

Wax Structures of *Scymnus louisianae* Attenuate Aggression From Aphid-Tending Ants

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ABSTRACT The cuticular wax structures of *Scymnus louisianae* J. Chapin larvae were investigated as a defense against ant aggression by *Lasius neoniger* Emery. The presence of wax structures provided significant defense against ant aggression compared with denuded larvae in that these structures attenuated the aggressive behavior of foraging ants. Furthermore, reapplication of wax dissolved in hexane partially restored defenses associated with intact structures, showing an attenuation of aggression based in part on cuticular wax components rather than solely on physical obstruction to ant mouthparts.

KEY WORDS *Scymnus louisianae*, *Aphis glycines*, *Lasius neoniger*, ant-tending

Many ants tend aphids and use the collected honeydew as a sugar source. In return, the aphids benefit from the tending ants (Way 1963, Hölldobler and Wilson 1990). In these systems, aphids can benefit through reduced predation (Banks 1962) and increased fitness (Flatt and Weisser 2000). The relationship between ants and aphids is often a mutualistic one but ranges from obligatory to facultative (Dixon 1998). Both arrangements have been shown to have benefits for aphids and ants with ecological consequences and are common in natural and in agricultural settings (Styrsky and Eubanks 2007). As a result, coccinellid larvae that feed on aphids often encounter tending ants and are subjected to ant aggression. Only a few coccinellids have been studied with regard to their ability to feed on aphids that are tended by ants (Völkl 1995; Völkl and Vohland 1996; Sloggett et al. 1998, 2002; Sloggett and Majerus 2000, 2003), including Coccinellidea in the genus *Scymnus* (Völkl and Vohland 1996). *Scymnus* beetle larvae possess a cuticular adaptation that is thought to aid in the avoidance of attack by aphid-tending ants. Specifically, the cottony wax structures of the larvae of two *Scymnus* beetles, *Scymnus nigrinus* Kugelann and *Scymnus interruptus* Goeze, have been observed to aid in defense by physically clogging mouthparts of aggressive ants (Völkl and Vohland 1996). Similarly, Lierre and Perfecto (2008) found that the sticky wax structures of the coccidophagous ladybird beetle larva *Azya orbiger* Mulsant clog the mouthparts of the aphid-tending ant *Azteca instabilis* F. Smith, resulting in decreased predation of ladybird larvae by tending ants.

Although most of the work on interactions between *Scymnus* sp. and ants has highlighted the effectiveness of

wax in promoting physical obstruction to the mouthparts of aphid-tending ants, little has been done to elucidate the role that wax plays in eliciting avoidance behavior or in attenuating aggression. Specifically, physical or chemical mimicry, camouflage, or repellency has not been looked at as a potential means of avoidance of ant aggression in the *Scymnus* beetles. It has been suggested that other myrmecophiles that exploit ant tended aphids are protected by chemical mimicry of their prey (Howard et al. 1990, Völkl and Mackauer 1993, Dettner and Liepert 1994, Liepert and Dettner 1996, Akino and Yamaoka 1998, Völkl and Mackauer 2000, Allan et al. 2002, Lohman et al. 2006). In these examples, the mimic benefits by being able to exploit ant attended individuals.

Several studies have investigated the role that larval wax structures play in avoidance of, or resistance toward, ant aggression with members in the tribe Scymnini (Richards 1980, Völkl and Vohland 1996, Agarwala and Yasuda 2001), as well as with other insects that possess similar wax structures (Eisner 1994, Kumar and Singh 1995, Lit et al. 1999, Smith 1999) or remove wax structures from their aphid prey to use as camouflage shields (Eisner et al. 1978). The function of these wax structures has been primarily attributed to protection against natural enemies and ants (Pope 1979, Richards 1980, Eisner 1994, Völkl and Vohland 1996, Agarwala and Yasuda 2001) and/or through benefits of UV light reflectance (Pope 1979). As in these aforementioned systems, *Scymnus louisianae* J. Chapin is able to forage on the cotton aphid, *Aphis gossypii* Glover, in the presence of the fire ant, *Solenopsis invicta* Buren (Vinson and Scarborough 1989), and the cornfield ant, *Lasius neoniger* Emery (unpublished data). Other beetles in the genus *Scymnus* have been studied for their ability to forage within ant attended systems. *Scymnus posticalis* Sicard is able to forage on *A. gossypii* while remaining undetected by the aphid-

