Context-dependent chemical communication: Alarm pheromones of thrips larvae

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When alarm pheromones vary with the danger from predation: Differential refuge seeking by thrips larvae?

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Abstract

Alarm signals are expected to vary with the level of danger, as shown for vocal alarm signals and recently also for chemical signals. Larvae of the Western flower thrips (*Frankliniella occidentalis*) are known to excrete anal droplets containing chemically well-defined alarm pheromone consisting of two compounds, decyl acetate and dodecyl acetate. With increasing levels of danger (predator species; encounter vs. attack), the chance that an excreted droplet contains alarm pheromone increases and moreover the two compounds increase in total and relative (% dodecyl acetate) amount. Here, we tested if the refuge-seeking response by receivers of the alarm signal reflects the context experienced by the sender, as defined by the attack of a predator species, even when those receivers do not experience any danger. Offering a silken spider-mite web on a leaf disc as a refuge, we analysed how often focal thrips larvae not exposed to predators nevertheless seek refuge after being presented with a filter paper containing alarm pheromone from another thrips larva attacked by a predatory mite or bug, or with a filter paper without pheromone. Because experience with predators might influence the response of thrips larvae to alarm pheromone, these experiments were done with thrips larvae that were naive or that had experience with predators. We found that thrips larvae rarely moved into the refuge when offered filter paper without anal droplets (negative control), but did so significantly more frequently when offered filter paper with anal droplets. Moreover, based on earlier measurement of the frequency of alarm pheromone in anal droplets, thrips larvae responded always to anal droplets excreted under attack by a predatory mite, but not always to anal droplets excreted under attack by a predatory bug. This suggests that thrips larvae respond to alarm pheromone in a context-dependent way.

*Unpublished manuscript*
Introduction

In many animal species, information about the presence of a predator in the vicinity is conveyed by alarm signals (Borden 1989; Ayasse et al. 2001). These signals can be vocal, chemical, visual or even mechanical (Lima and Dill 1990) and because danger may come in various forms, they are prone to be context-dependent (Blum 1996). For vocal alarm signals, there is ample evidence for context-dependent signals in that they vary with predator type or danger level (Sherman 1977; Seyfarth et al. 1980; Blumstein and Armitage 1997; Lima and Dill 1990; Furrer and Manser 2009). Whereas senders may adapt alarm to context, receivers adapt their response to the type of alarm call conveyed by the sender (Sih 1980; Lima and Dill 1990). For instance, Belding’s ground squirrels (Spermophilus beldingi) release signals that denote different levels of response urgency (Robinson 1980). The signals vary with the speed and distance of the approaching predator but not specifically with the type of predator (Robinson 1981). As ground squirrels live in open habitats and run to their burrows in response to any predator type, information about urgency may be more important than that on predator type (Blumstein and Armitage 1997; Furrer and Manser 2009). Another example of context dependent alarm calls is that of ring-tailed lemurs (Lemur catta), which are known to contain information on predator type in their alarm calls (Macedonia 1993). This makes sense because they live in complex habitats, are hunted in ways depending on predator type and tune their escape strategy accordingly (Macedonia 1993; Macedonia and Evans 1993; Furrer and Manser 2009).

