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## GLANDULAR HAIRS: PUPAL CHEMICAL DEFENSE IN A NON-NATIVE LADYBIRD BEETLE (COLEOPTERA: COCCINELLIDAE)

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**ABSTRACT** – *Subcoccinella vigintiquatuorpunktata*, an herbivorous coccinellid beetle of Eurasian origin, is now established in New England, as evidenced by the persistence of two central Connecticut populations from 1997–2001. The beetle’s pupal surface bears glandular hairs that produce a secretion known to consist principally of three polyazamacrolide alkaloids. We demonstrate that the secretion serves as a potent antipredator defense: contact with it elicited pronounced cleaning activity by the predatory ant *Crematogaster lineolata*. Additionally, application of the secretion to palatable food items rendered them unacceptable to the ant. This is one of few studies to examine the chemical defense of an introduced animal *vis à vis* a predator native to its new environment.

### INTRODUCTION

The biogeography of ladybird beetles (Coleoptera: Coccinellidae) has been strongly influenced by humans. Typically predaceous, both larval and adult coccinellids are widely viewed as effective natural enemies of agricultural pests including aphids, whiteflies, and scale insects (Hodek and Honek 1996, Obrycki and Kring 1998). As biological control agents, these predators have often been introduced to areas beyond their natural range. Beginning in 1874 with the introduction of *Coccinella undecimpunctata* (L.) to New Zealand from England in an aphid-control effort, there have been at least 768 attempts to introduce ladybird beetles to control insect pests (Dixon 2000). Although many such attempts have failed to establish a non-native coccinellid within a new region, some have nonetheless succeeded. As of the early 1980s, there were records of the importation of 179 coccinellid species into North America for biological control, coinciding with the establishment of 26 non-native species (16 apparently from intentional releases) (Gordon 1985).

Growing attention has been devoted to a number of alien coccinellid species in the northeastern United States and Canadian Maritime Provinces (Cormier et al. 2000, Ellis et al. 1999, Gordon and Vandenberg 1991, Hoebeke and Wheeler 1996, McCorquodale 1998, Wheeler

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1993a, Wheeler and Stoops 1996). The majority of these predatory species have Palearctic origins.

### *Subcoccinella vigintiquatuorpunctata*—two Connecticut populations

Not all recent introductions to the coccinellid fauna of the Northeast involve predators. *Subcoccinella vigintiquatuorpunctata* (L.), a member of the herbivorous subfamily Epilachninae, is a Eurasian species with a polyphagous diet (70 host species), including alfalfa (*Medicago sativa* L.) (Ali 1979). Apparently an accidental introduction, the beetle was first collected in North America in Pennsylvania in 1972 and New Jersey in 1973 (USDA 1974). Since then it has been reported from Maryland, Illinois, New York, Ohio, West Virginia, and Virginia (Gordon 1985, USDA 1975, Wheeler 1993b). In North America the beetle's diet is primarily restricted to bouncing bet (*Saponaria officinalis* L.) and several related caryophyllaceous species (Gordon 1985, USDA 1974, Wheeler 1993b, Wheeler and Henry 1981).

In an earlier investigation of this coccinellid's chemical ecology (Schroeder et al. 1998), we provided what appears to be the first published record of *S. vigintiquatuorpunctata* in New England. To obtain beetles for that and later studies, three central Connecticut sites were

