

Pollen Processing Behavior of Heliconius Butterflies: A Derived Grooming Behavior

Authors: Hikl, Anna-Laetitia, and Krenn, Harald W.

Source: Journal of Insect Science, 11(99): 1-13

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.011.9901

The BioOne Digital Library (<u>https://bioone.org/</u>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<u>https://bioone.org/subscribe</u>), the BioOne Complete Archive (<u>https://bioone.org/archive</u>), and the BioOne eBooks program offerings ESA eBook Collection (<u>https://bioone.org/esa-ebooks</u>) and CSIRO Publishing BioSelect Collection (<u>https://bioone.org/csiro-ebooks</u>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at <u>www.bioone.org/terms-of-use</u>.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.



Pollen processing behavior of Heliconius butterflies: A derived grooming behavior

Anna-Laetitia Hikl^{a*}, and Harald W. Krenn^b

University of Vienna, Department of Evolutionary Biology, Althanstrasse 14, A-1090, Vienna, Austria

Abstract

Pollen feeding behaviors of Heliconius and Laparus (Lepidoptera: Nymphalidae) represent a key innovation that has shaped other life history traits of these neotropical butterflies. Although all flower visiting Lepidoptera regularly come in contact with pollen, only *Heliconius* and *Laparus* butterflies actively collect pollen with the proboscis and subsequently take up nutrients from the pollen grains. This study focused on the behavior of pollen processing and compared the movement patterns with proboscis grooming behavior in various nymphalid butterflies using video analysis. The proboscis movements of pollen processing behavior consisted of a lengthy series of repeated coiling and uncoiling movements in a loosely coiled proboscis position combined with up and down movements and the release of saliva. The proboscis-grooming behavior was triggered by contamination of the proboscis in both pollen feeding and non-pollen feeding nymphalid butterflies. Proboscis grooming movements included interrupted series of coiling and uncoiling movements, characteristic sideways movements, proboscis lifting, and occasionally full extension of the proboscis. Discharge of saliva was more pronounced in pollen feeding species than in non-pollen feeding butterfly species. We conclude that the pollen processing behavior of *Heliconius* and *Laparus* is a modified proboscis grooming behavior that originally served to clean the proboscis after contamination with particles.

Keywords: evolution, feeding mechanism, Lepidoptera, proboscis movements, video analysis Abbreviations: NPF, non-pollen feeders; PF, pollen feeders Correspondence: a* antschal@gmail.com, b harald.krenn@univie.ac.at, *Corresponding author Editor: Todd Shelly was Editor of this paper. Received: 22 August 2010, Accepted: 21 January 2011 Copyright : This is an open access paper. We use the Creative Commons Attribution 3.0 license that permits unrestricted use, provided that the paper is properly attributed. ISSN: 1536-2442 | Vol. 11, Number 99 Cite this paper as:

Hikl A-L, Krenn HW. 2011. Pollen processing behavior of *Heliconius* butterflies: A derived grooming behavior. *Journal* of Insect Science 11:99 available online: insectscience.org/11.99

Journal of Insect Science | www.insectscience.org

Introduction

Nectar consuming butterflies come into contact with pollen while visiting flowers. When butterflies search for and consume nectar on flowers, pollen frequently adheres to the proboscis, head, or other body parts. However, only butterflies of the closely related neotropical genera Heliconius and Laparus (Lepidoptera: Nymphalidae) evolved a feeding technique in which amino acids are extracted from the pollen grains (Gilbert 1972; Boggs et al. 1981; Estrada and Jiggins 2002; O'Brien et al. 2003). Pollen as an additional source of nutrition is central in their life histories, and is linked to other elaborated life history traits such as extended longevity, mutualistic insect-plant interactions, uninterrupted ovogenesis, and cyanogenesis in the adults and larvae (Gilbert 1972, 1991; Boggs et al. 1981; Engler et al. 2000; Beltran et al. 2007).

Due to the high nutritional quality of pollen grains that contain amino acids, proteins, polysaccharides, lipids, and sometimes vitamins (Baker 1978; Baker and Baker 1986) there can be no doubt about the advantages of using pollen as a food source. Most flower visiting arthropods consume pollen by chewing and mastication, or they swallow whole pollen grains (e.g. Simpson 1955; Smith and Mommsen 1984: Crailsheim et al. 1992; Van Rijn and Tanigoshi 1999; Krenn et al. 2005, 2008; Momose 2005; Karolyi et al. 2009). However, butterflies, which possess a suctorial proboscis that serves to ingest fluids, need a special technique to utilize the nutritional content of pollen. The behavior is termed "pollen feeding", and the technique of nutrient acquisition is referred to as "pollen processing behavior" (Gilbert 1972) because the pollen is not ingested, but undergoes a special treatment on the outside of the proboscis by which the butterfly extracts nutrients from the pollen grains (Gilbert 1972; O'Brien et al. 2003; Krenn et al. 2009).

