

Pollen feeding in the butterfly *Heliconius charitonia*: isotopic evidence for essential amino acid transfer from pollen to eggs

Diane M. O'Brien^{1,2*†}, Carol L. Boggs¹ and Marilyn L. Fogel²

¹Center for Conservation Biology, Department of Biology, Stanford University, Stanford, CA 94305, USA

²Geophysical Laboratory, Carnegie Institution of Washington, 5251 Broad Branch Road NW, Washington, DC 20015, USA

Heliconius and *Laparus* butterflies exhibit a unique pollen-collecting behaviour that enhances lifespan and fecundity. The specific nutritional contribution of pollen, however, had not been previously demonstrated. We used stable isotope variation to trace the carbon flow into eggs from corn pollen provided experimentally to ovipositing female *Heliconius charitonia*, and to evaluate the use of isotopically contrasting nectar sugars in egg amino acids. The $\delta^{13}\text{C}$ of individual amino acids from pollen, larval host plant and the eggs from experimental butterflies was measured with gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), to evaluate amino acid transfer. The $\delta^{13}\text{C}$ of egg essential amino acids indicated a transfer of essential amino acids from pollen to butterfly eggs. However, the $\delta^{13}\text{C}$ of non-essential amino acids reflected the isotopic composition of the artificial nectar, indicating that *H. charitonia* synthesizes non-essential amino acids from dietary sugars. This, to our knowledge, is the first direct demonstration of amino acid transfer from pollen to butterfly eggs, and suggests that essential amino acids in particular are a key resource for extending lifespan and fecundity in *Heliconius* butterflies.

Keywords: *Heliconius*; pollen feeding; reproductive resource allocation; essential amino acids; compound-specific stable isotope analysis

1. INTRODUCTION

Butterflies and many moths capitalize on the sugar-rich nectar rewards that plants provide as incentives for pollination. However, nectar is an unbalanced food source, with abundant water and sugars but low concentrations of amino acids (Baker & Baker 1973; Baker 1975). Animals in nutritional steady-state must match nutrient use with nutrient intake; therefore, many nectarivorous animals supplement their diets with protein sources. For example, hummingbirds catch insects to meet their protein and mineral requirements (Brice 1992). Many butterflies and moths store proteins ingested during the larval phase for use in metamorphosis and reproduction (Wheeler *et al.* 2000; Pan & Telfer 2001). In addition, some butterflies select relatively amino acid-rich nectars (Alm *et al.* 1990; Erhardt & Rusterholz 1998; Mevischutz & Erhardt 2002) or ingest nitrogenous resources from mud puddles, dung or carrion (Beck *et al.* 1999; Hall & Willmott 2000). Despite these sources, adult butterflies are generally in negative protein balance and lose mass and nitrogen across their lifetimes (Boggs 1981a; Karlsson 1994; Boggs 1997; Stjernholm & Karlsson 2000). This unbalanced protein loss has been thought to contribute to the ephemeral nature of the adult lifestage in butterflies and moths (Dunlap-Pianka *et al.* 1977; Boggs 1987).

Butterflies of the closely related neotropical genera *Heliconius* and *Laparus* (Nymphalidae) supplement their

adult nectar diet with pollen (Gilbert 1972). Pollen feeding is a specialized behaviour in which butterflies repeatedly rub their proboscides over the anthers to collect pollen, which sticks to the proboscis as a mass (Gilbert 1972; Penz & Krenn 2000). The collected pollen mass is mixed on the proboscis with an exuded fluid and agitated for several hours. Pollen that is soaked releases a diversity of compounds, possibly via germination, including free amino acids and peptides (Linskens & Schrauwen 1969; Erhardt & Baker 1990). Thus, butterflies that re-ingest the fluid previously used to soak pollen could gain a dietary source of free amino acids. Labelled amino acids fed in solution to adult *Heliconius* butterflies appeared in both eggs and body structures within 1–2 days (Gilbert 1972; Boggs & Gilbert 1979; Boggs 1981b).

Heliconius longevity and age-specific fecundity patterns differ markedly from those typical of exclusively nectar-feeding butterflies (cf. Gilbert 1972; Dunlap-Pianka *et al.* 1977; Boggs 1986), and inclusion of pollen in the diet is necessary for normal life history (Dunlap-Pianka *et al.* 1977). For example, *Heliconius charitonia* in a free-flying greenhouse colony with access to pollen lived for an average of 35 days, with a maximum of 105 days (Boggs 1979), which is a similar lifespan to that recorded in the field (Cook *et al.* 1976). However, when offered only a 20% sucrose solution as adults, lifespan was reduced to an average of 21 days (maximum of 38 days; Dunlap-Pianka *et al.* 1977). Lifetime fecundity with pollen can be ca. 1000 eggs, at a daily oviposition rate of 9–18 eggs throughout the lifespan, depending on body size. Without pollen, lifetime fecundity drops to 70–350 eggs, and daily oviposition rate drops markedly after ca. 15–20 days (Dunlap-Pianka *et al.* 1977). Ovaries of females fed on

* Author for correspondence (dobrien@wellesley.edu).