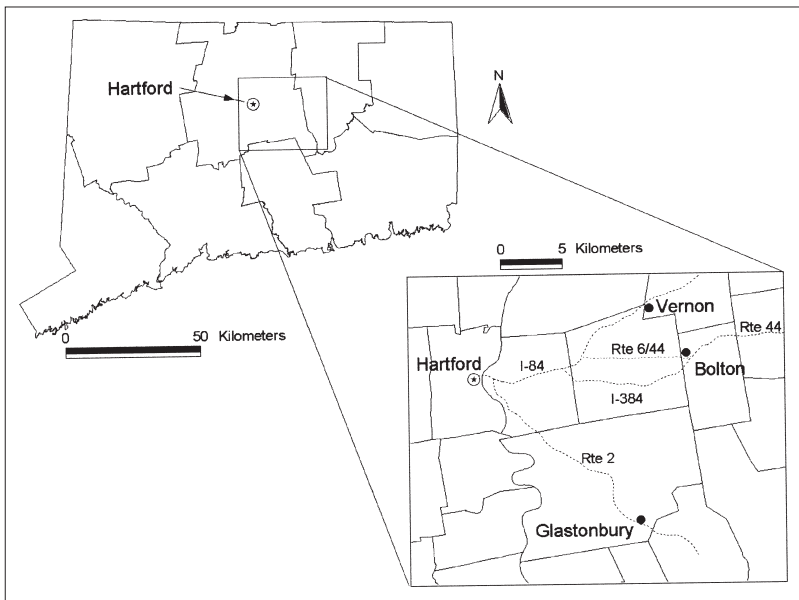


Figure 1. Location of Bolton and Glastonbury, Connecticut populations of *Subcoccinella vigintiquatuorpunctata*. State map delineates county boundaries, while enlarged section indicates municipal boundaries and major roadways. The Vernon site, although it contained a sizeable stand of the host plant, *Saponaria officinalis*, lacked the beetle.

examined from September 1997 to October 2001, typically once in the fall and once in the spring. Located in Bolton (N 41° 47.043', W 72° 27.910'), Tolland Co.; Vernon (N 41° 49.240', W 72° 30.027'), Tolland Co.; and Glastonbury (N 41° 39.682', W 72° 31.121'), Hartford Co. (Fig. 1), these field sites contained large patches of the beetle's host plant *S. officinalis*. During each visit, the presence or absence of immature (eggs, larvae, pupae [Fig. 2A-C]) or adult (Fig. 2E) *S. vigintiquatuorpunctata* was recorded following visual inspection and sweep netting of *S. officinalis* and surrounding vegetation.

From 1997 to 2001 the Bolton and Glastonbury sites each held a population of *S. vigintiquatuorpunctata* (Table 1, Fig. 1), with immature stages detected only in the spring and adults found in both spring and fall (Table 1). *S. officinalis* growing at both sites showed leaf tissue damage (Fig. 4E) characteristic of *S. vigintiquatuorpunctata* herbivory (Tanasijevic 1958, Wheeler and Henry 1981). Yearly inspection of the Vernon site (Fig. 1) detected neither the beetle nor signs of its herbivory (Table 1).

### *Subcoccinella vigintiquatuorpunctata*—pupal glandular hairs

The cuticular surface of the pupa in *S. vigintiquatuorpunctata* is covered with glandular hairs, each bearing a minute droplet of secretion at its tip. These hairs are interspersed among longer, non-secretory spines (Fig. 2C,D). Our earlier work (Schroeder et al. 1998) identified a mixture of three novel polyazamacrolide alkaloids (Fig. 3) as the secretion's primary components. Considering the biosynthetic origin of a structurally related alkaloid produced by another epilachnine

Table 1. Detection of *Subcoccinella vigintiquatuorpunctata* at central Connecticut field sites (Fig. 1) from 1997–2001. For a given season, observation dates are indicated (note that all three sites were not necessarily visited on a particular date). I = detection of immature stages (eggs, larvae, or pupae); A = detection of adults; ND = none detected; • = site not visited.