Butterflies of the genera Heliconius and Laparus actively collect pollen during flower probing and accumulate it on the outside of the basal third of the proboscis (Boggs et al. 1981; Penz and Krenn 2000), where specialized bristle-shaped sensilla retain the pollen load (Figure 1) (Krenn and Penz 1998). The pollen load is subsequently processed by coiling and uncoiling movements of the proboscis that can last several hours (Gilbert 1972, 1975; Boggs 1987). During this behavior, these butterflies release a fluid from the proboscis that is repeatedly imbibed and re-released. Amino acids are extracted from the pollen grains and taken up via the ingested fluid (O'Brien et al. 2003). During pollen processing, the pollen grains become damaged (Krenn et al. 2009) and their contents are released into the extracting fluid, which has been shown to be saliva (Eberhard et al. 2009a). After completion of pollen processing, the pollen falls off the proboscis (Gilbert 1972). Given that proteolytic enzymes in the saliva (Eberhard et al. 2007) are responsible for the extraction, the processing of pollen on the proboscis constitutes an unusual example of extra-oral digestion.

Although nitrogenous components of the pollen grains have long been regarded as the basis for the evolution of novel life history traits in *Heliconius* butterflies (Gilbert 1991), the evolutionary origins of the pollen processing behavior remain obscure. In this study, the proboscis movements of the pollen

processing behavior in species of Heliconius were described using video analysis and movements were compared to those of related nymphalid butterflies which were exposed to small pollen sized particles. In nymphalid butterflies, contamination of the body (antennae, proboscis, and eyes) with small particles is known to release grooming behavior performed by the mid-tibia (Jander 1966). However, observations of neotropical butterflies in the field (Nilic and Lintner, indicated that proboscis unpublished) movements also eliminate small particles from the proboscis, and that saliva was used to clean the food canal of the proboscis. Based on these observations, we hypothesized that pollen processing behavior is a derived proboscis grooming behavior that allows Heliconius butterflies to utilize the pollen adhering to the proboscis as a source of nutrient.

Materials and Methods

Analysis of pollen processing behavior in *Heliconius* butterflies

Videos of pollen processing behavior were recorded in Heliconius butterflies from a greenhouse population in Brackenridge Field Laboratory of the University of Texas (Austin, USA) in April 2008. A total of 3 hours and 40 min were recorded in 5 individuals of Heliconius cydno (Doubleday 1847) and 6 Heliconius melpomene individuals of (Linnaeus) (Lepidoptera: Nymphalidae). Prior to filming, these butterflies voluntarily collected pollen from flowers of Psiguria (=Anguria) (Curcurbitales: sp. Cucurbitaceae), a plant that is known to be a primary source of pollen for Heliconius butterflies (Gilbert 1972; Boggs et al. 1981; Murawski and Gilbert 1986; Estada and Jiggins 2002; Krenn et al. 2009).

Proboscis grooming behavior

The comparative study of the proboscis movements was carried out in the Tropical Research Station La Gamba in Costa Rica (8° 45' N, 83° 10' W; 81 m asl) between February to April 2007. The ecosystem diversity and the butterfly fauna of this region were described in Weissenhofer et al. (2008). The butterflies were caught with a net in the surrounding habitats of the station next to the Bosque Esquinas (Piedras Blancas National Park).

Proboscis movements were recorded in four pollen feeding species: Heliconius hecale (Fabricius), Heliconius sara (Fabricius), Heliconius pachinus (Salvin), and Laparus doris (Linnaeus), as well as in three nonpollen feeding nymphalid butterflies: Eueides lvbia (Fabricius), Drvas iulia (Fabricius), and Anartia fatima Fabricius. From each species, five to six individuals were tested; a total of 39 individuals were video recorded. In all butterflies, proboscis movements were triggered by contamination of the proboscis with small particles. Glass beads (diameter 106 μm and finer. Sigma. www.sigmaaldrich.com) were placed on the butterfly's proboscis using an insect pin in two subsequent trials. Each individual was subjected to the following sequence: (1) the butterfly was fed with water in an insectarium ten minutes before each trial; (2) glass beads were placed on the proboscis and the reaction was recorded for 20 min; (3) the proboscis was rinsed and the butterfly was set free. In some cases, when a butterfly constantly moved around or showed no reaction to the contamination material, the observation was stopped.

