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Cantharidin production in a blister beetle[1]

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Abstract. Cantharidin, a potent defensive chemical, is present in all ten life stages of the blister beetle *Epicauta funebris*. The first five larval stages accumulate cantharidin as they feed and grow in size. When disturbed, they exude cantharidin in a milky oral fluid, not in hemolymph which adult beetles reflexively discharge from leg joints. Two subsequent larval stages and the pupa do not feed, grow, regurgitate, or change in their defensive reserves (110 µg cantharidin/insect, regardless of sex). Adult beetles kept in isolation for 60–90 d exhibit a pronounced sexual dimorphism in cantharidin production: the male biosynthesizes about 17 mg of the toxin, representing 10% of his live weight, whereas the female actually loses most of her defensive reserves. But in the wild a female beetle repeatedly acquires cantharidin as copulatory gifts from her mates.

Key words. Coleoptera; Meloidae; *Epicauta funebris;* chemical defense; biosynthesis; terpenoid.

Blister bettles (Coleoptera, Meloidae) are notorious insects because they contain cantharidin, a defensive chemical that is reputed to possess aphrodisiac properties in humans[2]. The biosynthetic pathway for cantharidin in blister beetles is without known parallel[3]. Schmid's group documented cantharidin biosynthesis in several stages of *Lytta vesicatoria*, the Spanish fly, as well as male-to-female transfer of the substance in the seminal fluid[4].

Because this meloid species is difficult to culture, in most instances these researchers used wild adults of unknown age or limited numbers of laboratory-reared immatures that they were unable to sex. We have developed procedures to mass produce the blister beetle *Epicauta funebris* (formerly *E. pestifera*[5]) in our laboratory[2]. In addition, using external cuticular features, we can reliably determine the sex of coarctate larvae[6]. Exploiting these methodologies, we here report on the first tests that rigorously quantify cantharidin in all stages of a blister beetle. In addition, we describe the external release of cantharidin by meloid larvae for their defense and we document the sites of storage of cantharidin in larval and adult *E. funebris*.

We reared immature *E. funebris*, a meloid species common in Missouri, under controlled conditions (25 °C, 100% r.h., 24 h dark) in incubators. To determine typical cantharidin amounts by gas chromatographic analysis[7], five egg masses, in which the eggs were counted, and five representative larvae of each of the five early stages of this hypermetamorphic insect (T, triungulin; FG1, first grub one; FG2, first grub two; FG3, first grub three; FG4, first grub four) were weighed and frozen at −30 °C 1 d after molting. Larvae progressed in 15–17 d from T-larvae to FG4-larvae on a diet of *Melanoplus differentialis* grasshopper eggs. The remaining FG4-larvae (N = 90) molted in 7–10 d to the diapausing coarctate (C) larval stage, whereupon they were sexed. C-larvae were chilled at 5–7 °C for 180–250 d to terminate diapause and then warmed again to 25 °C for 13–25 d to stimulate development to second grub (SG) larvae, which in 7–12 d molted to pupae (P). After 14–16 d pupae metamorphosed into adult beetles. Five individuals of each sex representing the C, SG, and P stages were weighed and frozen 1 d after molting for cantharidin quantitation.

Adult beetles were individually isolated in plastic cages at 25–27 °C under continuous illumination and fed ad libitum an artificial diet[8]. To prevent incidental discharge of cantharidin by reflex bleeding, beetles were not handled during tests. On the basis of random assignment, twenty unmated adults of each sex were weighed and frozen when they entered one of four age groups: A1, 1–10 d; A2, 11–20 d; A3, 21–40 d; A4, 60–90 d. Cantharidin was extracted from each beetle and quantitatively analyzed by capillary gas chromatography[7].

As shown in figure 1, an individual T-larva (0.277 ± 0.021 mg, $\bar{X} \pm$ SEM) weighs as much as an unhatched egg (0.331 ± 0.046 mg). But as it feeds and molts (T-FG4 stages), the insect increases greatly in size until it enters diapause as a C-larva (160 ± 15 mg).