Year	Season (Date[s])	Field Sites		
		Bolton	Glastonbury	Vernon
1997	Fall (5, 6, 9, 13 September)	A	A	ND
1998	Spring (29 May, 2 June)	I, A	I, A	•
	Fall (19 September)	A	A	ND
1999	Spring (28 May)	•	I, A	•
	Fall (18 Sept., 3 October)	A	A	ND
2000	Spring (4 April, 28 May)	I, A	•	•
	Fall (6, 7 October)	A	•	ND
2001	Spring (4, 26 June)	I, A	I, A	•
	Fall (13 October)	A	A	ND

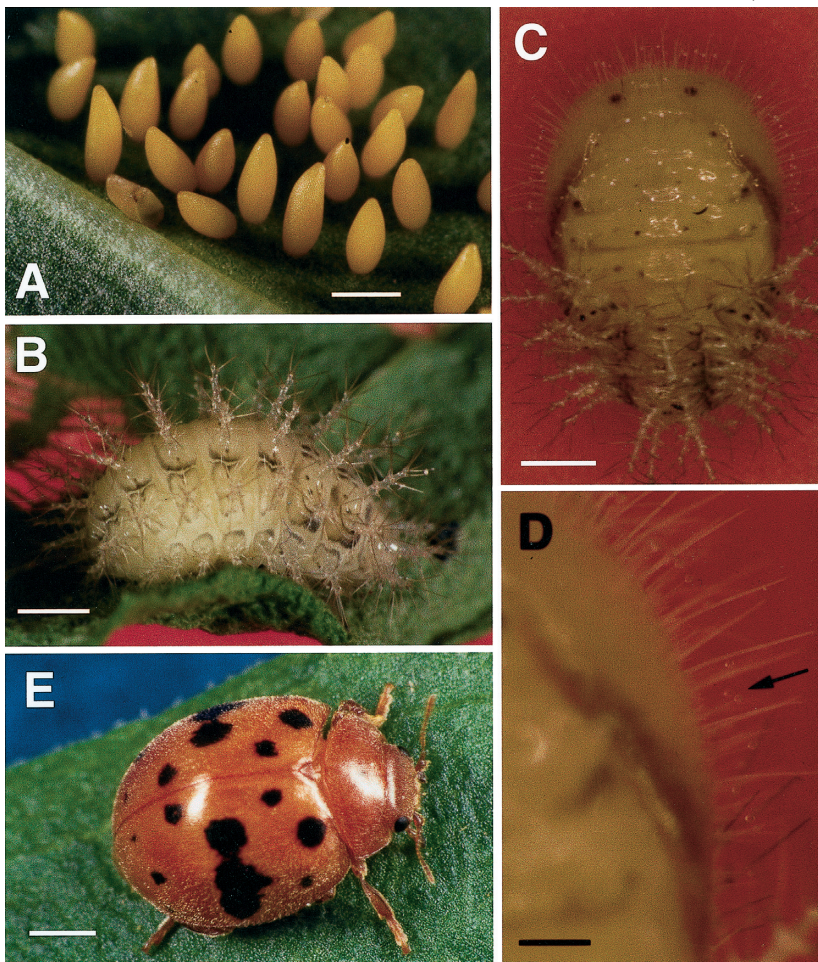


Figure 2. Life cycle of *Subcoccinella vigintiquatuorpunctata*. (A) Eggs on underside of *Saponaria officinalis* leaf. (B) Larva (ultimate instar, prepupal stage). (C) Pupa with shed larval cuticle adhering to posterior. Note the band of glistening secretory droplets slightly offset from the exposed pupal surface. (D) Close-up of pupal secretory hairs interspersed among longer non-secretory spines. Arrow indicates a droplet. (E) Adult beetle. Scale bars: A-C, E = 1 mm; D = 0.25 mm.

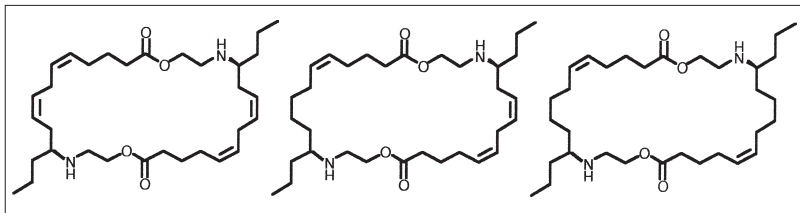


Figure 3. Molecular structures of the three dimeric polyazamacrolide alkaloids that represent the major constituents of the pupal secretion of *Subcoccinella vigintiquatuorpunctata*.



