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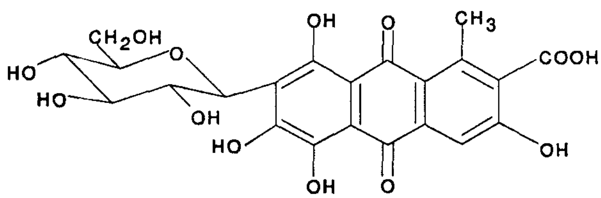
Defensive use of an acquired substance (carminic acid) by predaceous insect larvae[1]

T. Eisner[a], R. Ziegler[b], J. L. McCormick[c], M. Eisner[a], E. R. Hoebeke[d] and J. Meinwald[c]

[a]*Section of Neurobiology & Behavior, W 347 Mudd Hall, Cornell University, Ithaca (New York 14853, USA),* [b]*Department of Biochemistry, University of Arizona, Tucson (Arizona, 85721, USA),* [c]*Department of Chemistry, Cornell University, Ithaca (New York, USA), and* [d]*Department of Entomology, Cornell University, Ithaca (New York, USA)*
Received 25 January 1994; accepted 17 February 1994

Abstract. Larvae of two insects, a coccinellid beetle (*Hyperaspis trifurcata*) and a chamaemyiid fly (*Leucopis* sp.), feed on cochineal insects and appropriate their prey's defensive chemical, carminic acid, for protective purposes of their own. *H. trifurcata* discharges the chemical with droplets of blood (hemolymph) that it emits when disturbed; *Leucopis* sp. ejects the compound with rectal fluid. Ants are thwarted by these defenses, which are compared with the previously-described defense of a pyralid caterpillar (*Laetilia coccidivora*) that disgorges carminic acid-laden crop fluid. The defensive fluid of all three larvae contains carminic acid at concentrations spanning a range (0.2–6.2%) proven deterrent to ants. Many insects are known to appropriate defensive substances from plants. Insects that acquire defensive chemicals from animal sources may be relatively rare.
Key words. Quinone; Coccidae; Coccinellidae; Chamaemyiidae; Pyralidae.

Carminic acid (I), the well-known red dye from cochineal bugs (Homoptera; Coccidae, *Dactylopius* spp.), played a major role in the history of the textile industry and is to this day used for a variety of pigmentary purposes[2]. An anthraquinone, carminic acid has been shown to be a potent feeding deterrent to ants, a defensive property that may account for the evolution of the compound in cochineals[3]. Interestingly, not all insects are deterred by carminic acid. One species, the moth *Laetilia coccidivora* (Lepidoptera, Pyralidae), feeds as a larva on cochineals and uses the ingested carminic acid for defensive purposes of its own. When attacked, as by ants, the larva regurgitates carminic acid-laden crop contents, thereby effectively thwarting its assailants[3]. We here report that two other predators of cochineals, a beetle larva, *Hyperaspis trifurcata* (Coleoptera, Coccinellidae), and a fly larva, *Leucopis* sp. (Diptera, Chamaemyiidae), also use dietarily-acquired carminic acid for defense. The beetle larva sequesters the compound systemically and administers it to the enemy by reflex-bleeding, while the fly larva emits it by ejection of rectal fluid. These mechanisms are here described, and compared with that of *L. coccidivora.*



I

Scheme 1.

Materials and methods

The cochineal colonies (*Dactylopius confusus*) were collected on *Opuntia* cactus in the environs of Tucson, Arizona, in late summer and early fall. At that time of year the colonies consistently harbored all three larval predators. These were maintained singly in petri dishes with a small supply of cochineals. They fed voraciously on these, puncturing their bodies and imbibing the contents. Several individuals of each predator were raised to adulthood, providing specimens for identification. These are deposited as voucher specimens in lot 1213 of the Cornell University entomological collection. Our *Leucopis* sp., which we are assigning to the *ocellaris* group, may be a new species.

