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Authors: Davenport, Jason W., and Conner, William E.

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## Dietary alkaloids and the development of androconial organs in *Estigmene acraea*

Jason W. Davenport and William E. Conner

Department of Biology, Wake Forest University, Winston-Salem, NC 27109  
conner@wfu.edu

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### Abstract

Male salt marsh moths, *Estigmene acraea* (Lepidoptera: Arctiidae), possess inflatable androconial organs called coremata. Prior to mating males form aggregations and inflate their coremata en masse. The communal display attracts additional males and females for the purpose of mating. The coremata are known to carry the plant-derived dihydropyrrolizine, hydroxydanaidal. This pheromonal substance is derived from secondary plant chemicals called pyrrolizidine alkaloids found in the larval diet.

When *E. acraea* larvae were raised on semi-synthetic diets containing different levels of the pyrrolizidine alkaloid precursors the alkaloids triggered a pronounced morphogenetic effect. Adult males that fed on high levels of the pyrrolizidine alkaloid monocrotaline N-oxide (2500 µg) developed the largest coremata. Males that fed on lower levels of monocrotaline N-oxide (500 µg) or no alkaloid, while normal in body weight, had coremata that were progressively smaller and less robust. The size of the coremata and their commensurate pheromonal charge may have behavioral consequences in the unusual mating system of this species.

### Introduction

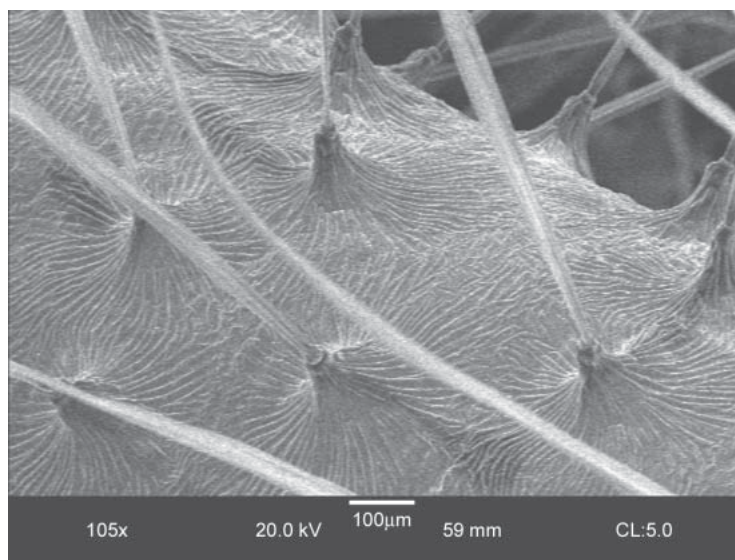
Androconial organs are common in moths and play an important role in their courtship (Birch, 1974, 1979; Phelan and Baker, 1987; Birch *et al.*, 1990). In tiger moths (Lepidoptera: Arctiidae) they often take the form of impressive inflatable tubes projecting from the ventral surface of the abdomen or from the genital valves (Birch and Hefetz, 1987; Hauser and Boppré, 1997; Weller *et al.*, 1999). In the tiger moth *Estigmene acraea* the coremata are composed of two medially curved, air-filled tubes that extend ventro-laterally from the intersegmental membrane between the seventh and eighth abdominal segments (Figure 1a). When fully inflated they are sparsely set with elongate scent scales projecting perpendicular to their surface (Figure 1b). The coremata of *E. acraea* have been shown to carry the dihydropyrrolizine, hydroxydanaidal (Figure 2a; Krasnoff and Roelofs, 1989), a chemical that is known to have pheromonal activity in some arctiid species (Conner *et al.* 1981; Davidson *et al.*, 1997; Iyengar *et al.*, 2001). Females of *Utetheisa ornatrix* prefer to mate with males with high levels of hydroxydanaidal, which provides information on the quality of the male as a mate (Iyengar *et al.*, 2001). The presence of hydroxydanaidal has been linked to the larval ingestion of dietary pyrrolizidine alkaloids (PAs) in *Utetheisa ornatrix* (Conner, 1981), *Cretonotus gangis* (Boppré and Schneider, 1985), *Phragmatobia fuliginosa*, *Pyrrharctia isabella*, and *Estigmene acraea* (Krasnoff and Roelofs, 1989). PAs are bitter tasting and hepatotoxic secondary chemicals that are thought to protect more than 300 plant species in

11 families from herbivory (Mattocks, 1986; Hartmann and Ober, 2000). Paradoxically, PAs have been shown to be phagostimulatory for the specialist feeder *Utetheisa ornatrix* (Bogner and Eisner, 1991) and some generalist feeders such as *Cretonotus gangis* (Boppré, 1990) that sequester the alkaloids. For *Utetheisa* the ingested alkaloids protect the larvae, pupae, and adult stages from predation (Eisner *et al.*, 2000 and references therein).

