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Mechanical damage to pollen aids nutrient acquisition in *Heliconius* butterflies (Nymphalidae)

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Abstract

Neotropical *Heliconius* and *Laparus* butterflies actively collect pollen onto the proboscis and extract nutrients from it. This study investigates the impact of the processing behaviour on the condition of the pollen grains. Pollen samples ($n = 72$) were collected from proboscides of various *Heliconius* species and *Laparus doris* in surrounding habitats of the Tropical Research Station La Gamba (Costa Rica). Examination using a light microscope revealed that pollen loads contained $74.88 \pm 53.67\%$ of damaged *Psychotria* pollen, $72.04 \pm 23.4\%$ of damaged *Psiguria/Gurania* pollen, and $21.35 \pm 14.5\%$ of damaged *Lantana* pollen (numbers represent median $\pm$ first quartile). Damaged pollen grains showed deformed contours, inhomogeneous and/or leaking contents, or they were empty. Experiments with *Heliconius* and *Laparus doris* from a natural population in Costa Rica demonstrated that 200 min of pollen processing behaviour significantly increased the percentage of damaged pollen of *Psychotria* compared to pollen from anthers ($P = 0.015$, $Z = -2.44$, Mann–Whitney U -test). Examination of pollen loads from green house reared *Heliconius* butterflies resulted in significantly greater amounts of damaged *Psiguria* pollen after 200 min of

processing behaviour compared to pollen from flowers ($P < 0.001$, $Z = -4.583$, Mann–Whitney U -test). These results indicate that pollen processing functions as extra oral digestion whereby pollen grains are ruptured to make the content available for ingestion.

Keywords

Pollen feeding; Extra oral digestion; *Heliconius*; Butterfly; Lepidoptera

Introduction

Butterflies regularly come into contact with pollen during flower-visits, but only the Neotropical butterflies of the genus *Heliconius* (Kluk, 1780) and *Laparus doris* (L., 1771) (both Nymphalidae) evolved a unique technique to utilize the nutritive value of pollen. In addition to nectar, they actively collect pollen from flowers using their proboscis (Gilbert 1972; Boggs et al. 1981; Estrada and Jiggins 2002). The proboscis of pollen feeding butterflies has a significantly greater number and length of sensory bristles in comparison to related non-pollen feeding nymphalids (Krenn and Penz 1998). Furthermore, pollen feeding butterflies perform lengthy flower probing movements with their proboscis while pollen is being collected and accumulated onto the proboscis (Penz and Krenn 2000; Krenn 2008). The pollen grains are suspended in a body fluid on the outside of the proboscis (Fig. 1a) and agitated for several hours by coiling and uncoiling movements of the proboscis (Gilbert 1972). During this processing behaviour essential amino acids are extracted from the pollen grains (O'Brien et al. 2003). The resulting liquid is ingested, while the pollen remains on the outer surface of the proboscis. After the period of processing, pollen is passively discarded. Amino acids from pollen constitute key resources for egg production, nuptial gifts by males, and longevity of adults (Gilbert 1972; Dunlap-Pianka et al. 1977; Boggs and Gilbert 1979; Boggs 1981; Cardoso and Gilbert 2007). In addition, pollen can provide precursor substances for the synthesis of cyanogenic glycosides in adult butterflies (Nahrstedt and Davis 1983) that might be the basis for the mimetic convergence of wing patterns in many species. Being a key innovation in the evolution of these insects, pollen feeding behaviour is central for the understanding of the *Heliconius* life history (review Gilbert 1991). Although many aspects of biology are well studied, the precise mechanism for obtaining nutrients from pollen has remained a mystery.

The aim of the study is to determine how pollen is altered during the processing behaviour and to elucidate the mechanism of pollen feeding. We hypothesize that pollen processing behaviour opens pollen grains permitting access to the nutrients within. To assess the effects of pollen processing we compared pollen samples removed directly from anthers with pollen processed by butterflies. This allowed us to determine whether pollen grains germinate, burst, or remain intact during pollen processing and whether the variously sized pollen are affected in the same way.

