 $\pm$ 50.7 sec., lacewing: 1108.4 $\pm$ 49.5 sec.) 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 concluded that the aphid alarm pheromone is not used as an arrestant cue for lacewing and ladybird predators, i.e. predators do not stay longer or search more intensively before leaving the plant, nor does it have a short-range attractant function.

IV and WWW conceived and designed the laboratory experiment. CJ and WWW conceived and designed the field experiment. IV conducted the laboratory and CJ the field experiment. CJ analyzed the data. CJ wrote the manuscript that was edited by WWW and approved by IV.

**Manuscript V**

Does the aphid alarm pheromone (*E*)- $\beta$ -farnesene act as a kairomone under field conditions?

**Joachim, C.**, Weisser, W. W.

*Status: submitted to Journal of Chemical Ecology*

This manuscript also addresses the fourth question by investigating the significance of EBF as a host/prey kairomone and long-range attractant cue for aphid natural enemies in the field, since several aphid natural enemies are believed to use EBF as a host/prey finding kairomone. While electroantennogram studies and olfactometer assays demonstrate that a variety of aphid natural enemies are capable of perceiving EBF or show an attractant behavioral response to EBF presence, these studies were mainly conducted in the laboratory using EBF amounts higher than natural occurring amounts. Therefore it is unclear, if EBF is used to locate a prey in the field when only natural amounts are present.

We therefore monitored the frequencies and durations of plant visits by aphid natural enemies in the field with long-term camera observations. By placing pheromone slow releaser with no, natural and unnatural high EBF concentrations next to small, fixated colonies of pea aphids in the field, it was analyzed if EBF presence altered predator dynamics and enemies' long-range foraging behavior. We counted thirteen groups of potential aphid natural enemies in 720 h of analyzed video material. The observation showed that EBF had no influence on the number of target (i.e., aphid colony) visits as well as the patch (i.e., monitored plant) and target residence times. The number of patch visits was, however, treatment dependent and increased in the presence of high concentrated EBF slow releaser.

We conclude that while there seems to be an attractant effect of high EBF concentrations on some aphid predators under specific environmental conditions and can hence be of great advantage in agricultural anti-pest management strategies, the kairomone effect of natural occurring EBF concentrations is highly doubtful and may be of less ecological importance.

CJ and WWW conceived and designed the experiment. CJ performed the experiment. CJ analyzed the data. CJ wrote the manuscript that was edited by WWW.

## Discussion

The aphid alarm pheromone (*E*)- $\beta$ -farnesene was subject of many studies since the early 1970s (Bowers et al. 1972). While the first studies mainly focused on the production and secretion of cornicle droplets, and the behavioral reactions of aphids perceiving the alarm signal, more and more studies looked at the attractive effect of EBF for aphid natural enemies (Hatano 2010; Verheggen 2008), the ecology of aphid alarm communication and the costs for the emitter and its colony members associated with such signaling (c.f. Vandermoten et al. 2012). Thus far, a detailed study on the emission dynamics and its influence on aphid-predator interaction has, however, been hindered due to elaborate pre-concentration techniques and only recently an emission time course pattern of alarm pheromone has been described for an attacked aphid individual (Schwartzberg et al. 2008).

This thesis enhances the knowledge and contributes to the understanding of the emission dynamics of the aphid alarm pheromone (*E*)- $\beta$ -farnesene and reveals its ecological implications under natural field conditions (see below): EBF emission dynamics and the colony response after perceiving the alarm pheromone are of complex nature and secretion of cornicle droplets cannot necessarily be equated with EBF emission. Further aphid alarm signaling displays great variations between predator species. And while (*E*)- $\beta$ -farnesene secretion bears several physiological costs, such as delaying offspring production, the ecological cost of attracting additional predators in the field is of less importance as previously expected.

## Alarm pheromone Emission Dynamics

Predation is an imminent threat and therefore an immediate alarm signal after an attack is crucial for aphid survival and inclusive fitness (Losey and Denno 1998). Hitherto, aphid alarm pheromone emission was believed to be closely connected to cornicle droplet production, i.e. the production of a cornicle droplet was equal to alarm pheromone emission (Mondor et al. 2000). The detailed study of the EBF emission is a new subject of aphid alarm pheromone research (Schwartzberg et al. 2008) and while the first analyses only was an initial approach to fathom EBF emission patterns, the majority of such dynamics remained unknown. Therefore the first part of the thesis investigates EBF emission dynamics in more detail, in particular if aphid-predator interactions and aphid escape response could be explained by the EBF emission dynamics.

## EBF Emission

Cornicle droplets are almost exclusively secreted after attacks that are fatal for the attacked aphid individual (Manuscript 3). Interestingly only a fraction of the EBF stored in an aphid's body is

set free in cornicle droplets after an attack by a predator (Manuscript 1): While pea aphids, for instance, contain  $89.70 \pm 11.11$  ng (*E*)- $\beta$ -farnesene, only 10 % to 16 % of the stored EBF volatilizes from the cornicle secretions (Manuscript 1). And while all aphids appear to produce and store alarm pheromone, up to 60 % of the emitted cornicle droplets do not contain alarm pheromone (Manuscript 1). Sometimes EBF is even present without droplet secretion; probably set free from body fluids due to the predator's feeding process (Manuscript 3). Therefore droplet production cannot be equated with alarm signaling or EBF emission. Also the likelihood of an aphid to produce cornicle droplets varies: While Mondor et al. (2000) found that almost 100 % of the pre-reproductive stages emitted cornicle droplets due to an attack, in Manuscript 1 only 30 % and 70 % secreted droplets after fatal ladybird and lacewing attacks, respectively. These significant differences between predator species lead to the suggestion that droplet production may be predator species dependent, because droplets are produced in higher frequencies when under lacewing attack than when under ladybird attack (c.f. Manuscript 1).

The emission of (*E*)- $\beta$ -farnesene of a single aphid after an attack follows a characteristic time course pattern (Fig. 2). With the new method of quantifying the amount of EBF in the headspace of an attacked pea aphid in two minute intervals with the help of rapid gas chromatography (zNose™) Schwartzberg et al. (2008) were the first to monitor and describe the emission of (*E*)- $\beta$ -farnesene in almost real time: An initial burst of the alarm signal, i.e. fast volatilization of EBF from the cornicle droplet resulting in an increase of alarm pheromone in the headspace, is followed by an exponential decline, i.e. the amount of EBF decreases slowly over time. The initial fast increase or burst of EBF is most likely the most important cue for the escape response in colony conspecifics, because they react within the first few seconds after perceiving the signal (Schwartzberg et al. 2008), before the peak emission is reached, at different EBF amounts in the surrounding air and even when EBF is already present in the headspace due to a previous attack (Manuscript 3). Further colony members stop their escape behavior before EBF declined completely in the headspace and at different concentration levels (Manuscript 3). Consequently, the escape behavior is most likely induced by an increasing EBF stimulus, i.e. a new burst, rather than total EBF amounts in the air and stops due to EBF habituation rather than depletion. The habituation assumption is in line with other studies that demonstrated that aphids are indeed capable of EBF habituation (de Vos et al. 2010). While the total number of colony members that display defense behavior is increased in EBF presence and seems to depend on the predator species attacking (Manuscript 3), Dill et al. (1990) proposed that the escape response is sensitive to the costs that the tactic evokes. Costs like giving up high quality feeding sites, walking to areas of unknown predation risks or dropping in a hostile environment decrease the chance of survival. When not under immediate attack, i.e. encountered or touched in course of an attack, conspecifics have the option to stay or to face the costs. This may be the reason why most

