Anti-predator responses to alarm pheromones in groups of young and/or old thrips larvae

de Bruijn, P.J.A.; Sabelis, M.W.; Egas, M.

DOI
10.1111/eth.12600

Publication date
2019

Document Version
Final published version

Published in
Ethology

Received: 18 October 2016 | Accepted: 28 January 2017

DOI: 10.1111/eth.12600

RESEARCH PAPER

WILEY ethology

Antipredator responses to alarm pheromone in groups of young and/or old thrips larvae

Paulien J. A. de Bruijn | Maurice W. Sabelis† | Martijn Egas

Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, the Netherlands

Correspondence
Martijn Egas, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, the Netherlands.
Email: egas@uva.nl

Funding information
ALW-NWO, Grant/Award Number: 818.01.018

Editor: J. Wright

Abstract

Many prey species suffer from different predators in the course of their ontogeny. Hence, the alarm signal a small prey individual sends can have a different meaning than the signal a large prey individual sends, both for small and for large receivers. Larvae of Western Flower Thrips face predators that attack only small larvae, or predators that attack small larvae and large larvae. Furthermore, thrips larvae release a two-component alarm pheromone, which varies in composition with larval age. Here, we study whether their response to alarm pheromone varies with composition of the pheromone. First, we confirmed that large and small larvae respond when nearby larvae of both sizes were prodded with a brush to induce alarm pheromone excretion. Subsequently, we tested whether thrips larvae of a given size respond differentially to alarm pheromone excreted by a small or large companion larva. We analyzed two types of behavior used in direct defense against a predator and one type of escape response. Only small (not large) larvae attempted to escape more frequently in response to excretions from a large larva. This difference in response could have been due to the alarm pheromone or to the companion larva in the vicinity. We subsequently tested for, but did not find, an effect of size of the companion larva on the behavior of the test larva when exposed to synthetic pheromone mimicking that of a large larva. Finally, we tested how pheromone composition affects antipredator behavior by exposing thrips larvae to synthetic pheromones differing in amount and ratio of the two components. Only for small larvae, we found significant changes in escape behavior with pheromone amount, and a trend with the ratio. Overall, we conclude that small thrips larvae respond differentially to alarm pheromones excreted by small and large larvae and that this differential response is due to differences in pheromone quantity and possibly also quality. Our results suggest that responses to alarm signals can vary with the chemical composition of those alarm signals.

KEYWORDS

alarm pheromone, antipredator behavior, context dependence, *Frankliniella occidentalis*

†deceased 7 January 2015.

1 | INTRODUCTION

The predation risk imposed by a predator on a prey individual often changes with prey size (e.g., Chase, 1999; Lima & Dill, 1990; Tonn, Paszkowski, & Holopainen, 1992). Larger individuals can be invulnerable to predators that effectively prey on smaller individuals of the same prey species and vice versa, whereas some predators are dangerous to prey of all possible sizes. Thus, if an alarm signal is sent by a small individual, it may convey information on a different danger level to another small individual than to a large one. So how can receivers of different sizes tell these differences in information apart if they have no clue of the sender's size? A possible solution to this problem may emerge if alarm signals vary consistently with prey size. Then, receivers may evolve an antipredator response that is balanced against other fitness-enhancing activities (Lima & Dill, 1990; Sih, 1980). Such context-dependent alarms and adaptive antipredator responses have been found for alarm cues in aquatic systems (e.g., Belden et al. 2000; Mirza & Chivers, 2002), but to the best of our knowledge, there are only two examples where alarm signals vary with ontogeny in terrestrial systems; First, colony foundresses and workers of the paper wasp *Polistes dominulus* (Christ) excrete alarm chemicals in different ratios in their venom, and workers respond differently to the pheromone of workers and that of foundresses (Bruschini, Cervo, Protti, & Turillazzi, 2008). Second, the amount and ratio of the two components of the alarm pheromone of Western Flower Thrips (*Frankliniella occidentalis*) (Insecta: Thripidae) varies with the age of the thrips larva emitting it (MacDonald, Hamilton, Jacobson, & Kirk, 2003). Here, we consider the second example in more depth by testing whether the response of thrips larvae to alarm pheromone varies with the age of the sender and receiver.

Thrips larvae have several features that make them suitable objects to study responses to chemical signals. First, the alarm pheromone is present in anal excretions that are released in the form of droplets of c. 1 nL (MacDonald et al., 2003) and the release of these so-called anal droplets can be observed. Second, the release of a droplet can be triggered by prodding a larva with a fine brush. Third, the chemicals constituting the alarm pheromone have been identified as decyl acetate and dodecyl acetate (Teerling, Pierce, Borden, & Gillespie, 1993), thus enabling the use of synthetic mimics of the alarm pheromone (de Bruijn, Egas, Janssen, & Sabelis, 2006; Teerling et al., 1993). Fourth, the variation in alarm pheromone described above concerns both the ratio and amount of decyl acetate and dodecyl acetate (MacDonald et al., 2003). Finally, thrips larvae exhibit easily observable antipredator responses when exposed to the alarm pheromone, such as walking away (Teerling et al., 1993), retreating into refuges (Venzon, Janssen, Pallini, & Sabelis, 2000), swinging their abdomen and producing an anal droplet, which they try to bring into contact with the integument and extremities of the predator (Bakker & Sabelis, 1987, 1989). These droplets are thought to be acidic, and when predators become contaminated with it, they give up attacking and retreat to groom (Bakker & Sabelis, 1989).

Thrips larvae commonly live in groups of mixed ages and—because their body size correlates well with age—also of mixed sizes. This is important because size matters to the predation risks that larvae experience (Bakker & Sabelis, 1987, 1989; Sabelis & Van Rijn, 1997). For example, predatory mites, which are c. 0.5 mm in size, are much more successful in attacking first-instar thrips larvae (c. 0.75 mm, see Table S2 and S3) than second-instar larvae (c. 1.0 mm, see supplement for materials and methods) (Bakker & Sabelis, 1987, 1989; Sabelis & Rijn, 1997), whereas predatory bugs (c. 2 mm) attack both instars equally successfully (Sabelis & Rijn, 1997). Given the variation in pheromone composition with age- and the size-dependent predation risk, the alarm pheromone excreted by small (first instar) and large (second instar) thrips larvae represents different information on the level of danger. However, to the best of our knowledge, nothing is known about responses of thrips to these different alarm signals.

