Why do larvae of *Utetheisa ornatrix* penetrate and feed in pods of *Crotalaria* species? Larval performance vs. chemical and physical constraints

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Abstract

Larvae of *Utetheisa ornatrix* (L.)(Lepidoptera: Arctiidae) are found mainly inside unripe pods of several alkaloid-bearing *Crotalaria* (Fabaceae) species. Although eggs are laid on the leaves, the larvae are usually found feeding on unripe seeds in the pods. In this work, we investigated the selective pressures that could explain why *U. ornatrix* larvae feed primarily on unripe pods with seeds and not on leaves. Our results showed that larval survivorship in the laboratory was unaffected by feeding on leaves or unripe seeds, and that larval development up to the pupal stage was better in larvae that fed on unripe seeds, although perforating unripe pods to reach seeds was costly in terms of survivorship. Females were also heavier when fed on unripe seeds, but there was no significant difference in the fecundity of females fed either of the two diets. Feeding on unripe seeds in pods had other benefits for *U. ornatrix*, including a lower predation rate for larvae that fed inside compared to larvae that fed outside the pods. Similarly, adults derived from larvae that fed on unripe seeds were preyed upon less frequently by the orb-weaving spider *Nephila clavipes* than were adults that fed on leaves. The latter benefit may be closely related to the high concentration of pyrrolizidine alkaloids in unripe seeds, which is about five times more than in leaves. These alkaloids are sequestered by the larvae and transferred to adults, which then become chemically protected. However, this chemical defence does not protect the larvae against ants such as *Ectatomma quadridens* and *Camponotus crassus*. Pods with unripe seeds that confer physical protection to larvae and pyrrolizidine alkaloids that confer chemical protection to adults limit the use of leaves by *U. ornatrix* larvae.

Introduction

Larvae of butterflies and moths are protected against predators in several ways (for reviews, see Baylis & Pierce, 1993; Bowers, 1993; Stamp & Wilkens, 1993). For example, unpalatability caused by the sequestration of chemicals from host plants or the *de novo* biosynthesis of deterrent compounds is a well-known defence strategy in Lepidoptera (see Trigo, 2000; Nishida, 2002; for reviews). Larvae of *Utetheisa ornatrix* L. (Lepidoptera: Arctiidae) sequester pyrrolizidine alkaloids (PAs) from their host plants, *Crotalaria* species (Fabaceae) and these alkaloids have been shown to protect different developmental stages of this insect against predators: larvae and adults are rejected by wolf-spiders (Eisner & Eisner, 1991), adults are released undamaged by the orb-weaving spider *Nephila clavipes* (Eisner, 1982), and eggs are rejected by coccinellid beetles (Dussourd et al., 1988), by larvae of the neuropteran *Ceraeochrysa cubana* (Eisner et al., 2000), and by the ant *Leptothorax longispinosus* (Hare & Eisner, 1993).

Utetheisa ornatrix larvae are found mainly inside unripe pods of several *Crotalaria* species, where they feed on unripe seeds, which contain the highest PA concentrations in these plants; leaves have substantially lower concentrations (Sharma et al., 1965; Johnson et al., 1985). As the female moths lay eggs on leaves, which are also available to the larvae for feeding, we investigated the pressures that lead to the preferential use of pods for food. Initially, we examined the performance of larvae, pupae, and adults derived from larvae that fed on leaves and unripe seeds of *Crotalaria*

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*Correspondence: E-mail: trigo@unicamp.br *pallida*, a common host plant in south-eastern Brazil. To determine whether there was a correlation between feeding on different parts of the host plants and protection against predation, we reared larvae on leaves and unripe seeds and then tested them against ants in laboratory and field assays. Subsequently, we reared larvae on two diets and tested the resulting adults against the orb-weaving spider *N. clavipes*. Finally, we assessed whether living inside pods of *C. pallida* offered physical protection to the larvae. The PA content of larvae and adults that had fed on one of the two diets was quantified to determine whether there was any correlation between alkaloid levels and extent of predation.

Materials and methods

Do different diets lead to differential performance?

