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# DIPLOMARBEIT

## Pollen processing behavior of *Heliconius* butterflies: A derived cleaning behavior

### Extrahieren von Pollenkörnern durch *Heliconius*-Falter: Ein abgeleitetes Putzverhalten

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)


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|---|---|
| Verfasserin: | Anna-Laetitia Hikl |
| Matrikel-Nummer: | 0008476 |
| Studienrichtung /Studienzweig Studienblatt): | Biologie/ Zoologie (lt. UG 2002) |
| Betreuer: | Dr. Harald Krenn |


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# Introduction

Nectar consuming butterflies come into contact with pollen while visiting flowers. When searching for nectar on the flowers and when consuming nectar, the pollen may become adhered on their proboscis, head or other body parts. However, only in the closely related genera *Heliconius* and *Laparus* butterflies has a pollen feeding technique evolved in which amino acids can be extracted from the pollen grains (Gilbert 1972, O'Brien *et al.* 2003, Beltran *et al.* 2007). The adult butterflies are alleged to be main pollinators of many of the cucurbit vines *Psyguria* and *Gurania* (Murawski and Gilbert 1986, Condon and Gilbert 1990). Observations on these butterflies and analysis of the adhered pollen loads on their proboscises showed large amounts of pollen grains from these plant species (Gilbert 1972, Murawski and Gilbert 1986, Estrada and Jiggins 2002).

This additional nutrition adaptation is central in their advanced life history 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, and 1991, Boggs *et al.* 1981, Brown 1981, Engler *et al.* 2000).

Due to the high nutritional quality of pollen grains (they contain amino acids, proteins, polysaccharides, lipids and sometimes vitamins), there can be no doubt about the advantages of using it as a food source (Baker 1978, Baker and Baker 1986, Boggs 1987). Other flower-visiting arthropods, pollinators and/or parasites such as hymenopterans, beetles, mites, spiders and larvae of different kinds of insects consume pollen by chewing and masticating the pollen grains (e.g. Simpson 1955, Smith and Mommsen 1984, Crailsheim *et al.* 1992, Van Rijn and Tanigoshi 1999, Krenn *et al.* 2005, Momose 2005). Butterflies have a proboscis which serves to ingest liquids, but in some species a special mechanism has evolved enabling them to feed on pollen and digest the contents of the grains (O'Brien *et al* 2003). The mechanisms that underlie this behavior are poorly understood and a detailed description of this special behavior is missing. This behavior is reported in the literature mostly under the designation 'pollen feeding' but it also can be found as 'pollen processing', 'pollen extraction' or 'pollen exploitation behavior'. In this

study I use the term pollen processing behavior because of the pollen undergoes treatment by the butterfly.

*Heliconius* butterflies collect pollen during flower probing and accumulate it on the basal third of the proboscis (Boggs *et al.* 1981). Following this the pollen processing begins by the repeated coiling and uncoiling movements of the proboscis that may last several hours. During the process, the butterfly repeatedly releases a clear liquid which is then repeatedly reabsorbed (Gilbert 1972 and 1975, Boggs 1987). Amino acids are extracted from the pollen grains during this behavior (O'Brien *et al.* 2003). After completion of pollen processing, the pollen load falls off the proboscis (Gilbert 1972). Some authors suggest that the liquid is regurgitated nectar or saliva (Gilbert 1972 and 1975, Boggs 1987), however, unpublished data of experiments with colored nectar and glucose testing revealed that this fluid is very similar to saliva (C. Boggs pers. comm., S. Eberhard pers. comm., A. Hikl pers. observ.). Subsequent analyses of the saliva of *Heliconius melpomene* showed that it contains proteolytic enzymes which in principle could be used for the extraction of the amino acids present in the pollen grains (Eberhard *et al.* 2007). By assuming that proteases in the saliva are responsible for the extraction of the amino acids in the pollen grains adhered to the proboscis, pollen processing behavior could be understood as a technique of extra oral digestion with a typical set of proboscis movements. As mentioned previously, butterflies normally come in contact with pollen during flower probing, and if they do, it can be supposed that some of the pollen grains may fall off the proboscis. In heavily contaminated proboscises a cleaning behavior can be expected. Our observations show that fluid is released from the tip of the butterfly's proboscis. This may have an important role in proboscis cleaning. In other butterflies the release of fluid from the tip helps to dilute dried up sugary fluids (Knopp and Krenn 2003).

Since pollen feeding is seen as key role in the advanced life-history of *Heliconius* and *Laparus* butterflies, understanding the mechanisms of this unique behavior could give insights on evolutionary processes and its adaptations. In this study we describe pollen processing behavior in various *Heliconius* species using video analysis. My aim was to determine whether there is a characteristic cleaning behavior in pollen feeding and non-pollen feeding butterfly species after the proboscis was contaminated with small particles (glass beads or pollen grains). In order to test if pollen processing could have evolved from such proboscis cleaning behavior, the

proboscis movements of the observed behaviors were compared. The hypothesis is that both pollen processing and proboscis cleaning behaviors include similar uncoiling and recoiling movements that differ only in the degree of proboscis extension. It is additionally postulated that fluid is released by the butterfly during the process. Moreover, it can be expected that a special pattern of movement should occur during pollen processing, and that this behavior represents an adaptation to utilize pollen as a nutritional resource. The present study includes video analysis of both behaviors (pollen processing and proboscis cleaning) in various *Heliconius* species and non-pollen feeding butterfly species from natural habitats in Costa Rica and from greenhouse colonies.

# Methods

## Study site

The data was collected between February to April 2007 in Costa Rica at the Tropical Research Station La Gamba (8°45' N, 83°10' W; 81 m asl.), Bosque Esquinas (Piedras Blancas National Park) in the Gulfo Dulce Region. Bosque Esquinas is a tropical wet forest (*sensu* Holdridge 1947) and is not subject to marked seasonality. This region has a high mean annual precipitation (about 6.000 mm), a high nutrient status and is known for its outstanding biodiversity (Weissenhofer *et al.* 2008a). The area consists mostly of primary forest, secondary forest of different ages, and agricultural land in use by the inhabitants of the small village La Gamba (see detailed map Weissenhofer *et al.* 2008b). Depending on the preferred habitat of the chosen butterfly species, All butterflies specimens were caught with a butterfly net in the preferred habitat of the species. Species were identified with the aid of DeVries (1987), and set free after completion of the experiments.

## Studied species

Nine butterfly species (5 or 6 individuals each) were chosen and their proboscis movements were studied: 6 pollen feeders from the genus *Heliconius*, i.e., *H. cydno, H.* (*Laparus*) *doris, H. hecale, H. melpomene, H. pachinus* and *H. sara*. While *H.*

*cydno* and *H. melpomene* were obtained from greenhouse populations at the University of Texas, all other *Heliconius* species were collected in the field (Tabs. I and II).

The closely related non-pollen feeding species, *Eueides isabella,* from the natural population in Piedras Blancas National Park was chosen for the experiments. *Dryas iulia*, a related non-pollen feeding Heliconiinae represents one of the basal genera of the subfamily. The non-pollen feeding species, *Anartia fatima*, was selected as the nymphalid out-group and was also collected in the field. In the study I used the system of the Heliconiinae by Beltran *et al.* (2007) where *H.* (*Laparus*) *doris* is part of the genus *Heliconius.* In contrast, the system introduced by Penz (1999) sees this species as a closely related sister genus of Attempts were made to collect a variety of different species from the field, as well as species from different clades of Heliconiini (Joron *et al.* 2006).

