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Authors: Jordan, Alex T., Jones, Tappey H., and Conner, William E.

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If you've got it, flaunt it: Ingested alkaloids affect corematal display behavior in the salt marsh moth, *Estigmene acrea*

Alex T. Jordan, Tappey H. Jones[1], and William E. Conner

Department of Biology, Wake Forest University, Winston-Salem, North Carolina 27106, USA
[1]Department of Chemistry, Virginia Military Institute, Lexington, Virginia 24450, USA
jordat1@wfu.edu

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Abstract

Plant-derived pyrrolizidine alkaloids play an important role in the biology of the salt marsh moth, *Estigmene acrea* (Lepidoptera: Arctiidae). They are phagostimulants for larvae and they stimulate the growth and development of adult male androconial organs called coremata. In this study, we have shown that the pyrrolizidine alkaloid monocrotaline N-oxide (MNO) fed to larvae also affects the courtship behavior of adult males. Males fed a diet containing MNO display their coremata while males fed on the same diet without alkaloid rarely display. This explains why it has been difficult to replicate field observations of the "lekking" behavior of this species in the laboratory where animals are frequently raised on commercially available diets devoid of alkaloids. Corematal inflation was observed in isolated males and in laboratory leks. The effect of larvae feeding on pyrrolizidine alkaloid on the reproductive behavior of adults suggests that this substance may modify the development of the moth's nervous system and contribute to their unusual dual mating strategies. MNO was also shown to be an adequate precursor for the production of the courtship pheromone hydroxydanaidal.

Keywords: Arctiidae, courtship pheromone, hydroxydanaidal, lek, pyrrolizidine alkaloid

This paper includes one video that can be accessed at http://insectscience.org/5.1

Introduction

Pyrrolizidine alkaloids (PAs) are plant allelochemicals based on the bicyclic nitrogen-containing pyrrolizidine ring (Figure 1a). Numerous plant species in the families Asteraceae, Boraginaceae, Fabaceae, and Orchidaceae contain significant concentrations of toxic PAs in their foliage, roots, and seeds that protect them from generalized herbivory. PAs are also found sporadically within the Apocynaceae, Celastraceae, Ranuculaceae, Rhizophoraceae, Santalaceae, Sapotaceae, and Convolvulaceae (Mattocks 1986; Hartmann and Witte 1995; Hartmann and Ober 2000). Some herbivorous insects, including many tiger moths (Arctiidae), can tolerate PAs and even sequester them (Conner et al. 1990; Nickisch-Rosenegk and Wink 1993). The stored chemicals protect their bearers from predators and parasites (Eisner and Eisner 1991; Hare and Eisner 1993; Cardoso 1997; Eisner et al. 2000; Eisner and Meinwald 2003; Singer et al. 2004) and serve as precursors to male courtship pheromones (Conner et al. 1981; Schultz et al. 1993).

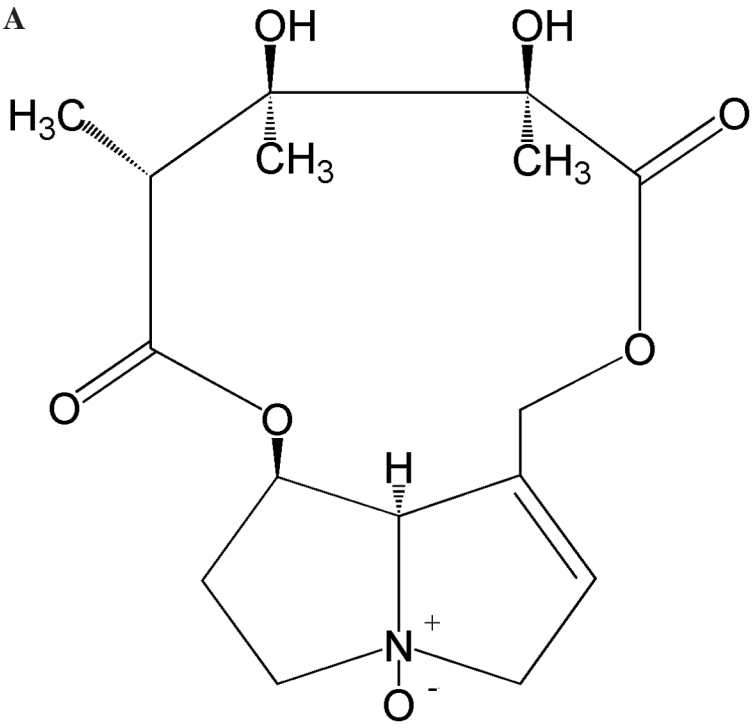
The reproductive biology of the polyphagous salt marsh tiger moth *Estigmene acrea* is closely tied to pyrrolizidine alkaloids found in some of their host plants. The larval stages have taste receptors specific for these compounds and display feeding preferences for diets containing them (Bernays et al. 2002a, 2002b). Larvae sequester the plant alkaloids and convert them to insect-specific forms (Hartmann et al. 2003). Adult males have coremata, that are inflatable androconial organs whose full development requires the presence of PAs in the larval diet (Davenport and Conner 2002). Males likewise produce courtship pheromones that are thought to be alkaloidal in origin (Krasnoff and Roelofs 1989; Hartmann et al. 2003).