(Attygalle et al. 1994, Attygalle et al. 1999), these polyazamacrolides are likely produced *de novo* by *S. vigintiquatuorpunctata*. Since the pupa is sedentary, typically attached to vegetation, one could envision that it might be particularly prone to predatory attack. In light of this and examination of the pupal secretion of two other epilachnines, *Epilachna varivestis* Mulsant (Attygalle et al. 1993) and *Epilachna*

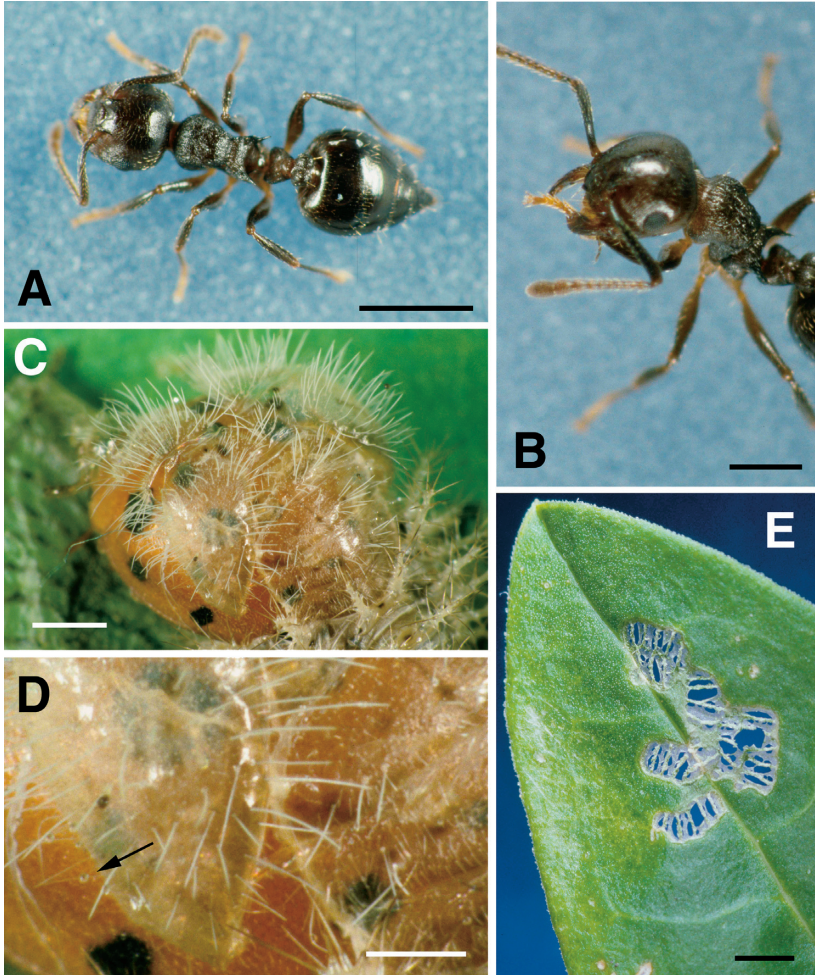


Figure 4. *Crematogaster lineolata*, *Subcoccinella vigintiquatuorpunctata*, and *Saponaria officinalis*. (A) *C. lineolata* worker. (B) *C. lineolata* worker cleaning left foreleg with mouthparts after using that leg to groom its antenna that had contacted the secretion-bearing cuticular surface of a *S. vigintiquatuorpunctata*. (C) Recently eclosed adult *S. vigintiquatuorpunctata* undergoing hardening of its exoskeleton within the confines of the shed pupal cuticle. (D) Enlarged portion of (C) showing shed pupal cuticle draped over left elytron. Arrow indicates a secretory hair bearing a droplet. (E) Characteristic damage to upper surface of a *S. officinalis* leaf resulting from *S. vigintiquatuorpunctata* herbivory. Scale bars: A, C = 1 mm; B, D = 0.5 mm; E = 2 mm.

