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RESEARCH ARTICLE

A multifaceted hemolymph defense against predation in *Diabrotica virgifera virgifera* larvae

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The physical and chemical aspects of *Diabrotica virgifera virgifera* larval hemolymph were quantitatively assessed against two predatory beetle species in the laboratory. Adult *Poecilus cupreus* and *Harpalus pensylvanicus* (Coleoptera: Carabidae) were fed pupae, second or third instar *D. v. virgifera* or a palatable surrogate prey, i.e., *Calliphora vicina* or *Sarcophaga bullata* larvae (Diptera: Calliphoridae, Sarcophagidae, respectively) of equivalent size. The ethanol-soluble fraction of third instar *D. v. virgifera* hemolymph was extracted and suspended in a 0.24 M sucrose solution and offered to *H. pensylvanicus* (using a sucrose only control for comparison). The mean duration until first consumption was recorded for each predator, as was the amount of time spent eating, cleaning, resting, or walking for 2 min post-attack (or 5 min for the sugar assay). Maggots and *D. virgifera* larvae and pupae were attacked equally by both predators. But upon attack, *D. v. virgifera* larval hemolymph coagulated onto the mouthparts of the predators, which they began vigorously cleaning. Predators ate the sucrose solution for significantly longer than hemolymph + sucrose solution, indicating the presence of deterrent chemicals in the hemolymph. This research suggests that *D. v. virgifera* larvae are defended from predation by sticky and repellent hemolymph. We hypothesize that this defense partially explains the widespread success of *D. v. virgifera* as an invasive pest.

Keywords: biological control; Carabidae; Chrysomelidae; maize; predation; rootworm

Introduction

Predator–prey interactions are mediated by numerous physiological and behavioral traits intrinsic to both predators and prey. Not the least of these factors are the physiological defenses of herbivorous insects, which often include defensive chemistry that renders an herbivorous prey physically sticky, repellent, or toxic (Pasteels, Grégoire, and Rowell-Rahier 1983). Defensive characteristics of these herbivorous insects become particularly important in subterranean systems, where competition among the diverse and abundant arthropod predator community results in intense predation pressure on soil-dwelling herbivores. Hemolymph-based defenses are one strategy that influences the relative strengths of trophic linkages between an insect and its diverse assemblage of predators.

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Many herbivorous insects are repellent to predators due to defensive chemistry. Often times, herbivores sequester secondary chemicals from their host plant as a means of defense. Other herbivores produce repellent chemicals *de novo* and store them in exocrine glands (Pasteels et al. 1983; Bowers 1992; Laurent, Braekman, and Daloze 2005). These repellent chemicals may be volatile, others are only repulsive to predators upon contact (Pasteels et al. 1983; Bowers 1992). Most often, repellent chemicals are present in gregarious herbivores that may benefit from group defense, and in those whose mobility is limited (such as in subterranean ecosystems) (Pasteels et al. 1983). Alongside the sequestration or autogenesis of toxic chemicals in their hemolymph, some herbivorous insects display a form of 'easy bleeding' (Boevé and Schaffner 2003) or 'reflex bleeding' (Happ and Eisner 1961; de Jong, Holloway, Brakefield, and de Vos 1991; Peck 2000) from weakened areas of the cuticle that increases the likelihood that predators will encounter offensive chemistry before attacking and killing the herbivore.

A poorly understood aspect of hemolymph defense involves its rapid coagulation onto the mouthparts of attacking predators that results in the physical binding of the mouthparts or the prolonged exposure of the predator to hemolymph-based repellent or toxic chemicals. Coagulation of insect hemolymph results from the cellular and humoral responses to wounds inflicted by attacking predators (Theopold, Li, Fabbri, Scherfer, and Schmidt 2002; Bidla, Lindgren, Theopold, and Dushay 2005; Agianian, Lesch, Losevac, and Dushay 2007; Haine, Rolff, and Siva-Jothy 2007; Lindgren et al. 2008). Coagulation seals the wound, restores the structural integrity of the exoskeleton, and protects the hemocoel from invasion by micro-organisms and particles (Haine et al. 2007). Rapid coagulation of invaded hemolymph can feasibly bind to the mouthparts of predators, thereby restricting their ability to continue chewing their insect prey. Moreover, when hemolymph contains repulsive or toxic sequestered chemicals, coagulation of the hemolymph to the mouthparts of predators could amplify its defensive properties.