We test the hypothesis that both small and large receiver larvae show differences in behavioral responses to alarm pheromone produced by a small or large companion larva. Because small larvae are more vulnerable to predation than large larvae, we expect small larvae to always respond to the alarm pheromone of both small and large larvae, whereas we expect large larvae to always respond to alarm pheromone of large larvae but less so to that of small larvae. We scored two types of behavior that thrips larvae use in direct defense against predators and one escape behavior. If larvae perceive alarm pheromone, this may indicate the presence of an attacking predator in the vicinity, but the receiving larva is not directly under attack. Hence, we expect that these larvae will not show an increase in defense behavior aimed at a predator, but will show an increase in escape behavior. Because small and large larvae release different amounts of alarm pheromones, we tested first whether alarm pheromone of larvae of different size invoked a response in all larvae, with a setup that was previously used to show that larvae do respond to alarm pheromone from large larvae (de Bruijn et al., 2006). Subsequently, we tested behavior of small and large focal thrips larvae before and after the induced release of an anal droplet by a small or large companion larva present in the same experimental arena. To control for differences between companion larvae other than the alarm pheromone they excrete, we also observed responses of focal larvae to synthetic pheromone. Finally, we tested whether the total amount or the ratio of decyl acetate to dodecyl acetate affected the response of focal thrips larvae.

2 | MATERIALS AND METHODS

2.1 | Cultures

Cucumber plants, *Cucumis sativus* (var. Ventura RZ, Rijk Zwaan, De Lier, the Netherlands), were grown, free of herbivores, in a climate room at 25°C, 70% relative humidity and L16:D8 photoperiod. We had two different cultures of Western Flower Thrips *Frankliniella occidentalis* Pergande, for our experiments; for the first culture, thrips were collected from cucumber plants in a commercial glasshouse in Pijnacker, the Netherlands, in February 2006. Thrips were subsequently reared in a climate box (25 °C, 60% RH, L16:D8) on cucumber leaves, cut to fit in a Petri dish on top of a layer of wet 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 such a cucumber leaf and pollen of *Typha latifolia* was provided on this leaf as additional food for the thrips. The adult females would lay eggs in this new leaf disk, and

after approximately a week, this would result in new adults and pupae and the procedure was repeated. Unfortunately, this culture collapsed when our research group moved to a new building in 2010. For the second culture, thrips were generously sent to us by Greet Steenhuis-Broers and Willem Jan de Kogel from Wageningen University in 2010. Before the thrips were sent to us, they had been kept on chrysanthemum. This new culture was reared in the same way as described above.

2.2 | Synthetic alarm pheromone

Synthetic alarm pheromone was prepared by dissolving decyl acetate (Alfa Aesar, Germany) and dodecyl acetate (>99% pure, Sigma-Aldrich, USA) in cyclohexane (98% pure, Sigma-Aldrich, USA). Four different pheromone blends were prepared in such a way that 1 μl of such a blend corresponded to the amount and/or ratio of the two pheromone components present in the anal droplet of one first- or second-instar larva. In the first blend, the total amount and ratio of the two components corresponded to that of the alarm pheromone of one second-instar thrips larva; (5 ng of each component in 1 μl; MacDonald et al., 2003). The second blend contained the total amount of pheromone released by one second-instar larva (10 ng), but in the ratio corresponding to the pheromone of first-instar larvae, 1:3 for decyl acetate: dodecyl acetate (MacDonald et al., 2003); hence, 1 μl contained 2.5 ng of decyl acetate and 7.5 ng of dodecyl acetate. The third blend contained the total amount of decyl acetate and dodecyl acetate as present in pheromone of a first-instar larva (0.6 ng, MacDonald et al., 2003), but the ratio of the two compounds was similar to the pheromone released by second-instar larvae (1:1). Hence, 1 μl of the third blend contained 0.3 ng of each component. In the fourth blend, the total amount and ratio of the two compounds corresponded to that of pheromone of a first-instar larva; therefore, 1 μl of the fourth blend contained 0.15 ng decyl acetate and 0.45 ng dodecyl acetate. In the experiments described below, either 1 μl of this solution of synthetic alarm pheromone was used or 1 μl of cyclohexane as a control.

2.3 | Response to natural pheromone of small and large larvae

Adult female thrips from the Wageningen culture were placed in groups of three to five on a rectangular leaf fragment of approximately 25 cm^2 and were allowed to oviposit for approximately one week. Subsequently, the females were removed. At this time, the leaf fragment harbored roughly 20 first- and second-instar larvae, of which we randomly selected up to five individuals for the experiment. We gently prodded a first-instar (small) or second-instar (large) larva once or twice within a second with a metal needle until it excreted an anal droplet and then dipped the needle in this droplet. We immediately challenged a first- or second-instar larva (haphazardly chosen) on another leaf fragment with this needle by repeatedly prodding it until an anal droplet was excreted, and we measured the time it took for this induced response to occur. As control treatment, we also challenged first- or second-instar larvae with a clean needle. Using this controlled method to test whether thrips larvae respond to the alarm pheromone, they are not exposed to cues from a predator that may also affect their response. We chose not to isolate thrips larvae for this test, because that involves moving them with a brush which usually results in excretion of an anal droplet, and most thrips larvae do not excrete another droplet for at least several hours afterward (de Bruijn, personal observation). In our procedure, most thrips larvae on leaf fragments where we collected excreted droplets are challenged after other thrips larvae from the same fragment excreted droplets. The latter may affect their response, but this is the same for all treatments in the experiment. To otherwise minimize recent experience with anal droplets, larvae used to measure the time until droplet excretion were selected from different leaf fragments than larvae used to excrete an anal droplet in which the needle was dipped. We analyzed the data using a one-way ANOVA.

