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Aristolochic acids affect the feeding behaviour and development of *Battus polydamas archidamas* larvae (Lepidoptera: Papilionidae: Troidini)

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Abstract. The feeding behaviour of specialist butterflies may be affected by the mechanical and chemical characteristics of the tissues of their host-plants. Larvae of the butterfly, *Battus polydamas archidamas* feed only on *Aristolochia chilensis*, which contains aristolochic acids. We studied the oviposition pattern of adults and the foraging of larvae of *B. polydamas archidamas* over time in relation to variations in hardness of the substrate and concentration of aristolochic acids in different plant tissues. We further tested the effect of two artificial diets containing different concentrations of aristolochic acids on larval performance. *B. polydamas archidamas* oviposited mostly on young leaves and the larvae fed on this tissue until the second instar. Third instar larvae fed also on mature leaves and fourth and higher instars fed also on stems. Young leaves are softer and contain higher concentrations of aristolochic acids than mature leaves, and stems are both harder and contain a high concentration of aristolochic acids. Larvae reared on artificial diets containing a high concentration of aristolochic acids suffered less mortality and were heavier than those reared on a diet with a lower concentration of aristolochic acids, which suggests they are phagostimulatory. A strategy of host use regulated by aristolochic acid content and tissue hardness is discussed.

INTRODUCTION

The exploitation of toxic plants as a source of food by insects was made possible by the evolution of mechanisms for detecting and dealing with plant toxins, a process eventually leading to insect specialization (Ehrlich & Raven, 1964; Barbosa, 1988; Jaenike, 1990; Agrawal & Dorken, 2001). Plant toxins, either intact or suitably modified inside the insect, can even become essential components of the insect's mating, defence and host location systems (Nishida, 2002; Sime, 2002; Murakami et al., 2003; Chachin et al., 2007).

The Lepidoptera are an important group for studying insect specialization because several families within this order are associated with particular host plants on account of their content of certain families of toxic chemicals. For example, Arctiidae are associated with various plant families that contain pyrrolizidinic alkaloids, Heliconidae and Acraenidae with cyanoglycoside-containing Passifloraceae, and Papilionidae, specifically the tribes Troidini and Zerynthiini, with species of the Aristolochiaceae, which are unique in containing aristolochic acids (Ehlrich & Raven, 1964; Nishida, 2002). Larvae of the genus *Battus* (Papilionidae, Troidini) are able to sequester aristolochic acids from their host-plants. The compounds remain in the body of the larvae and are transferred to the adults and their progeny (Sime et al., 2000; Nishida, 2002; Fordyce et al., 2005). Aristolochic acids are used in defence and in selecting oviposition sites by this genus of butterflies (Fordyce, 2000; Nishida, 2005).

Our study focuses on the interaction of adults and larvae of *Battus polydamas archidamas* (Boisduval, 1836) with *Aristolochia chilensis* Bridges ex Lindl., its only host-plant in central Chile (Pinto et al., 2009), and evaluates the effect of the chemical and physical features of this plant on the distribution of larvae on plants and their performance. We assessed: (i) the selection of oviposition substrates by females, (ii) the use of different plant tissues by larvae of *B. polydamas archidamas* during their development in the field, (iii) the aristolochic acid content of the tissues of *A. chilensis* and (iv) the effect of different concentrations of aristolochic acids in artificial diets on growth and mortality of larvae of *B. polydamas archidamas*.

MATERIAL AND METHODS

Oviposition sites and foraging of larvae in the field

Field work was done at Cuesta Lo Prado (15 km west of Santiago, 33°28′S, 70°56′W, 750 m above sea level) from mid-January to mid-March 2007. During this period, plants of *A. chilensis* bore fruit and leaves at different stages of development, but no flowers. Fifty six plants were examined and the number of egg clutches and where they were laid on the plants recorded. A subset of 32 plants of similar size and phenological stage, each bearing a single large egg clutch (10.0 ± 2.5 eggs per clutch), were marked. The larvae on each plant were monitored every three days and the percentage of larvae feeding on each type of tissue, i.e. young (apical) leaves, mature (basal) leaves and stems was recorded. This was continued until they reached the fifth day of the fifth instar.