Carminic acid was identified in *Leucopis* sp. by direct comparison with an authentic sample (Aldrich Chemical Co. Milwaukee, Wisconsin, USA). Comparison included thin layer chromatography (TLC) using n-butyl alcohol, water, acetic acid; high performance liquid chromatography (HPLC) on Supelco LC-18-DB and LC-SAX columns; ultraviolet/visible (UV/VIS) spectroscopy, and fast atom bombardment mass spectrometry (FAB-MS). For the *H. trifurcata* sample, comparisons via TLC, HPLC, and UV/VIS spectroscopy were carried out.

Quantification of carminic acid was effected by HPLC analysis using indole-3-acetic acid as an internal standard. Larval samples were diluted with known amounts of internal standard, and the amount of carminic acid was determined by using a calibration curve based on standards containing known amounts of carminic acid and indole-3-acetic acid.

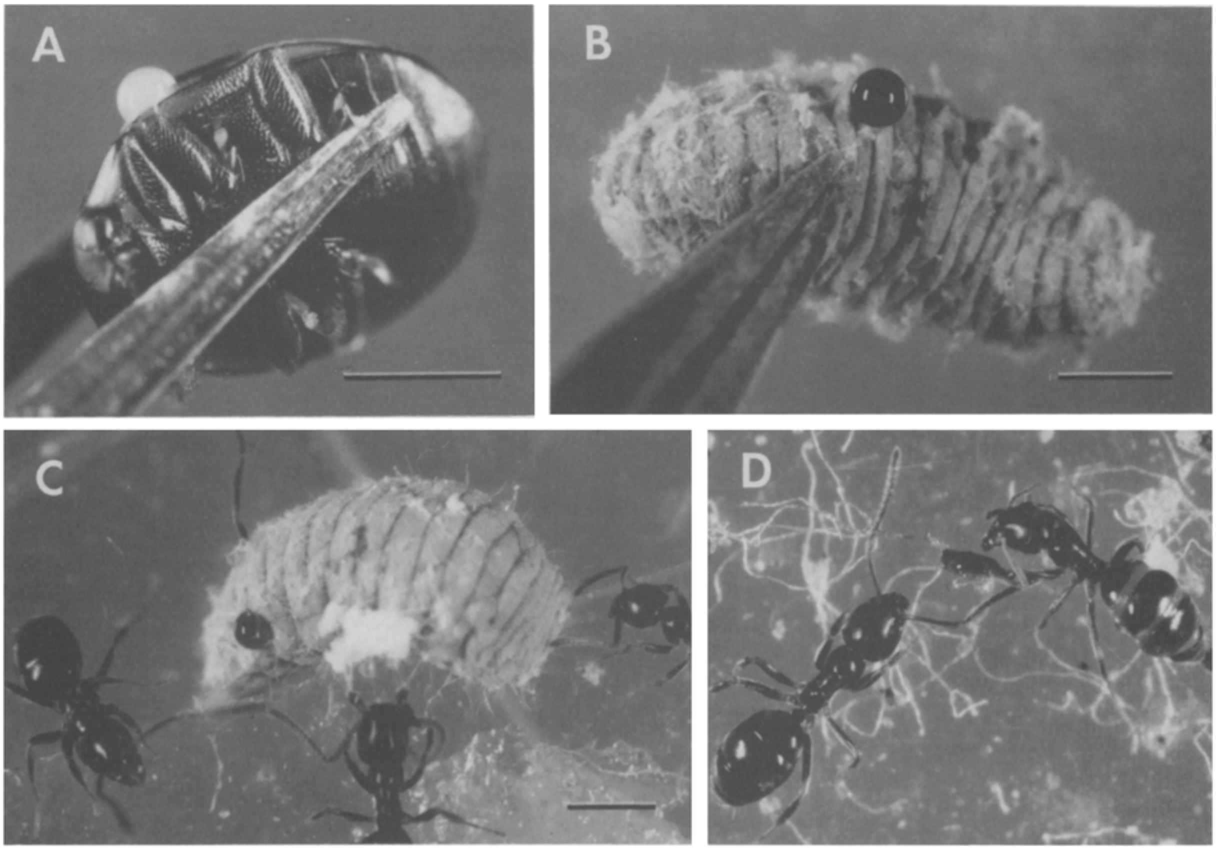


Figure 1. *H. trifurcata.*
A Adult beetle held in forceps, responding by reflex-bleeding from knee-joint of a left leg; the emitted fluid is white and presumably free of carminic acid;
B Larva, dorsal view, responding to poking by reflex bleeding from a site nearby; the droplet is dark red;
C Ants (*T. sessile*) confronting a larva; larva has reflex-bled in response to an ant's bite;
D Ant on right has attacked a larva and bears a semi-coagulated dab of the larva's blood on its left foreleg; ant on left has become stuck to that leg by its right antenna.
(Bars = 1 mm)

Results

Hyperaspis trifurcata. Observations were made on ca. 3 dozen mid-size to full-grown larvae. When individuals were poked with a probe or lightly pinched with forceps, they almost always responded by emitting one or several droplets of red fluid from the integument (fig. 1B). The emission was not necessarily from the site stimulated, but tended to occur from close to that site. There appeared to be no fixed points of emission. Effluence could occur from anywhere along the back or flanks of the larva. Dissection of larvae revealed the hemolymph to be red, leading to the conclusion that the emitted fluid is itself hemolymph and its discharge by the larva a form of reflex-bleeding. Examination of the surface of larvae by scanning electronmicroscopy (critical point-dried larvae, gold-coated) revealed no ready-made