2.4 | Responses to natural alarm pheromone & effect of companion larva

Small leaf disks (Ø10 mm) were cut from cotyledons of cucumber plants and served as experimental arenas. Two thrips larvae from the Pijnacker-culture were placed on each experimental arena. One was designated as "focal" larva, and its behavior was observed during the experiment. The other larva was designated as "companion" larva. To allow acclimatization of the larvae, the experimental arena with both focal and companion larvae was incubated in a climate room (25°C, 70% relative humidity, and L16:D8 photoperiod) for 16 hours. Approximately five minutes before the experiment, the experimental arena was placed on a larger leaf disk (Ø 24 mm), also cut from a cucumber cotyledon, which was placed in a Petri dish with a layer of wet cotton wool at the bottom. Five minutes appeared to be enough to allow thrips larvae to resume their feeding behavior (de Bruijn, personal observation). The larger leaf disk served as alternative to which the thrips larvae could escape from the experimental arena.

For experiments on behavioral responses to alarm pheromone, we scored two types of defensive behavior: the excretion of an anal droplet and the execution of abdominal swings (i.e., a characteristic movement where the thrips larva jerks its abdomen from one side to another; Bakker & Sabelis, 1987, 1989). In addition to these defensive behaviors, we also scored escape behavior, defined as thrips larvae moving off the experimental arena (smaller disk) onto the larger leaf disk. This escape behavior, however, was observed infrequently. Instead, we observed much more frequently that larvae move over the border of the experimental arena up to approximately half their body length, head first, yet move back to the experimental arena before they had fully moved off. We scored these partial crossings of the edge of the experimental arena (henceforth called "partial crossings") because they arguably relate to a tendency to leave the experimental arena. If a focal thrips larva escaped the experimental arena (smaller leaf disk) before a treatment was applied, the replicate was discarded. In case a thrips larva escaped from the experimental arena within two minutes after applying a treatment, the observation was terminated. These replicates were included in the analyses after correcting for the shorter observation time by calculating the rate of the observed behaviors (number of

scored behaviors divided by the observation time). Observations were made on 25 focal larvae per treatment. Thrips behavior was observed using a binocular microscope with a cold light source and was recorded and timed using the freeware event recorder EthoLog version 2.2.5 (Ottoni, 2000). This program is used to record the different types of behavior and the time at which they occurred.

All four combinations of small and large focal and companion larvae were tested. We induced the production of alarm pheromone (hereafter called natural alarm pheromone) by gently prodding the head of the companion larvae once or twice with a fine brush. To assess the role of cues coming from the companion larva other than the alarm pheromone, we added a control where we tested the response of the focal larva, in the presence of first- or second-instar companion larvae, to synthetic pheromone mimicking that produced by a second-instar larva. Furthermore, as a control we added only the solvent of the synthetic control, cyclohexane. In these two controls, we used a Gilson pipette to apply 1 μl of pheromone solution or cyclohexane on the experimental arena, away from the thrips larvae. Thrips were randomly assigned to the natural pheromone treatment or one of the two controls.

Because the observed antipredator behavior can also occur in the absence of alarm pheromone, we observed each focal larva for two minutes before and two minutes after application of a treatment. This enabled the detection of changes in droplet release by the thrips larva, which was subsequently used to test for the effects of the various treatments. To analyze behavioral differences due to companion larvae, changes in number of anal droplets released by individual larvae were analyzed using a generalized linear model (GLM) assuming a Poisson error distribution. Contrasts among treatments were assessed through model simplification (Crawley, 2007) and simplified models were compared with more extended models using the anova function in R. Furthermore, the standard assumptions on residual variation were checked.

Because the number of abdominal swings and partial crossings was zero-inflated, we analyzed the number of swings and partial crossings before and after the treatment separately using a nonparametric Kruskal–Wallis test (Siegel & Castellan, 1988). Because the groups of thrips larvae with the same instar as companion were treated identically before applying one of the pheromone treatments, we pooled before-treatment data for each of these categories of focal and companion larvae. Data obtained after application of the treatments were first tested with an overall Kruskal–Wallis test in R, and if this showed a significant effect of treatment, we performed a post hoc test correcting for multiple comparisons using the "pgirmess" package (Giraudoux, 2008).

We analyzed differences before and after applying treatments on the different behaviors separately, with the nonparametric Wilcoxon rank-sum test on the pooled data from all treatments. With respect to the first occurrence of the behaviors, data were subjected to a time-to-event Kaplan–Meier analysis (Hosmer & Lemeshow 1999).

All statistical analyses were carried out using R (R Development Core Team 2010). To avoid the possibility that outliers dominated the average parameter values, we removed data points more than three times the standard deviation away from the mean. In total, we removed 29 of 1800 data points. Outliers in the data are marked red in the supporting information (Table S4).

2.5 | Effect of amount and ratio of pheromone components on thrips behavior

Using only synthetic pheromone, we tested whether and how differences in amount and ratio of the two pheromone components influenced thrips behavior. For this, we used the same setup and tested the same behavior as described above (section *Responses to Natural Alarm Pheromone*), except that we used the Wageningen thrips culture and we always used a second-instar larva as a companion. Focal thrips larvae (either first or second instar) were subjected to one of the following five treatments: four different synthetic pheromone blends as described above and the solvent cyclohexane (all 1 μl). Assignment of thrips larvae to treatments was carried out using the Random() function in Excel (2003). The test was performed double blind, implying that the observer was unaware of the treatment applied. All statistical analyses were carried out as described above (section *Responses to Natural Alarm Pheromone*), except that the differences in abdominal swings and partial crossings after application of the treatments were analyzed with a GLM (because these data were not zero-inflated) with a quasi-Gaussian error distribution.