For chemical alarm signals, i.e., alarm pheromones, virtually nothing is known about context-dependent signalling (Verheggen et al. 2010; Blum 1996). There are examples of organisms that show inter-individual variation in chemical alarm pheromones, such as the pea aphid, Acyrthosiphon pisum (Pickett et al. 1992; Kunert et al. 2005; Podjasek et al. 2005), the paper wasp Polistes dominulus (Bruschini et al. 2008) and the Western flower thrips (de Bruijn et al. 2014b, in prep.). Pea aphids are known to respond to the alarm pheromone (E)-β-farnesene (Pickett et al. 1992) by increasing the production of the winged offspring (Podjasek et al. 2005) and they show a stronger response to the release frequency of the pheromone than to the amount of pheromone perceived (Kunert et al. 2005). Paper wasps respond more strongly to the pheromone produced by workers – which differs quantitatively – than to the pheromone produced by the foundresses (Bruschini et al. 2008). To the best of our knowledge, there is only one example showing that conspecific larvae tune their response to variation in chemical alarm pheromone, namely Western flower thrips larvae. Recently, we found that the composition of alarm pheromone from second-instar larvae of
Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), differs with the level of danger they experience (de Bruijn et al. 2014a, subm.). Thrips larvae may encounter different predators, varying in their voracity. For example, predatory bugs (*Orius laevigatus* (Fieber)) are successful in attacking all mobile stages (Sabelis and van Rijn 1997), whereas the much smaller predatory mites (*Iphiseius degenerans* (Berlese)) are more successful in attacking first-instar larvae and have little or no impact on all other stages. Despite the specialization of predatory mites on first-instar thrips larvae, both first- and second-instar larvae suffer most from predation by predatory bugs. The question we address here is whether Western flower thrips larval alarm pheromone produced under different levels of danger (attack by one or the other predator species) triggers different and adaptive antipredator responses in receiving larvae.

The alarm pheromone of Western flower thrips is present in anal excretions that are produced in the form of droplets – the so-called ‘anal droplets’ (MacDonald et al. 2003). Teerling et al. (1993) found that this pheromone consists of two compounds: decyl acetate (DAc) and dodecyl acetate (DDAc). De Bruijn et al. (2014a, subm.) found that anal droplets excreted by a second-instar larva during predator attack does not always contain alarm pheromone, and that alarm pheromone is released more frequently when the attacker is a predatory bug (69% of droplets contain pheromone) rather than a predatory mite (27.5% of droplets contain pheromone). Furthermore, when alarm pheromone is excreted, the ratio of decyl acetate and dodecyl acetate as well as the total amount of pheromone varies with the level of danger the sender encountered: dangerous vs. relatively harmless predator species, and encounter vs. attack. Alarm pheromone composition also varies with the instar of the sender (MacDonald et al. 2003), and first-instar larvae exhibit differential escape response to alarm pheromone of different instars (de Bruijn et al. 2014b, in prep.), in absence of a predator.

In this paper, we quantify the response of thrips larvae to anal droplets that are excreted by thrips larvae under attack by one or the other predator species, by scoring the frequency of refuge seeking. We use the silken web created by spider mites on a leaf as a refuge for the thrips (as in Venzon et al. 2000). Thrips and spider mites often co-occur on the same plants, and when threatened by predators, thrips larvae seek refuge in the web of spider mites where they are less vulnerable to predation by predatory bugs, as well as by predatory mites (Pallini et al. 1998). In absence of cues of these predators, thrips larvae prefer to stay outside of spider mite webs to avoid food competition and speed up development. In the presence of cues of these predators (but without a predator nearby) they
may move into the web refuge (Pallini et al. 1998; Venzon et al. 2000). We use the same setup to test whether thrips larvae seek refuge in response to anal droplets.

Unfortunately, the amount of pheromone (2-10 ng pheromone in a droplet of ca. 1 nl mostly containing water) excreted by individual thrips larvae is so small that we cannot simultaneously assess the presence of alarm pheromone in a droplet and the response to the droplet. Instead, to interpret our results, we assume that the probability that an anal droplet contains alarm pheromone and the composition of alarm pheromone are the same as we measured before (de Bruijn et al. 2014a). We hypothesize that thrips larvae always seek refuge when perceiving alarm pheromone, and hence more often when presented with anal droplets excreted by larvae under attack by predatory bugs rather than predatory mites.

**Materials and methods**

**Cucumber plants**
Cucumber plants (*Cucumis sativus* var. Ventura RZ, Rijk Zwaan, De Lier, The Netherlands) were grown from seeds, free of herbivores, in a climate room (25 °C, 70 ± 10% RH and L16:D8).

