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Modulation of Aphid Alarm Pheromone Emission of Pea Aphid Prey by Predators

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Abstract Recent studies on animal alarm signaling have shown that alarm calls generally are not 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 investigated variability in alarm signaling in aphids, group-living insect herbivores. Under attack, aphids release droplets containing a volatile alarm pheromone, (*E*)-β-farnesene (EBF), that induces specific escape behavior in conspecifics. We used a handheld gas chromatograph (zNose™), which allows real-time volatile analysis, to measure EBF emission by pea aphids, *Acyrthosiphon pisum*, under attack from different predators, lacewing or ladybird larvae. We demonstrate that aphid alarm signaling is 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. We, therefore, examined EBF amounts contained in these droplets and the aphid body. While all aphid bodies always contained EBF, many secreted droplets did not. Our experiments 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.

Keywords (*E*)-β-farnesene · *Acyrthosiphon pisum* · Alarm signal variation · zNose · Homoptera · Aphididae

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Introduction

In many animals, alarm communication in the presence of predators is a crucial adaptation for the reduction of predation risk and inclusive fitness (Zuberbühler, 2009). The signals are typically directed at conspecific receivers, such as mates or kin, and they are particularly common in group-living or colony-forming species (Zuberbühler, 2009). While in mammals and birds alarm signals are mainly visual or auditory, in the insect world they are predominantly chemical (Wyatt, 2003; Verheggen et al., 2010). One important feature of alarm signals that has received increasing interest is the conspicuous variation that exists within the alarm signal of many species (Macedonia and Evans, 1993; Zuberbühler, 2009). Some of this variation appears to be random, age-dependant, or influenced by environmental factors with no apparent underlying adaptive reason.

However, there is increasing evidence that in many species the variation in alarm signaling contains information on the type of predator attacking or the urgency of the threat (Templeton et al., 2005). Such examples only have been found in birds and mammals so far. In mammals, Diana monkeys, *Cercopithecus Diana*, and velvet monkeys, *Chlorocebus pygerythrus*, have distinct alarm calls for leopard, snake, and eagle predators (Seyfarth et al., 1980; Zuberbühler, 2001). In birds, superb starlings discriminate between aerial and terrestrial predators (Macedonia and Evans, 1993), and black-capped chickadees, *Poecile atricapillus*, encode information about the size of predators in variations of their alarm calls (Templeton et al., 2005). While more and more examples of such adaptive variation in alarm signaling are being described, little is known about variation in alarm signaling and the underlying causes of such variation in invertebrates, e.g., downregulation of signal intensity or predator suppression. In this study, we investigated variability in alarm signaling in an insect herbivore, and, in particular, whether alarm signaling varies depending on the species of predator attacking.

We chose aphids (Homoptera: Aphididae) as model organisms because chemical alarm signaling in aphids has been studied intensively since its description in the early 1970's (Bowers et al., 1972). Aphids are sap-sucking insect herbivores that live in colonies often consisting of clones due to parthenogenetic reproduction. When attacked by a predator, individuals can release a small droplet from their abdominal cornicles. This sticky droplet has direct defensive purposes when it glues the mouthparts of the enemy together, as well as indirect by also containing an alarm pheromone (Edwards, 1966). Such chemical alarm signaling is widespread among aphid species. However, the chemical nature and composition of the alarm pheromone varies between species (Francis et al., 2005). In many species, such as in the pea aphid, *Acyrthosiphon pisum*, the sesquiterpene (*E*)-β-farnesene (EBF) is the only compound (Nault and Bowers, 1974; Francis et al., 2005). 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). Emission of alarm pheromone, thus, increases indirect fitness by warning related conspecifics of the presence of a predator while the signaling individual dies (Byers, 2005).

There is evidence that emitting cornicle droplets is not only beneficial, but also can be costly for the aphid. These costs include the loss of a good feeding site or increased mortality risks when leaving the plant (McAllister et al., 1990). This also may be the reason why alarm pheromone emission does not occur at the moment of predator detection, but only after an aphid is physically attacked by the predator (Nault and Phelan, 1984; Mondor and Roitberg, 2004). Other costs include the attraction of natural enemies, as many aphid predators have been shown to perceive EBF and use it as a kairomone, including ants, lacewings, ladybirds, hoverflies, and parasitic hymenoptera (Hatano et al., 2008; Outreman et al., 2010).

From the perspective of an aphid prey, alarm signals are emitted that warn conspecifics (clones) of an immediate risk of predation. To increase the inclusive fitness of the sender they should be selected to warn the intended receiver efficiently. Due to potential costs, there is an emission trade-off: while increasing signal strength may increase the chance of warning the intended receivers, the signal may attract additional natural enemies, hence increasing the risk of predation. From the perspective of an aphid predator that has encountered prey, foraging success is likely to be higher if the emission of EBF is prevented or reduced by the predator, thus causing fewer prey to initiate escape reactions. Mondor and Roitberg (2004) have argued that attacked pea aphids 'scent-mark' predators, as alarm pheromone placed on a ladybird increased the chance of successful escape of aphids that were subsequently attacked by the predator. Further costs for aphid predators may arise through eavesdropping, similar to cases in other organisms with chemical alarm signals (Mathis et al., 1995), where the foraging success of the initial predator may decrease when potential competitors are attracted. Predators then generally suffer a reduction in feeding success, except in cases where several predators are needed to overcome a prey's defense, as in the case of large mammal herbivores (Mathis et al., 1995). Thus, predators themselves have an incentive to suppress or at least reduce prey alarm signaling, e.g., by fast consumption of the prey.

