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Real-Time Monitoring of (*E*)-β-Farnesene Emission in Colonies of the Pea Aphid, *Acyrthosiphon pisum*, Under Lacewing and Ladybird Predation

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Abstract Aphids (Homoptera) are constantly under attack by a variety of predators and parasitoids. Upon attack, most aphids release alarm pheromone that induces escape behavior in other colony members, such as dropping off the host plant. In the pea aphid, *Acyrthosiphon pisum* Harris (Aphididae), the only component of this alarm pheromone is the sesquiterpene (*E*)-β-farnesene (EBF). EBF is thought to act as a kairomone by attracting various species of parasitoids and predators including lacewings and ladybirds. Lately, it also was proposed that EBF is constantly emitted in low quantities and used by aphids as a social cue. No study has focused on emission dynamics of this compound over a long time period. Here, we present the first long-time monitoring of EBF emission in aphid colonies using real-time monitoring. We used a zNose™ to analyze the headspace of colonies of the pea aphid, under lacewing (Neuroptera: Chrysopidae) and ladybird (Coleoptera: Coccinellidae) predation, over 24 hr. We found no emission of EBF in the absence of predation. When either a ladybird adult or a lacewing larva was placed in an aphid colony, EBF was detected in the headspace of the colonies in the form of emission blocks; i.e., periods in which EBF was emitted alternating with periods without EBF emission. The number of emission blocks correlated well with the number of predation events that were determined at the end of each experiment. There was no circadian rhythm in alarm pheromone emission, and both predators were active during both night and day. Our results show that alarm pheromone emission pattern within an aphid colony is driven by the feeding behavior of a predator.

Keywords Aphid alarm pheromone · Emission activity · *Chrysoperla carnea* · *Coccinella septempunctata* · zNose™ · Circadian rhythm

Introduction

Chemical communication through volatile organic compounds is common among plants and animals (Dudareva et al. 2004; Müller-Schwarze 2006; Wyatt 2003). Semiochemicals are not released continuously, but their emission often is dependent on a trigger and emission lasts only for a short period of time. The amount of volatiles released depends on a number of factors, including the physiological state of the sender organism (Agelopoulos and Pickett 1998). Often, a low number of molecules is sufficient to trigger a specific behavior in the receiver, because both production and release of the signaling molecules and the olfactory system of the receiver have evolved to be highly selective and sensitive.

For detection, quantification, and identification of semiochemicals, several non-destructive methods that allow collection of volatiles from the headspace of living organisms are available (Torto 2004). Most commonly used are preconcentration techniques, in which headspace volatiles are first absorbed and pre-concentrated on organic polymers, such as activated charcoal or Tenax®, then desorbed, either by rapid heat or elution with solvent, and finally analyzed by gas chromatography (Agelopoulos and Pickett 1998). These methods lack real-time monitoring capability, due to compound

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handling, but real-time methods are available, including proton transfer reaction mass spectrometry (PTR-MS), using online measurements of reaction of H_3O^+ ions with a trace constituent (Hansel et al. 1995), and electronic noses, application-specific sensor systems based on chemical gas sensors, optical sensor systems, mass spectrometry, ion mobility spectrometry, gas chromatography, or a combination of these (Röck et al. 2008).

Aphids (Hemiptera: Aphididae) are sap-sucking insect herbivores that live in colonies, often consisting of clones, because of parthenogenetic reproduction. Aphids are attacked by a wide range of predators including lacewings (Boo et al. 1998; Zhu et al. 1999) and ladybirds (Al Abassi et al. 2000; Acar et al. 2001; Francis et al. 2004; Verheggen et al. 2007; Zhu et al. 1999). When attacked by a predator, individuals may release a small droplet from their abdominal cornicles containing an alarm pheromone (Edwards 1966). While the chemical composition of alarm pheromone varies among species (Francis et al. 2005), in many species such as the pea aphid, *Acyrthosiphon pisum* (Harris), the sesquiterpene (*E*)-β-farnesene (EBF) is the only component (Francis et al. 2005; Nault and Bowers 1974). When perceived by conspecifics, this alarm pheromone triggers various escape behaviors, ranging from withdrawal of the stylet, dropping off the host plant, kicking, or simply walking away (Dixon 1998).

