Evolutionary implications of pyrrolizidine alkaloid assimilation by danaine and ithomiine larvae (Lepidoptera: Nymphalidae)

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Summary. Larvae of danaine and ithomiine butterflies assimilate pyrrolizidine alkaloids painted on their larval host plants. This finding supports the theory of the common ancestral use of these compounds by Ithomiinae and Danainae.

Key words. Pyrrolizidine alkaloids; Ithomiinae; Danainae; phylogenetic relationships; assimilation of host-plant defensive chemicals.

Larvae of more than 95% of the species of Ithomiinae butterflies feed on plants of the family Solanaceae[1]. However, four primitive genera (*Tellervo, Tithorea, Elzunia* and *Aeria*) feed on Apocynaceae (tribe Parsonsieae), whose leaves may contain pyrrolizidine alkaloids (PAs)[2–5]. In some Ithomiinae, these alkaloids function as male pheromone precursors[6–10], and as a chemical defense against predation by the spider *Nephila clavipes*[11–13]. Larvae of *Tellervo* and *Tithorea* sequester PAs from their host plants[4, 5], while *Aeria* probably uses host plant compounds as precursors for PA biosynthesis[5]. The adults of Solanaceae-feeding Ithomiinae assimilate PAs from their food sources (nectar of Boraginaceae and Asteraceae) and decomposing leaves of Boraginaceae (mainly *Heliotropium*), since Solanaceae do not contain PAs[11–13]. It has been suggested that the ancestral host plant of the closely related Danainae and Ithomiinae[14, 15] contained PAs, leading to a dependence of these bufferflies on these alkaloids which has been retained up to the present[6, 7]. *Danaus plexippus* larvae assimilated PAs from a PA-free host plant which was treated with these alkaloids, suggesting that this danaine maintains the capacity for PA sequestration and storage probably used by its ancestors to obtain PAs from their host plants[16].

In this work we show that Ithomiinae larvae which feed on PA-free plants also retain the capacity for PA sequestration and storage. On the basis of this finding we discuss the possible common ancestry of Ithomiinae and Danainae, and of their larval host plants.

Material and methods

Eggs and larvae of species of Ithomiinae, Danainae, Pieridae, and their host plants (table 1) were obtained in the regions of Campinas and Jundiaí, São Paulo, Brazil. The Danainae were included as a closely related group (sister-group of Ithomiinae[15]) and the Pieridae as a control, since they do not utilize plants with PAs in their life cycle. Larvae were reared at room temperature (about 25 °C) on leaves of their respective food plants. The leaves were changed daily. The third instar larvae were fed daily with PA ascorbate painted on the host-plant leaves, until pupation. The PA ascorbate salt was prepared with the free bases of PAs from *Eupatorium laevigatum* (echinatine and three other non-identified alkaloids[5]) plus ascorbic acid (15:8 w/w). The PA ascorbate was utilized because it is water soluble (free bases are poorly miscible with water and soluble in organic solvents). PA N-oxides, which are soluble in water, could also be utilized, but the data on transformation from free bases to N-oxides would be lost. Controls were included for each butterfly species; they were given leaves painted only with an aqueous solution of ascorbic acid (50–100 μg). During the treatment, the feces of each larva were collected and placed in 2 ml of absolute methanol. After eclosion, the imago and the meconium were placed individually in separate vials with 2 ml of absolute methanol.

PA ascorbate and individual imagoes, meconia and feces were analyzed by a colorimetric method to determine the amount of PAs[12, 17–19]. One tenth of the total liquid volume was dried and oxidized for 25 min at 100 °C with 0.5 ml of 30% H_2O_2 + 5 mg/ml $Na_2P_2O_7$ diluted 1:200 with absolute MeOH. The samples were cooled to room temperature and isoamyl acetate (1 ml) plus acetic anydride (0.1 ml) were added. The samples were then heated at 100 °C for 2 min (Polonovski reaction), and then again cooled to room temperature, and treated with 1 ml freshly prepared modified Ehrlich reagent (8 ml of 20%