pores or slits such as one might have expected to be present for fluid egress. Following emission, the fluid becomes gummy and eventually hardens, as is typical for insect hemolymph. The body of the larva is covered with a fine 'dusting' of wax particles. While these particles may be a product of the larva itself, they may also stem from the cochineals, which secrete wax in copious quantity[4,5]. The dusting prevents the droplets of emitted fluid from sticking to, or spreading over, the larva's own integument, and facilitates the detachment of the droplets and their uptake by contacting objects. In full-grown larvae approaching pupation the emitted hemolymph is pink rather than intense red. Adults also reflex bleed, from the knee-joints as many coccinellid beetles do[6], but at that stage the hemolymph is white (fig. 1A).

Exposure to ants showed the emitted fluid to be effectively deterrent. Groups of 5–10 ants (*Tapinoma sessile*, from laboratory colonies collected near Ithaca, New York) were released into the petri dishes with individual larvae, and events were monitored with a photomicroscope. The results were consistent with each of 5 larvae thus tested. Most ants merely inspected the larvae without biting, but some bites occurred, and these invariably elicited droplet-emission by the larvae (fig. 1C). The droplets always emerged from close to the site bitten, with the result that the ants were in most cases wetted by the fluid. They invariably backed away after contact and, visibly contaminated, engaged in stereotyped cleansing activities (dragging of mouthparts against substrate; wiping of antennae with forelegs). As the fluid hardened, the ants became noticeably encumbered. Instances were noted where antennae became stuck to a leg or to the side of the head of an ant, or where ants became temporarily stuck to one another (fig. 1D).

For chemical purposes, individual larvae were 'milked' by causing them to reflex-bleed and taking up the emitted droplets in capillary tubes. One sample consisted of the pooled output (4.60 mg hemolymph) from 11 larvae (mostly full-grown; collective body mass =42.90 mg). This sample served for definitive characterization of carminic acid in the hemoplymph, and provided a figure (0.17% by mass) for mean carminic acid content of the fluid.