Chrysomelid beetles are all herbivorous and are well known for possessing repellent chemistry (Pasteels, Braekman, Daloze, and Ottinger 1982; Dettner 1987; Hilker, Eschbach, and Dettner 1992; Laurent et al. 2005; Pasteels, Daloze, de Bisseau, Termonia, and Windsor 2004). Within the Chrysomelidae, the Diabroticina are renowned for being pharmacophagous on cucurbitacins (Ferguson and Metcalf 1985; Ferguson, Metcalf, and Fischer 1985; Tallamy, Hibbard, Clark, and Gillespie 2005). They seek out plants in the Cucurbitaceae specifically for their defensive cucurbitacins. This is particularly interesting in that many species of Diabroticina do not use cucurbits as their host plant. For instance, *Diabrotica virgifera virgifera* LeConte is a severe pest of maize in North America and Europe (Krysan 1986; Miller et al. 2005; Moeser and Guillamaud 2009) whose larvae can only complete development on several species of Poaceae (Branson and Ortman 1970; Clark and Hibbard 2004). Nevertheless, adult *D. v. virgifera* are attracted to cucurbitacins, which they can sequester and divert into their eggs for protection from predators and pathogens (Tallamy et al. 1998; Tallamy, Gorski, and Burzon 2000). A diverse and abundant natural enemy community coincides with immature *D. v. virgifera* in maize fields (Lundgren, Nichols, Prischmann, and Ellsbury 2009; Toepfer et al. 2009). However, natural mortality of second and third instars is low, which has been identified as a major reason for the success of this pest species (Toepfer and Kuhlmann 2006). Under field conditions, predators clearly vary in their reliance on *D. virgifera* immatures as

prey (Lundgren, Prischmann, and Ellsbury in press), although mechanisms for why this occurs have not been identified. Here, we assessed laboratory-based behavioral observations to test the hypotheses that (1) immature stages *D. v. virgifera* have a defense mechanism against generalist predators, (2) immature stages vary in their defensive capabilities against generalist predators, and (3) defensive chemistry and the physical properties of the *D. v. virgifera* hemolymph contribute to its repellency.

Methods

Insects

Poecilus cupreus (Coleoptera: Carabidae) was captured from maize fields in the Delémont valley of northwestern Switzerland using dry pitfall traps (latitude, longitude: 47.368°, 7.332°). *Harpalus pensylvanicus* DeGeer (Coleoptera: Carabidae) was captured nocturnally at building lights amidst cropland in eastern Brookings, SD, USA (latitude, longitude: 44.340°, 96.790°). Predators were maintained in plastic containers containing moistened cat food and dampened field soil. *Poecilus cupreus* and *H. pensylvanicus* represent abundant carabids in maize fields in the major distribution areas of *D. v. virgifera* (Toepfer et al. 2009), i.e., in central Europe and in the Midwestern and Great Plains of the United States, respectively.

Diabrotica virgifera virgifera larvae were obtained from a continuous culture maintained at the North Central Agricultural Research Laboratory (NCARL), USDA-ARS. This colony is never exposed to cucurbitacins. Larvae were reared to the designated age on germinated corn seedlings (KWS Gavott D/MEI 2047/876 in Europe, Pioneer 38B85 in North America). At the time of the feeding assays, the larvae were separated gravimetrically from the corn tissue and soil substrate using screens through which larvae would fall into dampened paper toweling. Maggots, *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) in Switzerland and *Sarcophaga bullata* Parker (Diptera: Sarcophagidae) in the USA, were obtained commercially and reared to the desired size on beef liver.

Ontogenetic changes in D. v. virgifera *hemolymph defense*

The defensive capabilities of second instars, third instars, and pupae of *D. v. virgifera* to *P. cupreus* was evaluated using post-consumption behavioral observations. Prior to the assay, *P. cupreus* were housed individually in 100 mm diameter plastic Petri dishes containing only a water-saturated cotton wick, and were starved for 24 h. In these assays, *P. cupreus* adults ($n = 15$ per treatment) were randomly assigned to treatments fed an immature *D. v. virgifera* or an equivalent sized control maggot of *C. vicina*. Second instar *D. v. virgifera* and equivalent-sized *C. vicina* maggots weighed a mean $\pm$SEM of 2.89 ± 0.19 and 3.08 ± 0.38 mg, respectively. Third instar and equivalent-sized *C. vicina* weighed 11.87 ± 1.20 and 10.80 ± 0.62 mg, respectively. For the pupal assay, pupae of *D. v. virgifera* were compared with mortally frozen (and thawed) *C. vicina* that were size-equivalent to *D. v. virgifera* third instars.

