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SHORT COMMUNICATION

Context-dependent alarm signalling in an insect

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Abstract

Animals often respond to danger by raising alarm to inform others. Alarm signals come in many different forms, such as visual or mechanical display, sound or odour. Some animals produce vocal alarm signals that vary with the level of danger. For chemical alarm signals, virtually nothing is known about such context-dependent signalling due to a general notion that alarm pheromones have fixed compositions. Here, we show that larvae of the Western Flower Thrips (*Frankliniella occidentalis*) produce an alarm pheromone whose composition varies with the level of danger they face: the presence of a relatively harmless predator or a very dangerous predator, that is either actually attacking or not. The frequency of alarm pheromone excretion increases with the level of danger. Moreover, the composition of excreted alarm pheromone varies in the relationship between total and relative amount of the putative two components, decyl acetate (DAc) and dodecyl acetate (DDAc). When pheromone is excreted with a predator present but not attacking, the percentage DDAc increases with the total amount of pheromone. When a predator does attack, however, the relationship between percentage DDAc and total amount of pheromone is reversed. Taken together, the alarm signal of thrips larvae appears to be context dependent, which to our knowledge is the first report of context-dependent composition of an alarm pheromone.

Introduction

Many species are confronted with a variety of predators, some more dangerous than others. In order to successfully reproduce, individuals must avoid predation while simultaneously performing other activities such as foraging and mating (Lima & Dill, 1990). Hence, individuals face a trade-off between engaging in survival-enhancing antipredator behaviour and reproduction-enhancing behaviour (Lima & Dill, 1990). To avoid unnecessary investment in antipredator behaviour, an individual should be sensitive to the current level of predation risk (Robinson, 1980; Lima & Dill, 1990).

There are various ways in which a prey individual can detect that a predator is in the vicinity, such as the detection of cues from the predator (e.g. odours, sounds or vibrations) or the release of signals from conspecific prey individuals that can warn others of impending danger (e.g. vocal, visual or chemical signals). If predation risk is communicated by alarm signalling, natural selection tends to act on alarm signals so as to specify the type and level of danger in a context-dependent manner. Here, the context consists of sender, receiver and danger in the environment. There are examples of individual vertebrates that vary vocal alarm calls with context, such as vervet monkeys that make different calls for each of their main predators (Seyfarth *et al.*, 1980) or ground squirrels that change their alarm call depending on the urgency of the situation (Robinson, 1980; Furrer & Manser, 2009). For chemical alarm signals (alarm pheromones), however, virtually nothing is known about variation in alarm signals (but see Joachim *et al.*, 2013). This is

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[a]In loving memory of our dear friend and mentor Maurice Sabelis, who passed away on 7 January 2015.

striking because alarm pheromones are very common (Wyatt, 2003; Verheggen *et al.*, 2010).

We studied variation in the alarm pheromone of an insect, the Western Flower Thrips *Frankliniella occidentalis* (Pergande) (Insecta: Thripidae) in response to different types of danger. This thrips releases an alarm pheromone, consisting of two specific chemicals: decyl acetate (DAc) and dodecyl acetate (DDAc). These acetates are contained in droplets excreted from the rectum in response to artificial disturbance (Teerling *et al.*, 1993; Macdonald *et al.*, 2003) and – as we show in this article – it is also excreted in response to natural enemies. Amount of DAc plus DDAc (DAc + DDAc) and percentage of DDAc in the mixture of the two components (%DDAc) is known to vary with larval development: older thrips larvae release more DAc + DDAc and relatively more DAc than DDAc (Macdonald *et al.*, 2003). This raises the question whether the insect varies its quantity (DAc + DDAc) and quality (%DDAc) to specify different types of danger.

Here, we tested whether thrips larvae release different DAc + DDAc and/or %DDAc, depending on the type of predator they encountered or were attacked by. In nature, thrips larvae (0.5–1.2 mm in length) face different types of predators (Lewis, 1973), each posing a different threat level depending on their size relative to that of the thrips larva (Sabelis & Van Rijn, 1997). To represent two very different levels of danger, we selected predatory mites (*Iphiseius degenerans*, ~0.7 mm long) and predatory bugs (*Orius laevigatus*, ~2 mm long). Predatory mites are successful in attacking young (first-instar) larvae, but are much less successful or even harmless to older (second-instar) larvae (Bakker & Sabelis, 1987). Predatory bugs, however, are successful in attacking all developmental stages (Bakker & Sabelis, 1987; Sabelis & Van Rijn, 1997). To test for context-dependent release of alarm pheromone, we used second-instar thrips larvae with predatory mites as relatively harmless predators and predatory bugs as very dangerous predators. Second-instar larvae release enough pheromone per individual to analyse its qualitative and quantitative composition (Macdonald *et al.*, 2003).