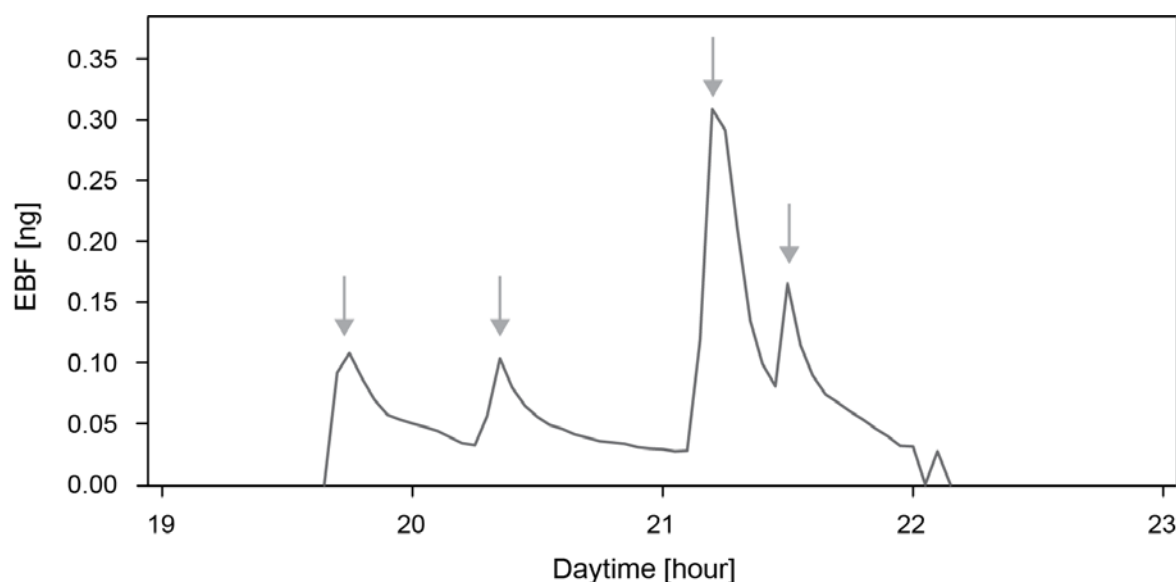
aphid colony conspecifics show escape behavior almost exclusively after lethal attacks and not after a predator only encountered an aphid (Manuscript 3).

By describing and comparing the emission curves, Schwartzberg et al. (2008) demonstrated that both the total EBF emission, i.e. the total amount of alarm pheromone emitted after the attack, and peak emission, i.e. the maximum amount of EBF measured in one of the measurement intervals, were higher in nymphs (1<sup>st</sup> to 4<sup>th</sup> instar) than in adult aphids. No difference was observed in the speed of emission, i.e. the time period between the onset of the predator attack and the peak emission (Schwartzberg et al. 2008). The emission patterns differ, however, not only between the instars, but also between the predator species attacking (Manuscript 1): While the emission curve after an attack is always of the same shape, as described above, the duration of aphid alarm signaling is increased and more EBF is emitted (total amounts) under lacewing larvae predation than after ladybird larvae attack.

When the emission of EBF is not only monitored in single aphids, but in aphid colonies, representing a more natural environment, the ideal shape of the emission curve is altered into different shapes (see Fig. 1 in Manuscript 2). As in single aphid observations, the EBF emission within an aphid colony is generally restricted to a certain period of time after a predation event has occurred. Based on the frequencies of predator attacks and EBF emission intensity (total EBF released, duration of emission), there is the possibility of overlapping emissions in natural predator-prey systems, i.e. while EBF generally dissipates in aphid colonies after an attack, due to e.g. airflow or wind, a new attack at the time point when EBF is still present in the headspace, can lead to an anew increase of total EBF amounts and an extension of EBF presence around the aphid colony under attack (Fig. 4, Manuscript 2, Manuscript 3). So, the alarm pheromone level in an aphid colony is not constant, but rather an up and down with a concentration of zero in-between.



Fig. 4 - Excerpt of an 24 h aphid alarm pheromone (E)- $\beta$ -farnesene (EBF) emission pattern, i.e. time course pattern of the amount of EBF measured in the headspace over a 24 h period, of an pea aphid colony under attack by a ladybird larva (data from ladybird replicate #7, Manuscript 2). Grey arrows indicate a predation event with subsequent EBF emission by the attacked aphid. Detailed information of the method can be found in Manuscript 2.



### Constant Release of EBF

Almohamad et al. (2008) were the first to describe a constant emission of alarm pheromone in aphid colonies that were not preyed upon. By collecting headspace volatiles of *Myzus persicae* (Sulzer) colonies on charcoal filters over a period of three hours they revealed a quadratic relationship between the amount of released EBF and aphid colony size.

A study conducted for this thesis, however, suggests that EBF is not constantly released in absence of aphid predators (Manuscript 2). Since EBF was measured in detectable amounts by the zNose<sup>TM</sup> at the beginning of almost each experimental replicate in Manuscript 2, right after placing the aphids into the experimental vessel, it seems possible that the relocation of aphids, even if it appears to be gently to humans, acts as drastic disturbance to aphids, resulting in EBF release. This finding is in accordance with other studies, where also no natural EBF was detected in non-preyed aphid colonies when exposed to synthesized deuterated EBF, as measured by charcoal filter trapping of volatiles with subsequent GC-MS analysis (Hatano et al. 2008a). Additionally, Verheggen et al. (2008b) also did not find any alarm pheromone in a setup where they measured the headspace of aphid infested plants, by trapping the headspace volatiles on an adsorbent filter with subsequent GC-FID analysis. While a handling effect is very reasonable, another potential reason for the difference

between these studies could be the aphid species, since Almohamad et al. (2008) used the green peach aphid *M. persicae* and the other studies the pea aphid *A. pisum*.

A constant emission of EBF would come with obvious costs, since it could (in-) directly affect the aphid colony fitness. First, aphids are known to habituate to EBF, i.e. displaying decreased escape behavior upon EBF detection after an attack, and are hence more likely to be consumed by predators (de Vos et al. 2010). Second, releasing even small amounts of pheromone can trigger unnecessary dispersal (Kunert et al. 2005). One often observed behavior of aphids after perceiving EBF is increased walking behavior, which leads to increased physical contact between aphids. As a consequence aphids are known to produce a higher proportion of winged morphs among their offspring (Kunert et al. 2005), that, as a cost, could leave potentially good feeding sites, i.e. disperse although they would not need to. Last, a constant release, even at small release rates, could have the cost of disclosing the presence of the colony to predators (Hatano et al. 2008b).

So, a constant release of EBF is associated with great costs. Acting as a social cue is therefore highly doubtful for the aphid alarm pheromone and would need further evidence.

### **Variation in EBF Emission**

Not only mammals and birds (i.a. Seyfarth et al. 1980; Templeton et al. 2005; Zuberbühler 2001), but also insects show the potential for variation in alarm signaling, since there are significant predator dependent differences in EBF emission dynamics and alarm signal response in aphids (Manuscript 1 - 3). Predator dependent variation in alarm signaling is believed to be dominant in species that are prey of a number of predators with different foraging and feeding behaviors that require different escape tactics (Macedonia and Evans 1993; Manser et al. 2002) and aphids are herbivore prey of several insect enemies (Hatano et al. 2008b).

Quantitative and qualitative variation in alarm signaling within aphid instars, i.e. EBF amounts in cornicle droplets significantly increase in pre-reproductive stages with the maximum in third and forth instar and then decrease again in reproductive or post-reproductive stages, can be explained by the more clustered colony structure and higher levels of predation of pre-reproductive stages, since young instar aphids are more likely to be killed by predators than adults (Mondor et al. 2000). This is in line with findings of Keiser et al. (2013), who found that pre-reproductive aphids that emit more EBF feed at plant structures of great predation risk by foliar foraging predators and they hence suggest that this provides inclusive fitness benefits for conspecifics.

Potential drivers for differences in emission patterns and EBF presence in cornicle droplets between predator species could be 1) the predator or 2) the attacked aphid. When the variation of alarm signaling between predator species is aphid driven, this would imply an evolutionary adaption to the type of predator attacking. Hence, aphid alarm signaling would either be egoistic and an individual fitness benefit (Edwards 1966), altruistic and hence an inclusive fitness benefits, as seen for kin selection (Maynard-Smith, 1965), or mutualistic and hence beneficial for both, the sending organism and the colony clone mates. It is important to distinguish if droplets and EBF emission is always intended for a receiver, if it is primary a defense function of if it is just the consequence of arousal (Zuberbühler 2008).

