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Predator responses to novel haemolymph defences of western corn rootworm (*Diabrotica virgifera*) larvae

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Abstract

Many herbivorous arthropods use defensive chemistry to discourage predators from attacking. This chemistry relies on the ability of predators to rapidly learn to recognize and avoid offensive stimuli. Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), employs multifaceted chemical defences in its haemolymph, which may contribute significantly to its success as a major economic pest. Here, we test the hypothesis that agrobiont predators can rapidly learn to recognize and avoid WCR larvae, and will thereby reduce their contribution to WCR suppression. In controlled feeding assays, the effectiveness of WCR haemolymph defences varied across three predator taxa (crickets, centipedes, and ants). Centipedes (Chilopoda: Lithobiidae) were minimally affected by WCR defences, but crickets [*Gryllus pennsylvanicus* Burmeister (Orthoptera: Gryllidae)] spent less time feeding on WCR than on an undefended control prey, house fly maggots. However, we uncovered no evidence indicating that experienced crickets rapidly learn to avoid WCR larvae, indicating that haemolymph defences offer few, if any, survival benefits for WCR. Colonies of ants [*Lasius neoniger* Emery (Hymenoptera: Formicidae)] switched from low worker participation in initial attacks on WCR to higher worker participation in subsequent attacks, indicating an attempt to overcome, rather than avoid, WCR haemolymph defences. These results suggest that a diverse assemblage of natural enemies will show a diverse array of behavioural responses to toxic pest prey, and highlight the importance of behavioural diversity in driving the function of natural enemy assemblages.

Introduction

Despite their small body and brain, arthropods are capable of surprising behavioural complexity (de Boer & Dicke, 2006; Giurfa, 2013). In recent years, there has been growing appreciation for the effects that this behavioural complexity can have on arthropod community dynamics (Abrams, 2010). For example, many arthropod predators display an ability to adaptively modify their foraging behaviour in response to recent and past foraging experiences (de Boer & Dicke, 2006; Zhang & Hui, 2014), and in response to real-time information on current foraging success (Blackledge & Wenzel, 2001; Welch et al., 2013). Adaptive use of information while foraging can shape the functional responses of predators to their prey, potentially causing dynamic shifts in trophic cascades and trophic web interactions (Schmitz & Suttle, 2001; Abrams, 2010).

However, although learning is adaptive for predators, it is not necessarily beneficial for societal or conservation purposes, such as suppression of pests or adaptation to invasive species. Many invasive and pest species are defended by toxins or armaments (Lundgren et al., 2009b), or have low nutritional quality for predators (Oelbermann & Scheu, 2002; Toft, 2005). Consequently, predators may learn to avoid attacking and consuming such prey, reducing top-down control of pests and potentially increasing invasibility of the ecosystem. For example, since the invasion of the toxic cane toad into Australia, several native vertebrate predators have learnt to avoid attacking cane toads, which reduces the negative effects of cane toad invasion on indigenous fauna, but also precludes control of the invasive toad by native

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predators (Webb et al., 2008; Greenlees et al., 2010; Nelson et al., 2011). On the other hand, a capacity to learn may enhance the effectiveness of top-down control by providing a means of overcoming or tolerating defences or nutritional deficits in their prey. For example, Robbins et al. (2013) discovered that increased exposure to toxic fire ants (*Solenopsis* spp.) increased consumption of fire ants by myrmecophagous lizards, likely due to an increased tolerance for fire ant venom. Many predatory arthropods have displayed similar learning abilities in the context of chemically or mechanically defended prey (e.g., Segura et al., 2007; Murphy et al., 2010; Costa & Reeve, 2011). In a diverse assemblage of natural enemies, a diverse array of learning responses may be expected, from aversion, to tolerance, to a total lack of learning; and each type of response can result in very different effects on the structure of trophic webs and pest-suppression potential of the natural enemy community. It is therefore important to determine whether natural enemies can learn to recognize suboptimal prey and whether learning will impede or facilitate pest consumption.