**Thrips**
Western flower thrips were collected from chrysanthemum plants in Wageningen, The Netherlands, in March 2010 and were reared in our laboratory on cucumber leaves in a climate room (25 °C, 60% RH, L16:D8) using the procedure described by de Bruijn et al. (2014c).

**Spider mites**
Spider mites (*Tetranychus urticae* Koch) were collected from cucumber plants in a commercial greenhouse in May 1994 and were reared in our laboratory on cucumber plants (Ventura RZ) in a climate room (25 °C, 60% RH, L16:D8).

**Predators**
Predatory bugs (*O. laevigatus*) were provided by Koppert Biological Systems and reared in the laboratory at 21 °C. The predators were kept in plastic boxes (40 x 25 x 25 cm³) covered with fine nylon gauze. We maintained the culture by providing the predatory bugs with eggs of *Ephestia kuehniella* Zeller as food and bean
pods (*Phaseolus vulgaris* L.) as oviposition substrate and a source of moisture. For the experiments only adult females were used.

Predatory mites (*I. degenerans*) were reared on plastic arenas (8 × 15 cm), placed on wet sponges in a plastic tray with water (Nomikou *et al.* 2003). The trays were kept in a climate room (25 °C, 60% RH, L16:D8). The predatory mites had access to water surrounding the arena and were fed with pollen of *Typha latifolia*. For the experiments only adult females were used.

**Response to alarm pheromone**

We created leaf arenas with refuges consisting of a spider mite web using the following procedure (Pallini *et al.* 1998). A cucumber leaf disc (24 mm diameter) was cut in such a way that the main vein was in the middle and divided the disc in two halves. The leaf disc was placed on wet cotton wool in a Petri dish (30 mm diameter). The main vein was covered with wet cotton wool and on one half of the leaf disc 30 adult female spider mites were released. The spider mites were left for two days to feed, oviposit and produce web. After two days the cotton wool and the adult mites were removed carefully with the use of a thin needle. While removing the spider mites, care was taken to minimize damage to the web they had produced. The eggs produced by spider mites (ca. 500 spider mite eggs) were left on the leaf disc because it would damage the web too much if we would remove them. During the experiments, thrips larvae had the possibility to feed on spider mite eggs, but feeding on eggs was not observed during the experiments. Hence our arena consisted of a leaf disc, half of which was damaged and covered with spider mite eggs, faeces and web, whereas the other half remained intact and clean. On the clean half we introduced one first-instar thrips larva. In this experiment, we tested responses of first-instar larvae to alarm pheromone in absence of a predator. First-instar larvae were used because they have been shown to vary their response with the composition of alarm pheromone in a similar set-up (de Bruijn *et al.* 2014b).

Subsequently, on a piece of filter paper (ca. 0.1 cm²) we collected an anal droplet produced by a second-instar larva that was under attack and deposited the filter paper on the clean half of the leaf disc. Second-instar larvae were used to excrete alarm pheromone because they have been shown to vary their alarm pheromone with the level of danger (de Bruijn *et al.* 2014a). Filter paper without excretions served as a negative control, whereas filter paper with excretions induced by artificially prodding the larvae with a fine brush served as a positive control. Previously we found that 69% of the droplets excreted when a larva was prodded with a brush contained alarm pheromone (de Bruijn *et al.* 2014a) and when alarm pheromone was found, it was present in high amounts. Finally, we recorded the position of the first-instar focal larva (clean half or webbed half) after 1, 5 and 30 min.
We used first-instar larvae without experience with predators, or with experience with a predatory mite, with a predatory bug, or with both. In order to give thrips experience, we introduced one predatory bug, two predatory mites or one bug and two predatory mites on a leaf disc containing thrips, and allowed the predators to feed on thrips overnight (12 h). For experiments, we used first-instar larvae from these leaf discs that had survived this predation setting.