EBF can be perceived by a number of natural enemies and has, therefore, been hypothesized to act as a kairomone, attracting predators and parasitoids to aphid colonies (Hatano et al. 2008). Aphid alarm signaling has been studied through observations of droplet excretion, gas chromatography/mass spectrometry analysis of droplet content, and headspace analysis using pre-concentration techniques and electronic noses. For EBF emission by pea aphids under attack, the frequency of droplet secretion (Mondor et al. 2000), the amount of alarm pheromone in a droplet (Joachim et al. 2013; Mondor et al. 2000), and the amount of EBF that volatizes after an attack (Joachim et al. 2013; Schwartzberg et al. 2008) vary considerably. Amounts of EBF released after an attack by ladybirds or lacewings range from <1 ng to almost 50 ng (Joachim et al. 2013; Schwartzberg et al. 2008). However, all these measurements after predation have been obtained in experiments in which a single aphid was exposed to a predator, or attacked by a human experimenter, and may not reflect the emission dynamics under more natural conditions, i.e., when a predator forages freely in an aphid colony. Because real-time measurement of alarm pheromone emission has not been possible until recently, little is known about the emission dynamics of EBF in aphid colonies. For example, there is no information on the temporal patterns of alarm pheromone emission. These might be expected to vary in a day because various aphid predators are supposed to have periods of activity or rest; e.g., ladybirds supposedly rest during the night, while lacewings are active when it is dark.

Recently, pre-concentration techniques have been used to analyze EBF emission in aphid colonies in the absence of predation, leading to the idea that EBF is released constantly in low concentrations from aphid colonies not attacked by predators (Almohamad et al. 2008; Verheggen et al. 2009). Over a three-hour observation period, EBF emission ranged from ca. 3–195 ng in colonies of 25–125 individuals (Fig. 1 in Almohamad et al. 2008). This constant release was suggested to serve aphids as an indicator for colony density (Almohamad et al. 2008; Verheggen et al. 2009).

In this paper, we used a zNose™ 4300 (Electronic Sensor Technology, Newbury Park, CA, USA), a handheld rapid gas chromatograph capable of repeated quantitative sampling of headspace odors (Kunert et al. 2002), to study the dynamic emission of EBF by pea aphids under attack by ladybird adults or lacewing larvae over a period of 24 hr. We asked the following questions: (1) Do aphids emit EBF when there is no attack by a predator? (2) Does the constant presence of a predator in an aphid colony result in continuous EBF release in an aphid colony? (3) Is there a difference in the daily patterns of EBF emission between aphid colonies attacked by ladybird or lacewing predators?

Methods and Materials

Experimental Plants and Animals Red clones of the pea aphid, originally collected in Bayreuth, Germany, were used in experiments. Aphids were reared on 2-wk-old dwarf broad bean plants, *Vicia faba* L. (The Sutton; Nickerson-Zwaan, UK), with an approximate height of 10–15 cm, grown in 10 cm-diam. flowerpots, and covered with air-permeable cellophane bags (18.5×39.0 cm, Unipack GmbH, Germany) to avoid aphid escape. Plants used in the experiment were all off similar size.

Eggs of lacewings, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), and ladybirds, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), were obtained from a commercial supplier (Katz Biotech AG, Baruth, Germany). Hatching larvae were reared on bean plants infested with pea aphids until they reached the second instar (lacewing) or the adult stage (ladybirds).

Rearing of aphids and plants, and experiments, took place under the same conditions (20 °C, 75 % RH, photoperiod 16: 8 L: D; light on at 6 am, light off at 10 pm).

Experimental Procedure We employed a split-brood design to control for any effect of previous rearing conditions on aphid alarm pheromone emission. By distributing individuals from one line equally among treatments, any variation due to rearing conditions should be equally distributed over all treatments (Kunert et al. 2005). To do this, we initiated 10 lines, by placing 10 adult foundress aphids (F_0 generation), randomly collected from a single population of the same clone, on 10 bean plants, where they were allowed to reproduce for 24 hr

before being removed. After 8–9 d, the offspring (F_1 generation) reached 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 hr. The resulting offspring (F_2 generation) were used for the experiment as soon as they reached third or fourth instar (after another 5–7 d). A split-brood design was achieved by distributing 20 F_2 individuals from one line among one replicate each of the ladybird and the lacewing treatment. In addition, five replicates without predators were set up from another five lines.

To start an experiment, bean plants without a predator were placed in an odorless 250 ml glass pot and 20 aphids (3rd–4th instar) were randomly chosen from a line and placed on the plant. Plants were positioned in a 3000 ml flange vessel (Schott, DN 150, flat bottom, 160×265 mm) with a three-necked lid (Schott, DN 150). Two openings of the flange vessel were sealed with a plug (custom made at MPI for Chemical Ecology, Jena, Germany; one with a tube connection and the other with two air-outlet holes). The third neck was covered with a septum plug (Gebr. Rettberg GmbH, Göttingen, Germany). The stainless steel inlet needle of the zNose™ was inserted through a small hole in this septum. Charcoal-filtered air was blown into the vessel by connecting an air pump (Thomas Memmingen, Germany) to a Rotilabo® charcoal filter (Carl Roth, Karlsruhe, Germany) and then to a 1/8″ PTFE tube that entered the vessel through the opening with the plug holding the tube connection. The tube reached to the bottom of the vessel, where it formed an O-ring that was perforated. The flow-rate into the vessel was adjusted to 200 ml min^{-1} using a flowmeter (Key Instruments, Trevose, PA, USA). Air left the chamber through the opening closed by the plug with air outlets. Thus, the air in the 3000 ml vessel was exchanged once about every 15 min. There was a slight overpressure in the vessel due to the small size of the air-outlet holes. After every replicate, the vessel was cleaned three times with boiling water.