The larvae used in the performance experiments were obtained from eggs laid by females collected in the field (open areas around UNICAMP, Campinas, São Paulo State, Brazil). The moths were housed in cardboard cylinders (9 cm in diameter × 20 cm high) containing vials with water and honey (20% aqueous solution). The eggs were collected daily and transferred to Petri dishes. After hatching, the larvae were transferred to plastic pots (4 cm wide × 5 cm high) and reared individually. Two plant parts (leaves and unripe seeds) were used to feed the larvae. Fifty larvae fed each diet were maintained in an acclimatized room (L12:D12, with L at 27 °C and D at 18 °C and about 70% r.h.) during their entire development. The rearing pots were cleaned daily to remove faeces and old food, and fresh food was added. The percentage of surviving larvae, larval development time, and pupal weight was recorded. After emergence, adults from larvae reared on the two diets were sexed according to Travassos (1946). Individual pairs for each diet were transferred to cardboard cylinders as described above and the eggs were collected daily and counted to determine female fecundity. The experiment ended when the female died. The survivorship between the two diets was compared by a bionomial Z-test, larval development and fecundity by Mann–Whitney U-test, and pupal weight between sex by two-way ANOVA (Zar, 1999).

Do different diets confer differential chemical protection?

To test whether the diet of the larvae influenced the chemical protection of larvae and adults against predators, the following three bioassays were done:

Bioassays with Ectatomma quadridens *ants in the field.* Larvae were reared on the two diets (leaves and unripe seeds) as described above. Fifth instar larvae were tested against the ant *Ectatomma quadridens* (Hymenoptera: Formicidae: Ponerinae). At the study site, *E. quadridens* frequently visited racemes of *C. pallida* to forage on its extrafloral nectaries (EFNs) (Guimarães, 2003; Guimarães et al., 2006). To test whether diet affected the protection of larvae against these ants, we gently placed one larva on the highest unripe pod of a raceme on which *E. quadridens* workers were foraging. The larvae were placed on the racemes, as ants are more abundant on these structures (94%) than on leaves (3%) (n = 29 plants; Guimarães, 2003; Guimarães et al., 2006), and larvae were found feeding mostly on seeds in the pods (98%) and rarely on leaves (2%) (n = 66 plants; Ferro, 2001). We monitored the raceme continuously until the ants' first attack on the larvae or for 15 min, if no attack occurred. Field observations suggested that this interval was twice the amount of time necessary for *U. ornatrix* larvae to perforate and enter an unripe pod. Ants apparently cannot attack the larvae once they are inside the pod. An attack was defined as any agonistic interaction between ants and larvae. The percentage of attacks on larvae feeding on two diets was analysed by comparison between two proportions (Zar, 1999).

Bioassays with Camponotus crassus *ants in the laboratory.* Because ants of the genus *Camponotus* are also abundant on EFNs of *C. pallida* (Guimarães, 2003; Guimarães et al., 2006), we did a laboratory test with *Camponotus crassus* (Hymenoptera: Formicidae). Fifth instar larvae were reared on leaves and unripe seeds as above. Colonies of *C. crassus* were maintained in glass tubes (18 cm long × 2.5 cm in diameter) and placed on a plastic square tray (20 × 20 × 8 cm) that was used as a nesting area. This tray was connected to another plastic circular tray (15 cm in diameter × 7 cm deep) used as the feeding area. The edges of both trays were coated with Fluon® (Fluon G, De Monchy I, Rotterdam) to prevent the ants from escaping. A 20% aqueous solution of honey was offered daily to the ants. Two days before the experiments, each colony was offered a palatable *Spodoptera frugiperda* larva (Lepidoptera: Noctuidae). Only colonies that ate this larva were used in the experiments. Fifth instar larvae were offered to colonies on *C. pallida* leaves placed in a deep vial with water to prevent desiccation of the plant. The percentage of predation was recorded after 24 h. If the larvae were not preyed on during this period, one control larva of *S. frugiperda* was immediately offered. If the ants ate the control larva within 24 h, the test was ended. However, if the control larva was not eaten, the test was discarded from subsequent analysis. Twenty larvae were used for each treatment. The statistical analysis was done as above.