† Present address: Department of Biology, Wellesley College, Wellesley, MA 02481, USA.

pollen show continual generation of new oocytes through time, whereas ovaries of females denied access to pollen degenerate with age in a manner similar to that seen in non-pollen-feeding heliconiines (Dunlap-Pianka *et al.* 1977). Boggs (1981a) showed that the frequency of pollen feeding by *H. charitonia* increased with decreasing abdominal nitrogen (independently of age), suggesting that pollen feeding contributes to nitrogen nutrition.

Given the clear effect of pollen feeding on reproduction and longevity in *Heliconius* and the availability of amino acids from pollen, researchers have suggested that amino acids are the key nutrient from pollen (Gilbert 1972; Boggs *et al.* 1981; Boggs 1987). Recent work has demonstrated that female hawkmoths synthesize the non-essential amino acids in eggs from nectar sugar, whereas essential amino acids derive exclusively from the larval diet (O'Brien *et al.* 2002). Thus, pollen has the potential to provide a critical resource to adult nectar-feeding Lepidoptera: essential amino acids.

Although pollen feeding has been hypothesized to explain the unusual life history of *Heliconius* butterflies, transfer of amino acids from pollen to eggs has not been previously demonstrated. Here, we traced carbon from pollen, nectar sugar and larval host plant into eggs, using naturally occurring differences in carbon isotope ratios between plants using C3 versus C4 photosynthesis (Farquhar *et al.* 1989). We used isotopically contrasting sources of nutrients from adult pollen and larval diets (C4 corn pollen versus C3 larval host plant) and isotopically contrasting nectar diets (C4 cane versus C3 beet sugar) to investigate the roles played by each of these three potential food sources in egg manufacture by *H. charitonia*. We measured the carbon isotope ratios of 14 amino acids from eggs, pollen and larval host plant, to evaluate whether specific pollen amino acids are incorporated into eggs, and to what extent (if any) egg amino acids are synthesized from nectar sugars.

2. MATERIAL AND METHODS

(a) *Butterflies*

Experimental *H. charitonia* came from a free-flying greenhouse colony founded from individuals collected in Costa Rica and Mexico, and maintained at Stanford University. Adults oviposit and larvae develop on potted *Passiflora caerulea* (Passifloraceae). Adults feed from flowers of potted *Lantana camara* (Verbenaceae) and *Psiguria umbrosa* (Cucurbitaceae). These natural sources of nectar and pollen were supplemented by feeders containing 1 : 3 v/v honey : water.

(b) *Experimental diets and sample collection*

Experimental females were collected as pupae, mated upon emergence and placed individually into 1.5 m × 0.9 m × 0.9 m cages. Forewing length (FWL), measured at emergence, was taken into account as butterflies were assigned to feeding treatments. Butterflies were assigned to one of four diets, based on sugar solution (either C3 or C4) and pollen treatment (either pollen-fed or pollen-deprived). Average FWL for pollen-fed and pollen-deprived butterflies was 38.5 ± 1.9 mm and 38.5 ± 3.7 mm, respectively.

Each cage contained a potted *P. caerulea* plant for oviposition and a feeder stand. Feeder stands offered 30% sucrose solution at artificial flowers, made from Eppendorf centrifuge tubes

painted with orange fingernail polish to resemble *P. umbrosa* flowers. All females were provided with sucrose solution *ad libitum*, made from either cane sugar ($\delta^{13}\text{C}^1 = -10.30$ parts per thousand (‰)) or beet sugar ($\delta^{13}\text{C} = -24.00$ ‰). These sugar solutions were made in a single batch, frozen in aliquots and changed daily. Females were introduced to the feeders for the first 2 days of adulthood, by using a pin to unroll the proboscis and place it in the feeder twice a day. Subsequently, females were observed feeding themselves.

Fresh pollen was presented daily to pollen-fed females, in similar artificial flowers on the same feeder stand. Pollen was collected each morning from greenhouse-reared corn plants (*Zea mays*; Poaceae). Although corn is not a natural pollen source for these butterflies, we used it here because it is isotopically distinct from the larval host plant and produces copious, easily collected pollen.

To ensure that pollen-fed females were actually feeding on pollen, pollen loads were monitored. The proboscis is not covered by the labial palps in *Heliconius*, and pollen loads can be assessed visually (Krenn & Penz 1998). Females' pollen load size was scored daily, using the scale of 0–3 of Boggs *et al.* (1981), where 0 is no pollen, and 3 is a very large pollen load. This scale reflects the number of pollen grains collected by the butterflies (Boggs *et al.* 1981). For a pollen-fed female to be included in our analyses, she must have collected pollen consistently over time, with pollen loads reaching 1.0 or more.