**Table I:** Studied butterfly species from natural habitats in Costa Rica and from a greenhouse population in Texas. The species were used for behavioral analysis of pollen processing and proboscis cleaning behavior (after Beltran *et al.* 2007).

Nymphalidae

Nymphalinae

*Anartia fatima* (Hübner, 1819)

Heliconiinae

Heliconiini

*Dryas iulia* (Fabricius, 1775)

*Eueides lybia* (Fabricius, 1775)

*Heliconius (Laparus) doris* (Linnaeus, 1771)

*Heliconius cydno** (Doubleday, 1847)

*Heliconius hecale* (Fabricius, 1775)

*Heliconius melpomene** (Linnaeus, 1758)

*Heliconius pachinus* (Salvin, 1871)

*Heliconius sara* (Fabricius, 1793)

* Greenhouse population

## Naturally performed pollen processing behavior of *Heliconius* butterflies which voluntarily collected pollen

Videos for the analysis of pollen processing behavior were recorded using *Heliconius* butterflies from a greenhouse population in Brackenridge Field Laboratory of the University of Texas, Austin. A total of 3 hours 40 min of butterfly videos were recorded with *H. cydno* (5 individuals) and *H. melpomene* (6 individuals), which voluntarily collected pollen from *Psiguria sp.* flowers in April 2008. A JVC GZ-MG37E hard disc camcorder was used to record for 20 minutes after the first mouthpart movements in each individual. All videos were saved on an external hard disc in .avi and MPEG formats and analyzed with The Observer XT software 2007 (Noldus IT) afterward.

A fine scale analysis was made from 2 min recordings (total N = 11) to code the behavioral traits in more detail. The analysis was made from the third to the fifth minute in all 20 minute recordings of pollen processing behavior.

## Experiments with pollen and glass beads

Four *Heliconius* species, *H. hecale, H. sara, H. pachinus* and *H.* (*Laparus*) *doris*, two related Heliconiinae *Eueides isabella* and *Dryas iulia*, and *Anartia fatima* as the nymphalid out-group were used for studying proboscis cleaning behavior. From each species between five to six individuals were tested in both experiments.

In order to compare response of the proboscis to contamination with small particles both glass beads and commercially available pollen was placed on the proboscis of various butterflies using an insect pin (Tab. II). To induce cleaning behavior without a chemical stimulus, the butterfly's proboscis was partially coated with small sterile glass beads (*Sigma*® diameter 106 µm and finer). In the second experiment commercially available pollen collected by bees was used (Carlisan Blütenpollen, Pronatura, Ebreichsdorf, Austria). Every individual was subject to the same experimental sequence. First they were put in an insectaria and fed with water. Ten minutes before each experiment the tested butterfly was fed again with water. Then all observed butterflies were subjected to the glass bead experiment. The glass beads and the bee-collected pollen for the second trial were placed on the individual proboscis and the behavior was video recorded. In some cases when a butterfly seemed to be under stress, (constantly moving around, going up and down) or when

there was no reaction to the contamination material the experiment was interrupted. The reaction of the butterfly was recorded in each experiment on video with a JVC GZ-MG37E hard disc camcorder for a period of 20 minutes after the first mouthpart movements were observed. Afterwards the video recordings were analyzed using The Observer XT 2007 software (Noldus IT).

## Video analysis by using "The Observer XT"

All butterflies were filmed in lateral or semi-lateral view to assure full detail in the behavioral analysis. The two distinct proboscis movements: *coiling-uncoiling* and *sideways movement* (Figs. 1 and 2), three different degrees of proboscis extensions, movements of the entire butterfly, as well as the release of fluid, were coded from all 20 min video recordings using "The Observer XT" software (© 2005 Noldus Information Technology) (Noldus 1991).

The proboscis extensions were coded into three different categories. These categories were chosen with a relatively wide-range because of the missing scale. In category (1) the proboscis is nearly coiled, thus the *coiling-uncoiling* movement is possible during proboscis extension (Fig 1 a. and c.). In this case the length of proximal part of the proboscis is either shorter than the diameter of the proboscis spiral in a totally coiled position or the diameter of the proboscis spiral does not exceed the diameter of the spiral in a totally coiled position by a quarter. All other partly uncoiled proboscis extensions were coded as category (2) (Fig 1 d.) and a totally uncoiled proboscis as category (3) (Fig. 1 b.).

Movement pauses were also coded and are described by not moving the entire proboscis for at least one second. To avoid loosing data on the duration of each behavior, all movements were coded as state events with a code for the start and the end of the behavior.

In addition a fine scale analysis, 2 min each, of video recordings of the *H. cydno* and *H. melpomene* butterflies which collected the pollen voluntarily was made to get a steady set of movements as a definition of pollen processing behavior.

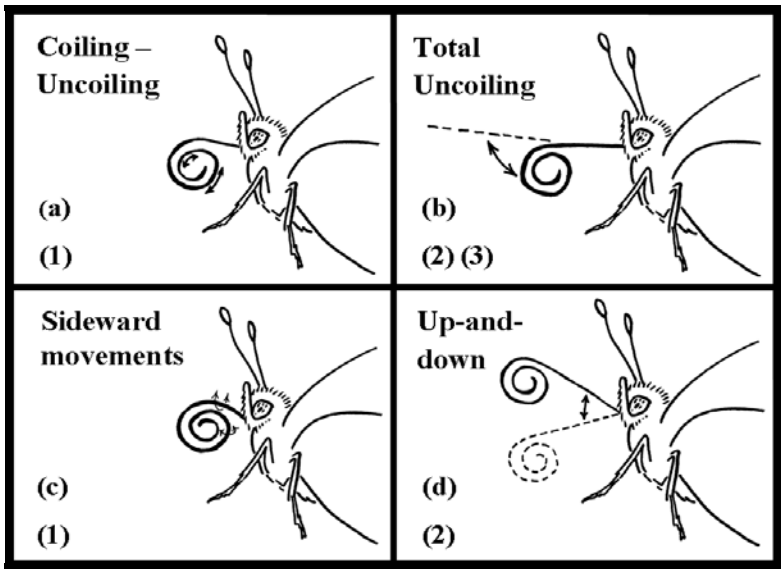


**Figure 1:** Types of proboscis movements (a-d) indicated by various proboscis extension categories (1-3).

(a) The *coiling-uncoiling* movement pattern, arrows show the inward and outward coiling movements of the proboscis. The proboscis is at its maximum extension, category (1), when the length of proximal part of the proboscis is shorter than the diameter of the coiled spiral.

(b) The *total uncoiling* movement pattern. Arrows show the uncoiling of the proboscis to its total extended position (dotted line). The partly coiled proboscis shows proboscis extension category (2) and the totally uncoiled proboscis shows proboscis extension category (3).

(c) The *sideways* movement. Arrows indicate lateral movements of the coiled proboscis. The proboscis is totally coiled during the proboscis extension category (1).

(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.


**Figure 2:** Photograph of *H. cydno* (from a greenhouse population) sitting calm and processing the pollen with its proboscis in extension category (1) (see Figure 1). (Photograph by S. H. Eberhard 2008)

## Statistical and behavioral analysis

Behavioral analysis was calculated with the software program "The Observer". The primer dataset was exported and statistics were calculated using SPSS software, Version 11.5 of the SAS System for Windows, © 2002.