3 | RESULTS

3.1 | Response to natural alarm pheromone of first- and second-instar larvae

Small and large larvae released an anal droplet earlier when challenged with a needle containing pheromone than when challenged with a clean needle (Figure 1, one-way ANOVA; small larvae: $F_{2,69} = 5.7$, $p = .005$; large larvae: $F_{2,76} = 11.4$, $p < .001$). For both types of larvae, there was no difference in response to a needle with alarm pheromone from a small larva or with alarm pheromone from a large larva (Tukey's post hoc test: small larvae $p = .92$, large larvae $p = .96$). Hence, thrips larvae respond to both types of alarm pheromone equally well.

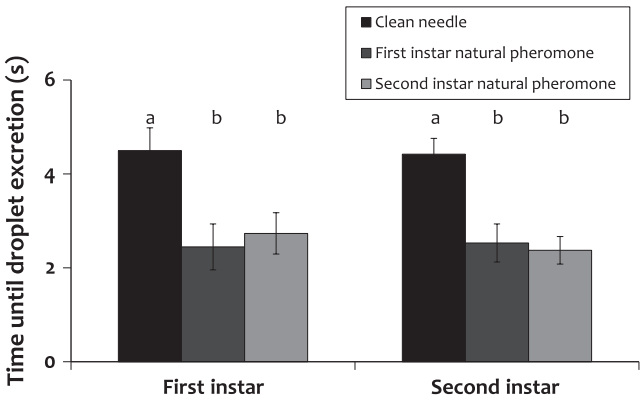

FIGURE 1 Response time of thrips larvae to a simulated attack. Shown is the average time (s) between attack with a needle and the release of an anal droplet by first- and second-instar larvae. The needle was either clean ($N = 31$) or had been dipped in first-instar ($N = 21$) or second-instar alarm pheromone ($N = 20$). Error bars represent standard errors. For each instar of focal larva, different letters indicate significant differences

TABLE 1 Results from GLM tests comparing the change in anal droplet release of a focal larva with treatment (natural pheromone, synthetic pheromone, or cyclohexane) in the vicinity of a first-instar versus second-instar companion thrips larva

Focal larva	Factor	
First instar	Companion	χ^2_1 = 3e-15, p = 1
	Treatment	χ^2_2 = 1e-14, p = 1
	Companion*treatment	χ^2_4 = 6.97, p = .14
Second instar	Companion	χ^2_1 = 8.9e-15, p = 1
	Treatment	χ^2_2 = 6.2e-15, p = 1
	Companion*treatment	χ^2_4 = 4.16, p = .38

TABLE 2 Results from Kruskal–Wallis tests comparing (a) the number of abdominal swings and (b) the number of partial crossings of a focal larva in the vicinity of a first-instar versus second-instar companion thrips larva

Focal larva	Treatment	
(a) Abdominal swings		
First instar	All treatments pooled	KW_5 = 7.09, p = .21
Second instar	All treatments pooled	KW_5 = 2.01, p = .85
(b) Partial crossings		
Before treatment		
First instar	All treatments pooled	KW_1 = 0.35, p = .55
Second instar	All treatments pooled	KW_1 = 0.05, p = .83
After treatment		
First instar	Natural pheromone	KW_1 = 7.70, p < .01
	Synthetic pheromone	KW_1 = 1.15, p = .28
	Cyclohexane	KW_1 = 0.88, p = .35
Second instar	Natural pheromone	KW_1 = 0.75, p = .39
	Synthetic pheromone	KW_1 = 1.61, p = .20
	Cyclohexane	KW_1 = 0.57, p = .45

3.2 | Responses to natural alarm pheromone and effect of companion larva

For both small and large focal larvae, the change in droplet release (from before to after the alarm pheromone treatment) did not vary significantly with treatment or with companion larva (Table 1). Also, the number of abdominal swings after application of the treatment did not vary significantly with treatment or with companion larva (Table 2a). For the number of times a focal larva partially crossed the border between the small and large leaf disk, the type of companion larva did not have a significant effect before treatments (small focal larvae; KW_1 = 0.35, p = .55; large focal larvae; KW_1 = 0.05, p = .83), but after treatments, small larvae displayed significantly more partial crossings when exposed to natural pheromone from a large larva than to that from a small larva (Figure 2, overall effect small larvae; KW5 = 17.6, p < .01; large larvae; KW5 = 24.1, p < .001; per treatment post hoc effects in Table 2b). If synthetic pheromone or only its solvent was released in the vicinity of a small or large companion larva, there was no significant difference in partial crossings (Table 2b). This shows that other cues from companion larvae play no role in triggering this type of response behavior. Hence, small thrips larvae respond differentially to pheromones produced by small or large larvae. The number of partial crossings by large larvae after exposure to natural pheromone, synthetic pheromone or cyclohexane did not vary significantly with the type of companion larva (Table 2b).

The number of anal droplets released, averaged over all treatments, was not significantly different before or after treatments (both instars, Table S2). The number of swings averaged over all treatments was lower after treatments than before treatments (bordering significance for first-instar larvae, significant for second-instar larvae, Table S2). The number of partial crossings averaged over all treatments was significantly higher after treatments than before treatments (both instars, Table S2).