A single zNose™ sample includes three phases: 1) sampling and trapping of volatiles, 2) discharge of the trapped compounds onto the column, followed by a specifically designed temperature-programmed elution with subsequent detection, and 3) recovery phase of the surface acoustic wave (SAW) detector (Kunert et al. 2002). The zNose™ was programmed to sample air within the vessel every 180 s., with a flow-rate of 30 ml min^{-1} for 60 s, drawing a total of 30 ml of air through its pre-concentration trap. Helium (Linde, Helium 6.0 T 10, ultra high purity) was supplied by a large gas cylinder.

After introducing the aphid colony to the vessel, emission from colonies was monitored for at least 1 hr before a predator was introduced. A single predator was transferred to the plant, and EBF emission was monitored over at least the next 24 hr. Experiments were started at any time during the day, between 8 am and 6 pm. At the end of an experiment, aphids were counted to calculate the number of predation events.

Calibration of the zNose™ Calibration was achieved with a heated desorber tube (3100 Vapor Calibrator, Electronic Sensor Technology, Newbury Park, CA, USA) attached to the Luer inlet of the zNose™. The SAW detector was set to 40 °C. A dilution series of 0.25, 0.5, 1, 2, 3, 4, 6, and 10 μg EBF/ml was made by dissolving EBF (Bedoukian Research Inc., Danbury, CT, USA) in methanol (Carl Roth Germany, 99.8 %). An aliquot (0.5 μl) of each diluted sample was injected into the heated tube (190 °C) with a syringe while the instrument was sampling (10 s, trapping the total amount of the injected solution). Volatized samples were eluted under the programmed conditions. Each concentration was tested at least five times. EBF was identified by comparison to synthetic standard. Regression analysis showed that the response of the SAW detector to EBF changed in a linear fashion. The calibration curve was described by $y(x)=3723.9x$, ($R^2=0.98$, $P<0.001$, $N=44$), where y = response of the SAW detector (in Hz) and x = amount (ng) 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. This corresponded to 0.027 ng EBF.

For calculating the total amount of aphid-emitted EBF, a dilution series of EBF in methanol at concentrations of 5, 10, 15, and 30 μg/ml was prepared. An aliquot (1.0 μl) of each diluted sample was applied with a syringe on a piece of filter paper (1 cm×1 cm) attached to a wooden stick, between the leaves of a broad-bean plant without aphids placed in the vessel, under the exact same conditions as the experiment. Each sample was tested four times, and the amount of EBF measured by the zNose™ was compared to the amount of EBF supplied into the vessel. The resonance frequency (Hz) of the SAW detector changed in a linear fashion with increasing EBF amounts. The calibration curve was described by $y(x)=0.0302x$, ($R^2=0.99$, $P=0.002$, $N=16$), where x = amount (ng) of alarm pheromone applied to the filter paper within the vessel, and y = amount (ng) of EBF detected by the zNose™. Below, we report only the adjusted EBF emission amounts.

Statistical Analysis Data were analyzed using R software 2.13.1 (www.r-project.org). All data are presented as mean ± standard error (SE). Means were compared using Welch's *t*-test. When normality and homogeneity of variances could not be achieved by transformation, a Wilcoxon rank sum test was performed. Correlation analysis was done using Spearman's rank correlation. Frequencies of emission-block types were analyzed following Crawley (2007, pp. 553-556). After fitting saturated generalized linear models (GLM), the interaction of interest was removed, and the updated model compared with the saturated model using ANOVA with a chi-square test.

Results

Predator Behavior In each of the ten replicates, lacewings consumed at least two aphids (4.3±0.7, range 2–8 individuals). Ladybirds consumed on average 5.2±2.2 aphids (N=10, range 0–18), not different from the lacewing treatment (Wilcoxon-test: W=65.5; P=0.233). Ladybirds killed aphids in six replicates. Excluding the four replicates in which no aphid was consumed, resulted in a mean of 8.7±2.9 (N=6) individuals consumed for the ladybird treatment, also not different from the lacewing treatment (Wilcoxon-test: W=25.5; P=0.615).