To analyse pheromone composition in single, rectally released droplets, we assessed DAc + DDAc using gas chromatography (GC). The single droplets collected and analysed were released by individual larvae either when a predator was attacking the larva or when the predator was present but not attacking. As a control for release of alarm pheromone upon contact with a predator, we prodded larvae with a brush and collected a single droplet per larva under 'attack' (brush contact). We recorded (i) which droplets contain DAc and DDAc, and if both were present, (ii) DAc + DDAc, and (iii) % DDAc of the droplets (cases where only DAc or DDAc were present did not occur).

Materials and methods

Host plants

All experiments and the rearing of thrips were conducted on 4-week-old cucumber plants, *Cucumis sativus* (var. Ventura RZ, Rijk Zwaan, De Lier, the Netherlands) grown in a greenhouse at 25 °C, 70% relative humidity, L16 : D8 photoperiod. Plants were kept insect and pathogen free (as far as visible symptoms are concerned) until they were used for the experiments or cultures.

Thrips

Western Flower Thrips, *Frankliniella occidentalis* Pergande, were collected from cucumber plants in a commercial greenhouse at Pijnacker, the Netherlands, in February 2006. Thrips were subsequently reared in a climate box (h × b × d: 50 × 50 × 60 cm; 25 °C, 60% RH, L16 : D8) on cucumber leaves, cut to fit in a Petri dish (diameter: 14 cm) on top of a layer of cotton wool that was put on the bottom of the Petri dish. Once a week, thrips pupae and adults from older leaves of the culture were put on the cucumber leaf and pollen of *Typha latifolia* was provided on this leaf as additional food for the thrips. From the eggs produced by the adult females, thrips larvae hatched. The emerging pupae and adults were then transferred to a new leaf in a new Petri dish to rear a next generation of thrips. This procedure was repeated to maintain a culture.

Predatory mites

A strain of the predatory mite *I. degenerans,* originally collected in Rabat, Morocco, was reared on a diet of *Typha* pollen in a climate box at 25 °C, 60% RH and L16 : D8. The rearing arenas consisted of a polyvinyl chloride (PVC) sheet (6 × 15 cm) placed on a wet sponge in a water-containing tray. The edges of the PVC sheet were covered with paper tissues that absorb water from the sponge underneath. These tissues served as a water source to the predatory mites and as a barrier to prevent escape from the PVC arena. Small threads of cotton placed on the PVC sheet served as a substrate for oviposition by the predatory mites. For the experiments, we used adult females, 8–15 days old since hatching and 0.7 mm in length.

Predatory bugs

Orius laevigatus (Fieber) were obtained from Koppert BV (Berkel en Rodenrijs, the Netherlands) and reared in plastic boxes (40 × 25 × 25 cm) covered with fine nylon gauze. Twice a week, the bugs were fed eggs of the flour moth *Ephestia kuehniella* (Zeller) and provided

with bean pods as an oviposition substrate and source of water supply. Boxes were lined with crumpled paper tissue to provide the juvenile bugs with places to hide from cannibalistic adults (Venzon *et al.*, 2000). For the experiments, we used adult females of an unknown age and c. 2 mm in length.

Experimental set-up

Leaf discs (diameter: 24 mm) with 5–10 second-instar thrips larvae were observed using a binocular microscope. These thrips larvae were presented with either a predatory mite or a predatory bug. We collected individual rectally excreted droplets with 80-mm capillary tubes (Hirschmann Laborgeräte, Eberstadt, Germany). The droplets were released by thrips larvae when a predator was present but had not (yet) attacked, or while being attacked by a predator. As a control, we placed 5–10 thrips larvae on a leaf disc without a predator, prodded them with a small brush and collected the droplet that was produced in response to brush contact (which required up to three times prodding).