Each *P. cupreus* was observed for at least 10 min after it was placed into an arena containing a prey item. The duration until the first attack was recorded; predators that did not attack the larvae were discarded from the assay. Following the attack, predator behaviors were recorded for 2 min. The duration that each predator spent

eating, cleaning their mouthparts, walking, and resting were recorded. Cleaning behavior is defined here as rubbing the mouthparts with palps and legs, and wiping the mouthparts on the Petri dish area.

Repellent properties of ethanol extracts of D. v. virgifera *hemolymph*

In two assays, the repellencies to *H. pensylvanicus* of third instar *D. v. virgifera* and the ethanol-soluble fraction of their hemolymph were evaluated. In the first assay, the post-attack response of *H. pensylvanicus* to third instar *D. v. virgifera* was measured using identical methods to those described for *P. cupreus*, except that *S. bullata* was used as the control prey species. In this assay, third instar *D. v. virgifera* and equivalent-sized *S. bullata* larvae weighed 12.00 ± 0.59 and 14.23 ± 1.96 mg, respectively.

In the second assay, hemolymph of *D. v. virgifera* third instars was filtered, incorporated into a sucrose solution, and its repellency to *H. pensylvanicus* was assayed. The cuticles of larvae ($n = 109$) were multiply pierced using a #3 insect pin, and larvae were allowed to bleed onto a piece of filter paper. The resulting hemolymph quickly coagulated onto the filter paper and was not soluble in ethanol or water. Approximately 33% of the larvae's mass was bled. These hemolymph-laden filter papers were agitated in 200 μL of an anticoagulant solution composed of 98 mM NaOH, 186 mM of NaCl, 17 mM Na_2EDTA, and 41 mM citric acid (pH 4.5) (Haine et al. 2007). This solution was vortexed into 2 mL 100% EtOH (Product #UN1170, Acros Organics, Geel, Belgium) for 15 s. The solution was then passed through a 2 cm column of celite to filter out proteinaceous matter from the hemolymph. The column was then rinsed with 1.5 mL of EtOH, and the filtered supernatant was dried under N_2. The dried product (32.29 mg) was resuspended in a 0.24 M sucrose solution (10% EtOH; 1.8 mL total volume), 50 μL of which was then offered to each *H. pensylvanicus*. The amount of hemolymph extract in 50 μL was roughly equivalent to the hemolymph in one *D. v. virgifera* third instar. A sucrose-only control solution was created using the exact methodology as described above (e.g., filtration and resuspension procedures), except there was no hemolymph included.

The feeding behavior of *H. pensylvanicus* fed sucrose and sucrose + hemolymph extract were recorded. The time until the first drink was recorded for each predator. Then the predators were observed for 5 min following the initiation of feeding, and the time devoted to eating, resting, and walking (activity) was recorded for each beetle.

Data analysis

For both *H. pensylvanicus* and *P. cupreus* fed prey, the time spent eating, cleaning, resting, and walking were compared between the *D. v. virgifera* and control treatments using independent Kruskal–Wallis non-parametric ANOVAs (SYSTAT Software 2004). Predators fed different prey life stage were analyzed separately. Similarly, the amounts of time spent eating, resting and walking by *H. pensylvanicus* fed sucrose or sucrose + hemolymph extract were compared using independent Kruskal–Wallis ANOVAs. Finally, the durations before initial consumption were compared between treatments using independent Kruskal–Wallis ANOVAs.

Results

Ontogenetic changes in D. v. virgifera *hemolymph defense*

Poecilus cupreus behaved differently when fed *D. v. virgifera* larvae from when they were fed *C. vicina* maggots (Figure 1). There was no significant effect of prey type on the time until first attack (second instar: $\chi^2_1 = 0.91$, $P = 0.34$; third instar: $\chi^2_1 = 1.45$, $P = 0.23$; Pupae: $\chi^2_1 = 3.26$, $P = 0.07$). But upon attack, *D. v. virgifera* larval hemolymph quickly coagulated onto the mouthparts of the predator, and they backed away from the prey vigorously cleaning their mouthparts. *Poecilus cupreus* fed second instar *D. v. virgifera* spent significantly more time cleaning their mouthparts ($\chi^2_1 = 7.13$, $P = 0.008$) and less time eating ($\chi^2_1 = 4.76$, $P = 0.03$) than those fed *C. vicina* maggots of equivalent size. *Poecilus cupreus* fed third instar *D. v. virgifera* spent significantly more time cleaning their mouthparts ($\chi^2_1 = 15.69$, $P < 0.001$) and less time eating ($\chi^2_1 = 8.44$, $P = 0.004$) than those fed *C. vicina* maggots. *Poecilus cupreus* fed pupae of *D. v. virgifera* or *C. vicina* maggots of equivalent size spent similar amounts of time eating ($\chi^2_1 = 0.002$, $P = 0.96$), and devoted no time to cleaning their mouthparts. There was no effect of treatment on the amount of time

