IMPORTANCIA DE LA QUÍMICA DE LA PLANTA EN LA INTERACCIÓN ENTRE *Battus polydamas archidamas* (Papilionidae) Y *Aristolochia chilensis* (Aristolochiaceae)

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INFORME DE APROBACION

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Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Magíster en Ciencias con mención en Ecología y Biología Evolutiva, en el examen de Defensa Privada de Tesis rendido el día 29 de diciembre de 2009

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A mi madre

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RESUMEN

Las mariposas de la tribu Troidini (Papilionidae) son especialistas en plantas de la familia Aristolochiaceae que contienen compuestos secundarios denominados ácidos aristolóquicos. Las larvas de *Battus polydamas archidamas* (Papilionidae) se encuentran en el campo en forma practicamente exclusiva sobre *Aristolochia chilensis* (Aristolochiaceae). Se estudió: i) los patrones de oviposición, los patrones de agregación larval y los patrones de forrajeo sobre los tejidos de la planta, en el campo; ii) la respuesta de adultos de ambos sexos hacia señales químicas y visuales de su planta hospedera; iii) el efecto de dos concentraciones (alta y baja) de AAs en dietas artificiales sobre larvas durante todo su desarrollo y iv) los patrones de secuestro en distintos tejidos de la larva de AAs en dietas artificiales, a lo largo del desarrollo larval. Se observó que: i) las hembras ovipositan sobre hojas jóvenes, la primera dispersión larval ocurre al final del tercer estadío, las larvas se alimentan de hojas jóvenes hasta el final del segundo estadío y luego amplían su dieta hacia los demás tejidos de la planta; ii) tanto adultos como larvas reconocen estímulos químicos volátiles de su planta hospedera; iii) los AAs en la dieta tienen un efecto fagoestimulante, que en los primeros estadíos se traduce en una alta mortalidad a concentraciones bajas y iv) el secuestro por las larvas aumenta a lo largo del desarrollo y dependiendo de la concentración de AAs en la dieta; los osmeterios parecen tener procesos más selectivos. Se discute la relación entre los datos de campo y de laboratorio.

ABSTRACT

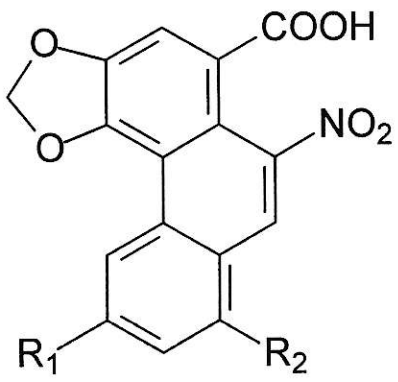
Butterflies of the Troidini tribe are specialists in plant of the Aristolochiaceae family characterized by contain secondary compounds named aristolochic acids (AAs). Larvae of *Battus polydamas archidamas* (Papilionidae: Troidini) are found in the field almost exclusively on *Aristolochia chilensis* (Aristolochiaceae). We studied i) the patterns of oviposition by adults, larval aggregation, and foraging in the field, ii) the response by adults of both sexes and larvae to chemical and visual signals of the host plant, iii) the effect of two concentrations (low and high) of AAs during larval development and iv) the patterns of sequestration of AAs from meridic diets in different tissues during larval development.,We observed that: i) females oviposited on young leaves, the first larval dispersion occurred at the end of the third instar, larvae fed on young leaves until the third instar and thereafter increased their diet bredth to include other plant tissues; ii) adults and larvae recognize chemical volatiles of their host plant; iii) the AAs in the diet showed a phagostimulant effect on larvae; this effect increased larval mortality in the younger instars and iv) the sequestration of AAs increased in relation to the concentration of AAs in the diet and larval instar and osmeterial glands showed more selective sequestration. Field and laboratory data will be discussed.

INTRODUCCIÓN GENERAL

La especialización ecológica en insectos puede ser el resultado de una eficiente explotación de plantas tóxicas como fuente de alimento y del desarrollo de mecanismos para detectar y lidiar con las toxinas de dichas plantas (Ehrlich & Raven, 1964; Barbosa, 1988; Jaenike, 1990; Agrawal & Dorken, 2001). Muchas familias dentro de los lepidópteros son especialistas en un rango restringido de plantas hospederas, caracterizadas por la presencia en ellas de ciertas familias de compuestos químicos tóxicos (Nishida, 2002; Schoonhoven, 2005). Por ejemplo, la familia Arctiidae está asociada a varias familias de plantas que contienen alcaloides pirrolizidínicos. Las familias Heliconidae y Acraenidae están asociadas a pasifloráceas que contienen glicósidos cianogénicos, y la familia Papilionidae, específicamente las tribus Troidini y Zerinthiini, están asociados a especies de plantas de la familia Aristolochiaceae, que contienen ácidos aristolóquicos (Ehlrich & Raven, 1964; Nishida, 2002). Las toxinas de las plantas en su forma intacta o convenientemente modificadas dentro del insecto, pueden convertirse en componentes esenciales de procesos ecológicos tales como defensa, selección de hospedero e incluso el apareamiento (Nishida, 2002; Sime, 2002; Murakami et al., 2003; Chachin et al., 2007).

El género *Battus* (Papilionidae: Troidini) se caracteriza por ser monófago,

alimentándose exclusivamente en plantas del género *Aristolochia* durante su desarrollo larval (Feeny, 1991; Weintraub, 1995). Los ácidos aristolóquicos que contienen estas plantas (Poonam et al., 2003) son secuestrados por las larvas durante su desarrollo, se mantienen en el cuerpo de las mariposas durante toda su vida y son transferidos a los huevos por adultos de ambos sexos (Urzúa & Priestap, 1985; Urzúa et al., 1987; Fordyce et al., 2005). Larvas y adultos utilizan los ácidos aristolóquicos como compuestos defensivos (Rothschild et al., 1970; Nishida & Fukami, 1989; Feeny, 1991, 1995; Fordyce, 2000, 2001; Sime, 2002) además de encontrarse entre los compuestos utilizados para seleccionar hospedero durante la oviposición (Papaj et al., 1992; Sachdev-Gupta et al., 1993). Los ácidos aristolóquicos se encuentran distribuidos en los tejidos de la planta de manera heterogénea (Fordyce, 2000), por lo que las larvas necesitan lidiar con diversos escenarios químicos que la planta les ofrece durante su desarrollo.



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

El proceso de selección de hospedero por este tipo de mariposas involucra el acercamiento hacia hospederos potenciales mediado en general tanto por factores visuales como por compuestos volátiles de la planta, y la evaluación de la calidad del sustrato en contacto con él. Estos procesos culminan con la aceptación o rechazo de las plantas dentro de la población local (Schoonhoven et al., 2005). En el caso de *Battus*, los ácidos aristolóquicos cumplen un importante rol durante la fase de contacto y evaluación. Sin embargo, debido a su escasa volatilidad, ellos no pueden ser detectados a distancia durante la fase de reconocimiento. Las señales químicas utilizadas durante la primera fase de la selección de hospedero no han sido identificadas. Ellas deberían ser importantes tanto para las larvas al momento de buscar nuevos individuos de hospedero durante su dispersión como para las hembras al momento de buscar lugares de oviposición (Tatar, 1991; Rausher, 1995; Fordyce, 2003).

En Chile Central, *Battus polydamas archidamas* se alimenta como larva y oviposita como hembra adulta sobre *Aristolochia chilensis*. Los ácidos aristolóquicos se encuentran en la planta en dos formas predominantes, el AAI y el AAII (Urzúa *et al.*, 1987) Se ha determinado que las larvas de las mariposas secuestran el 2% del total de ácidos aristolóquicos presentes en la planta (Urzúa & Priestap, 1985), aunque no se sabe cómo se distribuyen ellos en el cuerpo de la mariposa.

En esta tesis, me he planteado como evaluar: i) la atracción de adultos y larvas de *Battus polydamas archidamas* hacia *Aristolochia chilensis* mediada por señales químicas volátiles emitidas por la planta, ii) la importancia de características físicas y químicas de la planta sobre la distribución y desempeño de las larvas en su planta hospedera, y iii) los patrones de secuestro de ácidos aristolóquicos en distintos tejidos durante el desarrollo larval utilizando dietas artificiales que simulen distintos escenarios químicos ofrecidos por la planta.