If the variation in EBF emission is aphid driven, one hypothesis is that aphids are able to actively regulate the amount of alarm pheromone in the cornicle droplets in situations where the costs of alarm signaling outweigh the benefits. Since alarm pheromone presence in cornicle droplets displays a great variation (Manuscript 3), a potential trigger for aphids to regulate the pheromone content of their droplets, although highly speculative, could be the predator species itself: Is it a generalist or specialist? Is it a predator that will stay on a plant until the whole plant was observed and all aphids have been consumed or is it a predator that is just there for a flying visit? When the attacked aphid gets the information that the attacking predator is the type that just randomly searches for prey with a short residence time, based for instance on olfactory cues or feeding strategy, the attacked aphid may decrease or completely cease its EBF emission, since the risk that conspecifics drop from the host plant and may starve to death is greater than the benefit gained from warning (Dill et al. 1990). If the attacking predator, on the other hand, is the type that will stay on the plant for a longer period of time or until all aphids on a plant are consumed, it seems more reasonable to warn colony conspecifics. This adaption would favor the inclusive fitness, as shown for cornicle length (Mondor and Messing 2007) and scent marking predators (Mondor and Roitberg 2004). This hypothesis seems, however, not reasonable, since ladybirds are known to switch their foraging behavior from “extensive search” to a local “area-concentrated search” after having captured a prey (Nakamuta 1985), while the frequencies and amounts of EBF emitted by the attacked aphid under ladybird predation are lower as compared to lacewing predation (Manuscript 1, Manuscript 3). Not warning conspecifics would hence not be beneficial under ladybird predation, but rather increases the predation risk for colony conspecifics as the predator continues foraging. Increasing EBF emission or scent marking predators with cornicle secretions that contain alarm pheromone, as described by Mondor and Roitberg (2004), that display area concentrated search

behavior such as ladybirds, would seem more beneficial, when an aphid would indeed actively regulate the amount of EBF in cornicle droplets.

Another possibility is that the variation of EBF presence in cornicle droplets between predator species is adapted and optimized for self-defense, hence adapted to an individual fitness benefit. Aphids may actively change the composition of droplet components to the specific, predator dependent needs for self-defense, i.e. droplets have beneficial properties with/without EBF individually adjusted to the predator species attacking. Dixon (1958) proposed that releasing a droplet before a ladybird predator got hold on the attacked aphid, i.e. attacking it but not securing aphid body hold, can lead to an increased chance of escape, hence having a direct benefit. Contrary, in experiments made for this thesis cornicle droplets were almost exclusively secreted when the attacked aphid died (Manuscript 3), regardless of whether EBF was present or absent. Hence, although there are significant difference in EBF presence in droplets between predators (Manuscript 2, Manuscript 3), cornicle secretion in general has no direct benefit for the releasing individual, given the fact that the attacked aphid died in almost all cases. The study of Wu et al. (2010) were in line, by showing that cornicle droplets provide no direct fitness benefit against parasitoid wasps and should be considered as altruistic behavior, since the secretion increased with the number of colony conspecifics that can benefit. So, an individual adjustment of cornicle droplet components for self-defense benefits seems very unlikely when attacked by predators, see above, or parasitoids (Goff and Nault 1974; Wu et al. 2010). While secreting cornicle droplets does not reduce the probability of the sending individual to be consumed or parasitized, smearing cornicle droplets on predators or parasitoids, however, can reduce the rate of consumption or oviposition of colony members and hence could be considered as an kin-directed altruistic behavior (Mondor and Roitberg 2004; Wu et al. 2010).

A more likely possibility of aphid driven variation in EBF emission/presence, are physiological reasons. While all aphids contain EBF (Manuscript 1), there is great variation in EBF amounts released by individual aphids (Schwartzberg et al. 2008). Deformations in morphological structures that are associated with droplet production and EBF incorporation could lead to such variation in EBF emission. An inherent disability to secrete pheromone containing droplets could, however, not explain predator based differences in EBF presence or absence. Since the exact biosynthesis of (*E*)- $\beta$ -farnesene is still unknown (Vandermoten et al. 2012), a more detailed understanding of the mechanisms involved in how and when EBF is incorporated in cornicle droplets is required to verify this assumption.

If the predator is the driving force of the emission variation between predator species, there are two potential reasons how they could have an influence on the emission dynamics. The first possibility is that the feeding process of the attacking predator affects the EBF emission. For example, severely lacerating an aphid or damaging the secretory cell sac after an attack due to the predator's distinct feeding strategy, may lead to a decreased turgor pressure that may lead to a decreased amount of droplets secreted, hence fewer cases with EBF presence. There is, however, no difference in the number of droplets produced between aphid predators (Manuscript 3), although the EBF presence of cornicle secretions is predator depended (Manuscript 1). So, other internal injuries or tissue damages that originate from the feeding mode, in such way that no EBF can enter the droplet or is set free during the feeding period, either because the base of the siphunculi is injured, or because the injury prevents signal transduction to initiate EBF release, could cause the variation. Morphological studies of attacked aphids with previous EBF analysis would be needed to test this hypothesis.

Interestingly, live behavioral observations coupled with headspace EBF measurements showed that a rigorous feeding predator can cause an attacked aphid to leak body fluids with simultaneous EBF presence, while no cornicle droplets were secreted after a predator successfully attacked an aphid (Manuscript 3). This leads to the suggestion that also the predator's feeding mode could influence EBF presence or absence. The second hypothesis consequently is that the aphid natural enemies' foraging and feeding strategies evolved to control the emission of the aphid alarm pheromone. That aphid enemies are able to camouflage their attacks has already been shown for the predatory gallmidge *Aphidoletes aphidimyza* Rondani (Lucas and Brodeur 2001): The furtive feeding strategy of *A. aphidimyza*, by inconspicuously piercing the aphid's integument, injecting a paralyzing toxin and then feeding on the prey's body fluids, does not cause aphids to emit cornicle droplets, hence does not induce alarm signaling and prey dispersal, which is beneficial for the predator. Studies done for this thesis are along with this finding, since the specific foraging strategy and the feeding behavior of the predators used (lacewing larvae and ladybird larvae and adults, as described in the Materials and Methods section) match with the observed EBF emission patterns (Manuscript 1 - 3). While ladybirds rapidly consume their aphid prey within around 80 sec., the amount of alarm pheromone emitted by the attacked aphid is lower and the duration of emission shorter as in comparison with other predators (Manuscript 1), hence the dispersal of aphid colony conspecifics is decreased under ladybird predation (Manuscript 3). This leads to the suggestion that ladybirds consume or destroy most body parts that contain or produce EBF, hence suppression the emission. This would be beneficial for ladybirds, given that they generally consume many aphid individuals on the same plant (Hodek and Honěk 1996). As shown for other predator species, a fast-consumption behavior may have evolved in ladybirds to enhance the efficiency of feeding in aggregated prey

groups (Curio 1976) and to camouflage their attacks and stay inconspicuous on plants that host herbivore prey. Lacewings in contrast, as the other tested predator species in this thesis, show a different foraging and feeding strategy (Canard and Duelli 1984): They only consume a few aphids per day and slow down their movements and rest after successful feeding. Since they are piercing, sucking predators, slowly consuming their prey, the EBF signaling is increased in EBF amounts, emission durations and droplets containing EBF. Based on their feeding behavior there is probably little chance to suppress EBF emission and their resting behavior after prey consumption may most likely be an adaptation to the disturbance caused in the prey colony due to alarm signaling (Manuscript 1). Hence, there is also evidence that the predator itself may be the driving force for variation in aphid alarm signaling. A detailed comparison study of feeding strategies and alarm pheromone emission for several aphid enemies could shed light on the assumption of predator modulation of EBF emission.

Aphid alarm signaling is of complex nature and presumably influenced by multiple factors. While the benefits seem clear cut, since EBF emission warns colony conspecifics of impending danger, the underlying mechanisms, e.g. who is in control of emission variation or if alarm signaling is in favor of direct or indirect fitness benefits, are sophisticated. Having outlined the main hypothesis of predator dependent variations in EBF emission, one could speculate that EBF emission, from the aphid's point of view, is solely altruistic and for the benefit of the highly related colony members, since droplet production seems to have no benefit for the secreting aphid. The variation in EBF presence might further be most likely explained by the feeding strategies of the predators, whether they are a consequence of injuries in the attacked aphid or by evolutionary adapted predator foraging and feeding tactics to camouflage their attacks.

While this thesis gained more insight into aphid alarm signaling and EBF emission dynamics by using novel detection methods, more research on the topic of alarm signal variation is desirable to reveal the driving factors of emission variation and to verify these final, speculative assumptions. This could help to fully understand the dynamics of EBF signaling in a cost-benefit-framework.

## Kairomone Effect of EBF

When discussing the kairomone effect of the aphid alarm pheromone (*E*)- $\beta$ -farnesene and its ecological implication in a natural context it is important to consider the definition of a kairomone. Nordlund and Lewis (1976) defined a *kairomone* as

*“A substance, produced, acquired by, or released as a result of the activities of an organism, which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to the receiver but not to the emitter”.*

While semiochemicals are ideal for organisms to communicate and coordinate their behaviors, there is the possibility that such signals are overheard and exploited by organisms other than the intended conspecific receivers, hence bearing a cost for the sending organism (Vandermoten et al. 2012). Chemical cues mediating the foraging behavior of aphid natural enemies have been reviewed explicitly by Hatano et al. (2008b): Several aphid enemy species are able to perceive or show attractive behavioral response to EBF, displaying variation in larval and adult stages.