Initial Alarm Pheromone Emission after the Start of the Experiment Eighty percent of the aphid colonies emitted detectable amounts of EBF after being placed in the vessel where the experiment was conducted, before the predator was introduced. This emission lasted 46.1±7.9 min (range 0–134 min, N=25) and generally followed the pattern described in Fig. 1a (see also supplementary material S1). Mean emission during this initial time was 29.0±7.6 ng (range 0–157.40 ng, N=25). Only when this initial emission had ceased were predators introduced and the 24 hr observation period started.

Alarm Pheromone Emission of Colonies with and without Predator In the five control treatments (no predator introduced), no EBF emission was detected over the entire 24 hr. EBF emission was detected in every lacewing replicate, but only in five replicates under ladybird predation; i.e., in one of the six ladybird replicates in which aphids were consumed, no EBF was found in the headspace. In the lacewing treatment, the amount of EBF emitted over 24 hr was 34.4±9.8 ng (i.e., 8.0 ng of EBF emitted/aphid consumed). In the ladybird treatment, aphids emitted 27.7±15.2 ng EBF, not different from the lacewing treatment (Welsh's t-test: $t_{14.609}$=−1.28, P=0.220, data sqrt-transformed). Emission of EBF per aphid consumed was 5.3 ng. When the four ladybird replicates