*borealis* (Fabricius) (Schröder et al. 1998), we suggested that these droplets produced by *S. vigintiquatuorpunctata* play a defensive role (Schroeder et al. 1998). Here we present experimental evidence that the pupal secretion of *S. vigintiquatuorpunctata* indeed functions defensively *vis-à-vis* a native predatory ant that is sympatric with the beetle in its new environment. This is thus one of few studies to examine the effectiveness of an introduced animal's chemical defense against predators native to its new locale.

## METHODS

### **Bioassays with the predatory ant, *Crematogaster lineolata***

Native to the eastern United States, *Crematogaster lineolata* (Say) (Hymenoptera: Formicidae) (Fig. 4A,B) is a generalist predator that occurs sympatrically with the two populations of *S. vigintiquatuorpunctata* examined. Although no field observations of *C. lineolata* interacting with pupae of *S. vigintiquatuorpunctata* were made, this ant forages on vegetation (Johnson 1988) and therefore likely encounters the pupae.

*C. lineolata* colonies were collected in the field (Bolton, Manchester, Tolland, and Ashford [Trinity College Field Station at Church Farm], Connecticut) and were maintained in the laboratory in plastic boxes (56 x 41 x 22 cm, l x w x h) on a diet of chopped mealworms and honeywater.

### ***Bioassay I—Effect of pupal secretion on ant cleaning behavior.***

To determine whether contact with the pupal secretion of *S. vigintiquatuorpunctata* leads to an adverse response by *C. lineolata*, the cleaning behavior of ants was monitored before and after pupal contact. For each trial an individual ant was presented a single pupa. Three separate series of trials were conducted, each involving presentation of a different type of pupa: live pupa/secretion present (n = 27), dead pupa/secretion present (n = 15), and dead pupa/secretion absent (n = 16). In each series, three-day-old pupae were used. In the second and third series, the pupae were killed by freezing (3 h at -20 °C) before the trial; such pupae had returned to room temperature prior to presentation. In the third series, the secretion was removed by rinsing the pupa in a series of four 20 ml methanol baths; after the final rinse, adhering methanol was allowed to evaporate prior to pupal presentation. Characterization of the secretion's chemical composition (Schroeder et al. 1998) indicated that its dominant components are methanol- and dichloromethane-soluble.

Each trial took place in a Petri dish (5 cm diameter) with a textured floor to facilitate the ant's locomotion and Teflon-coated walls to confine the predator to the dish. The ant was allowed to acclimate to the

dish for  $\sim 2$  h prior to the trial. Trials consisted of two phases: an initial 5 min period with the ant alone, immediately followed by a 10 min period, begun with the placement of a *S. vigintiquatuor punctata* pupa into the ant's dish. Both phases were video-recorded through a dissecting microscope. A time track on the resulting image permitted quantification of the duration of behavioral episodes. There were occasional instances (typically amounting to  $\leq 1\%$  of each phase) during which the ant momentarily left the camera's field of view. Consequently, for a given trial, the *Pre-Contact period* was defined as 5 min, minus the cumulative period that the ant was invisible during the initial phase. The trial's *Post-Contact period* was similarly defined as 10 min, minus the period prior to the first ant-pupa contact, minus the cumulative period that the ant was invisible following that initial contact. The duration of the ant's cleaning behavior (i.e., time engaged in several related behaviors: antennal wiping with the forelegs, foreleg wiping with the mouthparts [Fig. 4B], dragging of the mouthparts on dish floor, or regurgitation) was separately determined for the Pre-Contact and Post-Contact period of each trial, thus permitting the paired comparison of the percentage of time spent cleaning by the ant before and after pupal contact.

