On the evolutionary arms-race between the moth *Utetheisa ornatrix* (Erebiidae: Arctiinae) and its Florida host, *Crotalaria pumila* (Fabaceae): chemical attraction and mechanical defense

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Abstract: While *Utetheisa ornatrix* larvae are able to develop through feeding only on the foliage of their hostplants in the genus *Crotalaria*, in later instars they are attracted to seeds, which are a richer source of alkaloids. Recently, it was demonstrated that seeds receive different degrees of mechanical protection from the larvae as provided by the surrounding pericarps. In the present paper we demonstrate that pods of *Crotolaria pumila*, a host native to the moth's range, attract larvae away from the foliage, which in turn slows down their development as they expend time and energy on breaking through the pericarps, instead of feeding. Hence, in this closer-to-natural scenario, gaining extra alkaloids through seed-feeding with its many demonstrated advantages for the adult moths can also place negative selective pressure on caterpillars. In *Crotalaria pumila*, the pods are small and scattered, so to understand how larvae locate them on a plant, we analyzed the volatile chemicals that are produced by these pods and compared them to those produced by the foliage and by pods of three other (non-native) *Crotalaria*: *C. spectabilis*, *C. lanceolata* and *C. pallida*, which are also widely utilized by the moths as hosts in Florida. The volatiles coming from the pods proved to comprise a much more diverse and complex mixture of chemicals than those coming from the foliage. Pods of *Crotalaria* species we tested produced species-specific chemical profiles, yet they had several compounds in common: <Propane, 2-methyl-1-nitro->, <(E)-beta-ocimene>, <Hexenyl acetate 3E->, <para-Ethyl acetophenone>, <4-Methyl-2-pentanone oxime>, <2-Methylpropanal oxime>, and <Indole>. It is likely that one of these compounds or a combination of several of them are cues that are used by larvae while searching for pods.

Key words: chemical ecology, plant defenses, trophic interactions, co-evolution, predation and parasitism

INTRODUCTION

Previously, it has been demonstrated that feeding on green seeds of *Crotalaria* (Fabaceae) that are removed from pods provides benefits to *Utetheisa ornatrix* (Erebiidae: Arctiinae) larvae in the form of faster development and larger resultant adult moths (Sourakov, 2015). However, if the larvae have to break through the pericarps to reach the seeds, the benefits can be reduced or negated depending on the species of hostplant. Brandon & Sourakov (2016) tested three groups of larvae on pods cracked open, closed pods and leaves and found that breaking through pods considerably slows down larval development on the native Florida species *Crotalaria pumila*, which presumably co-evolved with *U. ornatrix* for a considerable time. Here, that experiment is extended by testing diets of *C. pumila* leaves vs. leaves/closed pods, which is what the larvae are confronted with in nature. Faced with the readily available foliage, larvae would have to "make a decision" whether to crawl around searching for pods and expend energy and time by breaking through their tough pericarps to reach a few tiny and watery, but alkaloid-rich, seeds, or to complete their development on leaves alone. In this native Florida plant, the pods are too small to shelter larger larvae, so no additional benefit arises in the form of mechanical protection from the elements.

It has been well-determined that herbivores, predators and parasitoids cue in on specific volatiles (e.g., De Moraes *et al.*, 1998; Bush *et al.*, 2017). To better understand how pods attract larvae away from foliage, we conducted a limited study of volatiles emitted by both in *C. pumila* plants. For comparison, we also collected volatiles from the pods of three non-native *Crotalaria* species utilized by *U. ornatrix* in Florida (*C. spectabilis*, *C. lanceolata*, and *C. pallida*) and from pot-grown native *C. rotundifolia*.