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae; hereafter 'WCR'), is a major pest of maize in North America and Europe. The larval stages of WCR live in and feed on the roots of maize plants. Second and third instars of WCR (and other *Diabrotica* spp. larvae) display an elaborate system of chemical defences in their haemolymph, which includes a foul-tasting chemical to repel predators, and a rapid coagulation factor to ensnare a predator's mouthparts and impede its ability to feed (Wallace & Blum, 1971; Lundgren et al., 2009b, 2010). Lundgren et al. (2010) showed that these defences are effective against a range of predatory arthropods, increasing handling time of WCR prey by forcing predators to spend a great deal of time grooming and cleaning mouthparts, which distracts them from feeding on the WCR. Although a variety of natural enemies have been reported attacking and consuming WCR larvae in the field (Lundgren et al., 2009a), natural mortality of the chemically defended second and third instars is low (Toepfer & Kuhlmann, 2006), indicating that their defensive chemistry is an effective deterrent to predators. One likely explanation for this low rate of mortality is that short-term learning allows predators to rapidly recognize WCR larvae and avoid feeding on them after an initial exposure to their defensive chemistry. Here, we test the hypothesis that a range of predatory arthropods can rapidly learn aversion to WCR larval haemolymph defences in controlled feeding assays.

Materials and methods

Assay setup

We conducted memory-retention and functional-response assays to determine whether exposure to WCR haemolymph defences can induce predators to learn and avoid WCR larvae at the low encounter rates expected under field conditions. To assess predator learning, we observed behavioural responses to WCR larvae of naïve predators in an initial trial, and then recorded behavioural responses of the same predators in a follow-up trial. Changes in predator behaviour between initial and follow-up trials are indicative of learning responses. We made no attempt to condition or train predators to sensory stimuli, as our intention was to evaluate the predators' ability to recognize and learn from exposure to the prey itself. The lack of obvious aposematism in WCR larvae suggests that any associative learning would occur primarily through olfactory or gustatory cues. We therefore reasoned that changes in post-attack behaviour would be more likely than changes in attack/avoid probability. We also observed predator responses to an undefended control prey, third instars of the house fly [*Musca domestica* L. (Diptera: Muscidae); hereafter 'maggots'], which are similar in size and shape to third-instar WCR.

Study organisms

WCR larvae used in these trials were obtained from the continuous, non-diapause WCR colony maintained at the USDA Agricultural Research Service's North Central Agricultural Research Laboratory (NCARL) in Brookings, SD, USA (44°20′25.9″N, 96°47′17.3″W). The rearing protocols and conditions for this colony are reported in Branson et al. (1975). WCR larvae were obtained from the colony as newly emerged third instars, and used in trials within 1 week of their removal from the colony. Maggots were purchased from Beneficial Insectary (Redding, CA, USA) as third instars, and refrigerated for less than 5 days prior to their use in trials.

Three species of predator were assayed in this study: field crickets [*Gryllus pennsylvanicus* Burmeister (Orthoptera: Gryllidae)], stone centipedes (Chilopoda: Lithobiidae), and ants [*Lasius neoniger* Emery (Hymenoptera: Formicidae)]. These predators were chosen to expand on the range of predators evaluated by Lundgren et al. (2010), and to evaluate a spectrum of hunting and feeding modes. Crickets and centipedes were collected by hand from fields, lawns, and under rocks in Brookings, SD, USA. Intact ant colonies were located in the field at the same locality and assayed in situ. Voucher specimens of each species and from each ant colony were collected after

trials were completed, and deposited in the insect reference collection at NCARL.

Memory-retention trials

Memory-retention assays were conducted to assess the ability of predators to alter behavioural responses after initial exposure to WCR defences. Assays for crickets and centipedes were conducted in sterile, plastic Petri dishes (10 cm diameter, 2 cm high) under ambient laboratory conditions (30% r.h., 18 °C, photocycle L16: D8). After collection, predators were maintained in the laboratory for at least 24 h prior to trials. Crickets were kept in Petri dishes (10 cm diameter, 2 cm high), and centipedes were kept in specimen cups (8 cm diameter, 8 cm high) with leaf litter as a base. No food was provided during this time, but all predators were allowed an ad libitum supply of water. Centipede assays were conducted in a darkroom under red light, whereas cricket assays were conducted in full laboratory lighting. Each assay consisted of two trials: an initial trial and a follow-up trial. Each trial followed the protocols outlined in Lundgren et al. (2010).