GC analysis

In the periods of November 2009–March 2010 and February–June 2012, we collected and analysed a total of 544 individual excreted droplets for the presence and amount of the pheromone components DAc and DDAc. Each droplet comprises approximately 10 nL liquid (Macdonald *et al.*, 2003). By exerting a low level of air pressure, the droplet was removed from the capillary tube it was collected in and added to a solution of 3 μL internal standard (1 ng octyl acetate per μL hexane) and 2 μL n-octane in a 50-μL glass insert within a crimp-capped vial. Using a 7683 automatic injector, the entire volume of each extract was injected in a splitless inlet of a HP7890 gas chromatograph (GC) coupled with a high-resolution polar capillary column (DB-WAXetr [extended temperature range]; 30 m * 0.25 mm * 0.5 mm) and a flame ionization detector (FID). The GC was temperature-programmed from 60 °C (2 min hold) to 180 °C at 30 °C/min, then to 230 °C at 5 °C/min and finally to 250 °C at 20 °C/min, and the FID detector was held at 250 °C. Helium was used as the carrier gas. To exclude the possibility that contaminants in the GC or solvents occur at the same retention time as the pheromone components, before each GC sequence we measured a blank sample, a sample containing only hexane and a sample containing only octane. To assess column performance as well as check the retention times of each of the components, we injected the authentic standards of octyl acetate (> 99% pure, Sigma Aldrich, Zwijndrecht, the Netherlands), DAc (> 99% pure, Alfa Aesar, Lancashire, UK) and DDAc (> 99% pure, Sigma Aldrich) before each GC sequence as well. The amount of each pheromone component in each rectal droplet was calculated relative to the 3 ng of internal standard in each sample. To exclude background noise, we only used those samples that contained at least 0.1 ng DAc + DDAc.

Statistical analysis

To test for differences in number of droplets produced that contained DAc and DDAc between the five treatments, we applied *G*-tests for frequencies in 2×2 tables (Table 1).

To test whether DAc + DDAc or %DDAc were the same in all treatments (excluding the treatment 'attack' by brush), we used a generalized linear model with predator type and presence/absence of an attack as factors. To minimize residuals, we used a generalized linear model (GLM) assuming a quasi-Poisson distribution for DAc + DDAc, and a GLM assuming a normal distribution for %DDAc. This analysis was performed in R (R Development Core Team 2010).

To assess significant differences between two of the five treatments, we first pooled the data obtained under these two treatments and then compared the resulting model with the original model by calculating the contrast statistics (Crawley, 2007). If these two models were significantly different, we concluded that the two treatments had a significantly different effect.

We checked for possible relationships between DAc + DDAc and %DDAc for each of the treatments, using regression analysis. To test whether the slopes of regression lines differed from zero (Zar, 1999) and from each other (Sokal & Rohlf, 1995), an ANOVA was applied.

Table 1 χ^2 analyses of frequencies of droplets containing decyl acetate and dodecyl acetate (Fig. 1).