The mating system of *Estigmene acrea* apparently involves two distinct strategies. Willis and Birch (1982) describe aggregations of males at specific sites—leks—where they gather early in the evening and collectively evert their impressive bifid coremata for extended periods. Pheromones released by the coremata apparently attract both additional males and females for the purpose of mating (Willis and Birch 1982). Later in the evening the system reverts to a more typical female pheromone system in which the females release a hydrocarbon sex attractant (Hill et al. 1981) and males fly upwind to find and mate with them.

We here present evidence that corematal eversion can be studied in solitary males under laboratory conditions, that the male courtship pheromone hydroxydanaidal can be produced from larvally ingested monocrotaline N-oxide, and, most importantly, that the larvally ingested PAs affect the behavioral repertoire of adult *Estigmene acrea* males in ways consistent with the alternative mating strategies described by Willis and Birch (1982).

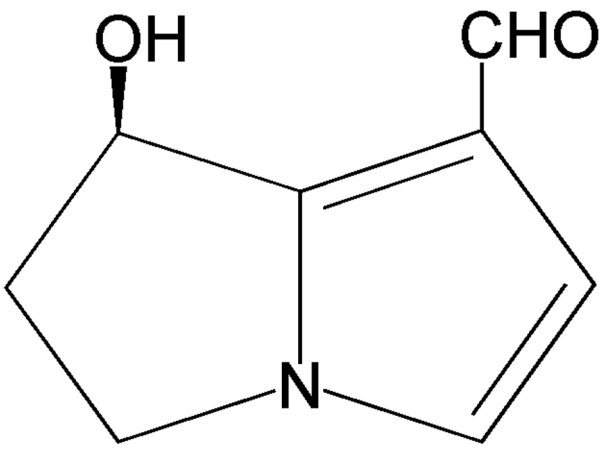
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A



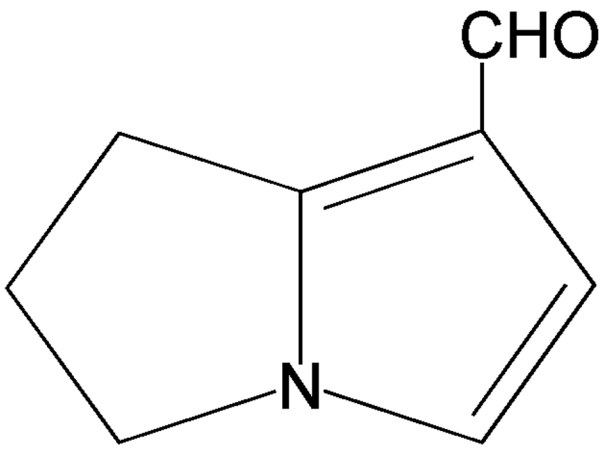
Monocrotaline N-oxide

B



Hydroxydanaidal

C



Danaidal

Figure 1. a (left top). Monocrotaline N-oxide. **b (left bottom)**. hydroxydanaidal. **c (above)**. danaidal