We collected anal droplets from second-instar larvae that were taken from the culture and hence had no experience with predators (cf. de Bruijn et al. 2014a). We collected the droplets on filter paper, while the second-instar larvae were under attack of a predator or were prodded by the experimenter using a fine brush. The predatory bug was released on the Petri dish and was allowed to feed on the second-instar larvae. As soon as the thrips larvae were under attack, they produced an anal droplet, which was then collected on a piece of filter paper by the experimenter. The predatory mite was not released in the Petri dish (the procedure used to collect droplets by de Bruijn et al. 2014a), but instead it was stuck on top of a fine brush. Using the fine brush the experimenter brought the predatory mite within reach of a second-instar thrips larva, until the predator touched the thrips larva with its legs. As soon as the larva excreted an anal droplet, it was collected on a piece of filter paper. Attention was paid to avoid the brush contacting the thrips larva. This procedure was used here, because it takes quite some time before a predatory mite attacks a thrips larva and we intended to control the time between placing a first-instar larva on the leaf disc with the web refuge and collecting the excretion on filter paper. All predators used were starved for 24 h.

In total, we conducted four experiments, one for each experience treatment of the focal thrips larva (no experience, experience with predatory bugs, with mites or with both). Each experiment had four attack treatments (alarm pheromone excreted under attack by predatory mite or predatory bug, negative control and positive control), and in each attack treatment we observed the response of 30 independent thrips larvae (except in the positive control of predator-naive thrips where we tested 50 individuals).

**Statistical analysis**

Using the open source program R (R Development Core Team 2010), we applied a Kaplan-Meier survivorship analysis (Hosmer and Lemeshow 1999) on the number of larvae in the clean area to compare the groups that were exposed to filter paper with anal droplets collected under attack of a predatory mite or a predatory bug, by prodding with a brush (positive control) or without anal droplets. Contrasts among treatments were assessed through exclusion of
treatment that by eye appeared to be the most deviant from the others until the survivorship analysis did not show a significant difference among the remaining treatments.

To assess whether thrips larvae always seek refuge quickly when perceiving alarm pheromone, the frequency of thrips larvae that move into the web within 5 min was compared with the frequency of alarm pheromone in anal droplets we found previously (69% of all droplet contain pheromone when thrips are under attack by a predatory bug, 27.5% when under attack by a predatory mite; de Bruijn et al. 2014a). Using 95% confidence intervals of these expected frequencies from a binominal distribution of 30 individuals, between 16 and 25 individuals are expected to seek refuge when presented with a filter paper containing an anal droplet excreted under attack by a predatory bug and between 4 and 13 individuals when presented with a filter paper containing an anal droplet excreted under attack by a predatory mite.

Focal larvae that had experience with one or two predators, had survived ca. 16 h in the vicinity of that predator(s). Hence, we may have selected for larvae that are somehow better able to survive predators nearby. This selection could be severe, especially near a predatory bug, because they kill numerous larvae in one night. Naive focal larvae had not been subjected to such a selection. Therefore, we do not analyse comparisons of refuge use data among the different experience treatments.

**Results**

*Naive thrips larvae*

When exposed to a filter paper with an anal droplet excreted by a second-instar thrips larva under attack by one of the two predator species or prodded with a brush (positive control), roughly 20-30% of the naive thrips larvae moved into the webbed area within 5 min, as opposed to none when exposed to a clean filter paper (negative control). After 5 min, some thrips larvae still moved into the web refuge, but these numbers were always comparable among all treatments including the negative control (see supplementary information). We found a significant overall effect of treatment on the rate of decline of thrips individuals that were on the clean half of the leaf disc (Figure 6.1; \( \chi^2 = 8.3, \text{d.f.} = 3, P = 0.040 \)), which was due to the negative control (there was no significant difference in refuge seeking among the other treatments; \( \chi^2 = 0.5, \text{d.f.} = 2, P = 0.77 \)). We conclude that naive thrips seek refuge when presented with anal droplets on the filter paper and not when presented with the filter paper only.
After 5 min, eight thrips larvae moved into the web when presented with a droplet excreted under attack by a predatory bug, which is less than expected (16–25 thrips larvae). When presented with a droplet excreted under attack by a predatory mite, six larvae moved in the web which is within the 95% confidence interval (4–13 thrips larvae).