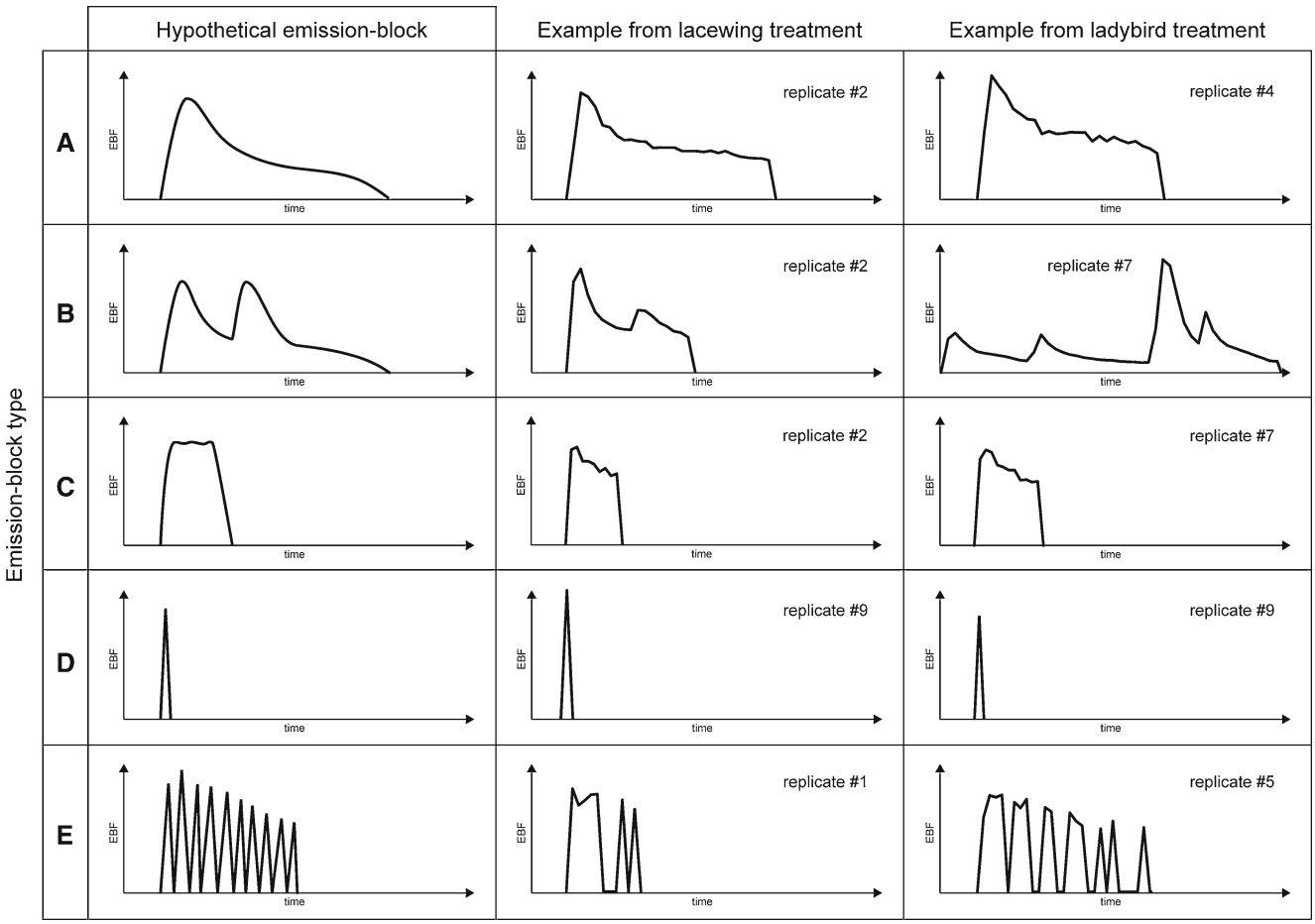

Fig. 1 Aphid alarm pheromone, (*E*)-β-farnesene (EBF), emission blocks derived from zNose™ chromatograms. The left column displays hypothetical EBF emission-block types, while the middle and right columns show recordings from replicates of lacewing and ladybird experiments, respectively. **a** Typical emission course with one peak, as described by Schwartzberg et al. (2008). **b** Two distinct emission blocks, in which the second block starts before EBF emission of the previous block had declined to zero. The end of the first block is not marked by a clear time-gap to the next peak, but by a new peak, higher than the last peaks of the previous emission block. **c** Emission block without a clear peak emission. **d** Emission block consisting of one solitary peak, with a time-gap before and after other emissions. **e** Non-continuous peaks forming an emission-block; only very few interruptions occur in an otherwise clearly defined emission block

without predation events were excluded from the analysis, there was still no difference in total EBF emission between the two predator treatments, likely due to high variability among replicates (Welsh's t-test: $t_{7.132}=0.10$, $P=0.926$, data sqrt-transformed).

When attacked by a predator, EBF emission by aphids generally followed a characteristic pattern (Schwartzberg et al. 2008): after an initial burst, EBF amount emitted declined exponentially over time. In the predator treatments, EBF emission was never continuous over the entire period after the introduction of the predator; i.e., there were always some times when no EBF was detected in the headspace. Visual inspection of the zNose™ chromatograms showed that EBF emission occurred in blocks of emission peaks; i.e., there were distinct periods in which EBF emission was recorded at each sampling interval of 3 min along with periods with no emission (see supplementary material S2). We derived five types of such emission-blocks (Fig. 1a–e).

An emission-block generally started with a high peak, occasionally preceded by a single low peak, followed by a block of peaks, usually with declining peak height (EBF amount) over time (Fig. 1a). The end of a block was marked by a clear gap to the next peak or by the appearance of a new peak, higher than the last peaks of the previous emission block (Fig. 1b). In cases in which there was a period of no EBF emission (no peaks), an emission block also could start with a minor peak before the main peak (Fig. 1c). In the extreme case, an emission block consisted of a single, isolated peak (Fig. 1d). Peaks also were considered to be part of the same emission block when there were only a few interruptions in an otherwise clear block of emissions (Fig. 1e). A key used to distinguish the emission-block types is presented in the supplementary material (S3). After visually grouping emissions into emission blocks for each replicate, all emissions were accounted for; i.e., there was no sampling time with EBF emission that could not be allocated to one of the five emission block types.

We tested the hypothesis that the number of emission blocks corresponded to the number of successful predation events, as assessed at the end of the experiment. Under lacewing predation, an emission block generally started with a high initial peak, followed by an exponential decline in emission, or it consisted of a single peak only. In contrast, under ladybird predation, there often was a strong increase at the beginning, followed by a period of constant emission and a harsh decline at the end (Fig. 1). The frequencies of the emission blocks for each block-type, as described above, were A=17, B=2, C=1, D=4, E=4 for lacewings, and A=3, B=3, C=2, D=4, E=6 for ladybirds. There was an interaction between predator species and emission block type ($\chi^2=9.57$, $df=4$, $P=0.048$). Thus, while lacewings caused mainly an A-block type, with a high initial peak and subsequent decline of peaks, ladybirds more often caused more irregular patterns of EBF emission. The mean number of emission blocks under lacewing predation was 2.8±0.4 (range 1–5), not different from the 1.8±0.8 (range 0–8) emission blocks observed under ladybird predation (Wilcoxon-test: $W=72.5$, $df=18$, $P=0.091$). The number of emission blocks correlated with the number of consumed aphids (Spearman's rank correlation: $S=335.35$, $r=0.747$, $P<0.001$, all 20 replicates pooled), with the number of consumed aphids always equaling or exceeding the emission-blocks in a replicate. More specifically, the number of emission blocks accounted for 65.1 % (34.6 %) of the number of consumed aphids under lacewing (ladybird) predation. The length of the emission blocks was 28.6±4.0 min (range 3–72 min) under lacewing predation, which was lower but not different from, the 44.5±9.4 min under ladybird predation (range 3–153 min, Welsh's t-test: $t_{4.219}=1.18$, $P=0.300$).