At the beginning of a trial, a predator was randomly assigned to a prey treatment (WCR or maggot). In cricket trials, the cricket was introduced into a clean Petri dish and allowed to acclimate before the prey was introduced within 1 min thereafter. Centipedes, however, were prone to escape from dishes when opened. To prevent centipede escapes, prey were introduced before centipedes; and centipedes consequently had no acclimation period. Data collection protocols are modified from Lundgren et al. (2010). After both predator and prey had been introduced, dishes were monitored for 10 min, or until the predator attacked the prey. Following an attack, the behaviour of the predator was recorded for 2 min. The 2-min post-attack interval was divided into 5-s subintervals. During each 5-s subinterval, the predator's behaviour was categorized as one of the following: (1) feeding on the prey; (2) reacting negatively to the prey (dropping the prey, backing away from the prey, or wiping coagulated haemolymph off mouthparts); or (3) any other behaviour (categorized as 'neutral' behaviours). Responses were scored as the total amount of time spent on a given behavioural category throughout the 2-min interval. Predator learning was assessed by comparing scores from initial and follow-up trials. For crickets, follow-up trials were conducted at one of two retention times after initial trials: 2 or 24 h. For centipedes, sample sizes were too small to allow two follow-up times, so all follow-up trials were run at 24 h after initial trials. Between trials, predators were returned to the containers in which they had been housed, and provided a fresh supply of water.

Field assays for ants

To evaluate learning by ants, assays were conducted on natural ant colonies in the field. Seventeen colonies of *L. neoniger* were located in lawns and fields in Brookings, SD, USA, and marked with a flag. Within each colony, a single mound with 1–2 burrow entrances was chosen and all trials for a single colony were conducted on the same mound. For ant assays, all trials were conducted during observed *L. neoniger* activity peaks (morning or evening). Each colony was exposed to both prey sequentially. Half of colonies were exposed to WCR larvae first, and the other half were exposed to maggots first. The second prey was not introduced until at least 30 min after the trial with the first prey ended and ant activity on the focal mound had returned to pre-trial levels. Follow-up trials were conducted 24 h later, and prey were introduced in the same order as in initial trials.

We considered colony-level responses of *L. neoniger* workers to WCR and maggot prey. Throughout the 10-min trial period, worker counts were taken at 1-min intervals. At each interval, we counted: (1) the total number of ants active on the focal mound and (2) the total number of ants simultaneously attacking (i.e., biting) the prey. If the prey was taken down into the nest and out of the observer's sight during the trial, at all time points thereafter, the number of attackers was scored as the highest number of simultaneous attackers observed during the trial. If at any time during the trial, the prey wandered more than ca. 5 cm from the mound, it was carefully picked up with forceps and placed at the edge of the mound again, unless it was being attacked by a group of ants (in which case it was not disturbed).

Functional-response assays

Predators may be incapable of learning to recognize a noxious stimulus after only a single trial: aversion learning may require repeated exposures over a relatively short period of time. Thus, predators exposed to noxious prey at higher rates should be expected to learn more rapidly, and decrease attack rates on the noxious prey. To further evaluate the effects of WCR defences on predator behaviour, assays were conducted to determine whether cricket predators would attack and consume multiple WCR larvae when given the opportunity. For these assays, crickets were collected from the field and maintained for 24 h on an L16:D8 photocycle under ambient laboratory conditions (30% r.h., 18 °C). No food was provided during this time, but crickets were allowed an ad libitum supply of water. To begin the trials, crickets were placed in Petri dish arenas (10 cm diameter, 2 cm high) with 1, 5, or 10 prey (either WCR or maggots), and survival of the prey in each of the six treatments was recorded 24 h after introduction to

assess the 24-h prey-consumption rate by crickets. Each treatment was replicated 10 times, and all crickets were assayed simultaneously. Assays were maintained under ambient laboratory conditions.

