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Sequestration of aristolochic acids from meridic diets by larvae of *Battus polydamas archidamas* (Papilionidae: Troidini)

CARLOS F. PINTO[1], ALEJANDRO URZÚA[2] and HERMANN M. NIEMEYER[1]*

[1]Laboratorio de Química Ecológica, Universidad de Chile, Casilla 653, Santiago, Chile
[2]Laboratorio de Química Ecológica, Universidad de Santiago de Chile, Casilla 40, C-33 Santiago, Chile

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Abstract. Larvae of the butterfly, *Battus polydamas archidamas* (Papilionidae: Troidini) feed exclusively on aristolochic acid (AAs)-containing *Aristolochia* species (Aristolochiaceae). The distribution of sequestrated AAs in the tissues (body, integument and osmeterial secretions) of *B. polydamas archidamas* larvae during their development, when fed on a meridic diet containing either a higher or lower concentration of AAs (AAI and AAII) than occurs naturally in the aerial tissues of their host plant, was determined. Accumulation of AAs in the body and integument was proportional to the weight of larvae and greater in the larvae that fed on the diet containing the higher concentration of AAs. Phenolic AAs (AAIa and AAIVa) not present in the diets were found in all larval tissues examined. Integument and body extracts had a higher AAI/AAII ratio than in the original diet and also a relatively high AAIa/AAIVa ratio, suggesting a preferred AAII to AAIa transformation in those larval tissues. In the osmeterial secretion, the value of the AAI/AAII ratio was similar to that in the diets and the AAIa/AAIVa ratio close to 1, which suggests that hydroxylation of AAI to AAIVa and of AAII to AAIa occur to similar extents. The higher accumulation of AAs and the relatively higher proportion of AAI, one of the most toxic AAs, in the integument, suggest that their role is to deter attacks by natural enemies.

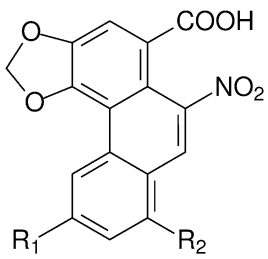
INTRODUCTION

Host plant specialization by insects is common in nature. Among the Lepidoptera, most species of butterflies oviposit and feed as larvae on a single plant species or on only a few genera or families (Nishida, 2002; Schoonhoven et al., 2005). This pattern is mostly attributed to certain groups of plant secondary chemicals that through time have resulted in the evolution of specific associations of butterflies with particular species of host plant (Ehlrich & Raven, 1964; Nishida, 2002). The nature of these chemicals is diverse and they are used by butterflies in various processes such as host selection, defence and even mating (Nishida, 2002; Opitz & Müller, 2009). For example, some Danaine and Ithomiine butterflies (Nymphalidae) are associated with pyrrolizidine alkaloids that occur in plants belonging to a wide variety of families (e.g. Asteraceae, Boraginaceae, Apocynaceae) (Nishida et al., 1991, 1996; Honda et al., 1997; Nishida, 2002). These compounds can be used as oviposition stimulants by females, as sex pheromones by males and for defence by larvae, which sequester them during their development (Nishida, 2002). When plant chemicals are sequestered by lepidopterans, their concentration in the insect body may be similar to that in the host plants or there is selective uptake, transformation and the original compounds may even be metabolized (Rothschild & Edgar, 1978; Seiber et al., 1980; Nishida, 2002).

The tribe Troidini (Papilionidae) is associated with a single plant family (Aristolochiaceae) (Weintraub, 1995) containing aristolochic acids (AAs), a group of 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acids unique to the family (Chen & Zu, 1987; Mix et al., 1982; Kumar et al., 2003). AAs occur in nature mostly as the non-phenolic compounds AAI and AAII, and also as phenolic compounds such as AAIa and AAIVa (Fig. 1), the former group being the most abundant in the host plant (Sime et al., 2000). AAs are sequestered by the larvae and are transferred to eggs by adults (Sime et al., 2000; Nishida, 2002; Fordyce et al., 2005). The genus *Battus* is known to use these chemicals for host selection and defence (Nishida & Fukami, 1989; Fordyce, 2000; Nishida, 2005; Pinto et al., 2009a).