Eggs from both sets of females were counted and collected daily, dried for at least 4 days at 50 °C and stored for analysis at room temperature in sealed Eppendorf tubes. Data from nine butterflies were analysed: five females fed on beet sugar, three of which were pollen-fed, and four females fed on cane sugar, one of which was pollen-fed. Thus, $n = 5$ and 4 for pollen-deprived and pollen-fed butterflies, respectively.

(c) *Bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis*

We measured the whole sample or 'bulk' $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of eggs and diet components using a CHNS analyser (CE Instruments, model NC2500) interfaced to a Finnigan Delta Plus XL isotope ratio mass spectrometer via the Finnigan Conflo II interface. Acetanilide standards run with each batch of samples gave standard deviations of less than or equal to 0.12‰ for $\delta^{13}\text{C}$ and less than or equal to 0.43‰ for $\delta^{15}\text{N}$. Between two and seven egg samples were analysed per female, spread evenly across her ovipositing lifetime. We also measured samples of both sugars and pollen, as well as two leaf samples from each larval host plant ($n = 10$), to evaluate inter-plant isotopic variability.

(d) *Compound-specific $\delta^{13}\text{C}$ analysis of amino acids*

We measured the isotopic composition of eggs ($n = 9$), host plant ($n = 1$) and pollen ($n = 1$) amino acids individually by using compound-specific stable isotope analysis (GC/C/IRMS: Silfer *et al.* 1991). Eggs were hydrolyzed to amino acids and derivitized to *N*-trifluoroacetic amino acid isopropyl esters, as described elsewhere (Fantle *et al.* 1999). Derivitized samples were injected into a Varian 3400 gas chromatograph (HP-1 Column), via a split/splitless injector in split mode. Separated amino acids in a continuous stream of helium carrier gas were converted to CO₂ gas via a combustion interface, and analysed with a Finnigan Delta Plus XL isotope ratio mass spectrometer.

We could resolve 14 amino acids using these methods, six of which are considered non-essential for insects (Hagen *et al.* 1984): alanine (ala), glycine (gly), serine (ser), proline (pro), aspartate (asx), and glutamate (glx); and seven of which are

considered essential: threonine (thr), valine (val), leucine (leu), isoleucine (ile), phenylalanine (phe), lysine (lys) and arginine (arg). Although tyrosine (tyr) can be synthesized from phenylalanine, it cannot be synthesized *de novo* from the sugars found in a nectar diet. Therefore, we include it among the amino acids classified as 'essential', indicating that its source cannot be sugar. Asparagine and glutamine are converted to aspartate and glutamate, respectively, during acid hydrolysis; therefore, these amino acids are indistinguishable.

(e) Calculating amino acid $\delta^{13}\text{C}$

Samples were run in triplicate during two batches of derivitization and analysis. Standards of known isotopic composition were derivitized and run in triplicate with each pair of samples analysed. Measurements were corrected for derivitization reagent carbon and amino acid specific fractionation (Silfer *et al.* 1991) and estimates of measurement standard error were calculated according to O'Brien *et al.* (2002). Measurement standard errors averaged 1.05‰ across all amino acids. These errors varied systematically among amino acids, ranging from an average minimum of 0.42‰ to an average maximum of 2.30‰ (glycine was consistently the most variable).

(f) Pollen composition

Voltage outputs (peak amplitude) from the Delta Plus isotope ratio mass spectrometer were used to evaluate the amount of amino acids present in the derivitized pollen sample (in μmol). We used data from standards to calculate $\mu\text{mol V}^{-1}$ corrections for each amino acid separately. Additionally, we soaked two samples of corn pollen (one fresh, one previously frozen at -80°C) for 24 h in distilled water (200 mg pollen per 40 ml). We filtered with a $0.5\ \mu\text{m}$ filter and analysed the filtrate using postcolumn, ninhydrin amino acid analysis (Smith 1997).

(g) Statistical analyses

All statistical analyses were performed in JMP 5.0 (SAS Institute, Inc, Duxbury Press). The effect of adult dietary sugar (C_3/C_4) on egg bulk $\delta^{13}\text{C}$ was tested with a nested ANCOVA with sugar type (C_3/C_4) and butterfly identity (nested within sugar type) as factors, and oviposition day as the covariate. The effect of pollen feeding on the bulk $\delta^{13}\text{C}$ values of eggs was also analysed using nested ANCOVA, with pollen feeding (yes/no), and butterfly identity (nested within pollen feeding) as factors, and oviposition day as the covariate.

Amino acid $\delta^{13}\text{C}$ data were analysed separately for essential and non-essential amino acids. In both cases, data were analysed using ANOVA, with amino acid identity, pollen feeding (yes/no) and sugar type (C_3/C_4) as factors. The normality of residuals was tested using a Shapiro–Wilks test with each application of ANOVA/ANCOVA, and in no case did they deviate significantly from a normal distribution.