Los resultados de mis investigaciones están resumidas en dos artículos que ya han sido publicados (capítulos I y II) y un manuscrito que está pronto a ser enviado para su publicación (capítulo III). Estos se insertan a continuación.

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Use of volatiles of *Aristolochia chilensis* (Aristolochiaceae) in host searching by fourth-instar larvae and adults of *Battus polydamas archidamas* (Papilionidae: Troidini)

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Key words. Host finding, aristolochic acids, host specialisation, host-plant volatiles, *Battus polydamas archidamas*

Abstract. Papilionid butterflies of the tribe Troidini are specialists on plants of the family Aristolochiaceae. The role of plant volatiles in host recognition by adult and larval stages of these insects remains unknown. We used *Battus polydamas archidamas* (Papilionidae: Troidini) and its host-plant, *Aristolochia chilensis* (Aristolochiaceae), to study: (i) the olfactory and electrophysiological responses of adults to headspace volatiles of the host-plant, (ii) the chemical composition of the headspace volatiles of the host-plant, (iii) the patterns of aggregation of larvae in the field in order to ascertain the time when they leave the plant where the eggs were laid, and (iv) the olfactory responses of solitary-feeding fourth-instar larvae to headspace volatiles of the host-plant. Larvae left their initial host-plant during the third or fourth instar. Host-plant headspace volatiles attracted fourth-instar larvae as well as adults; adult females were more responsive than males. Taken together, these results reveal changes in the responsiveness to host-plant volatiles during development, and provide an insight into the host-plant specialization of this butterfly.

INTRODUCTION

Host selection behaviour in insects consists of two consecutive phases: (1) search and recognition, usually ending with the finding of one or more potential host-plants, and (2) contact and evaluation, which ends with the acceptance or rejection of an individual plant within a local population of putative host-plants (Schoonhoven et al., 2005). Host selection is mediated by the integration within the insect central nervous system of numerous sensory inputs (Hansson, 2002; Bruce et al., 2005). Plant chemical signals can be of particular importance since plant tissues contain taxon-specific compounds or chemical blends, which can be used as cues during host selection (Nishida, 2002; Schoonhoven et al., 2005). Unique insect-plant associations can thus be established in which an insect species uses a single or a few host-plant species usually containing toxic qualitative defences (sensu Feeny, 1976; Bustamante et al., 2006) which are used by the insect for host recognition.

During the searching and recognition phase of host selection butterflies use visual and olfactory sensors located in their eyes and on their antennae, respectively (Hansson, 2002; Nishida, 2005). When contact is made with a plant, taste and mechanoreceptors located on the foretarsi and palpi are of paramount importance in the exploration of the plant surface, triggering the final decision on acceptance or rejection of the plant. Among the plant chemicals involved in the establishment of unique butterfly-plant associations are pyrrolizidine alkaloids in the Asteraceae, Fabaceae and Boraginaceae, which are important cues for many species of Arctiids, cyanoglycosides in Passifloraceae for heliconid and acraenid butterflies, iridoid glycosides in various plant families (i.e. Scrophulariaceae, Plantaginaceae, and Verbenaceae) for the common buckeye butterfly (*Junonia coenia*: Nymphalidae) (Nishida, 2002).

Among papilionids, the neotropical genus *Battus* is characterized by being monophagous and feeding only on plants of the genus *Aristolochia* during the larval stage (Feeny, 1991; Weintraub, 1995; Nishida, 2002). *Aristolochia* species contain toxic aristolochic acids (AAs) (Poonam et al., 2003) that are sequestered by the larvae and transferred to the eggs by adults of both sexes (Urzúa & Priestap, 1985; Urzúa et al., 1987; Fordyce et al., 2005). The aposematic larvae and adults use AAs as defensive compounds (Rothschild et al., 1970; Nishida & Fukami, 1989; Feeny, 1991, 1995; Fordyce, 2000, 2001; Sime, 2002). Aristolochic acids (Sachdev-Gupta et al., 1993) and also D-(+)-pinitol (Papaj et al., 1992) are among the plant cues used by females during the contact and evaluation phase of host selection when searching for a site for oviposition. To the best of our knowledge the plant chemical cues used by *Battus* females during the first phase of host selection are unknown.

On the other hand, large clutch sizes and aggregative feeding by larvae are characteristic of various families of Lepidoptera, papilionids among them (Sillén & Tullberg, 1988). For example, females of *Battus philenor* lay clutches of eggs on the leaves of *Aristolochia californica* and the larvae feed gregariously until the late third instar, when they disperse and feed solitarily (Tatar, 1991;

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Rausher, 1995; Fordyce, 2003). This raises the question – how do the dispersing larvae find new host-plants.

The host-plants of *Battus polydamas archidamas* (Boisduval, 1836), the only species of this genus in Chile, are species of *Aristolochia* (Peña & Ugarte, 1997). In this paper we explore the attraction of adults of *B. polydamas archidamas* to volatiles emitted by *Aristolochia chilensis* Bridges ex Lindl., describe the aggregation patterns of larvae in the field and determine the response of solitary feeding larvae to volatiles from *A. chilensis*.

MATERIAL AND METHODS

Battus polydamas archidamas and *Aristolochia chilensis*

Adults of *B. polydamas archidamas* oviposit on *A. chilensis* and *A. bridgesii* (Klotzsch) Duchart. (Aristolochiaceae), two perennial pipevines endemic to Chile (Marticorena & Quezada, 1985). The distribution range 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). Field work was done at Cuesta Lo Prado (33°28′S, 70°56′W, 750 m above sea level) 15 km west of Santiago.

Host recognition by adults of *B. polydamas archidamas*

Adult individuals of *B. polydamas archidamas* were caught in the field during January and February 2007, and the sexes kept separately in the laboratory. All adults tested were relatively young, as their wings were relatively undamaged (Tsubaki & Matsumoto, 1998), and were acclimatized for at least two days to the laboratory conditions before subjecting them to the tests. Tests were performed in a rectangular cubicle with white painted walls (4.4 m × 2.2 m sides, 2 m high) maintained at 25°C and a 16L : 8D photoperiod. A test stimulus was placed at one end of one of the short sides of the cubicle and a control stimulus at the other end. An adult butterfly was liberated near the centre of the opposite short side by an observer hidden behind a white screen. Test stimuli were a potted plant enclosed in a bell shaped glass jar provided with an inlet and an outlet (odour and visual stimuli) or a similar system covered with white paper (odour only stimulus). In both cases the control stimuli were similar but the pot contained only soil, i.e. lacked a plant. The inlets of the glass jars were connected to a compressed air cylinder containing synthetic air made from extra pure oxygen and nitrogen, with no detectable organic impurities, which had a regulator that provided a flow of 0.5 L of air/min through each jar. A test ended when a butterfly remained inactive for more than 5 min near the site of release, remained at one of the odour sources for more than 5 min, or was unable to make a choice between the stimuli offered within 15 min.

Collection and analysis of headspace volatiles of *A. chilensis*

Leaves and stems of *A. chilensis* (ca. 250 g fresh weight per sample, N = 3) were gathered and placed inside a glass jar with an inlet and an outlet. At the inlet, a compressed air cylinder was attached via an air flow regulator. Attached to the outlet was a glass column containing Porapak Q (30 mg). Volatile entrainment (5 h with an air flow of 0.5 L/min) commenced immediately after the plant samples were placed inside the glass jar. The volatiles adsorbed onto the Porapak Q columns were eluted with 500 µl of dichloromethane. These extracts were analysed using gas chromatography (GC, Hewlett Packard model HP5891) equipped with an Ultra 2 (25 m × 0.2 mm Ø, Agilent Technologies) capillary column and a mass spectrometric detector (Hewlett Packard model HP5972). Ionisation by electron impact (70 eV) was carried out at 280°C. The GC oven was programmed to remain at 50°C for 10 min, to increase up to 280°C at a rate of 5°C/min and then remain at 280°C for 5 min. The identification of compounds in the chromatographic profiles was achieved by comparison of their mass spectra with those in the NIST98 library database and confirmed by comparison of Kovats indexes with those of authentic standards or with values from the literature. Identifications were considered positive if the similarity index between experimental and library mass spectra was higher than 95%, and if the Kovats indexes did not differ by more than 5 units (differences were typically less than 3).