Coremata are deployed in either of two ways, depending on the nature of the mating system. In most species the coremata



**Figure 1.** Coremata of *Estigmene acraea*: A. Ventral view of inflated coremata of a field-collected male.



**Figure 1.** Coremata of *Estigmene acrea*: B. Scanning electron micrograph illustrating the elongate scales projecting from the surface of a fully inflated corema.

are inflated for a brief period just prior to copulating (Birch, 1974). In *Cretonotos gangis* and *C. transiens*, Asian arctiids, and *Estigmene acrea*, a species common in the New World, males congregate in the early evening and inflate their coremata for extended periods (Wunderer et al. 1986; Willis and Birch 1982). This behavior has been referred to as lekking (Willis and Birch, 1982; Alcock, 2000). Both males and females are attracted to the leks. Males join in the display, and females mate with the males. Boppré and Schneider (1985) demonstrated a remarkable morphogenetic effect of PAs in *Cretonotos*: the amount of PA

ingested by the larva determines the size of the adult's coremata; the more PA ingested, the larger the scent organ. The behavioral, ecological, and morphological similarities between *Cretonotos* and *Estigmene* suggested the possibility that *Estigmene* might exhibit a similar PA-dependent coremata development.

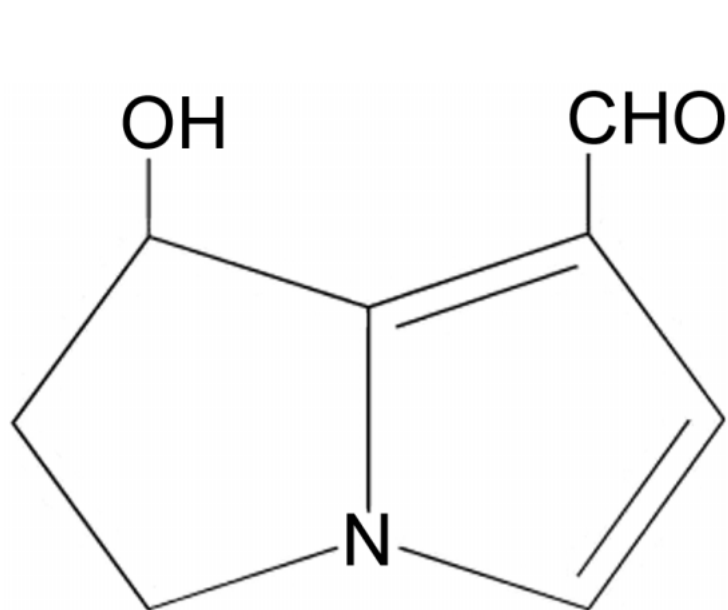
## Materials and Methods

### Insects

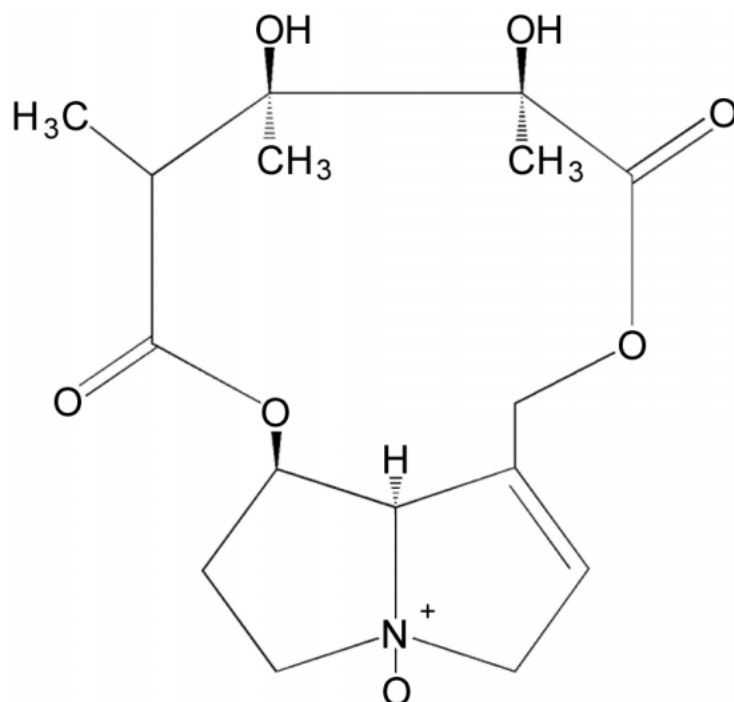
The adult *Estigmene acrea* examined in this experiment came from our laboratory culture, which was started with eggs collected near the Bonnet Carre Spillway in St. Charles Parish, Louisiana. Larvae were fed on a commercially available salt marsh caterpillar diet (Bioserv Inc., Frenchtown, NJ). The larvae used in this experiment were from the third generation of moths reared in our laboratory, none of which had previous exposure to PAs. This is important because PAs can be passed between generations via the eggs (Boppré and Schneider, 1985; Eisner *et al.*, 2000). The larvae, pupae, and adult moths were maintained at room temperature in continuous light. The larvae were raised in petri dishes (90 mm in diameter, 16 mm high) with between 1 and 10 larvae per dish depending on their size.