There is some evidence for quantitative and qualitative variation in alarm signaling within aphid species. For example, pre-reproductive, i.e., nymphal stages of the pea aphid are nearly twice as likely to emit cornicle droplets as adult or post-reproductive aphids, and the amount of EBF in a droplet is highest in second to fourth instar pea aphid nymphs (Mondor et al., 2000). A more recent study has shown that the amount of EBF contained in pea aphids depends on whether aphids grow up singly, a rare condition in nature, or in groups (Verheggen et al., 2009). In aphids, EBF is produced and stored at the bases of the siphunculi in modified oenocytes, then released in cornicle droplets in response to predator attack (Edwards, 1966; Chen and Edwards, 1972; Gut and van Oosten, 1985). EBF volatilizes upon emission of the droplets, and can then be perceived by other individuals. Variation in alarm pheromone signaling can, therefore, arise through a) the amount of EBF stored in aphids (Mondor et al., 2000), b) cornicle droplet size and/or concentration of EBF within the droplets (Mondor et al., 2000), and c) the speed of volatilization of EBF from the droplets, depending on the composition of the droplets controlling factors such as hardening (Edwards, 1966). These sources of variation may theoretically be independent of each other, because, for example, aphids may store large amounts of EBF but only emit a small

fraction of it upon attack by a predator. Thus, it is necessary to distinguish carefully between these sources of variation in studies of aphid alarm signaling.

Studies investigating the emission of aphid alarm pheromone have so far been hindered by the lack of technology available to quantify the amount of alarm pheromone emitted by a single aphid. Recently the zNose™ (Electronic Sensor Technology, Newbury Park, CA, USA) technology, 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 (Majerus, 1994; Schwartzberg et al., 2008). Schwartzberg et al. (2008) displayed the emission pattern of a single aphid under attack for the first time, and reported differences among instars and great variation within instars, based on attacks by single predator species.

In this paper, we used a zNose™ 4100 to analyze possible predator-dependent variations in pea aphid alarm signaling. Since aphid predators differ widely in their foraging and feeding behavior (van Emden and Harrington, 2007), the predators for this study were chosen to reflect this variety. Lacewing larvae (Neuroptera: Chrysopidae) overcome soft bodied aphid prey by piercing it with their mandibles and slowly consume it by sucking out the haemolymph while it is still alive (Canard and Duelli, 1984). In contrast, both adult and larval ladybirds (Coleoptera: Coccinellidae) consume an aphid almost entirely, often in less than 1 min (Majerus, 1994). These differences in foraging and feeding behavior may have consequences for aphids' escape tactics and survival and, therefore, be reflected in different types of alarm signaling. Here, we exposed aphids to either ladybird larvae or lacewing larvae to ask the following questions: 1) does aphid alarm pheromone emission differ between an attack by a lacewing larva or a ladybird larva? Based on the results of our headspace analysis we carried out two further experiments to ask the following questions: 2) do all aphids attacked by a predator secrete droplets containing EBF; and 3) are there aphids that do not store EBF in their body?

Methods and Materials

General Experimental Conditions Green pea aphids, *A. pisum*, originally collected in Jena, Germany, were reared on 3-wk-old broad bean plants, *Vicia faba*, variety The Sutton (Nickerson-Zwaan, UK) in 10 cm diameter plastic pots. Eggs of the ladybird, *Coccinella septempunctata* (Coleoptera—Coccinellidae), and first instars of the lacewing, *Chrysoperla carnea* (Neuroptera—Chrysopidae), were obtained from a commercial supplier (Katz Biotech AG, Baruth, Germany). Both predators were reared on broad bean plants infested with pea aphids. Ladybird larvae were used for experiments when they reached the fourth larval stage; lacewing larvae were used in their third stage. To prevent escape of aphids or predators, plants were covered with air-permeable cellophane bags (18.5×39 cm; Armin Zeller, Nachfolger Schütz & Co., Langenthal, Switzerland). Plants, aphids, and predators were kept in a climate chamber under constant environmental conditions (20 °C, 75 % relative humidity, photoperiod: 16:8 h L:D).

Rearing of Aphid Lines 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 is distributed equally over all treatments (Kunert et al., 2005). To do so, we initiated 15 lines by placing 15 adult aphids (F_0 generation), randomly collected from a single population consisting of the same clone, singly on 15 bean plants where they were allowed to reproduce for 24 h, before they were removed from the 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 experiment as soon as they reached the adult stage (after another 8 days). A split-brood design was achieved by choosing for each line one F_2 individual for the ladybird and one for the lacewing treatment, see below.

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 surface acoustic wave (SAW) detector was set to 40 °C. A dilution series was created by dissolving EBF (Bedoukian Research Inc., Danbury, CT, USA) in methanol (Carl Roth Germany, 99.8 %) at concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 3.0, 4.5, and 6.0 µl EBF ml^{-1}. 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 programmed conditions. Each concentration was tested at least five times. (E)-ß-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. The calibration curve was described by y(x)=2788.2x, where y=response of the SAW detector [Hz] and x=amount of EBF [ng] R^2=0.964, P<0.001, N=52.

