Saliva or Regurgitated Nectar? What Heliconius Butterflies (Lepidoptera: Nymphalidae) Use for Pollen Feeding

STEFAN H. EBERHARD,1,2 ANNA L. HIKL,1 CAROL L. BOGGS,3 AND HARALD W. KRENN1


ABSTRACT The Neotropical Heliconius butterflies are well known to supplement their nectar diet by active pollen collecting. They extract proteins and free amino acids from pollen grains, exhibiting a particular behavior that involves the use of a fluid of uncertain origin. It has been assumed that this fluid is either regurgitated nectar or saliva, because for anatomical reasons a butterfly is able to release only these two fluids through its proboscis. In an experimental approach, 27 individuals of Heliconius melpomene (L.) were given red-dyed sugar solution and subsequently we observed whether the fluid used in pollen feeding was dyed or not dyed. Because regurgitated nectar should contain sugar, fluid samples were taken from the proboscis of butterflies from natural populations in Costa Rica. Samples of 44 individuals from seven species were tested for the presence of fructose and glucose with the aid of aniline phthalate. This study is the first detailed investigation of the origin of the fluid used by Heliconius butterflies in pollen feeding. The results are discussed in terms of already existing hints in literature concerning the true nature of that fluid.

KEY WORDS heliconiines, Laparus, sugar assay, nutritional ecology

Butterflies of the genera Heliconius and Laparus actively collect pollen with their proboscises, in addition to nectar feeding. This unique behavior supplies these butterflies with proteins and free amino acids and is a key innovation for the sophisticated biology of pollen feeding heliconiines (Gilbert 1972, Gilbert 1991). Essential amino acids derived from pollen feeding have been shown to be transferred directly to eggs (O’Brien et al. 2003).

During pollen feeding, the pollen remains on the outer proboscis surface. A clear liquid is discharged, wherein pollen is processed for up to several hours by partial uncoiling and recoiling of the proboscis. During pollen processing the pollen grains become mechanically damaged, which aids nutrient acquisition for Heliconius butterflies (Krenn et al. 2009). For anatomical reasons, only saliva or regurgitated nectar can be released from the food canal of a butterfly’s proboscis (Eberhard and Krenn 2003), so the processing liquid must be one or the other, or a mixture of both. Gilbert (1972) had good reasons to suppose that the liquid used for pollen processing is regurgitated nectar. He referred to studies that indicated that germinating pollen placed in a sucrose medium released free amino acids and proteins (Stanley and Linskens 1965, Linskens and Schrauwen 1969).

However, insects in general use saliva to facilitate the uptake of food (Ribeiro 1995), and butterflies specifically are able to discharge saliva from the tip of the proboscis to dissolve solid or viscous substances (Knopp and Krenn 2003). Although the salivary glands of Heliconius do not differ in terms of anatomy and histology from those of other nonpollen feeding nymphalids (Eberhard and Krenn 2003), pollen feeders have disproportionately larger salivary glands (Eberhard et al. 2009). Combined with the fact that a greater amount of fluid is used for pollen feeding than for nectar feeding (Penz and Krenn 2000), this hints that saliva is important in pollen feeding. Insect saliva is an aqueous solution of different enzymes, depending on the mode of nutrition (Ribeiro 1995). Eberhard et al. (2007) detected protease activity in the saliva of Heliconius melpomene (L.), which may improve pollen digestion. In contrast, nectar is a solution of varying concentrations of different sugars, primarily sucrose, glucose, and fructose (Galetto and Bernardello 2005). In addition, germination of pollen grains in differently concentrated sugar solutions could not be observed (H.W.K., unpublished data).

In the current study, we use an experimental approach to determine whether pollen processing involves regurgitated nectar or salivary fluid or a mixture of both.