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Determination of hardness of plant tissues and extraction and chromatographic analysis of aristolochic acids

Another group of 30 plants of similar size and phenological stage as the above plants, but without egg clutches, was used to measure the hardness of young leaves, mature leaves and stems using a Shore A scale durometer (Fowler). The measurements were made on the abaxial surface of the leaves midway along the main vein and ca. 3 mm from it, and midway between two adjacent leaves in the case of stems. Three measurements were made on each plant at 3-week intervals during the study period.

Plant tissue samples from each of the 30 plants used for the hardness measurements (ca. 0.4 g of fresh weight of young and mature leaves and stems) were collected and weighed, and later macerated with 5 ml methanol (MeOH). Extracts were centrifuged for 6 min at 13,000 rpm (Heraeus, Biofuge 13), filtered through 0.45 µm Ø 13 mm filters (Millipore) and analysed using HPLC (Shimadzu LC-10A) coupled with UV-Vis detection at 250 nm using a C18 column (Phenomenex, 250 × 4.6 mm, 5 µm particle size). The mobile phase was a mixture (40 : 60) of 1% aqueous acetic acid and 1% acetic acid in acetonitrile flowing at 1 ml/min. The total concentrations of aristolochic acids (I and II) (Poonam et al., 2003) was determined by comparison with calibration curves constructed using standards isolated by preparative thin layer chromatography from a commercial mixture (Aldrich Chem Co.).

Rearing larvae in the laboratory

Eggs (ca. 400 for each diet) were collected in the field. The plant tissue around individual egg clutches was removed so that the neonates did not come into contact with chemicals of the plant. Each egg clutch was kept in a disposable Ø 35 mm Petri dish (Corning Glass Works) until eclosion. First instar larvae were transferred to new Petri dishes (Ø 55 mm) each containing a 25 × 15 × 10 mm (ca. 2 g) portion of artificial diet. The size of the containers was increased to Ø 60 mm for the third to fifth instar larvae and to Ø 110 mm for the sixth to seventh instar larvae so that the larvae could move more freely. The number of individuals per container was thus decreased from ca. 10 first instar larvae to one fourth instar larva. This simulated the aggregation patterns observed in the field (Pinto et al., 2009). Containers were kept at a photoperiod of 16L : 8D and a mean temperature of 23°C.

The composition of the meridic diet used was based on one used for *Battus philenor* (Fordyce & Nice, 2008). The basic diet incorporated 8% (dry w/wet w) of ground aerial tissue from a single batch of dried host plants; this resulted in a concentration of 0.62 mg aristolochic acids/g in the basic diet. As the aerial tissues of the host plant during our study contained a mean of ca. 1.5 mg/g fresh tissue, the basic diet was supplemented with different amounts of pure aristolochic acids I and II in the ratio present in the plant to produce two experimental diets, a low-aristolochic acid diet containing one-half the mean concentration in the host plant (0.75 mg/g fresh tissue) and a high-aristolochic acid diet containing twice that concentration (3 mg/g fresh tissue). Diets were prepared with a mixture of aristolochic acids I and II in order to mimic the conditions in the plant. The diets were prepared weekly and kept at 4°C. The instruments and larval containers were irradiated with UV light for 45 min before use, and all processing of diet and larvae was done in a laminar flow chamber to avoid contamination of the diets and larvae by fungi and other microorganisms. The containers were sealed with a Parafilm® (Pechiney Plastic Packaging, Chicago) membrane with two small holes that prevented desiccation of the diet but allowed the escape of excess humidity. The portions of diet offered to larvae were replaced every two days and the containers changed every 6 days.

The groups of larvae were monitored daily to determine the duration and mortality in each larval instar. In order to avoid mortality of the initial larval instars due to handling, larvae were weighed (Precisa Instruments AG) only after moulting to the third instar.