Experiment 1: Predator-Dependent EBF Emission Pattern

For this experiment, a single aphid was placed on the lower surface of an excised broad bean leaf embedded in 1.5 % agar in a Petri dish (diam 90 mm, height 20 mm) and allowed

to settle for 15 min in the inverted dish. For volatile collection, an air-collection chamber was constructed from a 4 ml glass vial (Macherey & Nagel, Düren, Germany) with its bottom cut-open. The vial with its lid and septum was connected to the zNose™ 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. A single zNose™ sample includes three phases (Kunert et al., 2002): 1) sampling and trapping of volatiles, 2) discharge of the trapped compounds onto the column followed by a specifically designed temperature-programmed elution with following detection, and 3) recovery phase of the SAW detector. So, after pre-concentration of the compounds on a Tenax® trap, the collected volatiles were transported to a DB5 column (1 m, film thickness 0.25 μm, ID 0.25 mm) for separation. Helium served as carrier gas at a flow rate of 3 ml min^{-1}.

A fourth instar ladybird larva or a third instar lacewing larva (last larval stages) was first weighed to test for any body size-dependant EBF emission effects and then placed in the vicinity of a feeding aphid on the leaf in the Petri dish. The air collection chamber attached to the zNose™ was then placed over predator and prey 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 and prey inside the collection system. Thus, any EBF emission before the predator attacked the aphid could be recorded.

The zNose™ was programmed to sample volatiles in the headspace at 2-min intervals. Sampling for 10 s at a flow rate of 30 ml min^{-1} implied that 5 ml air per measurement were extracted from the 4 ml chamber, i.e., more than the total volume of headspace air around the predator/prey pair was collected every 2 min to avoid EBF accumulation in the chamber. After fast elution under a programmed temperature gradient (from 40 °C to 180 °C at 5 °C s^{-1}), compounds were monitored and quantified by the SAW quartz microbalance detector. The temperature of the SAW detector was adjusted to 40 °C to obtain optimum sensitivity for quantification of EBF. Collections were terminated when the EBF signal intensity fell below 100 Hz.

In total, there were 32 and 26 replicates for lacewing and ladybird predator, respectively, using new leaves, aphids, predators, and glass vials each time. We calculated the following variables from the emission records: *duration of emission*—the total time during which EBF was detected after an attack, a multiple of 2 min intervals, *time to peak emission*—the time from the beginning of EBF emission to the maximum emission peak, *peak emission*—the highest amount of EBF emitted in any of the 2 min collection intervals following a predation event, and *total EBF emission*—the sum of all EBF peaks emitted by an aphid.

Experiment 2: Quantification of EBF in Cornicle Droplets After Attack by a Predator

Because in some replicates of Experiment 1 no EBF was detected in the headspace despite the fact that the predator killed the aphid, we carried out a second experiment to analyze the EBF content of cornicle droplets. For this experiment, 86 aphid lines were established as described above, and for each line one aphid was used for the ladybird treatment and one for the lacewing treatment.

A single aphid was placed on a broad bean leaf embedded in agar in a Petri dish. A predator larva was introduced near the aphid and allowed to attack the aphid. The cornicle droplet(s) were collected immediately with a short (~1 cm) glass capillary produced from Pasteur pipettes. The droplet-containing capillary was transferred into a glass micro-insert (100 μl, 31×0.6 mm) and stored in a 1.5 ml GC vial (both from Fisher Scientific GmbH, Schwerte, Germany). The capillary was covered with 15 μl of hexane containing 2.5 ng μl^{-1} β-caryophyllene (Sigma Aldrich, St. Louis, MO, USA) as an internal standard. Samples were stored at −20 °C until analysis.

EBF dissolved in hexane was analysed by injecting 2 μl 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^{-1}. 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^{-1}, and then increased further at 60 °C min^{-1} until 300 °C and kept for 2 min. Mass spectra of EBF and β-caryophyllene were compared to those in the National Institute of Standards and Technology and the Wiley libraries for identification of peaks.

Experiment 3: Quantification of EBF in Aphids

In order to test the hypothesis that all aphids contain EBF, whole-body extracts were made from 15 F_2 aphids, one from each of 15 lines initiated as described for Experiment 1. Aphids were carefully removed from their host plant using tweezers to avoid secretion of cornicle droplets, weighed to test for any effects of aphid size on EBF content, and immersed in 20 μl hexane containing 2.5 ng μl^{-1} β-caryophyllene as internal

standard. Both aphids and solvent were stored in glass inserts within 1.5 ml borosilicate vials at −20 °C for 24 h. Preliminary experiments showed that this is the sufficient time to extract all possible EBF from the aphids' body. Aphids then were removed from the inlet and the extract was kept under the same conditions until analysis.

For quantification of EBF, 2 µl extract of each aphid were analysed by GC-MS using the same method and equipment as described in Experiment 2 (see above).

Statistical Analysis

Statistical analyses were carried out using the statistical software R version 2.13.0 (www.r-project.org) or with PASW Statistics 18. All data are presented as mean ± standard error (SE). Means were compared using Welch's *t*-test (PASW). In case normality could not be achieved by suitable transformations, we used a Mann–Whitney *U*-Test (PASW). In cases where homoscedasticity could not be achieved, a Brunner-Munzel Test (BM-Test, *R*) was performed (Fagerland and Sandvik, 2009). For Experiment 1, we analyzed the effect of the predator species on the time-to-peak emission, peak emission, duration of emission, and total EBF emission using analysis of covariance (ANCOVA, *R*), with either predator weight or, for total EBF emission, the duration of emission as a covariate, and also tested for the interaction between covariate and predator species. Simple regression analyses were carried out using *R*.