Methods and Materials

Insects and alkaloid

Eggs of *E. acrea* were obtained from Drs. Michael Singer and Elizabeth Bernays in the Department of Entomology at the University of Arizona in Tucson. These came from a laboratory colony originally collected in St. Charles Parrish, Louisiana. PA-free (PA−) larvae were fed *ad lib* on commercially available Salt Marsh Caterpillar Diet (BioServ, www.bio-serv.com). PA-supplemented (PA+) larvae were fed the same diet but were also offered 1 ml of diet containing either 0.5 mg or 2.5 mg of the pyrrolizidine alkaloid monocrotaline N-oxide when they entered their final larval instar. The alkaloid was obtained from the Fairfield Chemical Company, Blythewood, South Carolina, and was certified 99% pure.

Behavioral measurements

Larvae, pupae, and adults were exposed to a 16hr light: 8 hr dark photoperiod. Newly eclosed adults were placed in transparent 16 oz plastic containers. The behavior of individuals of each sex and chemical background (20 PA+ males, 20 PA- males, 12 PA+ females and 12 PA- females) was viewed and recorded with an infrared sensitive video camera (Sony Model No. CCD-TRV65). Illumination was provided by an infrared light-emitting diode standard on the NightShot® capable camera. Adults were observed both singly in their plastic containers, in groups in large screened cages and outdoors in screened tents to simulate more natural conditions. Inflation of coremata were scored as all or none at one half hour intervals.

Chemical methods

The coremata of male *E. acrea* were everted by injecting their severed and ligated abdomens with air from a 1 ml disposable syringe. The inflated coremata were quickly snipped off and placed in a glass vial. For chemical analysis the coremata were extracted

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in a minimal amount of methylene chloride. Samples contained the coremata of 10 pooled individuals.

Gas chromatography-mass spectrometry was carried out in the EI mode using a Shimadzu (www.shimadzu.com) QP-5000 GC/MS equipped with a RTX-5, 30 m × .25 mm i.d. column. The instrument was programmed from 60 °C to 250 °C at 10°/min. Under these conditions, the dihydropyrrolizine hydroxydanaidal eluted at 15.3 min.

Results

Description and timing of behavior

Isolated virgin males (PA+) inflated their coremata with regularity. Initial inflation took several seconds, and some males pumped (inflated and deflated) their coremata repeatedly during an inflation bout (Video 1 available at http://www.insectscience.org/5.1/). The mean onset of inflation was 1.5 hours after the onset of the scotophase, and most males had ceased inflation 3 hours later. Males initiated inflation behavior on Day 2 after eclosion and continued in a nightly fashion through Day 6 (Figure 2a) when the experiment was terminated.

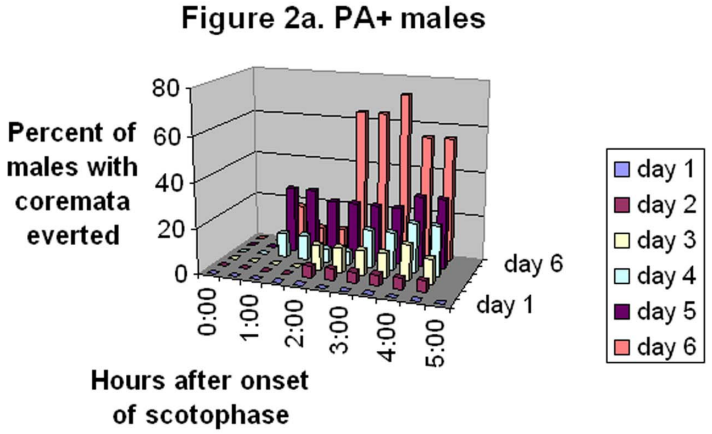
Isolated virgin females displayed typical female calling behavior–rhythmic exposure of the papillae anales and the openings of their sex pheromone glands (MacFarlane and Earle 1970; Conner et al. 1985). The mean onset of calling 1.5 hours after the onset of the scotophase was not significantly different from that of the males (Friedman ANOVA, Chi-Square = 0.333, df. 1, p = 0.564). Most females stopped calling approximately 3 hours later (Figure 3a).