In *Aristolochia chilensis* Bridges ex Lindl. the host of *Battus polydamas archidamas* (Boisduval, 1836) in central Chile, aristolochic acids occur mainly as AAI and AAII (Urzúa et al., 1987). In this plant the concentrations of AAs differ in the different parts of the plant and larvae consume different plant tissues at different stages during their ontogeny (Pinto et al., 2009b). AAs are sequestered by the larvae and have been found in wild imagoes and pupae of *B. polydamas archidamas* feeding on *A. chilensis*, but their distribution within the insect remains unknown (Urzúa el al., 1983, 1987). Furthermore, larval performance depends on the concentration of AAs contained in artificial meridic diets (Pinto et al., 2009b). In the present paper, the distribution of sequestrated AAs in the tissues of larvae of *B. polydamas archidamas* during their development was explored by feeding the larvae on meridic diets with two different concentrations of AAs.

* Author for correspondence; e-mail: niemeyer@abulafia.ciencias.uchile.cl



	R_1	R_2
I	H	OCH_3
II	H	H
I a	H	OH
IV a	OH	OCH_3

Fig. 1. Aristolochic acids present in larvae of *Battus polydamas archidamas*.

MATERIAL AND METHODS

Rearing of larvae on meridic diets

Battus polydamas archidamas is the only papilionid in Chile. It feeds and oviposits on two perennial endemic pipevines, *A. chilensis* and *A. bridgesii* (Klotzsch) Duchart. (Aristolochiaceae) (Marticorena & Quezada, 1985). The distribution of the butterfly coincides with the sum of the ranges of both plant species, i.e., the coastal and Andean mountain ranges of Chile between ca. 26°S and 35°S (Navas, 1976). Eggs of *B. polydamas archidamas* (ca. 400 for each diet treatment) and plant material of *A. chilensis* were collected at Cuesta Lo Prado (33°28´S, 70°56´W, 750 m above sea level) 15 km west of Santiago.

Larvae were reared in the laboratory using the method described by Pinto et al. (2009b). In brief, field collected egg clutches were allowed to hatch in Ø 35 mm-Petri dishes. First instar larvae were placed on a block of diet (ca. 2 g) in a Ø 55 mm-Petri dish. The diet was replaced every two days. As larvae grew, they were transferred to larger Petri dishes (Ø 60 mm for third to fifth instar larvae and Ø 110 mm for sixth and seventh instar larvae). The number of individuals per container was also decreased from ca. 10 first instar larvae to one fourth instar larva, thus simulating the aggregation patterns observed in the field (Pinto et al., 2009a). Containers were kept at a mean temperature of 23°C and 16L : 8D photoperiod.

In order to mimic different plant chemical scenarios, two experimental diets were prepared by adding AAI and AAII (Aldrich, St. Louis, MO) to a basic diet prepared following the method of Fordyce & Nice (2008) modified by Pinto et al. (2009b). The AAI/AAII ratio in both diets was that naturally present in the aerial tissues of plants at the stage when they are preferentially consumed in the field by larvae of *B. polydamas archidamas* (AAI: 55% and AAII: 45%) (Pinto et al., 2009b). The low aristolochic acid diet contained half the mean concentration of AAs in plants (0.75 mg/g fresh tissue) and the high aristolochic acid diet contained twice that concentration (3 mg/g fresh tissue).

Stability of AAI and AAII in diets was assessed by keeping a portion of the diet containing AAI and AAII in the percentages and concentrations mentioned above at room temperature for 5 days, re-isolating aristolochic acids as previously described (Urzúa et al., 1987) and analysing the mixture using HPLC (see below). There was no evidence that the aristolochic acids were converted into AAIa and AAIVa.

Content of aristolochic acids in larval tissues

Three types of larval tissues were sampled: integument, body after removal of the integument, and osmeterial secretion. Third, fourth, fifth and sixth instar larvae were used (ca. 15 samples/type of tissue/instar) to determine the distribution of the sequestered aristolochic acids in larvae throughout these stages in their development.

Individuals were sampled at the end of each of the four larval instars studied. They were starved for 24 h to avoid contamination with food remaining in the gut. Osmeterial secretions were collected using capillary tubes (0.5 µl, 32-mm long, SIGMA), which were placed in contact with everted glands (Sime at al., 2000) and the fluid so collected was dissolved in 0.5 ml of dichloromethane. Integument samples were obtained by briefly dipping individuals, previously killed by freezing, into 1 ml of dichloromethane at room temperature. Samples of the rest of the body were desiccated in an oven at 50°C, weighed and preserved in Eppendorf tubes inside plastic bags with silica gel until required for analysis by high performance liquid chromatography (HPLC).

