Biometrical evidence for adaptations of the salivary glands to pollen feeding in *Heliconius* butterflies (Lepidoptera: Nymphalidae)

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The Neotropical genus *Heliconius* (Nymphalidae) is unique among butterflies for its pollen-feeding behaviour. With the application of saliva, they extract amino acids from pollen grains on the outside of the proboscis. We predicted that the salivary glands of pollen-feeding Heliconiinae would show adaptations to this derived feeding behaviour. A biometrical analysis of the salivary glands revealed that pollen-feeding butterflies of the genus *Heliconius* have disproportionately longer and more voluminous salivary glands than nonpollen-feeding Nymphalidae. The first two components in the principal component analysis explained approximately 95% of the total variance. The size-dependent factor score coefficients of body length and salivary gland parameters were predominately represented on axis 1. They significantly discriminated pollen-feeding from nonpollen-feeding heliconiines on that axis. Factor score coefficients for the volume of the secretory region of the salivary glands separated heliconiines from the outgroup species. The detailed biometrical analysis of salivary glands features thus provides strong evidence that the secretory regions of the salivary glands are larger in pollen-feeding butterflies. We concluded that pollen feeding is associated with a high production of salivary fluid. © 2009 The Linnean Society of London, Biological Journal of the Linnean Society, 2009, 97, 604–612.


INTRODUCTION

By contrast to the majority of butterflies that feed exclusively on nectar or other fluids, the Neotropical butterflies of the genera *Heliconius* and *Laparus* utilize pollen grains as an important source of nitrogen (Gilbert, 1972; Brown, 1981). Phylogenetic studies based on morphology indicate that *Heliconius* and *Laparus* are not sister taxa, thus suggesting a convergent evolution of pollen feeding (Penz, 1999). However, investigations based on molecular data favour a single origin for pollen feeders (Beltrán et al., 2007). *Heliconius* butterflies actively collect pollen from the anthers of flowers. The pollen grains are admixed with a fluid to form a moist lump on the outside of the proboscis. There is strong evidence that this fluid used in pollen feeding is saliva (C. L. Boggs, S. H. Eberhard, A. L. Hikl & H. W. Krenn, unpubl. data). The pollen mixture is then agitated up to several hours by uncoiling and recoiling of the proboscis and in this process free amino acids and proteins are extracted from the pollen grains (Gilbert, 1972). Pollen feeding enhances the adult lifetime of *Heliconius* butterflies by up to 6 months (Gilbert, 1972). Presumably as a result of adults being supplied with nitrogenous compounds, the larval stages are short, and therefore the caterpillars are less exposed to predators and parasites (Gilbert, 1972, 1991). The direct transfer of essential amino acids from pollen to eggs has been demonstrated previously (O’Brien, Boggs & Fogel, 2003). One consequence of pollen feeding is constant egg production throughout the entire lifespan of adult females (Gilbert, 1972; Dunlap-Pianka, Boggs & Gilbert, 1977). Furthermore,
the nitrogen obtained from pollen apparently contributes to the production of cyanogenic glycosides, which increase the concentration of defensive chemicals in these butterflies (Nahrstedt & Davis, 1983).

Investigations of the mouthparts of *Heliconius* revealed subtle adaptations for the retention of pollen on the outer surface of the proboscis (Gilbert, 1972; Krenn & Penz, 1998), as well as modified flower-handling behaviour (Penz & Krenn, 2000). Adaptations of internal organs for the digestion of pollen can be expected, although they have yet to be demonstrated.

In Lepidoptera, the salivary glands are composed of a pair of convoluted tubes (Chapman, 1998). In adult nymphalids, they extend from the head through the thorax to the beginning of the abdomen and can be divided into five anatomically and histologically distinct regions, labelled R1–R5 (Fig. 1) (Eberhard & Krenn, 2003).

Insect saliva basically facilitates the uptake of food but also contains a wide range of enzymes (Ribeiro, 1995; Soyelu, Akingbohungbe & Okonji, 2007). In butterflies, saliva is not only admixed to fluid food, but also serves to dissolve solid or highly viscous substances (Kirbach, 1884; Wigglesworth, 1972; Knopp & Krenn, 2003). The ability to discharge saliva from the tip of the proboscis is provided by a salivary pump (Eberhard & Krenn, 2003). A functional model for saliva discharge and fluid uptake is provided by Eberhard & Krenn (2005).