Statistical analysis

Learning is a within-subjects effect. Therefore, in memory-retention assays, we looked for evidence of behavioural differences between initial and follow-up trials using general linear models with repeated measures. In cricket and centipede behavioural analyses, the within-subjects factor was the trial (two levels: initial vs. follow-up). In these assays, the behaviour category 'neutral' was observed infrequently (mean ± SE = 7.9 ± 1.5 s), so the remaining two categories, 'feeding' and 'reacting negatively', effectively obeyed a zero-sum rule (i.e., any change in the time spent in one category entailed a reciprocal change in the other category). Therefore, we evaluated only one response category, feeding, as this provides information on both responses. In the ant analysis, we evaluated three within-subjects factors: prey, trial (initial vs. follow-up), and trial minute (1–10). All analyses were conducted in SYSTAT, version 13 (SYSTAT Software, Chicago, IL, USA).

Results

Laboratory assays for crickets and centipedes

Data for cricket behavioural assays were analyzed using a general linear model with repeated measures incorporating two between-subjects factors: two prey species (WCR larvae and maggots) and two time intervals (2 and 24 h). A significant effect of prey species on time spent feeding by crickets was found (ANOVA: $F_{1,102} = 39.4$, $P<0.001$). Specifically, crickets fed longer on maggots than on WCR larvae, in both initial and follow-up trials (Figure 1), demonstrating that WCR larval defences do have a negative effect on feeding by crickets. However, the model uncovered no significant effects of trial (initial vs. follow-up: $F_{1,102} = 2.1$, $P = 0.15$) or time interval between trials ($F_{1,102} = 2.1$, $P = 0.16$), and interaction terms were all non-significant. Consequently, there is no evidence of learning by crickets in response to WCR larval defences.

Data for centipede behavioural assays were analyzed using a model incorporating one between-subjects factor, prey species. In this model, no significant effect of prey species was uncovered ($F_{1,20} = 0.62$, $P = 0.44$), and no significant within-subjects effect of trial was uncovered ($F_{1,20} = 1.29$, $P = 0.27$) (Figure 2). Negative reactions to WCR larvae were only observed in three of 13 WCR-fed centipedes (two in the initial trial, and one in the follow-up trial), indicating that WCR larval haemolymph defences have little effect on centipedes.

Field assays for ants

A total of 68 ant trials (34 initial and 34 follow-up) were analyzed, 52 of which resulted in successful prey capture. Rate of successful WCR capture was comparable to rate of successful maggot capture: out of 17 ant colonies, 12 (initial trials), and 13 (follow-up trials) successfully captured the WCR larva, whereas 13 and 14 successfully captured the maggot. Rate of success did not differ significantly between prey species for either initial trials or follow-up trials (Fisher's exact test: d.f. = 1, $P = 1.0$, in both cases), indicating that larval haemolymph defences did not greatly enhance WCR survival against ant predators.

Ant colonies exhibited prey-specific patterns of activity. In a general linear model with repeated measures, a significant main effect of prey species was observed (ANOVA: $F_{1,16} = 6.40$, $P = 0.022$): ant activity on the mound was higher in response to maggots than to WCR. We also found a significant interaction between prey species and time ($F_{9,144} = 8.92$, $P = 0.001$; Greenhouse–Geisser (G–G) correction for sphericity applied). Specifically, the number of ants active on the focal mound steadily

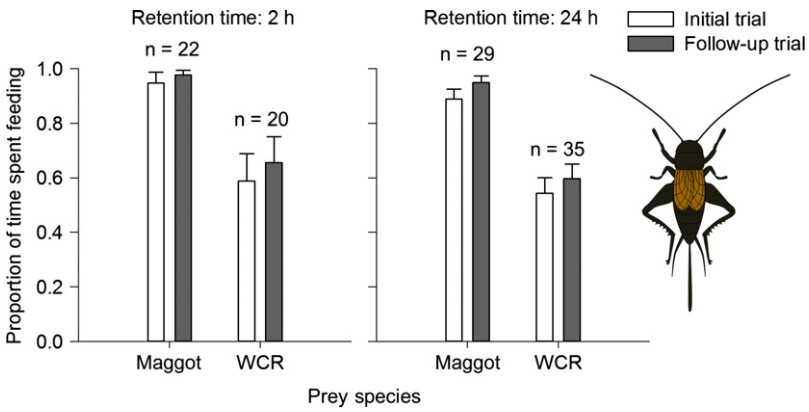

Figure 1 Average time (mean + SE) spent feeding on solitary, chemically defended western corn rootworm (WCR) larvae or undefended house fly maggots by crickets, *Gryllus pennsylvanicus*, in laboratory feeding assays, and potential for recognition of prey and learning after two memory-retention time intervals. Sample sizes appear above bars.