Methods

Field work and pollen preparation

Pollen loads were collected from proboscides of *Heliconius* and *Laparus* butterflies (Table 1) in the vicinity of the Tropical Research Station La Gamba (Puntarenas, Costa Rica); 8°45' N, 83°10' W; 81 m above sea level. This area hosts a broad spectrum of natural, secondary and anthropogenic habitats. The nearby Esquinas forest (Piedras Blancas National Parc) is one of the largest remaining stretches of pristine lowland rainforest along the Pacific coast of Central America. The region stands out by its high mean annual precipitation (about 6,000 mm), high nutrient status and striking levels of biodiversity (Weissenhofer et al. 2008).

Butterflies were captured with a net in secondary growth habitats along a dirt road and trails in the rain forests between February and April 2007, as well as in September 2007. Pollen loads (Fig. 1a) were removed from the proboscis using an insect pin and then the butterflies were released immediately. Microscope slides of pollen loads were prepared in the field. Pollen grains were directly washed off from the insect pin in a small drops of glycerin onto glass slides. The slides were covered with cover glasses and sealed with nail enamel. Table 1 lists the species and number of butterflies from which pollen samples were taken and on which pollen processing tests were conducted. Both sexes were equally included in all investigations.

Analyses of the composition of pollen loads and pollen condition

Pollen preparations were studied using a Nikon Eclipse 100 light microscope. Photographs of pollen grains were taken with a Samsung V50 camera attached to the microscope. In 60 pollen load samples all pollen grains were counted and examined. In most samples, more than 90% of the pollen grains could be determined to genus level using the determination guide book of Roubik and Moreno (1991). Pollen of *Psiguria* (Neck. ex Arn.) and *Gurania* (Schltdl.) Cogn. (Cucurbitaceae) could not be distinguished as both are very similar and form tetrads consisting of four single pollen grains. In 72 pollen samples the first 50–100 pollen grains of the three predominant plant genera within the samples i.e., *Psychotria* (L.) (Rubiaceae), *Lantana* L. (Verbenaceae) and *Psiguria/Gurania* were classified as “intact” or “damaged”. Grains were classified as “intact” if the exine was intact and if their content was as homogeneous as in pollen from the anthers of the respective flower. Pollen grains were classified as “damaged” if they were deformed, had inhomogeneous content, or if the pollen exines were broken and/or empty (Fig. 1b-d). Only preparations which contained more than 50 pollen grains of at least one of these three predominant plants were taken into consideration. Observations of flowering plants near the station suggest that pollen originates from *Psychotria elata* (Sw.) Hammel and/or *P. poeppigiana* Müll. Arg., *Lantana camara* L., as well as from *Psiguria warscewiczii* (Hook. F.) Wunderlin or *Gurania makoyana* (Lem.) Cogn., all of which occur in the study area (Weber 2001).

Preliminary studies showed that glycerin is an appropriate fluid to preserve pollen without changing its shape or condition. Ten pollen preparations from the first study period

(February to April 2007) were recounted a year later, but no changes in the condition of the pollen grains could be observed.

Impact of pollen processing on pollen condition

To study the effect of the processing behaviour on the condition and shape of the pollen, we used pollen from *Psychotria elata* (Rubiaceae), one of the natural pollen sources of *Heliconius* and *Laparus* in the habitats around the station. Butterflies of various species (Table 1) were netted between 09.00 and 12.00 h in September 2007. Butterflies ($n = 17$) without pollen loads were returned to the station where the experiments were performed in a net cage. Pollen was removed from the anthers of fresh *P. elata* flowers. This pollen was experimentally placed on the proboscis using a dissection pin. Only butterflies that started proboscis movements were included in the experiments. After 200 min, 13 pollen loads could be removed from the proboscides and prepared for light microscopy. A time period of 200 min was chosen since the natural pollen processing time has been found to be several hours (Gilbert 1972). In parallel, some pollen taken directly from anthers was placed on microscope slides as controls ($n = 10$). The condition of pollen grains was classified using a light microscope according to the criteria described above. All butterflies were marked on one wing to prevent double testing and released. A non-parametric Mann–Whitney *U*-test was used to test for differences between experimental and control pollen preparations in percent of damaged pollen observed.