Individual ants and beetle pupae were used in only a single trial. Pupae that were presented live were examined for adult emergence, as was a group of control pupae that were not used in trials. Ant viability was examined at 48 h past the completion of each trial. Additionally, the viability of ants in a separate group that was not subjected to *S. vigintiquatuor punctata* pupae was also monitored at 48 h.

#### ***Bioassay II—Effect of pupal secretion on ant foraging behavior.***

To determine whether secretion isolated from *S. vigintiquatuor punctata* pupae served as a deterrent to foraging colonies of *C. lineolata*, the following experiment was undertaken. To obtain material for each trial, the secretion was rinsed off an individual pupa via drop-wise addition of 100  $\mu$ l of dichloromethane that drained into a vial with a conical concavity. This solvent was then evaporated under nitrogen and the remaining residue was resolubilized in 5  $\mu$ l of dichloromethane. This volume was then topically applied (drop-wise) to a food item readily acceptable to *C. lineolata* — an egg of the hemipteran *Oncopeltus fasciatus* (Dallas), reared on commercial sunflower seeds, rather than the bug's cardenolide-laden natural diet of milkweed (*Asclepias* sp.) seeds. A treated egg thus received one pupal equivalent of the secretion, while a control egg simply received the volume (5  $\mu$ l) of dichloromethane equal to that used to apply secretion. Within a trial, eggs were presented as a pair, with the treated and control egg set at diagonally opposite corners of a glass coverslip (22 x 22 mm). Trials were conducted with four different colonies of *C. lineolata* maintained in the laboratory as de-



scribed above. Within a colony's box, the coverslip was placed at the center of a circular platform (8.8 cm diameter) to which the ants had been previously trained to forage on chopped mealworm pieces. Several fresh mealworm pieces were added to the coverslip to maintain foraging activity at the site. During each trial, the feeding platform was examined at 1-min intervals for 1 h. Eggs were considered removed, if the foragers had carried them off the platform. Sixteen trials were performed, four with each colony.

### **Deposition of Voucher Specimens**

Voucher specimens of adult *S. vigintiquatuorpunctata* are deposited at the Smithsonian Institution (National Museum of Natural History), Harvard University (Museum of Comparative Zoology), Yale University (Peabody Museum), and Cornell University (Cornell Insect Collection). Voucher specimens of *C. lineolata* are deposited at the Museum of Comparative Zoology.

### **Statistics**

Non-parametric statistics were performed using StatView (v 4.5). Frequency data were analyzed with a G-test, employing the Williams correction (Sokal and Rohlf 1995). When the results of multiple tests were considered, an experiment-wide  $\alpha = 0.05$  was maintained using the sequential Bonferonni procedure (Rice 1989).

## **RESULTS**

### **Bioassays with the predatory ant, *Crematogaster lineolata***

**Bioassay I—Effect of pupal secretion on ant cleaning behavior.** When pupal secretory droplets were present, contact with a *S. vigintiquatuorpunctata* pupa resulted in increased ant cleaning activity, regardless of whether the pupa was alive or dead (Fig. 5). However, when droplets were absent, post-contact cleaning did not differ from background (Fig. 5). Regurgitation and mouth wiping were only observed following ant contact with secretion-bearing pupae (in 56% of trials with live and 27% of trials with dead pupae), but never following contact with secretion-free pupae or during the pre-contact phase of trials.

All live pupae used in trials produced viable adult beetles, as did 16 control pupae that had no ant contact. All ants used in trials were alive 48 h later. Likewise, no mortality was observed at 48 h for 16 ants not used in trials.