Pollen-feeding behaviour can last several hours, and the occurrence of protease in the saliva of *Heliconius melpomene* is thought to be important in the feeding process (Eberhard et al., 2007). During pollen collection and extraction, saliva is released frequently and in a larger amount than in nonpollen-feeding species (Penz & Krenn, 2000). This suggests that saliva plays a crucial role in the extraction of free amino acids and proteins from pollen grains. Because of the importance of saliva in this context, the saliva producing organs (i.e. the salivary glands) are of particular interest. Because the anatomy and histology of the salivary glands of pollen feeders and non-pollen feeders are equivalent (Eberhard & Krenn, 2003), and because a higher amount of saliva is needed for pollen feeding, it is hypothesized that salivary glands are larger in pollen-feeding heliconines. To verify this hypothesis, we performed a biometrical comparison of the salivary glands of pollen feeders and related nonpollen-feeding butterflies.

**Figure 1.** A, location of salivary glands in a *Heliconius* butterfly. B, schematic illustration depicting only one of the paired salivary gland (proportions not true to scale). Each gland is divided into five regions, R1–R5 (left); the secretory regions (R1–R2) consist of three parts (hemisphere, frustum, and cylinder) (right). R1 comprises approximately 10% of the secretory regions (R1–R2). Nomenclature of the salivary gland regions *sensu* Eberhard & Krenn (2003). R1, bulbous secretion region; R2, tubular secretion region; R3, salivary duct; R4, salivary reservoir; R5, salivary outlet tube; dl, duct length; gl, gland length; tl, total length.
Because allometric relationships of salivary glands must be assumed, and because percentages or size-specific indices seldom eliminate the influence of body size (Packard & Boardman, 1999), we used multivariate data analyses. The present study comprises the first attempt to search for internal morphological features that can be interpreted as adaptations to pollen-feeding behaviour in Heliconius butterflies.

MATERIAL AND METHODS

ANIMALS

Five species of Nymphalidae were used for this investigations: H. melpomene (Linnaeus 1758), Laparus doris (Linnaeus 1771), Dryas julia (Fabricius 1775), Dione junö (Cramer 1779), and Vanessa cardui (Linnaeus 1758). All species belong to the subfamily of the Heliconiinae, except V. cardui which belongs to the Nymphalinae (Harvey, 1991). Heliconius melpomene (N = 14) and L. doris (N = 13) are pollen feeders, whereas D. julia (N = 11) and D. junö (N = 10) feed exclusively on nectar; V. cardui (N = 11) serves as an outgroup species and also feeds on nectar only.

The butterflies were kept in a greenhouse (under a 12 : 12 h light/dark cycle, relative humidity in the range 40–90%, and at approximately 25 °C) of the University of Vienna. They were fed with artificial nectar which mimics the nectar of Lantana camara (Alm et al., 1990) and with Butterfly Nectar (The Birding Company). Additionally, H. melpomene was provided with pollen (Carlisan Blütenpollen, Pronatura GmbH). In all species, both sexes were present.

PRODUCTION OF PERMANENT MICROSCOPICAL PREPARATIONS OF THE SALIVARY GLANDS

To produce microscopical preparations of the whole salivary glands, butterflies were narcotized with CO₂ and then frozen (−40 °C). After defrosting, the body length was measured with a sliding calliper. Because the tips of the forewing were occasionally damaged or worn, we used body length to estimate body size.

Specimens were submerged in water for dissection. The strongly convoluted glands were untangled; right and left glands were separated in 35% ethanol solution. The glands were spread out on a microscope slide, embedded in polyvinylacetophenol without dehydration, covered with a cover glass, and dried at 40 °C for no less than 24 h.

MEASURING THE SALIVARY GLANDS

To measure the length of the salivary glands, drawings of the microscopical preparations were made using a Nikon SMZ-10 stereomicroscope equipped with a drawing-tube. Similarly, drawings for the measurements of the diameters of the salivary glands were made with a Reichert Diavar microscope equipped with a drawing-tube. Detailed descriptions of anatomy and histology of the salivary glands of nymphalid butterflies are provided elsewhere (Eberhard & Krenn, 2003).

To diminish errors of measurement, each drawing of the total length of the salivary glands (Fig. 1) was measured twice and the mean value calculated. The total length of the salivary gland was measured from the bifurcation of the right and left glands (within R5) to the blind end in R1. The gland length (i.e. the secretory part of the salivary gland), which corresponds to regions R1 and R2, was calculated as total length minus duct length (Fig. 1). The latter corresponds to R3, R4 and the paired part of R5 (Fig. 1). In the majority of individuals, both salivary glands (left and right) were prepared successfully and also both glands were measured. From a few specimens, only one gland could be prepared and measured. In cases where both glands were measured, we selected, with the aid of a computer-generated random number, either the right or left gland for the statistical analysis.