To test if predators differed in the fraction of droplets produced that contained EBF, contingency tests (*Chi-square, R*) were performed. A binominal test was performed to test whether aphids contain alarm pheromone or not (*R*).

Results

Experiment 1: Predator-Dependent EBF Emission Pattern

The general shape of the emission curve corresponded to the pattern described by Schwartzberg et al. (2008): after attack of a predator, the amount of EBF in headspace quickly rose to a maximum followed by declining slowly (S1, S2). However, despite having the same shape, emission patterns differed significantly in several aspects between predators. No EBF was ever emitted before the attack. An attack is defined here as a direct approach with consequent capture of the aphid. In all our replicates, the first encounter between aphid and predator resulted in an attack and subsequent consumption of the aphid.

Predator weight differed between *C. septempunctata* and *C. carnea*. Mean ladybird larval weight was 7.75±0.54 mg (range 3.8–8.6 mg) and higher than the weight of lacewing larvae (6.20±0.32 mg, range 4.8–12.4 mg, Welch's *t*-test: $t_{29.83}=-2.18$, $P=0.037$).

Interestingly, for 17 out of the 33 replicates with lacewing larvae (51.52 %) and for 6 out of the 26 replicates with ladybirds (23.08 %), no EBF was detected in the headspace despite the attack and subsequent consumption of the aphid by the predator. Except when indicated otherwise, we only used those 16 replicates with lacewing larvae (henceforth lacewings) and 20 replicates with ladybird larvae (henceforth ladybirds) where EBF emission was detected to calculate total EBF emission, peak emission and time to peak emission.

The EBF emission patterns of aphids attacked by lacewing larvae or ladybird larvae differed in several aspects (Fig. 1). Time to peak emission was 5.50±0.50 min (range 4–36 min) when aphids were attacked by a lacewing, significantly earlier than for ladybirds (9.63±2.03 min, range 2–12 min, ANCOVA: $F_{1,34}=6.33$, $P=0.017$, data ln-transformed). The time to peak emission was not affected by predator weight (ANCOVA: $F_{1,34}=0.55$, $P=0.464$) and the interaction between predator species and predator weight was not significant (ANCOVA: $F_{1,34}=0.25$, $P=0.621$).

The peak emission after lacewing attack (range 0.09–2.20 ng) was more than twice as high as the peak after attack by a ladybird (range 0.07–0.75 ng, ANCOVA: $F_{1,34}=9.98$, $P=0.003$, data ln-transformed, Fig. 2a, S1, S2). The peak emission was independent of predator weight (ANCOVA: $F_{1,34}=0.39$, $P=0.539$). The interaction between predator weight and predator species was not significant for peak emission (ANCOVA: $F_{1,34}=1.00$, $P=0.324$): In lacewings, peak emission declined with increase in predator weight, while under ladybirds this decline was weak.

The duration of emission, measured as the time from the beginning of emission to the sampling interval when EBF detection dropped below 100 Hz (0.036 ng), was four times higher after lacewing attack (range 44–232 min) than after ladybird larva attack (range 10–70 min, ANCOVA: $F_{1,34}=87.65$, $P<0.001$, Fig. 2b). Predator weight was negatively correlated with the duration of emission (ANCOVA: $F_{1,34}=8.13$, $P=0.008$). The interaction between predator species and predator weight was significant (ANCOVA: $F_{1,34}=16.20$, $P<0.001$): While the duration of emission strongly declined with predator weight for lacewings, this decline was weak with ladybirds (Fig. 3a).

Total EBF emission was on average almost six times higher after lacewing attack (range 2.07–28.67 ng) than after ladybird attack (range 0.27–9.19 ng, ANCOVA: $F_{1,34}=40.62$, $P<0.001$, total EBF emission ln-transformed, Fig. 2c). Total EBF emission was independent of predator weight (ANCOVA: $F_{1,34}=2.32$, $P=0.138$, total EBF emission ln-transformed) and also the interaction between predator weight and predator species was not significant (ANCOVA: $F_{1,34}=1.19$, $P=0.283$, Fig. 3b). While for lacewings there was a trend of decreasing total EBF emission with increasing predator weight, there was clearly no relationship for ladybirds (Fig. 3b).