Additional analyses were made of the milkings of 6 individual larvae. These ranged from medium-sized (2.00 mg) to full-grown (4.62 mg), and their fluid output was taken up in capillaries and weighed. The results are given in the table, top row. The carminic acid content of the fluid varied over more than an order of magnitude. Observation had told us that the younger larvae, whose hemolymph is the most intensely colored, should contain the higher levels of carminic acid. This held true [the two highest percentages (4.25 and 1.81%) pertained to the smallest larvae (2.73 and 2.00 mg, respectively), while the lower percentages (0.74, 0.46, 0.37, 0.17) corresponded to larger larvae (3.37, 4.62, 3.52, 4.53 mg, respectively)]. We attribute the low percent value obtained with the initial pooled sample to the fact that it consisted mostly of blood from older larvae.

Leucopis sp. Observations were made on some 2 dozen larvae. When probed or pinched these responded promptly by emitting a large droplet of red fluid from the anus (fig. 2B). The larvae are typically covered with waxy powder and silken threads, both probably stemming from the cochineal prey (cochineals produce both wax and silk; *Leucopis* larvae divested of their coating failed to regenerate the cover if confined without cochineals). The emitted fluid remained globular without spreading over the larva and flowed readily onto contacting objects. The fluid remained mobile on exposure to air and dried without becoming gummy. Only two larvae were tested (in the same manner as *H. trifurcata* larvae) with *T. sessile* ants. Both were eventually induced to discharge anal fluid (in one case this was clearly in response to an ant bite; in the other, it seemed to be triggered by the mere scurrying of ants over the larva's body). In both instances ants became contaminated and, after desisting from the attack, engaged in immediate cleansing activities.

In larvae cleared of their investiture, the malpighian tubules could be seen, dark-red in color, through the semi-translucent body wall (fig. 2C). Dissection of larvae showed the tubules (fig. 2D) to be the only inner organs thus intensely colored. Dissection also revealed presence of a capacious rectal pouch, the presumed storage sac for the defensive fluid. Indeed, dissection of a larva killed by freezing, without previously having been caused to discharge, showed the rectal pouch to be replete with dark-red fluid.

A sample of anal effluent from a number of larvae, taken up in capillary tubes, provided material for definitive characterization of carminic acid in the fluid. For quantitative purposes, an additional 8 'milkings' from individual larvae were analyzed. These were stimulated by probing, and their anal effluent was taken up in capillary tubes and weighed. The results are given in the table, bottom row.

One further sample, consisting of two isolated malpighian tubules from a larva, was also analyzed. The weight of this sample was too low to be detected by our balance (<20 μg). Net carminic acid content of this sample was 1.9 μg, or in the order of at least 10% of tubule mass.

Laetilia coccidivora. Our earlier study of this pyralid larva[3] was with populations from central Florida. We found the caterpillar to be common also in the cochineal colonies from Arizona (adults raised from these larvae proved definitively to be *L. coccidivora*). Cursory experimentation with the larvae showed these to behave precisely as those from Florida. They responded to disturbance by disgorging crop fluid, and they flexed their front end so as to deliver their effluent with accuracy onto whatever instrument was used to provoke them.

Percent carminic acid in larval effluent, in relation to effluent mass, and larval body mass

	Larval mass (mg)	Effluent mass (mg)	Carminic acid in effluent (% by mass)
H. trifurcata	3.46 ± 0.42 [2.00–4.62]	0.28 ± 0.07 [0.10–0.48]	1.30 ± 0.46 [0.17–4.25]
Leucopis sp.	3.30 ± 0.22 [2.16–3.93]	0.15 ± 0.03 [0.05–0.30]	1.80 ± 0.70 [0.15–6.17]

Figures give mean ± SEM, and range in brackets. Effluent in the case of *H. trifurcata* is emitted blood (hemolymph); in the case of *Leucopis* sp. it is discharged anal fluid.