When the ability to perceive EBF is tested using electroantennograms (EAG), low amounts up to 100  $\mu$ g did not elicit any reaction in scouts of the ant *Lasius niger* (L.) but 1000  $\mu$ g did (Verheggen et al. 2012). The EAG response of two tested lacewing species differed: Male and female adults of the green lacewing *Chrysoperla carnea* (Stephens) showed a neurological response towards (*E*)- $\beta$ -farnesene at concentrations between 0.1 and 1000  $\mu$ g (Zhu et al. 1999). The highest response was, however, observed at a dose of 1000  $\mu$ g. For adults of the Asian lacewing *Chrysopa cognata* (McLachlan), in contrast, no EAG response was obtained, when amounts between 1 and 10,000  $\mu$ g were tested (Boo et al. 1998). Adults of the ladybird species *Coccinella septempunctata* L., *Coleomegilla maculata* (Degeer) and *Harmonia axyridis* (Pallas) showed response to EBF in both, EAGs and single cell recordings when exposed to concentrations ranging from 1 ng – 10  $\mu$ g, 0.1 ng – 1  $\mu$ g or when 10  $\mu$ g were tested, respectively (Al Abassi et al. 2000; Verheggen et al. 2007; Zhu et al. 1999). The electrophysiological activity for EBF was also confirmed for naïve females of the parasitoid wasp *Aphidius ervi* Halliday (Du et al. 1998). Coupled gas chromatography-electroantennography revealed that *A. ervi* showed positive response to EBF at concentrations of 1  $\mu$ g and 10  $\mu$ g, respectively. Also the syrphid fly *Episyrphus balteatus* (Degeer) displays significant EAG response in EBF presence between 1  $\mu$ g and 1,000  $\mu$ g, but no response at concentrations between 1 ng and 100 ng, using increments by x10 (Verheggen et al. 2008a). Thus, the ability to perceive EBF has been confirmed for several important aphid natural enemies at different concentration level.

When behavioral assays were conducted to show a positive behavioral response towards EBF or test if it is used as an attractant or arrestant, mainly olfactometer experiments were performed. While tending the aphid species *Chaitophorus populicola* Thomas adults of the ant *Formica subsericea* Say displays a positive behavioral response, i.e. raising and extending the antennae, opening the mandibles, and starting to walk, when exposed to 2 ng to 2 µg EBF, but not when exposed to 0.2 ng (Nault et al. 1976). Also adult scouts of *L. niger* was attracted and orientated towards plants where pure aphid alarm pheromone was released in unknown concentrations by a rubber septum in a two-choice bioassay (Verheggen et al. 2012). When exposed to cornicle droplets that containing EBF of one individual of *C. populicola*, the Argentine ant *Linepithema humile* (Mayr) exhibit increased aggression and double the number of visits to an aphid colony (Mondor and Addicott 2007), hence showing a positive behavioral change after exposition to the alarm pheromone. When the utilization of EBF as a prey finding kairomone was tested for males and females of the three ground beetle species *Pterostichus melanarius* Illiger, *Harpalus rufipes* Degeer and *Nebria brevicollis* Fabricius using a four-arm olfactometer *P. melanarius* and *H. rufipes* both showed a positive response to one of two synthetic EBF extracts (Kielty et al. 1996). The extracts were produced the same way following Dawson et al. (1982) by passing nerolidol through an evacuated column of alumina, treated with pyridine. The fact that both extracts were a blend of different β-farnesene isomers, one of the two extract contained an isomeric mixture of the start-compound nerolidol, and that the two carabid species displayed positive behavior to another synthetic extract respectively, leads to the suggestion that other compounds besides (*E*)-β-farnesene may have had an influence on the behavior. *N. brevicollis* however, did not show any response to both extracts. No attraction was observed to 10 mg EBF for adults of the lacewing *C. cognata* when presented in Y-tube olfactometer assays (Boo et al. 1998). Also field trapping of *C. carnea* was not increased when 50 mg EBF were presented on cotton roll dispensers in alfalfa fields (Zhu et al. 2005). Almost all tested ladybird species displayed a positive response towards EBF emission. First and second instars of the ladybird *Adalia bipunctata* (L.) were attracted to EBF when presented at the concentration of 1 µg and 2 µg or 2 µg, respectively (Francis et al. 2004; Hemptinne et al. 2000). Also adult *A. bipunctata* were attracted by concentrations of 2 µg EBF in four-arm olfactometer assays (Francis et al. 2004). Adult seven-spot ladybirds (*C. septempunctata*) were significantly attracted by 10 µg EBF (Al Abassi et al. 2000), but when applied at concentrations of 200 ng it does not alter the locomotary pattern of the local search behavior (Nakamuta 1991). Also field trapping of *C. maculata* was not increased when 50 mg EBF were presented on cotton roll dispensers in alfalfa fields (Zhu et al. 2005). Further *H. axyridis* adults did not change their foraging behavior when exposed to the cornicle secretion of five pea aphids (Mondor and Roitberg 2000). Adults of *Hippodamia convergens* Guérin-Méneville, in contrast, perceive and orient towards 1 µL EBF when applied to a piece of filter



paper in an eight-arm olfactometer (Acar et al. 2001). Naïve and experienced adult females of the parasitoid wasp *A. ervi* show significantly increased oriented flight behavior when exposed to 10 µg EBF in wind tunnel bioassays (Du et al. 1998). And while naïve adult females of *A. uzbekistanicus* Luzhetskii were not attracted to EBF at concentrations of 0.6 µg, 1.4 µg and 2.9 µg EBF, but to 5.7 µg, experienced females were attracted to lower concentrations starting at 1.4 µg (Micha and Wyss 1996). Virgin females of *Diaeretiella rapae* (McIntosh) displayed different positive behavioral changes when exposed to EBF concentrations of 0.3 µg, 3.0 µg and 30 µg, respectively (Foster et al. 2005). And while experienced *D. rapae* were attracted to 40 EBF secreting cabbage aphids (*Brevicoryne brassicae* (L.)) in Y-olfactometers, naïve females did not (Moayeri et al. 2014). Alarm pheromone concentrations of 2.4 µg, 24 µg and 119 µg elicited a positive response in adult females of *Lysiphelebus testaceipes* (Cresson), where the parasitoids significantly decreased their host examination times (Grasswitz and Paine 1992). When applied in concentrations of 2.4 ng, 24 ng and 240 ng there was, however, no difference to the control. Also adult females of *Praon volucre* (Haliday) were significantly attracted to EBF volatiles in an Y-tube olfactometer (Micha and Wyss 1996). The syrphid fly *E. balteatus* increased the time of flight and acceptance of the host plant when exposed to 40 µg EBF (Verheggen et al. 2008a) and 1<sup>st</sup> instar larvae are attracted to EBF volatiles in an four-arm olfactometer (Francis et al. 2005b).

Besides application of pure EBF or cornicle secretion also crushed aphid species, consequently releasing the stored alarm pheromone, were attractive for the ant *Lasius niger*, the ladybirds *Adalia bipunctata* and *Harmonia axyridis*, the parasitoid *Lysiphelebus testaceipes*, and 1<sup>st</sup> instar *Episyrphus balteatus* larvae (Francis et al. 2004; Francis et al. 2005b; Grasswitz and Paine 1992; Verheggen et al. 2012; Verheggen et al. 2007).

A detailed list of studies, to the best of my knowledge, checking for a kairomone effect of EBF can be found in Tab. 2.

So, based on the studies conducted so far, there is strong evidence that (*E*)-β-farnesene acts as a host or prey finding kairomone or foraging cue in many aphid natural enemies, hence bearing a potential cost for the sender by disclosing its presence and position within a habitat. This evidence is, however, weak because of at least two reasons:

1. The experimental design of these studies was often not optimized for revealing the ecological relevance of (*E*)-β-farnesene as a kairomone in natural predator-prey interactions. Measuring electroantennogram responses only gives information of the potential of predator to use EBF as a kairomone, but does not reveal if the natural enemy actually

employs EBF as a chemical signal in aphid localization or general foraging. Olfactometers assays, in contrast, do reveal the true behavioral response to a chemical compound, but here the amounts used were often well above natural emission rates and have hence no ecological implication. Realistic field studies with natural emission amounts, in contrast, would provide a better way to clarify the ecological role and importance of EBF.