Diameters were measured only from the secretory part (R1–R2) (Fig. 1B, left) of the salivary glands. The largest diameter of region R1 was recorded. The diameter of the cylindrical part (R2) was determined as the mean value of nine measurements (three measurements each at approximately 1%, 50%, and 90% of the length of the secretory part). The diameters were required to calculate the volumes of the secretory parts of the salivary glands. On each drawing, the corresponding scales were plotted to calibrate the computer program. All drawings were measured on a drawing tablet (Jandel Scientific) using Sigma-Scan™ computer software, version 3.90 (Jandel Scientific).

To calculate the volume, the glandular part of the salivary gland was notionally divided into three geometrical bodies: a hemisphere, a circular frustum (both belong to R1), and a cylinder (R2) (Fig. 1B, right). Because previous investigations indicated that the bulbous secretory region (R1) always comprises approximately 10% of the total length of the secretory part of the salivary glands (R1 and R2) in H. melpomene, D. julia, and V. cardui (Eberhard & Krenn, 2003), the length of R1 was calculated as exactly 10% of the secretory part.

To estimate the measurement error, the total length of the salivary gland of a randomly chosen microscopic preparation was measured ten times. The error was less than 0.4%.

STATISTICAL ANALYSIS

The investigated species were pooled into three groups: (1) pollen feeders (H. melpomene, L. doris); (2)
nonpollen-feeding heliconiines (D. julia, D. juno); and (3) outgroup (V. cardui). The statistical analyses were performed mainly using SPSS, version 15.0 (SPSS Inc.). A multivariate data analysis was performed using principal component analysis (PCA) and linear discriminant analysis (DA). For allometric reasons, the data for the multivariate data analyses were transformed by means of the common logarithm. For the PCA, five biometrical parameters were used: body length, total length of the salivary gland (R1 up to the paired part of R5), cylinder volume, frustum volume, and hemisphere volume. The significance of factor score coefficients was checked by a random permutation test (10,000 permutations each; software package of computer-intensive statistics; Nemeshkal, 1999). For principal components with eigenvalues greater than 1, a Mann–Whitney U-test was performed to test significant differences of the factor scores among the three different groups. The test results were corrected according to Bonferroni inequality.

The DA was based on Z-transformed factor scores previously extracted from the original data by the orthogonalization process of PCA. The discriminant scores were gained using a linear regression which was performed including the pollen feeders and the nonpollen feeders and excluding the outgroup. The values for the discriminant function resulting from the linear regression (numbers rounded) were: $D = 1.505 - 0.199x_1 + 0.096x_2 - 0.021x_3 - 0.271x_4 + 1.199x_5$ (coefficient of determination: $R^2 = 0.666$). In a second step, the discriminant scores of the outgroup were calculated by inserting them into the discriminant function. Subsequently, a DA was performed in SPSS, version 15.0, using the calculated discriminant scores. With this method, it was possible to determine whether the outgroup individuals would be classified as ‘pollen feeders’ or as ‘nonpollen feeders’. The discriminant scores of the groups were normally distributed which was verified by the Kolmogorov–Smirnov test. Moreover, an $F$-test was performed to determine the homogeneity of variance of the discriminant scores between pollen feeders and nonpollen feeders and, to test for significant differences, a $t$-test was performed.

RESULTS

LENGTHS AND VOLUMES OF THE SALIVARY GLANDS

The salivary glands of the pollen-feeding H. melpomene and L. doris are longer and more voluminous compared to the nonpollen-feeding D. julia and D. juno, as well as the outgroup species V. cardui (Fig. 2, Table 1). The mean body length of the pollen feeders is 17.60% greater compared to the nonpollen feeders and 30.39% greater compared to the outgroup. However, the total length of the salivary glands is 49.67% greater in the pollen feeders than in the nonpollen feeders; the pollen feeders have a 39.95% greater salivary gland length compared to the outgroup. Moreover, pollen feeders have a 88.37% greater volume of the secretory region of the salivary gland compared to the nonpollen feeders and a 58.36% greater volume compared to the outgroup (Table 1). It can be concluded that the salivary glands of pollen-feeding nymphalids are disproportionately longer, resulting in a greater volume than in those of nonpollen-feeding butterflies.

The mean value of the body length of the pollen feeders (24.47 ± 1.36 mm; $N = 27$) is greater than the average body lengths of the nonpollen feeders (20.81 ± 1.56 mm; $N = 21$) and the outgroup (18.76 ± 1.21 mm; $N = 11$) (Table 1). The same is true for the mean values of the salivary gland length measurements: total length, duct length, and gland length (i.e. in pollen feeders, those lengths are greatest). By contrast, the outgroup has greater mean values of the salivary gland length measurements compared to the nonpollen feeders (Table 1).