HPLC analysis of aristolochic acids

Dichloromethane extracts of osmeterial secretions and integuments were directly injected (20 µl) into the HPLC. The desiccated bodies were ground to a fine powder in a mortar and then 500 µl of methanol were added and the sample macerated for 50 min. This extract was centrifuged for 6 min at 13,000 rpm and the supernatant directly injected into the HPLC (20 µl).

The non-phenolic and phenolic AAs fractions were analysed using HPLC (Waters 600) with a reverse-phase Symmetry column (5 µm particle size; 25 × 0.46 cm). Gradient elution was performed using a mobile phase consisting of 0.1% acetic acid in water (solution A) and 0.1% acetic acid in acetonitrile (solution B) as follows: 0–5 min, isocratic elution with 70% A / 30% B; 5–45 min, linear gradient from 70% A / 30% B to 55% A / 45% B. A Waters 2996 diode-array-detector (DAD) was used to detect the aristolochic acids and their spectra were recorded at wavelengths between 200 and 800 nm. The UV spectra and retention times of all AAs detected were coincident with standards of AA-I, AA-II, AA-Ia and AA-Iva, previously isolated from *A. chilensis*, *A. argentina* and *B. polydamas archidamas* (Urzúa et al., 1983; Priestap, 1987). Quantification was based on areas of peaks in chromatograms taken at 254 nm. A dilution series of standard solutions was prepared from stock solutions of standards and all solutions of standards and samples were stored at 5°C. Calibration lines were obtained by plotting areas of peaks against the concentrations of the standards; these lines were used to determine the concentrations of the AAs in the samples.

Data analysis

The relationships between total AAs present in different tissues of individual larvae and their weights were analysed using one way ANOVAs of the ratio between total AA content and weight of larvae, because the results were not normally distributed (Conover & Iman, 1981; Sokal & Rohlf, 1995; McDonald, 2009). Total non-phenolic and phenolic AAs present in different larval tissues were compared using one way ANOVA of ranks of ratios followed by post-hoc Dunn's tests. The ratios of non phenolic AAs (AAI/AAII) and phenolic AAs (AAIa/AAIVa) in different tissues were assessed using one way ANOVA on ranks followed by post-hoc Dunn's tests; additionally, these ratios were analysed separately for tissue obtained from larvae feeding on the high-AAs and the low-AAs diets and the results for each diet showed the same pattern as that obtained using the combined data. The relationships between AAs content and weight of larvae were analysed using Spearman correlations.

RESULTS

The increase in quantity of AAs per individual with increase in larval weight (all instars pooled together) significantly depended on the concentration of AAs in the diet in the case of the body and integument extracts (H =

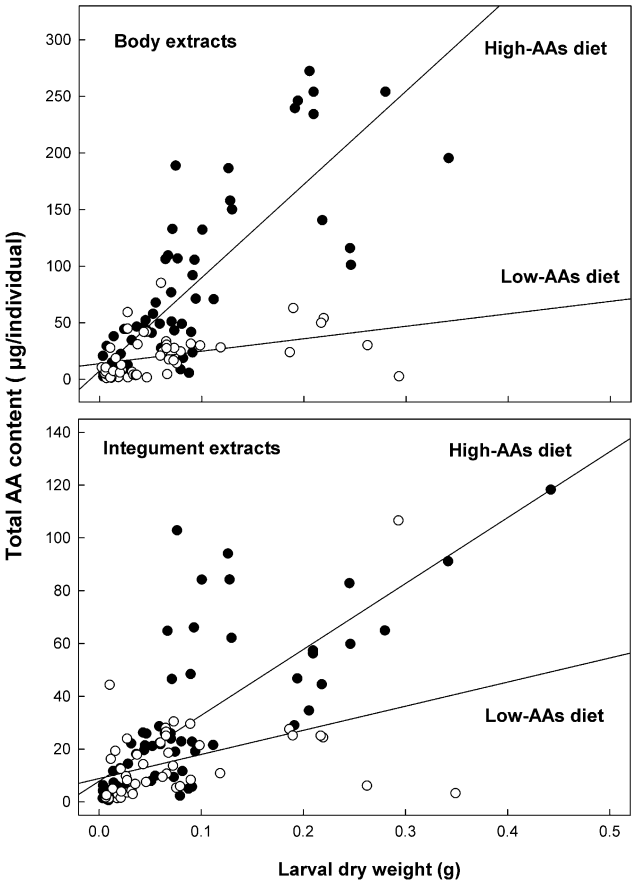


Fig. 2. Correlations between AA content (µg/individual) of the extracts of the integument and body, and the dry weight of the larvae of *Battus polydamas archidamas* fed on diets containing low (empty circles) and high concentrations (dotted circles) of AAs. Spearman correlations for body extracts (low-AAs diet: r = 0.479, P < 0.001; high-AAs diet: r = 0.832, P < 0.001,) and for integument extracts (low-AAs diet: r = 0.490, P < 0.001; high-AAs diet: r = 0.796, P < 0.001).