Daily Patterns of EBF Emission Under lacewing predation, EBF emission was observed during both day and night, but with a higher emission rate during the day. On average, 71.6±11.8 % of all sampling intervals with EBF detection during the 24 hr observation period occurred in the photophase, between 6 am and 10 pm (Fig. 2). Slightly over 50 % of emission events fell in the 8 hr range between 2 pm and 10 pm. Under ladybird predation, EBF emission was almost exclusively found during daytime, and only 3.4±2.8 % of emission events (replicates without any emission excluded) occurred between 10 pm and 6 am. More than 75 % of emission events occurred between 2 pm and 10 pm.

Discussion

In this paper, we present the first extended monitoring of real-time alarm pheromone emission from aphid colonies under attack by different predators. There are four main results from our study: 1) there was no evidence for a constant release of EBF in aphid colonies that had no contact with a predator; 2) EBF emission of aphid colonies under attack by a predator never occurred continuously over the entire observation time; 3) emission of EBF was always restricted to a clearly defined period of time, allowing emission blocks to be defined; and 4), there was no evidence, based on EBF emission patterns, for differences between the two predator species in diurnal patterns of activity.

Absence of EBF Emission in Aphid Colonies without Contact to Predators In contrast to other studies (Almohamad et al. 2008; Verheggen et al. 2009), there was no evidence of constant release of alarm pheromone from aphid colonies in the absence of predation; no detectable amounts of EBF were