### PA feeding

All larvae were raised under the same conditions until they reached their final larval instar. On the first day after molting into the final larval instar they were provided with the pyrrolizidine alkaloid monocrotaline N-oxide (Figure 2b). One experimental group (PA+) received 500 µg of monocrotaline N-oxide per individual. A second experimental group (PA5+) was offered 2500



**Hydroxydanaidal**



**Monocrotaline N-oxide**

**Figure 2.** A. Hydroxydanaidal.

**Figure 2.** B. Monocrotaline N-oxide.



µg. The control group (PA-) received diet containing no alkaloid. Monocrotaline N-oxide was mixed into the diet so that a milliliter block of diet contained 500 µg. The PA+ group was given one block containing monocrotaline and four blocks of alkaloid-free diet. The PA5+ group was given five blocks of the monocrotaline N-oxide diet, and the control group received five blocks of the control diet. After the blocks of diet were consumed, each larva was placed back on PA-free diet until it pupated. The levels of alkaloid made available to these insects are comparable to those used in previous experiments with *Cretonotus* (Boppré and Schneider, 1985) and to levels readily available to larval arctiids fed on their natural hostplants (Nickisch-Rosenegk and Wink, 1993).

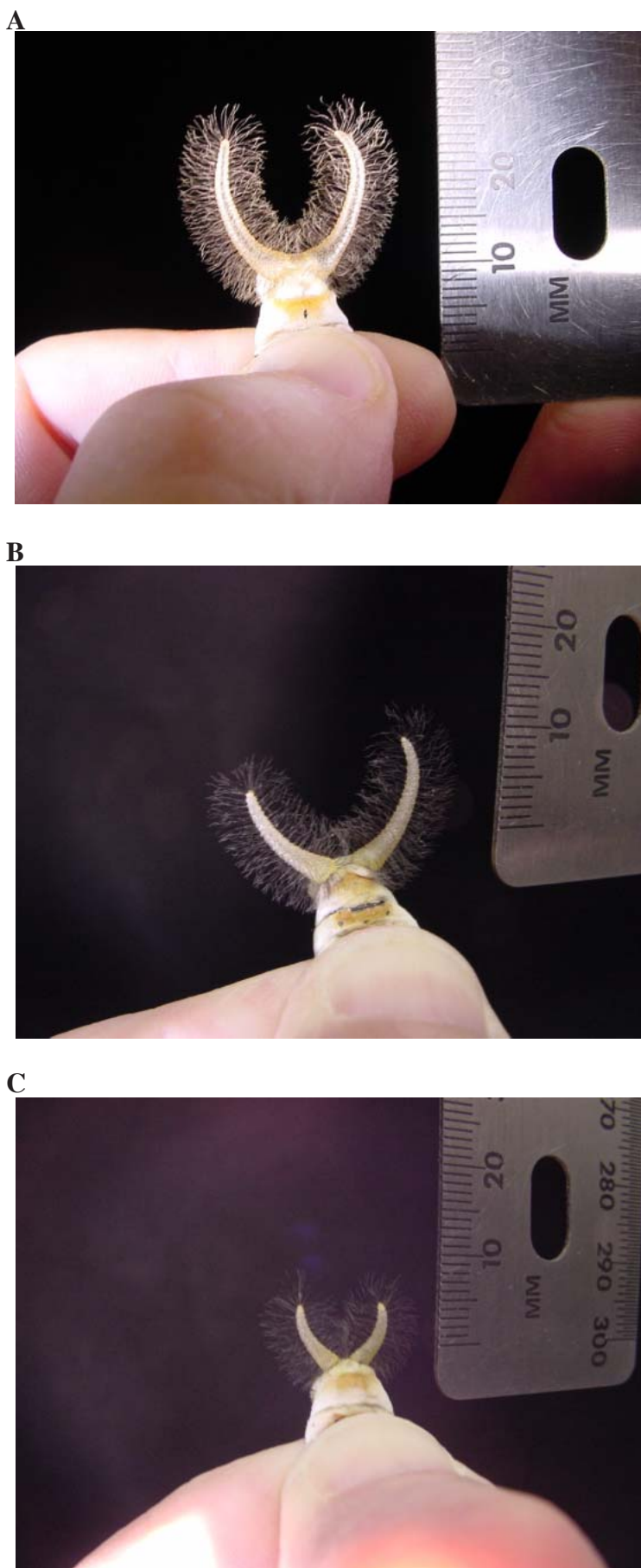
#### Coremata measurements

Upon eclosion into an adult, each male *Estigmene* was placed in a plastic cup (1 oz). Two days after eclosing the moths were weighed and the coremata were examined. The coremata were obtained by removing the abdomen of the freshly killed moth with scissors. The needle of an air-filled 10 ml syringe was inserted into the anterior cut end of the abdomen and ligated in place. Air was gently forced into the abdomen to inflate the coremata. If necessary, additional pressure was applied by pinching the abdomen. The coremata were deemed fully inflated when all of the coremata scales had separated at the distal tips of the coremata. At this level of inflation the coremata were promptly photographed side by side with a calibration scale.

Digital images of the coremata were enlarged and measurements were obtained from the images. The overall length of a coremata was measured from the distal tip of the structure, along the centerline, to the middle of the arch that marks the proximal end of the coremata tube. Scale length was determined by measuring from the outer edge of the coremata to the tip of the three longest hairs. Width of the coremata was obtained by measuring the width of the coremata 2 mm from the proximal end of the coremata branch. Both the left