**Bioassay II—Effect of pupal secretion on ant foraging behavior.** Topical application of *S. vigintiquatuorpunctata* pupal secretion to *O. fasciatus* eggs afforded protection against foraging colonies of *C.*

*lineolata*: only 3 of 16 treated eggs were removed by foragers, whereas all 16 control eggs were taken ( $G_{\text{adj}} = 26.49$ ,  $P < 0.001$ ). Moreover, for every trial, removal of the control egg preceded the removal of the treated egg, if the latter was removed at all (Sign test,  $P < 0.001$ ). Removal times (mean  $\pm$  1 S.E.) for control and treated eggs were  $26.5 \pm 2.9$  min ( $n = 16$ ) and  $45.7 \pm 2.3$  min ( $n = 3$ ), respectively.

## DISCUSSION

Ants are common enemies of ladybird beetles (Majerus 1994). The pupal secretion of the introduced coccinellid *S. vigintiquatuorpunktata* deters the predatory advances of *C. lineolata*, an ant native to the beetle's new range in southern New England, as evidenced through bioassays measuring both the ants' cleaning and foraging behaviors. Elevated cleaning by *C. lineolata* after contact with pupae (either alive or dead) possessing secretory droplets, indicates that the secretion is an irritant, while the absence of ant mortality 48 h post contact suggests that the material has limited toxicity. The secretion itself, independent of any behavioral response by the pupa, is sufficient to elicit ant cleaning, as seen by increased cleansing behavior following contact with dead pupae bearing secretion. Apart from the secretion, the pupa does not appear to have mechanical properties (e.g., the non-secretory spines) that account for the ants' adverse reaction: contact with the secretion-

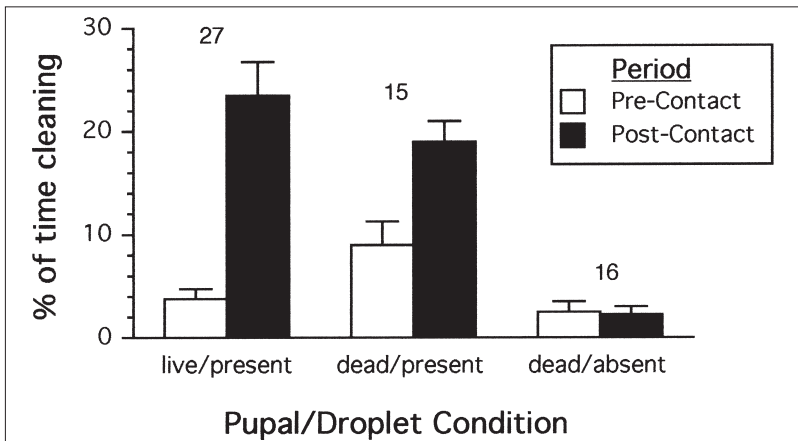


Figure 5. Ant (*Crematogaster lineolata*) cleaning behavior before and after contact with three types of *Subcoccinella vigintiquatuorpunktata* pupae. Cleaning was elevated following contact with live pupae with droplets present ( $Z = -4.52$ ,  $P < 0.0001^*$ ) and dead pupae with droplets present ( $Z = -2.50$ ,  $P = 0.01^*$ ). However, cleaning was unaffected by contact with dead pupae with droplets absent ( $Z = -0.60$ ,  $P = 0.55$ ). Data are plotted as mean  $\pm$  1 S.E., with the number of trials present above columns. Results of Wilcoxon Signed-Rank tests are provided: P-values accompanied by an asterisk are significant at an experiment-wide  $\alpha = 0.05$ .

free pupa did not alter ant cleaning behavior. Moreover, regurgitation and wiping of mouthparts, extreme manifestations of cleaning, were only displayed following the ants' contact with secretion-bearing pupae. Furthermore, topical application of *S. vigintiquatuorpunctata* pupal secretion to edible food items markedly reduced their acceptability to foraging colonies of *C. lineolata*.