3. RESULTS

(a) Lifespan and fecundity

Fecundity in pollen-fed and pollen-deprived butterflies was 161 ± 81 and 97 ± 43 eggs, respectively, and lifespan was 34 ± 11 and 28 ± 14 days, respectively ($n = 9$). These differences were consistent in direction with previous studies on *H. charitonia* (Dunlap–Pianka *et al.* 1977), but were not significant. Neither fecundity nor lifespan were affected by sugar type. Pollen load size (when pollen was collected) averaged 0.4 ± 0.1 ($n = 4$ females) and collecting frequency was $0.5 \pm 0.1\ \text{day}^{-1}$.

(b) Bulk egg $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$

Pollen was heavier in both $\delta^{13}\text{C}$ ($-10.74 \pm 0.06\text{‰}$) and $\delta^{15}\text{N}$ ($1.30 \pm 0.56\text{‰}$) than larval host plant ($\delta^{13}\text{C}$ ($-29.89 \pm 0.04\text{‰}$) and $\delta^{15}\text{N}$ ($0.40 \pm 0.16\text{‰}$)). Data are replicate measures of single samples. Because the difference in $\delta^{15}\text{N}$ between pollen and host plant is less than 1‰, we do not attempt to use it as a dietary tracer.

Differences in $\delta^{13}\text{C}$ between the eggs laid by females fed cane (-10.30‰) or beet (-24.00‰) sugar reflect incorporation of sugar carbon into eggs. Eggs laid by females fed cane sugar contained significantly more ^{13}C than those laid by females fed beet sugar (-19.29‰ versus -26.17‰ , $F_{1,26} = 353.0$, $p < 0.0001$). The $\delta^{13}\text{C}$ of all eggs increased with age, indicating increasing use of sugar carbon in eggs ($F_{1,26} = 39.9$, $p < 0.0001$; O'Brien *et al.* 2000).

Differences in egg $\delta^{13}\text{C}$ between pollen-fed and pollen-deprived females indicate use of pollen carbon in egg manufacture. Pollen feeding, in contrast to nectar feeding, had no effect on whole-egg $\delta^{13}\text{C}$.

Finally, individual females varied significantly in $\delta^{13}\text{C}$ ($F_{7,26} = 11.6$, $p < 0.0001$). As larvae remained on the same host plant for the duration of development, variation associated with butterfly identity may be related to isotopic differences among individual larval host plants. Host plants (*P. caerulea*) varied modestly but highly significantly in $\delta^{13}\text{C}$ (mean \pm s.d.: $\delta^{13}\text{C} = -29.15 \pm 0.89\text{‰}$ one-way ANOVA, $p < 0.0001$).

(c) Amino acid $\delta^{13}\text{C}$

We measured amino acid $\delta^{13}\text{C}$ in egg samples from nine females, taken from as late in life as possible to maximize the opportunity for incorporation of pollen-derived nutrients. We also measured amino acid $\delta^{13}\text{C}$ in the larval host plant and in corn pollen. We evaluated the effect of pollen-feeding and sugar type on essential and non-essential amino acid $\delta^{13}\text{C}$ separately, using ANOVA (table 1). The essential amino acids from eggs of pollen-fed butterflies were an average of 3‰ heavier than those of pollen-deprived butterflies ($p < 0.0001$; figure 1; tables 1 and 2), indicating transfer of essential amino acids from pollen to eggs. Using a mixing equation (table 2), we calculate that *ca.* 17% of the carbon in egg essential amino acids derived from pollen, with individual essential amino acids ranging from 13% to 20% pollen-derived carbon (table 2). There was no influence of sugar type on the $\delta^{13}\text{C}$ of essential amino acids (table 1).

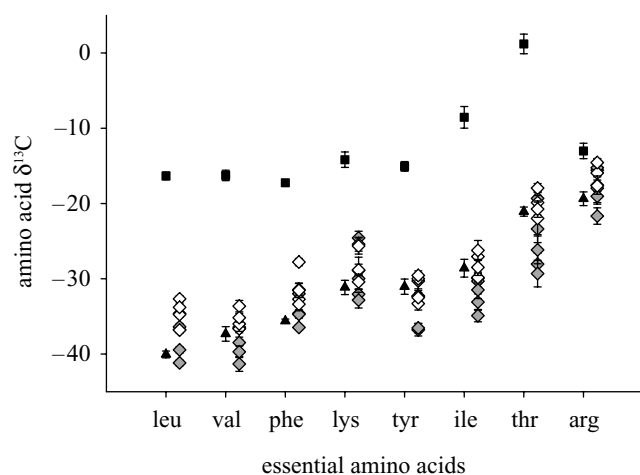
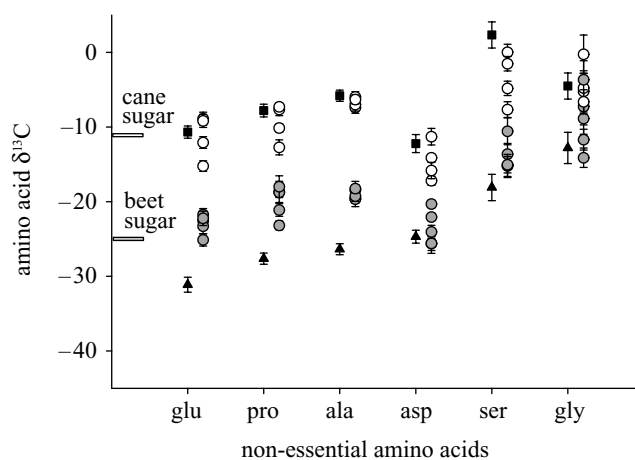
By contrast, pollen feeding had no effect on the $\delta^{13}\text{C}$ of non-essential egg amino acids (figure 2; table 1). However, sugar type (C_3 versus C_4), had a large, highly significant effect on egg non-essential amino acid $\delta^{13}\text{C}$ (-18.2‰ versus -8.2‰ , respectively; tables 1 and 3). This effect signifies that much of the carbon in these amino acids derives from the sugars in the adult nectar diet. The percentage of carbon from the diet ($\%C_{\text{sugar}}$) can be evaluated by the expression