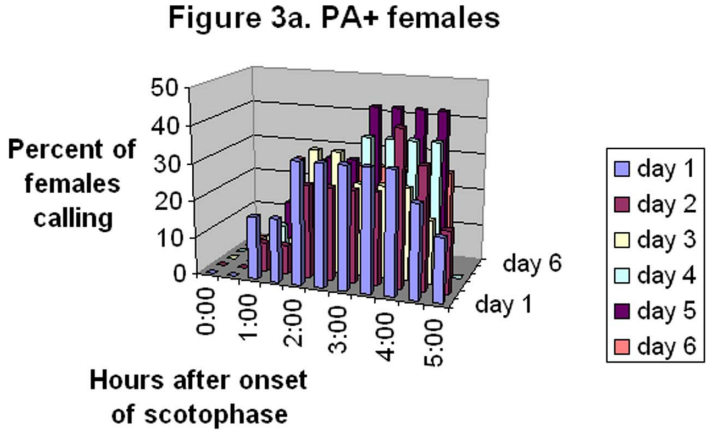
Chemistry dependence on alkaloid

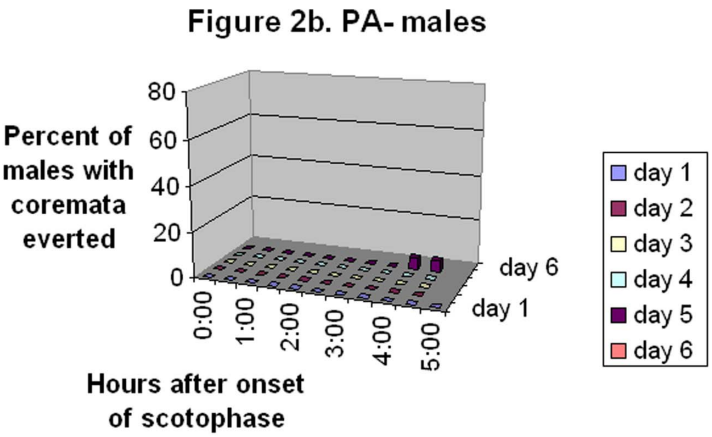
The coremata of males reared on the diet with 0.5 or 2.5 mg of monocrotaline N-oxide produced two known dihydropyrrolizines: hydroxydanaidal (Figure 1b) was the major component along with trace amounts of danaidal (Figure 1c, 4a). Their mass spectra were compared with that of an authentic sample of 7R (−) hydroxydanaidal. A third related but as yet uncharacterized alkaloid derivative was also detected as a trace component in some samples (not shown). Males raised on the diet devoid of pyrrolizidine alkaloid produced no detectable dihydropyrrolizines in their coremata (Figure 4b). All compounds with a retention time greater than 19 minutes were fatty acids and their methyl esters typical of insect samples.

Behavioral dependence on alkaloid

Males that had been fed the larval diet supplemented with monocrotaline N-oxide (2.5 mg) regularly inflated their coremata







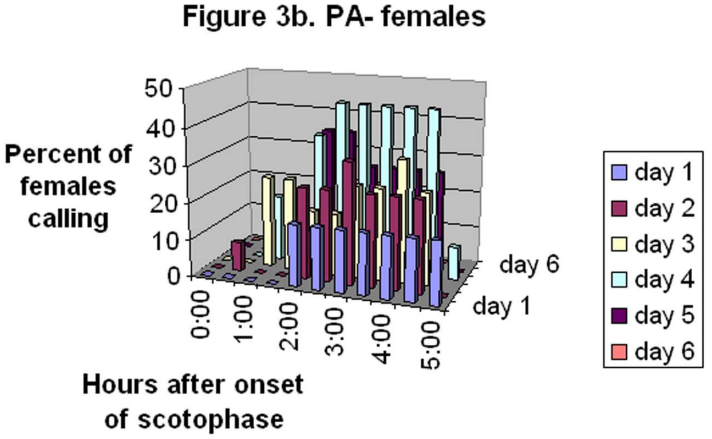


Figure 2. a. Timing of corematal inflation in isolated PA-supplemented (2500 µg) males (n = 20). b. Timing of corematal inflation in isolated PA-free males (n = 20).