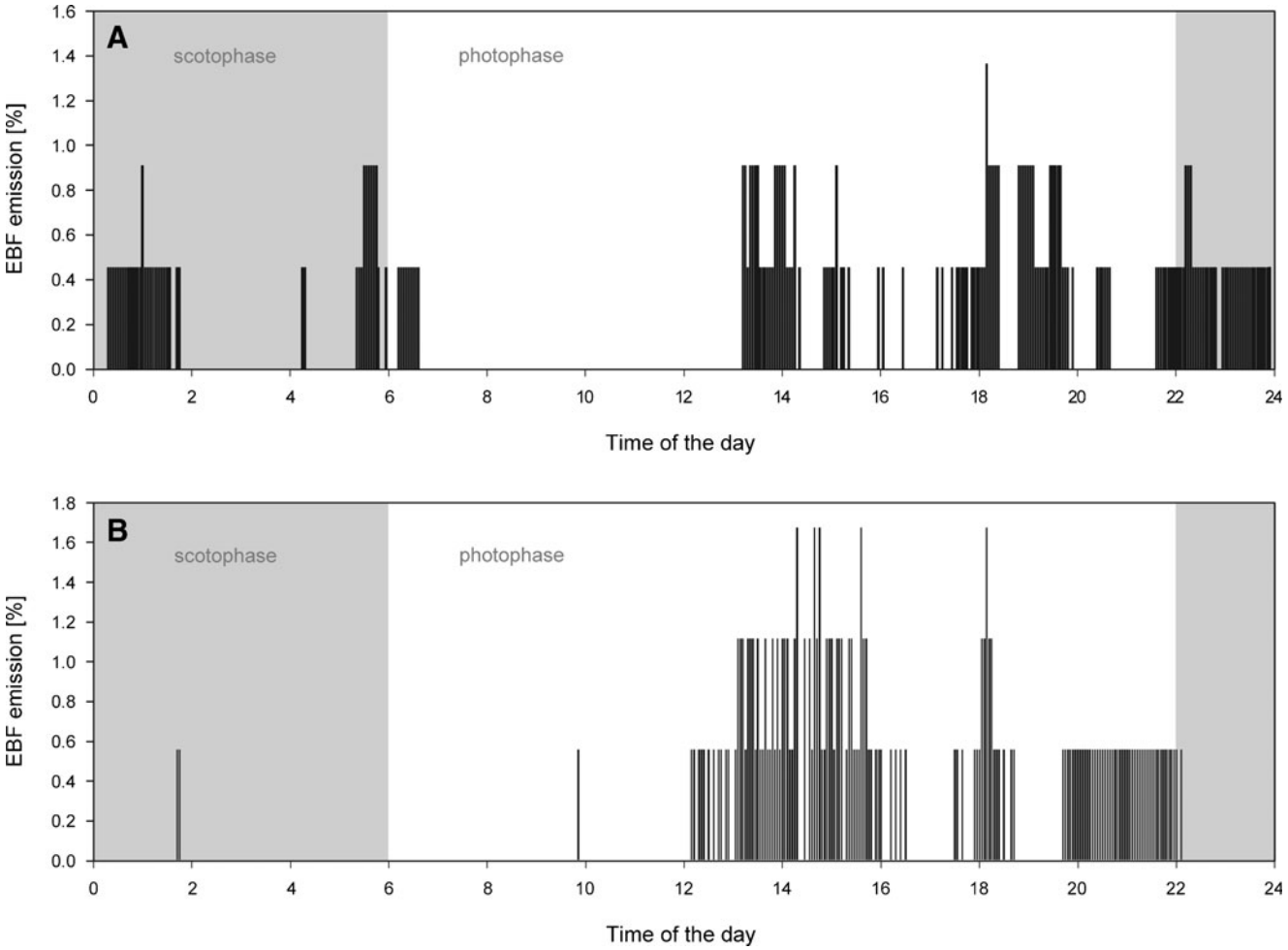

Fig. 2 Daily patterns of aphid alarm pheromone emission in pea aphid colonies under predation by (**A**) larvae of the green lacewing *Chrysoperla carnea* or (**B**) adults of the 7-spotted ladybird *Coccinella septempunctata*. Data from all replicates summed (lacewing $N=10$, ladybird $N=5$, replicates with zero emission excluded). Bars represent the percentage of daily EBF emission. For each time point it was calculated by counting the number of replicates in which EBF emission was recorded at that time, multiplying the number by 100, and dividing the product by the total number of sampling times with EBF emission summed over all replicates with a particular predator. Thus, daily activity was based on EBF occurrence and not on the amount of EBF emitted. Gray background indicates scotophase (lights off), white background indicates photophase (lights on)

present in the headspace of aphid colonies when there was no predator foraging in the colony. In the presence of a predator, EBF was detected only when predation (determined by aphid counts after the experiment) occurred; in the absence of predation (i.e., in replicates in which ladybirds did not feed on aphids), no EBF emission was detected. A low, constant release, reported by Almohamad et al. (2008), would have been detected by the zNose™. In support of our finding, Hatano et al. (2008), using pre-concentration techniques, found no detectable amounts of endogenous EBF in the headspace of pea aphid colonies treated with deuterated EBF or hexane. Constant emission of EBF, even at low amounts, might be costly, as it could have direct and indirect effects on the fitness of aphid colonies, such as habituation to the stimulus (de Vos et al. 2010), unnecessary dispersal (Kunert et al. 2005), or disclosing the presence of colonies to predators (Hatano et al. 2008). Although it is believed that EBF is emitted only after an attack, we detected EBF at the beginning of each experimental replicate, after placing plants with aphids into the vessel. It is possible that the relocation of plants acted as a disturbance to the aphids, resulting in EBF release. It also is possible that when aphids were relocated to new plants or experimental vessels, traces of cornicle droplets contaminated the tools (e.g., forceps or brushes). Thus, by repeatedly transferring aphids, EBF accumulated in the experimental setup (supplementary material S1). Had we started a 24 hr EBF recording immediately after placement of colonies in the vessel, total emission over 24 hr would have been higher than that recorded, and present even in replicates without predators. Thus, care should be taken with handling aphids, and measurements should be made only after a certain time period has elapsed.

EBF Release is not Continuous Aphids have been shown to restrict certain behaviors to certain times of the day, i.e., in a circadian rhythm. The emission of sex pheromone, for example, is restricted to daytime and is synchronized with flight activity of males (Eisenbach and Mittler 1980; Stewart-Jones et al. 2007; Thieme and Dixon 1996). Many aphid species also show strong preferences for movement during daytime (Narayandas and Alyokhin 2006). In our experiment with aphid predators, aphid alarm pheromone was not continuously released, but emission was also not restricted to a certain time of the day (Fig. 2, supplementary material S2).

EBF Emission Occurs in Discrete Emission Blocks Our chromatograms revealed that EBF emission occurs in discrete, discernible bouts. The general shape of an EBF emission curve after an attack is characterized by a fast increase of EBF in the headspace, followed by a slow decline (Joachim et al. 2013; Schwartzberg et al. 2008). While previous studies were made in short trials using single aphids and a predator, this general pattern is also apparent in the 24 hr chromatograms in our study (Fig. 1a). Lacewings, which pierce an aphid's cuticula with their mandibles, slowly consume an aphid, yielding an emission profile of Type "A" block-type, with peaks of decreasing height (Fig. 1a), resulting in a long overall duration of the emission blocks, with high overall amounts of EBF emitted (Joachim et al. 2013; Schwartzberg et al. 2008). Ladybirds, in contrast, consume an aphid quickly, resulting in variable emission scenarios (often emission block-type E, but also all other block types, Fig 1e), with the total amount of EBF emitted lower than for lacewing larvae.