$$\%C_{\text{sugar}} = \frac{(\delta^{13}\text{C}_{\text{C}_4 \text{ egg AA}} - \delta^{13}\text{C}_{\text{C}_3 \text{ egg AA}})}{(\delta^{13}\text{C}_{\text{C}_4 \text{ sugar}} - \delta^{13}\text{C}_{\text{C}_3 \text{ sugar}})} \times 100\%$$

(O'Brien *et al.* 2002). On average, *ca.* 73% of non-essential amino acid carbon derives from sugar feeding in these females (table 3).

Table 1. Effect of pollen and sugar type on $\delta^{13}\text{C}$ for both essential and non-essential egg amino acids (ANOVA). (d.f., degrees of freedom; SS, sums of squares.)

effect	essential amino acids ($n = 72$)				non-essential amino acids ($n = 54$)			
	d.f.	SS	F_{ratio}	p	d.f.	SS	F_{ratio}	p
amino acid identity	7	2866	70	< 0.0001	5	1030	27	< 0.0001
pollen (yes/no)	1	175	30	< 0.0001	1	2.6	0.3	0.5641
sugar (C3/C4)	1	22	4	0.0545	1	1160	150	< 0.0001
error	62	361			46	356		

Figure 1. Essential amino acid $\delta^{13}\text{C}$ from pollen, larval host plant and eggs. Amino acid values from pollen and host plant are indicated by black squares and triangles, respectively. Open diamonds indicate eggs laid by pollen-fed butterflies, whereas grey diamonds indicate eggs laid by butterflies not fed pollen.Figure 2. Non-essential amino acid $\delta^{13}\text{C}$ from pollen, host plant and eggs. Amino acid values from pollen and host plant are indicated by black squares and triangles, respectively. Open circles indicate eggs laid by butterflies fed cane sugar, whereas grey circles indicate eggs laid by butterflies fed beet sugar. The isotopic values of cane and beet sugar are indicated by the bars on the y-axis.Table 2. Essential amino acid $\delta^{13}\text{C}$ (‰) from host plant (*Passiflora caerulea*), pollen (*Zea mays*) and eggs from pollen-fed ($n = 5$) and pollen-deprived ($n = 4$) *Heliconius charitonia*.

essential amino acid	host plant (H)	pollen (P)	P - H	pollen-deprived (PD)	pollen-fed (PF)	PF - PD	corn essential amino acids ^{a,b} (%)
leu	-40.1	-16.3	23.7	-37.8 ± 2.6	-34.6 ± 1.8	3.2	13
val	-37.3	-16.3	21.1	-38.6 ± 2.1	-35.5 ± 1.3	3.1	15
phe	-35.6	-17.2	18.4	-34.0 ± 2.1	-31.5 ± 2.5	2.5	14
lys	-31.2	-14.2	17.0	-29.8 ± 3.3	-27.7 ± 2.4	2.1	12
tyr	-31.1	-15.1	16.0	-33.8 ± 2.9	-31.5 ± 1.8	2.3	15
ile	-28.6	-8.6	20.1	-32.1 ± 2.1	-28.0 ± 1.6	4.0	20
thr	-21.1	1.2	22.3	-25.4 ± 4.0	-20.3 ± 1.7	5.1	23
arg	-19.4	-13.0	6.4	-18.0 ± 2.6	-16.6 ± 1.5	1.5	23
average			18.1			3.0	17

^a Calculated as $(\text{PF} - \text{PD})/(\text{P} - \text{H}) \times 100$.

^b These percentages are presented without error because host plant and pollen are not experimentally replicated.