Materials and Methods

Experiment 1: Dyed Nectar. To check if the fluid used in pollen feeding is regurgitated nectar, 27 individuals from a H. melpomene greenhouse population were allowed to feed on nectar, which had been dyed with a red food coloring. Subsequently, pollen of ei-
ther pumpkin (*Cucurbita pepo* L.) or sunflower (*Helianthus annuus* L.) was placed on the proboscis by using a needle. These species’ pollen was chosen because the size is similar to pollen used in the wild. In most cases, the butterfly immediately released a droplet of fluid from the proboscis and started pollen-processing behavior (Fig. 1). The color of this fluid was recorded. A few individuals did not start processing pollen; thus, no fluid release was observable. Some butterflies were tested with pollen from both plant species (Table 1).

**Experiment 2: Sugar Content of Fluid.** We performed two different tests for sugar in the regurgitated fluid, by using only those individuals that exhibited processing behavior and fluid release.

In test 1, 15 individuals of *H. melpomene* were tested in total (10 females, four males, and one undetermined gender). The individuals were isolated overnight and provided with water next morning. After a butterfly had ceased feeding, its proboscis was rinsed twice with distilled water to remove any remaining sugar. Then, glass beads (≈106 μm in diameter) were placed on the proboscis with a needle. Most butterflies started pollen processing behavior and released a clear liquid. This mixture of liquid and glass beads was rinsed off the proboscis into a vessel with 0.05 ml of distilled water. To test for sugar, a small amount of the liquid was applied with a micropipette onto a precoated sheet for thin layer chromatography (TLC). The TLC sheet was put on a hot-plate (45°C) until the applied liquid spots were dried. This procedure was repeated until the whole sample from an individual was applied on one spot on the TLC sheet. Three control samples (distilled water and 3 and 0.3% sugar solution) were applied in the same way. The TLC sheet was then sprayed with aniline phthalate and heated at 125°C. Aniline phthalate reacts only with reducing sugars, such as glucose and fructose (not with sucrose) during heat exposure. Presence of any kind of reducing sugar in the sample results in a color reaction. In preliminary tests, even a 0.05% glucose solution resulted in a color reaction.

Test 2 was performed similarly, but without first isolating and putting the individuals on sugar solution. The proboscis was only rinsed with distilled water if it was contaminated with pollen. In total, 12 individuals (eight females and four males) were tested; these individuals also had been used in test 1.

**Experiment 3: Field Samples.** In total, 44 *Heliconius* and *Laparus* butterflies were netted at the Tropical Research Station La Gamba, Puntarenas, Costa Rica (8° 45' N, 83° 10' W; 81 m above sea level) in February 2007. The butterflies were put in a net cage, and fluid samples were extracted by putting glass beads on the proboscis. When a butterfly performed pollen-processing behavior, the mixture of released fluid and glass beads was rinsed off with distilled water into a small vessel. Afterward, the butterflies were released where they had been caught. The fluid sample from each individual was applied onto a filter paper (seven individuals each) by using a micropipette. The filter papers were dried at 50°C for 12 h and then stored in an airtight container with silica gel. Those filter papers were tested for reducing sugars using aniline phthalate as described above. The threshold for detecting reducing sugars with this method is between 0.1 and 1% reducing sugar solution.

### Results

**Experiment 1: Dyed Nectar.** In total, 33 trials were performed with 27 *H. melpomene* individuals, which had been fed red-dyed nectar. In 18 trials with pumpkin pollen, the fluid droplets released on the proboscis during pollen processing were crystal clear and thus recorded as undyed (Table 1). No droplets were released in four further trials with pumpkin pollen. In five tests using sunflower pollen, the fluid droplets also were undyed and in six tests no fluid was released. Red dyed fluid was never visible on the proboscis (Table 1).

**Experiment 2: Sugar Content of Fluid.** In test 1, butterflies were isolated overnight and then fed a sugar solution before the test. Three fluid samples

---

**Table 1.** *H. melpomene* (N = 27) from a laboratory population were tested for the color of the released fluid during pollen processing after intake of dyed sugar solution (experiment 1)

<table>
<thead>
<tr>
<th>Tested with</th>
<th>Pumpkin pollen</th>
<th>Sunflower pollen</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals tested</td>
<td>22</td>
<td>11</td>
<td>33</td>
</tr>
<tr>
<td>Dyed fluid released</td>
<td>18</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Total no. of tests</td>
<td>4</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