Behavioral analysis

A hard disc camcorder (JVC GZ-MG37E, www.jvc.com) was used to record 20 min after the first mouthpart movements in each individual (N = 50). All videos were saved on an external hard disc in avi AVI and MPEG formats. All movements of each butterfly were analyzed using "The Observer XT" software (© 2005 Noldus Information Technology) (Noldus 1991). All butterflies were filmed in lateral or semi-lateral views to ensure a detailed behavioral analysis. Distinct patterns of proboscis movements were distinguished, i.e., coiling, uncoiling and sideways movement, degrees of proboscis extensions, as well as release of fluid (Figure. 2).

The proboscis extensions were coded into three categories. In category (I), the proboscis was tightly coiled; the proximal part of the proboscis was extended less than the diameter of the proboscis spiral in a totally coiled position (Fig. 2A) (Video). In category II, the diameter of the loose proboscis spiral and exceeded the diameter in coiled position by more than a quarter (Figure 2B). In category III, the proboscis was more or less straightly uncoiled (Figure 2D).

A fine scale analysis was made from 2 min recordings of the pollen processing behavior in H. cydno (N = 5) and H. melpomene (N = 6). These analyses included only the third to the fifth minute in all 20 min recordings of pollen-processing behavior to obtain a standard set of proboscis movements that natural characterize pollen processing behavior (Figure 3). In this detailed behavioral analysis, the movements of the proboscis were broken down and coded into individual movements (i.e. coiling, uncoiling, up, down) (Figure 3).

Periods of no movement (i.e. pauses) were coded as well. A pause was defined when the entire proboscis did not move for at least one second. To avoid losing data on the duration of each behavior, all movements were coded as state events in Observer XT with a code for the start and the end of the behavior. The primer dataset was exported, and statistics were calculated using SPSS software, Version 11.5 of the SAS System for Windows 2002. Since the butterflies moved freely during the video recordings, some individuals turned to a position from which the proboscis movements could not be observed. These time periods made up less than 15% (except two individuals with approximately 45%) of the total observation time, and were included as "movements not observable" in the data analyses.

Non-parametric statistics were used to compare the data. Significance of the comparisons was set at p < 0.05 level.

Results

Pollen processing behavior

During pollen processing behavior, individuals of *H. cydno* and *H. melpomene* sat motionless with their wings closed upwards. They moved the proboscis to handle pollen



from Psiguria sp. flowers that adhered to the coils during previous foraging bouts (Figure 1). Pollen processing behavior was a repeated sequence of partly coiling and uncoiling movements of the proboscis (Figure 2A). At the same time, saliva was released and mixed with the collected pollen load. A repetitive sequence of the several proboscis movements, i.e. coiling, uncoiling, up and down (Figure 3) was regularly detected in pollen processing behavior of H. cydno and H. melpomene. Depending on the size of the pollen load, the proboscis was coiled from a fully coiled to a loosely coiled position (equivalent to extension category I and II). During the coiling movement, the number of coils increases, and the proboscis spiral became tighter. During uncoiling the number of coils decreases, and the diameter of the proboscis spiral widens. At the same time, the entire proboscis is raised upwards at its joint to the head and is lowered afterwards, resulting in an up and down movement sequence.

Due to the size of the pollen load, some specimens were unable to fully coil the proboscis into resting position. They processed the pollen in proboscis extension category II. Proboscis extension category III was never observed during pollen processing behavior (Figure 4).

The characteristic cycle of proboscis pollen-processing movements during are displayed in Figure 3 as an example of the fine scale analysis. An upward movement of proboscis was accompanied the bv simultaneous and quick uncoiling, after which a lengthier period of coiling followed. Coiling behavior occupied the majority of total time (60.5 %) per 2 min (N = 11). A rapid downward movement of the proboscis initiated proboscis coiling, after which the whole pattern would begin anew with the upward uncoiling movements (Fig. 3). During the uncoiling motions of the proboscis, the inner coils were in contact with the outer coils and they slid over the pollen load spreading the saliva released from the proboscis.

The proboscis movement cycles in the fine scale analysis showed a mean frequency of 44.4 ± 21.1 times per minute for coiling and uncoiling and 45.1 ± 25.9 times per minute for up-and-down movements (Figure 3). In the up-and-down movement pattern, the proboscis spiral was elevated at the joint to the head up to 45° over the horizontal plane, after which it was subsequently lowered.

In addition, sideways movement of the proboscis was found 13 times in a total of 220 min with a mean total duration of 0.54% per 20 min of video recordings of the pollen processing behavior (Figure 5). During the sideways movement, the degree of coiling remained constant, but the proboscis spiral turned to the left and right sides alternatively.

The release of saliva was observed during pollen processing behavior. Salivary fluid was released from the proximal part and the tip of the proboscis to form a thick suspension of pollen. The liquid fraction was sucked in, and again, saliva was released and added to the pollen load.