# Results

## I. Pollen processing behavior

Pollen processing behavior is a repeated sequence of partly coiling and uncoiling movements of the proboscis (Fig. 1). It is described in *H. cydno* and *H. melpomene* individuals (total N = 11) which voluntarily collected pollen from flowers of *Psiguria sp.* from a greenhouse population at the University of Texas. The typical movements found in pollen processing behavior are *coiling-uncoiling* and *up-and-down* movements. In addition, a clear fluid is released continuously and mixed up with the collected pollen load.

During pollen processing behavior, the butterflies sit calmly with their wings closed upwards, only moving their proboscises when a pollen load sticks to the outer coil (Fig. 2).

Depending on the size of the pollen load, the proboscis is recoiled between 2 and 3 coils. The pollen load size is the reason why the proboscis cannot be coiled completely. Fluid is released while coiling and uncoiling.

In a fine scale video analysis of naturally performed pollen processing behavior (total N =11; of 2-min videos), a repetitive locomotory pattern of the four movements *coiling*, *uncoiling*, *up* and *down* (Fig. 3) was detected.

During *coiling* the number of coils increase and the proboscis spiral becomes tighter in lateral view. The opposite effect happens while *uncoiling* where the numbers of coils decreases. During *uncoiling*, the diameter of the proboscis spiral widens and/or the proboscis is extended at the joint to the butterfly's head. The entire proboscis rises up and lowers at its base during the *up-and-down* movement pattern. As shown in Figure 3 the movement patterns of *coiling* and *uncoiling* are different from the *up* and *down* patterns and can occur simultaneously.

The characteristic movement cycles displayed in Figure 3 starts with the *upward* motion: The proboscis commences to move upward, and after a few hundredths of a second, a very quick *uncoiling* movement is immediately followed by a longer coiling period. *Coiling* is the movement requiring the most amount of time. In this period the proboscis also moves down rapidly and the whole locomotory pattern starts again with the *upward* movement.

This pattern was shown in both fine scale analysis of naturally performed pollen processing behavior of *H. cydno* (N = 5) and *H. melpomene* (N = 6).
Due to the *uncoiling* motions of the proboscis, the inner coils contact the outer coils and slide over the pollen load spreading the released fluid.

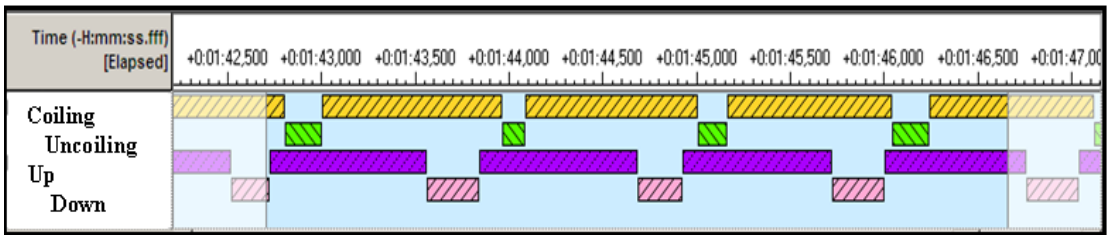


**Figure 3:** Representation of proboscis movements analyzed in the software program "The Observer XT" of naturally performed pollen processing behaviour of *H. cydno.* Four proboscis movements are represented: *coiling*, *uncoiling*, *up* and *down*. The *coiling* and *uncoiling* motions occur simultaneously with *up* and *down* movements of the proboscis. All movements are repeated times in the same order. Screenshot shows 4 seconds.

Ongoing cycles of *coiling* and *uncoiling* movements and *up* and *down* movements show a mean frequency of 44.4 (+ 21.1/ - 33.9) times per minute for *coiling* and *uncoiling* and 45.1 (+ 25.9/- 27.6) times per minute for up and down movements (Fig. 3).
In the *up-and-down* movement pattern, the proboscis spiral is elevated at the joint to the head up to 45° over the horizontal plane, after which it is subsequently lowered.
*Sideways* movement could be distinguished as a third pattern of motion. It was observed only a few times in 20 min video recordings of pollen processing behavior (Fig. 5). The degree of coiling remains constant in this movement pattern but the proboscis spiral turns to the left and right sides alternatively.
When the proboscis coils and uncoils, fluid is released from the proximal part of the proboscis, sometimes also from other areas and the tip. This fluid is mixed with the pollen to a thick paste. The liquid part of this mixed mass is sucked in and again fluid is released and added to the pollen load. The release of a liquid was observed many times in pollen processing behavior. But since these butterflies collected the pollen on their own, these pollen loads were too big to locate a single fluid drop on the proboscis or to measure the duration of its appearance.

Because of the large size of the pollen load, some butterflies were unable to totally coil the proboscis and process the pollen in proboscis extension category (1). Proboscis extension category (3) was never observed in association with pollen processing behavior (Fig. 6).

## II. Proboscis movements after contamination with pollen or glass beads

All butterflies performed similar movements of the proboscis regardless whether bee-collected pollen or glass beads were experimentally placed onto the proboscis. This means that there was no cleaning behavior which was exclusively found after contamination with bee-collected pollen or with glass beads. In addition, there was no significant difference (in the total number or total duration in % per 20 min video recordings) between the experiments with glass beads and bee–collected pollen as well as in the observed species and in the nutrition categories (pollen feeders, non-pollen feeders and the nymphalid out-group). Therefore, further analysis and comparisons were made between pollen processing behavior and the data set of the experiments with bee pollen.

Three patterns of proboscis movements could be distinguished in video analysis during cleaning behavior. The proboscis was coiled and uncoiled similar to pollen processing behavior. This means that the proboscis was coiled and recoiled to a spiral from the tip to its proximal region. And this was performed in all proboscis extension categories, so the proboscis can be partly or totally coiled and uncoiled (Fig. 1 a. and b.). *Up-and-down* movements were found but only coded in fine scale analysis of pollen processing behavior.

In addition, the coiled proboscis was repeatedly moved to the lateral sides. This *sideways* movement consisted of series of turning motions to the left and to the right side. Maximal duration of these swinging sideways movements was up to 70 sec in *A. fatima*. *Sideways* movements were never performed in proboscis extension category (3). Details of the total numbers and total durations of *coiling-uncoiling* and *sideways movement* of all observed butterfly species are presented in Tab. II.

During cleaning behavior, fluid drops appear on the proboscis tip and, additionally, in *Heliconius* species in the proximal region. Fluid discharge leads to a moist proboscis surface in most of the observed butterflies. However, all observed butterfly

species released a liquid from their proboscis, but only the pollen feeding *Heliconius* species released a fluid in form of a drop from their proximal proboscis section so that they could be counted. In non-pollen feeding butterfly species only small amounts of fluid occurred and no drops were found. Since it was not possible to quantify the amount of fluid released in the non-pollen feeders, further comparisons and analysis are restricted to the *Heliconius* species.

There was no significant difference between the total number (Mann-Whitney-U-test: Z = -0.289; P = 0.772), the total duration (Mann-Whitney-U-test: Z = - 0.133; P = 0.894) and the mean duration (Mann-Whitney-U-test: Z = -0.216; P = 0.829) of all fluid drops of all observed *Heliconius* species (species, N = 4; individuals, N = 22) per 20 min video recordings between the two contamination materials. *H. (Laparus) doris* had the most drops observed in both experiments (mean ~ 20.2 drops/20 min with glass beads and mean ~ 14.0 drops/20 min with bee pollen). The highest mean duration of drops was observed in *H. pachinus* after glass bead contamination with 54.1 sec per 20 min video recordings and for bee-collected pollen with 30.6 sec per 20 min video recordings. In all *Heliconius* species it was observed that up to 3 drops of fluid were present at the same time on the proximal part of the proboscis.