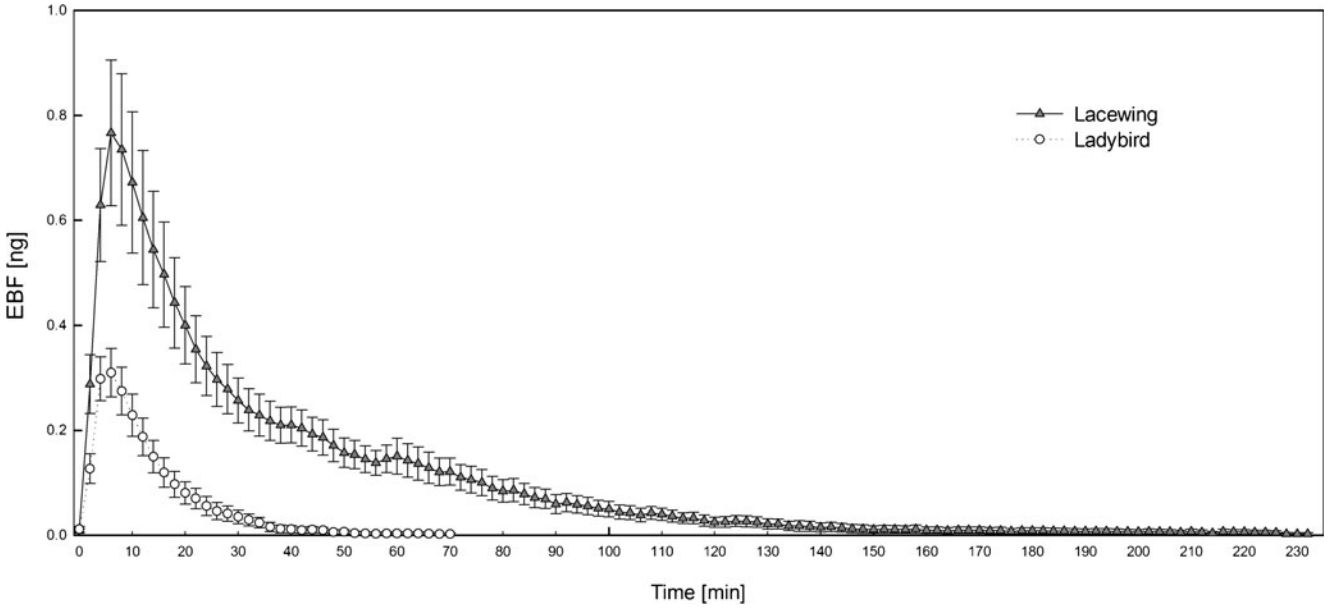

Fig. 1 Time course of aphid alarm pheromone (EBF) emission after attack by a lacewing larva or ladybird larva. For each time point, amounts emitted were averaged over all replicates with a lacewing larva (N=16) or a ladybird larva (N=20). Runs with zero EBF emission were excluded

When duration of emission rather than predator weight was included as a covariate in the analysis, there was a positive relationship with total EBF emission (ANCOVA: $F_{1,34}$= 42.29, P<0.001 total EBF emission ln-transformed, Fig. 3c). The EBF emission remained significantly different between predators (ANCOVA: $F_{1,34}$=118.94, P<0.001, total EBF emission ln-transformed) just as the interaction between the duration of emission and predator species (ANCOVA: $F_{1,34}$= 29.66, P<0.001, total EBF emission ln-transformed). When runs with zero EBF emission were included, total EBF emission was still positively correlated with the duration of emission (ANCOVA: $F_{1,58}$=259.89, P<0.001). EBF emission was still different between predators (ANCOVA: $F_{1,58}$=34.22, P< 0.001) but the interaction between duration of emission and predator species was then not significant (ANCOVA: $F_{1,58}$= 0.06, P<0.810).

Experiment 2: Quantification of EBF in Cornicle Droplets After Attack by a Predator

The quantification of EBF in cornicle droplets after attack by lacewings and ladybirds showed that not all droplets contained aphid alarm pheromone. The predator had a significant influence on the presence of EBF in cornicle droplets (*Chi-square* test: χ^2=12.26, df=1, P<0.001), whereby droplets were more likely to contain EBF after a lacewing attack. In 23 of 71 cases (32.4 %) and 52 of 84 cases (61.9 %), the

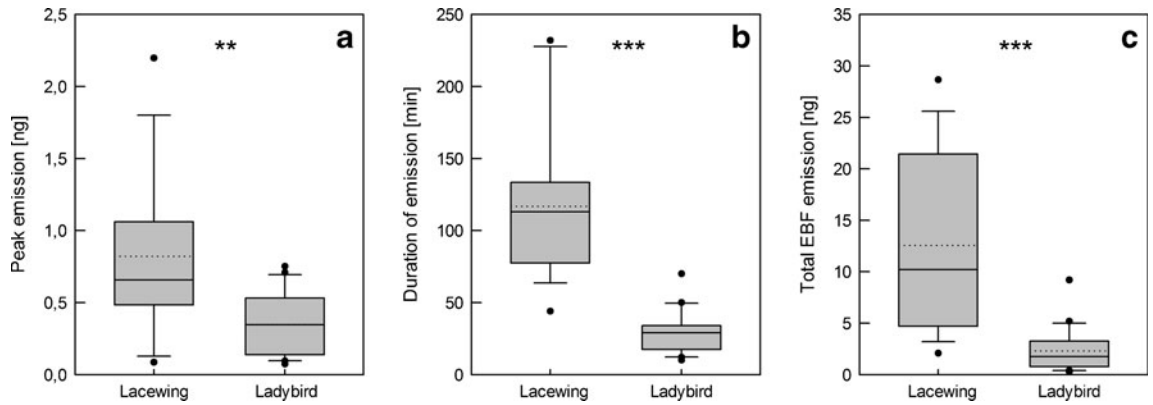

Fig. 2 Emission of the aphid alarm pheromone (E)-β-farnesene (EBF) by aphids after attack by a lacewing larva or ladybird larva in Experiment 1. (**a**) Peak emission (**b**) Duration of emission (**c**) Total EBF emission. *Box plots* show the median value (*solid line*), the 25th and 75th percentile; the *error bars* below and above the box indicate the 10th and 90th percentile, respectively. *Dotted lines* indicate means; *black dots* indicate outliers; *=P≤0.05, **=P≤0.01, ***=P≤0.001