(d) Composition of amino acids released from corn pollen

Proline was the most abundant amino acid in corn pollen (14%), with glx, gly and ala of similar abundances (10–13%). Asx, ser, leu, lys, thr, ile and val were all 5–7% of the hydrolysate, with phe, tyr and arg making up 1–4%. Soaked corn pollen released 0.045 and 0.078 nmol amino acids mg^{-1} , for fresh and frozen pollen, respectively. Proline was released disproportionately by corn

pollen when soaked, comprising 80–85% of all amino acids in solution. Lysine, threonine and alanine were the next most abundant amino acids released by soaked pollen (2–4% abundance). The remaining amino acids ranged from 0 to 2% molar abundance.

4. DISCUSSION

The pollen-feeding behaviour of *Heliconius* butterflies has intrigued researchers for decades, because it presents

Table 3. Non-essential amino acid $\delta^{13}\text{C}$ (‰) from larval host plant (*Passiflora caerulea*), pollen (*Zea mays*) and eggs from C3 and C4 sugar-fed *Heliconius charitonia*.

non-essential amino acid	host plant	pollen	C3-fed eggs	C4-fed eggs	%C _{sugar} ^a
glu	-31.13	-10.68	-23.0 ± 1.3	-11.3 ± 3.0	85 ± 31
pro	-27.68	-7.80	-19.9 ± 2.2	-9.5 ± 2.5	77 ± 34
ala	-26.37	-5.81	-19.0 ± 0.7	-6.7 ± 0.6	90 ± 9
asp	-24.69	-12.22	-23.5 ± 2.3	-14.6 ± 2.5	65 ± 35
ser	-18.10	2.33	-13.9 ± 2.0	-3.5 ± 3.4	76 ± 40
gly	-12.81	-4.51	-9.1 ± 4.0	-4.2 ± 2.8	36 ± 50

^a Calculated as $(\text{C4}_{\text{egg}} - \text{C3}_{\text{egg}})/(13.7\%) \times 100$.

a case study for how an unusual nutritional resource may affect resource allocation in a model system (Gilbert 1972; Dunlap-Pianka *et al.* 1977; Boggs *et al.* 1981; Boggs 1987; Krenn & Penz 1998; Estrada & Jiggins 2002). This study directly demonstrates amino acid transfer from pollen to eggs for the first time, to our knowledge, in *Heliconius* butterflies, supporting the hypothesis that pollen feeding provides a source of amino acids for use in reproduction. Essential amino acids from the eggs of pollen-fed butterflies were enriched in ^{13}C compared with those from the eggs of pollen-deprived butterflies, reflecting carbon input from corn pollen. This enrichment averaged 3‰ over all essential amino acids, indicating that *ca.* 17% of egg essential amino acids derived from pollen in these butterflies. Although this is a substantial carbon signal from pollen, it indicates that stored larval dietary reserves are still supplying most of the egg essential amino acids. Interestingly, the bulk egg $\delta^{13}\text{C}$ did not reflect carbon input from pollen. Thus, a nutrient-specific approach was required to detect nutrient allocation in this relatively complex system.

Previous studies have demonstrated striking effects of pollen feeding on life history in *H. charitonia*, including more than a doubling of maximum lifespan and several-fold increases in mean fecundity (Dunlap-Pianka *et al.* 1977). Given these effects, it is somewhat surprising that the extent of pollen utilization in egg manufacture measured here was not higher. Pollen loads collected in this study were consistent with other estimates from laboratory studies (Dunlap-Pianka *et al.* 1977; Boggs *et al.* 1981). However, the composition of amino acids or other nutrients released by corn pollen may differ from that provided by natural pollen sources, such as *P. umbrosa*. The observation that pollen-fed and pollen-deprived butterflies did not differ significantly in survival and fecundity in this study supports the idea that corn may be a lower-quality pollen source than those used by *H. charitonia* in nature. If so, the transfer of amino acids from pollen to butterflies documented in this study may underestimate the amino acid yield of pollen collection in the wild.

The effectiveness of pollen collection may also be higher in the field, where both the amount of pollen collected and the plant species from which it is collected vary among the *Heliconius* species, within species between the sexes, across ages, or space and time (Boggs 1981b; Cardoso 2001; Estrada & Jiggins 2002). For example, *Heliconius cydno* and *H. hecale* are more reliant on pollen than *H. charitonia* (Boggs *et al.* 1981), females generally collect more pollen than do males and pollen load size increases with age (Boggs & Gilbert 1979). The identity of the

pollen used depends in part on the habitat preference of the particular butterfly species and the flowers available in that habitat. In one study, pollen loads on wild *H. charitonia* were three times larger than those measured in this study (Boggs *et al.* 1981). Thus, wild *H. charitonia* may, on average, obtain more essential amino acids from pollen than this study suggests. Furthermore, more pollen-reliant species such as *H. cydno* or *H. hecale* might use pollen essential amino acids to a greater degree than *H. charitonia* (Boggs *et al.* 1981).