Electroantennograms recorded from the antennae of *B. polydamas archidamas*

Electroantennograms were recorded in order to determine the role of volatiles in the attraction of the adults to their host-plant. Antennae of male and female adults of *B. polydamas archidamas* were excised and their tip and base immersed in glass electrodes filled with Dicardio-Gel® (Difem Pharma). The antenna was continuously flushed with a stream of moistened air passing through an aeration tube placed 15 mm away from the antenna. The tube had a 2 mm hole. The treatment stimuli were prepared by placing 3 µl of a dichloromethane solution of plant headspace volatiles on a piece of filter paper (10 × 15 mm), which was placed inside a Pasteur pipette. The blank control consisted of a piece of filter paper plus 3 µl of dichloromethane. The stimuli were delivered to an antenna by inserting the end of the Pasteur pipette in the hole in the aeration tube and abruptly flushing it with 1 ml of air by means of a stimulus controller CS-05 (Syntech, Hilversum, The Netherlands). The signal was amplified using a high impedance amplifier, and stored and analysed by a PC equipped with an IDAC-card and the program AutoSpike 32, both from Syntech (Hilversum, The Netherlands).

Aggregation patterns of larvae of *B. polydamas archidamas* in the field

During February and March 2007, a total of 114 plants of *A. chilensis* in the study area were examined and their size, phenological stage and presence of egg clutches of *B. polydamas archidamas* recorded. Thirty-two plants of similar size and phenological stage, and bearing a single large egg clutch (10.0 ± 2.50 eggs per clutch) were chosen and marked. Plants were monitored every three days until larvae reached the fifth instar stage. The sizes of the egg clutches were recorded and the number of both aggregated and solitary larvae determined at the end of each larval stage. A larva was considered solitary if it was more than 30 cm away from another larva. The number of dead larvae within a 1 m radius circle around the focal plant were also counted at each observation period.

Host recognition by larvae of *B. polydamas archidamas*

Eggs of *B. polydamas archidamas* were collected during February 2007 and reared in a laboratory chamber maintained at 27°C and a 16L : 8D photoperiod. The larvae were fed *ad libitum* with fresh stems with leaves of *A. chilensis* provided three days until the fourth instar. Their behaviour was observed in a white Plexiglas 3-sided rectangular arena (6 cm wide, 4 cm high, 60 cm long) whose top was covered with white cotton mesh. The ends of the arena were also covered with white cotton mesh, which permitted volatiles from the treatment and control stimuli placed near the ends to diffuse into the arena. A small opening in the centre of the cotton mesh that covered the top allowed the introduction of larvae into the system. The floor of the arena was divided along its length into three zones; the central zone from which the larvae moved towards either of the two stimulus sources, and the two zones at the ends (10 cm from

TABLE 1. Results of olfactory tests on female and male adults and larvae of *Battus polydamas archidamas*. Frequency of individuals in zones permeated with stimuli A and B were compared using a Chi-squared procedure.

Stimulus A	Stimulus B	Adults in A	Adults in B	Adults not choosing	P
A. Bioassays with female adults					
Exposed potted plant	Exposed pot	33	5	1	< 0.001
Covered potted plant	Covered pot	32	4	0	< 0.001
B. Bioassays with male adults					
Exposed potted plant	Exposed pot	22	7	4	< 0.01
Covered potted plant	Covered pot	22	9	2	< 0.05
Stimulus A	Stimulus B	Larvae in A	Larvae in B	Larvae in neutral zone	P
C. Bioassays with larvae					
Potted plant	Pot without plant	32	0	0	< 0.001
Extract of headspace volatiles from plant on filter paper	Solvent on filter paper	17	0	3	< 0.001
Extract of headspace volatiles from plant on filter paper	Potted plant	10	9	1	n.s.
Nothing	Nothing	0	0	20	n.s.

the ends of the arena). Entry into and occupation of one of the latter was assumed to indicate attraction to that stimulus. The position of each larva after 20 min was noted. All tests were performed under the same conditions of temperature and lighting as in the rearing chamber, with diffuse illumination provided from above the arena. The treatments were: a fresh potted plant surrounded by a cylinder of black cotton mesh, and a dichloromethane extract of headspace volatiles from a fresh plant presented on filter paper. The corresponding controls were a pot surrounded by a cylinder of black cotton mesh and filter paper spotted with dichloromethane. In addition, there was a control in which no stimulus sources were placed near the ends of the arena. Each test was terminated if the test larva spent more than 5 min in the central zone or in either of the stimulus zones, or more than 15 min without making a selection.

Data analysis

The frequency with which males and females selected covered and exposed plants relative to pots with soil were compared using Chi-squared tests. The time to recognition of covered and exposed host-plant by males and females were compared using a non-parametric two-way ANOVA (the Scheirer-Ray-Hare extension of the Kruskal-Wallis test, Sokal & Rohlf, 1995) followed by post-hoc Tukey tests (Sokal & Rohlf, 1995). EAG results (differences in intensity of the signals from the antenna stimulated by headspace volatiles and solvent), aggregation patterns in the field (percentages of individuals that aggregated in the different larval stages), and mortality (percentage mortality in each larval stage) were compared using a one way ANOVA of ranked data (Kruskal-Wallis test) followed by post-hoc Tukey tests. Olfactory tests of larvae (number of individuals in the two stimulus zones) were compared using Chi-squared tests.

RESULTS

Host recognition by adults of *B. polydamas archidamas*

Males and females prefered the plant over the other stimulus, both when the plant was exposed and covered by a cloth (Table 1, A and B). The times taken to choose volatile-emitting exposed and non-exposed plants did not differ ($H = 0.13$, $P = 0.625$), but there were significant differences between sexes ($H = 29.01$, $P < 0.001$) and in the interaction between treatment and sex ($H = 9.31$, $P < 0.001$) (Fig. 1). Females both recognised host-plants and arrived at exposed plants relative to non-exposed plants faster than males. EAG tests confirmed sex-dependent sensitivity, females being more sensitive than males to volatiles from their host-plants [mV, median (interquartile range); females ($N = 22$): 4.36 (2.93–6.54), males ($N = 22$): 1.41 (0.62–2.04); ANOVA: $H = 23.84$, $P < 0.001$].

Composition of the headspace volatiles of *A. chilensis*

Table 2 summarises the analysis of the headspace volatiles of intact plants of *A. chilensis*. A total of 53 compounds were identified, corresponding to 96.4% of the total mixture of compounds. The mixture consisted mainly of monoterpenes (38.8%), esters (17.1%), sesquiterpenes (16.8%), green leaf odours (15.3%), hydrocarbons (3.3%) and miscellaneous compounds (5.1%).

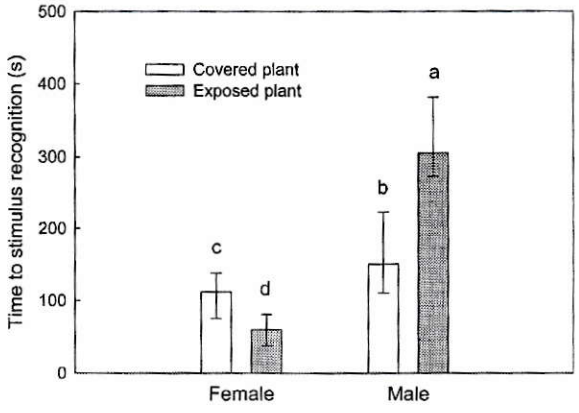

Fig. 1. Time to host-plant recognition by males and females of *Battus polydamas archidamas*. Plant volatiles were from an exposed or a covered plant (median and interquartile range of data is plotted).

TABLE 2. Volatiles in the headspace of plants of *Aristolochia chilensis*.