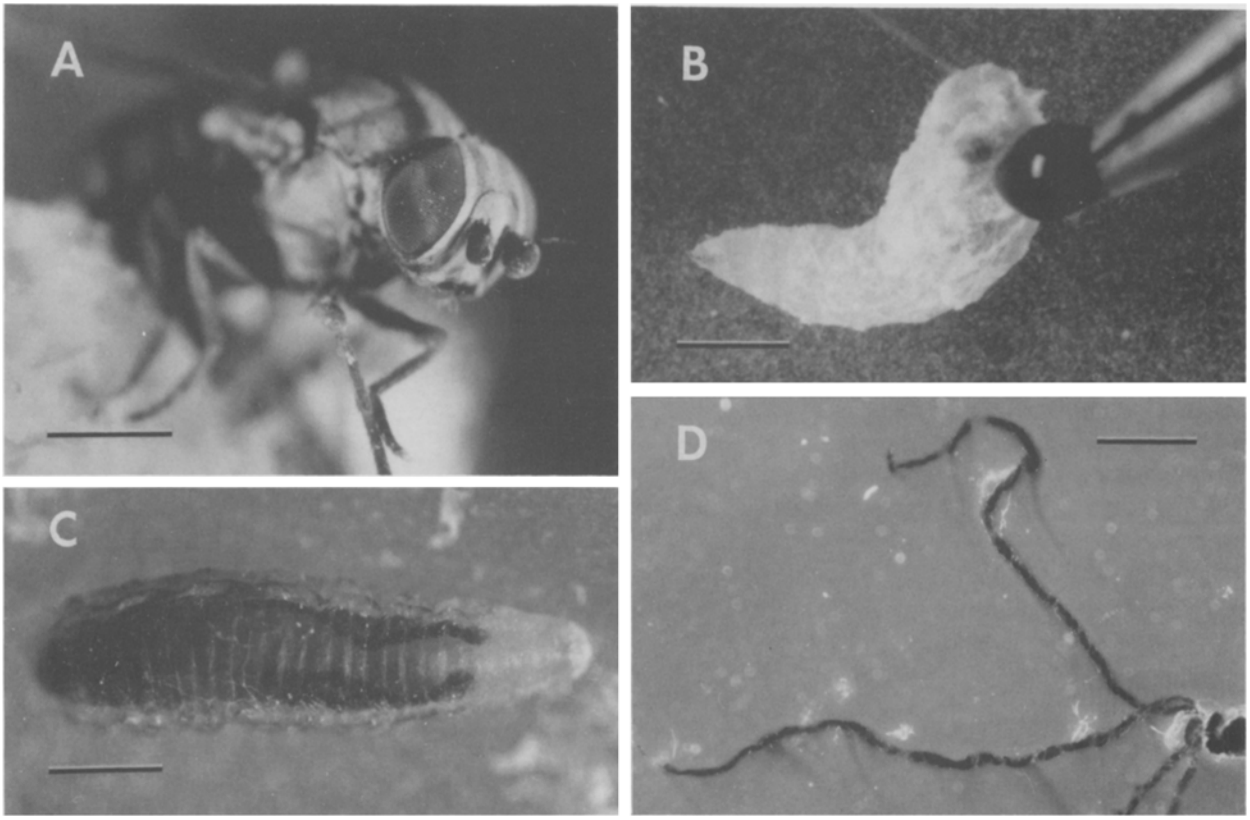


Figure 2. *Leucopis* sp. *A* Adult fly;
B Larva, responding to poking by ejecting a droplet of dark-red rectal fluid onto the probe;
C Larva (divested of exogenous coating) showing dark-red malpighian tubules projecting forward on each side of the midline;
D Isolated malpighian tubules, in saline solution; red pigment, presumably carminic acid, can be seen diffusing into the medium.
Bars = 1 mm (*A*, *C*), 0.5 mm (*B*), 0.05 mm (*D*)

Discussion

The fluid-emission mechanisms of *H. trifurcata* and *Leucopis* sp. appear to be defensive. In both larvae the mechanism is activated by disturbance, and in both cases the fluid contains a compound, carminic acid, of proven anti-insectan potency[3]. The experiments with ants, while not attesting directly to the deterrency of carminic acid, did demonstrate that the effluents of both larvae are protective. Ants are ubiquitous, and most probably figure as natural enemies of the larvae. Whether other insect predators are also deterred by carminic acid remains unknown. One enemy appears able to cope with *Leucopis* sp. Our Arizona cochineal colonies, in the laboratory, gave rise to several individuals of a parasitoid wasp, which turned out to be a possibly new species of *Pachyneuron* (Hymenoptera, Pteromalidae). One species of this genus, *P. mucronatum*, is a known parasitoid of *Leucopis* in the USA and Mexico[7].