24.077, df = 1, P < 0.001 and H = 5.875, df = 1, P < 0.05, respectively) but not in the osmeterial secretion (H = 3.389, df = 1, P = 0.06). Fig. 2 shows the significant correlations between the quantity of AAs per individual in the body and integument extracts, and larval weight.

Quantity of non-phenolic and phenolic AAs differed among tissues (H = 116.327; df = 5; P < 0.001) and was lower in osmeterial secretion (P < 0.05) than in the integument and body extracts (Fig. 3).

The ratio of non-phenolic AAs (AAI/AAII) differed among tissues (H = 170.114; df = 2; P < 0.001) and was lower (P < 0.05) in osmeterial secretion than in the integument and body extracts (Fig. 4). The ratio of phenolic AAs (AAIa/AAIVa) also differed among tissues (H = 87.058; df = 2; P < 0.001) and was also lower (P < 0.05) in osmeterial secretion than in the integument and body extracts (Fig. 4). Furthermore, the ratio of non-phenolic AAs (AAI/AAII) in osmeterial secretion was similar to that in the original meridic diet (55 : 45), whereas in the integument and body extracts the AAI/AAII ratio was approximately ten times higher than that in the original diet (Fig. 4).

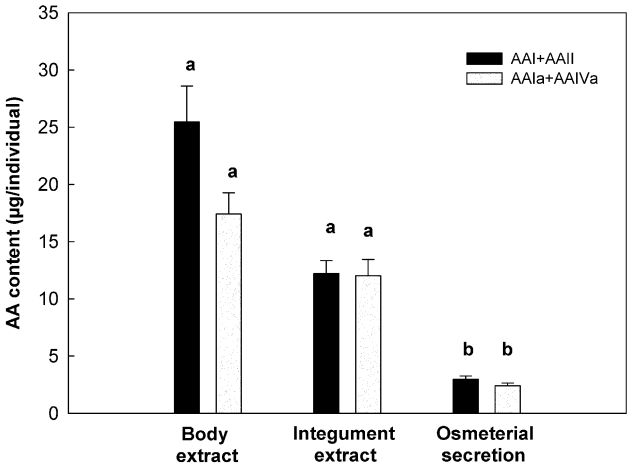


Fig. 3. Sum of the non-phenolic and phenolic AAs present in body extracts, integument extracts and osmeterial secretion of larvae of *Battus polydamas archidamas.* Different letters above the columns indicate significant differences (P < 0.05, Dunn tests).

DISCUSSION

The quantity of AAs in body and integument increased with increase in the weight of larvae and was more pronounced when the larvae were fed on the diet containing the higher concentration of AAs (Fig. 2), which suggests a passive uptake of AAs from the diet if weight gain by larvae is proportional to the amount of diet ingested. This is likely since swallowtail larvae tolerate the compounds