accumulated over the 10-min observation interval when the prey was a maggot, but remained constant over time when the prey was a WCR larva (Figure 3). The main effect of trial (initial vs. follow-up) on ant activity was non-significant ($F_{1,16}$ = 0.57, P = 0.46), as was the interaction effect between trial and prey ($F_{1,16}$ = 0.114, P = 0.74), indicating that there was no change in ant activity between trials for either prey (i.e., no evidence of learning).

When we only considered the subset of ants that were directly participating in the attack on the prey, a different dynamic was observed. There was a significant interaction effect between prey and trial ($F_{1,16}$ = 10.21, P = 0.006): a shift in the number of simultaneous attackers was observed in response to WCR prey, but not in response to maggot prey (Figure 4). In maggot assays, the number of simultaneous attackers increased over the 10-min interval for both initial and follow-up trials (main effect of minute: $F_{9,144}$ = 16.6, P<0.001, after G–G correction), and there was no significant difference between trials (trial*minute interaction effect: $F_{9,144}$ = 0.81, P = 0.61). However, in WCR assays, there were significant effects of both trial ($F_{1,16}$ = 10.71, P = 0.005) and minute ($F_{9,144}$ = 11.19, P<0.001, G–G correction applied) on the number of simultaneous attackers, and a marginally significant interaction between trial and minute ($F_{9,144}$ = 2.64, P = 0.068, after G–G correction). In initial trials, the number of attackers remained roughly constant throughout the 10-

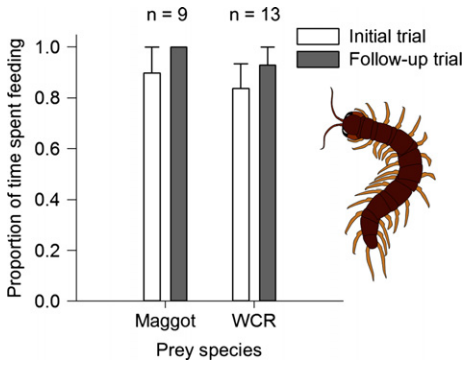

Figure 2 Average time (mean + SE) spent feeding on solitary chemically defended western corn rootworm (WCR) larvae or undefended house fly maggots by lithobiid centipedes in laboratory feeding assays, and learning response after 24 h. Note, in follow-up trials with maggots, there was no variance: all centipedes fed for the entire 2 min. Sample sizes appear above bars.