Data analysis

The frequency of oviposition on different plant tissues (young leaves, mature leaves and stems) was compared using a Chi-squared test. Concentration of aristolochic acids in different plant tissues was compared using a one way ANOVA for ranked data (Kruskal-Wallis test) followed by a post-hoc Tukey test. The hardness of different plant tissues over time was compared using a one way repeated measure ANOVA followed by post-hoc Tukey tests. The proportion of individuals from a clutch of eggs found on different plant tissues in the field over time were compared using a two-factor ANOVA with repeated measures of both factors; the data were ranked, the Scheirer-Ray-Hare correction applied (Sokal & Rohlf, 1995) and the Holm-Sidak test used for post-hoc comparisons. In the diet experiments, the percentage mortality of individuals in each replicate and the weight of the larvae from the third instar onwards were compared using a two-way non-parametric ANOVA (the Scheirer-Ray-Hare extension of the Kruskal-Wallis test, Sokal & Rohlf, 1995) followed by post-hoc Holm-Sidak tests with diet and larval instar as factors.

RESULTS

Oviposition and feeding sites of larvae in the field

A total of 78 egg clutches were found. They occurred mostly on young leaves and to a lesser extent on mature leaves and stems (78.2, 16.7 and 5.1% on young leaves, mature leaves and stems, respectively; $X^2 = 11.2$, $P < 0.005$). Larvae were distributed heterogeneously on the different types of plant tissue ($H_{plant\ tissue} = 47.806$, $P < 0.005$) (Fig. 1) and there was a progressive increase in the use of mature leaves and stems by the older larval stages ($H_{larval\ instar\ x\ plant\ tissue} = 57.959$, $P < 0.005$). The first larval instar fed mostly on the cuticle of young leaves; shortly before moulting to the second instar they began feeding on all the tissues of young leaves. Second instar larvae

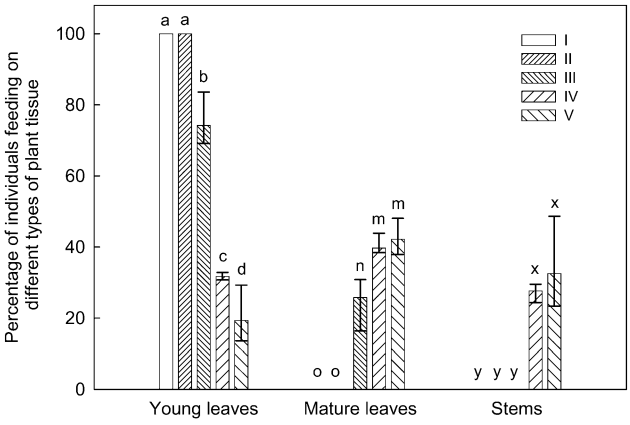

Fig. 1. Percentage (median and interquartile range of data are plotted) of larvae (instars: I, II, III, IV, V) of *Battus polydamas archidamas* feeding on young and mature leaves, and stems of the host plant. Different letters above the columns within each type of tissue indicate significant differences ($P < 0.05$, Holm-Sidak tests).

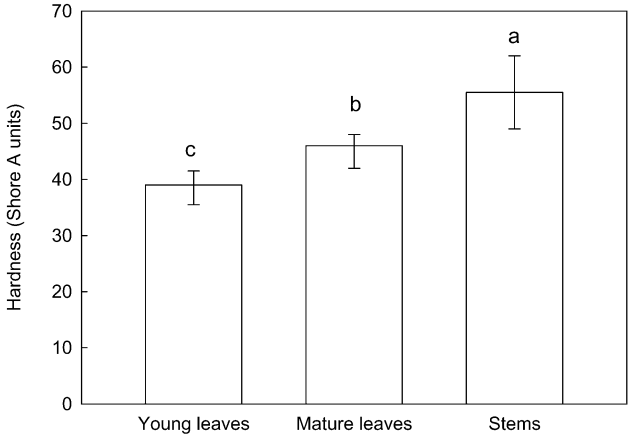

Fig. 2. Relative hardness of tissues of *Aristolochia chilensis* (median and interquartile range of data are plotted). Different letters (a, b and c) above columns indicate significant differences ($P < 0.05$, Tukey tests).

also fed exclusively on young leaves. Third instar larvae also ate mature leaves, and fourth and fifth instar larvae fed on young leaves, mature leaves and stems.