Bioassays with Nephila clavipes *spiders.* Larvae were reared on the two diets as above. After emergence, adults were killed by freezing and kept at –20 °C for a maximum of 1 week until the bioassay. The bioassay against the orb-weaving spider *Nephila clavipes* (Araneae: Tetragnathidae) was performed in the field as described by Vasconcellos-Neto

& Lewinsohn (1984) and Trigo et al. (1993). The spider's response (predation or liberation) was recorded. A freeze-dried adult of the palatable moth *S. frugiperda* was later offered to the spider; if the moth was accepted, then the bioassay was validated, otherwise the test was discarded. The statistical analysis was done as above.

Do pods confer physical protection?

We used a field experiment to examine whether the habit of living inside pods conferred physical protection to *U. ornatrix* larvae. We collected young plants from the areas described above and transferred each one to a 50-l pot. When each plant began to produce pods, its architecture was simplified, leaving only four branches with leaves; all pods were cut from the branches. The plants were divided into four treatments: (i) plants with 10 non-bored unripe pods protected against predators, (ii) plants with 10 non-bored unripe pods not protected against predators, (iii) plants without unripe pods but that were protected, and (iv) plants without unripe pods and without protection. For treatments 1 and 2, non-bored unripe pods, obtained daily from plants at field, were attached to a branch with help of an adhesive ribbon. A piece of adhesive ribbon was attached to a branch of treatments 3 and 4. Protection against possible predators (e.g., wasps, bugs, mantids, ants, and birds) was assessed as described by Nogueira-de-Sá & Trigo (2002). Plants were protected individually by exclusion cages (1 × 1 × 1 m) made of PVC tubes of 13 mm covered by a fine mesh, thus forming a closed cage with tissue walls. We also put masking tape around the base of the plant and applied Tanglefoot^{TM} (Tanglefoot Co., Grand Rapids, MI, USA) to the tape. Only third instar larvae feeding on leaves were used, as bioassays with ants showed that there was no significant difference in the larval protection offered by the two diets (see below). The bioassays were done in an open area of the campus at UNICAMP, where *C. pallida* occurs naturally, and consisted of a randomized block design (Petersen, 1985), with each block containing four plants placed 1 m apart. The experiment was done for 15 consecutive 24-h periods (blocks), in which each of the four plants received each of the four treatments. The number of larvae remaining after each treatment was recorded after 24 h. For the subsequent period, a new set of larvae replaced the old ones and bored pods were replaced by non-bored ones as above. Data on disappearance were arc sin transformed to improve their normality.

Analysis of pyrrolizidine alkaloids (PAs)

Sets consisting of 10 units of two plant parts (leaves and unripe seeds) of *C. pallida* were placed separately in vials containing 2 ml of methanol (MeOH) immediately after being collected. The PA levels were analysed qualitatively by gas chromatography-mass spectrometry (GC-MS) as described by Trigo et al. (1996). Quantitative analyses were done by GC-MS in SIM mode (ion m/z 120) using pure integerrimine isolated from *Senecio brasiliensis* (Asteraceae) (see Klitzke & Trigo, 2000) as the calibration compound, as the main alkaloids present in *C. pallida* are integerrimine and usaramine (Ferro, 2001). The comparison between PA amount in leaves and unripe seeds was performed by t-test (Zar, 1999). In the same way, larvae, pupae, and adults feeding on different diets were also analysed for PA amount. A two-way ANOVA was performed to compare PA amount between development stages and diets (Zar, 1999).

Results

Performance of *Utetheisa ornatrix*

Larval survivorship was not significantly greater on leaves than in unripe seeds (Table 1). However, larval development

Table 1 Larval performance in *Utetheisa ornatrix* larvae reared on different parts of *Crotalaria pallida*. The values are the mean ± SE followed by sample size in parentheses

	Leaves	Unripe seeds	Statistical analysis
Survivorship at pupation (%)	56 (50)	38 (50)	Binomial test Z = 1.80, P = 0.07
Larval development (days)	25.92 ± 0.37 (28)	21.05 ± 0.38 (19)	Mann–Whitney U-test Z(U) = 4.98, P<0.001
Pupal weight of males (g)[1]	0.190 ± 0.005 (11)a	0.189 ± 0.006 (5)a,b	Two-way ANOVA
Pupal weight of females (g)[1]	0.168 ± 0.005 (14)b	0.192 ± 0.006 (9)a	Sex: $F_{1,35} = 2.65$, P = 0.11 Diet: $F_{1,35} = 3.92$, P = 0.06 Sex*diet: $F_{1,35} = 4.491$, P = 0.04
Fecundity (number of eggs/female)	244.40 ± 37.60 (10)	297.75 ± 102.15 (4)	Mann–Whitney U-test Z(U) = 0.36, P = 0.73

[1] Different letters following the pupal weights indicate significant differences at $\alpha<0.05$ (Tukey HSD test).

was significantly faster in unripe seeds than on leaves. Pupae of females feeding on unripe seeds were significantly heavier than those feeding on leaves; in males, no difference was verified. There was no difference in the fecundity of females fed either of the two diets.