MATERIALS AND METHODS

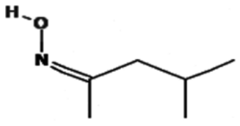
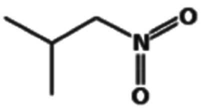
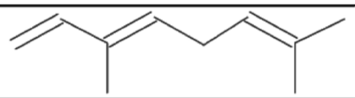
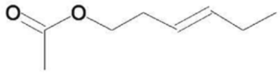
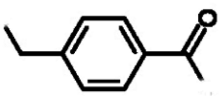
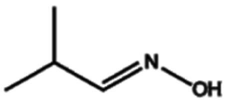
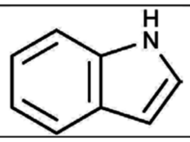
A split-brood design was used in the feeding experiment: the parental female of *U. ornatrix* was collected on the campus of the University of Florida and eggs were obtained on January 17, 2017. Larvae (N=50), were initially raised on foliage of *Crotalaria pumila* (potted plants were grown outside, ahead of time, from seed in one-gallon pots). When larvae reached 1 cm in length, they were split randomly into two groups: one continued to be fed on leaves, while the other was given not only leaves but also beans to feed on. Larvae were held three per 2 oz cup; the cups were placed in checker-board manner to minimize possible influence of the environment. Cups were changed every two days together with diet, which was maintained in ample supply. Ten days later, the larvae were frozen and weighed using a Mettler Toledo AL104 analytical balance and a paired *t*-test was used to compare larval weights. The volatiles emitted by pods from *Crotalaria pumila* were collected and analyzed using a technique based on Heath & Manukian (1992). Samples were placed in a glass volatile collection chamber (34 cm long and 4 cm outside diameter) with a glass frit inlet and a glass joint outlet and a single port collector base. Dry charcoal-filtered air was supplied to one end of the chamber and pulled for one hour. Three collections were done (mid-day, 11:00 to 14:00 (light), afternoon, 14:00 to 17:00 (light), and night, 17:00 to 20:00 (dark)) by vacuum (0.5 l/min) over the pods and through a volatile collection filter containing 50 mg of HayeSepQ at the other end of the chamber. Volatiles from plants without pods were collected by placing Teflon bags around branches of living plants growing naturally on the University of Florida campus, and in the lab by placing branches of potted *C. pumila* and *C. rotundifolia* plants into 12 cm x 6 cm (ID) open-bottom chambers flushed by a gentle stream of carbon-filtered air, in which collection filters were attached to the top and the bottom. Volatiles were collected for 6 hours at mid-day by pulling air through the chamber and collection filter at a flow of 0.2 l/min. Trapped volatiles were removed from the filter by eluting with 150-µl dichloromethane and analyzed by electron impact gas chromatography-mass spectrometry (HP 6890 gas chromatograph coupled to an HP 5973 MS detector). One µl of the sample was injected using a splitless injector (injector purge at 0.5 min) onto an HP-5MS dimethylpolysiloxane column (30 m × 250 µm (i.d.) × 0.25 µm film, Agilent Technologies, Palo Alto, CA). The GC oven was programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a constant flow carrier gas of 35 cm/min. Volatiles were tentatively identified by comparison of mass spectra with mass spectra libraries.

RESULTS

Larval weights were significantly greater (P=0.008) in the group fed on leaves-only (group A, Fig. 1) compared to that fed leaves and pods (group B): 115±40 mg (N=25) versus 86±32 mg (N=22 (3 larvae died in this group)), respectively. The group B larvae were observed throughout the experiment to target pods as a clearly preferred food-choice, despite the need to expend energy on chewing through them.

The volatiles coming from the pods comprised a much more diverse and complex mixture of chemicals than those coming from the foliage, which consisted of typical green plant volatiles: <4,8,12-Tetradecatrienal,5,9,13-trimethyl> ($C_{17}H_{28}O$; Mol. weight: 248) and <Neryl acetone> ($C_{13}H_{22}O$; Mol. weight: 194.31). While pods of *Crotalaria* species we tested produced unique chemical profiles, they had several compounds in common (Table 1). It is likely that one of these compounds, or a combination of several of them, contain a cue that is used by *Utetheisa ornatrix* larvae in their search for pods.

Table 1. Volatiles produced by pods of all four *Crotalaria* species examined in this study.

Compound	**Structure**	**Formula**	**Mol. Weight**
4-Methyl-2-pentanone oxime		$C_6H_{13}NO$	115.1735
Propane, 2-methyl-1-nitro-		C_4H_9NO2	103.1198
(E)-beta-ocimene		$C_{10}H_{16}$	136.238
Hexenyl acetate 3E-		$C_8H_{14}O_2$	142.198
Para-Ethyl acetophenone		$C_9H_{10}O_2$	150.177
2-Methylpropanal oxime		C_4H_9NO	87.12
Indole		C_8H_7N	117.15