Some movement pauses occurred in most individuals between the movement cycles, described above. Pauses were observed with a mean duration of 4.18 sec, which represented about 16.06% of the 2 min fine scale analysis.

Proboscis movements after contamination with glass beads

The proboscis grooming behavior included coiling and uncoiling, up and down, and sideways movement (Video). These movements were performed in various proboscis extension categories (Figure 2). The proboscis sometimes was uncoiled into fully extended position after contamination with glass beads (Figure 4).

In addition, the coiled proboscis was regularly moved to the lateral sides. This sideways movement consisted of a series of repeated turning motions to the left and right sides. Maximal duration of these lateral swinging movements continued up to 70 sec in *A. fatima*. Sideways movements were never performed when the proboscis was uncoiled (extension category III).

During grooming behavior, saliva discharge led to a moist proboscis surface in most of the observed specimens. Although all observed butterfly species released a liquid from the proboscis, only the *Heliconius* species released enough to form well-defined drops that could be counted. In *Heliconius* species, 1-3 drops of saliva were present at the same time on the proboscis. *Laparus doris* produced the highest number of drops of all observed species (mean 20.2 drops/20 min with glass beads) after proboscis contamination. The highest mean duration of drops was observed in *H. pachinus* after glass bead contamination with 54.1 sec per 20 min video recordings.

Pollen processing behavior versus proboscis grooming behavior

All proboscis movements of pollen processing and proboscis grooming were compared in 20 min video recordings (Figures 4-6) (Table 1) and checked for the presence of fluid. All butterflies exhibited the same principle pattern of proboscis movements during pollen processing and proboscis grooming behaviors, including the distinct pauses in which the proboscis remained motionless and the discharge of salvia.

Regardless of feeding category (PF, NPF), the differences between pollen-processing and

able 1. Proboscis movements of pollen processing (*) and proboscis grooming behavior.					
			Median percentages of proboscis movements per 20 min video ± first quartile		
				Sideways	Movement
Feeding category	Species	Ν	Coiling-uncoiling	movement	pauses
Pollen processing behavior					
PF	H. cydno	5	96.75 ± 27.17	0.0 ± 0.25	3.25 ± 4.04
PF	H. melpomene	6	65.42 ± 58.25	0.54 ± 1.5	18.75 ± 37.65
Proboscis grooming behavior					
PF	H. hecale	5	43.17 ± 40.54	6.58 ± 9.33	40.92 ± 43.29
PF	H. pachinus	6	12.46 ± 16.54	2.38 ± 8.25	76.58 ± 20.31
PF	H. sara	5	19.92 ± 23.04	6.17 ± 4.75	62.08 ± 24.83
PF	L. doris	6	45.75 ± 53.46	4.08 ± 8.35	39.0 ± 48.0
NPF	E. lybia	5	15.0 ± 31.21	4.0 ± 8.0	76.08 ± 33.04
NPF	D. iulia	6	16.42 ± 15.15	8.29 ± 12.88	74.54 ± 20.23
NPF	A. fatima	6	10.13 ± 20.67	7.0 ± 12.33	76.42 ± 26.38

Median \pm first quartile of total durations in % per 20 min video of the analysed movement categories (coiling-uncoiling and sideways movement and movement pauses).

Observations of pollen processing (*) were performed in the greenhouse of the University of Texas, all other proboscis grooming experiments were conducted in the field in Costa Rica.

PF = pollen feeding Helicniini, NPF = non pollen feeding Nymphalidae

proboscis grooming behaviors mainly concerned (1) degree of proboscis extension (Figure 4), (2) movement pauses, and (3) sideways movements (Table 1).

The proboscis sometimes was uncoiled into the fully extended position only during proboscis grooming behavior (Figure 4), but never in pollen processing behavior.

During pollen processing, Heliconius butterflies performed nearly uninterrupted coiling-and-uncoiling movements together with a few pauses (Figure. 5). Pauses in movement characterized the grooming behavior of butterflies from both feeding categories (PF, NPF). The duration of movement pauses was significantly higher in grooming behavior of butterflies with an experimentally contaminated proboscis compared to pollen processing behavior of Heliconius butterflies (Table. 1) (Kruskal-Wallis, $\chi^2 = 24.081$, df = 2, *p* < 0.0001). The pauses in proboscis movements occurred mostly when the proboscis was in the coiled position (extension category I). No pauses of movement were observed in the uncoiled proboscis of extension category III.