Compound	RI [a]	% [b]
Hexanal	796	1.04
Ethyl butyrate	800	0.38
(*E*)-2-Hexenal	850	2.40
(*Z*)-3-Hexen-1-ol	861	10.96
1,3-Dimethylbenzene	866	1.82
(*E*)-2-Hexen-1-ol	869	0.89
1,2-Dimethylbenzene	889	0.88
Nonane	900	0.58
α-Thujene	925	2.04
α-Pinene	932	4.38
Sabinene	973	6.90
β-Pinene	977	2.37
β-Myrcene	990	0.84
1,3,5-Trimethylbenzene	992	0.88
Decane	1000	1.13
(*Z*)-3-Hexen-1-ol, acetate	1007	4.38
α-Terpinene	1017	0.46
p-Cymene	1025	1.53
Limonene	1029	4.95
1,8-Cineol	1032	1.15
(*Z*)-Ocimene	1039	7.86
α-Ocimene	1048	1.82
γ-Terpinene	1060	1.41
cis-β-Terpineol	1070	0.58
p-Cresol	1078	0.46
Methyl benzoate	1096	0.98
Undecane	1099	0.55
Linalool	1100	0.50
Nonanal	1103	0.50
3-Methy-3-butenyl isovalerate	1116	1.31
Allo-ocimene	1129	1.92
Ethyl benzoate	1172	0.26
4-Terpineol	1181	0.17
(Z)-3-Hexen-1-ol, butyrate	1186	1.27
Methyl salicylate	1198	5.18
Decanal	1205	0.47
(*Z*)-3-Hexen-1-ol, 2-methylbutyrate	1232	0.51
(*Z*)-3-Hexen-1-ol, isovalerate	1235	0.27
(*Z*)-3-Hexen-1-ol, hexanoate	1380	0.54
Hexyl hexanoate	1385	0.29
Tetradecane	1400	0.37
β-Caryophyllene	1433	4.67
γ-Elemene	1442	0.80
β-Gurjunene	1447	1.88
(*E*)-β-Farnesene	1458	0.49
α-Humulene	1467	0.78
Alloaromadendrene	1475	1.36
Germacrene D	1494	1.46
Ledene	1500	0.78
β-Bisabolene	1513	1.63
δ-Cadinene	1533	2.97
(*Z*)-3-Hexen-1-ol, benzoate	1577	1.80
Heptadecane	1700	0.63
Identified compounds (%)		96.40

[a] Relative retention indices on an Ultra 2 column relative to *n*-alkanes; [b] Peak areas relative to total peak area (means of three samples).

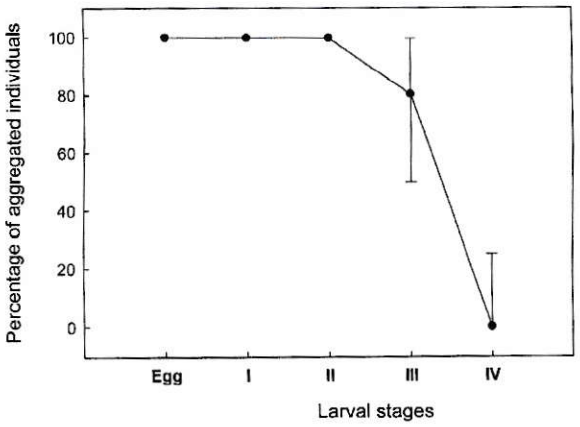

Fig. 2. Mean percentage of individuals that aggregate in the different stages of development of *Battus polydamas archidamas* (median and interquartile range of data are plotted).

Aggregation patterns of larvae of *B. polydamas archidamas* in the field

There were significant differences in the mean percentage of individuals that aggregated during development from egg through the different larval stages ($H = 120.44$, $P < 0.001$). All aggregated from the egg to second instar stage, and then the incidence of aggregation decreased from the third to fourth instar, when most of the individual larvae were found feeding solitarily (Fig. 2). There were significant differences in mortality ($H = 31.49$, $P < 0.001$), with most larvae dying during the first larval stage; differences in mortality between the other stages were not significant. Dead larvae showed symptoms of desiccation, and no parasitization or predation events were observed.

Host recognition by larvae of *B. polydamas archidamas*

Fourth instar larvae were used because this is the instar that predominantly disperses from the plant on which they are born. Larvae were preferentially attracted to volatiles emitted by plants and extracts of headspace volatiles collected of the plant presented on filter paper. When both stimuli were offered the larvae were equally attracted to both, but showed no selection when no stimuli were offered (Table 1, C).

DISCUSSION

Behavioural and EAG bioassays showed that adults of *B. polydamas archidamas* respond from a distance to volatile chemicals emitted by their host-plant, *A. chilensis*. Larvae of *B. polydamas archidamas* disperse in the field at the end of the third instar or beginning of the fourth instar, and solitary-feeding fourth instar larvae also respond to the volatile chemicals emitted by their host-plant.

The fact that adults use olfactory cues to find their host-plant in the laboratory suggests that when searching for host-plants in nature they are able to find their hosts even in the presence of volatiles emitted by other plants; in other words, the mixture of volatiles present in the envi-

ronment does not mask the cue emitted by their host-plant, which supports the coincidence detection theory proposed by Bruce et al. (2005). However, the volatile compounds identified are not particular to *A. chilensis*. Provided there is not a unique *A. chilensis* compound(s) undetected among the non-identified peaks in the chromatograms (ca. 3%), then it is likely this species uses a particular blend of volatiles as a cue when searching for a host-plant, as proposed by the ratio-specific odour recognition theory (Visser, 1986).

The behavioural bioassays showed that the plant volatiles attracted both male and female adults, females more so than males (Fig. 1). EAGs confirmed the sensitivity of antennae of both sexes to host-plant volatiles, female antennae being more sensitive than those of males. While the attraction of females is explained by their need to find oviposition sites, that of males may be explained by their need to identify mating zones. Although the ultimate infochemicals involved in mating are sex pheromones, their effect is modulated by host-plant semiochemicals in the mating environment (Landolt et al., 1994; Lilley & Hardie, 1996; Ochieng et al., 2002).

The dispersal of larvae of *B. polydamas archidamas* occurred between the end of the third instar and beginning of the fourth instar; thereafter, the larvae were solitary. Arguments put forward to explain larval aggregation include thermoregulation, feeding stimulation, defence and avoidance of the induced responses of the plant (Denno & Benrey, 1997; Bryant et al., 2000; Hunter, 2000; Tullberg et al., 2000; Fordyce & Shapiro, 2003). The several hypotheses proposed to account for dispersal, include the disappearance of the benefits of group feeding when the intraspecific competition for leaf area between the large larvae becomes intense, the avoidance of potential predators, increase of the ability of larvae to defend themselves, the depletion of resources and avoidance of cannibalism (Stamp, 1986; Inouye & Johnson, 2005). Dispersing larvae need to find other host-plants and continue feeding. The fourth instar larvae seem to have developed the sensory system necessary for them to be able to recognise a host-plant, and at that point, they may have also sequestered sufficient AAs in their tissues (Sime et al., 2000) to deter predators.

Our results indicate that olfactory cues are used at three instances during the ontogeny of *B. polydamas archidamas*: when females search for an oviposition site, when large larvae search for new host-plants, and possibly when males search for a mate. Thus, olfactory receptors appear to be coordinated to respond to *A. chilensis* volatiles during host searching by solitary larvae and adults. This accords with the idea of a congruency in sensory mechanisms involved in oviposition and larval feeding recorded for other papilionids (Nishida, 2005). The volatile chemicals that attract *B. polydamas archidamas* to its host-plant remain to be identified.

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

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Key words. Lepidoptera, Papilionidae, *Battus polydamas archidamas*, *Aristolochia chilensis*, aristolochic acid content, foraging substrate, larval development

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

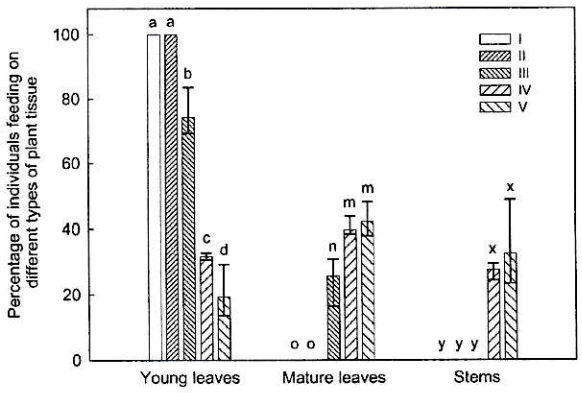

Fig. 1. Percentage (median and interquartile range of data are plotted) of larvae (instars: I, II, III, IV, V) of *Battus polydamas archidamas* feeding on young and mature leaves, and stems of the host plant. Different letters above the columns within each type of tissue indicate significant differences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

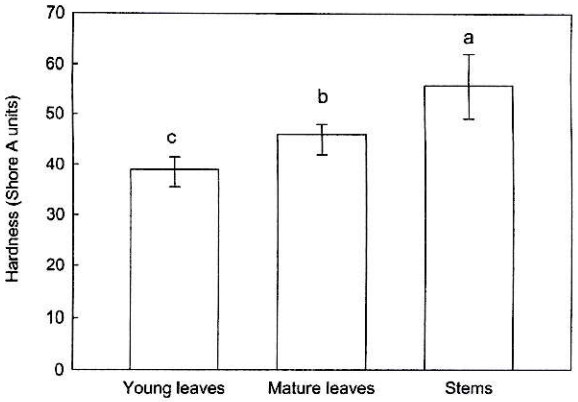

Fig. 2. Relative hardness of tissues of *Aristolochia chilensis* (median and interquartile range of data are plotted). Different letters (a, b and c) above columns indicate significant differences ($P < 0.05$, Tukey tests).