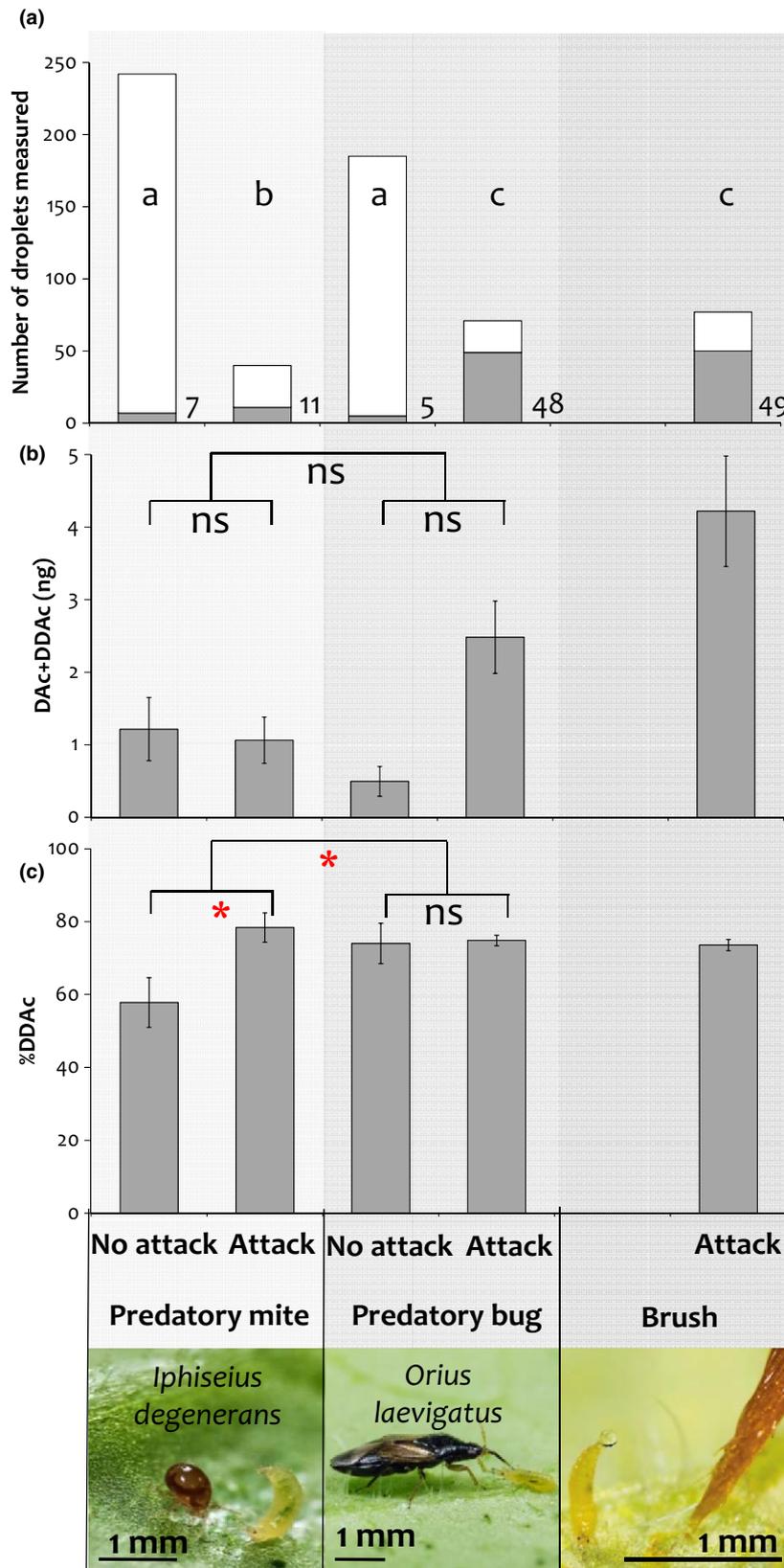|		No attack		Attack		
		Predatory mite	Predatory bug	Predatory mite	Predatory bug	Brush
No attack	Predatory mite	–	$G_1 = 0.01\ P = 0.91$	$G_1 = 23.43\ P < 0.001$	$G_1 = 142.76\ P < 0.001$	$G_1 = 137.18\ P < 0.001$
	Predatory bug		–	$G_1 = 22.40\ P < 0.001$	$G_1 = 129.91\ P < 0.001$	$G_1 = 124.56\ P < 0.001$
Attack	Predatory mite			–	$G_1 = 18.20\ P < 0.001$	$G_1 = 16.01\ P < 0.001$
	Predatory bug				–	$G_1 = 0.13\ P = 0.69$
	Brush					–

Fig. 1 Presence of decyl acetate (DAc) and dodecyl acetateDDAc in rectal droplets excreted by Western Flower Thrips. (a) Number of droplets containing the acetates (grey bars) and the number of droplets containing no acetates (white bars). (b) Total amount of DAc and DDAc measured in the droplets containing these acetates (DAc + DDAc). (c) Percentage DDAc of the total DAc and DDAc measured (% DDAc). The horizontal axis shows the different treatments (predatory mite attack/no attack, predatory bug attack/ no attack, brush attack). The pictures under the horizontal axis illustrate thrips larvae attacked by predatory mite, predatory bug and brush. Note the droplet release of the larva under brush attack. Bars in panel (a) with different letters display significantly different proportions ($P < 0.05$) of droplets containing the acetates in different treatments, and the numbers to the right side of these bars indicate the number of droplets in which acetates were found. Bars in panels (b) and (c) indicate mean values ($\pm$ SEM); ns = not significant; *$P < 0.05$.