Heliconius butterflies showed by far the shortest total duration of sideways movements in pollen-processing behavior (Figure 6). A long duration of sideways movement was found in all individuals tested with glass beads, and was characteristic of grooming behavior. The total duration of sideways movement was higher during proboscis grooming behavior in both groups (pollenfeeding and non-pollen feeding butterflies) compared to pollen-processing behavior of *Heliconius* (Kruskal-Wallis, $\chi^2 = 13.240$, df =2, *p* < 0.001).

Discussion

Pollen-feeding in Heliconius butterflies is regarded as a key evolutionary feature in their life-histories (Gilbert 1972, 1991; Brown 1981; Boggs et al. 1981; Beltran et al. 2007). These insects gather pollen from flowers, but do not ingest it. Instead, the butterflies extract amino acids from pollen by destroying the grains during the pollen-processing behavior which is performed by the proboscis (Krenn et al. 2009). In this study, the stereotypic pattern of proboscis movements that occur during pollen-processing were analyzed. Pollencharacterized processing was bv uninterrupted, lengthy coiling-and-uncoiling movements and up-and-down motions of the proboscis. Contamination coiled of a butterfly's proboscis with glass beads triggered proboscis grooming behavior, which is similar in many aspects to pollen processing behavior. Coiling-and-uncoiling movements occurred but pauses of movement, lateral sideways motions and uncoiling to the fully extended position of the proboscis, were proboscis regularly observed during grooming.

All butterflies released some amount of fluid during proboscis grooming and pollenprocessing behaviors. However, drops of saliva were only observed on the proboscis in *Heliconius* and *Laparus* butterflies during grooming behavior. Apparently, when the pollen load on the proboscis was very large, drops of the fluid were not visible. Instead, the fluid immediately mixed with the collected pollen during pollen-processing.

The presence of fluid on the proboscis tip was observed during pollen collecting on flowers (Penz and Krenn 2000), as well as during

pollen and glass bead processing in the first investigation of pollen feeding by Heliconius butterflies (Gilbert 1972). A recent study proved that this fluid is saliva (Eberhard et al. 2009a). It has been established that the salivary glands of Heliconius are larger than in other nectar feeding butterflies and in related genera (Eberhard et al. 2009b). The present results indicate that saliva is also used for proboscis grooming, which could be the evolutionary origin of pollen processing behavior. In Heliconius butterflies, a special salivary pump was detected that serves for the two-way flow of fluid in the food canal of the proboscis (Eberhard and Krenn 2003). Release of saliva is not restricted to Heliconiinae and was also found in fruit feeding butterflies, which dilute dried up fruit juice before taking it up (Knopp and Krenn 2003). Likewise, the uptake of pyrrholizidin alkaloids from withering plants in Ithomiinae Danainae (Boppré and 1981, 1983) presumably involves the release of saliva from the proboscis tip.

The proboscis movements can be explained by the functional mechanism of the lepidopteran proboscis (reviewed in Krenn 2010). According to this model, the elasticity of the cuticle coils the proboscis into a loosely coiled spiral. Further coiling movements are caused by contractions of the intrinsic galeal muscles within the lumen of the proboscis. In Heliconiinae, these intrinsic galeal muscles were found to be particularly numerous and they extend further into the tip region than in other Nymphalidae (Krenn and Mühlberger 2002; Bauder and Krenn 2009). The coiling and uncoiling movement involve two different mechanisms, the intrinsic galeal muscles causes coiling and the elasticity uncoils the proboscis into a loose spiral. A hydraulic mechanism is responsible for further uncoiling, which results from increased haemolymph generated by pressure compressing the stipes pumps in the butterfly's head (Schmitt 1938; Bänziger 1971; Krenn 1990; Wannenmacher and Wasserthal 2003). The sideways movements of the proboscis are probably caused by alternative contractions of intrinsic galeal muscles in the two proboscis halves. Similar lateral movements have been observed during the proboscis assembly after emergence from the pupae (Krenn 1997). Sideward movements characterize the last phase of proboscis assembly in which the galeae shift against each other until the linking structures of the proboscis halves are locked. The observed up and down movements are probably due to contraction extrinsic galeal muscles in the basal proboscis joint and an antagonistic stipital muscle (Eastham and Eassa 1955; Krenn 1990, 2000). The latter proboscis movements are regularly seen during probing movements of the proboscis in all Lepidoptera (Krenn 1990; Penz and Krenn 2000; Krenn 2008). Thus, in principle, all individual movements of pollen processing or proboscis grooming behavior can be performed by all butterflies.

We conclude that pollen processing behavior of *Heliconius* and *Laparus* originated from grooming behavior, similar to that found in other nymphalids, but was modified by (1) the loss of the sideway bending of the coiled proboscis, (2) loss of full uncoiling, and (3) increasing periods of coiling and uncoiling motions. A similar hypothesis has been proposed for the evolution of pollen collecting behavior in bees. In these insects, leg movements are primarily responsible for pollen manipulation (i.e. pollen uptake, loading and unloading). They have been shown to represent evolutionarily derived grooming movements, which are similar to

cleaning behavior (Jander 1976; Michener et al. 1978).