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

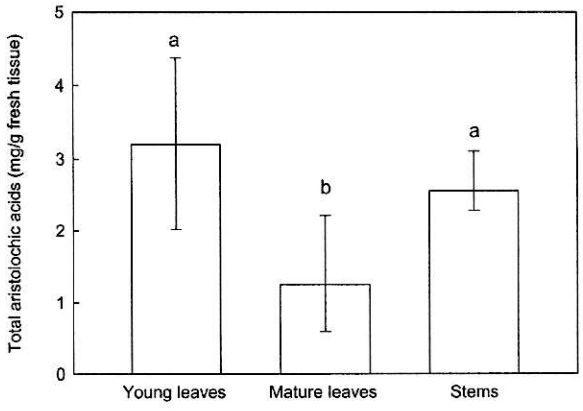

Fig. 3. The total concentration of aristolochic acids (I and II) in young and mature leaves, and stems of *Aristolochia chilensis*. Different letters (a, b and c) above columns indicate significant differences ($P < 0.05$, Tukey tests).

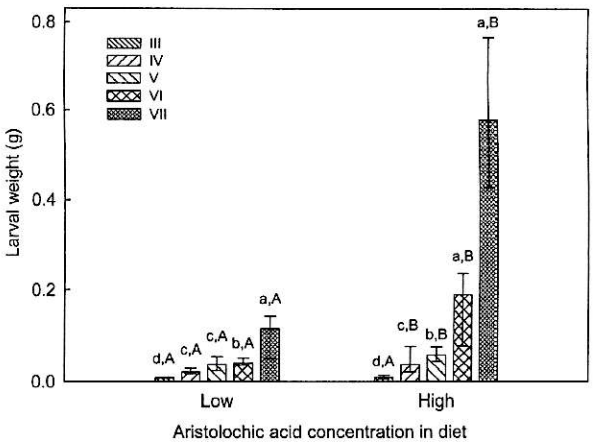

Fig. 4. The weight of larvae reared on a meridic diet containing either half or twice the concentration of aristolochic acids recorded in the aerial tissue of their host plant. Different letters above the columns indicate significant differences ($P < 0.05$, Tukey tests); low-case letters refer to intra-diet 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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SEQUESTRATION OF ARISTOLOCHIC ACIDS BY *Battus polydamas archidamas* LARVAE IN MERIDIC DIETS

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ABSTRACT

Larvae of the butterfly, *Battus polydamas archidamas* (Papilionidae: Troidini) feed exclusively on aristolochic acid-containing *Aristolochia chilensis* (Aristolochiaceae). We explored the patterns of sequestration of AAs in larval tissues (body extracts, integument and osmeteria) of *B. polydamas archidamas* along their development through the use of three meridic diets containing different concentrations of aristolochic acids (AAs). The comparison of AAs profiles in body extracts and in integument extracts showed a pattern of passive sequestration directly related with larval development and the concentration of AAs in the diet. In osmeteria, the

pattern observed suggests the occurrence of selective uptake. In all cases the appearance of phenolic AAs (AAIa and AAIVa) in the tissues imply the occurrence of processes that modify the original AAs offered in the meridic diets (AAI and AAII).

INTRODUCTION

Host plant specialization by insects is a frequent phenomenon in nature. Among the Lepidoptera, most species of butterflies oviposit and feed on a single plant species or are associated with only a few genera or families (Nishida, 2002; Schoonhoven, 2005). This pattern is mostly attributed to certain groups of chemicals that through time has led the evolution of specific associations with distinct host plants (Ehlrich & Raven, 1964; Nishida, 2002). The nature of these chemicals is diverse, as it is their role and the intensity of their effect in the interaction between Lepidoptera and their host plants (Nishida, 2002). For example, some Danaine use pyrrolizidine alkaloids (PAs) of the host plant as oviposition stimulants by females, as sex pheromones by males and they are sequestered by larvae during their development, since the plant produce this chemical as induced defense (Allomone), PAs are retained throughout all life stages and are passed to progeny (Nishida *et al.*,1991; Nishida, 1994/1995; Nishida *et al.*,1996; Honda *et al.,* 1997), In contrast, some Nymphalinae store iridoid glycosides (IGs) only during larval and pupal stages and eliminate them at adult eclosion (Bowers & Puttick, 1986). Secondary compounds are used by butterflies in

various processes such as host selection, defence and even mating (Nishida, 2002; Opitz & Müller, 2009). When plant chemicals are sequestered by lepidopterans, the concentration in their bodies may follow that in their host plants or selective uptake, transformation or even metabolization of the original compounds consumed may take place (Rotschild & Edgar, 1978; Seiber *et al.,* 1980; Nishida, 2002).

The tribe Troidini (Papilionidae) is associated with a single plant family (Aristolochiaceae) (Weintraub, 1995) characterized by containing aristolochic acids (AAs), a group of nitrophenanthrenes unique to the family (Chen & Zu, 1987; Mix *et al.,* 1982). Aristolochic acids in nature occur as phenolic compounds (AA Ia and AA IVa) and non-phenoliccompounds (AAI and AAII), the latter being the most common abundant in the host plant (Sime *et al.,* 2000). AAs are sequestered by the larvae and remain in the body during all the life cycle since they are transferred to eggs by adults (Sime *et al.,* 2000; Nishida, 2002; Fordyce *et al.,* 2005). The genus *Battus* is known for using these chemicals for host selection and defence (Nishida & Fukami, 1989; Fordyce, 2000; Nishida, 2005; Pinto *et al.*, 2009[b]).

In *Aristolochia chilensis*, the host of *Battus polydamas archidamas* in central Chile, aristolochic acids occur mainly as AAI and AAII. Some 2% of the total plant AAs consumed by larvae are sequestered but their distribution in the insect remains unknown (Urzúa & Priestap, 1985). Moreover, the plants present tissue-dependent concentrations of AAs, Larvae consume different plant tissues along their ontogeny (Pinto *et al.*, 2009a) and larval

performance has been shown to depend on the concentration of AAs contained in artificial meridic diets (Pinto *et al.,* 2009b). In the present paper we explore the patterns of sequestration of AAs in larval tissues of *B. polydamas archidamas* along their development through the use of meridic diets. Three concentrations of AAs in the diet were used with the aim to investigate their effect on the pattern of allocation of AAs in the larvae.

METHODS AND MATERIALS

Larval rearing in meridic diets

Battus polydamas archidamas is the only Papilionid in Chile. It feeds and oviposits on *A. chilensis* and *A. bridgesii* (Klotzsch) Duchart. (Aristolochiaceae), two perennial endemic pipevines (Marticorena & Quezada, 1985). The distribution range 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 in January 2007.

Larval rearing in the laboratory and basic diet composition was performed following the methods mentioned by Fordyce & Nice (2008) and Pinto *et al.,* (2009b). In order to mimic different plant chemical scenarios, three experimental diets were generated, adding a mixture of pure

aristolochic acids I and II in the ratio present in the plant as a whole. The low aristolochic acid diet contained half the mean concentration in the host plant (0.75 mg/g fresh tissue), the medium aristolochic acid diet contained ca. the natural concentration in the plant (1.5 mg/g fresh tissue) and the high aristolochic acid diet contained twice that concentration (3 mg/g fresh tissue).

Concentration of aristolochic acids in larval tissues

Three types of larval tissues were sampled: osmeterial fluids, integument and body after the extraction of the integument. We used larvae from third to sixth instars (ca. 15 samples/type of tissue) in order to describe the sequestration pattern of aristolochic acids along larval development.