We found that amino acids released by corn pollen soaked in distilled water were dominated by proline. These results are consistent with data showing that proline is the primary amino acid released from the pollen of four other plant species, when soaked in sugar solution (Erhardt & Baker 1990). However, this seems to contradict what the butterflies are actually gaining from pollen: essential amino acids, but not non-essential amino acids (and not, apparently, proline). These results thus suggest that butterflies enhance amino acid release from pollen, beyond the effect of soaking alone. Or potentially, they may indicate that abundant amino acids like proline are used as a nitrogen source, but not a carbon source.

The isotopic composition of the artificial nectar diet strongly affected the $\delta^{13}\text{C}$ of non-essential amino acids. This result indicates that most non-essential egg amino acids are synthesized from the sugars in the nectar diet, using amine groups from endogenous reserves or from pollen amino acids. The extent of *de novo* synthesis varied among the non-essential amino acids, from *ca.* 90% synthesis in alanine to *ca.* 40% in glycine. Both the total extent of non-essential amino acid synthesis and the pattern of variation among amino acids are consistent with previously collected data on a nectar-feeding hawkmoth (O'Brien *et al.* 2002). The similarity between these two distantly related lineages suggests that extensive synthesis of non-essential egg amino acids from dietary sugar may be a widespread phenomenon in nectar-feeding Lepidoptera.

The present work demonstrates that *H. charitonia* use essential amino acids released from collected pollen in egg manufacture, and that they synthesize non-essential amino acids from nectar sugars. The result strongly suggests a mechanistic basis for the enhanced longevity and reproduction exhibited by pollen-feeding *Heliconius* and *Laparus* butterflies. Although numerous butterflies seek fluid sources of nitrogen and/or amino acids (Alm *et al.* 1990; Erhardt & Rusterholz 1998; Hall & Willmott 2000; Mevi-Schutz & Erhardt 2002), many of these sources are relatively dilute. The expansion of a butterfly's feeding

niche into pollen collection may provide richer and/or more constant sources of amino acids than exclusively nectar-feeding butterflies have available. By this argument, pollen feeding may be one strategy in a suite of amino acid seeking behaviours in foraging butterflies: a behavioural and nutritional innovation with important implications for life history.

The authors thank E. Forwand, L. Kropuenske, I. Kulahci, A. Leidner, A. McMillan, K. Primus and J. Schwann for their help rearing butterflies and collecting samples. D. Goodman and V. Walbot gave advice on raising corn and collecting pollen. S. Clarke suggested helpful improvements to the manuscript. This work was supported by NSF grant (IBN) 99-83044.

ENDNOTE

$^1\delta X = [((R_{\text{sample}} - R_{\text{std}})/R_{\text{std}}) \times 1000]$, where X is the heavy isotope of N or C, and R is the ratio of heavy-to-light isotopes (standards = Pee Dee Belemnite (C) and atmospheric N_2 (N)).