Table 1. Butterflies and their larval host plants

Butterflies	Larval host plants
Pieridae	
Ascia monuste	*Brassica oleracea* (Cruciferae)
Danainae	
Danaus plexippus	*Asclepias curassavica* (Asclepiadaceae)
Danaus gilippus	*idem*
Ithomiinae (Apocynaceae-feeding)	
Aeria olena	*Prestonia coalita*
Ithomiinae (Solanaceae-feeding)	
Methona themisto	*Brunfelsia uniflora*
Hypothyris euclea	*Solanum mauritianum*
Mechanitis polymnia	*Solanum concinnum*
Mechanitis lysimnia	*Solanum aculeatissimum*
Prittwitzia hymenaea	*Solanum caavurana*

Table 2. Amount of total bases, percent of N-oxide and percent of total bases assimilated in imagoes of butterflies whose larvae fed on leaves painted with PA ascorbate

Species	Total bases in imago (μg)	% N-oxide in imago (in relation to total bases)[a]	% Total bases assimilated in imago[b]
	$\bar{x} \pm$ sd (n)	$\bar{x} \pm$ sd	$\bar{x} \pm$ sd
Ascia monuste	0 (10)	0	0
D. plexippus	148 ± 72 (11)	54 ± 16	20 ± 8
D. Gilippus	86 (1)	57	57
A. olena	56 ± 19 (3)	55 ± 9	NC
M. themisto	20 ± 2 (3)	53 ± 10	NC
H. euclea	14 ± 4 (9)	62 ± 17	16 ± 8
M. polymnia	22 ± 4 (3)	44 ± 40	14 ± 2
M. lysimnia	20 ± 11 (5)	68 ± 22	NC
P. hymenaea	39 ± 15 (3)	61 ± 17	40 ± 29

[a] The percent of N-oxide in the PA ascorbate solution was about 7%.
[b] NC: not calculated.

methanolic BF_3 + 72 ml of absolute EtOH + 1.4 g dimethylaminobenzaldehyde). After heating at 60 °C for 5 min, the samples were cooled to room temperature and diluted to 3.8 ml in a 1-cm cuvette with redistilled acetone. The absorbance was read on a Micronal B-280 instrument at 561.5 nm. The amounts of total bases and of N-oxide were calculated by the following equations[19]: μg total bases = abs × 333 and μg N-oxide = abs × 200. The N-oxide percent in relation to total bases (in ascorbate form) ingested by the larvae was calculated.

Results

Table 2 shows the total bases, the percent of total bases assimilated and the percent of N-oxide in imagoes whose larvae fed on leaves treated with PA ascorbate. Both danaine and ithomiine imagoes incorporated the alkaloids; the pierid *Ascia monuste* failed to assimilate PAs. When the larvae had received no PAs (only ascorbic acid solution), no alkaloids were found in the imagoes, meconium or feces. No PAs were demonstrated in the analyses of meconium and feces of any species of Danainae and Ithomiinae after feeding on PA ascorbate; however, the pigments of the feces could have interfered with the colorimetric analyses. Feces of *Ascia monuste* did contain PAs. The meconium of this butterfly was not analyzed. Imagos of Danainae and Ithomiinae whose larvae fed on host plants with PA ascorbate showed a percentage of N-oxide ranging from 44 to 68%. However, only 7% was present as N-oxide in the PA-ascorbate applied to the leaves.

Discussion

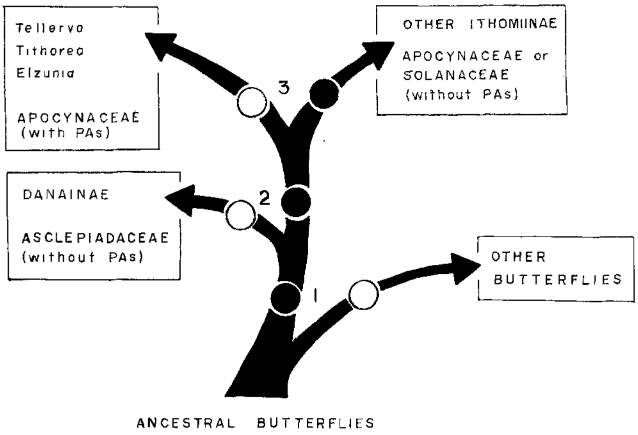
Relationships between Ithomiinae/Danainae and their ancestral host plants. The association of Ithomiinae and Danainae with PA-containing plants has been explained by two different evolutionary hypotheses.