A similar procedure was performed in a large tropical insectary at the Brackenridge Field Laboratory at the University of Texas at Austin in March 2008, where a population of *Heliconius* butterflies was kept under semi-natural conditions with flowering plants of *Psychotria poeppigiana*, *Lantana camara*, *Psiguria tabascensis* Donn. Sm. and *P. bignoniacea* (= *triphylla*) (Poepp. and Endl) Wunderlin and *Gurania insolita* Cogn. (Cucurbitaceae). For the tests, 17 *Heliconius* butterflies kept in net cages were provided with *Psiguria* flowers and allowed to collect pollen from these flowers for about 1 h in the morning. Subsequently, they were isolated in a small net cage for 200 min. The pollen loads were removed from the proboscis and glycer in preparation slides were produced as described above. Pollen control preparations ($n = 23$) were made directly from the flowers. Afterwards, pollen grain conditions were classified as “intact” or “damaged” (see above) using a light microscope. Determination of pollen condition was repeated 10 times on several slides to estimate the error in counting which resulted in less than 3% for a total of more than 300 pollen grains.

Results

Pollen spectrum and pollen condition of field collected samples

All pollen grains (12–4,122 per slide, mean $641.1 \pm 1,049$ standard deviation) from the pollen loads of *Heliconius* butterflies and *Laparus doris* were determined to generic level in 60 samples. The majority of pollen (in total 85.31%) belonged to three plant genera; i.e., *Psychotria* (Rubiaceae) (36.76%), *Lantana* (Verbenaceae) (28.15%) and *Psiguria/Gurania* (Cucurbitaceae) (20.4%). Pollen of *Tournefortia angustifolia* Roem. and Schult. (Boraginaceae) amounted to 11.76%; less than 3% belonged to eight other plants,

determined as *Coccoloba* (Browne 1756) (Polygonaceae), one species of Convolvulaceae, three species of Asteraceae and three unidentified plant species.

The three most common pollen types were classified as “intact” or “damaged” (Fig. 1b-d) in samples which contained more than 50 pollen grains ($N = 72$). From these, 43 samples contained *Psychotria* pollen of which on $74.88 \pm 53.67\%$ (median $\pm$ first quartile) was damaged. In 29 samples, *Lantana* pollen was encountered that was damaged to $21.35 \pm 14.5\%$ (median $\pm$ first quartile). *Psiguria/Gurania* pollen tetrads were found in 26 samples with a percentage of 72.04 ± 23.4 (median $\pm$ first quartile) of damaged pollen grains (Table 2). The pollen exines and the cytoplasm content was affected in the same way regardless of the pollen type. One butterfly had only intact pollen in the pollen load. In five pollen samples more than 90% of the pollen grains were classified as damaged.

The control pollen samples from flowers of *Psychotria* and *Lantana* showed significantly fewer damaged pollen in comparison to the butterfly pollen loads ($P < 0.001$, Mann–Whitney U -test) (Table 2). No control preparation for *Psiguria/Gurania* could be made since no flowering plants were found.

Pollen processing behaviour damages pollen grains

The impact of the pollen processing behaviour on the condition of pollen grains was tested experimentally in the field station and under semi-natural conditions in the tropical insectary.

Pollen from the anthers of *Psychotria elata* was placed on the outside of the proboscis of 17 butterflies from various species (Table 1). After 200 min, four individuals had lost their pollen; one pollen sample contained less than 50 pollen grains and was excluded from further analysis. The remaining 12 pollen samples were examined and compared to 10 control preparations. The pollen loads from butterflies contained significantly more damaged pollen grains than the samples from the anthers ($P = 0.015$, $Z = -2.44$, Mann–Whitney U -test) (Table 3). Only in two microscopic slides the proportions of intact versus damaged pollen grains were similar to the controls. In both cases the butterflies had ceased proboscis movements soon after onset of the experiments.

Similar tests were conducted using a greenhouse population of *Heliconius* (Table 1). These butterflies freely collected pollen from male flowers of *Psiguria tabascensis* and *P. bignoniacea* presented in plastic centrifuge tubes. After 200 min the 17 samples from proboscides contained $90.71 \pm 32.88\%$ (median $\pm$ first quartile) of damaged pollen which was significantly higher than in the preparations from anthers ($P < 0.001$, Mann–Whitney U -test) (Table 3). In four butterflies, however, the percentages of damaged pollen were lower than 20% and thus within the range of the control preparations.