Subsequently its body weight remains relatively constant for nearly 8 months even though it undergoes two molts (C-SG-P stages). As a young adult, the A1 stage, it resumes feeding and quickly gains about 100 mg. Thereafter a beetle continues to feed for many weeks, the A2-A4 stages, yet its mass does not change significantly (263 ± 19 mg). No sexual difference in mass was detected.

Amounts of cantharidin increase tremendously in larvae as they progress through five feeding stages (T-FG4),

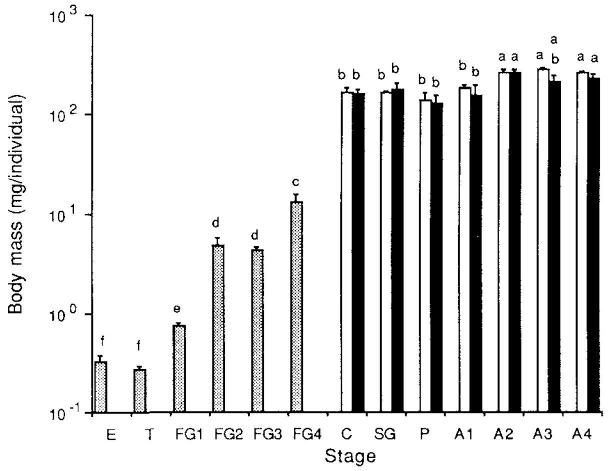

Figure 1. Mean (±SEM, N = 5) body mass of the life stages of unsexed (grey), male (white), or female (black) *Epicauta funebris*. Stage abbreviations are: E, egg; T, triungulin larva; FG1, first grub one larva; FG2, first grub two larva; FG3, first grub three larva; FG4, first grub four larva; C, coarctate larva; SG, second grub larva; P, pupa; A1, adult age 1–10 d; A2, adult age 11–20 d; A3, adult age 21–40 d; A4, adult age 60–90 d. Log transformed data met the assumption of equal variances. Rectangles with the same letter above are not statistically different from each other using Student-Newman-Keuls multiple range (SNK) test at the 0.05 level of significance[18].

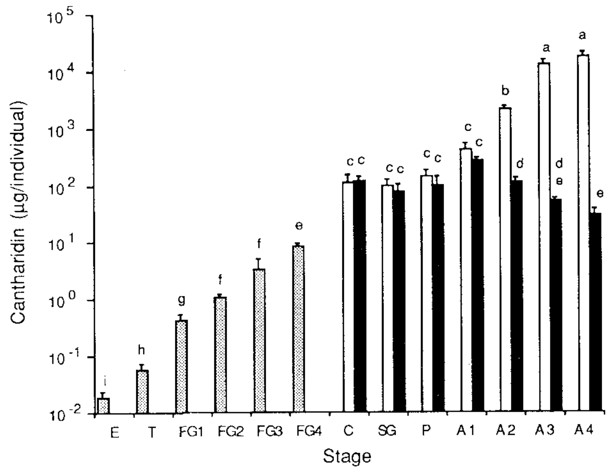

Figure 2. Mean (±SEM, N = 5) cantharidin content of the life stages of *Epicauta funebris*. Abbreviations of stages and statistical analysis are the same as in figure 1.

rising from 0.019 ± 0.005 μg in an egg to 110 ± 30 μg in a C-larva that has just entered diapause (fig. 2). Thereafter immature beetles in the SG and P stages have static amounts of cantharidin and there is no statistically significant difference between the sexes. But, as shown in figure 2, a profound sexual difference develops in adult beetles. Isolated adult males daily accumulate several hundred micrograms of cantharidin after they begin to feed, so at age 60–90 d (the A4 stage) they possess 16.9 ± 3.6 mg of the substance. We were astonished to find 30.3 mg cantharidin in one male (291 mg live weight). Because water comprises half the body mass in *E. funebris* adults[9], we estimate that cantharidin can amount to 20% of the dry matter in a male beetle. On the other hand, isolated adult females steadily lose cantharidin, so at age 60–90 d (A4 stage) they possess only 29.4 ± 8.3 μg of the toxin. These results dramatically confirm radiolabeling studies with *L. vesicatoria*[4] that demonstrate a sexual dimorphism in cantharidin biosynthesis in adult meloids.