Individuals were sampled at the end of larval instars. They were starved for 24 h to avoid contamination by remains of food in the gut and then killed by freezing (after withdrawing osmeterial secretions from the third instar onwards).

Osmeterial secretions were collected with capillary tubes (0.5 µl, 32mm long, SIGMA) in contact with the everted gland (Sime *at al.*, 2000) and the fluid was dissolved in 0.5 ml of 95% dichloromethane. Integument samples were obtained by briefly dipping the individuals, previously killed by freezing, in 1 ml of dichloromethane at room temperature. Samples of the rest of the body were dissicated in an oven at 50° C and preserved in Eppendorf tubes inside plastic bags with silica gel until HPLC analysis.

HPLC analysis of aristolochic acids

Dichloromethane extracts of osmeterial secretions and integuments were directly injected (20 ul) in an HPLC. The desiccated bodies were ground in a mortar until obtaining a fine powder and 500 µl of methanol were added to macerate the sample for 50 min. This extract was centrifuged for 6 min at 13000 rpm and the supernatant directly injected into an HPLC (20 µl).

The non-phenolic and phenolic AAs fractions were analyzed by analytical HPLC (Waters 600), using a reverse-phase Symmetry column (5 µm particle size; 25 x 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. Detection was accomplished with a Waters 2996 diode-array-detector (DAD) and 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 (1), AA-II (2), AA-Ia (3), and AA-IVa (4) previously isolated from *A. chilensis, A. argentina* and *B. polydamas archidamas* (Priestap, 1987; Urzúa *et al.,* 1983). Quantification was based on peak areas 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 ºC. Calibration lines were obtained by plotting peak areas against the concentrations of the standards; these lines were used to determine the concentrations of the AAs in the samples.

Data analysis

Concentrations of each aristolochic acid (µg/individual), I, II, Ia and IVa were compared individually with two-way non parametric ANOVAs, using larval instar and type of meridic diet as factors (Conover & Iman, 1981; Sokal & Rohlf, 1995), followed by post-hoc Holm-Sidak tests.

RESULTS

Integument extracts

The quantity of AAs sequestered increased along larval development and with the concentration of AAs in the meridic diet (Fig. 2). Concentrations of most AAs (µg/individual) showed significant differences with respect to the type of diet, the larval instar and the interaction of both factors (**AAI:** $F= 33.955$, $df = 2$, $p< 0.001$; $F= 265.081$, $df = 3$, $p< 0.001$ and $F= 3.554$, $df = 6$, $p< 0.005$, respectively; **AAIa:** $F= 184.606$, $df = 2$, $p< 0.001$; $F= 14.331$, $df = 3$, $p< 0.001$ and $F= 2.658$, $df = 6$, $p< 0.05$, respectively; and **AAIVa:** $F= 227.334$, $df = 2$, $p< 0.001$; $F= 18.114$, $df = 3$, $p< 0.001$ and $F= 2.757$, $df = 6$, $p< 0.05$, respectively). AAII showed significant differences only in relation to the type of diet (**AAII:** $F= 3.760$, $df = 2$, $p< 0.05$; $F= 1.156$, $df = 3$, $p= 0.317$ and $F= 2.071$, $df = 6$, $p= 0.059$, respectively). AAI was the most abundant aristolochic acid present in integument extracts and showed differences among diets ($p< 0.05$) excluding the combination between medium and high diets; the second most abundant AA was AAIa, with a pattern of differences similar to AAI; AAIVa was the third in concentration and showed differences

between all diet combinations ($p < 0.05$), and AAII was the least abundant and did not show any differences between diets.

Body extracts

As in the case of integument extractions, the concentration of AAs in body extracts increased along larval development and was also affected by the concentration of AAs in the diet (Fig. 3) Significant differences were observed among diets in each analysis performed to individual AAs ($p < 0.05$) Concentrations of AAs (µg/ individual) in body extracts were significatively associated with the type of diet, larval instar and the interaction of both factors (**AAI:** $F = 78.537$, $df = 2$, $p < 0.001$; $F = 258.174$, $df = 3$, $p < 0.001$ and $F = 2.738$, $df = 6$, $p < 0.05$, respectively; **AAII:** $F = 71.419$, $df = 2$, $p < 0.001$; $F = 172.465$, $df = 3$, $p < 0.001$ and $F = 3.060$, $df = 6$, $p < 0.01$, respectively; **AAIa:** $F = 60.520$, $df = 2$, $p < 0.001$; $F = 175.071$, $df = 3$, $p < 0.001$ and $F = 3.967$, $df = 6$, $p < 0.001$, respectively; and **AAIVa:** $F = 50.943$, $df = 2$, $p < 0.001$; $F = 158.010$, $df = 3$, $p < 0.001$ and $F = 4.105$, $df = 6$, $p < 0.001$, respectively). AAI was the most abundant aristolochic acid in body extracts, showing differences between all diet combinations ($p < 0.05$), followed by AAIa, AAIVa and AAII, with the same pattern of differences among diets.

Osmeterial secretions

Sequestration of AAs in osmeterial secretions showed a more complex pattern. A high variation in the pattern of sequestration along larval development was observed and only the normal diet showed a passive

pattern of increasing concentration along larval development (Fig. 4). AAI was the most abundant aristolochic acid in the osmeterial fluid and showed differences among diets ($p < 0.05$) except for the combination of normal and high diet; AAIa was the second AA in concentration and showed differences between all diets ($p < 0.05$); AAII was the third in concentration and showed differences between normal and high diets and low and high diets ($p < 0.05$); and AAIVa was the least abundant and did not show differences between low and high diets. Concentration of AAs (µg/ individual) (I, II, Ia and IVa) in osmeterial secretions were significantly associated with the type of meridic diet and larval instar but not with the interaction of both factors (**AAI:** $F = 9.651$, $df = 2$, $p < 0.001$; $F = 22.301$, $df = 3$, $p < 0.001$ and $F = 1.845$, $df = 6$, $p = 0.127$, respectively; **AAII:** $F = 23.858$, $df = 2$, $p < 0.001$; $F = 3.207$, $df = 3$, $p < 0.05$ and $F = 0.997$, $df = 6$, $p = 0.414$, respectively; **AAIa:** $F = 12.730$, $df = 2$, $p < 0.001$; $F = 13.837$, $df = 3$, $p < 0.001$ and $F = 2.138$, $df = 6$, $p = 0.083$, respectively; and **AAIVa:** $F = 5.413$, $df = 2$, $p < 0.05$; $F = 11.870$, $df = 3$, $p < 0.001$ and $F = 0.948$, $df = 6$, $p = 0.440$, respectively).

Discussion

The comparison of AAs profiles in body extracts and in integument extracts showed a pattern of passive sequestration directly related with larval development and the concentration of AAs in the diet. On the other hand, the pattern observed in osmeterial fluids suggests the occurrence of selective uptake. In all cases, phenolic AAs (AAIa and AAIVa) not present in the diets

appear in larval tissues. The occurrence of phenolic and non phenolic AAs has been reported in larvae of *B. polydamas archidamas* feeding on *A. chilensis* (Urzúa *et al.* (1983) and *Aristolochia argentina* (Urzúa *et al.*, 1987). This finding suggest the occurrence of processes that modify the original AAs offered in the meridic diets, for example by demethylation of AAI or hydroxylation of AAII to produce the AAIa, and hydroxylation of AAI to produce AAIVa. A pattern of accumulation of AAs similar to those we report was found in larvae of *Battus philenor* fed with *Aristolochia. macrophylla* (Sime, 2000); this author proposed the occurrence of selective conversion of AAII given the low proportion of this compound in the samples, or the existence of selective accumulation of AAI to be transformed into the other metabolites.

Experiments with butterfly predators showed that birds were deterred from feeding on rice grains impregnated with AAI (Nishida & Fukami, 1989). The defensive role of AAs has also been tested on potential arthropod predators and found to increase the mortality or modify the behaviour of the attacker (Fordyce, 2001; Sime, 2002). In *B. polydamas archidamas* we found a large concentration of aristolochic acids in the integument extracts: almost half of the AAs contained in the rest of body extracts. This pattern could be related with a selective allocation of defenses to the surface in order to prevent the initial attack of large predators and also as a signal of toxicity for small predators that use sensorial organs to determine the quality of the potential host. Tyler *et al.*,(1994) describes the existence of various

parasitoids of Papilionidae; nevertheless, we did not observe any event of parasitoidism or predation by animals in larvae or eggs during field work.