Figure 3. a. Timing of calling behavior in isolated PA-supplemented (2500 µg) females (n = 20). b. Timing of calling behavior in isolated PA-free females (n = 20).

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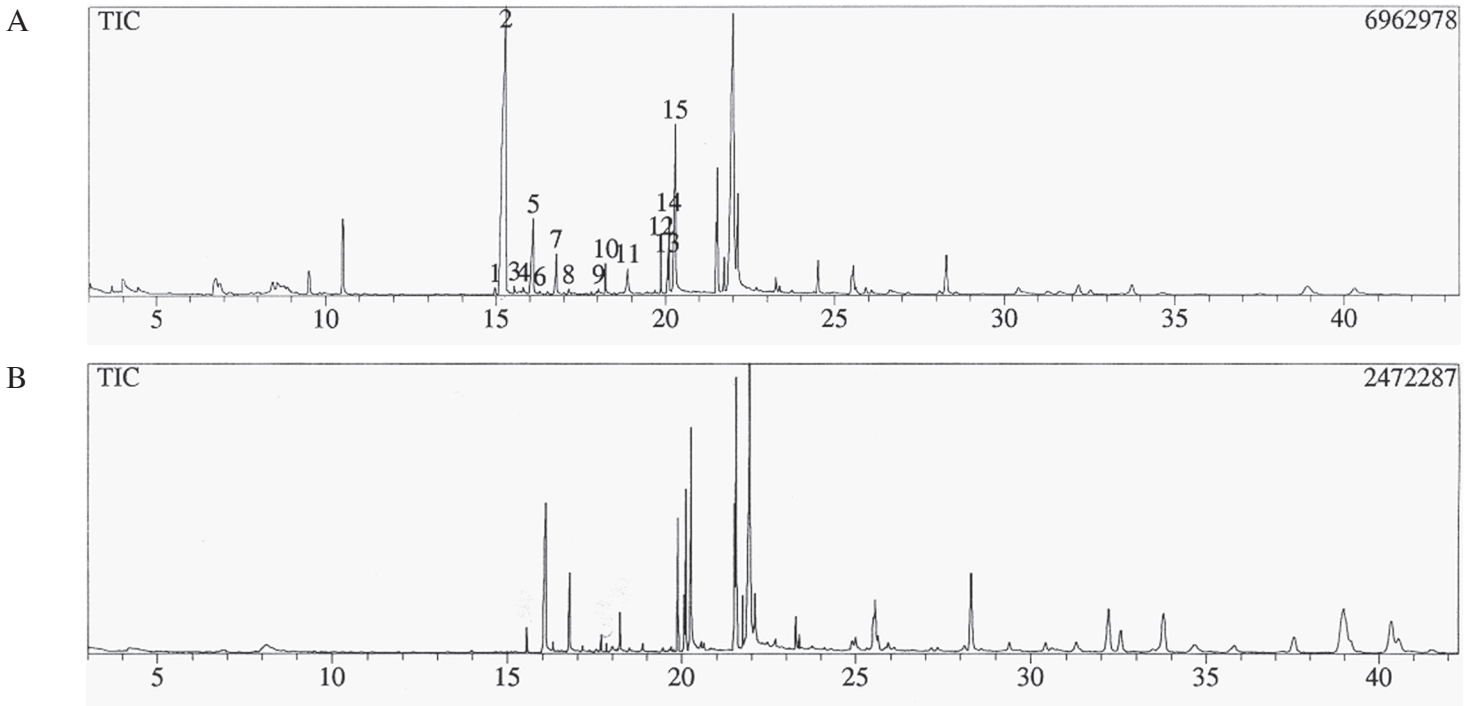


Figure 4. a. Gas chromatographic trace of corematal extract from 10 PA+ (500 µg) males. Peak number 2 = hydroxydanaidal. Peak number 1 = danaidal. b. Gas chromatographic trace of corematal extract from 10 PA-free males. Note that peaks with retention times near 15 minutes are missing.