No differences between the two contamination materials and the coded proboscis extension categories were found in the non-pollen feeders, except in *D. iulia.* In this species, differences were found in the total number of occurrences of category (1) (Z = -2.330; P = 0.020), in the mean duration of category (1) (Z = -2.169; P = 0.030) and in the total number of occurrences of category (2) (Z = -2.082; P = 0.037).

**Table II:** Mean, standard deviation, minimum and maximum values in total number and total duration in % per 20 min videotapes of the analyzed movements *coiling-uncoiling* and *sideways movement* of all observed butterfly species (calculated to two decimal places). Values in parenthesis show minimum and maximum values. PF = Pollen feeders, NPF = Non-pollen feeders, TX = Experiment was conducted in the greenhouse of the University of Texas with original pollen, CR = Experiment was conducted in the field in Costa Rica with bee pollen.

| | | | | Mean, minimum and maximum values of proboscis movements per 20 min video tapes | | | |
|---|---|---|---|---|---|---|---|
| Experiment | Feeding Category | Species | N | Total Number *Coiling-Uncoiling* | Total Duration *Coiling-Uncoiling* in % | Total Number *Sideways movement* | Total Duration *Sideways movement* in % |
| TX | PF | *H. cydno* | 5 | 15.2 ± 13.39 (7 – 39) | 86.75 ± 20.68 (50.08 – 98.17) | 0.2 ± 0.45 (0 – 1) | 0.1 ± 0.22 (0 – 0.5) |
| TX | PF | *H. melpomene* | 6 | 35.83 ± 27.24 (7 – 77) | 64.25 ± 27.98 (30.83 – 99.33) | 2.17 ± 2.32 (0 – 6) | 0.92 ± 1.32 (0 – 3.5) |
| CR | PF | *H. doris* | 6 | 59.17 ± 7.17 (55 – 73) | 51.69 ± 14.73 (33.67 – 71.75) | 19.5 ± 10.05 (6 – 33) | 12.93 ± 8.75 (0.83 – 23.17) |
| CR | PF | *H. hecale* | 5 | 44.6 ± 17.76 (28 – 73) | 18.03 ± 12.81 (9.25 – 38.75) | 12.4 ± 7.44 (3 – 20) | 8.77 ± 4.07 (3.58 – 13.5) |
| CR | PF | *H. pachinus* | 6 | 51.83 ± 36.91 (19 – 120) | 19.54 ± 18.07 (4.75 – 55.08) | 21.67 ± 9.46 (12 – 33) | 8.57 ± 3.82 (3.08 – 14.25) |
| CR | PF | *H. sara* | 5 | 43.4 ± 19.66 (14 – 62) | 19.83 ± 11.9 (7.17 – 35.25) | 16.4 ± 6.31 (9 – 23) | 11.75 ± 7.88 (3.83 – 21.58) |
| CR | NPF | *E. isabella* | 5 | 21.2 ± 29.52 (0 – 73) | 14.63 ± 12.30 (0 – 29.08) | 9.2 ± 7.05 (4 – 21) | 6.97 ± 5.09 (1.17 – 14.08) |
| CR | NPF | *D. iulia* | 6 | 30.17 ± 18.04 (12 – 55) | 14.57 ± 12.23 (4.17 – 36) | 15 ± 9.86 (2 – 27) | 12.07 ± 7.82 (2.08 – 19.75) |
| CR | NPF | *A. fatima* | 6 | 35.67 ± 40.90 (3 – 98) | 8.10 ± 10.0 (1.58 – 27.75) | 14.06 ± 13.81 (0 – 37) | 12.15 ± 10.31 (0 – 28.83) |

## III. Comparison of pollen processing behavior and proboscis cleaning behavior

Video analysis of all observed butterfly species in all experiments showed that the butterflies performed three distinct proboscis movements: *coiling-uncoiling*, *sideways* and *up-and-down*, as well as distinct pauses in which the proboscis remains motionless (Fig. 1). To test our hypothesis that both behaviors include a coiling and recoiling in different proboscis extension categories and that a special derived movement or movement pattern is found in pollen processing behavior, we compared all observed behaviors in total number and duration in % per 20 min video recordings (Tabs. II and III). The presence of the observed fluid was compared between these two behaviors.

The number and duration of movement pauses was significantly higher in all butterflies with bee-collected pollen compared to butterflies which showed naturally performed pollen processing behavior (Kruskal-Wallis: $\chi^2 = 24.811$; df = 3; $P < 0.0001$). Butterflies which naturally performed pollen processing behavior were most active in the observed time and spent by far the least amount of time in pauses per 20 min video recordings (PF *Heliconius* TX: 14.08%; PF *Heliconius* CR: 58.77%; NPF Heliconiinae CR: 73.73%; NPF Nymphalidae CR: 71.83% of 20 min). This pauses in movement were performed mostly in the extension category (1), when the proboscis is totally coiled into a spiral, which is regarded as the resting position of the proboscis (Krenn 1990). No pauses in movement were observed in proboscis extension category (3).

Three classes of butterflies were tested in the experiments: pollen feeders, non-pollen feeders and the out-group species. The display or performance of pollen processing or proboscis cleaning behavior among these classes was significantly different in the total number of *coiling-uncoiling* movements (Kruskal-Wallis, $\chi^2 = 10.390$; df = 3; $P < 0.016$) and highly significant different in the total duration of this behavior (Kruskal-Wallis, $\chi^2 = 26.293$; df = 3; $P < 0.0001$) (Fig. 3).

*Heliconius* butterflies which voluntarily collected pollen in the greenhouse of the University of Texas were more active in performing the *coiling-uncoiling* movement, which can be demonstrated in nearly 100% of the observed 20 min (Fig. 4).

A comparison of the statistics concerning the amount and duration of proboscis movements in all species is presented in Tab. II. The total amount of *sideways*

movement was significant higher ($\chi^2$ = 22.836; df = 3; P < 0.0001) and also the total duration of *sideways* movement was higher in proboscis cleaning behavior ($\chi^2$ = 22.316; df = 3; P < 0.0001). Details are shown in Table II. Naturally performed pollen processing behavior showed by far the smallest total number and the shortest total duration of this behavior (Fig. 5). The high number of *sideways* movement was found in all tested species regardless whether bee-collected pollen or glass beads were used.

Apart from the total number of *coiling-uncoiling* movements, all total numbers and total durations of all observed proboscis movements showed significant or highly significant differences between the species. The same test between all species in which the proboscis was experimentally contaminated with bee-collected pollen (pollen feeders and non-pollen feeding species) revealed only significances in the total duration of *coiling-uncoiling* and motionless pauses. The same applies also to the *Heliconius* species. No significant difference was found between the non-pollen feeding butterflies. The data is tabulated in Tab. III.

Since all proboscis extensions categories are dependent on each other and since category (2) shows the highest variation among the butterfly classes, tests were only performed for extension category (2) for differences (Fig. 6). A comparison of the pollen feeders with original and with bee collected pollen showed significant differences in the total duration of extension category (2) per 20 min (Z = -3.108; P = 0.002). Comparison of the pollen feeding *Heliconius* species with the non-pollen feeding species of Heliconiinae which were contaminated with bee pollen revealed a significant difference (Z = -3.103; P = 0.002). Likewise, compared to the nymphalid out-group with bee collected pollen, significant differences were also found in the total duration of extension category (2) (Z = -3.230; P = 0.001). In the experiment with bee collected pollen (Fig. 6), significant differences occur between the *Heliconius* species and 1) the non-pollen feeding Heliconiinae (Z =1.891; P = 0.059) and 2) the nymphalid out-group (Z =-2.828; P = 0.005).