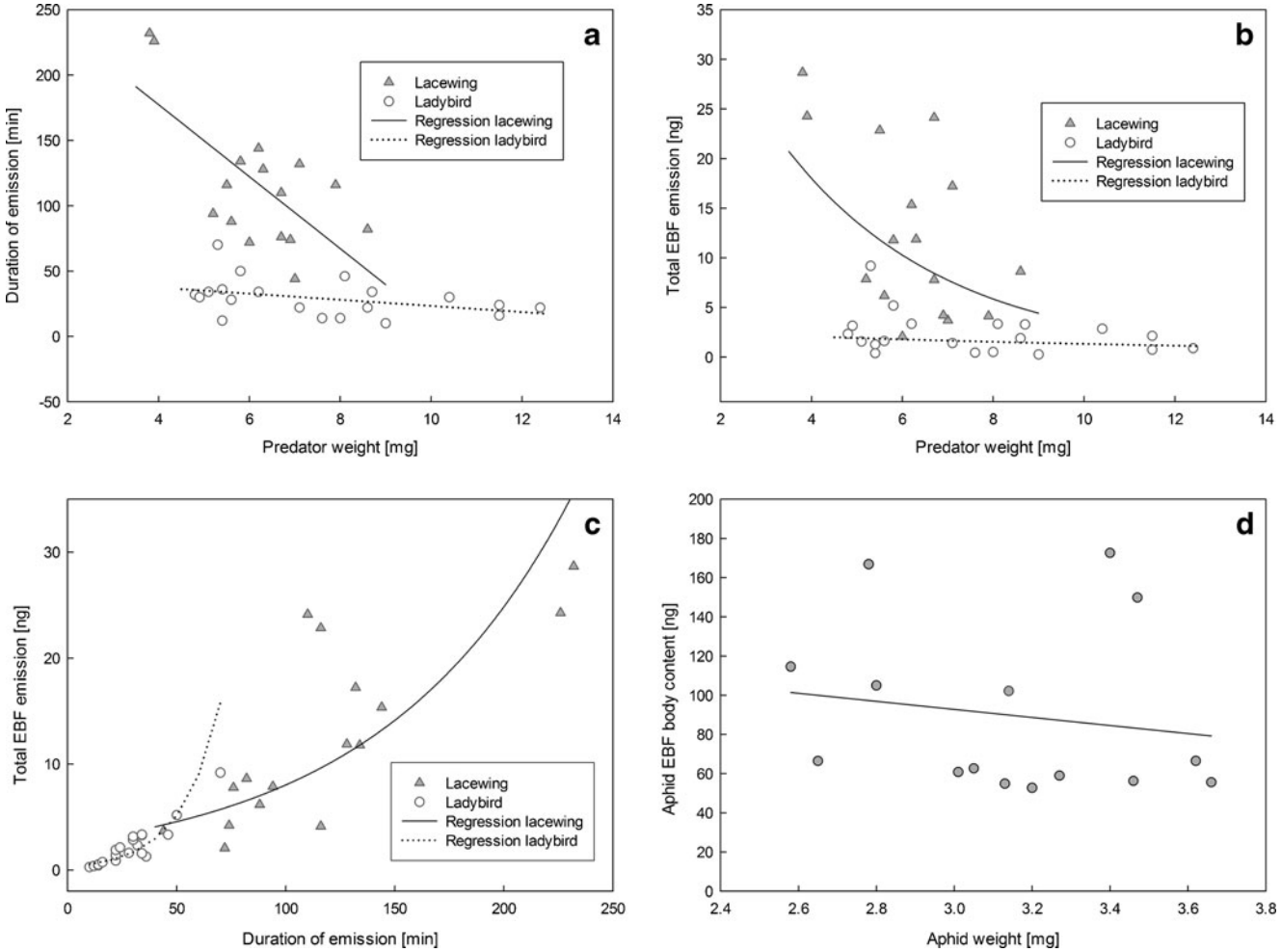

Fig. 3 The relationships between predator weight, duration of emission and total EBF emission when aphids were attacked either by a lacewing larva or by a ladybird larva in Experiment 1 and the relationship between EBF body content of individual aphids and the aphid's weight in Experiment 3. (**a**) Duration of emission vs. predator weight. Regression equations: Lacewing y=287.63−27.56x (R^2=0.46, P=0.004), ladybird y=46.748−2.345x (R^2=0.15, P=0.087) (**b**) Total EBF emission vs. predator weight. Regression equations of ln-transformed data of total EBF emission (data back-transformed for figure): Lacewing y=4.015−0.281x (R^2=0.21, P=0.077), ladybird y=1.01933−0.07362x (R^2=0.04, P=0.415) (**c**) Total EBF emission vs. duration of emission. Regression equations of ln-transformed data of total EBF emission (data back-transformed for figure): Lacewing y=0.95454+0.01129x (R^2=0.55, P<0.001), ladybird y=−1.16363+0.05606x (R^2=0.77, P<0.001) (**d**) Alarm pheromone body content of single aphids vs. aphid weight (R^2=−0.027, P=0.56). See text for explanation

droplets contained no EBF when pea aphids were attacked by lacewing larvae and ladybird larvae, respectively.