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Fig. 1. A wax-covered ladybird beetle larva, *S. louisianae*, feeding on a soybean aphid, *A. glycines*, on the leaf of a soybean plant, *G. max*.

tending ant *Lasius niger* (Kaneko 2002); in return, *S. posticalis* is thought to benefit through reduced intra-guild predation. Similarly, *Scymnus interruptus* and *Scymnus nigrinus* are able to forage on *Aphis fabae* Scopoli in the presence of both *L. niger* and *F. polyctena*. In this case, the authors found that *Scymnus* spp. densities were actually higher in ant-attended resources than in unattended ones (Völkl and Vohland 1996).

The larvae of the ladybird beetle *S. louisianae* are capable of preying on soybean aphids, *Aphis glycines* Matsumura, in the presence of the aphid tending ant *Lasius neoniger* while eliciting little to no aggressive action by ants. *S. louisianae* is a promising natural enemy for *A. glycines* and shows potential for applications in biological control of this pest (Brown et al. 2003). Furthermore, *S. louisianae* may prove to be especially productive in controlling *A. glycines* in fields that are colonized by *L. neoniger*. In this study, we investigate the wax structures of *S. louisianae* larvae (Fig. 1). We show that beetle larvae attenuate aggression from aphid-tending ants by testing whether this protection is afforded by the waxy nature of its cuticular structures.

Materials and Methods

Beetle larvae used in bioassay experiments were reared on colonies of *A. glycines* on soybean plants, *Glycine max* L. Merrill, maintained in a greenhouse at $25 \pm 5^\circ\text{C}$, 15:9 L:D, and ambient humidity under high-pressure sodium vapor illumination. Aphids were reared on young soybean plants, variety AG5602 (Asgrow, St. Louis, MO), in 10-cm peat pots and watered as needed with 0.02% 20:20:20 N:P:K. The aphid colony was established at the University of Kentucky using aphids obtained from the University of Illinois (courtesy D. Voegtlin, Illinois Natural History Survey), collected from a field population in Illinois. Beetles were collected from naturally occurring field pop-

ulations in Kentucky and were allowed to propagate freely on aphid-infested plants. Third- and fourth-instar beetle larvae were periodically removed and stored at -80°C in sealed petri dishes for use in bioassays.

Our bioassay was conducted on soybeans planted in mid-May. Field populations of aphids were established by inoculating *G. max* with cut stems containing 50–200 aphids. These aphid-infested stems were placed on the upper portion of 4-wk-old plants in the field. Roughly 20–30 plants were inoculated in a “V” pattern throughout our 70 by 70-m field plot. Aphids were allowed to develop for 5 wk before experimental procedures were performed. After this 5-wk period, many of the inoculated plants had established populations of aphids and naturally occurring aphid-tending *L. neoniger*.

We created four treatment manipulations of the beetle larvae’s waxy cuticle; mechanically removed, hexane wash, reapplied in hexane, and intact wax. (1) In the mechanically removed treatment, wax was removed from beetle larvae by an air-blasting technique using a charcoal filtered stream of compressed air followed by mechanical removal using a fine paintbrush. This manipulation effectively removed all visible wax from each beetle larva. (2) For the hexane wash treatment, all visible wax was first removed from the larva using compressed air as described above, and the larva was further washed with hexane through a series of steps. First, 1–2 ml of hexane was pipetted over each larva, followed by swirling in 1.5 ml of hexane for 60 s. Larvae were removed from vials and rinsed with an additional 1–2 ml hexane via pipette. (3) For the reapplied in hexane treatment, wax was mechanically removed using stainless steel forceps. Larvae were swirled in a 1-dram vial with 1.5 ml hexane for 60 s. While the larvae were still moist with hexane, wax removed from other individuals was reapplied onto the dorsal surfaces of larvae, causing the wax to dissolve into residual solvent on the insect cuticle. Thus, no traces of wax were visible under a dissecting microscope. (4) Finally, the intact wax treatment, which served as a control, consisted of fourth-instar larvae with the wax left fully intact on the surface of the larval cuticle. Charcoal-filtered air was lightly passed over the wax structures to control for possible air-stream effects. A total of 60 larvae were used to make 15 replicates of the four treatments. After treatment preparation, all larvae were stored frozen at -20°C for 20–50 h before bioassay.