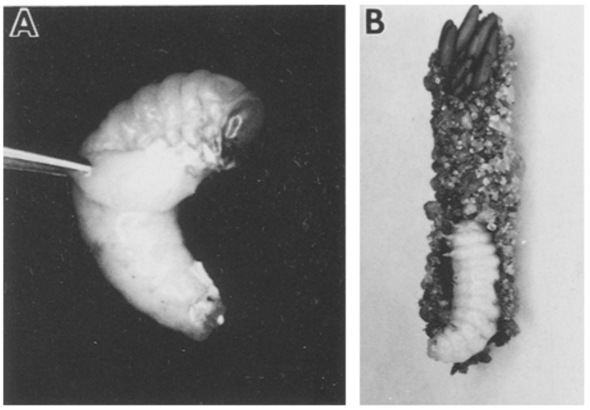

Figure 3. *A Epicauta funebris* FG4 larva, pinched with forceps, responding by regurgitating milky fluid onto its integument and the instrument. *B* Larva feeding within pod of grasshopper eggs. The egg pod has been cut open at both ends to expose the larva and uneaten eggs. Length of each larva is 13 mm.

Knowing that adult beetles deter attacks of many predators by discharging droplets of cantharidin-laden blood reflexively from their leg joints[10], we reasoned that meloid larvae might exhibit a similar defensive response. We discovered that feeding larvae (T-FG4 stages), instead of autohemorrhaging when pinched or poked, slowly rear their heads and emit milky fluid from the mouth that spreads to anoint the integument (fig. 3A). To test the idea that the regurgitated liquid contains cantharidin, we made chemical analyses of oral effluent collected from FG4 stage larvae by pinching them with forceps and placing pieces of filter paper over their mouths so as to trap the disgorged fluid. Not only is cantharidin present in the oral effluent, but the amount of this substance regurgitated by one larva (4.84 ± 4.61 μg/larva, N = 5) is equivalent statistically (Mann-Whitney test, $P > 0.05$) to the amount of toxin contained in an undisturbed grub (8.51 ± 1.00, N = 5). Storage of cantharidin by larvae in the alimentary canal was confirmed by analysis of tissues dissected from larvae (N = 3) frozen slowly to prevent regurgitation. Most (83%) of each grub's cantharidin is localized in the digestive system (8.13 ± 1.62 μg/larva), not in the rest of the body (1.70 ± 0.64 μg/larva). The presence of cantharidin in extracts of regurgitated fluid and larval tissues was confirmed by electron impact mass spectrometry[2]. We failed to detect cantharidin in fat body and rudimentary reproductive systems isolated by dissection from both male and female FG4-larvae. Microscopic examination of dissected specimens did not reveal any diverticular pouches or other storage organs in the alimentary canal. Although we have no information on enemies of immature meloids, it seems clearly advantageous if a larva feeding within a grasshopper egg pod in the soil, as depicted in figure 3B, when threatened physically would turn its body so as to deliver toxic effluent near the afflicted site, much as is done by other insect larvae that possess oral defenses[11].

To determine the internal distribution of cantharidin in adult *E. funebris*, virginal beetles (N = 13 of each sex) selected randomly at ages 2–88 d were frozen and dissected into three subsamples (male third pair of accessory glands or female spermatophoral receptacle, the rest of the reproductive system, and the body minus reproductive system), each of which was submitted to chemical analysis[7]. We found that males sequester cantharidin preferentially in the third pair of accessory glands (fig. 4A). After age 20 d (the equivalent of the A3 and A4 stages), approximately 80% of the substance in a male beetle reposes in these long, tubular structures that snake through the abdomen beside the gut. In contrast, the amount of cantharidin in the rest of a male's reproductive system and elsewhere in his body increases at a slower rate.

The site of cantharidin biosynthesis is not known. Oxygen labeling studies performed by us imply that the post-farnesyl steps in the cantharidin pathway occur outside the reproductive system, suggesting the accessory sex glands in *E. funebris* males may act more or less as a 'cantharidin kidney' to sequester this terpenoid[2]. In vitro biosynthetic studies designed to test this idea are in progress in our laboratory.