The hemolymph of *H. trifurcata* and the anal effluent of *Leucopis* sp. contain carminic acid at remarkably similar concentrations (1.30% and 1.80%, respectively). These concentrations are in a range (10^{-1}–10^{-2} M) known to be highly deterrent to ants[3]. Even at their lowest detected values (0.17% for *H. trifurcata*; 0.15% for *Leucopis* sp.) the concentrations were higher by an order of magnitude than the threshold of deterrency of carminic acid to ants (10^{-3}–10^{-4} M)[3]. Interestingly, the carminic acid concentration in the oral effluent of *L. coccidivora* ($2.7 \pm 0.3\%$; range 2.2–3.3%)[3] is roughly in line with those in the *H. trifurcata* and *Leucopis* sp. effluents. In cochineals themselves, the carminic acid concentration ranges from 1.5% (adult female) to 3.0% (newborn)[3].

The discharge mechanisms of the three cochineal predators – oral emission, reflex bleeding, and anal emission – are schematically represented in figures 3 and 4.

Little question remains about the operation of the *L. coccidivora* defense (figs 3A, 4A): the carminic acid ejected by the larva represents a presumably freshly ingested chemical disgorged directly from the crop, without having been previously absorbed and cycled systemically by the larva[3].

In *H. trifurcata* (figs 3B, 4B) we assume carminic acid to be absorbed enterically, probably in the midgut, and to be taken up directly into the hemolymph, where it is

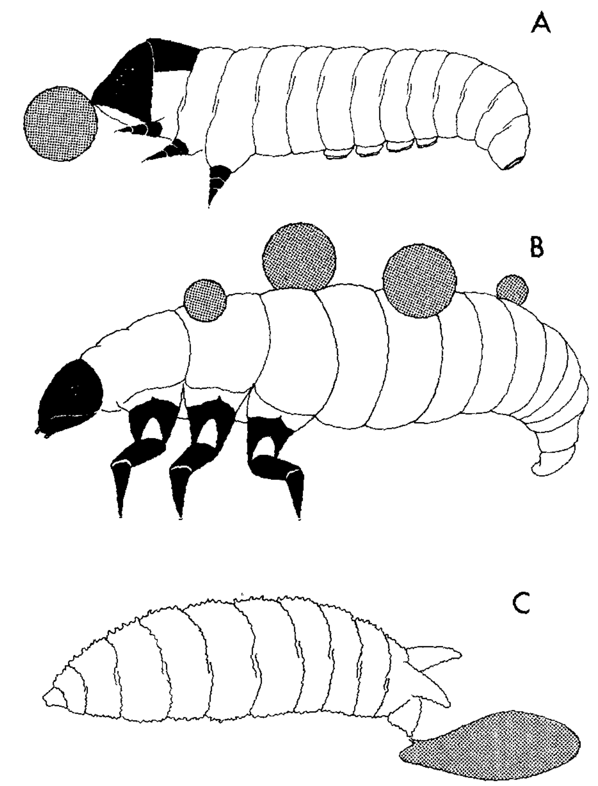


Figure 3. Defensive strategies of the three larval predators of cochineals.
A L. coccidivora – oral discharge; *B H. trifurcata* – reflex bleeding; *C Leucopis* sp. – anal discharge.
A early instar; *B, C* mid-size; Bar = 1 mm