Beetle larvae were presented to aphid-tending ants in a randomized complete block design, with the plant as the blocking factor. Larvae from each of the four treatments were placed on plant stems one at a time. The order of treatment placement on plants was randomized for each aphid colony. Larvae were placed on a petiole of a soybean plant in an area colonized by aphids and actively tended by ants. Preference was given to areas on petiole crotches or horizontal petioles. This placement was necessary to prevent beetles from falling off plants before interaction with *L. neoniger*. After a trial had been completed, an untested

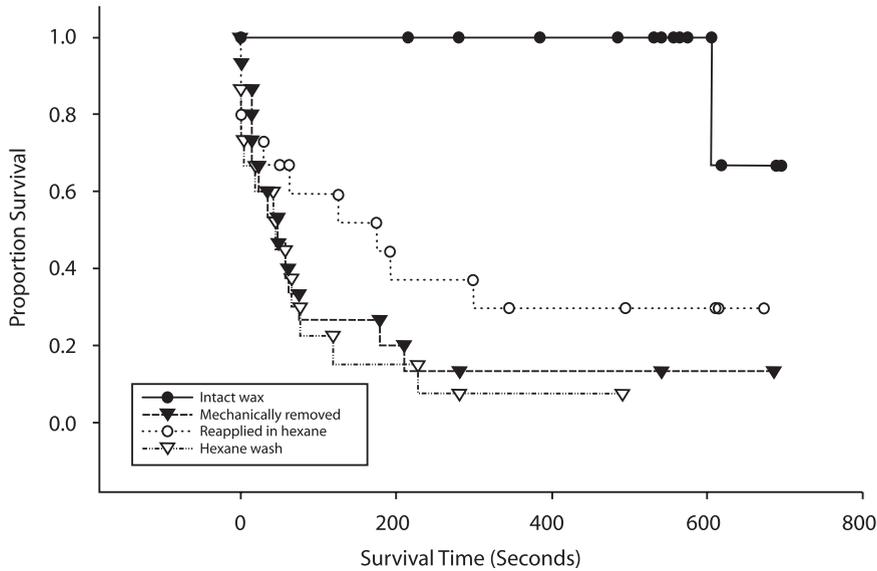


Fig. 2. Survival analysis of *S. louisianae* subject to interaction with *L. neoner* on soybeans. Survival is measured as the time a larva remained on the plant before it was removed by an ant.

beetle from one of the remaining treatments was introduced onto the same plant in the same spot. This continued until all the treatments had been tested. Subsequent replicates were tested on new plants using untested beetles. Plants were selected for bioassay based on having high densities of *A. glycines* (>1,000 per plant) and actively tending populations of *L. neoner*. The bioassay was conducted over a 5-d period in mid-July. Within this period, two to four replications were conducted on any given day.

Ant-beetle interactions were recorded using a digital video camera. All ant antennations, attacks, and beetle removals were recorded from video, and the following variables were calculated: (1) number of antennations before first clasp event, (2) time to first clasp event, (3) time of removal from plants by tending ant or observer, and (4) number of beetles remaining 10 min after the start of the experiment. An action was deemed aggressive if an ant clasped onto the wax or the body of a beetle larva. If a larva was clasped by the mandibles of an ant and released, it was also recorded as an aggressive action.

Avoidance of ant aggression by *S. louisianae* was determined as the duration of time spent on a plant between initial contact and aggressive action by an aphid-tending ant. To account for differential finding rates between treatments, aggression was also determined as the number of times a beetle was antennated before an aggressive action. By this means, a beetle that was antennated several times before being attacked was seen as being able to avoid aggression more than a beetle attacked after fewer contacts. Data were considered "right censored" (Lawless 2003), because beetle treatments that remained unmolested were terminated at 10 min and removed by hand. Because beetles were removed by two different means, by hand or by ants, a survival analysis was used to sepa-

rately analyze for (1) the time at which ants acted aggressively toward a beetle larva and (2) the time at which the trial ended. Analyses were performed using the PROC LIFETEST procedure in SAS (SAS Institute 2000). Treatments were deemed significantly different at $\alpha = 0.05$ using the likelihood ratio and log rank post hoc tests for exponentially and nonexponentially distributed data, respectively. Survival distributions were tested for exponential distribution using PROC UNIVARIATE procedure in SAS (SAS Institute 2000). The number of beetle larvae remaining at the end of the experiment was tested against treatment using 2 by 2 Fisher exact tests in JMP.

