

Pollen-feeding delays reproductive senescence and maintains toxicity of *Heliconius erato*

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Abstract

Dietary shifts may act to ease energetic constraints and allow organisms to optimise life-history traits. *Heliconius* butterflies differ from other nectar-feeders due to their unique ability to digest pollen, which provides a reliable source of amino acids to adults. Pollen-feeding has been associated with prolonged adult lifespan and increased fertility, yet there is a lack of empirical data demonstrating how pollen consumption influences key fitness traits, including chemical defences and adult body weight, as well as fertility over their elongated lifespan. Here, we investigated the effect of pollen-feeding on fertility, weight and chemical defences, as well as offspring defences, controlling for butterfly age and sex. Recently emerged *Heliconius erato* butterflies of similar size were fed for 14 or 45 days on one of three diets: sugar solution only, or sugar solution replenished with either amino acid supplement or pollen. At the end of the experiment, oviposition assays were performed to evaluate fertility, and afterwards all butterflies and eggs were weighed and used for quantification of cyanogenic glucosides (CG). We found that there is an age-specific and sex-specific effect of pollen-feeding on butterfly weight, with both the sugar-only and amino-acid supplement diets reducing the weight of old females (45d), but not young females (14d) or males of any age. Females fed only sugar significantly reduced their egg-laying through adulthood, whereas females that had access to pollen maintained their fertility. Diet had a significant effect on the maintenance of the chemical defence of females, but not males. Curiously, even though females that have access to pollen were heavier, more toxic and laid more eggs, this did not translate into improvements in offspring defences, as eggs from butterflies of all ages and diet treatments had similar CG content. Our results emphasise the importance of controlling for age-specific and sex-specific effects in studies of life-history evolution and demonstrate that dietary novelty can relax energetic constraints.

1 Introduction

2 Survival and reproductive success are the two major components of Darwinian fitness, and as with all
3 life-history traits, they are under strong selective pressures. Nevertheless, species cannot evolve to
4 live forever and reproduce continuously (there are no “Darwinian demons” (Law, 1979)) due to
5 physiological and energetic constraints that create trade-offs between life-history traits (Healy, Ezard,
6 Jones, Salguero-Gómez, & Buckley, 2019). Yet, dietary shifts may ease energetic constraints, which
7 could in turn allow organisms to optimise multiple fitness traits simultaneously (Swanson et al., 2016).

8 One striking case of dietary innovation is provided by the pollen feeding *Heliconius* butterflies (Gilbert
9 1972; Young and Montgomery 2020). Butterflies typically require water and sugars during adulthood,
10 which can be acquired either from rotten fruits (fruit-feeders) or nectar produced by flowers (nectar-
11 feeders) (Krenn, 2008). Butterflies of the *Heliconius* genus differ from other nectar-feeders due to
12 their ability to additionally collect and digest pollen while feeding on nectar (Gilbert 1972; Young and
13 Montgomery 2020). Although many insects can eat pollen (e.g. bees as well as some beetles, sawflies,
14 mirids, thrips, flies and moths) (Wäckers, Romeis, & Van Rijn, 2007), *Heliconius* are the only butterflies
15 known to actively collect and digest pollen grains. This is probably explained by the necessity of
16 specific adaptations for mechanical and chemical digestion of pollen to make its nutrients available
17 for absorption (Johnson & Nicolson, 2001).