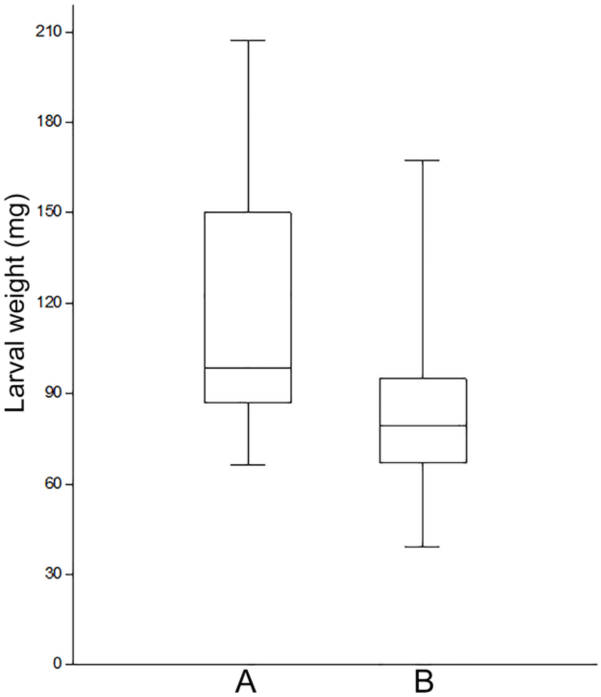


Fig. 1. Weight difference between larvae that fed for 10 days on (A) foliage of *Crotalaria pumila* vs. (B) pods/leaves (P=0.008, N=47)). While feeding on seeds removed from pods normally speeds up the development, slower growth was due to the mechanical protection provided by pericarps.

DISCUSSION

We provide here evidence that the volatiles released by the foliage of *Crotalaria pumila* are different from those produced by pods. At present it is not clear which, if any, of these volatiles are used as cues by the larvae. The fact that pods of different *Crotalaria* species produce different volatiles is not surprising, as the chemistry of these plants is known to differ (e.g., Martins *et al.*, 2015). Possibly there is a common component between pods and foliage (perhaps a derivative of the pyrrolizidine alkaloids found in both) that larvae cue in on, but we were unable to identify it. It is clear, however, from our limited study that there are many additional volatile components that would allow caterpillars to distinguish between foliage and pods and to cue in to identify the latter.

Larvae were slowed down in their development by the diet of unbroken *C. pumila* pods, the penetration of which is time-consuming and energetically expensive (Brandon & Sourakov, 2015), and the experimental design used in the present study replicates a scenario closer to natural conditions. The results support the idea that there is indeed ongoing ‘warfare’ between the plant and *U. ornatrix* larva. While *Crotalaria* have been ineffective against *U. ornatrix* in terms of the secondary chemicals that defend the plants against herbivores in general, since this moth’s caterpillars are able to sequester the toxins for their own benefits, mechanical ‘warfare’ is still apparently ongoing. The combined mechanical defenses provided collectively by pericarps seem to have a strong potential to at least partially reduce the benefits (PAs and better nutrition) accrued by caterpillars feeding on seeds. In addition to the pericarps’ mechanical defense, the small size of *C. pumila* pods and the high water contents of the seeds translate into smaller per-pod nutritional benefits. Finally, although the caterpillars are chemically protected, this protection is not effective against some natural enemies, such as tachinid flies, *Lespesia aletiae*, which can take a toll of at least 12% on mature *U. ornatrix* larvae (Sourakov, pers. obs.). Evolving smaller and more scattered pods (compared to many non-native *Crotalaria*) can be another way in which native hostplants combat *U. ornatrix* infestation, as the pods are too small to shelter caterpillars from their enemies and other adverse environmental conditions.

Crotalaria spectabilis, C. lanceolata and C. pallida are non-native legumes introduced to the New World from Asia and Africa in the 20th century. All three have spread rapidly throughout the southeastern United States, and while originally they were introduced as cover crops, they are toxic to birds and large mammals, including cattle. They are highly invasive in many natural habitats and thus need to be controlled. Fortunately, since the plants’ introduction, *Utetheisa ornatrix* has adopted them as hostplants. The moth’s caterpillars not only effectively feed on exotic *Crotalaria* but also, by destroying their seeds, limit their growth. This is, therefore, a great example of natural control of invasive organisms by a native species, and understanding these trophic interactions, and especially what signals the moth uses to find and select suitable hostplants, is not only of the theoretical but also of practical importance.