2. More important, while these studies performed experiments with variable EBF concentrations, they generally used EBF amounts that were much higher than the amounts typically emitted in natural aphid-predator interactions. Before defining a substance as a particular semiochemical, one should always know the natural release or emission rates of a substance (Byers 1988). When discussing the function of a substance it is important to consider the definitions with respect to the implicit quantitative qualification, since substances could cause a specific reaction when presented in unnatural high concentrations but are irrelevant in natural occurring concentrations and have hence no ecological relevance (Byers 1988). Recent studies showed that the amounts emitted by a single pea aphid can range from 0.27 to 48.85 ng (Manuscript 1, Schwartzberg et al. 2008), while the mean emission found by Schwartzberg et al. (2008) for aphids attacked by lacewing larvae was  $16.33 \pm 1.54$  ng and the mean emission found in the study presented in Manuscript 1 was  $12.55 \pm 2.14$  and  $2.29 \pm 0.46$  for lacewing and ladybird larvae, respectively. So, the alarm pheromone amounts used in the studies on the kairomone effect of EBF should be within a plausible range of the maximum amounts, considering the number of possible aphid attacks by natural enemies per hour.

Tab 2 - List of studies on the kairomone effect of the aphid alarm pheromone (E)- $\beta$ -farnesene tested in several aphid natural enemy species. The list comprehends the tested aphid enemy species, the test methods, the amount of EBF (when applied manually), the source of EBF, the experimental outcome whether the enemy species showed a response towards EBF (yes) or not (no) and the reference of the study.

Enemy taxa	Aphid enemy species	Enemy life stage	Test method	EBF amount ( $\mu$ g)	Other EBFsource	Aphid species	Plant species	Significant reaction	Reference
Ants	<i>Formica subsericea</i>	adult	behavioral observation (aphid tending)	0.0002				no	Nault et al. (1976)
		adult	behavioral observation (aphid tending)	0.002, 0.02; 0.2; 2				yes	Nault et al. (1976)
	<i>Lasius niger</i>	adult scout	EAG	1; 10; 100				no	Verheggen et al. (2012)
		adult scout	EAG	1000				yes	Verheggen et al. (2012)
		adult scout	4-arm olfactometer		5 crushed aphids	<i>Aphis fabae</i>	-	yes	Verheggen et al. (2012)
		adult scout	two-choice bioassay	?				yes	Verheggen et al. (2012)
Ground beetles	<i>Linepithema humile</i>	adult	behavioral observation (colony entrance)		cornicle secretion from 1 aphid	<i>Chaitophorus populicola</i>	<i>Populus fremontii</i>	yes	Mondor and Addicott (2007)
	<i>Pterostichus melanarius</i>	adult	4-arm olfactometer	? (mix with other components)				yes	Kielty et al. (1996)
	<i>Harpalus rufipens</i>	adult	4-arm olfactometer	? (mix with other components)				yes	Kielty et al. (1996)
	<i>Nebria brevicolis</i>	adult	4-arm olfactometer	? (mix with other components)				no	Kielty et al. (1996)
Lacewings	<i>Chrysoperla carnea</i>	adult	field trapping	50,000				no	Zhu et al. (1999)
		adult	EAG	0.1 - 1000				yes	Zhu et al. (1999)
	<i>Chrysopa cognata</i>	adult	EAG	1 - 10,000				no	Boo et al. (1998)
		adult	Y-tube olfactometer	10,000				no	Boo et al. (1998)
Ladybirds	<i>Adalia bipunctata</i>	1 <sup>st</sup> instar	4-arm olfactometer	1; 2				yes	Hemptinne et al. (2000)
		1 <sup>st</sup> instar	4-arm olfactometer		40 mg crushed aphids	<i>Acyrtosiphon pisum</i>	-	yes	Hemptinne et al. (2000)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer	2				yes	Francis et al. (2004)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer		0.25 $\mu$ g crushed aphids	<i>Acyrtosiphon pisum</i>	<i>Vicia faba</i>	yes	Francis et al. (2004)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer		0.25 $\mu$ g crushed aphids	<i>Myzus persicae</i>	<i>Vicia faba</i>	yes	Francis et al. (2004)

Ladybirds (continued)		2 <sup>nd</sup> instar, adult	4-arm olfactometer	2				yes	Francis et al. (2004)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer		0.25 µg crushed aphids	<i>Myzus persicae</i>	<i>Brassica napus</i>	yes	Francis et al. (2004)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer		0.25 µg crushed aphids	<i>Myzus persicae</i>	<i>Sinaphis alba</i>	yes	Francis et al. (2004)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer	0	0.25 µg crushed aphids	<i>Brevicoryne brassicae</i>	<i>Brassica napus</i>	no	Francis et al. (2004)
		2 <sup>nd</sup> instar, adult	4-arm olfactometer	0	0.25 µg crushed aphids	<i>Brevicoryne brassicae</i>	<i>Sinaphis alba</i>	no	Francis et al. (2004)
	<i>Coccinella septempunctata</i>	4 <sup>th</sup> instar	search time		1 crushed aphid	<i>Acyrtosiphon pisum</i>	-	yes	Stubbs (1980)
		Adult	EAG	0.001 – 10				yes	Al Abassi et al. (2000)
		Adult	2-way olfactometer	10				yes	Al Abassi et al. (2000)
		Adult	behavioral observation (search pattern)	0.2				no	Nakamuta (1991)
	<i>Coleomegilla maculata</i>	Adult	EAG	0.1 - 1000				yes	Zhu et al. (1999)
		Adult	field trapping	50,000				no	Zhu et al. (1999)
	<i>Harmonia axyridis</i>	Adult	EAG	10				yes	Verheggen et al. (2007)
		Adult	4-arm olfactometer		20 crushed aphid	<i>Myzus persicae</i>	-	yes	Verheggen et al. (2007))
		Adult	behavioral observation (search pattern)		cornicle secretion from 5 aphids	<i>Acyrtosiphon pisum</i>	<i>Vicia faba</i>	no	Mondor and Roitberg (2000)
Parasitoids	<i>Hippodamia convergens</i>	Adult	8-arm olfactometer	0.001				yes	Acar et al. (2001)
	<i>Aphidius ervi</i>	adult female (naïve)	EAG	1 - 10				yes	Du et al. (1998)
		adult female (naïve)	wind tunnel bioassay	10				yes	Du et al. (1998)
		adult female (experienced)	wind tunnel bioassay	10				yes	Du et al. (1998)
		adult female (naïve)	two-choice bioassay		cornicle secretion from 10 aphids	<i>Acyrtosiphon pisum</i>	<i>Vicia faba</i>	yes	Battaglia et al. (1993)
	<i>Aphidius uzbekistanicus</i>	adult female (naïve)	Y-tube olfactometer	0.6; 1.4; 2.9				no	Micha and Wyss (1996)
		adult female (naïve)	Y-tube olfactometer	5.7				yes	Micha and Wyss (1996)
		adult female (naïve)	Y-tube olfactometer		5 cornicle droplets in 10 µl solution	<i>Sitobion avenae</i>	-	no	Micha and Wyss (1996)
		adult female (experienced)	Y-tube olfactometer	0.6				no	Micha and Wyss (1996)