For the timing of release of the first anal droplets or abdominal swings, no significant effect of treatment was detected (see Fig. S1 and

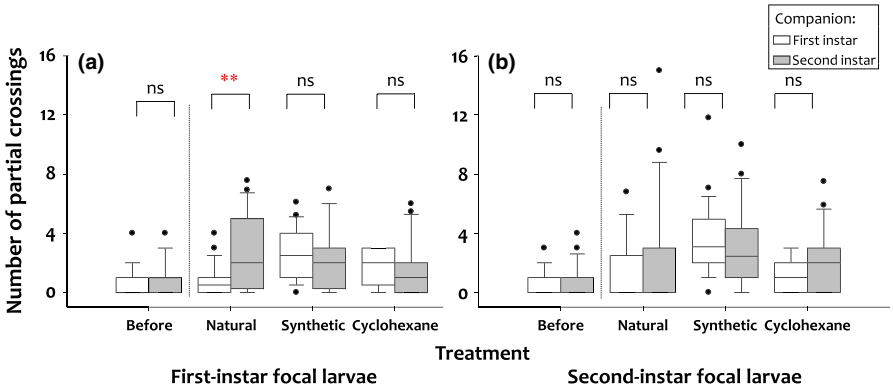

FIGURE 2 Number of partial crossings, in response to natural pheromone or control treatments. Shown are box plots of the numbers of crossings before treatment (pooled for all treatments, N = 75) and after treatment (release of natural pheromone, synthetic pheromone, or cyclohexane, N = 25 each). Focal larvae were either first-instar larvae (panel a) or second-instar larvae (panel b) and were in the company of either first-instar (white boxes) or second-instar larvae (gray boxes). Boxes indicate the second and the third quartile, horizontal lines separating the boxes indicate the medians, whiskers above and below the box indicate the 90th and 10th percentiles, dots indicate outliers, ns indicates not significant, and ** indicates p < .01 [Colour figure can be viewed at wileyonlinelibrary.com]

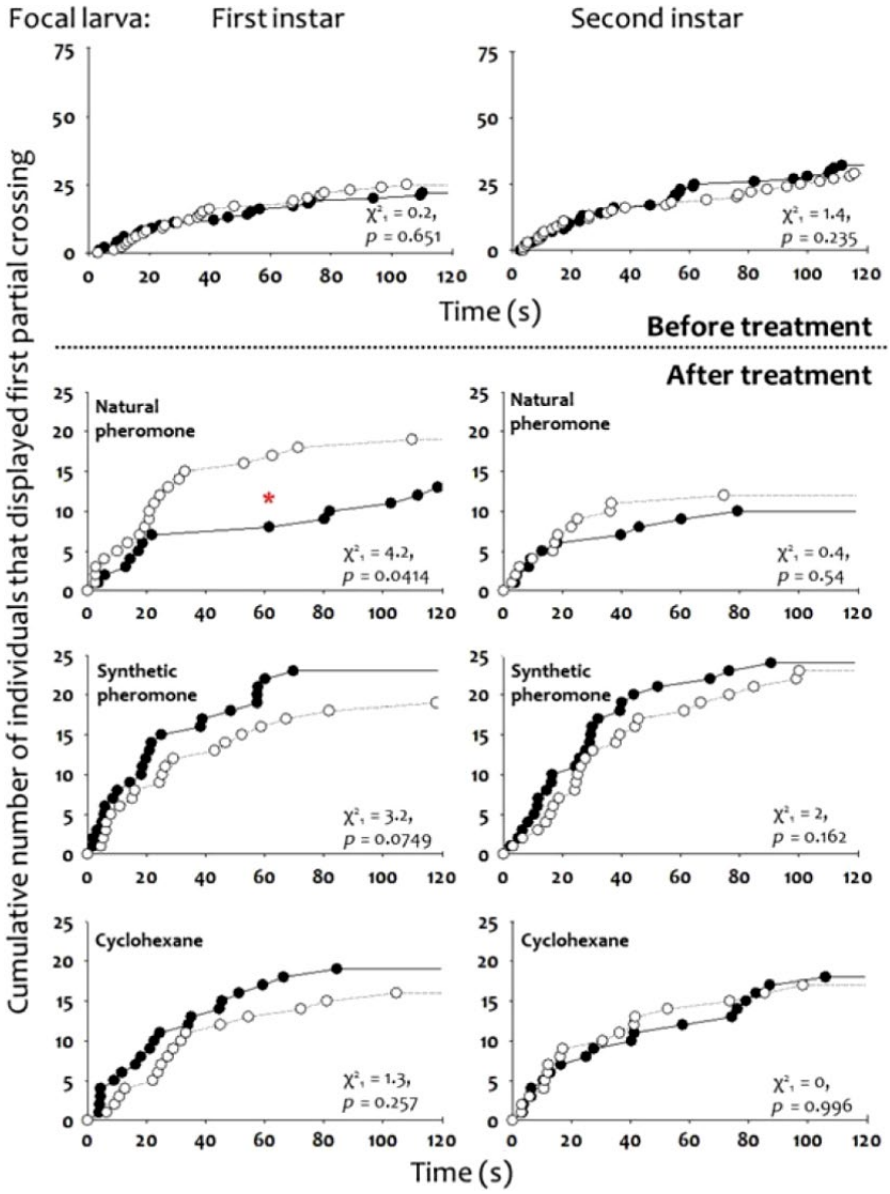

FIGURE 3 Timing of first partial crossing, in response to natural pheromone or control treatment. Shown is the increase of the number of individuals that has partially crossed the edge of the experimental disk over time (s) before treatment (pooled for all treatments, $N = 75$) and after treatment (release of natural pheromone, synthetic pheromone or cyclohexane, $N = 25$ each). Note that the y-axes are scaled to the maximum number of individuals that could have partially crossed (75 before treatments and 25 after treatments). Focal larvae were either first instar (left column of graphs) or second instar (right column of graphs). Companion larvae were either first instar (black circles) or second instar (white circles). * indicates $p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

S2). First-instar larvae partially crossed earlier when exposed to natural pheromone of large companion larvae than when exposed to that of small companion larvae ($\chi^2_1 = 4.2$, $p < .05$) (Figure 3). Thus, partial crossings did not only occur more frequently, but also earlier.