The evolutionary origin of pollen feeding in butterflies has involved behavioral modifications like proboscis grooming (shown in this study) and flower visiting (Penz & Krenn 2000; Krenn 2008), as well as morphological adaptations of the proboscis (Krenn & Penz 1998) and the salivary glands (Eberhard & Krenn 2003; Eberhard et al. 2009b). Which of these modifications arose first in the evolution of pollen feeding is still unknown. However, the hypothesis of the evolutionary origin of pollen processing behavior from proboscis grooming could be a valuable key toward understanding the pollen puzzling evolution of feeding. Utilization of pollen has allowed Heliconius butterflies and the closely related species, Laparus doris, to enter a novel adaptive zone in the evolution of their life histories.

Acknowledgements

We thank the Costa Rican Ministerio del Ambients y Energia, which kindly granted research permits. Further, we thank Stefan and Monika Eberhard for help with field work and the staff of the Tropical Research Station La Gamba, Costa Rica, for providing excellent facilities. We thank John Plant (Vienna) for checking the English of the manuscript. Special thanks go to Larry E. Gilbert (Section of Integrative Biology Brackenridge Field Laboratory, University of Texas, Austin) for his assistance and advice. Use of the Greenhouse Facilities at BFL was made possible by grants to LEG. This study was funded by the Austrian Science Fund (FWF-Project 18425-B03).

References

Baker HG. 1978. Chemical aspects of the pollination biology of woody plants in the tropics. In: Tomlinson PB, Zimmermann MH, Editors. *Tropical Trees as living Systems*, pp. 57-82. Cambridge University Press.

Baker HG, Baker I. 1986. The occurrence and significance of amino-acids in floral nectar. *Plant Systematics and Evolution* 151: 175–186.

Bänziger H. 1971. Extension and coiling of the lepidopterous proboscis- a new interpretation of the blood-pressure theory. *Mitteilungen derSchweizerischen entomologischen. Gesellschaft* 43: 225-239.

Bauder J, Krenn HW. 2009. Muskelanordnung im Rüssel von pollenfressenden und nektrasaugenden Heliconiini (Lepidoptera: Nymphalidae). *Entomologica Austriaca* 16: 159-160.

Beltran M, Jiggins CD, Brower AVZ, Bermingham E, Mallet J. 2007. Do pollen feeding, pupal mating and larval gregariousness have a single origin in *Heliconius* butterflies? Inferences from multilocus DNA sequence data. *Biological Journal of the Linnean Society* 92: 221-239.

Boggs CL, Smiley JT, Gilbert LE. 1981. Patterns of pollen exploitation by *Heliconius* butterflies. *Oecologia* 48: 284-289.

Boppré M. 1981. Adult Lepidoptera "feeding" at withered Heliotropium plants (Boraginaceae) in East Africa. *Ecological Entomology* 6: 449-452. Brown Jr. KS. 1981. The biology of *Heliconius* and related genera. *Annual Review of Entomology* 26: 427-456.

Crailsheim K, Schneider LHW, Hrassnigg N, Bühlmann G, Brosch U, Gmeinbauer R, Schöffmann B. 1992. Pollen consumption and utilization in worker honeybees: dependence on individual age and function. *Journal of Insect Physiology* 38: 409–419.

Eastham LES, Eassa YEE. 1955. The feeding mechanism of the butterfly *Pieris brassicae* L. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*. 239: 1-43.

Eberhard SH, Krenn HW. 2003. Salivary glands and salivary pumps of adult butterflies (Nymphalidae, Lepidoptera). *Zoomorphology* 122: 161-167.

Eberhard SH, Hrassnigg N, Crailsheim K, Krenn, HW. 2007. Evidence of protease in the saliva of the butterfly *Heliconius melpomene* (L.) (Nymphalidae, Lepidoptera). *Journal of Insect Physiology* 53: 126-131.

Eberhard SH, Hikl A-L, Boggs CL, Krenn HW. 2009a. Saliva or regurgitated nectar? What Heliconius butterflies (Lepidoptera: Nymphalidae) use for pollen feeding. *Annals of the Entomological Society of America* 102/6: 1105-1108. Eberhard SH, Nemeschkal HL, Krenn HW. 2009b. Biometrical evidence for adaptations of the salivary glands to pollen feeding in *Heliconius* butterflies (Lepidoptera: Nymphalidae). *Biological Journal of the Linnean Society* 97: 604-612.