Results

After a beetle larva was placed on a stem, a *L. neoner* ant would eventually approach and antennate the larva. Antennating ants would do one of two things: they would either clasp onto the larva with their mandibles or disregard the larva and continue foraging along the plant. When an ant clasped onto a beetle, the ant would proceed to lift or pull the beetle from the immediate vicinity. If successful, beetles were carried from the spot of initial placement to an unknown location off of the plant. In some cases larvae were clasped and even tugged by ants, but not moved from their location. In these occurrences, ants eventually left larvae after a period of mandibular clasping. Nevertheless, clasping actions serve as a method of removal and were classified and recorded as an aggressive action.

Intact larvae had significantly higher survival than all other treatments as measured by both time (Fig. 2) and number of antennations (Fig. 3; $P < 0.001$). Ants responded differently toward intact wax larvae than to those from which the wax structures had been me-

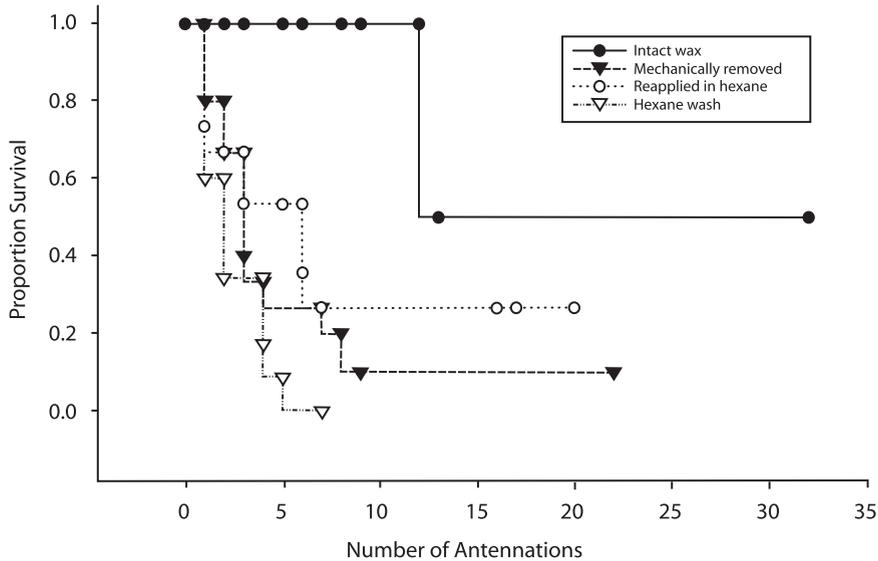


Fig. 3. Survival analysis of *S. louisianae* subject to interaction with *L. neoneiger* on soybeans. Survival is measured as the number of antennations by ants on a larva before it was removed.

chanically removed. Among intact wax larvae, there was only one occurrence where an ant clasped a larva with its mandibles. In all other cases, after initial and subsequent antennations by ants, these wax-covered larvae remained unmolested. Ants simply evaluated larvae by antennating them with no associated aggressive action. In all other treatments, only a few beetles remained unmolested by ants for the 10-min test period (Fig. 4).

Redissolving wax onto hexane-washed individuals partially restored protection to *S. louisianae* larvae. Survival values of treatments reapplied in hexane and hexane wash were significantly different from one another with respect to time (Fig. 2) and antennations (Fig. 3). The treatments mechanically removed and

hexane wash were not significantly different from one another (Figs. 2 and 3), indicating a minimal effect of hexane as a disturbance in our bioassay. The number of beetle larvae remaining on plants at the end of the experiment was significantly different between treatments. More intact wax larvae remained than reapplied in hexane (Fig. 4; $P = 0.0032$). We did not see significantly more reapplied in hexane larvae remaining than hexane wash individuals ($P = 0.96$) at the end of the experiment.