Cantharidin is lost by aging virgin females mostly from their somatic tissues (fig. 4B). The spermatophoral receptacle, ovaries, and remainder of the reproductive tract lose little of the toxin. This means that an old, virginal female not only has small cantharidin reserves, but also that levels of the defensive substance in her blood and other peripheral tissues are profoundly diminished. Therefore, with time isolated *E. funebris* males become more potent defensively whereas females tend to become defenseless, with the male's chemical armament exceeding that of the female by a ratio greater than 200:1.

Contrary to what one would expect from our results, both sexes of *E. funebris* in the wild have comparable amounts of cantharidin (164 ± 90 µg/beetle)[12]. This is because the female beetle is rearmed chemically during repeated matings. In each protracted copulation the male deposits a spermatophore containing many micrograms of cantharidin in her spermatophoral receptacle[2]. Afterwards the male replenishes his defensive reserves in a few days[2]. The fate of the male's deposit once it is inside the female's body is not fully understood, but about one-third of the donated cantharidin is quickly absorbed into her body fluids[2]. In *E. funebris* little cantharidin is deposited with the eggs, but other meloid species evidently endow their embryos with much of the toxin[13].

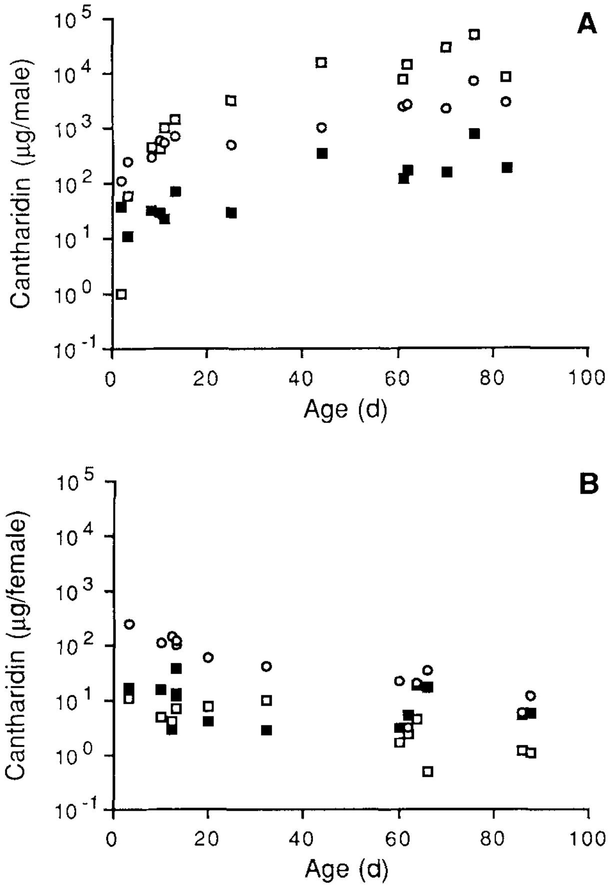

Figure 4. Distribution of cantharidin within *A* male and *B* female *Epicauta funebris* adults as a function of age when held in isolation. Three tissue samples are denoted for each individual: (□) male third pair of accessory glands or female spermatophoral receptacle, (■) the rest of the reproductive system, and (○) the body minus the reproductive system. Regression analysis[18] of log transformed data yielded the following results. *A*: □, $y = 2.040 + 0.0331x$ ($p < 0.025$, $R^2 = 0.629$); ■, $y = 1.351 + 0.0146x$ ($p < 0.01$, $R^2 = 0.771$); ○, $y = 2.394 + 0.0155x$ ($p < 0.001$, $R^2 = 0.857$). *B*: □, $y = 1.027 - 0.0116$ ($p < 0.01$, $R^2 = 0.668$); ■, $y = 1.036 - 0.0029$ ($p > 0.05$, $R^2 = 0.058$); ○, $y = 2.239 - 0.0161$ ($p < 0.01$, $R^2 = 0.777$).

Production of cantharidin in meloids is probably a physiologically expensive process. Although we know little about such costs, extensive studies of production of defenses against herbivores by plants[14] and insect sequestration of phytochemicals for defense[15] suggest that blister beetles should allocate defenses to maximize individual inclusive fitness. Selection may favor male blister beetles that not only biosynthesize and store much cantharidin, but those that effectively transfer the defensive chemical in the spermatophore to the female. Selection in females may be toward individuals that invest both physiologically in reproduction and behaviorally in choosing big males with abundant defensive reserves. Although instances of sexual size selection in meloid populations have been reported[16], our results with *E. funebris* suggest that females choose males randomly with regard to body size[17].