**Table III:** Results for all Mann-Whitney-tests made for proboscis movements in pollen feeding (PF) and non-pollen feeding (NPF) butterfly species. TX = Experiment was conducted in the greenhouse of the University of Texas with original pollen, CR = Experiment was conducted in the field in Costa Rica with bee pollen.

| Pairs Mann-Whitney-tests | P-value | Z-value |
|---|---|---|
| *Coiling-uncoiling*, total number | | |
| PF *Heliconius* CR & NPF Heliconiinae CR | 0.006 | -2.734 |
| PF *Heliconius* CR & NPF Nymphalidae CR | n. s. | - |
| PF *Heliconius* CR & PF *Heliconius* TX | 0.007 | -2.696 |
| NPF Heliconiinae CR & NPF Nymphalidae CR | n. s. | - |
| NPF Heliconiinae CR & PF *Heliconius* TX | n. s. | - |
| NPF Nymphalidae CR & PF *Heliconius* TX | n. s. | - |
| *Coiling-uncoiling*, total duration | | |
| PF *Heliconius* CR & NPF Heliconiinae CR | 0.049 | -1.967 |
| PF *Heliconius* CR & NPF Nymphalidae CR | 0.007 | -2.687 |
| PF *Heliconius* CR & PF *Heliconius* TX | < 0.0001 | -3.704 |
| NPF Heliconiinae CR & NPF Nymphalidae CR | n. s. | - |
| NPF Heliconiinae CR & PF *Heliconius* TX | < 0.0001 | -3.841 |
| NPF Nymphalidae CR & PF *Heliconius* TX | 0.001 | -3.317 |
| *Sideways movements*, total number | | |
| PF *Heliconius* CR & NPF Heliconiinae CR | n. s. | - |
| PF *Heliconius* CR & NPF Nymphalidae CR | n. s. | |
| PF *Heliconius* CR & PF *Heliconius* TX | < 0.0001 | -4.525 |
| NPF Heliconiinae CR & NPF Nymphalidae CR | n. s. | - |
| NPF Heliconiinae CR & PF *Heliconius* TX | < 0.0001 | -3.618 |
| NPF Nymphalidae CR & PF *Heliconius* TX | 0.027 | -2.294 |
| *Sideways movements*, total duration | | |
| PF *Heliconius* CR & NPF Heliconiinae CR | n. s. | - |
| PF *Heliconius* CR & NPF Nymphalidae CR | n. s. | - |
| PF *Heliconius* CR & PF *Heliconius* TX | < 0.0001 | -4.539 |
| NPF Heliconiinae CR & NPF Nymphalidae CR | n. s. | - |
| NPF Heliconiinae CR & PF *Heliconius* TX | < 0.0001 | -3.814 |
| NPF Nymphalidae CR & PF *Heliconius* TX | 0.01 | -2.604 |

| | | |
|---|---|---|
| Movement pauses, total number | | |
| PF *Heliconius* CR & NPF Heliconiinae CR | n. s. | - |
| PF *Heliconius* CR & NPF Nymphalidae CR | n. s. | - |
| PF *Heliconius* CR & PF *Heliconius* TX | 0.004 | -2.905 |
| NPF Heliconiinae CR & NPF Nymphalidae CR | n. s. | - |
| NPF Heliconiinae CR & PF *Heliconius* TX | n. s. | - |
| NPF Nymphalidae CR & PF *Heliconius* TX | n. s. | - |
| Movement pauses, total duration | | |
| PF *Heliconius* CR & NPF Heliconiinae CR | 0.056 | -1.909 |
| PF *Heliconius* CR & NPF Nymphalidae CR | n. s. | - |
| PF *Heliconius* CR & PF *Heliconius* TX | < 0.0001 | -4.086 |
| NPF Heliconiinae CR & NPF Nymphalidae CR | n. s. | - |
| NPF Heliconiinae CR & PF *Heliconius* TX | < 0.0001 | -3.973 |
| NPF Nymphalidae CR & PF *Heliconius* TX | 0.001 | -3.216 |

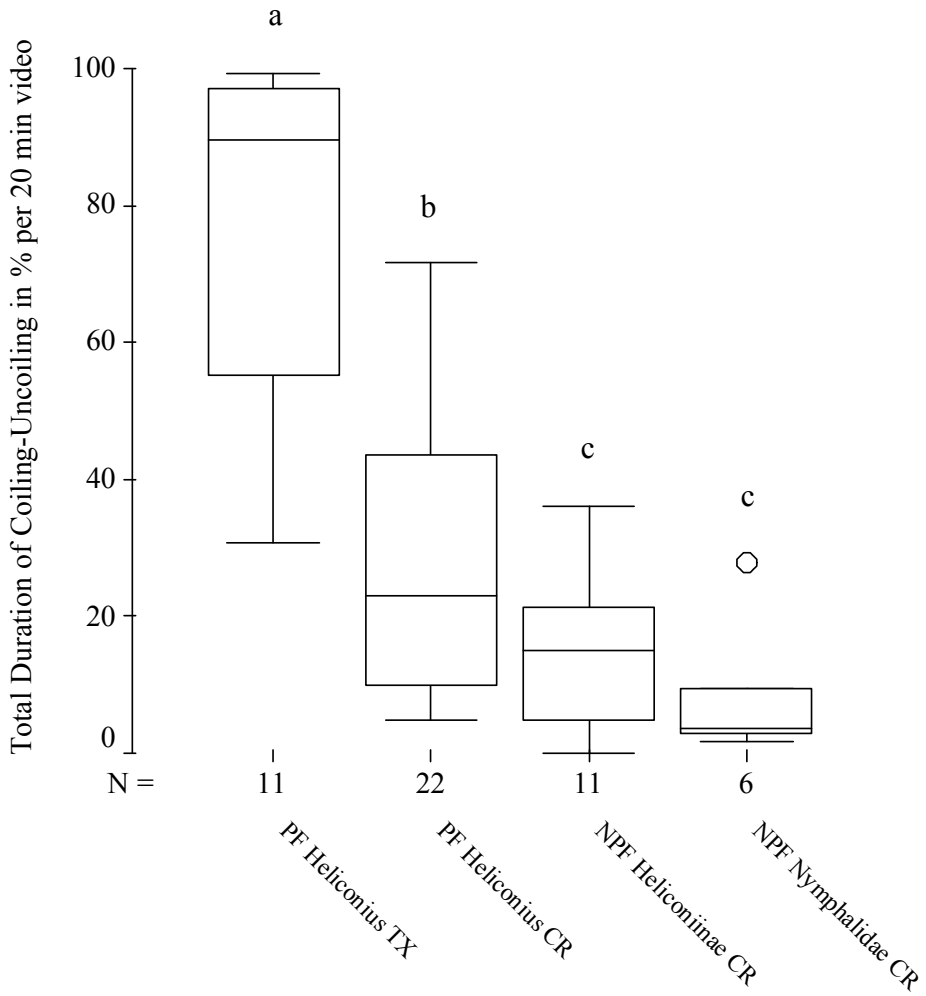


**Figure 4:** Total Duration in % of the *coiling-uncoiling* movement among butterfly classes in 20 min videotapes.

PF *Heliconius* TX = *H. melpomene, cydno* = Pollen feeders from University of Texas with original pollen, PF *Heliconius* CR = *H. pachinus, hecale, sara* = pollen feeders from Costa Rica with bee-collected pollen, NPF Heliconiinae CR = *D. iulia, E. lybia* = non-pollen feeders from Costa Rica with bee-collected pollen, NPF Nymphalidae CR= *A. fatima* = Nymphalid out-group from Costa Rica with bee-collected pollen, CR = Experiment was made in the field in Costa Rica, TX = Experiment was made in the greenhouse of the University of Texas. Circles symbolizing outliers. Letters show the significant difference of Mann-Whitney-U-test (Tab. III).