A defensive role is attributed also to osmeterial glands of Troidini butterflies, since they are partly effective in deterring small potential predators (Stamp, 1986). The presence of deterrent and toxic compounds has been reported in other Troidini osmeterial fluids (Honda, 1980, 1983b). Furthermore, the presence of AAs in this organ is also widespread in the tribe; however, their actual ecological role is not well established (Nishida, 1995b; Sime, 2000). Personal observations of larval behaviour suggest that the strong odor released by this organ, extruded by the larvae when they feel attacked, could work as an alerting signal via associative learning with toxicity, as suggested by Nishida (2002); however, the question still remains whether the presence of AAs in this organ is related to a detoxification mechanism.

The patterns of allocation of secondary compounds in larval tissues of *B. polydamas archidamas* supports the idea of the defensive role of AAs; nevertheless, the results also showed the occurrence of changes in the original secondary compounds offered by the host plant, that are allocated and transformed in differential way depending on the larval tissue. It may be suggested that some organs, such as the osmeterium, could work in a different way with respect to the presence of AAs. Experimental approaches to test the ecological role of AAs in this organ are needed.

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Legends to figures:

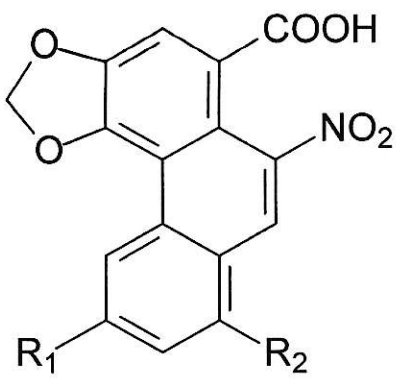
Fig. 1. Aristolochic acids present in larvae of *Battus polydamas archidamas*.

Fig. 2. Aristolochic acids present in the integument of larvae of *Battus polydamas archidamas*.

Fig.3. Aristolochic acids present in body extracts (after extraction of compounds present in the integument) of larvae of *Battus polydamas archidamas*.

Fig. 4. Aristolochic acids present in osmeterial secretions of larvae of *Battus polydamas archidamas*.

Figure 1



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

Figure 2

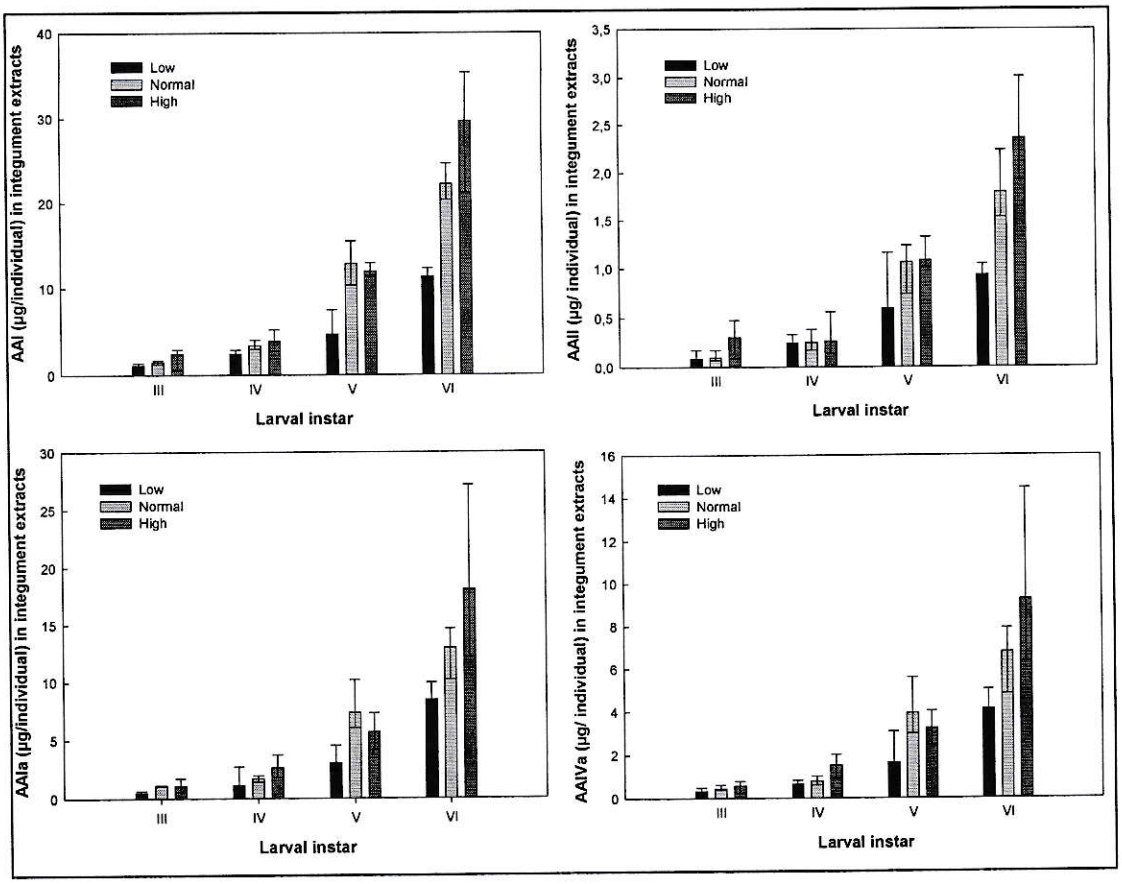

AAI (µg/individual) in integument extracts
AAII (µg/ individual) in integument extracts
AAIa (µg/individual) in integument extracts
AAIVa (µg/ individual) in integument extracts
Low
Normal
High
III
IV
V
VI
Larval instar