REFERENCES

- Alm, J., Ohnmeiss, T. E., Lanza, J. & Vriesenga, L. 1990 Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. *Oecologia* **84**, 53–57.
- Baker, H. G. 1975 Sugar concentrations in nectars from hummingbird flowers. *Biotropica* **7**, 37–41.
- Baker, H. G. & Baker, I. 1973 Amino-acids in nectar and their evolutionary significance. *Nature* **241**, 543–545.
- Beck, J., Muhlenberg, E. & Fiedler, K. 1999 Mud-puddling behavior in tropical butterflies: in search of proteins or minerals? *Oecologia* **119**, 140–148.
- Boggs, C. L. 1979 Resource allocation and reproductive strategies in several heliconiine butterfly species. PhD thesis, University of Texas at Austin, USA.
- Boggs, C. L. 1981a Nutritional and life-history determinants of resource allocation in holometabolous insects. *Am. Nat.* **117**, 692–709.
- Boggs, C. L. 1981b Selection pressures affecting male nutrient investment at mating in heliconiine butterflies. *Evolution* **35**, 931–940.
- Boggs, C. L. 1986 Reproductive strategies of female butterflies: variation in and constraints on fecundity. *Ecological Entomol.* **11**, 7–15.
- Boggs, C. L. 1987 Ecology of nectar and pollen feeding in Lepidoptera. In *Nutritional ecology of insects, mites and spiders* (ed. F. Slansky & J. C. Rodriguez), pp. 369–391. New York: Wiley.
- Boggs, C. L. 1997 Reproductive allocation from reserves and income in butterfly species with differing adult diets. *Ecology* **78**, 181–191.
- Boggs, C. L. & Gilbert, L. E. 1979 Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science* **206**, 83–84.
- Boggs, C. L., Smiley, J. T. & Gilbert, L. E. 1981 Patterns of pollen exploitation by *Heliconius* butterflies. *Oecologia* **48**, 284–289.
- Brice, A. T. 1992 The essentiality of nectar and arthropods in the diet of the anna's hummingbird (*Calypte anna*). *Comp. Biochem. Physiol.* **101A**, 151–155.
- Cardoso, N. Z. 2001 Patterns of pollen collection and flower visitation by *Heliconius* butterflies in southeastern Mexico. *J. Trop. Ecol.* **17**, 763–768.
- Cook, L. M., Thomason, E. W. & Young, A. M. 1976 Population structure, dynamics and dispersal of the tropical butterfly *Heliconius charitonia*. *J. Anim. Ecol.* **45**, 851–863.
- Dunlap-Pianka, H., Boggs, C. L. & Gilbert, L. E. 1977 Ovarian dynamics in heliconiine butterflies: programmed senescence versus eternal youth. *Science* **197**, 487–490.
- Erhardt, A. & Baker, I. 1990 Pollen amino acids—an additional diet for a nectar feeding butterfly? *Pl. Syst. Evol.* **169**, 111–121.
- Erhardt, A. & Rusterholz, H. P. 1998 Do peacock butterflies (*Inachis io* L.) detect and prefer nectar amino acids and other nitrogenous compounds? *Oecologia* **117**, 536–542.
- Estrada, C. & Jiggins, C. D. 2002 Patterns of pollen feeding and habitat preference among *Heliconius* species. *Ecol. Entomol.* **27**, 448–456.
- Fantle, M. S., Dittel, A. I., Schwalm, S. M., Epifanio, C. E. & Fogel, M. L. 1999 A food web analysis of the juvenile blue crab, *Callinectes sapidus*, using stable isotopes in whole animals and individual amino acids. *Oecologia* **120**, 416–426.
- Farquhar, G. D., Ehleringer, J. R. & Hubick, K. T. 1989 Carbon isotope discrimination and photosynthesis. *A. Rev. Pl. Physiol. Pl. Mol. Biol.* **40**, 503–537.
- Gilbert, L. E. 1972 Pollen feeding and reproductive biology of *Heliconius* butterflies. *Proc. Natl Acad. Sci. USA* **69**, 1403–1407.
- Hagen, K. S., Dadd, R. H. & Reese, J. 1984 The food of insects. In *Ecological entomology* (ed. C. B. Huffaker & R. L. Rabb), pp. 79–112. New York: Wiley.
- Hall, J. P. W. & Willmott, K. R. 2000 Patterns of feeding behaviour in adult male riordinid butterflies and their relationship to morphology and ecology. *Biol. J. Linn. Soc.* **69**, 1–23.
- Karlsson, B. 1994 Feeding-habits and change of body-composition with age in 3 nymphalid butterfly species. *Oikos* **69**, 224–230.
- Krenn, H. W. & Penz, C. M. 1998 Mouthparts of *Heliconius* butterflies (Lepidoptera: Nymphalidae): a search for anatomical adaptations to pollen-feeding behavior. *Int. J. Insect Morphol. Embryol.* **27**, 301–309.
- Linskens, H. F. & Schrauwen, J. 1969 The release of free amino acids from germinating pollen. *Acta Bot. Neerl.* **18**, 605–614.
- Mevi-Schutz, J. & Erhardt, A. 2002 Can *Inachis io* detect nectar amino acids at low concentrations? *Physiol. Entomol.* **27**, 256–260.
- O'Brien, D. M., Schrag, D. P. & del Rio, C. M. 2000 Allocation to reproduction in a hawkmoth: a quantitative analysis using stable carbon isotopes. *Ecology* **81**, 2822–2831.
- O'Brien, D. M., Fogel, M. L. & Boggs, C. L. 2002 Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. *Proc. Natl Acad. Sci. USA* **99**, 4413–4418.
- Pan, M. L. & Telfer, W. H. 2001 Storage hexamer utilization in two lepidopterans: difference correlated with the timing of egg formation. *J. Insect Sci.* **1**, 2, 1–9.
- Penz, C. M. & Krenn, H. W. 2000 Behavioral adaptations to pollen-feeding in *Heliconius* butterflies (Nymphalidae, Heliconiinae): an experiment using Lantana flowers. *J. Insect Behav.* **13**, 865–880.
- Silfer, J. A., Engel, M. H., Macko, S. A. & Jumeau, E. J. 1991 Stable carbon isotope analysis of amino-acid enantiomers by conventional isotope ratio mass-spectrometry and combined gas-chromatography isotope ratio mass-spectrometry. *Analyt. Chem.* **63**, 370–374.
- Smith, A. J. 1997 Postcolumn amino acid analysis. *Meth. Mol. Biol.: Protein Sequencing Protocols* **64**, 139–146.
- Stjernholm, F. & Karlsson, B. 2000 Nuptial gifts and the use of body resources for reproduction in the green-veined white butterfly *Pieris napi*. *Proc. R. Soc. Lond. B* **267**, 807–811. (DOI 10.1098/rspb.2000.1075.)
- Wheeler, D. E., Tuchinskaya, I., Buck, N. A. & Tabashnik, B. E. 2000 Hexameric storage proteins during metamorphosis and egg production in the diamondback moth, *Plutella xylostella* (Lepidoptera). *J. Insect Physiol.* **46**, 951–958.