For lacewing attacks, cornicle droplets contained on average 14.98±2.04 ng EBF (range 0.21–52.54 ng), not significantly different from the 12.55±2.14 ng (range 0.16–53.81 ng) measured in the headspace in Experiment 1 (Welch's t-test: $t_{43.178}$=−0.83, P=0.41). When runs and droplets with zero EBF were included in the analysis, there was still no difference between EBF amounts in cornicle droplets (10.13±1.61 ng, N=71) and headspace measurements (BM-Test: tBM=−1.62, 6.08±1.51 ng, df=64.80, P=0.111).

For ladybird attacks, the amount of EBF contained in cornicle droplets (9.51±2.40 ng, range 2.07–28.67 ng) tended to be higher than the amount detected by the zNose™ (2.29±0.46 ng, range 0.27–9.19 ng), but the difference was not significant (BM-Test: tBM=−1.99, df=49.85, P=0.052). However, when runs and droplets with no EBF were included in the analysis, the amount contained in droplets (3.62±1.04 ng, N=84) was higher (U-Test: U=764.00, df=108, P=0.012) than amounts calculated from headspace measurements by the zNose™ (1.76±0.40 ng).

The total amount of EBF contained in droplets emitted after lacewing predation was higher when compared to the EBF amount present in droplets after ladybird attack (BM-Test: tBM=−2.02, df=70.141, P=0.048). This was still the case when droplets with no EBF were included in the analysis (BM-Test: tBM=−4.39, df=137.114, P<0.001).

Droplet number emitted after predator attack was recorded for some of the replicates. There was no difference in droplet number between attacks by different predator species: for lacewings mean droplet number was 1.85± 0.06 droplets (N=39), while for ladybirds it was 1.76± 0.07 droplets (N=37, BM-Test: tBM=0.97, df=70.442, P=0.336).

Experiment 3: Quantification of EBF in Aphids

Interestingly, despite the great variation in EBF presence in secreted droplets and headspace measurements, alarm pheromone was found in all aphids when EBF was directly extracted from aphid bodies (89.70±11.11 ng, range 52.68–172.59 ng; Binominal Test: N=15, P<0.001). Total EBF in the aphid body was independent of aphid weight (Regression: R^2=−0.027, N=15, P=0.56, Fig. 3d). Under lacewing attack, pea aphids secreted droplets that contained on average only 16.70 % of the total EBF contained in the body. Under ladybird attack, droplets contained only 10.60 % of the amount of EBF found in an aphid body. The amount collected by the zNose™ in the headspace samplings was on average 14.0 % of the alarm pheromone content found in the aphid body when the aphid was attacked by a lacewing larva, and 2.55 % when attacked by a ladybird larva.

Discussion

The main results from our study are: 1) aphid alarm signalling differs when different predator species attack the aphid, with longer and more EBF emission under lacewing predation than under ladybird attack; 2) total EBF emission depends on predator feeding time; 3) only a fraction of the EBF stored in an aphid's body is set free in cornicle droplets after attack by the predator; 4) all aphids appear to produce and store alarm pheromone, yet a relatively large fraction of individuals, up to 60 %, emit cornicle droplets that do not contain alarm pheromone, despite the fact that the attacks are lethal. The differences between the predators were surprisingly strong; however, it is the absence of EBF in cornicle droplets that is most surprising, because no previous study has reported an absence of EBF in cornicle droplets. The general assumption has been that all droplets secreted by aphids after an attack contain aphid alarm pheromone, although the amounts may vary (Mondor et al., 2000; Schwartzberg et al., 2008). Below, we discuss possible reasons for the variability observed.

The seven-spot ladybird *C. septempunctata* and the green lacewing *C. carnea* differ greatly in their foraging behavior and food intake. While *C. septempunctata* larvae (and also adults) consume aphids quickly and completely, such that few remains of the aphids are left when consumption stops, lacewing larvae feed longer on aphids and the aphid continues to be alive also for much longer. These feeding strategies appear to be reflected in the EBF emission pattern of the aphid prey; the duration of alarm pheromone emission was longer after lacewing than after ladybird attack. Because the total amount of alarm pheromone emitted was positively correlated with the duration of emission and hence feeding time, attacks by lacewings resulted in a stronger overall EBF signal than attacks by ladybirds. Even the emission peak after the predator first seized the aphid was higher for lacewing than for ladybird attacks. It, therefore, appears that differences in feeding mode underlie the observed large differences in EBF emission patterns: aphids undergoing ladybird predation emitted only about one sixth of the total amount of EBF compared with those undergoing lacewing predation in about a quarter of the time.