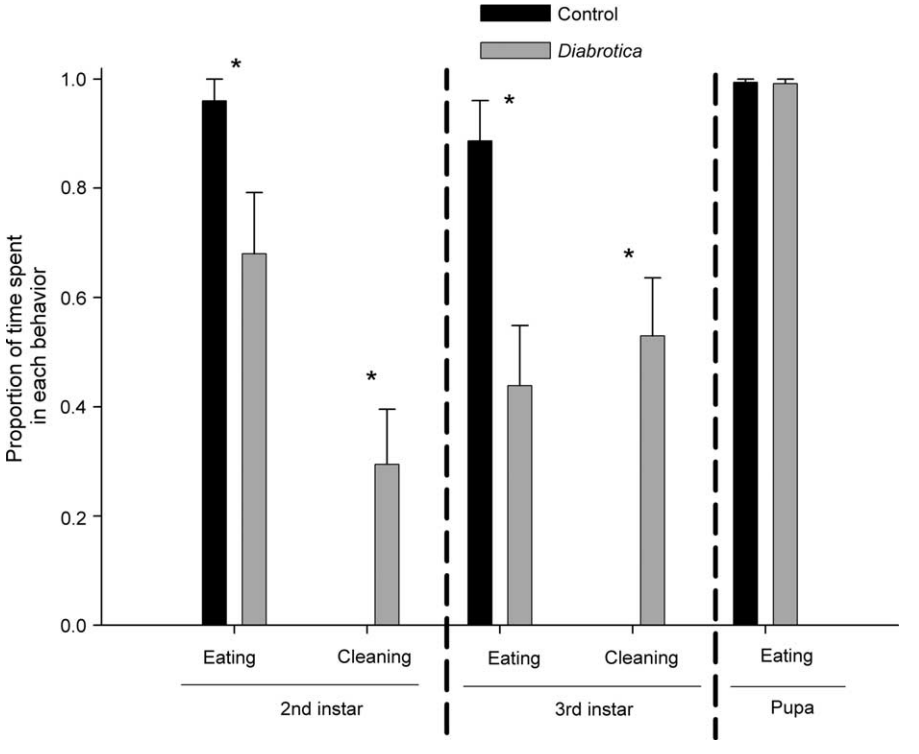

Figure 1. The mean proportion of 2 min observations spent eating or cleaning by *Poecilus cupreus* fed *Diabrotica v. virgifera* second instars, third instars, and pupae or the control food (*Calliphora vicina* maggots of equivalent size and vigor). Beetles fed maggots spent no time cleaning themselves. Asterisks denote significant differences in time between the control and *Diabrotica* treatments for each behavior ($\alpha = 0.05$, Kruskal–Wallis non-parametric ANOVA); error bars denote SEM. For all treatments, $n = 15$.

predators spent walking (second instar: $\chi^2_1 = 0.90$, $P = 0.34$; third instar: $\chi^2_1 = 0.90$, $P = 0.34$; Pupae: $\chi^2_1 = 0.002$, $P = 0.96$).

Repellent properties of ethanol extracts of D. v. virgifera *hemolymph*

The physical (e.g., rapid coagulation) and chemical properties of *D. v. virgifera* larval hemolymph deterred feeding by *H. pensylvanicus* (Figure 2). There was no affect of prey type on the time until initial attack ($\chi^2_1 = 0.008$, $P = 0.93$); mean $\pm$ SEM time to first attack was 253.7 ± 48.9 s for those fed *S. bullata* larvae and 235.3 ± 43.9 s for *D. v. virgifera* third instars. *Harpalus pensylvanicus* spent significantly less time feeding ($\chi^2_1 = 20.72$, $P < 0.001$) and more time cleaning ($\chi^2_1 = 23.68$, $P < 0.001$) when fed *D. v. virgifera* larvae than the maggots. Walking and resting activities were unaffected by treatment (walking: $\chi^2_1 = 1.67$, $P = 0.20$; resting: $\chi^2_1 = 0.55$, $P = 0.46$). Adding the ethanol-extracted component of *D. v. virgifera* hemolymph to a sucrose solution did not affect its attractiveness