18 A number of adaptations were probably necessary to allow *Heliconius* to digest pollen (Harpel et al.,
19 2015; Smith et al., 2016; Smith et al., 2018; Cicconardi et al., 2023). Pollen grains collected in the
20 elongated proboscis of these butterflies are humidified with salivary secretions, aided by the co-option
21 of a “grooming behaviour” (coiling and uncoiling of the proboscis for some minutes to hours) (Gilbert
22 1972; Krenn et al. 2009; Hinkl and Krenn 2011). Pollen-feeding is not observed in other genera of the
23 *Heliconiini* tribe and arose in the *Heliconius* genus, with an independent loss in the *aoede* clade (four
24 species that were previously classified as the *Neruda* genus) (Turner 1976; Beltrán et al. 2007; Kozak
25 et al. 2015; Cicconardi et al., 2022). As *Heliconius* is the most speciose genus of the tribe, their novel
26 ability to use pollen has likely contributed to their diversification, opening new niches to be exploited
27 (i. e. through habitat partitioning, foodplant preference, foraging behaviour) and providing them with
28 the energetic resources necessary for the maintenance of complex traits (Estrada and Jiggins 2002;
29 Montgomery et al. 2016; Young & Montgomery, 2020; Couto et al. 2022). Indeed, the pollen-feeding
30 behaviour of *Heliconius* butterflies has been associated with several aspects of their biology that
31 diverge from the other heliconiine genera, including an elongated adult-lifespan (Dunlap-Pianka et al.
32 1977), prolonged fertility (Boggs et al. 1981; O’Brien et al. 2003), enlarged mushroom-bodies
33 (Montgomery et al. 2016), foraging site fidelity (Moura, Corso, Montgomery, & Cardoso, 2022) and
34 increased adult toxicity (de Castro et al. 2020)

35 Lepidopteran generally acquire most, if not all, of their nutrients during larval feeding. By supplying
36 butterflies with amino acids, pollen feeding may have decoupled this partition (Boggs, 2009), providing
37 a mechanism for further investment in adult behavioural strategies. Indeed, while most Lepidoptera
38 tend to live relatively long lives as larvae and shorter lives as adults, *Heliconius* adults that have access
39 to pollen can live up to 6 months, which is much longer than the regular average life-span of other
40 heliconiines (~1 month)(Brown, 1981), despite a similar larval period (Hebberecht, Melo-Flórez,
41 Young, McMillan, & Montgomery, 2022). Alongside this increased longevity, *Heliconius* butterflies
42 also maintain their fecundity for longer than other heliconiines, such as *Dryas iulia*, showing limited
43 evidence of reproductive senescence, unless deprived of pollen (Dunlap-Pianka et al., 1977). This
44 prolonged fertility is energetically costly: a female butterfly can lay up to 9-18 eggs a day and they can
45 live for many months, such that total resources allocated to oviposition exceed their own body mass.
46 Indeed, O’Brien et al. (2003) used isotopic labelling to demonstrate the direct transfer of essential

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47 amino acid from pollen ingested by females to their eggs. Males also contribute to the cost of fertility
48 by transferring nuptial gifts to the female during mating (Boggs and Gilbert, 1979; Cardoso, Roper, &
49 Gilbert, 2009) which can exceed 5% of male body weight, and pollen resources may also be used for
50 this purpose (Boggs, 1990). Although the relationship between diet, body weight, fertility and
51 longevity seems obvious, there is a lack of empirical data about how pollen-feeding affects weight
52 maintenance and how is this associated with the prolonged fertility of these butterflies.

53 Finally, the evolution of pollen feeding has also been associated with toxicity, a critical trait for
54 chemically defended aposematic butterflies. *Heliconius* tend to have higher total concentrations of
55 cyanogenic glucosides (CG) than other heliconiines (de Castro et al. 2019; Sculfort et al. 2020) and
56 mature adults have higher concentrations than larvae and young adults (Nahrstedt and Davis 1983;
57 de Castro et al. 2020). This is unusual in aposematic butterflies, which normally acquire their chemical
58 defences from plants during larval feeding and therefore have more toxins as final instar larvae
59 (Nishida, 2002). Whereas larvae of *Heliconius* balance between CG biosynthesis and sequestration
60 from their obligatory Passifloraceae hostplants (de Castro et al. 2018; de Castro et al. 2021), adults
61 can only biosynthesize these defence compounds, for which they need the amino acids valine and
62 isoleucine (de Castro et al. 2020). It has been hypothesized that *Heliconius* butterflies use the essential
63 amino acids from pollen for CG biosynthesis (Nahrstedt and Davis 1983). However, studies comparing
64 the CG content of young *Heliconius* butterflies fed only sugar to those whose diet was supplemented
65 with amino acids/pollen did not show any significant differences (Nahrstedt and Davis 1985; Cardoso
66 and Gilbert 2013). This suggests that *Heliconius* butterflies might biosynthesize CGs initially using
67 amino acids acquired during the larval stage, with resources from pollen-feeding only used later in
68 adulthood.