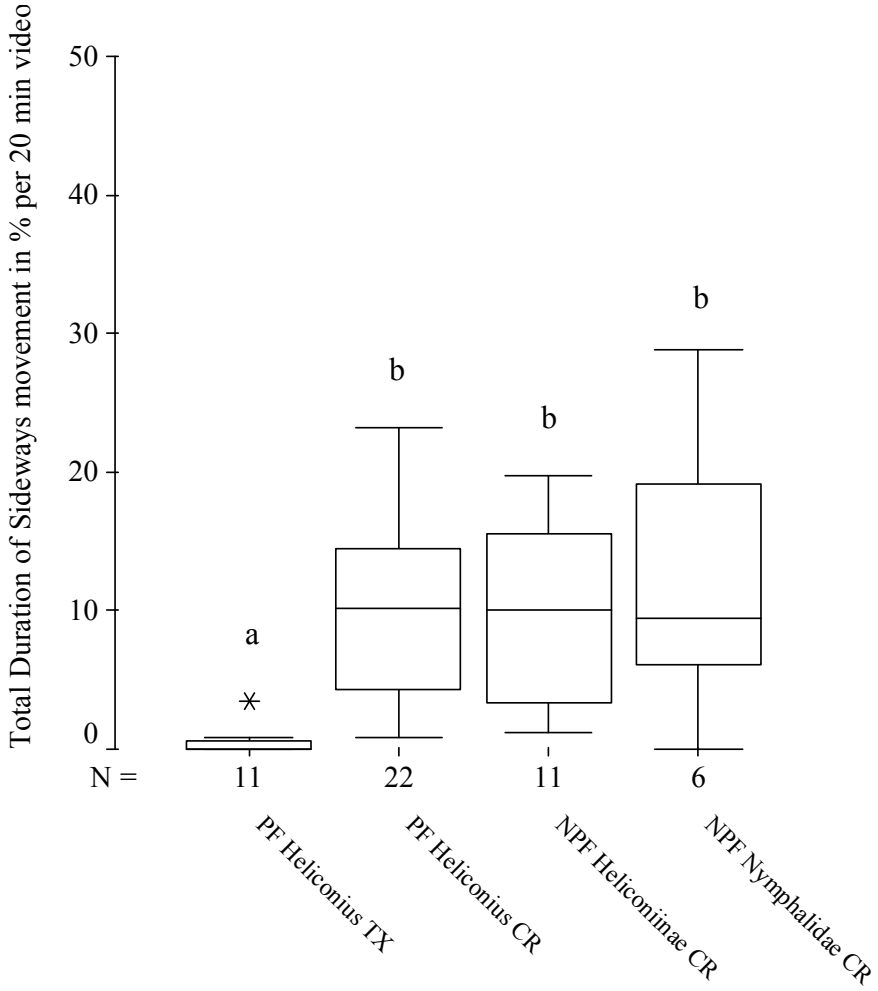


**Figure 5:** Total duration in % of *sideways* movement among the butterfly classes in 20 min videotapes.

PF *Heliconius* TX = *H. melpomene, cydno* = Pollen feeders from University of Texas with original pollen, PF *Heliconius* CR = *H. pachinus, hecale, sara* = Pollen feeders from Costa Rica with bee-collected pollen, NPF Heliconiinae CR = *D. iulia, E. lybia* = Non-pollen feeders from Costa Rica with bee-collected pollen, NPF Nymphalidae CR = *A. fatima* = Nymphalid out-group from Costa Rica with bee-collected pollen, CR = Experiment was conducted in the field in Costa Rica, TX = Experiment was conducted in the greenhouse of the University of Texas. Stars symbolize extreme values. Letters show the significant difference of Mann-Whitney-U-test (Tab. III).

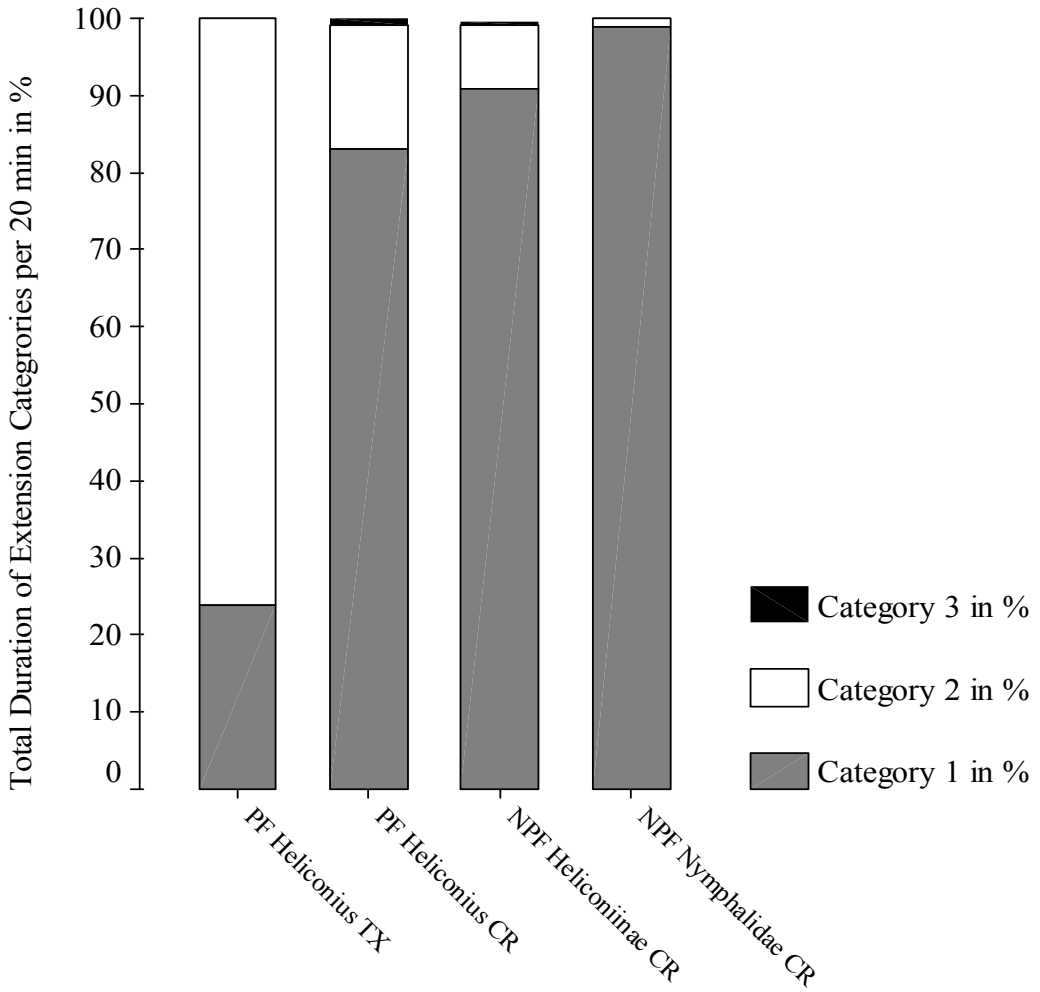


**Figure 6:** Total duration in % of observed proboscis extension categories (1, 2 and 3) per 20 min videotapes among butterfly categories with original or bee pollen.
PF *Heliconius* CR = *H. pachinus, hecale, sara* = pollen feeders from Costa Rica with bee-collected pollen, NPF Heliconiinae CR = *D. iulia, E. lybia* = Non-pollen feeders from Costa Rica with bee-collected pollen, NPF Nymphalidae CR = *A. fatima* = Nymphalid out-group from Costa Rica with bee-collected pollen, PF *Heliconius* TX = *H. melpomene, cydno* = pollen feeders from University of Texas with original pollen, CR = Experiment was conducted in the field in Costa Rica, TX = Experiment was conducted in the greenhouse of the University of Texas.