Syrphid flies	Parasitoids (continued)	adult female (experienced)	Y-tube olfactometer	1.4; 2.9; 5.7				yes	Micha and Wyss (1996)
		<i>Diaeretiella rapae</i> virgin females	behavioral observation (misc. foraging parameters)	0.3. 3.0, 30				yes	Foster et al. (2005)
		adult female	GC-EAG		Transgenic, EBF producing plant	-	<i>Arabidopsis thaliana</i>	yes	(Beale et al. 2006)
		adult female	behavioral observation (foraging time)		Transgenic, EBF producing plant	-	<i>Arabidopsis thaliana</i>	yes	(Beale et al. 2006)
		adult female (naïve)	Y-tube olfactometer		40 secreting aphids	<i>Brevicoryne brassicae</i>	<i>Brassica oleracea</i>	no	Moayeri et al. (2014)
		adult female (experienced)	Y-tube olfactometer		40 secreting aphids	<i>Brevicoryne brassicae</i>	<i>Brassica oleracea</i>	yes	Moayeri et al. (2014)
		adult female (naïve)	two-choice bioassay (oviposition)		4 secreting aphids	<i>Brevicoryne brassicae</i>	<i>Brassica oleracea</i>	no	Moayeri et al. (2014)
		adult female (experienced)	two-choice bioassay (oviposition)		4 secreting aphids	<i>Brevicoryne brassicae</i>	<i>Brassica oleracea</i>	no	Moayeri et al. (2014)
		<i>Lysiphelebus testaceipes</i> adult female (naïve)	Y-tube olfactometer	5.7				no	Micha and Wyss (1996)
		adult female	two-choice bioassay		cornicle secretion from 10-15 aphids	<i>Rhopalosiphum padi</i>	-	yes	Grasswitz and Paine (1992)
		adult female	two-choice bioassay		cornicle secretion from 10-15 aphids	<i>Aphis nerii</i>	-	no	Grasswitz and Paine (1992)
		adult female	behavioral observation (oviposition)	0.0024, 0.024, 0.24				no	Grasswitz and Paine (1992)
		adult female	behavioral observation (oviposition)	2.4, 24, 119				no	Grasswitz and Paine (1992)
		adult female	behavioral observation (oviposition)		homogenates of 2 aphids	<i>Rhopalosiphum padi</i>	-	yes	Grasswitz and Paine (1992)
		adult female	behavioral observation (oviposition)		homogenates of 2 aphids	<i>Aphis nerii</i>	-	no	Grasswitz and Paine (1992)
		<i>Praon volucre</i> adult female (experienced)	Y-tube olfactometer	5.7				yes	Micha and Wyss (1996)
		<i>Episyrphus balteatus</i> 1 <sup>st</sup> instar	4-arm olfactometer	2				yes	Francis et al. (2005b)
		1 <sup>st</sup> instar	4-arm olfactometer		250 mg crushed aphids	<i>Acyrtosiphon pisum</i>	<i>Vicia faba</i>	yes	Francis et al. (2005b)
		1 <sup>st</sup> instar	4-arm olfactometer		250 mg crushed aphids	<i>Megoura viciae</i>	<i>Vicia faba</i>	yes	Francis et al. (2005b)
		1 <sup>st</sup> instar	4-arm olfactometer		250 mg crushed aphids	<i>Aphis fabae</i>	<i>Vicia faba</i>	yes	Francis et al. (2005b)
		adult females and males	EAG	0.001 - 0.1				no	Verheggen et al. (2008a)
		adult females and males	EAG	1 - 1,000				yes	Verheggen et al. (2008a)
		adult females and males	behavioral observation (oviposition)	40		<i>Acyrtosiphon pisum</i>	-	yes	Verheggen et al. (2008a)

**EBF as a Kairomone in Prey Localization**

Host or prey localization in aphid natural enemies generally follow a hierarchical behavioral pattern before encountering a prey (Vinson 1976): The first step is the long-range localization of a habitat where prey might be present. The second step is then short-range localization of the prey itself within the chosen habitat. Since many predatory and parasitic insects utilize chemical cues for both steps of prey localization (Carde and Bell 1995; Fellows et al. 2005), the aphid alarm pheromone EBF could consequently be utilized as a potential kairomone for both, long and short range localization.

To investigate if aphid natural enemies utilize (*E*)- $\beta$ -farnesene in one of these localization steps at ecological relevant concentrations, hence bearing a potential cost for the sender, two assumptions were hypothesized and eventually tested (Manuscript 4, Manuscript 5):

- I. Aphid natural enemies utilize (*E*)- $\beta$ -farnesene as a kairomone for long-range attraction under natural emission conditions.
- II. Aphid natural enemies utilize (*E*)- $\beta$ -farnesene as a kairomone for short-range attraction and as an arrestant cue under natural emission conditions.

***Long Range***

In contrast to positive EAG perception results and positive behavioral assays of aphid enemies towards EBF in the laboratory (Tab. 2), no increase in natural enemy visits or patch residence times were observed when EBF is presented in the field with natural emission rates (Manuscript 5). By placing small artificial aphid colonies on plants next to EBF slow releaser, these findings suggest that aphid natural enemies do not use the aphid alarm pheromone (*E*)- $\beta$ -farnesene as a kairomone for host/prey localization in the field under natural conditions. Despite the usage of exaggerated vs. natural pheromone amounts, this difference between laboratory and field studies is also in line with the study of Zhu et al. (1999): While adults of the ladybird *Coleomegilla maculata* showed active EAG response to EBF, there was no difference in field trappings between control and EBF treatments, where visual sticky traps were equipped with cotton roll dispensers impregnated with 50 mg EBF in an alfalfa field. So laboratory studies that show an attractant effect of EBF, even when high doses were used, may not resemble attraction under natural field conditions.

Nevertheless, applying exaggerated EBF amounts in the field resulted in increased total plant visits of aphid enemies (Manuscript 5). This was, however, only the case when the group of natural

enemy was included in the analysis as an independent variable, i.e. the frequencies of appearances per replicate was individually accounted for each enemy group, but not when the analysis was done independent of the particular predator species. This is in accordance with studies that found that application of high EBF amounts, i.e., 1 mg in controlled-release dispensers and  $0.45 \mu\text{g h}^{-1}$  as set free by slow releasers, respectively, can attract additional predators, such as syrphid flies, ladybirds and parasitoid wasps (Aphidiidae), in agricultural fields and can hence decrease the infestation pressure of crop (Alhmedi et al. 2010; Cui et al. 2012). So while certain natural enemies show an attractive behavior towards high concentrations of EBF in the field, this has no ecological implication for EBF being a kairomone per definition, since natural concentrations do not show any effect.

Instead of using EBF as a kairomone for long-range attraction, other chemical cues seem more beneficial under natural conditions. The reason is that aphid natural enemies, as all other insect predators and parasitoids, face the 'reliability-detectability' problem (Vet and Dicke 1992): While volatiles directly emitted by herbivores, e.g. EBF, give reliable information about their presence, these cues are often only present in low, inconspicuous concentrations. Plant-derived volatile compounds, in contrast, can be detected more easily due to their greater quantities, but are less reliable. While it has been shown for several parasitoids that they are attracted to undamaged host plants most of the aphid enemies seem not to use these kind of plant derived volatiles as a long range attractant cue (c.f. Hatano et al. 2008b). Prey induced plant volatiles seem to convey more reliable information of the presence of aphid colonies while also detectable over a long range. There are several studies that demonstrate that plants change their emission profile under herbivory and that aphid parasitoids are attracted by such volatile cues (Du et al. 1998; Guerrieri et al. 1999; Powell et al. 1998). Herbivore induced changes can be highly specific and differ in total quantities of volatiles released (Turlings et al. 1998) or in different compositions of the volatile blend (De Moraes et al. 1998; Du et al. 1998). Laboratory studies on the attraction of aphid predators to herbivore induced plant volatiles have, however, often failed to show an effect (Hatano et al. 2008b), but they are believed to frequently visit plants to feed on nectar or pollen.

### **Short Range**

After a natural enemy located a habitat where potential prey might be present, it generally switches its foraging behavior to short-range localization of the prey itself within the habitat (Fellows et al. 2005; Hatano et al. 2008b). Also here, EBF seems not to be utilized as a foraging cue or short range kairomone, at least in two important aphid predators – the larvae of green lacewings *Chrysoperla carnea* and the seven-spot ladybird *Coccinella septempunctata* (Manuscript 4). When natural emission amounts are applied to a piece of filter paper next to a fixated aphid on a plant, EBF

did not cause the predators to change their foraging behavior in the benefit of EBF. Particular value was put on the distinction between the potential *arrestant* and potential *attractant* role of EBF. While *attractant* stimuli can direct the searching forager to areas containing prey, i.e. guiding the forager towards the prey, the perception of *arrestant* stimuli results in a reduction in distance or area covered per unit of time, i.e. increasing the residence time of a forager in the area where prey is present (Fellows et al. 2005). EBF did not trigger the predators to stay and search longer or more intensively within the habitat; also did the predators not find the fixated aphid in higher frequencies in EBF presence as compared to the control (where no EBF was applied). Consequently (*E*)- $\beta$ -farnesene seems to have neither an arrestant nor an attractant influence on these aphid predators. Therefore aphid alarm signaling does not represent a cost for the sender and its colony by attracting additional predators in the field.

However, aphid natural enemies utilize various other chemical and physical cues to locate a suitable herbivore prey on the host plant (Hatano et al. 2008b). Important physical cues for aphid predators and parasitoids encompass visual and locomotive stimuli (Hatano et al. 2008b). Coccinellid adults for instance are believed to be able to discriminate between an aphid and non prey objects under light conditions by their size and shape with a visual perceptive distance between 2-7 mm (Nakamuta 1984). Color seems to be an important foraging cue in many ladybird species (Harmon et al. 1998; Mondor and Warren 2000) and adults and larvae can perceive color contrast and orientate towards objects having the sharpest color contrast against the background (Khalil et al. 1985). Parasitoid wasps are also known to use color in host evaluation (Michaud and Mackauer 1994), but also use locomotive cues, such as aphid movement (Michaud and Mackauer 1995).