Discussion

The analysis of the pollen loads of free flying *Heliconius* and *Laparus doris* from nature, experimental results from natural populations, and observations from a green house population, unequivocally demonstrated that processing behaviour damages pollen. We

conclude that pollen damage enables these heliconiine butterflies to access the nutrients inside. Pollen processing is not simply a passive diffusion process as suggested by Erhardt and Baker (1990), and probably not an induction of pre-germination (Gilbert 1972), in fact, the grains are broken up and the cytoplasm is partly disintegrated in a high percentage of grains. In this way the pollen is lost for pollination after the processing behaviour. Since pollen remains on the outside of the proboscis, the pollen processing behaviour is an example of extra oral digestion. The fluid released during pollen collecting and processing is not regurgitated nectar (Boggs 1987) and is most likely saliva (C. Boggs, S. Eberhard and A. Hikl, unpublished data). While no histological specialization of the salivary glands has been demonstrated (Eberhard and Krenn 2003), they were found to be disproportionally longer in *Heliconius* and *Laparus* than in related non-pollen feeding nymphalids (Eberhard et al. 2009). Recently, it was proven that the salivary fluid of *Heliconius* contains proteases (Eberhard et al. 2007). We conclude that the proteolytic activity causes the pollen damage during processing behavior.

Support for this conclusion is our observation that pollen collected by butterflies is more dramatically disrupted than pollen which was experimentally placed onto the proboscis. These results correspond with the fact that pollen collecting behaviour itself involves increased salivary discharge (Penz and Krenn 2000). Furthermore, some butterflies ceased processing behaviour during the experiments. In these cases, the condition of pollen grains was similar to that of control preparations (i.e. only 10–20% of damaged pollen). Pollen damage and thus access to pollen content appears to be related to the duration of pollen processing but whether the processing time is correlated with the percentage of damaged pollen grains is not known.

In pollen loads from butterflies at La Gamba Station, more than 85% of the pollen belonged to 3 genera of plants; i.e. *Lantana* (Verbenaceae), *Psychotria* (Rubiaceae), and *Psiguria/Gurania* (Cucurbitaceae), all are well-known pollen source plants of *Heliconius* and *Laparus doris* in various regions of the Neotropics (Boggs et al. 1981; Murawski 1986; Cardoso 2001; Estrada and Jiggins 2002). Pollen tetrads from probably both Cucurbitaceae plants were found in more than half of the pollen loads although no flowering plants were observed during the study periods probably because these vines flower in the canopy (Condon and Gilbert 1990). This underlines the preference of at least some *Heliconius* species for *Psiguria* or *Gurania* flowers. The majority of pollen loads also contained small proportions of pollen from other plants. Previous studies indicated that the majority of pollen within the pollen loads originated from few plants (obviously from the pollen source plants); whereas small amounts of a number of additional pollen was interpreted to come from flowers which were visited for nectar feeding (Boggs et al. 1981; Murawski 1986; Estrada and Jiggins 2002).

Previous studies discussed specializations of taxonomic groups of *Heliconius* with respect to pollen size, viz. small and large pollen grains (Boggs et al. 1981; Cardoso 2001; Estrada and Jiggins 2002). In the present study the majority of pollen samples contained a mixture of both pollen grain sizes regardless of butterfly species. Very few samples contained only large sized pollen from *Psychotria* and/or *Psiguria* or only small pollen from *Lantana* and/or *Tournefortia* and no further analysis was conducted. However, our results indicate same

impacts of the pollen processing on the variously sized pollen. All pollen types were damaged in a similar way, and processing behaviour of all *Heliconius* species caused the opening of pollen exines and a loss of the cytoplasmic content.

Studies of pollen flow in natural populations of *Psiguria warscewiczii* (Cucurbitaceae) indicated that *Heliconius* butterflies are more effective pollinators of this plant than co-occurring hummingbirds, and that they are the primary pollinator of this plant (Murawski and Gilbert 1986; Murawski 1987). However, the present results indicate rapid damaging of pollen on the proboscis, and it is concluded that much pollen is lost for pollination after the butterflies commence with processing behaviour. Murawski (1987) found that most effective pollination occurred in the morning, and that pollen deposited on the floral stigma of *P. warscewiczii* was derived from recently visited plants likely before the processing behaviour began (Murawski 1986).