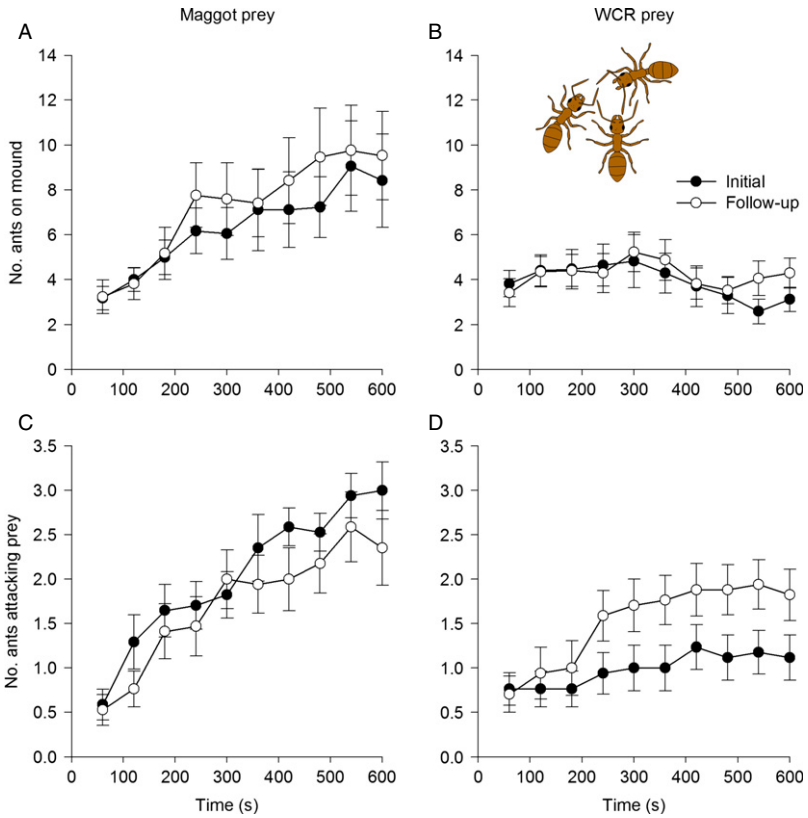

Figure 3 Foraging activity of *Lasius neoniger* ants in response to solitary, chemically defended WCR larvae and undefended house fly maggots in field feeding assays before and after initial experience with the prey. Mean (± SE) (A,B) total number of ants active on the focal mound over time, and (C,D) number of ants simultaneously attacking the prey over time (n = 17 colonies in all treatments).

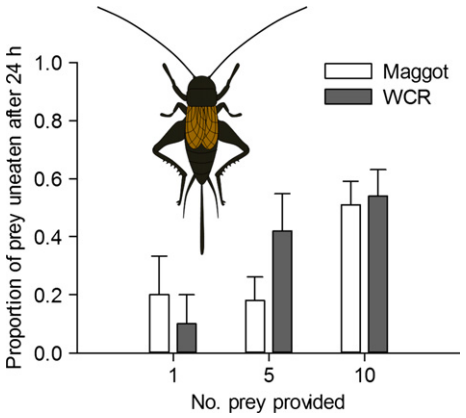

Figure 4 Proportional survival of WCR larvae and house fly maggots (mean + SE) in groups of different sizes after 24 h of predation by a *Gryllus pennsylvanicus* cricket in laboratory functional-response assays (n = 10 replicates in each treatment).

min observation interval; but increased over the 10-min interval in follow-up trials, indicating a shift in behavioural response to WCR larvae 24 h after a single exposure.

Functional-response assays for crickets

Data for cricket functional-response assays were analyzed in a multinomial logistic regression model incorporating prey species and prey number as factors explaining the likelihood of an individual WCR larva or maggot surviving 24 h. In these assays, survival of prey was found to be significantly affected by the initial number of prey (coefficient $\pm$ SE = -0.26 ± 0.01, $P = 0.008$). Specifically, proportional survival of prey was higher in larger prey group sizes (Figure 4). However, the effect of prey species was non-significant (-1.08 ± 0.80, $P = 0.18$), and the interaction between prey species and prey number was also not significant (0.08 ± 0.09, $P = 0.38$). This indicates that increased survival at larger population densities was the result of predator satiation, and WCR haemolymph defences did not offer additional survival benefits over maggots.

Discussion

In controlled feeding assays, we observed variation among three predator species in their response to WCR larval haemolymph defences. The effectiveness of the haemolymph defences varied across predator species, and elicited learning responses in some, but not all, predators. Because of this variability in effectiveness, no generalizable predictions can be made concerning the potential effects of WCR larval defences on the biological control potential of natural enemies. A diverse assemblage of natural enemies can therefore be expected to display a diverse range of responses to a toxic pest.