Characteristics of plant tissues

Hardness of young leaves, mature leaves and stems differed significantly ($H = 49.41$, $P < 0.001$) but not among samples of the same tissue collected on different occasions. Young leaves were softer than mature leaves and stems the hardest tissue (Fig. 2).

The total concentration of aristolochic acids in the different plant tissues differed significantly ($H = 17.86$; $P < 0.001$) with the young leaves and stems richer in aristolochic acids than mature leaves (Fig. 3).

Mortality and weight of larvae reared on diets with low and high concentrations of aristolochic acids

Three supernumerary moults (VI, VII and VIII) were observed on both diets. The mortality of larvae was significantly associated with the concentration of aristolochic acids, the developmental stage of the individual

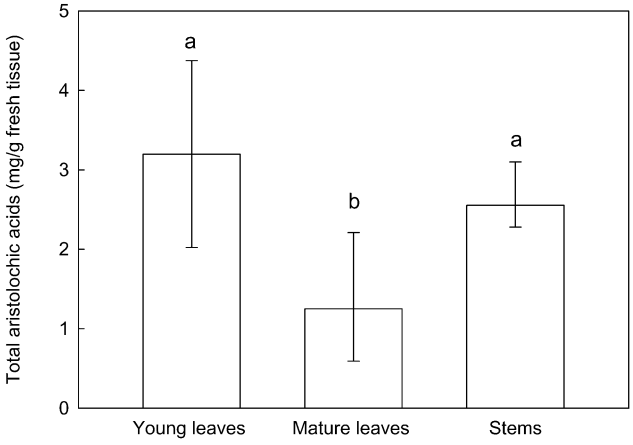

Fig. 3. The total concentration of aristolochic acids (I and II) in young and mature leaves, and stems of *Aristolochia chilensis*. Different letters (a, b and c) above columns indicate significant differences ($P < 0.05$, Tukey tests).

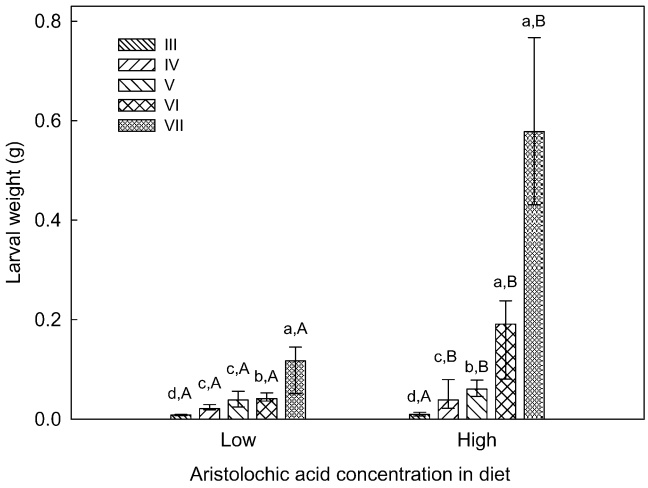

Fig. 4. The weight of larvae reared on a meridic diet containing either half or twice the concentration of aristolochic acids recorded in the aerial tissue of their host plant. Different letters above the columns indicate significant differences ($P < 0.05$, Tukey tests); low-case letters refer to intra-diet comparisons and capital letters refer to inter-diet comparisons.

and the interaction of the developmental stage with aristolochic acid concentration ($H = 16.73$, $P < 0.001$; $H = 52.01$, $P < 0.001$; and $H = 14.57$, $P < 0.01$, respectively). Larval mortality was highest on the low-aristolochic acid diet. On both diets, mortality was highest in the first two instars and approached zero in the sixth instar.