The average volume of the entire secretory part of the salivary gland, which corresponds with R1 and R2...
The biometric parameters of the investigated butterfly species, including mean ± SD and range values, are presented in Table 1. The principal component analysis (PCA) of the biometric variables extracted from the correlation matrix resulted in five principal components. The first two components, which cumulatively explain 94.758% of the total variance, can be classified as size- and shape-components. The volume of the cylinder displays the highest value within the factor score coefficients, followed by the total length of the salivary gland, the frustum volume, the body length, and the hemisphere volume. The separation of the pollen feeders from the nonpollen feeders is highly significant (Z = -5.289; Table 1).
Table 2. Component matrix (factor score coefficients), eigenvalues, and explained total variance of salivary gland parameters

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylinder volume</td>
<td>0.931**</td>
<td>-0.214</td>
<td>0.079</td>
<td>-0.284*</td>
<td>0.009</td>
</tr>
<tr>
<td>Total length</td>
<td>0.926**</td>
<td>-0.242</td>
<td>-0.280*</td>
<td>0.076</td>
<td>0.013</td>
</tr>
<tr>
<td>Frustum volume</td>
<td>0.889**</td>
<td>0.455**</td>
<td>-0.019</td>
<td>0.007</td>
<td>-0.041</td>
</tr>
<tr>
<td>Body length</td>
<td>0.782**</td>
<td>-0.559**</td>
<td>0.200</td>
<td>0.189</td>
<td>-0.001</td>
</tr>
<tr>
<td>Hemisphere volume</td>
<td>0.552**</td>
<td>0.826**</td>
<td>0.084</td>
<td>0.073</td>
<td>0.028</td>
</tr>
<tr>
<td>Explained variance %</td>
<td>68.635</td>
<td>26.123</td>
<td>2.648</td>
<td>2.540</td>
<td>0.054</td>
</tr>
<tr>
<td>Cumulative variance %</td>
<td>68.635</td>
<td>94.758</td>
<td>97.406</td>
<td>99.946</td>
<td>100.000</td>
</tr>
</tbody>
</table>

*Significant (P < 0.05), **Highly significant (P < 0.01).

Extraction method: principal component analysis. Five components were extracted. The raw data were previously transformed by means of the common logarithm. To test for significance of the factor score coefficients, a random permutational test was performed (number of permutations: 10 000).

DISCRIMINANT ANALYSIS

The discriminant scores of the pollen feeders and the nonpollen feeders are normally distributed (Kolmogorov–Smirnov test, pollen feeder: Z = -0.509; P = 0.611), whereas the separation is highly significant between the outgroup and either the pollen feeders (Z = -4.651; P < 0.0001) or the nonpollen feeders (Z = -4.583; P < 0.0001). A Bonferroni correction resulted in no changes to the results of the discriminations between the groups. The analysis of the scatterplot at the species level revealed that the overlap of pollen feeders and nonpollen feeders is caused by *H. melpomene* and *D. julia*. The *U*-test, however, indicates a significant separation of the two species (Z = -2.628; P = 0.008).

**DISCUSSION**

Previous investigations of the salivary glands in *H. melpomene*, *D. julia*, and *V. cardui* revealed no sub-
Table 3. Classification of pollen-feeding and nonpollen-feeding butterflies gained by discriminant analysis of the morphometry of salivary glands

<table>
<thead>
<tr>
<th>Butterfly feeding groups</th>
<th>Predicted group membership</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pollen feeder</td>
<td>Nonpollen feeder</td>
</tr>
<tr>
<td>Pollen feeder</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>Nonpollen feeder</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>Ungrouped cases</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Pollen feeder</td>
<td>85.2%</td>
<td>14.8%</td>
</tr>
<tr>
<td>Nonpollen feeder</td>
<td>9.5%</td>
<td>90.5%</td>
</tr>
<tr>
<td>Ungrouped cases</td>
<td>0.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

Of the originally grouped cases, 87.5% were correctly classified. The ungrouped cases correspond to the outgroup. All outgroup individuals were correctly grouped among the nonpollen feeders.

stantial anatomical and histological differences (Eberhard & Krenn, 2003). However, the results obtained in the present study indicate that the salivary glands of the pollen-feeding species are disproportionately larger than in closely related nonpollen-feeding Heliconiinae, as well as in more distantly related Nymphalinae. It is concluded that the larger salivary glands are an adaptation to pollen feeding because this mode of feeding requires higher amounts of saliva than pure nectar feeding (Penz & Krenn, 2000).