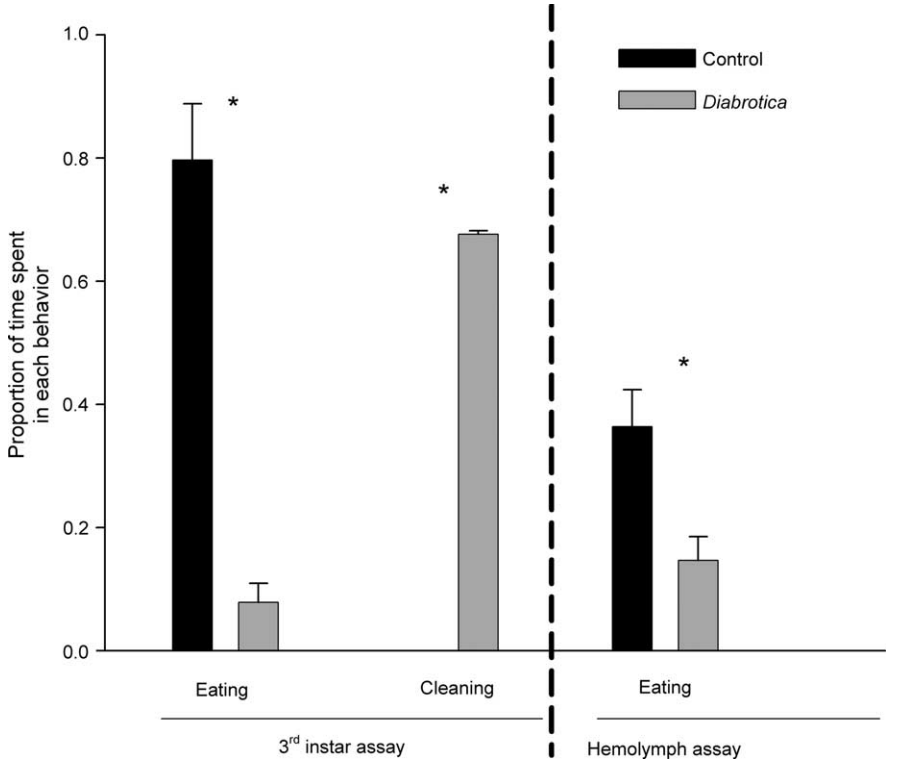

Figure 2. The mean proportion of time spent eating or cleaning by *Harpalus pensylvanicus* fed *Diabrotica v. virgifera* third instars (left) or ethanol-extracted hemolymph or the control food (*Sarcophaga bullata* maggots of equivalent size and vigor and sugar solution, respectively). Beetles fed maggots and the sugar solutions spent no time cleaning themselves. Asterisks denote significant differences in time between the control and *Diabrotica* treatments for each behavior ($\alpha = 0.05$, Kruskal–Wallis non-parametric ANOVA); error bars denote SEM. In the hemolymph assay, $n = 15$ and 16 for the *Diabrotica* and control treatments. In the third instar assay, $n = 14$ and 15 for the control and *Diabrotica* treatments.

to *H. pensylvanicus* ($\chi^2_1 = 0.35$, $P = 0.55$); 166.1 ± 36.02 s until first consumption of sucrose and 183.1 ± 35.9 s for consumption of sucrose + hemolymph solutions. Adding hemolymph to the sucrose solution significantly reduced the amount of time spent feeding by *H. pensylvanicus* ($\chi^2_1 = 10.26$, $P = 0.001$). Walking and resting activities were unaffected by the treatment (walking: $\chi^2_1 = 2.08$, $P = 0.15$; resting: $\chi^2_1 = 0.83$, $P = 0.36$). Beetles in the sucrose assays did not clean their mouthparts, which is why this behavior is not compared.

Discussion

This study proved that *Diabrotica virgifera* larval hemolymph chemically and physically defends them from attack by predators. The two carabid predators equally recognized *D. v. virgifera* larvae or pupae and alternative prey larvae as food, but were only repelled after they attacked a *D. v. virgifera* larva. After biting a *D. v. virgifera* larva, the predator would back away from it (often within seconds of attacking it), with their mouthparts ensnared in sticky hemolymph. The predator immediately began cleaning its mouthparts vigorously, sometimes for up to an hour, and the predators were reluctant to revisit the *D. v. virgifera* larvae (within 15-min post-attack observation periods; J.G.L., unpublished data). In contrast, predators fed maggots and *D. v. virgifera* pupae often ate for the entire observation period feeding.