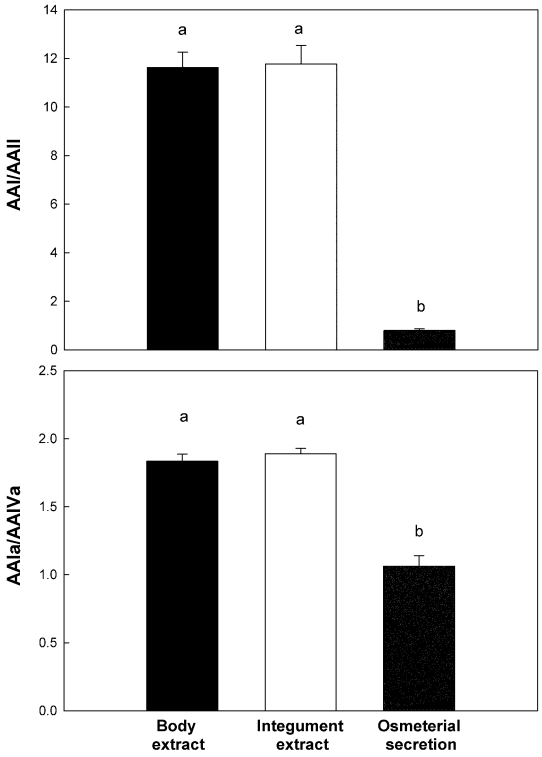


Fig. 4. Ratios of non-phenolic AAs (AAI/AAII) and phenolic AAs (AAIa/AAIVa) present in different tissues of larvae of *Battus polydamas archidamas*. Different letters above the columns indicate significant differences (P < 0.05, Dunn tests).

characteristic of their host plants when added to diets (Miller & Feeny, 1989).

Phenolic AAs (AAIa and AAIVa), not present in the diets, were found in all the tissues examined, indicating a detoxification process that modifies the AAs ingested, e.g. hydroxylation of AAII to produce AAIa, and hydroxylation of AAI to produce AAIVa. Interestingly, both in the integument and body extracts there was a higher AAI/AAII ratio than in the diet. This suggests a preferred AAII to AAIa transformation, resulting in the relatively higher AAIa/AAIVa ratio in those two tissues, although preferential excretion of AAIVa or conversion into other undetected metabolites is also possible. In the osmeterial secretion, the ratio of AAI/AAII is similar to that in the diets and that of the AAIa/AAIVa ratio close to 1, which indicates that both hydroxylation reactions occur to similar extents. Adults of *Battus philenor* fed on *Aristolochia macrophylla* during their larval development have similar ratios of AAs (Sime et al., 2000), which is attributed to the selective conversion of AAII as there is a low proportion of this compound in the samples, or the selective uptake of AAI and its transformation into phenolic AAs. The results presented support the first of these two hypotheses. Pupae of *B. polydamas archidamas* fed on *A. chilensis* during larval development also have a higher AAI/AAII ratio than is present in the plant (Urzúa el al., 1987); however, in this case the ratio of phenolic AAs may partly reflect the presence of these compounds in the plant.

Birds that are likely to feed on butterflies avoid feeding on rice grains impregnated with AAI (Nishida & Fukami, 1989). The defensive role of AAs has also been tested using potential arthropod predators and shown to increase the mortality or cause the attacker to modify its behaviour (Fordyce, 2000; Sime, 2002). In *B. polydamas archidamas*, both phenolic and non-phenolic AAs accumulated in integument and body extracts to similar extents (Fig. 3). Considering the relative contributions of integument and body to insect weight, this indicates a higher concentration of AAs in the integument than in the body. This pattern of accumulation could be related to a selective allocation of defenses to the body surface in order to deter attack by parasitoids or predators and also to signal toxicity as shown by Sime (2002) for the role of AAs in the integument of *Battus philenor* attacked by the parasitoid *Trogus pennator*.

AAs exhibit a wide range of biological activities (Arlt et al., 2002; Balachandran et al., 2005; Mei et al., 2006; Shibutani et al., 2007). Non-phenolic AAs are generally more active than phenolic AAs, in particular, AAI is among the most toxic of AAs (Lajide et al., 1993; Bachalandran et al., 2005). AAI is the most abundant AA both in integument and body extracts, which indicates that non-phenolic AAs may have a defensive role.

A defensive role is usually attributed to the osmeterium of Troidini butterflies, since it is partly effective in deterring small predators (Stamp, 1986; Bowers, 1993; Sime, 2002). The presence of deterrent and toxic compounds is reported in the osmeterial secretions of several Troidini (Honda, 1980, 1983; Omura et al., 2006). The presence of AAs in this organ is also widespread in the tribe Troidini; nevertheless, their ecological role is not well established (Nishida, 1995; Sime et al., 2000). Personal observations on the behaviour of larvae of *B. polydamas archidamas* suggest that the strong odour of the substances released by these organs, which are extruded by the larvae when attacked, could function as an alerting signal via associative learning with toxicity, as suggested by Nishida (2002).

The patterns of allocation of secondary compounds to the larval tissues of *B. polydamas archidamas* support the idea that AAs have a defensive role; nevertheless, the results also indicate that the secondary compounds present in the diet were allocated and transformed in different ways depending on the larval tissue. There is now a need for experiments to test the ecological role of the AAs in the different larval tissues.

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