3.3 | Effect of amount and ratio of pheromone components on thrips behavior

For both small and large larvae, the change in anal droplet release and the number of abdominal swings did not significantly depend on the amount of pheromone offered or on the ratio of the two components (Table 3). The amount of synthetic alarm pheromone had a significant effect on the partial crossings of small larvae (Figure 4, $F_{2,120} = 3.4$, $p = .04$). The ratio of the two components in the alarm pheromones did not significantly affect this behavior of small larvae ($F_{1,119} = 1.8$, $p = .19$), but there was a trend toward more partial crossings in response to mixtures where the ratio mimicked that of a large larva

TABLE 3 Results from GLM tests comparing (a) the change in anal droplet release and (b) the difference in abdominal swings of a small or large focal larva with amount or ratio of synthetic pheromone components

Focal larva	Factor	
(a) Anal droplets		
First instar	Amount	deviance = .01, $df = 2$, $p > .99$
	Ratio	deviance = .35, $df = 1$, $p = .55$
Second instar	Amount	deviance < .001, $df = 2$, $p = 1$
	Ratio	deviance < .001, $df = 1$, $p = 1$
(b) Abdominal swings		
First instar	Amount	$F_{2,125} = .78$, $p = .45$
	Ratio	$F_{1,125} = .33$, $p = .57$
Second instar	Amount	$F_{2,124} = .29$, $p = .75$
	Ratio	$F_{1,124} = .81$, $p = .37$

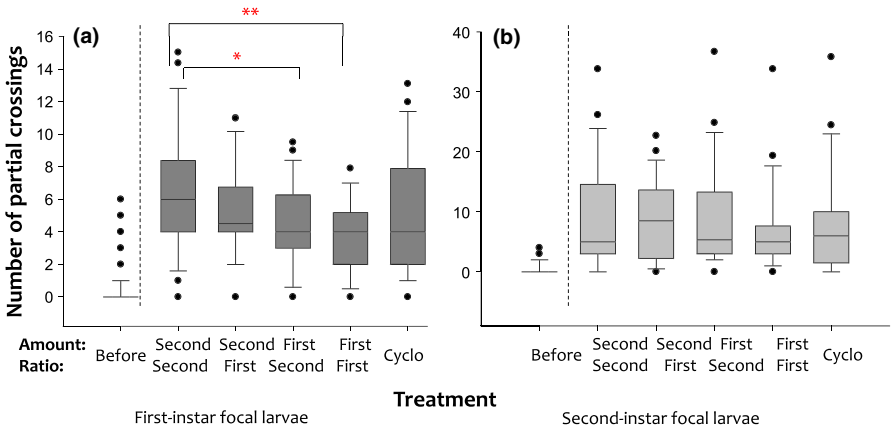

FIGURE 4 Number of partial crossings in response to different blends of synthetic pheromone. Shown are box plots of numbers of partial crossings before treatment ("Before"; pooled for all treatments, $N = 125$) and after treatment (various blends of synthetic pheromone or cyclohexane, "Cyclo", $N = 25$ each). Synthetic pheromone blends were systematically varied to mimic known amounts and/or ratios of alarm pheromone components produced by first- or second-instar larvae (coded on the horizontal axis with "First" and "Second," respectively). Note that these blends include the mimics of first- and second-instar alarm pheromone. Focal larvae were either first instar (panel a) or second instar (panel b). Boxes indicate the second and the third quartile, horizontal lines separating the boxes indicate the medians, whiskers above and below the box indicate the 90th and 10th percentiles, dots indicate outliers, * indicates $p < .05$, and ** indicates $p < .01$ [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 4). For partial crossings of large larvae, we found no significant effect of amount or ratio (Figure 4, amount: $F_{2,119} = 0.14$, $p = .87$; ratio: $F_{3,119} = 0.12$, $p = .73$).

The number of anal droplets released, averaged over all treatments, was not significantly different before or after treatments (both instars, Table S3). The number of swings averaged over all treatments was significantly lower after treatments than before treatments (both instars, Table S3). The number of partial crossings averaged over all treatments was significantly higher after treatments than before treatments (both instars, Table S3). With respect to first occurrence of droplets, abdominal swings, and partial crossings, no significant effects of concentration or ratio of components were detected (see Fig. S3, S4 and S5).

4 | DISCUSSION

We investigated alarm communication in Western Flower Thrips by addressing the following three questions: First, do both small and large larvae respond to alarm pheromones excreted by small and large larvae? Second, do thrips show differential behavioral responses to alarm pheromone produced by a small or a large companion larva? Third, does the amount of pheromone or the ratio of the two compounds affect antipredator behavior? Below we discuss these three questions, compare our results with what is known about thrips and their defense behavior, and address the scope for context-dependent alarm signaling in thrips.

4.1 | Evidence for the perception of natural alarm pheromone

Thrips larvae responded to an anal droplet excreted by a small or a large larva (Figure 1). We found a similar behavioral effect, called "priming," in an earlier study using large larvae only and showed that this priming was caused by the alarm pheromone in the anal droplet (de Bruijn et al., 2006). For large larvae, the priming effect of anal droplets excreted by large larvae is similar in the previous and the present paper. The priming effect on large larvae and small larvae is also similar. Hence, the priming by droplets of small larvae suggests that large and small larvae can perceive alarm pheromone of small larvae.

4.2 | Evidence for differential responses to alarm pheromone of small and large larvae

Small larvae show stronger responses when exposed to alarm pheromone from large larvae than to that from small larvae (Figure 2, Table 2b). Large larvae do not show differential responses to alarm pheromone from small or large larvae (Figure 2, Table 2b). Neither small nor large larvae seem to show increased partial crossings to natural alarm pheromone of a small companion larva (Figure 2). These results are in contrast with our expectation that small larvae would always respond to pheromone of small and large larvae, whereas large larvae would always respond to pheromone of large larvae, but only sometimes to that of small larvae. What could explain this stronger response of small larvae to an alarm pheromone of an instar other than their own? To the best of our knowledge, predators that form a threat to large larvae always form a threat to small larvae as well (but not always vice versa) and those predators are more voracious to small larvae than predators that attack only small larvae. Hence, small larvae should always respond to alarm pheromone of large larvae. Why large larvae do not differentiate between alarm pheromone from small and large larvae remains unclear. The lack of response of small and large larvae to alarm pheromone excreted by small larvae recorded here suggests either that our setup did not provide thrips larvae a chance to display the antipredator behavior they would normally display when perceiving alarm pheromone or that thrips larvae do not change their behavior when perceiving an alarm signal of a small larva under attack.