Figure 3

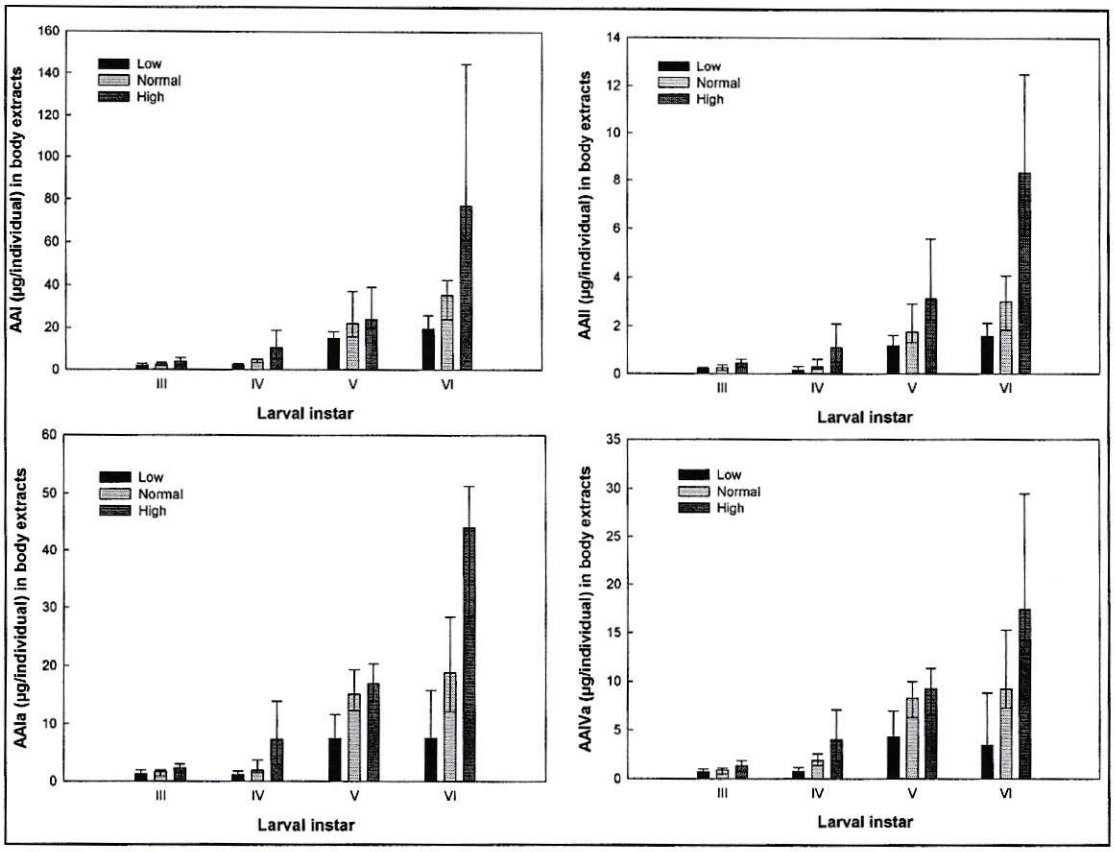
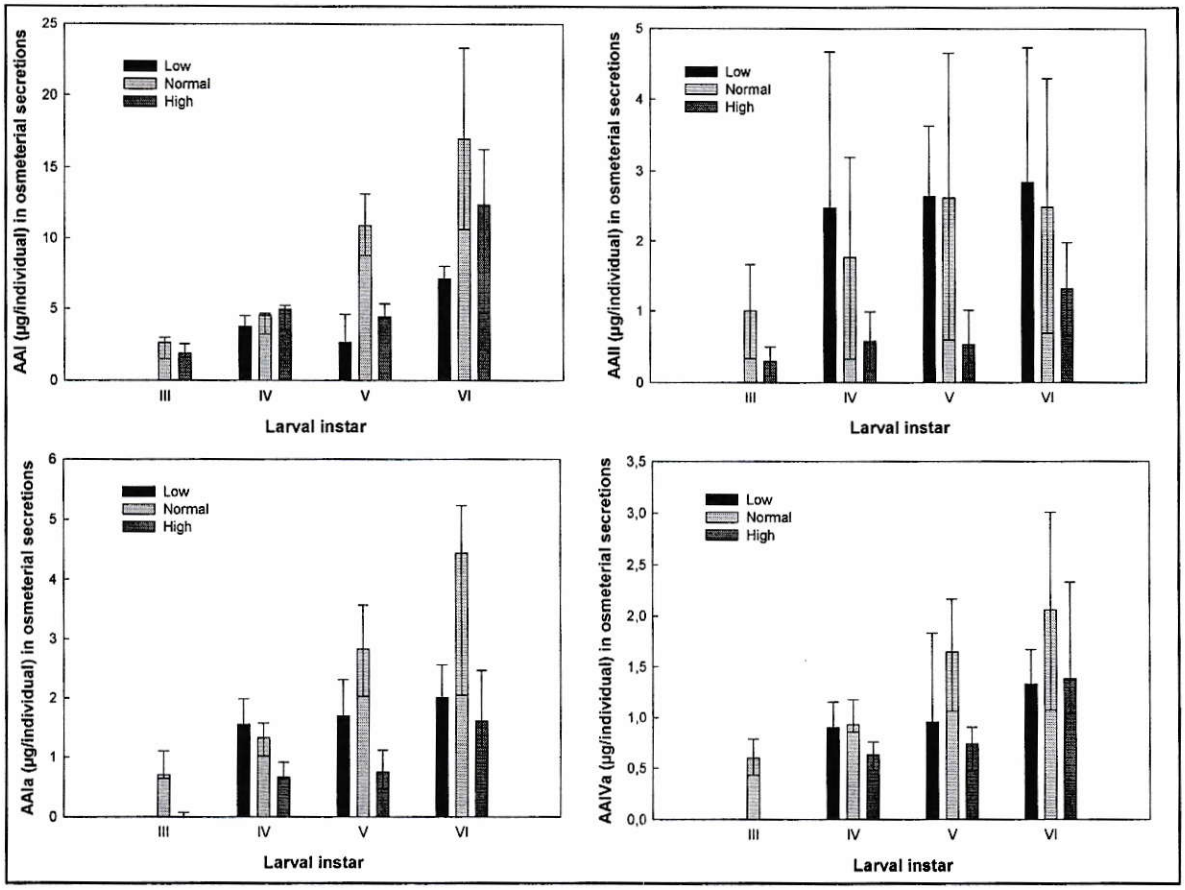

AAI (µg/individual) in body extracts
AAII (µg/individual) in body extracts
AAIa (µg/individual) in body extracts
AAIVa (µg/individual) in body extracts
Low
Normal
High
III
IV
V
VI
Larval instar

Figure 4


AAI (µg/individual) in osmeterial secretions
Low
Normal
High
Larval instar
III
IV
V
VI
AAII (µg/individual) in osmeterial secretions
AAIa (µg/individual) in osmeterial secretions
AAIVa (µg/individual) in osmeterial secretions

CONCLUSIONES GENERALES

B. polydamas archidamas oviposita mayoritariamente en hojas jóvenes de *A. chilensis*. Las larvas permaneces agregadas durante los primeros tres estadíos larvales alimentándose casi exclusivamente de hojas jóvenes. Luego, las larvas se disgregan convirtiéndose en individuos solitarios y expandiendo su dieta a otros tejidos, tales como hojas maduras y tallos. Las hojas jóvenes fueron las de menor dureza pero con mayor concentración de AAs en comparación con las hojas maduras, mientras que los tallos mostraron ser los más duros y contener altas concentraciones de AAs. Los patrones de alimentación observados en las larvas se verían afectados al inicio de su desarrollo por las características físicas de la planta, que ejerce limitaciones de acceso al resto de los tejidos de las plantas; este efecto va desapareciendo a medida que la larva se desarrolla y sus mandíbulas adquieren la fortaleza para lidiar con tejidos más duros.

Altos niveles de mortalidad y bajo peso larval son los resultados obtenidos de las larvas criadas en dietas artificiales con baja concentración de AAs. El mayor incremento del peso larval en dietas con altas concentraciones de AAs sugiere un efecto fagoestimulante de los AAs. La mortalidad en el primer estadío fue significativamente más alta en la dieta de baja concentración de AAs (87%) comparada con la mortalidad observada en la dieta con alta concentración de AAs (57%), lo cual se asemeja a patrones naturales de mortalidad descritos en la literatura (Zalucki et al., 2002). Esto estaría relacionado con un bajo nivel de reconocimiento del sustrato alimenticio en las dietas con baja concentración de AAs.

Los bioensayos conductuales y de electroantenografía (EAG) mostraron que tanto los adultos (hembras y machos) como las larvas de *B. polydamas archidamas* usan los compuestos químicos volátiles emitidos por la planta durante el reconocimiento del hospedero a distancia; sin embargo, ninguno de los compuestos volátiles identificados resultaron particulares de *A. chilensis*. Las mariposas utilizarían mezclas específicas de volátiles como señales para encontrar su planta hospedera.

Un patrón pasivo de secuestro de AAs fue observado en las larvas criadas en dietas artificiales, en directa relación con el nivel de desarrollo larval y la concentración de AAs en las dietas. La transformación de AAs no fenólicos en fenólicos muestra una de las posibles estrategias utilizadas por las larvas frente a las defensas naturales de su planta hospedera para limitar su acción tóxica. Sin embargo, tejidos tales como las glándulas osmeteriales mostraron patrones distintos al secuestrar posiblemente de forma selectiva los AAs. La mayor concentración observada en la cutícula de las larvas podría estar relacionada directamente con su defensa al concentrar compuestos tóxicos en la superficie que las tornen impalatables.

En conclusión, los resultados han mostrado que el olfato juega un rol importante en tres instancias durante la ontogenia de *B. polydamas archidamas*: a) cuando las hembras buscan un lugar donde oviponer, b) cuando las larvas se tornan solitarias y buscan nuevas plantas para alimentarse y c) posiblemente cuando los machos buscan sitios de apareamiento. Los receptores olfativos parecen estar intrínsecamente coordinados para utilizar los compuestos volátiles emitidos por *A. chilensis* para encontrar hospederos a lo largo de toda la ontogenia de la mariposa. Por otro lado, la combinación de altos niveles de AAs y tejidos suaves pueden ser

determinantes al momento de seleccionar un sustrato de oviposición por parte de las hembras, siendo los beneficios de esta selección un mejor desempeño larval de su progenie al menos durante los primeros estadíos de desarrollo. Los patrones de secuestro de AAs observados generan nuevas preguntas acerca de las funciones que cumple cada tipo de tejido larval durante este proceso y su rol ecológico.

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