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Scientific Note: new records of *Marpesia chiron* (Lepidoptera: Nymphalidae) from Jamaica

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Marpesia chiron F. has a wide distribution stretching from southern Texas to Brazil and Argentina, Cuba and the Isle of Pines, Hispaniola, Puerto Rico and Jamaica (Smith *et al.*, 1994). The species is common on the Central American mainland and is sometimes referred to as the Common Dagger-tail (Riley, 1975). Breeding populations have been recorded on three of the islands of the Greater Antilles, Cuba and the Isle of Pines, where it is fairly common, on Hispaniola where populations are localized, and on Puerto Rico where sightings are rare and breeding populations, if they exist, are highly localized (Smith *et al.*, 1994). However, no breeding population has been reported from Jamaica. Records from Jamaica are rare and include sightings/collections in 1929, 1931 and 1951 (Brown & Heineman, 1972); all except one record are from the north-central region of the island; the other is from the southeast (Fig. 1). It is generally believed that species might be a migrant to the island (Brown & Heineman, 1972; Smith *et al.*, 1994). *Marpesia chiron* is one of the two *Marpesia* species on Jamaica, the other being *M. eleuchea pellenis* (Godart). *Marpesia eleuchea* is widespread across Jamaica but is generally uncommon (Brown & Heineman, 1972; Smith *et al.*, 1994).

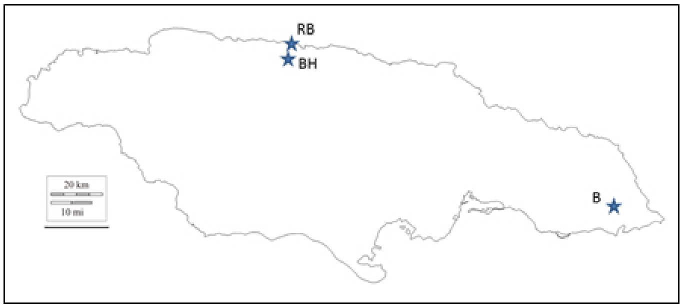

Fig. 1. Locations from which *M. chiron* has been recorded on Jamaica: Rio Bueno (RB); Baron Hill (BH); and Bath (B).

On January 31, 2016, a single specimen of *M. chiron* was observed at Rio Bueno, north-central Jamaica. This specimen was in excellent condition, and appeared to be fresh (Fig. 2). One week later, February 7, the species was again observed. As many as three individuals were observed at any one time, but the number was clearly greater than this as several individuals seen flying alone could be distinguished by distinct wing marks. No fresh specimens were observed on this second visit, instead, all had significant wing wear – a varying number of wing chips, loss of tails, and even significant loss of the hind wing.

Interestingly, Rio Bueno was the site of the 1951 record. The repeated records from Rio Bueno, and the fresh specimen on January 31, suggests the possibility of a breeding population. The suggestion by Brown & Heineman (1972) that "a breeding colony should be sought by resident collectors" seems even more applicable at this time to this location. A rapid survey of the area revealed the presence of species of *Ficus* (potential hosts plants, Srygley *et al.*, 2014), but no larvae were found.

The occurrence of potential host plants makes the establishment of breeding populations highly plausible. Srygley *et al.* (2014) noted that this species feeds on several members of the family Moraceae, including some species non-native to the Neotropics. Adams (1970) recorded 17 species in 9 genera in that family of plants occurring on Jamaica, including some species of known host plants of *M. chiron*, such as *Artocarpus het-*

Fig. 2. *Marpesia chiron* from Rio Bueno, Jamaica.

erophyllus Lam. and *Brosimum alicastrum* Sw. Srygley *et al*. (2014) indicated that there is evidence of specialization on food plants by different populations; this specialization might reduce the chances of a migrating population becoming established on Jamaica.

Marpesia chiron is well known for its migration and it has been known to travel hundreds of kilometres over water (Dudley & Syrgley, 2008). Jamaica is only 140 km from Cuba, hence the island is within the dispersive range of *M. chiron*, especially in the presence of northeast trade winds. The re-occurrence of records suggest a regular migration pathway, assuming no breeding population is present.

The butterflies were observed in a clearing at the edge of a dry limestone forest. Shortly after sunrise they basked in the sun but later spent most of their time sitting head down and aggressively patrolling. The presence of other members of the species initiated intense responses, and individuals repeatedly exchanged what appeared to be the prime perching sites. Other butterfly species were also attacked. Nectaring was not observed.

There is clearly a need for careful study of the Rio Bueno site in an attempt to establish if a breeding population exists. The search should be extended beyond Rio Bueno given that two of the previous sightings (1929 and 1931) were from Baron Hill (Brown & Heineman, 1972), approximately 4.5 km to the south of this site.

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