In the latter case, a behavioral response may require additional cues of predation, such as cues elicited by the predator (as shown for thrips by Venzon et al., 2000) or cues from wounded conspecifics (this latter type of cue is commonly found in aquatic predator–prey systems, for example, see Chivers & Smith, 1998).

We found no differential response to alarm pheromone in other aspects of antipredator behavior (Tables 1,2a). Focal larvae also did not perform more partial crossings in the presence of a large companion larva than in the presence of a small companion larva before treatments, or after exposure to synthetic alarm pheromone of fixed composition (Figure 2, Table 2b). Hence, the cue they responded to after treatments was the pheromone, and not any other cue related to the companion larva. To test whether the presence of a companion larva has any effect on a focal larva, focal larvae should be presented with synthetic alarm pheromone in the presence or absence of a companion larva. We did not perform these tests, because we focused on the hypothesis that thrips larvae perceive a difference between natural alarm pheromone produced by small or large larvae.

4.3 | Does response depend on ratio or amount of pheromonal components?

Given that small thrips larvae display more antipredator behavior in response to alarm pheromone of large larvae than to that of small larvae, we also investigated whether this effect can be attributed to the difference in amount of pheromone or the difference in the ratio of the two components. We found that the total amount of the two components had a significant effect on the number of partial crossings small larvae make, but their ratio of the two compounds in the mixture did not. However, the strong response to the solvent cyclohexane may have masked subtle effects of the ratio of the components. Indeed, there is a trend for small larvae to respond more strongly to mixtures with the ratio mimicking alarm pheromone of large larvae compared to mixtures with the ratio mimicking alarm pheromone of small larvae (as seen in Figure 4). Therefore, we suggest that the ratio of pheromone components does matter to the response of small thrips larvae.

4.4 | Do responses to natural and synthetic pheromone correspond?

Throughout this article, we assumed that the alarm pheromone consists of two components. However, we cannot exclude the presence of other components in the pheromone in concentrations below the detection threshold of analytical equipment, but which might cause a behavioral response of thrips larvae. To exclude that such components have a large effect on thrips behavior, we tested whether the synthetic pheromone elicits a response mimicking that of natural pheromone. Small larvae made significantly more partial crossings when exposed to synthetic blends aimed to mimic alarm pheromone of large larvae than that of small larvae (one-way ANOVA; $F_{1,48} = 7.21$ $p < .01$, Figure 4), which corresponds to our results using natural pheromone of these thrips larvae (Figure 2a). Large larvae did not make more partial crossings when exposed to synthetic blends mimicking alarm pheromone of large larvae than that of small larvae (one-way ANOVA; $F_{1,48} = 0.37$, $p = .55$, Figure 4), which again corresponds to our results found using natural pheromone (Figure 2b). Hence, the natural pheromone and its synthetic analog seem to have a similar effect on the response of thrips larvae.

4.5 | Comparing results with known antipredator behavior

Our results are in agreement with what is known of thrips antipredator behavior. In an attempt to defend themselves, thrips larvae release anal droplets and swing their abdomen when contacted by a predator (Bakker & Sabelis, 1989; Teerling et al., 1993). In the absence of contact with a predator, such antipredator behavior is expected to occur at a lower frequency. Indeed, when thrips larvae were subjected to natural pheromone, we did not observe an increase in release of anal droplets (Table 1, Table S3), and a decrease in the number of abdominal swings (Table 2a, Table S3). However, we did observe an increase in the frequency of partial crossings (Figure 2, Table S3). We interpret the latter behavior as an increased tendency to avoid contact with a predator by leaving the area where alarm was raised.

4.6 | Scope for context-dependent signals

Context-dependent alarm signals allow receivers to respond adaptively to predation risk (Blum, 1996). In this article, we show that small thrips larvae respond differentially to alarm pheromone excreted by small larvae or large larvae and that this differential response could be explained by differences in amount of pheromone and possibly its composition. If the amount of pheromone perceived by the receiver thrips would decrease with increasing distance from the sender, we would expect differential responses with increasing distance between sender and receiver. For a thrips larva, however, to be able to distinguish between two signals without knowing the distance between itself and a sender, the signals should not only differ in amount, but also in other aspects, such as the ratio of the two components. We did find a trend for first-instar larvae to respond more strongly to mixtures where the components had the ratio of second-instar alarm pheromone. Thrips larvae in this experiment responded not only to the synthetic pheromones, but also to the solvent used (Figure 4), which could have masked significant effects of the ratio of the components.

Context-dependent responses to alarm signals are known for vocal alarm calls (e.g., Furrer & Manser, 2009; Seyfarth, Cheney, & Marler, 1980; Sherman, 1977). Chemical alarm signals (alarm pheromones), however, have hardly been studied with respect to the extent to which conspecifics respond to intraindividual variation in pheromones. In invertebrates, we are aware of only one other example (of paper wasps) where the composition of alarm pheromone and the response to it varies (Bruschini et al., 2008). In line

with this, the eusocial thrips Kladothrips intermedius displays caste differences in response to anal droplets excreted by different life stages (De Facci, Svensson, Chapman, & Anderbrant, 2013), and such anal droplets differ in chemical composition (De Facci et al., 2014). Our results add a second example of adjusted response to changes in alarm pheromone of an individual insect: The composition of alarm pheromone changes with the age of a thrips larva (MacDonald et al., 2003), and here, we found that the response of small larvae changes with the composition of alarm pheromone. Moreover, sending thrips larvae are able to vary the ratio of decyl acetate and dodecyl acetate as well as the amount of pheromone with the level of danger they perceive (de Bruijn, Egas, Sabelis, & Groot, 2016). Hence, together with these earlier findings, our results suggest that sender and receiver thrips change their behavior with the level of danger and thereby display context-dependent alarm communication.