Edgar[6,7] suggested that the necessity for PAs is derived from an ancient association of Ithomiinae/Danainae ancestors with their ancestral larval host plants, probably Apocynaceae. These plants, in the ancestral environment, may have contained both cardiac glycosides (CG) and pyrrolizidine alkaloids (PAs). As a result of intense herbivory by insects (including larvae of these ancestral butterflies which evolved dependence on these secondary metabolites for sex pheromones and chemical defense), the ancestral plants differentiated into three main lines; one containing only CG (Asclepiadaceae), another only PAs (Boraginaceae and Asteraceae), and the third with neither (Solanaceae). After this divergence, each line had a smaller number of herbivorous species specialized on it. The ancestral butterflies branched into two groups: the Danainae fed upon Asclepiadaceae and Moraceae (plants with only CG) and Ithomiinae adapted to use Solanaceae (plants without PAs or CG). Adults of both continued to satisfy their PA dependence by collecting these compounds from Boraginaceae and Asteraceae. Some primitive genera in both groups continued to feed on Apocynaceae (plants with PAs and/or CG).

Boppré[20] proposed an alternative hypothesis – an adaptation to PA-containing plants independent of larval host plants. The ancestral adults of Danainae and Ithomiinae butterflies obtained their nutrition from plants of various species. Those that could tolerate toxic nectar containing PAs were favorably selected by protection against predation, becoming pharmacophagous[21]. Consequently, aposematic traits appeared, followed by evolution of Müllerian mimicry. The next step was the use of PAs or their derivatives for sexual communication, since females were attracted by the odors of PA derivatives and thus avoided breeding with Müllerian co-mimic species. Butterflies which could later use PAs or CG-plants as larval host plants would not only have adults which were better protected, but would also gain protection for the larvae.

We have supposed that in the ancestral environment the larvae could sequester and store PAs from the larval host plant, in accordance with Edgar's hypothesis, competing with other larvae on the same plants which also sequestered these compounds (such as Arctiid moths). For those individuals which also possessed the capacity to assimilate PAs from nectar sources as adults, the necessity for the larvae to feed on PA-containing host plants might be diminished. Much larger amounts of PAs could be accumulated by the highly mobile adults from abundant flowers. In this way, Ithomiinae larvae expanded their diet, invading Solanaceae, a new host-plant group which was probably relatively free of herbivore pressures because of an abundance of toxic compounds. The sympatric occurrence of Solanaceae with the ancestral host plant helped the radiation of the Ithomiinae.

Our results indicate that the supposed capacity to sequester and store PAs is present in larvae and adult stages of individual Ithomiinae, in either a latent or a functional state. Thus, the Ithomiinae adopted two main strategies to obtain PAs which were not mutually exclusive: a) primitive Ithomiinae (genera *Tellervo, Tithorea* and probably *Elzunia*) obtain PAs from their larval host plants (Parsonsieae), with the adults visiting PA-contain-



Acquisition of PAs and phylogenetic relationships in Danainae and Ithomiinae butterflies. Apomorphic traits (solid circles in each branching): 1) sequester PAs and cardenolides from ancestral host plants (probably Parsonsieae: Apocynaceae); 2) incorporate only PAs (not CG) from larval host plant; and 3) do not incorporate PAs from larval host plant but obtain PAs from adult food sources.

ing plants only occasionally; and b) other Ithomiinae, whose larvae use plants without PAs (Solanaceae), sequester the alkaloids by visits to flower nectar of Asteraceae and decomposing leaves of Boraginaceae. In Campinas, adults of both sexes of *Tithorea harmonia pseudethra* rarely visit nectar sources containing PAs[5]. The capacity for PA assimilation is only used when PAs are present in the larval host plant or adult food sources. Larvae of more advanced Ithomiinae have maintained the capacity to assimilate PAs throughout the evolutionary periods, even with a switch of host plants from Apocynaceae to Solanaceae. The capacity for PA assimilation by Ithomiinae larvae is ontogenetically related to the capacity for PA assimilation of the adults. If, in the ancestral environment, the larvae had lost this capacity, the adults would also not have had it. However if, after acquisition of this capacity, the adults had lost it, the larvae would have lost it, too. In other words, the capacity for PA assimilation by larvae, at the present time, is maintained by selective pressures (predation and sexual selection) on the adults. Thus we suggest that the sequestering of PA by larvae from their host plant in actual evolutionary time is a plesiomorphic character, while PA acquisition in the adult would be apomorphic (fig.).

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