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2 McCormick, J. P., and Carrel, J. E., in: Pheromone Biochemistry, p. 307. Eds G. D. Prestwich and G. J. Blomquist. Academic Press, Orlando 1987.

3 McCormick, J. P., and Carrel, J. E., in: Biosynthesis and Metabolism of Secondary-Metabolite Natural Products, in press. Ed. R. Petroski, Plenum Press, New York 1992.

4 Schlatter, C., Waldner, E. E., and Schmid, H., Experientia *24* (1968) 994; Meyer, D., Schlatter, C., Schlatter-Lang, I., Schmid, H., and Bovey, P., Experientia *24* (1968) 955; Sierra, J. R., Woggon, W.-D., and Schmid, H., Experientia *32* (1976) 142; Peter, M. G., Thesis, University Zürich 1973.

5 Pinto, J. D., Univ. Calif. Publs Ent. *110* (1991) 186.

6 Berrios-Ortiz, A., Selander, R. B., J. Kans. ent. Soc. *45* (1972) 377; McCairel, M. H., Thesis, University Missouri-Columbia 1988.

7 Carrel, J. E., Doom, J. P., and McCormick, J. P., J. Chromat. biomed. Appl. *343* (1985) 411. Sensitivity was better than 0.25 μg cantharidin per sample.

8 Chippendale, G. M., and Cassatt, K., in: Handbook of Insect Rearing, p. 257. Eds P. Singh and R. F. Moore. Elsevier, Amsterdam 1985.

9 Carrel, J. E., Wood, J. M., Yang, Z., McCairel, M. H., and Hindman, E. E., Envir. Ent. *19* (1990) 1283.

10 Carrel, J. E., and Eisner, T., Science *183* (1974) 755; Carrel, J. E., Thesis, Cornell University 1971.

11 Eisner, T., Johnessee, J. S., Carrel, J. E., Hendry, L. B., and Meinwald, J., Science *184* (1974) 996; Morrow, P. A., Bellas, T. E., and Eisner, T., Oecologia *24* (1976) 193.

12 Blodgett, S. L., Carrel, J. E., and Higgins, R. A., Envir. Ent. *20* (1991) 776.

13 Carrel, J. E., Eisner, T., and McCormick, J. P., in preparation (1992).

14 Gulmon, S. L., and Mooney, H. A., in: On the Economy of Plant Form and Function, p. 681. Ed. T. J. Givnish. Cambridge University Press, Cambridge 1986; Fagerström, T., Larsson, S., and Tenow, O., Funct. Ecol. *1* (1987) 73, Fagerström, T., Am. Nat. *133* (1989) 281.

15 Duffey, S. S., A. Rev. Ent. *25* (1980) 447; Cohen, J. A., J. chem. Ecol. *11* (1985) 85; Rowell-Rahier, M., and Pasteels, J. M., J. chem. Ecol. *12* (1986) 1189; Scudder, G. G. E., Moore, L. V., and Isman, M. B., J. chem. Ecol. *12* (1986) 1171; Bowers, M. D., and Collinge, S. K., J. chem. Ecol. *18* (1992) 817.

16 McLain, D. K., Am. Midl. Nat. *107* (1982) 396; Snead, J. S., and Alcock, J., Evolution *39* (1985) 1123; Pinto, J. D., and Mayor, A. J., Ann. ent. Soc. Am. *79* (1986) 597; Alcock, J., and Hadley, N. F., J. Kans. ent. Soc. *60* (1987) 41; Brown, W. L., Anim. Behav. *40* (1990) 901.

17 Carrel, J. E., Breden, F., Carson, S., and Briesacher, S. L., in preparation (1992).

18 SAS Institute Inc., SAS User's Guide: Basics, 3rd Edn, p. 55. SAS Institute Inc., Cary, N. C. USA 1985; SAS Institute Inc., SAS User's Guide: Statistics, Version 5 Edition, p. 117. SAS Institute Inc., Cary, N. C. USA 1988.