In contrast to EBF, other chemicals have been shown to act as a kairomone in aphid enemies' short range prey localization. Honeydew, for instance, provides reliable information of aphid presence, but is believed to be only effective over a short range and unlikely to act over long distances until plants become infested heavily (Hatano et al. 2008b). In coccinellids, for instance, honeydew acts as a contact kairomone, where ladybird larvae display an alternate foraging behavior after they have been in close (physical) contact with honeydew (Buitenhuis et al. 2004; Carter and Dixon 1984; Ide et al. 2007). The amount or concentration of honeydew, however, is believed to have no influence on ladybird foraging (Carter and Dixon 1984). Aside from ladybirds, also other aphid natural enemies, such as parasitoid wasps and lacewings, have been shown to induce intensive area-restricted foraging behavior or increase their rate of oviposition on direct contact with honeydew or, for syrphid flies (Diptera – Syrphidae), after perceiving honeydew volatiles (Budenberg 1990; Budenberg and Powell 1992; Budenberg et al. 1992; McEwen et al. 1993; Purandare and Tenhumberg 2012).



### ***Functionality of EBF as a Kairomone***

But why is EBF not utilized as a kairomone (as defined above) by aphid natural enemies? There are several reasons why natural occurring concentrations of the aphid alarm pheromone (*E*)- $\beta$ -farnesene might not be suitable cue for prey detection. First, aphid alarm pheromone emission should be naturally selected for inconspicuousness to avoid parasitism and predation (Vet and Dicke 1992). From an observer's point of view this is the case for (*E*)- $\beta$ -farnesene, since it is present exclusively in the event of a predator attack, and not even after every single attack (Manuscript 3). Further the pheromone is only emitted in low quantities, not amplified by the colony (Manuscript 1; Hatano et al. 2008a; Schwartzberg et al. 2008; Verheggen et al. 2008b). So, the amount of alarm pheromone in the headspace of an aphid colony is very limited. While this sets limitation to the detection ability of aphid enemies, EBF is also believed to degenerate fast when in contact to air (Kourtchev et al. 2009; Pinto et al. 2007). The chance of being transported over a long range before disappearing is therefore questionable under natural conditions, and Kourtchev et al. (2009) argue that EBF might only have an impact on a local scale, close to its emission source. A short range dissipation, due to ozone degradation, plant structure and wind turbulence, is therefore more likely and would give natural enemies only limited possibilities to utilize it as a kairomone. Second, EBF gives no reliable information of aphid presence. While EBF emission implies a predation event at the source of emission, it further indicates that the colony is probably disturbed and potentially scattered due to escape behavior, e.g. dropping from the host plant or walking away (Dixon 1998; Wohlers 1981). EBF acts only as an indicator that aphids had been present at some point, but are not necessarily still present at the source of emission at the time of the receiver's arrival. In reverse, when EBF is absent in the air, a predator cannot assume that there are no aphids in close vicinity whatsoever, but rather that there is no additional aphid natural enemy attacking an aphid. Hence, the utilization of chemical signals solely disclosing prey existence or presence, such as prey odors or prey waste products, should preferentially be selected for by natural predators, instead of signals triggering dispersal at the source of emission. Third, relying on EBF as a kairomone cue may be also a disadvantage for the receiver in terms of competition. Since EBF is emitted only after attacks, the presence of EBF may also indicate the presence of a competitor or intraguild predator at the source of emission. Since intraguild predation is very common among aphidophagous predators (Lucas 2005), competition might have a direct influence on the receivers foraging success and also instant survival (Keddy 2001). Thus, utilizing cues that imply the chance of competition and increase mortality risks should not be selected for. The deterrent effect of alarm pheromone for competing enemies in terms of survival has been demonstrated for the predatory gallmidge *Aphidoletes aphidimyza* Rondani (Hatano 2010): Larvae of *A. aphidimyza* display a furtive feeding strategy which does not cause aphids to emit cornicle droplets, hence preventing the aphids to display alarm

pheromone induced escape behavior such as moving around. When alarm pheromone is, however, perceived by the gallmidge larvae, they leave the plant, since this indicates the presence of another predator in the close vicinity, consequently increasing the risk of falling prey to intraguild predation. By being beneficial to both, the sender, i.e. aphid, and the receiver, i.e. gallmidge, EBF has the ability to function as a *synomone* in this specific interaction (Dicke and Sabelis 1988; Nordlund and Lewis 1976), but again not as a kairomone.

In contrast to the aphid alarm pheromone (*E*)- $\beta$ -farnesene the exploitation of alarm pheromones has, however, been demonstrated in other predator-prey systems: Allan et al. (1996) showed that the cursorial spider *Habronestes bradleyi* (Zodariidae), a specialist predator of the meat ant *Iridomyrmex purpureus* (Smith) is attracted by natural occurring amounts of its alarm pheromone 6-methyl-5-hepten-2-one, emitted by ant workers engaged in agonistic interactions. As another example *Orius tristicolor* White and the predator mite *Amblyseius cucumeris* (Oudemans) change their foraging behaviour when perceiving the alarm pheromone of the western flower thrip, *Frankliniella occidentalis* (Pergande), utilizing it as a prey-finding kairomone and as an arrestant cue (Teerling et al. 1993). Hence, other studies are able to clearly induce specific reactions to an alarm pheromone when applied at natural concentrations.

Although the utilization of EBF as a kairomone seems highly unlikely in a natural ecological context (Manuscript 4, Manuscript 5), the attraction of some natural enemies to high or exaggerated EBF amounts is proven by some studies, and hence without question (see above). Even in observations made during this thesis, although not significant, there was a slight trend for some aphid predators to be attracted by unnaturally high, i.e. exaggerated, EBF amounts (Manuscript 5). In treatments where around 1000 ng EBF were set free per hour by alginate slow releasers, Polistinae (Hymenoptera: Vespidae) and adult syrphid flies (Diptera: Syrphidae) showed increased visits of plants where the pheromone was released – the attractant effect of exaggerated EBF amounts in the field could, however, generally not be confirmed. So although one cannot refer to EBF as a kairomone, it has the ability to work as an attractant in unnatural concentrations. A potential reason why some aphid enemies show attractant behavior to exaggerated EBF amounts can be found in the study of Vandermoten et al. (2011). They showed that the English grain aphid, *Sitobion avenae*, and two aphid natural enemies (the marmalade hoverfly *Episyrphus balteatus* and the multicolored Asian lady beetle *Harmonia axyridis*) utilize highly conserved odourant-binding proteins for the recognition of (*E*)- $\beta$ -farnesene, while orthologous genes are absent in other insect species. This indicates that

these proteins evolved for the detection of the same alarm pheromone in three distinct insect orders. The presence of these proteins in the predators might, however, be a relic of aphids' evolutionary adaption to avoid eavesdropping by natural enemies in minimizing EBF emission, since it EBF is not utilized in a natural emission framework. Despite that, it has been shown, that unnaturally high concentrations of a chemical substance can alter a receiver's behavior in a way natural concentrations would not: By releasing different ratios and concentrations of the sex pheromone and its components of different moth species, Roelofs (1978) for instance showed, that the behavioral response to a sex pheromone can change from attraction to inhibition at unnaturally high concentrations.

However, since some studies cannot confirm a long range attractant effect of unnaturally high concentrations of EBF in the field (e.g. Manuscript 5, Zhu et al. 1999), but some can (e.g. Alhmedi et al. 2010; Cui et al. 2012) the attractant effect of exaggerated EBF amounts may not be a general phenomenon, but rather dependent on 1) the concentration of EBF applied, i.e., how overdosed the releasers are, since e.g. the releasers used in Manuscript 5 were still less concentrated than that of e.g. Heuskin et al. (2012) that were used by Cui et al. (2012), or 2) the environmental setting, i.e., vegetation, time of the year, climatic region, since some studies were conducted in agricultural crop fields (c.f. Alhmedi et al. 2010; Cui et al. 2012; Zhu et al. 1999) and others on fallow grassland (c.f. Manuscript 5), some in Europe (c.f. Manuscript 5, Alhmedi et al. 2010) and others in China (c.f. Cui et al. 2012) or the USA (c.f. Zhu et al. 1999). Especially the environmental setting can have a major impact on the predator diversity and community (Russell 1989).