Results of the feeding assays suggest that there is a chemical deterrent present in the hemolymph in addition to its coagulative properties. Sticky secretions that function in arthropod defense often consist of proteins, mucilages, waxes, or resins and are often found in subterranean arthropods with limited dispersal capabilities (Pasteels et al. 1983). In this case, the coagulative nature of the *D. v. virgifera* larval hemolymph bound the predators' mouthparts, and blocked the oral cavity. Its effectiveness may have been exacerbated by the repellent chemistry found within the hemolymph to produce such a stark reaction in the predator. *Harpalus pensylvanicus* fed twice as long on sugar solution alone than sugar solution containing the ethanol-soluble chemical fraction of *D. v. virgifera* hemolymph. Adult *D. v. virgifera* are chemically defended against predation by cucurbitacins sequestered from their adult host plants (Tallamy et al. 2005). Congeners protect their eggs by coating them with these defensive chemicals during oviposition (Tallamy et al. 1998). However, the *D. v. virgifera* culture used in this study has never experienced cucurbitacins, and this research represents a novel, multifaceted hemolymph defense of unknown origin for this herbivore. *Zea mays* possesses several secondary chemicals, some of which are used by *D. v. virgifera* larvae to locate their host plants, e.g., hydroxamic acids (Bjostad and Hibbard 1992; Xie et al. 1992). Whether *D. v. virgifera* larvae sequester defensive chemicals from *Z. mays*-derived secondary chemicals or synthesize these chemicals *de novo* as in other chrysomelids (Pasteels, Eggenberger, Rowell-Rahier, Ehmke, and Hartmann 1992; Feld, Pasteels, and Boland 2001) merits further attention.

It appears that *D. v. virgifera* hemolymph changes in its defensive characteristics ontogenetically. In contrast to the larvae, pupae were readily consumed by the carabid predators, suggesting a physiological shift in the defensive properties of the hemolymph analogous to what is observed in other insects (Pasteels et al. 1983; Bowers 1992). *Diabrotica v. virgifera* pupate within a soil-encased cocoon, and so

may be physically protected from predators. Numerous carabid predators readily consume adult *D. v. virgifera* from this culture in the laboratory without adverse reaction (J.G. Lundgren, personal observation), and there are numerous reports of carabid beetles consuming *D. v. virgifera* adult beetles under field conditions (Kirk 1971, 1973, 1975), lending credence to the notion that *D. v. virgifera* adults are not protected by this hemolymph defense. Finally, although the carabid attacks on *D. v. virgifera* larvae were often brief, they were severe and often resulted in the deaths of the larvae. Larvae often survived for 24 h (when the observation was ceased) when less severe attacks by other predators and punctures with an insect pin were inflicted (J.G. Lundgren, personal observation). Thus, the hemolymph defense may or may not prevent the death of the attacked *D. v. virgifera* larva. However, *Diabrotica virgifera* eggs and larvae are aggregated under field conditions (Ruesink 1986; Toepfer, Ellsbury, Eschen, and Kuhlmann 2007) and evolutionary benefits of the repellent hemolymph may be best realized within the context of group defense (Pasteels et al. 1983).

The factors that dictate the strength of trophic interactions between herbivores and a diverse community of predators are poorly understood in subterranean food webs. Numerous natural enemies co-occur with and consume *D. v. virgifera* eggs and larvae under field conditions (Toepfer et al. 2009), but these species vary in the strength of their reliance on *D. v. virgifera* as prey (Lundgren et al. in press). We hypothesize that the relative contributions of each predator species to the suppression of this invasive pest will be strongly influenced by their relative susceptibility to the defensive properties of the larval hemolymph.

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References

Agianian, B., Lesch, C., Losevac, O., and Dushay, M.S. (2007), 'Preliminary Characterization of Hemolymph Coagulation in *Anopheles gambiae* Larvae', *Developmental and Comparative Immunology*, 31, 879–888.

Bidla, G., Lindgren, M., Theopold, U., and Dushay, M.S. (2005), 'Hemolymph Coagulation and Phenoloxidase in *Drosophila* larvae', *Developmental and Comparative Immunology*, 29, 669–679.

Bjostad, L.B., and Hibbard, B.E. (1992), '6-Methoxy-2-benzoxazolinone: A Semiochemical for Host Location by Western Corn Rootworm Larvae', *Journal of Chemical Ecology*, 18, 931–944.

Boevé, J.-L., and Schaffner, U. (2003), 'Why Does the Larval Integument of Some Sawfly Species Disrupt so Easily? The Harmful Hemolymph Hypothesis', *Oecologia*, 134, 104–111.

Bowers, M.D. (1992), 'The Evolution of Unpalatability and the Cost of Chemical Defense in Insects', in *Insect Chemical Ecology*, eds. B.D. Roitberg and M.B. Isman, Dordrecht, The Netherlands: Springer, pp. 216–244.