(Figure 2a). Males deprived of the alkaloid (PA−) inflated their coremata only rarely (Figure 2b). A Friedman ANOVA showed this difference to be significant (Chi-Square = 5.0, df. = 1, p = 0.025). PA+ animals also showed a correlation between age and willingness to inflate the coremata, with older males being more prone to inflation (Spearman Correlation, $p < 0.01$). In contrast, PA- males did not show a significant age effect.

In the tent and large cages, PA+ males everted their coremata individually or in small groups. In one case eversion by a male appeared to stimulate synchronous eversion by a second male. In the tent we observed 5 PA+ males and 5PA- males in the presence of 5 PA- females. We replicated the experiment 4 times. PA+ males mated on four occasions and all did so by attracting females to their corematal display. 3 PA- males mated and in all cases they were attracted to calling females. Using a Fisher Exact Probability Test this is result is significantly different from random (p = 0.028). It should be noted that all females used for these observations were PA-.

The calling behavior of female *E. acrea* did not depend on their PA background (Friedman ANOVA; Chi-Square = 1.0, df. = 1, p = 0.317), and there were no detectable differences between PA+ and PA− females in the onset or daily duration of calling (Friedman ANOVA, Chi-Square = 0.0, df = 1, p = 1.0) (Figures 3a and 3b).

Discussion

The courtship behavior of *E. acrea* under laboratory conditions was identical with that described by Willis and Birch (1982) in all but one respect. Willis and Birch found that the males formed leks early in the evening and that females called significantly later, while the calling periods of the males and females in our study overlapped. This difference may be the result of strain differences (California vs. Louisiana), differences in methods (e.g., gradual photoperiodic transitions in nature vs. abrupt photoperiodic transitions in the lab), or genetic differences arising from laboratory rearing.

The effect of pyrrolizidine alkaloids on the development of the pheromone disseminating structure, on the production of the pheromone itself, and now on the behavior associated with pheromone dissemination, is consistent with the two alternative mating strategies observed by Willis and Birch (1982). Although more observations need to be made we speculate that males that consume alkaloids during their larval development may utilize a strategy that involves attracting females to them. Males that do not find an alkaloid-containing plant as larvae revert to a more primitive strategy in which they compete for females by following a pheromone plume to their mates. This was what we observed in our simulated field conditions (the outdoor tent). Males with PAs exposed their coremata and attracted females to them, and males deprived of alkaloid did not expose their coremata and mated by pursuing females.

How did this dual strategy arise? There are several possible scenarios. The lek-based strategy may be an evolutionary legacy dating from a time when *E. acrea* was a specialist feeding only on PA-containing plants (Krasnoff and Roelofs 1990). There is evidence that some polyphagous arctiine arctiids evolved from specialist feeders (Weller et

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al. 1999). Or the lek strategy may have evolved as a form of sex role reversal. If the nuptial gifts offered by males to females in spermatophores (Dussourd et al. 1988) are sufficiently large, one would predict a shift in the mating systems from females attracting males to males attracting females (Gwynne and Simmons 1990). Finally alkaloid-finding males may have evolved a new strategy for competing for females by tapping into females' preexisting propensity to respond to alkaloid derivatives in their hostplants (Grant et al. 1989; Bogner and Boppré 1989). Additional experiments will be necessary to differentiate among these hypotheses.

We are confident that the effects of PA diet on corematal eversion are truly behavioral. The coremata of PA-deprived males, albeit small, have the same functional connections to the tracheal system as those of PA-laden males, and they can be manually inflated with the same pressures. The effects of alkaloids on the mating behavior of *E. acrea* have interesting developmental implications. In addition to altering the developmental blueprint of the coremata, pyrrolizidine alkaloids may also influence the development of the nervous system in ways that influence reproductive behavior. We do not know at what level (sensory, integrative, or motor) this effect is mediated. In another context PA background has been shown to shape sensory responses. The taste receptors of larval *E. acrea* reared in the absence of PAs are much less sensitive to PAs than those of larvae fed a PA-supplemented diet (Chapman et al. 2003). Perhaps PAs stimulate a similar shift in the physiology and behavior of adults. Whatever the mechanism, it is clear that the relationship between pyrrolizidine alkaloids and tiger moths like *Estigmene* is even more intimate than previously imagined.

Acknowledgments

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