Nevertheless, the utilization of EBF in anti pest management strategies can be of economic and agricultural interest. It has been shown that transgenic *Arabidopsis thaliana* (L.), that are capable of producing and emitting EBF, can increase the foraging time in parasitoids (Beale et al. 2006). The continuous alarm pheromone perception in aphids reared on transgeneic *A. thaliana* further leads to a habituation of aphids to their alarm pheromone EBF that results in a decreased escape response, hence lower survival rate (de Vos et al. 2010). The fact that habituated aphids are more likely to be eaten by their predators seems to be a promising control strategy (de Vos et al. 2010) and while the emitted amounts of *A. thaliana* range from undetectable to over 880 ng per hour (Beale et al. 2006), these amounts might be too small for attracting additional predators in the field (Manuscript 5). A combination of transgenic plants (to habituate the aphid pest) and an easy, cost effective application of alginate EBF slow releaser (to attract additional enemies), as produced and optimized by Heuskin et al. (2012), could be a promising approach in anti pest management strategies for aphids - especially in times with a general trend towards organic farming and the wish to enhance the use of biological insecticides (Zehnder et al. 2007). Future work on the amounts and the environmental

framework for application of exaggerated EBF amounts with and without transgenic plants could shed light on the exploitation of the aphid alarm pheromone for organic pest management.

## Conclusion

On the example of aphids, this thesis contributes to the understanding of alarm communication in insects. By utilizing the novel technique of rapid gas chromatography to measure headspace volatiles of individual aphids and aphid colonies under attack, as initially introduced by Schwartzberg et al. (2008), it gains fundamental new insight in the basic processes of aphid alarm pheromone emission and particularly in the emission dynamics of single aphids and aphid colonies with and without predation. While it demonstrates great variation in aphid alarm signaling, and is therefore in line with previous studies on this topic, it exceeds these studies by answering the questions, asked in the Introduction, which arose from previous research on aphid alarm signaling and its associated costs and benefits:

1. Are the dynamics of (*E*)- $\beta$ -farnesene emission pattern affected by the predator species that attacks the aphid?

There are differences between predator species in (*E*)- $\beta$ -farnesene emission dynamics after an aphid was attacked, i.e. total pheromone amounts, emission duration, height of peak emission. However, it is still unclear what can explain these differences. While the attacker as well as the attacked may affect alarm signal variation, as discussed in this thesis, more studies are essential to further understand this interesting aspect of aphid alarm signaling.

2. How are (*E*)- $\beta$ -farnesene emission dynamics shaped in aphid colonies with and without predation?

While there is no evidence for a constant release of EBF in aphid colonies, as described by previous studies, EBF emission of aphid colonies under attack is always restricted to clearly defined periods of time, never occurs continuously and is most likely driven by the feeding behavior of the predator species foraging on the plant. Further, there is no circadian rhythm in alarm pheromone emission.

3. How do (*E*)- $\beta$ -farnesene emission dynamics affect aphid-predator interaction?

Aphid alarm signaling is of complex nature, but combining live behavioral observations with real-time headspace measurements is a promising approach to fathom it. While the attacked individual almost always dies, independent of EBF or droplet presence or absence, EBF can be

absent in droplet presence and present in droplet absence. The colony escape response is induced by increasing EBF stimuli rather than total pheromone amounts, stops due to EBF habituation and is more dependent on the predator attacking rather than EBF presence.

4. Do aphid natural enemies utilize (*E*)- $\beta$ -farnesene as a cue in the field?

(*E*)- $\beta$ -farnesene is not used as an arrestant cue nor has it a short- or long-range attractant function for important aphid natural enemies in the field when presented in natural occurring amounts. Hence EBF seems to have no ecological implications under natural conditions and cannot be considered a kairomone in natural settings. When, however, presented in exaggerated amounts EBF can play a promising role in agricultural pest management strategies, since it has the potential of attracting additional aphid natural enemies.

Consequently, the present work provides evidence to understand the aphid alarm pheromone emission dynamics in more detail and to elucidate the role of aphid alarm signaling in predator-prey interaction. However, several new questions arose, concerning EBF incorporation, perception distance and longevity of EBF, and should be addressed in future research:

- When and how is (*E*)- $\beta$ -farnesene incorporated in the cornicle droplets?
- How stable is (*E*)- $\beta$ -farnesene? How fast does it evaporate in the field and over what distance does it play a role in aphid defense behavior?
- Do aphid colony conspecifics react to (*E*)- $\beta$ -farnesene or a breakdown product?
- Is (*E*)- $\beta$ -farnesene stable enough to pass the predator's digestive track and leave cues on the host plant, hence increase the inclusive fitness of aphid alarm signaling, not only by smearing events, but also by their feces?
- What are ideal concentrations of (*E*)- $\beta$ -farnesene to attract additional aphid enemies in the field and what is the ideal environmental setting in which EBF could be used for agricultural pest management?

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## Curriculum Vitae

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### Education

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| 05/2011-12/2013 | PhD student at Technische Universität München, Germany.<br>Proceeding of Thesis – Costs and benefits of alarm signaling in aphids                   |
| 09/2010-04/2011 | PhD student at Friedrich-Schiller-University Jena, Germany.<br>Thesis – Costs and benefits of alarm signaling in aphids                             |
| 06/2008         | Diploma /Mc in biology at the University of Hohenheim, Germany. Diploma thesis - Biodiversität und Palökologie fossiler Insekten des Randecker Maar |
| 12/2007-05/2008 | Graduand at the State Museum of Natural History Stuttgart, Germany  |
| 09/2003-06/2008 | Biology student at the Department of Biology, University of Hohenheim, Germany. Major in zoology, animal ecology and parasitology                   |

### Conference contributions

- Presentations
  - **Joachim, C.** and Weisser, W.W. (2013): Aphid-predator interactions: Costs and Benefits of Alarm Communication in Aphids. *43rd Annual Meeting of the Ecology Society of Germany, Austria and Switzerland*, University of Potsdam, Potsdam, DE.
  - **Joachim, C.**, Hatano, E. and Weisser, W.W. (2012): Aphid-predator interactions: The emission of alarm pheromone in an aphid colony under predation. *11th IMPRS Symposium*, MPI for Chemical Ecology, Dornburg, DE.
  - **Joachim, C.** (2008): Fossil insects from the Randeck Maar. 5. Bonner Paläoentomologen-Treffen, Bonn, DE.
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- Posters
  - **Joachim, C.**, Linse, C. and Weisser, W.W. (2011): Competition for mutualists between plants and aphids, and the role of aphid alarm pheromone. *10th IMPRS Symposium*, MPI for Chemical Ecology, Dornburg, DE
  - Rasser, M.W., Böttcher, R., Ebner, M., Göhringer, F., **Joachim, C.**, Kovar-Eder, J., Nebelsick, J.H., Roth-Nebelsick, A., Schoch, R., Schweigert, G. & Ziegler, R. (2010): Das Randecker Maar: Ökologische und klimatische Proxies eines untermiozänen Kratersees). *80th Annual Meeting of the Paläontologischen Gesellschaft München*, München, DE.
  - **Joachim, C.** (2008) - Biodiversity of fossil insects from the Early Miocene of the Randeck Maar (Swabian Alb, SW Germany). *Molasse Meeting 2008*, Stuttgart, DE.

## List of Publications

**Joachim C.**, Weisser W.W. (2013) Real-time monitoring of (E)- $\beta$ -farnesene emission in colonies of the pea Aphid, *Acyrtosiphon pisum*, under lacewing and ladybird predation. *Journal of Chemical Ecology* 39(10): 1254-1262.

Rasser, M.W., Bechly, G., Böttcher, R., Ebner, M., Heizmann, E.P.J., Höltnke, O., **Joachim, C.**, Kern, A.K., Kovar-Eder, J., Nebelsich, J.H., Roth-Nebelsick, A., Schock, R.R., Schweigert, G. and Ziegler, R. (2013): The Randeck Maar: Palaeoenvironment and habitat differentiation of a Miocene lacustrine system. *Palaeogeography, Palaeoclimatology, Palaeoecology* 392: 426-453.

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Rasser, M.W., Battenstein, M., Dynowski, J.F., Friedrich, J-P., Göhringer, F., Grein, M., **Joachim, C.**, Kamenz, M., Kapitzke, M., Koblizek, P., Lehmkuhl, A., Nebelsich, J.H., Rieter, M. and Roth-Nebelsick, A. (2012): Ein neues Sedimentprofil vom Randecker Maar (Unter/Mittelmiozän, Schwäbische Alb). *Jahresbericht und Mitteilungen des oberrheinischen geologischen Vereins*.

**Joachim, C.** (2010): Biodiversität und Palökologie fossiler Insekten des Randecker Maar (Unter-Miozän, SW-Deutschland). *Documenta Nature* 179:1-108.