Although we have yet to establish that the pupal secretion's primary components (three polyazamacrolide alkaloids, Fig. 3) account for the observed effects on the ants, they seem likely candidates. Epilachnene, a structurally similar alkaloid, produced by the glandular hairs on the pupa of *E. varivestis* (Attygalle et al. 1993), has known deterrent properties to insect predators (Rossini et al. 2000). Topical application of a related alkaloid, a trimeric polyazamacrolide found in the pupal secretion of *E. borealis* (Schroder et al. 1998), renders normally palatable food items unacceptable to *C. lineolata* (unpub. data, Smedley et al.).

Benefits of the pupal defense may even extend into the adult stage of *S. vigintiquatuorpunctata*. Adult coccinellids, like other holometabolous insects, emerge from the pupa with a soft exoskeleton. It frequently requires an hour or more for teneral adult coccinellids to expand their wings and harden their cuticle (Majerus 1994). Insects are particularly vulnerable to predation during this period. We have observed that adult *S. vigintiquatuorpunctata* frequently undergo cuticular hardening while draped in the shed pupal exoskeleton (Fig. 4C). In addition to the pupal cuticle imparting physical protection, droplets of secretion remain (Fig. 4D), conceivably affording chemical defense.

The non-native *S. vigintiquatuorpunctata* seems established in Connecticut, considering the persistence of its Bolton and Glastonbury populations from 1997–2001. Although our 1997 observations form the basis of what appears to be the first published account of *S. vigintiquatuorpunctata* in New England (Schroeder et al. 1998), it is likely that the beetle has existed here for a longer period. The northern New Jersey site where the species was detected in 1973 is fewer than 40 km from southwestern Connecticut and within 175 km of the two populations described here. The average rate of dispersal for four species of adventive coccinellids now established on Cape Breton Island was estimated to range from 30 to 400 km/yr (McCorquodale 1998). Assuming that *S. vigintiquatuorpunctata* spread at a similar rate, it may well have reached Connecticut prior to the 1980s. Examination of museum collections may thus yield New England specimens of *S. vigintiquatuorpunctata* pre-dating 1997.

The observation of both immature forms and adults in the spring and adults alone in the fall for the two Connecticut populations of *S. vigintiquatuorpunctata* is consistent with Wheeler and Henry (1981). These researchers found that in Pennsylvania the beetle over-wintered

as adults that reproduced the following spring, with the resulting adult offspring typically entering reproductive diapause until the next spring.

There is growing concern that predaceous coccinellids introduced into North America may have negative impacts on native ladybird beetles. Recent studies have in fact suggested links between declines in native coccinellid populations and the establishment of certain exotic species (Colunga-Garcia and Gage 1998, Elliott et al. 1996, Wheeler and Hoebeke 1995). It seems unlikely that the herbivorous *S. vigintiquatuor punctata* will have a major negative impact on natural ecosystems in North America, given that its primary host plant *S. officinalis* was itself introduced from Europe in colonial times (Gleason 1963, Mitich 1990). The establishment of *S. vigintiquatuor punctata* may have an indirect impact, however. Wheeler and Henry (1981) found *S. vigintiquatuor punctata* larvae harboring a parasitoid, a eulophid wasp (*Tetrastichus* sp.), which may itself be introduced with the beetle. If this wasp can parasitize native epilachnines (i.e., *E. varivestis* and *E. borealis*), it could impact these beetles. Precedence for such interactions exists: the broad host range of a non-native, tachinid parasitoid of gypsy moth [*Lymantria dispar* (L.)] larvae, has resulted in considerable mortality for certain native North American lepidopterans (Boettner et al. 2000).

Although release from their natural predators has long been considered a factor leading to the proliferation of exotic species within a new environment (Elton 1958, Mack et al. 2000), few studies have specifically examined the effectiveness of the chemical defenses of introduced animals against predators native to their new locale (e.g., Crossland and Azevedo-Ramos 1999, Formanowicz and Brodie 1985, Punzo and Lindstrom 2001, Smedley et al. 2002). The current study thus adds to this growing body. Effective defense against newly encountered predatory species is clearly one factor that could facilitate establishment of an introduced species.

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