Predation bioassays

There was no significant difference in the frequency with which *E. quadridens* attacked larvae that were fed seeds (100%) or leaves (83%) (comparison between two proportions – binomial test: Z = 1.48, P = 0.14; n = 12 for each diet). The larvae were attacked between 3 s and 13 min after being introduced onto the racemes. When attacked, the larvae generally fell to the ground. Three larvae that were fed seeds and one that was fed leaves were killed by the ants. Two larvae that were fed leaves perforated and entered the pods, thus escaping attack by ants.

In the *C. crassus* ant bioassay, 80% of the larvae that were fed either diet were preyed upon (n = 20 for each diet). The orb-weaving spider *N. clavipes* preyed significantly more on adults from larvae that were fed leaves (36%) than on adults from larvae that were fed unripe seeds (3%) (comparison between two proportions – binomial test: Z = 3.23, P = 0.001; n = 30 for each diet).

Physical protection bioassay

The disappearance of larvae was significantly influenced by the presence of pods. Plants that were not protected against predators and those that did not have pods showed the highest percentage of disappearance (75.3%) when compared with the other treatments (18.0% in protected plants with pods, 36.7% in protected plants without pods, and 42.7% in unprotected plants with pods) (Table 2).

Table 2 Percentage of disappearance (mean ± SE) of *Utetheisa ornatrix* larvae and the corresponding randomized block, one-way ANOVA (arcsine transformed). The values in the top table are the mean ± SE of 15 replicates

Treatment	Disappearance[1]
Unprotected plants with pods	42.67 ± 5.39a
Protected plants with pods	18.00 ± 2.43b
Unprotected plants without pods	75.33 ± 5.42c
Protected plants without pods	36.67 ± 3.03a

ANOVA

Source	SS	d.f.	MS	F-ratio	P
Between treatments	21.306	3	4046	29.65	<0.001
Between times (blocks)	3439	14	246	1.80	0.071
Error	5730	42	136		

[1] Different letters indicate significant differences at α<0.05 (Tukey HSD test).

Chemical analyses of plants and insects

The quantitative analysis of PAs in plants showed that unripe seeds (0.240 ± 0.096 μg mg^{-1}, n = 10) had a significantly higher content of alkaloids than leaves (0.054 ± 0.018, n = 10) (t-test: t = 1.91, d.f. = 9.66, P = 0.04). Regardless of the developmental stage, adult moths that fed on seeds in their larval stage had a significantly higher level of PAs than those that fed on leaves in their larval stage (Table 3).

Discussion

Although larval survivorship up to pupation was similar in leaves and unripe seeds, other results obtained in this study suggested that feeding on unripe seeds enhanced larval performance when compared with larvae that fed on leaves. Larval development time was significantly shorter for larvae that fed on unripe seeds. The larvae of holometabolous insects that have a longer development time are subject to heavy predation and parasitism (see Benrey & Denno, 1997, and references therein). In addition, females that fed

Table 3 Total pyrrolizidine alkaloid content (in μg/mg) and the corresponding two-way ANOVA of the developmental stages of *Utetheisa ornatrix* reared on different part of *Crotalaria pallida*. The values in the top table are the mean ± SE of 10 replicates

	Leaves[1]	Unripe seeds[1]
Larvae	0.060 ± 0.037a	0.881 ± 0.650b
Pupae	0.049 ± 0.013a	1.862 ± 1.685b
Adult males	0.038 ± 0.014a	0.987 ± 0.351b
Adult females	0.006 ± 0.002a	0.446 ± 0.308b

ANOVA

Source	SS	d.f.	MS	F-ratio	P
Between developmental stages	5.544	3	1.848	0.425	0.736
Between diets	20.253	1	20.253	4.659	0.034
Developmental stages*diet	5.048	3	1.683	0.387	0.763
Error	312.985	72	4.347		

[1] Different letters indicate significant differences at α<0.05 (Tukey HSD test).

on unripe seeds were heavier than those that fed on leaves, although there was no significant difference in fecundity.