**Experienced thrips larvae**

When thrips larvae had survived on a leaf disc where a predatory bug, two predatory mites or both predator species had preyed on conspecific thrips larvae for one night, and subsequently tested for their response to pheromones, we found an overall effect of treatment (experience with predatory bug: $\chi^2 = 19.1$, d.f. = 3, $P<0.001$; experience with predatory mites: $\chi^2 = 14.3$, d.f. = 3, $P = 0.003$; experience with both predators: $\chi^2 = 10.2$, d.f. = 3, $P = 0.017$), that mainly emerged from the treatment where larvae were exposed to a filter paper without an anal droplet (**FIGURES 6-2, 6-3 and 6-4**). Larvae did not show significantly different refuge seeking behaviour when exposed to an anal droplet excreted at bug attack, positive control or mite attack (experience with predatory bug: $\chi^2 = 4.5$, d.f. = 2, $P = 0.10$; experience with predatory mites: $\chi^2 = 5.7$, d.f. = 2, $P = 0.059$; experience with both predators: $\chi^2 = 1.1$, d.f. = 2, $P = 0.58$).

**FIGURE 6-1** Percentage of predator-naive thrips larvae remaining outside the refuge after exposure to anal droplets. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments, except when larvae were prodded with a brush, where N = 50. Treatments indicated with different letters exhibit significant difference in the frequency of refuge use.
CHAPTER 6

**FIGURE 6-2** Percentage of thrips larvae remaining outside the refuge after exposure to anal droplets. Larvae had survived for 12 h in the vicinity of a predatory bug. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments. Treatments indicated with different letters exhibit significant difference in the frequency of refuge use.

**FIGURE 6-3** Percentage of thrips larvae remaining outside the refuge after exposure to anal droplets. Larvae had survived for 12 h in the vicinity of predatory mites. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments. Treatments indicated with different letters exhibit significant difference in the frequency of refuge use.
After 5 min, the number of thrips larvae that moved into the web when presented with a droplet excreted under attack by a predatory bug was 8 (experience with predatory bug), 13 (experience with predatory mite), and 8 (experience with both predators). All three numbers are less than expected. When presented with a droplet excreted under attack by a predatory mite, 15 (experience with predatory bug), 11 (experience with predatory mite), and 9 (experience with both predators) moved into the web. These numbers are not different from what was expected, except for the predatory bug experience treatment, where it is more often than expected.

**Discussion**

By the experiments presented here, we investigated how thrips larvae respond to anal droplets putatively containing alarm pheromone in different frequencies and compositions, aiming to test whether they show differential refuge seeking behaviour. Since we evaluated the thrips response to anal droplets only and in absence of the agents (predators, brush) that induced their release, all information focal larvae receive must be contained within the anal droplets released by the sending thrips larvae and not by the predator or injured thrips. The results
showed that thrips larvae, irrespective of their experience, seek refuge in response to odours from anal droplets and do not seek refuge in the negative control. We found no difference in their refuge-seeking response among odours from anal droplets produced under the different types of attack (FIGURES 6-1 – 6-4). A simple explanation for the lack of differentiation in response to droplets excreted under attack by different predators is that thrips respond to the anal droplets, regardless of their content. However, it is known that thrips respond to alarm pheromone (MacDonald et al. 2002; de Bruijn et al. 2006) and that some anal droplets have a higher chance of containing alarm pheromone than others (de Bruijn et al. 2014a). Hence, it seems unlikely that our results can be explained exclusively by the presence of an anal droplet in absence of the alarm pheromone.