Discussion

When a *L. neoneiger* worker approached and evaluated intact larvae by tapping their antennae on the surface of the cuticular wax structures, they rarely engaged in aggressive behavior. In our system, however, as ants approach larvae, they make contact with their antennae first, and then with their legs, and rarely act in an aggressive manner. This is not the case with other native and non-native ladybird beetles, where tending ants readily attack these foraging predators (Finlayson et al. 2009). These observations suggest that *S. louisianae* are using their wax as protection against aggression by *L. neoneiger*. However, this defense is not because of a physical obstruction of ant mouthparts but rather to other qualities of the wax structures that attenuate aggression. Larvae with wax reapplied in hexane were considerably more defended than larvae that had their wax structures removed either physically or using solvent. This suggests that properties of the wax, and not just the ability of the wax structures to obstruct ant mouthpart function, are playing a role in this protection by altering the behavior of *L. neoneiger*. Although the presence of wax structures attenuate aggression by foraging ants, we do not know if the wax acts as a deterrent, as seen in other

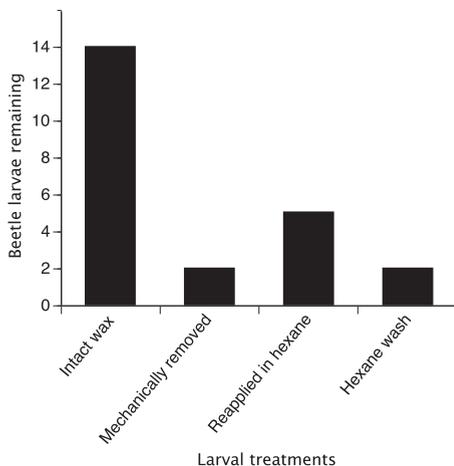


Fig. 4. Number of beetle larvae remaining on the plant from the original 15 for the total duration of the trial period (≈ 10 min) that have escaped aggression from *L. neoneiger*.

interactions between beetles and ants (Eisner et al. 1986, Dettner 1987), or if the wax acts to disguise the beetle larvae, as seen in systems where chemical mimicry has been shown as an aid in protection of aphid parasitoids from aphid-tending ants (Liepert and Dettner 1996).

We chose to analyze ant aggression using a survival analysis in addition to a comparison of beetles remaining at the end of the experiment to take into account the effects of the treatments throughout the time that larvae were vulnerable to ant aggression. Using survival analyses, we tested survival (ability of beetle larvae to remain unattacked by ants) in terms of both the duration that beetles were able to remain on a plant before an aggressive encounter and the number of antennations by ants before an aggressive encounter. We felt that it was important to test for the number of antennations by ants before an aggressive encounter to control for differential discovery rates between treatments. For instance, if one treatment was more apparent to foraging ants, it might be discovered sooner, and hence attacked sooner. Analyzing by number of antennations before aggressive encounter better describes the role of the treatments in their ability to attenuate aggression while removing variability in discovery time.

Although reapplication of the wax back to solvent-washed larvae attenuated aggression by ants compared with solvent-washed individuals, it did not restore the protective benefits provided by intact wax. This was the case when performing a survival analysis on both time and number of antennations. This indicates that, whereas reapplication does partially restore this defense, it does not do so completely, and it is likely that other factors are responsible for the observed attenuation of ant aggression. It is possible, for instance, that the physical feel of the pliable waxy tufts is not recognized by tending ants as the cuticle of a predator, acting as a tactile camouflage (Bloom 1975, Pasteur 1982); however, this is only speculative. Regardless, other nonchemical attributes could account for some of the protective benefits of the larval wax structures; therefore, we cannot conclude that non-physical attributes alone are responsible for attenuated aggression.

Previous studies on these beetles and other aphidophagous predators that produce wax structures have noted the benefits of protection against aphid-tending ants (Völkl and Vohland 1996, Liere and Perfect 2008); however, this is the first time that waxy coverings have been shown to attenuate aggression. Eisner et al. (1978) described a similar interaction with Chrysopid predators that remove wax from their aphid prey and place it on their dorsum to mimic their prey's cuticle. Although similar in effect, these Chrysopids do not produce the wax themselves but rather collect it from their prey. The ability of *S. louisianae* to produce a covering that attenuates aggression is the first known for a ladybird beetle. Furthermore, ants will readily attack denuded larvae on contact, but they do not attack wax-covered larvae. This suggests that the

wax is serving to attenuate aggression by ants rather than serving as a physical obstruction with the main purpose being clogging of ant mouthparts long enough to escape predation (Völkl and Vohland 1996, Liere and Perfect 2008). The ability of dissolved and then reapplied wax to partially restore these properties also points toward a nonphysical barrier as a mechanism for this attenuation of aggression. To test this more thoroughly, studies into potentially active chemical components of the wax that may serve as a chemical mimic or camouflage would be needed.

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