The correlation between the number of EBF emission blocks and the number of aphids consumed was high. Interestingly, while EBF was never detected in replicates without aphid consumption, there were replicates, for both lacewings and ladybirds, in which there were more aphids consumed than emission blocks observed: This suggests differences in the probability of aphids emitting alarm pheromone after an attack (Joachim et al. 2013). EBF is produced and stored at the base of the siphunculi in oenocytes cells, and is excreted within droplets (Chen and Edwards 1972; Edwards 1966; Gut and van Oosten 1985). Joachim et al. (2013) found that all aphids analyzed contained EBF. However, EBF emission was not as closely correlated to cornicle droplet secretion as previously thought (Mondor et al. 2000), and many droplets did not contain EBF (Joachim et al. 2013). Secreted cornicle droplets were more likely to contain EBF after lacewing attack than after ladybird attack (Joachim et al. 2013). While many factors, such as the amount of EBF in the aphid, number and size of secreted droplets and composition of the droplet may influence the presence and volatilization of EBF, it is presently unclear why, as found in the current study, there is such great variation in EBF emission.

Of particular note in our study were two replicates of ladybird predation, in which only two or three aphids were consumed. In these cases, the emission blocks were longer (max: 153 min, mean: 84.0±24.7 min) than in the other three ladybird replicates in which more aphids were consumed (max: 60 min, mean: 29.3±5.2 min). Their shape (Fig. 1e) and extended duration may indicate smearing events, in which the cornicle droplet is daubed on the predator, causing it to carry and display alarm pheromone while searching for further prey (Mondor and Roitberg 2004), thus warning other colony members to escape, resulting in decreased foraging success by the predator and a reduction in the number of aphids consumed.

Despite the similarity of the emission-blocks to the patterns of EBF emission in trials with single aphids and predators (Joachim et al. 2013; Schwartzberg et al. 2008), our study hints at the variability of EBF emission in the headspace under natural conditions. Aphid alarm signaling varies due to a number of factors. There is evidence for quantitative and qualitative variation in alarm signaling between different aphid instars (Mondor et al. 2000), and in response to aphid colony size during juvenile development (Verheggen et al. 2009). Because we used aphids of more or less the same instar, under identical rearing conditions, these sources of variation cannot explain the variability observed in our study. Other sources of variation are the absence of EBF in cornicle droplets after predator attack, or differences in amounts emitted for the same type of predator attacking (Joachim et al. 2013). These likely contribute to the wide variety of emission blocks observed here and may have contributed to the shorter length of emission blocks in our study compared to those recorded in Joachim et al. (2013) or Schwartzberg et al. (2008) as well as to the lack of difference in total EBF emitted in response to the two predator species. In addition, it is also likely that the amount of EBF detected was influenced by abiotic factors, such as the air volume of the sampling device, adsorption to the plant or degradation (Pinto et al. 2007).

Predator Influence on Daily Patterns of EBF Emission *Coccinella septempunctata* possesses a strong circadian rhythm in its activity patterns, with lower locomotion activity during night and an activity maximum between 9 am and 4 pm (Nakamuta 1987), under a 16:8 L:D photoperiod (day: 4 am to 8 pm, light on; night: 8 pm to 4 am, light off; i.e., a 2 hr time shift compared to our experiment). While a ladybird searches for, and feeds on aphids at night, it consumes more prey during the day. This circadian rhythm has been suggested to be the consequence of restricted night vision (Harmon et al. 1998). Under daylight, ladybirds are capable of visual prey recognition within a distance of 7–8 mm, but in darkness,

orientation toward prey is not achieved even at distances of <2 mm, but only after physical contact (Nakamuta 1984). Our results support these findings, with slight modifications: we recorded an activity maximum between 2–10 pm. Although the number of aphids consumed by ladybirds was more than twice that of the number of emission blocks, foraging at night seems unlikely. The inability to recognize prey visually in the dark likely would lead to increased prey handling, resulting in an increased chance for an aphid to escape and/or increased emission of alarm pheromone, which was not detected in our headspace analyses. In contrast to the ladybird, the lacewing *C. carnea* searches a plant for prey randomly (Bond 1980), but also is thought to be able to locate prey, from a distance of up to 50 mm, using cues from aphid honeydew or sex pheromone (Kawecki 1932; Zhu et al. 2005). Relatively little is known about the lacewing's diel foraging activity. Lacewing larvae can search for food in complete darkness (Canard and Duelli 1984), albeit with a decreased predation rate compared to under light (Rosenheim et al. 1999). Our evidence supports that *C. carnea* indeed searches and consumes prey during the scotophase. Based on EBF emissions (Fig. 2), nearly a quarter of the attacks on aphids occurred during night, although the most active foraging period was the afternoon.

In summary, the zNose™ provided novel insight into aphid-predator interactions through monitoring of alarm pheromone emission under real-time conditions. Further studies linking EBF emission dynamics to aphid behavior are likely to shed more light on real-time interactions between aphids and predators.

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