Specific foraging and feeding behavior of the predators used in this study match with the observed EBF emission patterns. For ladybirds at least, a feeding strategy that includes fast consumption of the prey and thereby limits the amount of alarm pheromone emitted may be beneficial, given that ladybirds need to feed on many individuals during the day and often feed on many individuals on the same plant. As in other predator species, a fast-consumption behavior may have evolved to enhance the efficiency of feeding in aggregated prey groups (Curio, 1976). Therefore, it is beneficial for the ladybird if a group of aphids does not disperse. In contrast, lacewing larvae only consume a few aphids per day and slow down their movements and rest after feeding (Canard and Duelli, 1984). Due to the feeding mode, there presumably is little chance for lacewings to suppress aphid EBF emission, and the rest after aphid consumption may in fact be an adaptation to the disturbance caused in the prey colony (in addition to any physiological needs for digestion). As a caveat, our set-up was optimized to measure precisely the amount of EBF emitted into the headspace. Hence, the small volume of the vial precluded detailed behavioral observations of the interactions between predator and prey. Studies linking such observations to patterns of behavioral interactions are, however, needed to understand more precisely which behaviors of predator or prey results in the release of more or less aphid alarm pheromone, including the aphid body part first attacked by the predator.

While the differences in EBF emission patterns caused by the two predator species were consistent, there was nevertheless large variation in the amounts released by individual aphids. Such variation also has been reported by e.g. Mondor et al. (2000) or Schwartzberg et al. (2008) who stimulated aphids to produce droplets by either a blunt probe or lacewing larval attack, respectively. Surprisingly, there

was also a significant fraction of individuals that did not emit any EBF. While not quantified in our experiment, the impression during the observations was that all attacked individuals emitted cornicle droplets. To rule out any failure to detect EBF with the zNose™ we separately analyzed the cornicle droplets of aphids attacked by predators. As this also showed the absence of EBF in a significant fraction of individuals, we further analyzed the body content of EBF, also in a separate cohort. These experiments showed that all aphids store EBF in their bodies and suggested that individuals where no EBF was detected in the headspace after predator attack probably emitted cornicle droplets that did not contain any EBF.

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). By retracting a cornicle muscle that is attached to the top and base of the siphunculi, a valve-like flap at the top of the cornicle can be opened and release the content of secretory cells forming a droplet *via* turgor (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). EBF is believed to be produced *de novo* and thought to be linked to the juvenile hormone biosynthesis from (E,E)-farnesyl-pyrophosphate (Gut and van Oosten, 1985; Mondor et al., 2000). There are a number of possible reasons for the absence of alarm pheromone in the secreted cornicle droplets. A simple hypothesis is that aphids failed to produce droplets containing EBF for physiological reasons (e.g., due to deformations in the ultrastructure of secretory cells). A more detailed understanding of the mechanics that regulate the release of EBF from the base of the siphunculi is required to rule out this hypothesis. A second hypothesis is that predators affect EBF emission by modulating the EBF presence in the droplet or the cornicle droplet size and number. For example, severely lacerating an aphid or damaging the cornicle secretory cell sac after an attack could cause a decrease of the aphid's turgor pressure that may lead to smaller droplet sizes or numbers. We saw no difference in droplet number between predator species, but due to the experimental design and the fast course of action, estimating the droplet size while allowing the predator to feed was not possible. However, if the injury is such that no EBF can enter the droplet before emission, either because the base of the siphunculi is injured, or because the injury prevents signal transduction to initiate EBF release, then there would be some predator control over EBF emission. Behavioral observations coupled with morphological studies are needed to test this hypothesis. A third hypothesis is that pea aphids are able to regulate the alarm pheromone content in cornicle droplets and that a downregulation of alarm signaling is adaptive. Such downregulation only has advantages, however, if the costs of alarm signaling outweigh the benefits. Further tests with aphids placed in different environments including differing aphid colony sizes are needed to test if aphids are able to modulate actively the release of aphid alarm pheromone. Such ability to regulate the EBF content of cornicle droplets could then also explain the considerable variation in the amounts of EBF emitted among individuals. Downregulation, however, only has advantages if the costs of alarm signaling outweigh the benefits. The following costs are conceivable: a) physiological production costs that can lead to lower reproductive success (Mondor and Roitberg, 2003); b) trade-offs with other functions of cornicle droplet such as the smearing of predator mouthparts (Dixon, 1998); c) attraction of further natural enemies to the plant (Hatano et al., 2008); and d) costs to conspecifics that interrupt their feeding (e.g., Roitberg et al., 1979). Because an attacked aphid will die in the majority of cases when grabbed by a predator, physiological costs (option a) are an unlikely explanation for a modulation of EBF emission. Evidence that e.g., a high EBF content compromises the 'gluing' effect of cornicle secretion (option b) is so far missing and requires insight into the mechanics of EBF release. Option b and c are unlikely to lead to zero emission. The avoidance of attacks by additional predators attracted by EBF (option c), may play a role for a downregulation of EBF release, but it is unlikely to lead to zero emission. Such zero emission would only be favored if the future risk of predation by eavesdropping predators is greater than the risk posed by the predator that is already foraging in the colony. Option d) similarly is unlikely to select for zero emission. If an aphid is disturbed enough to produce a cornicle droplet, it is unlikely that this threat is perceived as being a threat only for the aphid itself but not for the conspecifics.

We have shown 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. It is believed that variation in alarm signaling is dominant in species preyed upon by various predators that require different escape behaviors (Macedonia and Evans, 1993; Manser et al., 2002), thus there may well be the potential for an adaptive adjustment of alarm signaling in aphids as well as in other insects. Future work should clarify if attacked aphids actively modulate the amount of EBF emitted and whether the observed variability in alarm signaling is adaptive.

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