Crickets (*G. pennsylvanicus*) spent less time feeding on WCR larvae than on an undefended control prey, house fly maggots, demonstrating the unpalatability of WCR larvae for crickets. Crickets showed no change in this behaviour when retested 2 or 24 h later, indicating that they did not learn from their previous experience. However, previous work indicates that *Gryllus* crickets are capable of learning and remembering conditioned olfactory stimuli for many weeks (Matsumoto & Mizunami, 2000, 2002a,b). In addition, Simoes et al. (2012) found that another orthopterous insect, a *Schistocerca* locust, is capable of rapid aversive learning through associative training. The disagreement between the present study and the previous work can be explained by the difference in methodology: here, we made no attempt to train crickets with a conditioned stimulus, to understand the dynamics of learning under more natural stimulus-training conditions. Our results indicate that, if crickets are capable of recognizing and learning to avoid WCR larvae in the field, it will not occur after only a single exposure. In addition, crickets that were offered groups of WCR larvae readily attacked and consumed them at rates comparable to the rate of attack on undefended maggot prey, indicating that even repeated exposures to WCR defences over a relatively short span of time are insufficient to induce learned aversion in crickets. In the field, as in our trials, stimulus-training conditions will be sub-optimal, so crickets will likely display poor learning and retention, and this sub-optimal learning may consequently be of benefit to WCR suppression by precluding learned aversion to WCR defences.

It is noteworthy that the majority of centipedes in our assays showed no negative reaction to WCR defences. Lundgren et al. (2010) observed a similarly reduced effect of WCR defences on wolf spiders. It is therefore tempting to suggest that certain common aspects of the feeding process of these predators, such as venom or extra-oral digestion, provide some means of circumventing WCR defences. However, the feeding methods of centipedes and spiders are not identical: centipedes in our trials were observed to chew their prey and ingest the entire body, whereas spiders are obligate fluid-feeders and extra-oral digesters (reviewed in Cohen, 1995). Thus, the similarities in tolerance for WCR larval defences may only be superficial, and not attributable to a common mechanism. Nevertheless, given that many arthropod predators use venom and extra-oral digestion, the hypothesis that these feeding tactics facilitate consumption of toxic WCR larvae merits further investigation.

Ants are a dominant presence in many agricultural fields, and may be important predators of WCR (Kirk, 1981). Anecdotally, WCR defences appear to be at their most effective when used against individual ants. In this study, we observed that WCR larvae readily autohaemorrhaged when antennated or touched by ants. Furthermore, individual ants that came in contact with haemolymph were strongly repelled and often ensnared by the haemolymph. Unlike crickets or centipedes, ants did not pierce the cuticle of their prey during their attack, and autohaemorrhage is therefore required to expose ant predators to the defensive chemistry. It is thus our belief that the autohaemorrhage response is specifically a defence against ant predators. Undoubtedly, the defensive chemistry would have facilitated WCR escape from solitary ant workers foraging away from their nests. However, because our assays were conducted in close proximity to ant nests, where ant activity was bound to be high and pheromonal communication quick and efficient, the effectiveness of WCR defences was lower. Most ant colonies in our study successfully subdued WCR larvae and carried them down into their nests in spite of their defensive chemistry. It is noteworthy that the number of attackers on WCR increased between trials, whereas the general activity of ants on the mounds did not. That is, no additional workers were recruited from within the nest, but a greater proportion of those workers that were already active on the surface participated in the attack. This suggests that the increase in simultaneous attackers was most likely accomplished by an increased behavioural tendency for individual workers to attack upon encounter, rather than by increased recruitment via alarm pheromones. This implies that *L. neoniger* ants are capable of single-trial learning (cf. Foubert & Nowbahari, 2008; Josens et al., 2009). Henaut et al. (2014) observed mutual avoidance between ants and web-building spiders after only a single antagonistic encounter, suggesting that these arthropod predators are indeed capable of such rapid aversion learning. In our study, the response to WCR differed in type from the strong, numerical response observed in trials with maggot prey. Maggots tended to writhe about and fight back when attacked, which likely induced ants to recruit large numbers of workers to aid in the capture of the maggot. In contrast, WCR larvae tended to remain motionless when attacked, and no swarming or recruitment response was induced.

The heterogeneity of predator responses to noxious prey in this study highlights the importance of understanding the role of trait-mediated interactions within natural enemy-pest food webs. An assemblage of natural enemies is a mosaic of behavioural, ecological, and physiological phenotypes that all interact in unique ways with target pests. Here we show that an herbivore's single defence mechanism can trigger a variety of different responses in different species of natural enemies. Heterogeneous behavioural interactions such as these may lead to non-intuitive and non-linear effects on trophic webs, and their effects on the biological control potential of a natural enemy assemblage are difficult to predict without concrete data.

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