Results

In total, we collected and analysed 612 droplets, 120 of which contained DAc and DDAc (Fig. 1). The probability that a droplet contained DAc and DDAc was higher when the larva excreting the droplet was actually attacked by predators (see Fig. 1a, Table 1). When a predator was not attacking, this probability was less than 1 : 30. Moreover, DAc and DDAc were found more often when the attacking predator was a dangerous predatory bug compared to a relatively harmless predatory mite.

The average DAc + DDAc released in the droplets differed depending on the type of attack (Fig. 1b, Table 2) (GLM: brush vs. mite attack; $F_{1, 116} = 5.3$, $P = 0.02$, brush vs. bug attack; $F_{1, 116} = 4.3$, $P = 0.04$). The DAc + DDAc was higher, although not significantly, in two cases: (i) when thrips were attacked by predatory bugs, as compared to when the predatory bugs were only present but not attacking (Fig. 1b) and (ii) when attacked by the predatory bug as compared to when attacked by the predatory mite (Fig. 1b). Thus, thrips are able to release different DAc + DDAc depending on the context they experience.

When DAc and DDAc were present in excreted droplets, they consisted of c. 75% DDAc in all treatments, except for the treatment where a relatively harmless predatory mite was present but not attacking. In that

Table 2 GLM analyses of effects of predator type, occurrence of an attack and their interaction on %dodecyl acetate (DDAc) and decyl acetate (DAc) + DDAc in all treatments, excluding brush 'attack'.

	%DDAc	Dac + DDAc
Predator	d.f. = 1, 69; $P = 0.195$	d.f. = 1, 69; $P = 0.094$
Attack	d.f. = 1, 68; $P = 0.004$	d.f. = 1, 68; $P = 0.236$
Predator*Attack	d.f. = 1, 67; $P = 0.026$	d.f. = 1, 67; $P = 0.208$

case, the %DDAc dropped to 55% (Fig. 1c, Table 2). Thus, thrips larvae released DAc and DDAc in different proportions depending on whether a predatory mite attacked or not (Fig. 1c, Table 2). In addition, thrips larvae released DAc and DDAc in different proportions depending on which of the two predators was present without attacking.

Using regression analysis, we checked for possible relationships between DAc + DDAc and %DDAc in each of the treatments (Fig. 2). There was a reversal in the slope of regression line: when the predators were present but not attacking, the %DDAc increased with DAc + DDAc released, whereas the %DDAc decreased with DAc + DDAc released when the predators attacked ($F_{1, 14} = 9.8$, $P < 0.01$ for the predatory mite, $F_{1,48} = 2.8$, $P = 0.1$ for the predatory bug).

Fig. 2 Regression analysis of quantity and quality of the two acetates decyl acetate (DAc) and dodecyl acetate (DDAc). Scatter plots and linear regressions of %DDAc (y-axis) against DAc + DDAc (x-axis). Different panels relate to different treatments, indicated above and right of the panels. Each dot represents the results from a single droplet. The slopes of the regression lines for a predatory bug or mite without an attack do not differ significantly ($F_{1, 8} = 0.4$, $P = 0.6$), but do differ significantly when a bug or mite is attacking ($F_{1, 55} = 13.7$, $P < 0.001$). The slopes also differ significantly between attack by a predatory mite and when thrips are prodded by a brush ($F_{1, 56} = 12.2$, $P < 0.001$). There is no significant difference in slopes for droplets produced under attack by a predatory bug or a brush ($F_{1, 93} = 0.4$, $P = 0.5$). For other comparisons between slopes, see the main text.
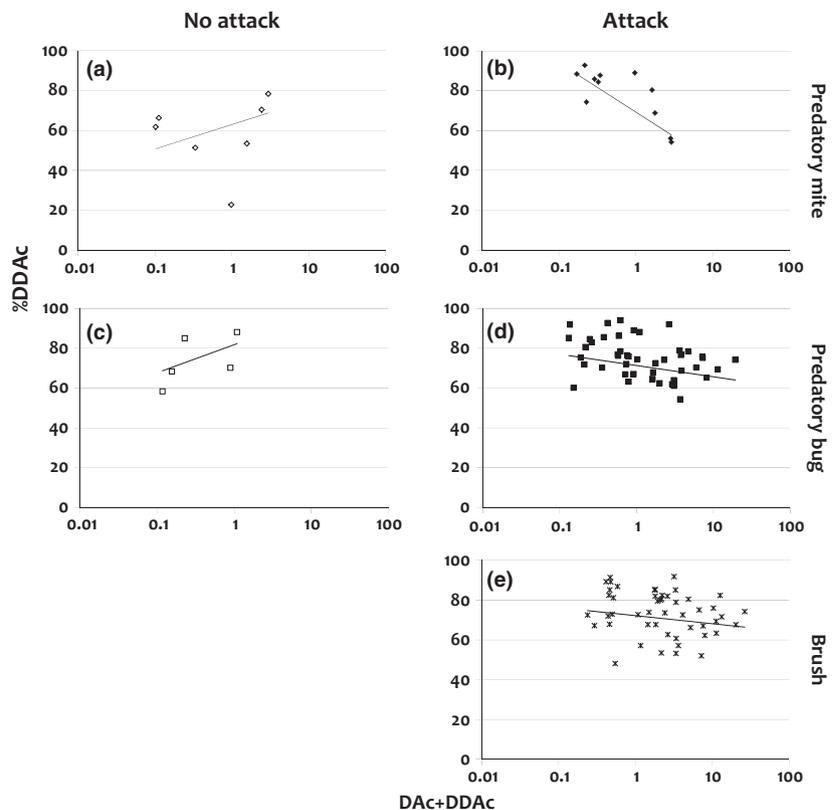
Discussion