Although our results showed a benefit in feeding on unripe seeds, under normal conditions in the wild, *U. ornatrix* larvae do not enter the pods immediately after hatching. The first larval instars remain around the apex of the leaves, and only in the second or third instar do the larvae enter the pods (JR Trigo, pers. obs.). Our laboratory experiments showed that feeding on pods had a high cost for first instar larvae of *U. ornatrix,* as the survivorship of larvae feeding on closed unripe pods was 4%, whereas larvae feeding on opened unripe pods had a 60% survivorship (Ferro, 2001). The time required to perforate the pods probably did not increase the mortality rate of first instar larvae, as the major predators on *C. pallida* (ants) did not patrol leaves efficiently. Indeed, Guimarães (2003) and Guimarães et al. (2006) found that termite baits glued to pods were removed by ants more frequently than those glued to leaves.

Extrinsic factors, such as predation, can also increase the fitness of larvae feeding on unripe seeds. When resting on pods, the level of predation was high, independent of the diet consumed by *U. ornatrix*. Similar results were found in laboratory bioassays with *C. crassus* Guimarães (2003) observed in the field that when larvae were found out of the pods by ants, such as *Camponotus* and *E. quadridens*, they were quickly preyed upon or expulsed. PAs did not appear to defend larvae against ant predation. In contrast, Dyer & Floyd (1993) and Dyer (1995) showed that the best explanation for rejection of the larvae of several lepidopteran species by *Paraponera clavata* ants was the larval chemical composition. Hare & Eisner (1993) reported that monocrotaline protected the eggs of *U. ornatrix* against the ant *L. longispinosus*. Based on our results, it is difficult to affirm that the ants studied here were not affected by PAs. Predator response may be dose-dependent [see Brower et al. (1968) for cardenolides and Silva & Trigo (2002) for PAs], and the concentration of PAs in the larvae may not have been sufficient to elicit an aversive response in the ants.

Whatever the role of PA in larval defence against ants, predation on larvae was lower in racemes with pods containing unripe seeds than on plants without pods. By feeding inside the pods, *U. ornatrix* larvae are efficiently protected against ants and other natural enemies. When larvae need to move to other pods to find food, they generally do so when ant activity is low (e.g., at sunrise – JR Trigo, pers. obs.).

Feeding on unripe seeds inside pods increased the sequestration of PAs when compared to larvae that fed on leaves (Conner et al., 1990), enhancing the fitness of *U. ornatrix* due to chemical protection (Eisner & Meinwald, 1995, and references therein). This high PA content increased the chemical protection of adults against the orb-weaving spider *N. clavipes*; several authors have demonstrated this for various butterflies and moths (Eisner, 1982; Brown, 1984, 1985, 1987; Masters, 1990; Trigo et al., 1993, 1996; Orr et al., 1996). This type of protection can also be extended to other predators, such as wolf-spiders (Eisner & Eisner, 1991), lizards (Masters, 1992), and birds (Masters, 1992; Cardoso, 1997).

Feeding on diets with a high PA content also increases the probability of a male finding a mate. Males with large amounts of PAs to give to females during mating produced more progeny due to protection of females, being preferred by females that could recognize this quantity during courtship. Conner et al. (1981, 1990), studying *U. ornatrix*, suggested that PA-derived pheromone (hydroxydanaidal) was used by females as a measure of the capacity of PA incorporation by males, and these compounds, on the other hand, also represent a measure of the potential capacity of chemical defence. In addition, females would use these PAs in their own defence or defence of their offspring by chemical protection of eggs (see Dussourd et al., 1988). Brown (1984, 1985) and Dussourd et al. (1989) suggested that similar mechanism occurs also in other PA specialist butterflies, such as Itomiinae and Danainae. In conclusion, we suggest that physical (boring into seed pods) and chemical (feeding on a diet rich in PA) factors exert selective pressures that limit the feeding life history of *U. ornatrix*.

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