Branson, T.F., and Ortman, E.E. (1970), 'The Host Range of Larvae of the Western Corn Rootworm: Further Studies', *Journal of Economic Entomology*, 63, 800–803.

Clark, T.L., and Hibbard, B.E. (2004), 'Comparison of Nonmaize Hosts to Support Western Corn Rootworm (Coleoptera: Chrysomelidae) Larval Biology', *Environmental Entomology*, 33, 681–689.

de Jong, P.W., Holloway, G.J., Brakefield, P.M., and de Vos, H. (1991), 'Chemical Defence in Ladybird Beetles (Coccinellidae). II. Amount of Reflex Fluid, the Alkaloid Adaline and Individual Variation in Defence in 2-Spot Ladybirds (*Adalia bipunctata*)', *Chemoecology*, 2, 15–19.

Dettner, K. (1987), 'Chemosystematics and Evolution of Beetle Chemical Defenses', *Annual Review of Entomology*, 32, 17–48.

Feld, B.K., Pasteels, J.M., and Boland, W. (2001), '*Phaedon cochleariae* and *Gastrophysa viridula* (Coleoptera: Chrysomelidae) Produce Defensive Iridoid Monoterpenes *De Novo* and are Able to Sequester Glycosidically Bound Terpenoid Precursors', *Chemoecology*, 11, 191–198.

Ferguson, J.E., and Metcalf, R.L. (1985), 'Cucurbitacins: Plant Derived Defense Compounds for Diabroticites (Coleoptera: Chrysomelidae)', *Journal of Chemical Ecology*, 11, 311–318.

Ferguson, J.E., Metcalf, R.L., and Fischer, D.C. (1985), 'Disposition and Fate of Cucurbitacin B in Five Species of Diabroticites', *Journal of Chemical Ecology*, 11, 1307–1321.

Haine, E.R., Rolff, J., and Siva-Jothy, M.T. (2007), 'Functional Consequences of Blood Clotting in Insects', *Developmental and Comparative Immunology*, 31, 456–464.

Happ, G.M., and Eisner, T. (1961), 'Hemorrhage in a Coccinellid Beetle and its Repellent Effect on Ants', *Science*, 134, 329–331.

Hilker, M., Eschbach, U., and Dettner, K. (1992), 'Occurrence of Anthraquinones in Eggs and Larvae of Several Galerucinae (Coleoptera: Chrysomelidae)', *Naturwissenschaften*, 79, 271–274.

Kirk, V.M. (1971), 'Biological Studies of a Ground Beetle, *Pterostichus lucublandus*', *Annals of the Entomological Society of America*, 64, 540–544.

Kirk, V.M. (1973), 'Biology of a Ground Beetle, *Harpalus pensylvanicus*', *Annals of the Entomological Society of America*, 66, 513–518.

Kirk, V.M. (1975), 'Biology of *Pterostichus chalcites*, a Ground Beetle of Cropland', *Annals of the Entomological Society of America*, 68, 855–858.

Krysan, J.L. (1986), 'Introduction: Biology, Distribution, and Identification of Pest *Diabrotica,*' in *Methods for the Study of Pest Diabrotica*, eds. J.L. Krysan and T.A. Miller, New York, NY: Springer-Verlag, pp. 1–23.

Laurent, P., Braekman, J.-C., and Daloze, D. (2005), 'Insect Chemical Defense', *Topics in Current Chemistry*, 240, 167–229.

Lindgren, M., Riazi, R., Lesch, C., Wilhelmsson, C., Theopold, U., and Dushaya, M.S. (2008), 'Fondue and Transglutaminase in the *Drosophila* Larval Clot', *Journal of Insect Physiology*, 54, 586–592.

Lundgren, J.G., Nichols, S., Prischmann, D.A., and Ellsbury, M.M. (2009), 'Seasonal and Diel Activity Patterns of Generalist Predators Associated with *Diabrotica virgifera* Immatures (Coleoptera: Chrysomelidae)', *Biocontrol Science and Technology*, 19, 327–333.

Lundgren, J.G., Prischmann, D.A., and Ellsbury, M.M. (in press), 'PCR-based Analysis of the Predator Community Associated with a Subterranean Herbivorous Insect', *Ecological Applications.*

Miller, N., Estoup, A., Toepfer, S., Bourguet, D., Lapchin, L., Derridj, S., Kim, K.S., Reynaud, P., Furlan, L., and Guillemaud, T. (2005), 'Multiple Transatlantic Introductions of the Western Corn Rootworm', *Science*, 310, 992.