Our experiments show that thrips larvae release a different quantity (DAc + DDAc) and/or quality (%DDAc) of the known compounds of alarm pheromone, DAc and DDAc, depending on the type of predator they are exposed to and depending on whether this predator actually attacked the thrips larva or not. These observations support our hypothesis that alarm signals of an insect can specify the level of danger and are thus context dependent. To the best of our knowledge, this is the first report of context-dependent composition of an alarm pheromone. Together with the recent finding that aphids can change the release and quantity of their one-compound alarm pheromone (E-β-farnesene) (Joachim *et al.*, 2013), this suggests that variation in alarm pheromone composition may be widespread.

We do not know how the thrips recognize the predator or estimate the level of danger. The recognition of a predator may be mediated by scent either from the prey consumed by the predator, from the predator itself or from both. Thrips larvae exposed to odours from predatory bugs have been shown to exhibit more frequent escape responses when the predatory bugs had previously been fed thrips larvae compared to predatory bugs fed on eggs of flour moths (Venzon *et al.*, 2000). Hence, dietary cues may play a role in predator recognition by thrips larvae. The predators in our experiments had never eaten thrips larvae as prey in their lifetime, as the predatory bugs were reared on flour moth eggs and the predatory mites were reared on pollen. In our experiments, however, we cannot fully exclude a role of prey cues in predator recognition, as the diets of the predators differed. Apart from scent, touch may be another recognition cue for thrips larvae, not only to recognize the predator but also to recognize the level of danger. This is suggested by our finding that, in terms of DAc + DDAc and %DDAc, thrips larvae responded similarly to an attack by a predatory bug as to an 'attack' with a brush (Fig. 1).

Our study adds to only few studies providing evidence for phenotypic plasticity in chemical communication processes, each of which requires its own timescale. For pheromone-mediated aggregation (Bashir *et al.*, 2003) and sexual attraction of males (Groot *et al.*, 2010) and females (Kent *et al.*, 2008), changes in signal composition can take longer than those required for signalling danger via alarm pheromone, because the type of danger may change at such a short timescale. We found that, in response to either a relatively harmless or a very dangerous predator, actually attacking or not, thrips larvae vary the acetate composition of rectally excreted droplets in seconds or minutes after a thrips larva was exposed to its enemy. Thus, our results suggest that thrips have control over the composition of acetates they excrete.

We conclude that our experiments support the hypothesis that the thrips chemical alarm signal carries information on the level of danger, and suggest that an individual thrips can vary its pheromone composition depending on context, at a timescale similar to the examples of context-dependent signalling in vertebrate vocal alarm calls (Seyfarth *et al.*, 1980; Furrer & Manser, 2009). We propose that context-dependent alarm signalling by means of chemical alarm signals is widespread.

Acknowledgments

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