and right coremata tubes were measured for all criteria. The mean of the two tubes was then calculated and used in all further comparisons. All measurements were taken in a double blind fashion. Statistical analyses were carried out using SPSS for Windows Release 11.0.1 (SPSS Inc.) or Statistica Kernal Release 5.5 (StatSoft Inc.). All data are presented as means  $\pm$  one standard deviation.

#### Results

The effects of the different levels of PAs ingested were readily apparent when the coremata were inflated (Figure 3). There was a clear positive effect of the level of PA ingested on the size of the coremata (Table 1). An ANOVA comparing the overall length of the coremata was significant ( $p < 0.001$ ). The PA5+ coremata were 25% longer than the PA+ coremata, which in turn were 23% longer than the PA- coremata (Duncan Multiple Range Test,  $p < 0.01$ ). A positive PA treatment effect was also found for the width of the coremata tubes and scale length (ANOVA,  $p < 0.01$  in both cases). The coremata width of the PA5+ group was significantly larger than the corresponding measurement in the PA- group (Duncan Multiple Range Test,  $p < 0.01$ ). Neither the PA5+ group nor the PA- group was significantly different from the PA+ group in coremata width. Scale length was significantly different between the PA5+



**Figure 3.** Effects of larval ingested monocrotaline N-oxide on coremata development in *Estigmene acraea*. (top) PA5+ male, (middle) PA1+ male, (bottom) PA- male. All digital photos are adjusted to the same magnification.

group and the PA- group (Duncan Multiple Range Test,  $p<0.05$ ), but the PA- and PA5+ groups were not significantly different from the PA+ group ( $p>0.05$ ; Table 1).

An ANCOVA comparing corematal length to the log of the body mass (fresh weight) with the PA level as a factor (recommended by one reviewer) showed a strong effect of PA feeding on corematal length ( $p<10^{-6}$ ) with no significant effect of body mass on corematal length ( $p=0.60$ ) indicating that PAs are not influencing the size of the coremata indirectly through an effect on body mass. In fact there is a trend towards small body masses with the highest level of PAs (Table 1) which suggests a possible tradeoff between body mass and corematal size.

The log of the body mass data easily met the assumption of parallelism for the ANCOVA and came very close to meeting the assumption of homogeneity of variances (Cochran's C,  $p = 0.044$ ). This small deviation from the assumptions should not influence the robustness of the overall levels of significance or our conclusions (Sokal and Rohlf, 1995).