For these tests, pumpkin pollen (*C. pepo*) and sunflower pollen (*H. annuus*) were used. Six individuals were tested with both species’ pollen.
from 15 tested *H. melpomene* individuals displayed a positive color reaction with aniline phthalate, indicating that sugar was present. In 12 fluid samples no reducing sugar could be found (Table 2). For comparison, all control sugar solutions of 3 and 0.3% exhibited the brownish staining for a positive reaction of reducing sugars with aniline phthalate after heating. In test 2, butterflies were not isolated before the test. All fluid samples from the 12 *H. melpomene* individuals displayed a negative color reaction (Table 2). No reducing sugar could be detected in the fluid, whereas the control sugar solutions (3 and 0.3%) exhibited a positive reaction.

**Experiment 3: Field Samples.** Fluid samples from the proboscis of 44 butterflies from seven species were tested for reducing sugars (Table 3). No color reaction was detectable with aniline phthalate, indicating that no reducing sugars were present in the fluid released to process the glass beads (Table 3).

**Discussion**

All three experiments clearly indicate that pollen feeding butterflies do not use regurgitated nectar for pollen processing. None of the tested *H. melpomene* individuals released a red dyed fluid during pollen feeding, although they had been fed with red-dyed nectar before (Table 1). The possibility that the food coloring was chemically destroyed in the butterfly’s digestive tract and that therefore no dye could be detected seems unlikely, because the droppings of the butterflies were red colored for hours after the trials were performed.

In addition, the tests for the presence of sugar in the released fluid gave unambiguous results. In all tests without feeding the butterflies before fluid sampling, no sugar was detected. This indicates that neither lab populations of *H. melpomene* nor natural populations of various *Heliconius* species and *Laparus doris* (L.) use regurgitated nectar for pollen processing. However, sugar was present in three fluid samples from butterflies that were fed on cotton soaked with a sugar solution before sampling. Because the butterflies’ tarsi came into contact with sugar, we cannot exclude the possibility that rapidly moving legs might have touched the proboscis. This may have caused contamination with sugar before the fluid sample was removed. Furthermore, it cannot be entirely excluded that the butterflies may have released small amounts of nectar through the proboscis during experimental handling. So, the positive sugar samples are not proof for regurgitated nectar.

The food canal of the butterflies’ proboscis is connected with the sucking pump in the head and leads into the esophagus. Beneath the sucking pump, the salivary gland opens via a salivary pump into the food canal (Eberhard and Krenn 2003). Therefore, if regurgitated nectar is excluded as the pollen processing fluid, the only fluid that may enter the food canal is saliva. Moreover, the oral valve, which is situated in butterflies between the food canal and the sucking pump, should prevent the reflux of nectar into the proboscis (Eberhard and Krenn 2005).

The ability of butterflies to discharge a fluid from the proboscis (Knopp and Krenn 2003) and the fact that *Heliconius* needs a comparatively high amount of fluid for pollen processing (Penz and Krenn 2000) suggested the crucial role of saliva in pollen feeding. In previous studies, protease activity was detected in the pollen processing fluid (Eberhard et al. 2007), and it is very unlikely that those proteases originate from nectar. A biometric study of the salivary glands showed that pollen-feeding heliconiines have disproportionately larger glands than comparable nymphalid species (Eberhard et al. 2009). The larger salivary glands of pollen feeding species were interpreted as an adaptation to pollen feeding (Eberhard et al. 2009). Taken together with the results presented in this study, we conclude that the fluid used in pollen feeding probably is saliva.

**Acknowledgments**

We thank S. Schmid, S. Suette, and D. Senkpiel for help in performing the experiments and the staff of the Tropical Research Station La Gamba, Costa Rica, for providing excellent facilities. The Costa Rican Ministerio del Ambientes y Energia kindly granted research permits. We also thank the Department of Ecohphysiology and Functional Anatomy of Plants (University of Vienna) for granting us permission to a greenhouse. The study was funded by the Austrian Science Fund (FWF-Project 18425-B03).

**References Cited**


Received 18 May 2009; accepted 8 September 2009.