Moeser, J., and Guillamaud, T. (2009), 'International Cooperation on Western Corn Rootworm Ecology Research: State-of-the-art and Future Research', *Agricultural and Forest Entomology*, 11, 3–7.

Pasteels, J.M., Braekman, J.C., Daloze, D., and Ottinger, R. (1982), 'Chemical Defence in Chrysomelid Larvae and Adults', *Tetrahedron*, 38, 1891–1897.

Pasteels, J.M., Grégoire, J.-C., and Rowell-Rahier, M. (1983), 'The Chemical Ecology of Defense in Arthropods', *Annual Review of Entomology*, 28, 263–289.

Pasteels, J.M., Eggenberger, F., Rowell-Rahier, M., Ehmke, A., and Hartmann, T. (1992), 'Chemical Defense in Chrysomelid Leaf Beetles', *Naturwissenschaften*, 79, 521–523.

Pasteels, J.M., Daloze, D., de Bisseau, J.-C., Termonia, A., and Windsor, D.M. (2004), 'Patterns in Host-plant Association and Defensive Toxins Produced by Neotropical Chrysomeline Beetles', in *New Developments in the Biology of Chrysomelidae*, eds. P. Jolivet, J.A. Santiago-Blay and M. Schmitt, The Hague, The Netherlands: SPB Academic Publishing, pp. 669–676.

Peck, D.C. (2000), 'Reflex Bleeding in Froghoppers (Homoptera: Cercopidae): Variation in Behavior and Taxonomic Distribution', *Annals of the Entomological Society of America*, 93, 1186–1194.

Ruesink, W.G. (1986), 'Egg Sampling Techniques', in *Methods for the Study of Pest Diabrotica.*, eds. J.L. Krysan and T.A. Miller, New York, NY: Springer-Verlag, pp. 83–100.

SYSTAT Software (2004), 'SYSTAT 11', Richmond, CA: Systat Software, Inc.

Tallamy, D.W., Whittington, D.P., Defurio, F., Fontaine, D.A., Gorski, P.M., and Gothro, P.W. (1998), 'Sequestered Cucurbitacins and Pathogenicity of *Metarhizium anisopliae* (Monilales: Moniliaceae) on Spotted Cucumber Beetle Eggs and Larvae (Coleoptera: Chrysomelidae)', *Environmental Entomology*, 27, 366–372.

Tallamy, D.W., Gorski, P.M., and Burzon, J.K. (2000), 'The Fate of Male-derived Cucurbitacins in Spotted Cucumber Beetle Females', *Journal of Chemical Ecology*, 26, 413–427.

Tallamy, D.W., Hibbard, B.E., Clark, T.L., and Gillespie, J.J. (2005), 'Western Corn Rootworm, Cucurbits and Cucurbitacins', in *Western Corn Rootworm: Ecology and Management*, eds. S. Vidal, U. Kuhlmann and C.R. Edwards, Wallingford, UK: CAB International, pp. 67–94.

Theopold, U., Li, D., Fabbri, M., Scherfer, C., and Schmidt, O. (2002), 'The Coagulation of Insect Hemolymph', *Cellular and Molecular Life Sciences*, 59, 363–372.

Toepfer, S., and Kuhlmann, U. (2006), 'Constructing Life-tables for the Invasive Maize Pest *Diabrotica virgifera virgifera* (Col., Chrysomelidae) in Europe', *Journal of Applied Entomology*, 130, 193–205.

Toepfer, S., Ellsbury, M.M., Eschen, R., and Kuhlmann, U. (2007), 'Spatial Clustering of *Diabrotica virgifera virgifera* and *Agriotes ustulatus* in Small-scale Maize Fields without Topographic Relief Drift', *Entomologia Experimentalis et Applicata*, 124, 61–75.

Toepfer, S., Haye, T., Erlandson, M., Goettel, M., Lundgren, J.G., Kleespies, R.G., Weber, D.C., Cabrera Walsh, G., Peters, A., Ehlers, R.-U., Strasser, H., Moore, D., Jackson, J.J., Keller, S., Vidal, S., and Kuhlmann, U. (2009), 'A Review of the Natural Enemies of Beetles in the Subtribe Diabroticina (Coleoptera: Chrysomelidae): Implications for Sustainable Pest Management', *Biocontrol Science and Technology*, 19, 1–65.

Xie, Y.S., Arnason, J.T., Philogene, B.J.R., Atkinson, J., and Morand, P. (1992), 'Behavioral Responses of Western Corn Rootworm Larvae to Naturally-occurring and Synthetic Hydroxamic Acids', *Journal of Chemical Ecology*, 18, 945–957.