Discussion

The levels of alkaloid fed in this experiment span the range readily available to arctiids from their natural hostplants (Rothschild et al., 1979; Nickisch-Rosenegk and Wink, 1993; Trigo et al., 1993). The ingested PA monocrotaline N-oxide had a clear effect on the ontogenetic trajectory of the coremata in the *Estigmene acrea* males that we studied. The results of the ANCOVA indicate that the effect was specific to the corematal tissue and was not a function of overall body mass. Others who have studied *Estigmene acrea* have not seen the effect of PAs on corematal development that we report. Willis and Birch (1982), for example, stated, “*E. acrea* shows no readily discernible natural variation in the size of male coremata with diet...” They presented no quantitative data to support this statement, and it is possible that all of the males in their study had roughly equal access to PAs either from their larval feeding or passed to them from their mothers. Krasnoff and Roelofs (1989) likewise reported that PAs had no effect on the males of *Estigmene acrea*. Like Willis and Birch, however, they presented no supporting data, so it is difficult to evaluate their claim. It is also possible that the morphogenetic effect varies across populations that were different in all three studies.

The molecular mechanism by which the PAs exert their

morphogenetic effect is unknown. In *Cretonotus*, which shows a similar effect at comparable PA concentrations, high titers of ecdysone in the pupal stage are essential for corematal development regardless of the level of PAs available to larvae (Schmitz et al., 1989). This ecdysone effect is expected since the coremata are derived from specific larval anlage, the commitment and development of which are frequently stimulated by ecdysone in insects (Nijhout, 1994). How PAs modulate the development of the imaginal disks is not clear.

The specific attractant released by the coremata of *Estigmene* is unknown as well, but comparison with related arctiids indicates that it is likely that it is PA-derived, and if not hydroxydanaidal, a related compound. We have found the level of hydroxydanaidal in the coremata of *Estigmene acrea* to be highly correlated with the amount of PA in the larval diet (Jordan, Jones, and Conner, unpublished). Our findings suggest that the overall size of the coremata, and thus the surface area for pheromonal release, is greater in males with access to PAs. The increased surface area combined with the greater amounts of pheromone should affect the strength of a pheromonal signal downwind of a male. If this is so, it could theoretically provide a mechanism by which females could choose males with high levels of PAs from a considerable distance downwind of the lek. Males so chosen could provide the female with a spermatophore rich in alkaloids for her protection and the protection of her offspring, as *Utetheisa ornatrix* males have been shown to do (Dussourd et al., 1991 ; González et al., 1999). Males with large coremata may produce more attractive pheromonal signals and serve as foci for lek formation. Less well-endowed males may join leks to combine their signals with those of more attractive males or to intercept incoming females. These aspects of the courtship of *Estigmene* are speculative and remain to be investigated.

*Estigmene acrea* is common throughout much of the United States (Covell, 1984; Ferguson, 2000) and is occasionally of economic importance (Metcalf and Metcalf, 1993). Although it is considered a generalist feeder its list of foodplants include some species that are know to contain PAs (Tietz, 1972). The strong morphogenetic effect of pyrrolizidine alkaloids on corematal development in this species argues that PAs play a critical role in the reproductive biology of this insect. In keeping with this *E. acrea* has recently been shown to have a preference for PA-containing food – a preference that is likely mediated by PA receptors located on the larval galea (Bernays et al., 2002a,b). Because of its ready availability and extraordinary reproductive physiology *Estigmene acrea* is an excellent model for the study of chemically mediated lekking behavior in insects.

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**Table 1.** Effect of ingested PAs on the dimensions of the coremata in *Estigmene acrea*. All values shown are means  $\pm$  standard deviations (n = 10).

	PA-free	500 $\mu$ g (PA+)	2500 $\mu$ g (PA5+)
Corematal length (mm.)	12.2 $\pm$ 1.9 <sup>a</sup>	15.0 $\pm$ 1.7 <sup>b</sup>	18.8 $\pm$ 2.0 <sup>c</sup>
Corematal width (mm.)	2.1 $\pm$ 0.1 <sup>a</sup>	2.4 $\pm$ 0.3 <sup>ab</sup>	2.9 $\pm$ 0.3 <sup>b</sup>
Scale length (mm.)	3.6 $\pm$ 0.4 <sup>a</sup>	3.8 $\pm$ 0.3 <sup>ab</sup>	4.2 $\pm$ 0.3 <sup>b</sup>
Body mass* (mg.)	417 $\pm$ 60 <sup>a</sup>	420 $\pm$ 68 <sup>a</sup>	392 $\pm$ 26 <sup>a</sup>

All values are expressed as means  $\pm$  standard deviation (n = 10 in each case)

<sup>a</sup>Means sharing a superscript within a row are not significantly different from each other at  $p<0.05$  using a Duncan Multiple Range Test. See text for exact p values.

\* Measured as fresh weight

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