Larval weight was associated with the concentration of aristolochic acid in the diet, developmental stage and their interaction ($H = 56.24$, $P < 0.001$; $H = 47.30$, $P < 0.001$; and $H = 16.05$, $P < 0.005$, respectively). From the IV instar onwards larvae reared on the low-aristolochic acid diet were lower in weight than those reared on the high-aristolochic acid diet. The mean final weight of the VII instar was almost five times greater on the high-aristolochic acid diet than on the low-aristolochic acid diet (Fig. 4).

DISCUSSION

Oviposition by *B. polydamas archidamas* occurred mostly but not exclusively on young leaves of *A. chilensis* and the first and second instar larvae were only found feeding on young leaves. The apparent discrepancy between site of occurrence of egg clutches and of first instars may be due to death of first instar larvae while moving to more palatable tissues. Older larvae also include mature leaves and stems in their diet. Young leaves were softer and contained a higher concentration of aristolochic acids than mature leaves, whereas stems were harder and contained a high concentration of aristolochic acids. Hardness and age of leaves are important for young larvae, which preferred young leaves with little sclerophylly (Rausher, 1980). A similar pattern of feeding is described for the lepidopteran *Ostrinia nubilalis* (European corn borer) feeding on maize, a plant characterised by the presence of benzoxazinoid hydroxamic acids, which are toxic for the larvae (Niemeyer, 2009). Thus, third instar larvae of *O. nubilalis* prefer immature tissues

characterised by a combination of a relative absence of physical defenses and higher nutritional value, in spite of high levels of benzoxazinoid hydroxamic acids (Bergvinson et al., 1995); as the larvae mature and can cope with tougher tissues they prefer older tissue (Mao et al., 2007). Thus, it is likely that the feeding patterns of first and second instar larvae of *B. polydamas archidamas* is affected by tissue hardness because of developmental constraints associated with their buccal apparatus and physiology, and that the importance of tissue hardness decreases when these constraints cease to exist. In spite of the similarities in the biology of these two species it is possible that other factors important for larval feeding, such as water content and nitrogen availability, covary with tissue hardness (Scriber & Slansky Jr., 1981).

Artificial diets allow one to study the effect of aristolochic acids on larval performance in the absence of hardness. The experiments revealed that larvae feeding on diets containing a low concentration of aristolochic acids suffered higher levels of first-instar mortality and were lower in weight from the fourth instar onwards than larvae fed on diets containing a high level of aristolochic acids. The positive effects of high concentrations of aristolochic acids in the diet suggest they have a phagostimulant effect. Aristolochic acids in diets are known to increase larval weight in *B. polydamas* (Miller & Feeny, 1989) and trigger the acceptability of plant tissues by *Battus* species (Nishida & Fukami, 1989). Early instars of lepidopteran larvae are known to be sensitive to changes in their environment, particularly chemical ones (Zalucki et al., 2001) and mortality of early larval instars is on average close to 50% (Zalucki et al., 2002). In our experiments, mortality of first instar larvae was 87% on the low-aristolochic acid diet and only 57% on the high-aristolochic acid diet. Hence, it may be argued that hatchling larvae may not have recognised the low-aristolochic acid diet as a food source because the aristolochic acid concentration was only half that found in plants. Interestingly, mortality of first instar larvae reared on the high-aristolochic acid diet did not differ substantially from expectation based on reports in the literature (Zalucki et al., 2002).

The combination of high levels of aristolochic acids (Sachdev-Gupta et al., 1993) and tissue softness may in part determine the selection of oviposition sites by females of *B. polydamas archidamas*. The most likely benefit of choosing young leaves for oviposition is the increased survival and growth of the larvae during their early stages of development, when they are less mobile and more prone to suffer from environmental stresses.

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