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

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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,

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when they disperse and feed solitarily (Tatar, 1991; 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

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.

two stimulus sources, and the two zones at the ends (10 cm from 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%).

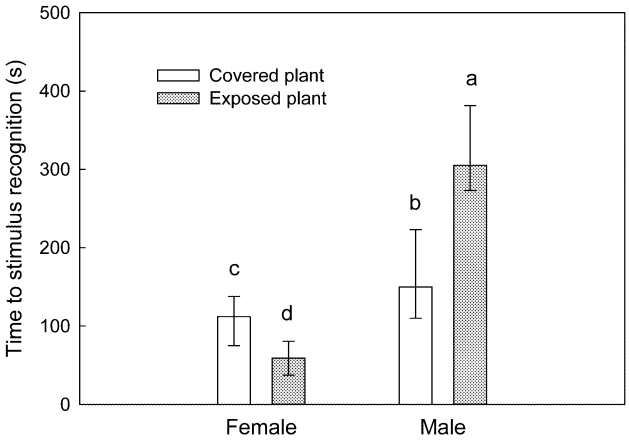

Fig. 1. Time to host-plant recognition by males and females of *Battus polydamas archidamas*. Plant volatiles were from an exposed or a covered plant (median and interquartile range of data is plotted).

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).

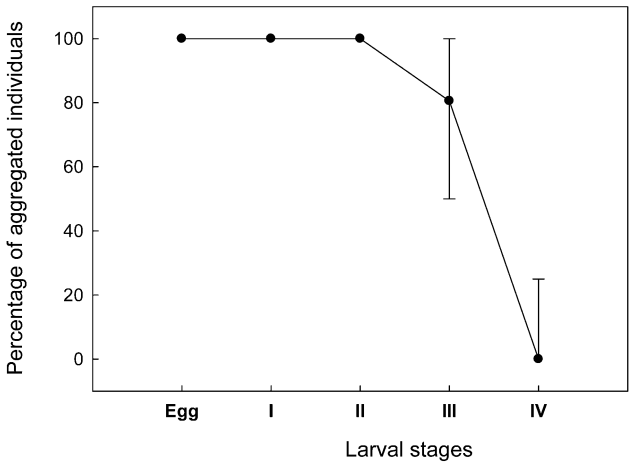

Fig. 2. Mean percentage of individuals that aggregate in the different stages of development of *Battus polydamas archidamas* (median and interquartile range of data are plotted).

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 info-chemicals 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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