ACKNOWLEDGEMENTS

PdB was supported by a grant from the Netherlands Organization for Scientific Research (NWO, project number 818.01.018). We thank Tim Bulters for practical help with experiments; Arne Janssen for statistical advice and extensive comments on the manuscript, and Tessa van der Hammen, Roos van Maanen, Paul van Rijn, Nicola Tien, and Michiel van Wijk for many helpful discussions that improved this study.

REFERENCES

Bakker, F. M., & Sabelis, M. W. (1987). Attack success of *Amblyseius mckenziei* and the stage related defensive capacity of thrips larvae. *Netherlands Journal of Zoology*, *37*, 437.

Bakker, F. M., & Sabelis, M. W. (1989). How larvae of *Thrips tabaci* reduce the attack success of phytoseiid predators. *Entomologia Experimentalis et Applicata*, *50*, 47–51.

Belden, L. K., Wildy, E. L., Hatch, A. C., & Blaustein, A. R. (2000). Juvenile western toads, Bufo boreas, avoid chemical cues of snakes fed juvenile, but not larval, conspecifics. *Animal Behaviour*, *59*, 871–875.

Blum, M. S. (1996). Semiochemical parsimony in the Arthropoda. *Annual Review of Entomology*, *41*, 353–374.

de Bruijn, P. J. A., Egas, M., Janssen, A., & Sabelis, M. W. (2006). Pheromone-induced priming of a defensive response in western flower thrips. *Journal of Chemical Ecology*, *32*, 1599–1603.

de Bruijn, P. J. A., Egas, M., Sabelis, M. W., & Groot, A. T. (2016). Context-dependent alarm signalling in an insect. *Journal of Evolutionary Biology*, *29*, 665–671.

Bruschini, C., Cervo, R., Protti, I., & Turillazzi, S. (2008). Caste differences in venom volatiles and their effect on alarm behaviour in the paper wasp *Polistes dominulus* (Christ). *Journal of Experimental Biology*, *211*, 2442–2449.

Chase, J. M. (1999). Food web effects of prey size refugia: Variable interactions and alternative stable equilibria. *The American Naturalist*, *154*, 559–570.

Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Ecoscience*, *5*, 338–352.

Crawley, M. J. (2007). *The R book*. Chichester (England): John Wiley and Sons Ltd.

De Facci, M., Svensson, G. P., Chapman, T. W., & Anderbrant, O. (2013). Evidence for caste differences in anal droplet alarm pheromone production and responses in the eusocial thrips *Kladothrips intermedius*. *Ethology*, *119*, 1118–1125.

De Facci, M., Wang, H.-L., Yuvaraj, J. K., Dublon, I. A. N., Svensson, G. P., Chapman, T. W., & Allerbrant, O. (2014). Chemical composition of anal droplets of the eusocial gall-inducing thrips *Kladothrips intermedius*. *Chemoecology*, *24*, 85–94.

Furrer, R. D., & Manser, M. B. (2009). The evolution of urgency-based and functionally referential alarm calls in ground-dwelling species. *The American Naturalist*, *173*, 400–410.

Giraudoux, P. 2008: pgirmess: data analysis in ecology. R package version 1.5.8. http://pagesperso-orange.fr/giraudoux. Dd 03 Oct 2014

Hosmer, D. W. Jr, & Lemeshow, S., (1999).*Applied survival analysis: regression modeling of time to event data*. New York, NY: John Wiley and Sons, Inc.

Lima, S. L., & Dill, L. M. (1990). Behavioural decisions made under the risk of predation: a review and prospectus. *Canadian Journal of Zoology*, *68*, 619–640.

MacDonald, K. M., Hamilton, J. G. C., Jacobson, R., & Kirk, W. D. J. (2003). Analysis of anal droplets of the western flower thrips *Frankliniella occidentalis*. *Journal of Chemical Ecology*, *29*, 2385–2389.

Mirza, R. S., & Chivers, D. P. (2002). Brook char (*Salvelinus fontinalis*) can differentiate chemical alarm cues produced by different age/size classes of conspecifics. *Journal of Chemical Ecology*, *28*, 555–564.

Ottoni, E. B. (2000). Etholog 2.2 - a tool for the transcription and timing of behavior observation sessions. *Behavior Research Methods, Instruments, & Computers*, *32*, 446–449.

R Development Core Team (2010) *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. ISBN 3-900051-07-0, URL http://www.R-project.org/

Sabelis, M. W. & van Rijn, P. C. J. 1997: Predation by insects and mites. In: *Thrips as crop pests* (Lewis, T. ed). London: CAB International, pp. 259–354

Seyfarth, R. M., Cheney, D. L., & Marler, P. (1980). Vervet monkey alarm calls - semantic communication in a free-ranging primate. *Animal Behaviour*, *28*, 1070–1094.

Sherman, P. W. (1977). Nepotism and evolution of alarm calls. *Science*, *197*, 1246–1253.

Siegel, S., & Castellan, N. J. (1988). *Non-parametric statistics for the behavioural sciences*, 2nd ed. New York: McGraw-Hill International Editions.

Sih, A. (1980). Optimal behavior: can foragers balance two conflicting demands? *Science*, *210*, 1041–1043.

Teerling, C. R., Pierce, H. D., Borden, J. H., & Gillespie, D. R. (1993). Identification and bioactivity of alarm pheromone in the western flower thrips, *Frankliniella occidentalis*. *Journal of Chemical Ecology*, *19*, 681–697.

Tonn, W. M., Paszkowski, C. A., & Holopainen, I. J. (1992). Piscivory and recruitment: mechanisms structuring prey populations in small lakes. *Ecology*, *73*, 951–958.

Venzon, M., Janssen, A., Pallini, A., & Sabelis, M. W. (2000). Diet of a polyphagous arthropod predator affects refuge seeking of its thrips prey. *Animal Behaviour*, *60*, 369–375.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: de Bruijn PJA, Sabelis MW, Egas M. Antipredator responses to alarm pheromone in groups of young and/or old thrips larvae. *Ethology*. 2019;125:73–81. https://doi.org/10.1111/eth.12600