# Discussion

Pollen feeding in *Heliconius* butterflies is held to be a key evolutionary feature in their advanced life-history (Gilbert 1972, Brown 1981, Boggs 1981). These insects gather pollen from flowers with a special behavior, termed pollen processing, by which amino acids are extracted from pollen grains. In this study the stereotypic pattern of proboscis movements which occur during pollen processing were analyzed. As a result, differences and similarities to proboscis cleaning behavior were detected (Figs. 1 and 3). Contamination of a butterfly's proboscis with glass beads or pollen grains triggers the particular pattern of proboscis movements which are similar in many aspects to pollen processing behavior. As expected *coiling-uncoiling* movements occurred in both sets of behavior, but they were performed in pollen processing with a much higher total duration (Fig. 4). This means that butterflies processing the pollen performed this motion pattern with greater activity. This was previously described in studies of pollen processing behavior as a repeated coiling and uncoiling of the proboscis (Gilbert 1972, Boggs 1981). After collecting pollen grains under semi-natural conditions there are only short motionless pauses occur during pollen processing. In contrast to proboscis cleaning, the motionless pauses were observed at different time intervals and the *coiling-uncoiling* movement was not performed in such a high total duration in the examined videos as in pollen processing behavior. Since relatively little is known about proboscis cleaning behavior in butterflies, a description of this movement pattern is given for the first time, in the present investigation.

All butterfly species release some amount of fluid during proboscis cleaning and pollen processing. However, drops of this fluid were only observed and counted on the proximal part of the proboscis in *Heliconius* and *Laparus* butterflies. *Heliconius* species which naturally performed pollen processing behavior exuded a fluid. Apparently, when the pollen load on their proboscis is very large, e.g. pollen load size 3 as classified by Boggs *et al.* (1981), drops of the fluid are not visible. Instead the fluid immediately mixes in with the collected pollen. That is why it was only possible to observe the presence of a fluid and not to count the number of drops.

The presence of a fluid during pollen processing was also observed in previous investigations, but never quantified (Gilbert 1972, Penz and Krenn 2000). The fluid

is most likely saliva (Boggs 1987, C. Boggs pers. comm., S. Eberhard pers. comm., A. Hikl pers. observ.). It is known that the salivary glands of *Heliconius* are larger than in other nectar feeding butterflies and in its sister genera (Eberhard *et al.* 2009). Another argument in favor of the salivary hypothesis is the presence of protease in the saliva which would be necessary to extract the amino acids out of the pollen (Eberhard *et al.* 2007). Therefore, I expect that saliva is used for proboscis cleaning in various butterfly species which could have important implications for the evolution of pollen processing behavior. I was able to observe pollen feeding species releasing fluid in quantifiable amounts, shown by the number of drops, on the proximal part of the proboscis. Undoubtedly, this could have lead to further adaptations in the proboscis morphology of *Heliconius* butterflies, for example, the salivary pump, which is a special structure in the *Heliconius* butterfly's head (Eberhard and Krenn 2003). The movements of *coiling* and *uncoiling*, which are typical in pollen processing behavior, were exhibited in all *Heliconius* species not only during pollen processing but also during proboscis cleaning behavior. This is one of the obvious similarities between these behaviors (Fig. 4). The *sideways* movement is an individual component of proboscis cleaning behavior (Fig. 5) and could hardly be observed in pollen processing. Another component of cleaning behavior not observed during pollen processing behavior was a total uncoiling of the proboscis, described as extension category (3). Here the butterfly's proboscis is raised higher than in the other proboscis extension categories. The other proboscis extension categories were observed in both compared behaviors. Since a unique movement pattern was found for proboscis cleaning behavior, it is clearly possible to distinguish pollen processing behavior from proboscis cleaning behavior (Fig. 5). Therefore, I conclude that pollen processing behavior is derived from a modified cleaning behavior which lost a particular movement pattern. Interesting is that a similar hypothesis has been proposed for the evolution of pollen collecting behavior in bees. Here, it is however leg movements which are primarily responsible for pollen manipulation (i.e., pollen uptake, loading and unloading). They have been shown to represent evolutionarily derived grooming movements which is similar to a cleaning behavior (Jander 1976, Michener et al. 1978).

The mechanisms of movements found in pollen processing behavior are based on the functional model for proboscis movements: The elasticity coils the proboscis in a loosely coiled position and specific coiling movement then is caused by the intrinsic

galeal muscles, which were experimentally demonstrated by Krenn (2000) and electrophysiological examined by Wannenmacher and Wasserthal (2003). *Coiling* movements caused by intrinsic galeal muscles seem to be particularly in high number in Heliconiinae (Krenn and Mühlberger 2002).

A hydraulic mechanism is responsible for uncoiling of the butterfly's proboscis, which is due to increased hemolymph pressure in the proboscis lumen generated by compressing the stipes pumps (Schmitt 1938, Bänzinger 1971, Krenn 1990, Wannenmacher and Wasserthal 2003). To summarize, for typical *coiling-uncoiling* movement of pollen processing behavior two different mechanisms are necessary, for coiling contraction of the intrinsic galeal muscles and for uncoiling a hydraulic mechanism caused by hemolymph pressure. This is caused and created by compression of the maxillary structures which connects the proboscis to the butterfly's head.

The *sideways movement*, typical for proboscis cleaning is probably caused by contraction of galeal muscles in one proboscis half. A similar movement was described in the proboscis assembly in Lepidoptera as anti-parallel movements of the galeae and slight sideward movements (Krenn 1997). These movements characterize the last phase of assembly where the proboscis is tightly coiled while it is repeatedly moved from side to side. This means that the galeae shift against each other and the linking structures of the proboscis half are locked. Since fluid release was observed in all species and especially in *Heliconius* in various proboscis regions, the possible reason of this motion may be the locking and tightening of the two galeae with each other. The additionally observed movement category of *up-and-down* movements are probably due to contraction and extension of extrinsic galeal muscles in the basal proboscis joint and an antagonistic stipital muscle (Eastham and Eassa 1955, Krenn 1990). This movement was observed on many occasions, and it occurs simultaneously along with the coiling movements. It is also expressed in flower probing behavior to push the proboscis deeper into or pull it out of the floral corolla tube (Krenn 1990, Penz and Krenn 2000). Due to these previous studies of proboscis movements in butterflies and hawkmoths, the mechanics of pollen processing behavior can be explained by the action of various proboscis muscles, and the function of the performed motions can be adequately discussed.