Engler HS, Spencer KC, Gilbert LE. 2000. Preventing cyanid release from leaves. *Nature* 406: 144-145.

Estrada C, Jiggins CD. 2002. Patterns of pollen feeding and habitat preference among *Heliconius* species. *Ecological Entomology* 27: 448-456.

Gilbert LE. 1972. Pollen feeding and reproductive biology of *Heliconius* butterflies. *Proceedings of the National Academy of Sciences USA* 69: 1403 - 1407.

Gilbert LE. 1991. Biodiversity of a Central American *Heliconius* community: pattern, process, and problems. In: Price PW, Lewinsohn TM, Fernandes GW, Benson WW, Editors. *Plant-animal interactions: evolutionary ecology in tropical and temperate regions*, pp. 403-427. John Wiley & Sons.

Jander R. 1976. Grooming and pollen manipulation in bees (Apoidea): The nature and evolution of movements involving the foreleg. *Physiological Entomolology* 1: 179-194.

Jander U. 1966. Untersuchungen zur Stammesgeschichte von Putzbewegungen von

Tracheaten. Zeitschrift für Tierpsychologie 23: 799-844.

Karolyi F, Gorb SN, Krenn HW. 2009. Trapping pollen by the moist mouth: Structure and function of the mouthparts in the flower visiting beetle *Cetonia aurata* (Scarabaeidae, Coleoptera). *Arthopod-Plant Interactions* 3: 1-8.

Knopp MCN, Krenn HW. 2003. Efficiency of fruit juice feeding in *Morpho peleides* (Nymphalidae, Lepidoptera). *Journal of Insect Behavior* 16: 67-77.

Krenn HW. 1990. Functional morphology and movements of the proboscis of Lepidoptera (Insecta). *Zoomorphology* 110: 105-114.

Krenn HW. 1997. Proboscis assembly in Lepidoptera – a once in a lifetime sequence of events. *European Journal of Entomology* 94: 495-501.

Krenn HW. 2000. Proboscis musculature in the butterfly *Vanessa cardui* (Nymphalidae, Lepidoptera): settling the proboscis recoiling controversy. *Acta Zoologica* (Stockholm) 81: 259-266.

Krenn HW. 2008. Feeding behaviors of neotropical butterflies (Lepidoptera, Papilionoidea). In: Weissenhofer A, Huber W, Mayer V, Pamperl S, Weber A, Aubrecht G, Editors. *Natural and Cultural History of the Golfo Dulce Region, Costa Rica*, pp. 295-304. Stapfia 88, Oberösterreichische Landesmuseen. Krenn HW. 2010. Feeding mechanisms of adult Lepidoptera: structure, function, and evolution of the mouthparts. *Annual Review of Entomology* 55: 307-327.

Krenn HW, Penz CM. 1998. Mouthparts of *Heliconius* butterflies (Lepidoptera: Nymphalidae): A search for anatomical adaptations to pollen–feeding behavior. *International Journal of Insect Morphology and Embryology* 27(4): 301-309.

Krenn HW, Mühlberger N. 2002. Groundplan anatomy of the proboscis of butterflies (Papilionoidae, Lepidoptera). *Zoologischer Anzeiger* 241: 369-380.

Krenn HW, Plant JD, Szucsich NU. 2005. Mouthparts of flower-visiting insects. *Arthropod Structure & Development* 34: 1-40.

Krenn HW, Gereben-Krenn B-A, Steinwender BM, Popov A. 2008. Flower visiting Neuroptera: mouthparts and feeding behaviour of *Nemoptera sinuata* (Nemopteridae). *European Journal of Entomology* 105: 267-277.

Krenn HW, Eberhard MJB, Eberhard SH, Hikl A-L, Huber W, Gilbert LE. 2009. Mechanical damage to pollen aids nutrient acquisition in *Heliconius* butterflies (Nymphalidae). *Arthopod-Plant Interactions* 3(4): 203-208.

Momose K. 2005. Beetle Pollination in Tropical Rain Forests. In: Roubik DW, Sakai S, Hamid Karim AA, Editors. *Pollination Ecology and the Rain Forest, Sarawak*

Studies. Ecological Studies 174: 104-110. Springer Science + Business Media, Inc.

Michener CD, Winston M, Jander R. 1978. Pollen manipulations and related activities and structures in bees of the family Apidae. *The University of Kansas Science Bulletin* 51(19): 575-601.

Murawski DA, Gilbert LE. 1986. Pollen flow in *Psiguria warscewiczii*: a comparison of *Heliconius* butterflies and hummingbirds. *Oecologia* 68: 161-167.

Noldus LPJJ, 1991. The Observer: a software system for collection and analysis of observational data. *Behavior Research Methods, Instruments, and Computers* 23: 415–429.