69 Here, we explore how a dietary novelty can ease energetic constraints on life-history traits, using
70 pollen feeding *Heliconius* as a case study. We investigate the effect of pollen-feeding on *H. erato* body
71 weight, chemical defences, and fertility controlling for sex and age, and specifically comparing young
72 adults (14d) with mature adults (45d). We therefore tested the hypothesis that mature butterflies that
73 only had access to sugar during adulthood would have lower fertility, body weight and depleted
74 chemical defences.

75 **Methods**

76 Rearing conditions of *H. erato* stock population

77 All experiments were performed using individuals from a stock population of *H. erato demophoon*
78 kept at University of Cambridge. This population was sourced from Panamá city (Panama) and has
79 been kept under insectary conditions for about 7 years. Adults were kept in breeding cages (60x60x90
80 cm) containing plants of *Passiflora biflora* for oviposition, as well as flowering *Lantana sp.* and few
81 *Psiguria sp.* for adult feeding. Cages included feeders with artificial nectar made from 10% sucrose
82 solution (m/v) with 1.5% (m/v) Vetark Critical Care Formula (CCF). *P. biflora* shoots with eggs were
83 collected from the breeding cages and used to set up larval cages. Larvae were fed with fresh *P. biflora*
84 shoots *ad libitum* until pupation. Larval cages were checked every other day and encountered pupae
85 were transferred to pupal cages, where pupae were hung under a stick covered with a microfiber cloth
86 Freshly emerged individuals in the pupal cages were transferred to breeding cages. All cages are kept
87 at 25–28 °C, 60–80% humidity and 12h day/night cycle.

88 Experimental Design and Diet treatments

89 Recently emerged adults (0–1 day after eclosion) were transferred to the experimental cages
90 (60x60x90 cm). Only adults that had morphologically healthy, with uncrumpled dry wings were used

91 in these experiments. In addition, only individuals with a forewings between 3.0 to 3.5 cm in length
92 were used to control for potential size effects. One experimental cage was set up for each treatment
93 (diet/age) and each had initially 8 males and 8 females (N=16). Butterflies that died during the first
94 week of experiment were replaced to control for density. Butterflies were placed on feeders when
95 added into the experimental cages to ensure that they would be able to find their food source. Each
96 experimental cage received one of the diet treatments: 1) three feeders with artificial nectar made of
97 10% sucrose; or 2) three feeders with artificial nectar made of 10% sucrose + 1.5% amino acid
98 supplement (CCF); or 3) three feeders with artificial nectar made of 10% sucrose and freshly collected
99 *Lantana* flowers, as a natural source of pollen. Butterflies were fed *ad libitum*, with feeders and
100 flowers were replaced every other day. Males and females in each treatment were allowed to mate
101 freely. Experimental cages were kept for 14 days to assess the importance of amino acid on young
102 butterflies and for 45 days to assess this effect on mature butterflies. All other heliconiines live for ~1
103 month, therefore 45 days is the beginning of an adulthood period that is specific of mature *Heliconius*
104 butterflies. All experimental cages were kept at the same environmental conditions used for
105 husbandry (25-28 °C, 60-80% humidity and 12h day/night cycle). The protein concentration of *Lantana*
106 pollen extracts and the CCF supplement was determined using the Pierce method (Supplementary
107 Methods, Table S3).

108 Fertility assays

109 At the end of the experiments, female butterflies were individually assayed for oviposition to evaluate
110 the effect of the diet treatments on fertility, while males were kept in the experimental cages until
111 sample collection. For the fertility assays, female butterflies were transferred into individual cages
112 (30x30x40 cm) containing their previous diet (one feeder per cage, with one flower bouquet for the
113 pollen treatment) and a *P. biflora* cutting with 5 expanded leaves for oviposition. After 48h of assay,
114 eggs were counted, weighed, and collected for further analyses.