Pollen processing behavior is probably originated from a proboscis cleaning behavior; it was modified by the loss of *sideways movements* and by performing

longer periods of *coiling-uncoiling* motions. Similar evolutionary pathways can be proposed for the morphological adaptations of the proboscis to pollen collecting or in the different performances of flower handling behavior in the pollen feeding *Heliconius* species compared with its sister genera and other Nymphalids (Krenn and Penz 1998, Krenn 2008). These key behavioral modifications can be regarded as an adaptation to optimize the adherence of pollen on the butterfly's proboscis and for the utilization of pollen as a source for amino acids.

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# Summary

Pollen feeding behavior of *Heliconius* and *Laparus* (Nymphalidae) represents a key innovation in the advanced life-history of these butterflies. Although flower visiting nectar feeding butterflies regularly come in contact with pollen, only *Heliconius* and *Laparus* actively collect pollen with their proboscis and process it subsequently. This study focuses on the behavior of pollen processing and compares the movement patterns with proboscis cleaning behavior. By using video analysis with The Observer XT 2007 software (Noldus IT), a comparison of behaviors between different butterfly species was made. The pollen processing behavior was analyzed and described for first time in detail. Fundamental proboscis movements are a repeated coiling and uncoiling of the proboscis occurring in both pollen processing and proboscis cleaning behavior in all studied species. A higher number of repetitions and total duration in the observed time of the movement *coiling-uncoiling* was recorded for pollen processing behavior. The release of a digestive fluid, similar to saliva, was observed frequently in both feeding behaviors. The proboscis cleaning behavior included a characteristic *sideways movement* of the proboscis which was expressed in both pollen feeding and non-pollen feeding butterflies. Therefore we conclude that pollen processing behavior is a derived proboscis cleaning behavior with the loss of the particular *sideways movements* and by adding performing *coiling-uncoiling* movements in continuously repeated and longer total durations.

**Keywords:** *Heliconius,* pollen processing behavior, cleaning behavior, proboscis movements, video analysis, comparative behavioral analysis

# Zusammenfassung

**Extrahieren von Pollenkörnern durch *Heliconius*-Falter: Ein abgeleitetes Putzverhalten**

Neotropische Tagfalter der Gattung *Heliconius* (Nymphalidae) sind die einzigen Schmetterlinge, die Pollen als zusätzliche Nahrungsquelle nützen, wodurch ihre außergewöhnliche Lebensweise begründet werden kann. Der Pollen wird aktiv beim Blütenbesuch gesammelt und bleibt am Rüssel haften. In einem anschließenden Bearbeitungsverhalten werden die enthaltenen Aminosäuren mit Hilfe einer klaren Körperflüssigkeit und charakteristischen Rüsselbewegungen aus den Pollenkörnern extrahiert. Auch andere Nektar fressende Schmetterlinge kommen bei der Nahrungsaufnahme in Kontakt mit Pollen, zeigen jedoch nicht dieses außergewöhnliche Verhalten. Bei diesen Tagfaltern stellt der anhaftende Pollen eine Verunreinigung des Rüssels dar und sollte deshalb ein Rüsselputzverhalten auslösen.

Im Rahmen dieser Studie wurde erstmals das Verhalten des Pollenbearbeitens detailliert beschrieben und mit den Bewegungen eines Rüsselputzverhaltens verschiedener Tagfalterarten verglichen. Durch Videoanalyse und unter Verwendung des Programms „The Observer XT“ wurden die verschiedenen Verhaltensweisen untersucht, verglichen und statistisch ausgewertet. Die in Vorstudien beobachtete Rüsselbewegung *Ein- und Ausrollen* (*coiling-uncoiling*) wurde wie erwartet beim Pollenbearbeiten und Rüsselputzverhalten, beobachtet. Beim Bearbeiten von Pollenkörnern zeigen Tagfalter jedoch dieses Verhalten insgesamt häufiger und auch länger im beobachteten Zeitraum. Als charakteristisch für ein Rüsselputzverhalten wurde eine *Seitwärtsbewegung* (*sideways movement*) beschrieben, die fast ausschließlich bei dieser Verhaltensweise beobachtet wurde. In beiden Verhaltensweisen wurde die Bewegung *Ein- und Ausrollen* sowie die häufige Abgabe einer Flüssigkeit, wobei es sich wahrscheinlich um Speichel handelt, beobachtet. Trotzdem sind Pollenbearbeiten und Rüsselputzverhalten durch die charakteristische Bewegung *Seitwärtsbewegung* deutlich voneinander unterscheidbar.

Die Ergebnisse lassen den Schluss zu, dass das Bearbeiten des Pollens der Schmetterlingsgattung *Heliconius* von einem Rüsselputzverhalten ableitbar ist. Vermutlich ist die Verhaltensweise *Seitwärtsbewegung* als Rüsselbewegung bei der

Pollenbearbeitung verloren gegangen und stellt jedoch eine wichtige Funktion beim Putzen des Schmetterlingsrüssels dar.

Anna-Laetitia Hikl
Löwengasse 9/11
A-1030 Wien
Tel: 0043 (0)699 190 54 335
antschal@gmail.com

## *Curriculum Vitae*

**Personal Information:**

Date of birth: January 22, 1982
Place of birth: Vienna
Nationality: Austria
Marital status: Single
Driving license: Yes

**Education:**

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|---|---|
| Since 2001 | Studies of Biology, University of Vienna |
| 1992 - 2000 | High School, Vienna |

**Experience and Skills:**

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| Languages | German, English, Spanish, Italian |
| 2009 July | Presentation of poster "Pollen processing behaviour of *Heliconius* butterflies: A derived cleaning behaviour?" at Joint Meeting of Association for Tropical Biology and Conservation & Society for Tropical Ecology 2009 in Marburg. |
| 2009 February | Data collection for project "Foraging for pollen grains: differences in *Psiguria* flower handling times of *Heliconius* and *Laparus* butterflies depending on the presence of pollen grains" |

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| | at La Selva field station (Organisation for Tropical Studies) |
| 2008 August | Presentation of poster "Motion analysis of butterflies: Investigation of the evolutionary origin of pollen feeding behaviour of Heliconius butterflies (Nymphalidae)" at Measuring Behaviour 2008 in Maastricht. |
| 2008 February | OTS-1 field course "Tropical Ecology" (Organisation for Tropical Studies) |
| 2007 July | Presentation of poster "Pollen processing behaviour of *Heliconius* butterflies" at "Biobutterfly 2007", 5th Conference on the Biology of Butterflies in Rome |
| 2007 February | Field work for Master thesis "Pollen processing behaviour in *Heliconius* Butterflies", Field Station La Gamba, Costa Rica, University of Vienna |
| 2006 February | Practical training for Tropical Ecology, Field Station La Gamba, Costa Rica, University of Vienna |
| 2005 August | Zoological-Botanical Excursion to Costa Rica, University of Vienna |
| 2004 August | Voluntary service, Field Station La Gamba, Costa Rica, University of Vienna |
| 2003 August | Practical training for Nature Conservation, Turkey, University of Vienna |

**Previous Scientific Work:**

| | |
|---|---|
| 2009 February | Tutor, Field course "Ecology and behaviour of insects and birds in Costa Rica", University of Vienna |
| 2007 May | Tutor, Field course "Diversity of Central Europe habitats", University of Vienna |
| 2006 August | FWF-Project: P 18425 B03. Assisstant Project Manager |