O'Brien DM, Boggs CL, Fogel ML. 2003. Pollen feeding in the butterfly *Heliconius charitonia*: isotopic evidence for essential amino acid transfer from pollen to eggs. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 270: 2631– 2636.

Penz CM, Krenn HW. 2000. Behavioral adaptations to pollen–feeding in *Heliconius* butterflies (Nymphalidae, Heliconiinae): An experiment using *Lantana* flowers. *Journal of Insect Behavior* 13: 865–880.

Schmitt JB. 1938. The feeding mechanism of adult Lepidoptera. *Smithsonian Misccellaneous Collections* 97: 1-28.

Simpson J. 1955. The significance of the presence of pollen in the food of worker

larvae of the honeybee. *Quarterly Journal of Microscopical Sciences* 96: 117–120.

Smith RB, Mommsen TP. 1984. Pollen feeding in an orb-weaving spider. *Science* 226: 1330–1332.

Van Rijn PCJ, Tanigoshi LK. 1999. Pollen as food for the predatory mites *Iphiseius degenerans* and *Neoseiulus cucumeris* (Acari: Phytoseiidae): Dietary range and life history. *Experimental and Applied. Acarology* 23 (10): 785-802(18).

Wannenmacher G, Wasserthal LT. 2003. Contribution to the maxillary muscles to proboscis movement in hawkmoths (Lepidoptera: Sphingidae) – an electrophysiological study. *Journal of Insect Physiology* 49: 765-776.

Weissenhofer A, Huber W, Mayer V, Pamperl S, Weber A, Aubrecht G. 2008. *Natural and Cultural History of the Golfo Dulce Region, Costa Rica*, Stapfia 88, Oberösterreichische Landesmuseen, Linz.



Figure 1. Heliconius cydno sitting motionless and processing pollen with coiling and uncoiling proboscis movements. The coiled position of the proboscis was coded as extension category I; (greenhouse population in Brackenridge Field Laboratory of the University of Texas; photo: courtesy of S. H. Eberhard.). High quality figures are available online.

Coiling-uncoiling 2 A (I) Total uncoiling B (I) (II) (III) Sideways movement C (I) (I) (II) (II) (I) (II) (II)

Figure 2. Proboscis movements in the loosely coiled and uncoiled positions; extension categories I-III. (A) The coiling and uncoiling movement pattern; arrows show directions of movements of the proboscis. In category I, the uncoiled proximal part of the proboscis is shorter than the diameter of the proboscis spiral. (B) The uncoiling movement; arrows show the uncoiling of the proboscis to its totally extended position (dotted line). The loosely coiled proboscis shows extension category II and the uncoiled proboscis shows extension category III. (C) The sideways movement. Arrows indicate lateral movements of the coiled proboscis. (D) The up and down movement pattern; arrows show the upward and downward motions of the proboscis; it is raised and lowered at the joint to the head. High quality figures are available online.



Figure 4. Position of the proboscis during pollen processing in pollen feeding *Heliconius* butterflies (N = 11) and during proboscis grooming behavior in pollen feeding (PF) Heliconiini (N = 22) and non-pollen feeding (NPF) Nymphalidae (N = 17). Total duration in percent of observed proboscis extension categories I, II, and III per 20 min video recordings. High quality figures are available online.

Journal of Insect Science | www.insectscience.org



Hikl and Krenn

Figure 3. Fine scale analysis of pollen processing behaviour in *Heliconius cydno.* Screenshot showing an example of 4 sec from Observer XT. Four proboscis movements are represented, i.e. coiling, uncoiling, up and down. The coiling and uncoiling motions occured simultaneously with the up and down movements of the proboscis. All movements were repeated four times in the same order. High quality figures are available online.



Figure 5. Duration of the coiling-uncoiling movements in percent of 20 min video. Coiling and uncoiling movements lasted longer in pollen processing than in grooming behavior (Mann-Whitney U-test, Z = -3.591, P < 0.0001), no significant difference of duration during grooming behavior between *Heliconius* (grooming PF) and non-pollen feeding Nymphalidae (grooming NPF) (Mann-Whitney U-test, Z = -1.785, P = 0.222). ** = highly significant; n. s. = not significant. High quality figures are available online.



Figure 6. Duration of sideways movements in percent of 20 min video. Sideways movements were performed longer in proboscis grooming than in pollen processing (Mann-Whitney U-test, Z = -3.218, P = 0.003). There was no difference between pollen feeders (PF) and non-pollen feeders (NPF) in grooming behavior (Mann-Whitney U-test, Z = -0.71, P = 1.434). ** = highly significant; n. s. = not significant. High quality figures are available online.