115 Sample collection, metabolite extraction and HPLC-MS conditions

116 The weight of each butterfly was recorded at the end of the experiment (14 days or 45 days). 8 males
117 and 8 females of freshly emerged butterflies (unfed, after 0-1 day of eclosion) were also weighed and
118 collected as a baseline. Afterwards, butterflies were collected in 1 mL methanol 80% (v/v) for chemical
119 analyses. All samples were kept at -20 °C until further processing. For the metabolite extraction,
120 butterfly samples were homogenized (1mL methanol 80% (v/v)) using a porcelain mortar and pestle.
121 Egg samples were homogenised in 300 µL methanol 80% (v/v) into their own collection tube using a
122 small pestle. Extracts were centrifuged at 14,000 *g* for 5 min, filtered (45 µm) and collected for
123 analyses in a LC-Orbitrap-MS/MS. LC-MS methods and analyses were conducted as described in de
124 Castro et al. (2019). The *de novo* biosynthesized CGs linamarin, lotaustralin and epilotaustalin were
125 quantified in the analysed samples, which had no other CGs. The absolute amount of each compound
126 in each sample was calculated using the peak area of their sodium adduct applied to a regression curve
127 established using pure standards. Raw chemical data as well as quantification methods can be found in
128 <https://doi.org/10.17863/CAM.92867>

129 Statistical analyses

130 Statistical analyses and plots were performed in R. Shapiro-Wilko test was used to analyses if the
131 variables were normally distributed (Table S1) and Levene's test for the homogeneity of the variances
132 (Table S2). ANOVA was used to evaluate the effect of diet, age and sex, as well as their interaction, on
133 butterfly weight (Table 1, Table S3 for females only). Tukey HSD was used for pairwise comparisons
134 between the different diet:age treatments in males and females. ANOVA was used to examine the

135 effect of diet and age on CG per laid egg with Tukey HSD for pairwise comparisons. Kruskal-Wallis was
136 used on variables that were not normally distributed: to analyse the effect of age and diet on laid eggs;
137 and the effect of diet, age and sex on butterfly CG content.

138 **Results**

139 Females are more affected than males by the lack of pollen

140 Age, diet and sex significantly influenced the body weight of *H. erato* butterflies (Table 1). There was
141 also a significant interaction between age and sex, which indicates that the body weight of males and
142 females were differently affected through adulthood (Table 1). Indeed, overall females were heavier
143 than males and they were more affected by the absence of [nitrogen-amino acids](#) on their adult diet
144 (Figure 1). Mature females had lower weights by mid adulthood without access to nitrogen, but adult
145 diet did not affect the weight of mature males (Figure 1). Mature females that had access to pollen
146 were heavier than mature females fed sugar only, which had lower weights than freshly eclosed
147 females (Figure 1). Males and females eclosed with similar weight (0d) (Figure S1).

148 **Table 1.** Effect of diet, age and sex on weight (grams
149 per individual) of *H. erato* butterflies. The variables
150 that have a significant effect on butterfly weight are
151 marked in bold, with a * near their p value ($p > 0.05$).
152 Diet treatments: sugar only, sugar + amino acid
153 supplement, and sugar + pollen. Sex: female and
154 male. Age: young (14d) and mature(45d).
155

Variables	Three-way ANOVA
Diet	F₂₋₉₅= 4.597, p= 0.012*
Age	F₁₋₉₅= 7.287, p= 0.008*
Sex	F₁₋₉₅= 25.055, p= 2.5 x 10⁻⁶*
Diet:Age	F ₂₋₉₅ = 2.016, p= 0.139
Diet:Sex	F ₂₋₉₅ = 0.486, p= 0.617
Age:Sex	F₁₋₉₅= 4.520, p= 0.036*
Diet:Age:Sex:	F ₂₋₉₅ = 1.791, p= 0.172

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160 Access to pollen only affects the chemical defences of females