Heliconiine phylogeny based on morphological features advocates two independent evolutionary origins of pollen feeding (Penz 1999). A recent study of the phylogenetic relationships of Heliconiini inferred from mtDNA and nuclear genes demonstrated a single origin of pollen feeding and loss of this trait in the stem lineage of *Neruda* Turner 1976 (Beltrán et al. 2007). The present study does not lend differential support to either of these phylogenetic hypotheses, although the evolutionary loss of such a valuable ability would seem surprising. Rather, we show that the same mechanism of extra oral digestion can be assumed for all *Heliconius* species and *Laparus doris*, since the pollen grains are processed and damaged in the same way by all studied species.

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Fig. 1.

a *Heliconius pachinus* with pollen load on coiled proboscis. **b–d** Intact pollen and damaged pollen (arrows) of the three most common plants in the examined pollen samples. **b** Pollen tetrads of *Psiguria/Gurania* (Cucurbitaceae). **c** *Psychotria* sp. (Rubiaceae). **d** *Lantana camara* (Verbenaceae)

Table 1

Butterflies species and numbers of pollen samples

Butterfly species	Numbers of examined pollen loads		Numbers of pollen processing tests	
	Determination of pollen spectrum	Intact versus damaged pollen	<i>Psychotria elata</i> pollen	<i>Psiguria</i> pollen
<i>Heliconius hewitsoni</i> Hewitson 1875	4	10	2	
<i>Heliconius sara</i> (Fabricius 1793)	3	15	8	
<i>Heliconius melpomene</i> (Linnaeus 1758)	7	5	3	4
<i>Heliconius cydno</i> Doubleday 1847				4
<i>Heliconius cydno</i> × <i>H. melpomene</i> hybrids				9
<i>Heliconius pachinus</i> Salvin 1871	34	32	1	
<i>Heliconius hecale</i> (Fabricius 1776)	9	8	2	
<i>Laparus doris</i> (Linnaeus 1771)	3	2	1	
Total numbers (<i>n</i>)	60	72	17	17

Studies were performed with natural populations of butterflies in the Tropical Research Station La Gamba (Costa Rica); the tests with *Psiguria* pollen were done in Brackenridge Field Laboratory, University Texas at Austin (USA)

Table 2

Pollen samples collected from the proboscis of *Heliconius* species and *Laparus doris* (list of species see Table 1) contain a significantly higher percentage of damaged pollen compared to samples collected from anthers of the same flowers

Plant species	Pollen from proboscides of butterflies ^a		Pollen from flowers		P-value in Mann–Whitney U-test
	Median percentages of damaged pollen ± first quartile	n	Median percentages of damaged pollen ± first quartile	n	
<i>Psychotria elata/poeppigiana</i> (Rubiaceae)	74.88 ± 53.67	43	10.35 ± 9.4	10	<0.001 Z = -4.888
<i>Psiguria/Gurania</i> sp. (Cucurbitaceae)	72.04 ± 23.4	26	–	–	
<i>Lantana camara</i> (Verbenaceae)	21.35 ± 14.5	29	9.21 ± 7.55	10	<0.001 Z = -3.482

^a H. hewitsoni, H. sara, H. melpomene, H. pachinus, H. hecale, *Laparus doris*

Table 3

Pollen samples after 200 min of processing behaviour of *Heliconius* butterflies contain significantly higher proportions of damaged pollen grains than control samples gained directly from anthers

Plant species	Processed pollen		Pollen from flowers		<i>P</i> -value in Mann–Whitney U-test
	Median percentages of damaged pollen ± first quartile	<i>n</i>	Median percentages of damaged pollen ± first quartile	<i>n</i>	
<i>Psychotria elata</i> (Rubiaceae) ^a	33.2 ± 17.03	12	10.35 ± 9.4	10	0.015 <i>Z</i> = −2.44
<i>Psiguria tabscensis</i> or <i>P. bignoniacea</i> (Cucurbitaceae) ^b	90.71 ± 32.88	17	7.89 ± 5.22	23	< 0.001 <i>Z</i> = −4.583

^aTested in *H. hewitsoni*, *H. sara*, *H. melpomene*, *H. pachinus*, *H. hecale*, *L. doris*; in Tropical Research Station La Gamba (Costa Rica)

^bTested in *H. melpomene*, *H. cydno*, *H. melpomene* × *cydno*; in Brackenridge Field Laboratory, University Texas at Austin