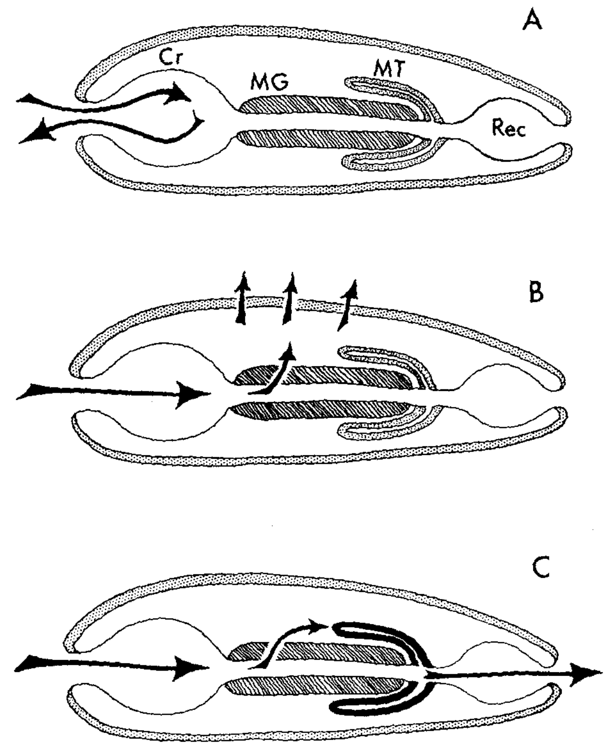


Figure 4. Comparable to figure 3, showing the postulated routes (arrows) of ingested carminic acid in the three predators. Anatomical details are schematic: Cr = crop; MG = midgut; MT = malpighian tubules: Rec = rectum.

held for eventual defensive use. Whether the carminic acid is stored extracellularly in the hemolymph or also within cells, remains unsettled. Also unexplained is the mechanism by which the hemolymph of the larva is emitted through the integument. How the larva is able to effect controlled, localized emission of hemolymph, through a cuticle that is not obviously perforate, remains a mystery. The fact that the adult *H. trifurcata* also reflex-bleeds, but appears not to utilize carminic acid, is interesting. Adult coccinellids are known to synthesize a wide array of defensive toxins[8–10]. We presume *H. trifurcata* to be no exception, and to contain a number of such chemicals in its adult blood, as alternatives to carminic acid.

Perhaps least clear is the mechanism of emission of *Leucopis* sp. (figs 3C, 4C), since there is no ready explanation for the apparent involvement of the malpighian tubules in the process. We envision the mechanism as involving, first, absorption of carminic acid in the midgut, then, concentration of the substance in the malpighian tubules, and finally, secretion of the compound by these tubules into the rectum. But why the circuitous route? We suggest that carminic acid absorption in the midgut might be inevitable, and that the malpighian tubules might simply be performing their normal excretory role of ridding the larva of what could well be a potentially burdensome substance. Such a routing system could be viewed as having set the stage for the evolution of a defense entailing anal emission of carminic acid. The one chemical assay made of malpighian tubules revealed presence of carminic acid at a level well in excess of that in cochineals, a finding consistent with the notion that carminic acid is concentrated in the tubules. It would obviously have been desirable to have had sufficient larvae for further quantitative assay of tubules and, for that matter, of other anatomical parts of the larva.

Many insects utilize defensive chemicals that they acquire from the diet[11]. Most documented cases involve insects that sequester compounds from plants. *L. coccidivora*, *H. trifurcata*, and *Leucopis* sp. are exceptional in that they obtain their defensive substance from an animal source. Moreover, they exemplify variation on a theme. They share the same insect prey, appropriate the same protective compound, and put that compound to use by discharging it as part of a liquid effluent. They differ in that each uses a different effluent, which it discharges in its own unique way. Given that they are members of different orders and have doubtless evolved their defenses independently, it should come as no surprise that they have achieved similar adaptive ends in somewhat unequal ways.

Acknowledgments. Study supported by grants AI02908 (Eisner), AI12020 (Meinwald), and AI26905 (Ziegler) from NIH. We thank J. G. Franclemont for identification of *L. coccidivora*, James Hare for providing the ant colonies and Ian Baldwin for comments on the manuscript.

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