Our measurements show that the analysed sample of pollen-feeding heliconines had significantly larger bodies than their nonpollen-feeding counterparts used for comparison (Table 1). Because all of the investigated parameters of the salivary glands apart from the volume of the hemisphere are greater in pollen feeders, it is conceivable that the enlargement of the salivary glands could simply be the result of the larger body size. To remove allometric relationships, we performed multivariate data analyses with logarithmically-transformed data to identify cryptic relationships among the salivary gland parameters.

All factor score coefficients of the first component extracted by PCA are positive and, as revealed in a random permutational test, they are highly significant (Table 2). Thus, an increase in body length would lead to an increase in all salivary gland parameters; thus, the first component can be characterized as 'size factor'. Component 1 explains 68.6% of the total variance; therefore, the remaining variance is assumed to be largely uninfluenced by body size. Component 2 explains 26.1% of the total variance, and the highest factor score coefficients are hemisphere volume, body length, and frustum volume, which all are highly significant (Table 2). Positive signs of factor score coefficients of hemisphere and frustum volume indicate that they belong together and, indeed, both belong to the blind enlarged end of the salivary gland's secretory region (R1), which also differs histologically from the rest of the gland (Fig. 1) (Eberhard & Krenn, 2003). Regarding component 2, an increase of body size would also lead to an increase of the cylindrical part of the salivary gland because both parameters have the same algebraic sign (i.e. negative) (Table 2). According to Hakim & Kafatos (1974), this region in the hawkmoth Manduca sexta is mainly responsible for fluid production. If this holds true for butterflies, it would infer that pollen feeders secrete higher amounts of fluid because they have larger cylinder volumes than other groups. However, this is just a tendency because the factor score coefficient is not significant ($P = 0.122$). The remaining three components are difficult to interpret in a biologically meaningful way but, together, they explain only a small portion of the total variance (5.2%).

The factor scores of components 1 and 2 together explain a major part of the total variance (94.76%). Component 1 (i.e. the 'size factor') separates the pollen feeders significantly from both the nonpollen feeders and the outgroup (Fig. 3). Component 2 separates the outgroup from the two other groups significantly, but not the pollen feeders from the nonpollen feeders (Fig. 3). Thus, the outgroup individuals and the nonpollen feeders are similar with regard to their size depending parameters, although the pollen feeders and the nonpollen feeders are more similar in the proportions of the salivary gland parameters. Because pollen feeding requires larger amounts of saliva for an effective extraction of proteins from pollen grains, natural selection has favoured the pollen feeders that enlarged their salivary glands as an adaptation to the particular mode of pollen feeding.

Although pollen feeding has been regarded as a key innovation for the radiation of Heliconius butterflies (Gilbert, 1991), behavioural or anatomical correlates have been difficult to demonstrate (Krenn & Penz, 1998; Penz & Krenn, 2000). Results of the classification extracted by the DA indicate that almost 90% of the originally grouped cases were classified correctly, and differences between the salivary glands of these two groups are not the result of chance. The results obtained in the present study provide the first evidence for an adaptation of an internal organ to pollen collecting and processing and complement previous studies on morphological and behavioural adaptations to pollen feeding in butterflies (Krenn & Penz, 1998; Penz & Krenn, 2000). Beyond that, our study shows that biometric and multivariate data analyses of internal organs, such as salivary glands, can be a
very useful tool for discovering cryptic adaptations. Our investigation is the first attempt to sample data in such a way and to interpret it with respect to the special form of nutrition in Heliconius butterflies.

The proboscis of pollen-feeding butterflies acquired a new role in the pollen extracting process beyond that of nectar sucking (Gilbert, 1972). Although the proboscises of these species lack unique features, they exhibit significantly longer bristle sensilla compared to nonpollen-feeding species (Krenn & Penz, 1998). Similarly, their flower-handling behaviours differ only in the sequence of probing movements, which was shown to be important for pollen accumulation on the proboscis (Penz & Krenn, 2000). Modifications in the length and number of bristle shaped sensilla of the proboscis, as well as modifications in flower-visiting behaviour, created a new form–function relationship, and these modifications most likely possess fitness relevance. Similarly, the present results can be interpreted as a subtle modification of existing features that probably evolved in context with pollen feeding. Features of Heliconius butterflies that are responsible for their unique ability to exploit pollen include a mosaic of small changes in proboscis morphology, flower probing behaviour, the occurrence of proteases in the saliva (Eberhard et al., 2007), and a size dependent increase of the salivary glands.

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