To interpret thrips refuge-seeking response to alarm pheromone, we had to make assumptions on pheromone presence in anal droplets, because we were unable to simultaneously measure pheromone in an anal droplet and the response of another thrips larva to that same droplet. We assumed that the proportions of anal droplets with pheromone is the same as measured earlier (de Bruijn et al. 2014a), i.e., 69% in the droplets derived from predatory bug attack and the positive control (brush attack), and 27.5% in droplets derived from predatory mite attack. Under this assumption, bug-induced droplets did not always trigger refuge-seeking behaviour to both naive and experienced thrips larvae, whereas all mite-induced droplets did. In the case where thrips have experience with a predatory bug and are presented with a droplet excreted under attack by a predatory mite, thrips larvae sought refuge significantly more often than 27.5% of the replicates. In this specific treatment therefore, our assumption on the percentage of excreted droplets containing pheromone appeared to be underestimated. Notwithstanding, our results suggest that thrips larvae seek refuge at different rates when presented with alarm pheromone excreted under attack by a predatory bug or predatory mite.

We found that thrips larvae move in the web less often than expected when they perceive droplets excreted under attack by a predatory bug. What could be the cause of this observation? When perceiving variation in alarm pheromone, thrips larvae might differentiate their response. Differentiation in anti-predator behaviour has been shown for various prey species that adapt their escape behaviour to the hunting strategies of the predator they face (Marler 1967; Cheney and Seyfarth 1990; Macedonia and Evans 1993; Furrer and Manser 2009). The predators used in our study have a different hunting strategy as well: predatory mites do not have eyes (although they can perceive light; Evans 1992) and hunt by smell and touch (personal observations, PJAdB), but predatory bugs
respond to movement while hunting (personal observations, PJAdB). Hence the response of first-instar thrips larvae not to move into the web when perceiving alarm pheromone excreted by a larva under attack of a predatory bug, might be adaptive when larvae are preyed upon earlier while moving when a predatory bug is nearby. Such an effect of movement on predation risk is not expected for thrips under attack of a predatory mite.

Varying an alarm signal with the context of the sender, enables receivers of this signal to respond adaptively. Adaptive responses are known for vocal alarm signals, but scarce for chemical alarm signals. Our results suggest that thrips larvae can distinguish between chemical alarm signals collected in different contexts and hence can tune their response to the signal they perceive.

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Differential refuge seeking by thrips larvae?


Supplementary information

**Figure S6-I** Percentage of predator-naive thrips larvae remaining outside the refuge 30 min after exposure to anal droplets. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments, except when larvae were prodded with a brush, where N = 50. Treatments indicated with different letters exhibit significant difference in the frequency of refuge use.
FIGURE S6-II Percentage of thrips larvae remaining outside the refuge 30 min after exposure to anal droplets. Larvae had survived for 12 h in the vicinity of a predatory bug. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments. Treatments indicated with different letters exhibit significant difference in the frequency of refuge use.

FIGURE S6-III Percentage of thrips larvae remaining outside the refuge 30 min after exposure to anal droplets. Larvae had survived for 12 h in the vicinity of predatory mites. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments. Treatments indicated with different letters exhibit significant difference in the frequency of refuge use.
Differential refuge seeking by thrips larvae?

**FIGURE S6-IV** Percentage of thrips larvae remaining outside the refuge 30 min after exposure to anal droplets. Larvae had survived for 12 h in the vicinity of both predatory bug and mites. Different lines and symbols represent the four treatments: attack of a predatory bug (blue line with diamond markings), attack of a predatory mite (red line with squares), positive control (green line with triangles), negative control (purple line with crosses). N = 30 for all treatments, except when larvae were attacked by a predatory mite, where N = 29. No significant differences were found.