161 Males and females increased their CG content after eclosion (Figure S2) and kept their defences
162 through adulthood, indicating that they intensively biosynthesize these compounds. Curiously, diet
163 only affected the CG content of females (Figure 2. Kruaskal-Wallis, Females: $X^2= 6.35$, $p= 0.048*$;
164 Males: $X^2= 2.115$, $p= 0.347$), with butterflies having access to amino acids (supplement or pollen)
165 showing greater CG content than those fed sugar alone. Young and mature butterflies of both sex had
166 similar CG content (Figure 2. Kruaskal-Wallis, Females: $X^2= 1.441$, $p= 0.23$; Males: $X^2= 0.198$, $p= 0.656$).

167
168 Access to pollen delays reproductive senescence

169 Adult diet affected egg laying in mature butterflies of *H. erato* (Figure 3. Kruskal-Wallis, $X^2= 0.569$, $p=$
170 $0.017*$), but not in young ones (Figure 3. Kruskal-Wallis, $X^2= 0.569$, $p= 0.752$). Young females (14d) laid
171 similar numbers of eggs regardless of their diet. In contrast, mature females (45d) that had access to
172 pollen laid more eggs than butterflies that had access to sugar only, or sugar + supplement. This
173 indicates that access to pollen delays reproductive senescence in *Heliconius*.

174 In contrast to our expectations, adult diet did not affect parental allocation in the chemical defences
175 (CG) of their eggs. Eggs of young (ANOVA, $F_{2-10}= 0.56$, $p= 0.588$) and mature butterflies (ANOVA, $F_{2-17}=$
176 0.09 , $p= 0.914$) had similar concentrations of CG regardless of the diet of their parents.

177 The nutritional uniqueness of pollen

178 Although the CCF supplement had far more proteins (651.70 ± 19.97 μg per mg DW) than the *Lantana*
179 flower extract (pollen and nectar) (1.71 ± 0.45 μg per mg DW) (Table S3), it did not lead to

180 improvements in the butterfly fitness traits. Thus, pollen might have an amino acid profile that fits
181 better the nutritional needs of *Heliconius* and/or have them in a more accessible way (free amino acids
182 instead of proteins/peptides).

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187 **Discussion**

188 When does access to pollen start to be important and for whom?

189 Pollen-feeding eases energetic constraints and allows *Heliconius* to optimize multiple life-history traits
190 simultaneously, aiding the maintenance of fertility, body weight and chemical defences during their
191 prolonged adult-lifespan (Fig. 1, 2 and 3), as we hypothesized. Nevertheless, we found age-specific
192 responses to this dietary novelty, as access to pollen has an effect on old butterflies (45d) of *H. erato*,
193 but not on the young ones (14d). This is the first time to our knowledge that the effect of pollen
194 deprivation on multiple life-history traits of *Heliconius* has been evaluated controlling for age. Studies
195 supplementing the diets of other nectar-feeding long-lived nymphalids (*Polygonia c-album*, *Maniola*
196 *jurtina*) with amino acids have not found an improvement in life-history traits (Karlsson and Wickman
197 1989; Grill et al. 2013). Our results therefore emphasize that adaptations were required to make use
198 of pollen-derived amino acids in *Heliconius* butterflies (Dunlap-Pianka et al. 1977; Boggs et al. 1981).

199 The age-effects also reveal that the balance between larval and adult derived resources changes over
200 the life course (Boggs, 2009). It is possible that the physiology of young *Heliconius* butterflies, including
201 their fertility, initially relies mostly on resources acquired during larval feeding, as in the vast majority
202 of butterflies and moths. Nevertheless, as *Heliconius* butterflies live relatively long adult lives, the
203 reservoir of larval derived resources likely becomes depleted over time, such that the presence of
204 amino acids in their adult diet becomes a crucial factor for the maintenance of the homeostasis. This
205 is consistent with field data showing that older *Heliconius* butterflies generally collect more pollen
206 than the young ones (Boggs et al., 1981; Boggs et al, 1990), which may indicate greater motivation as
207 larval resources deplete.

208 This implies that studies evaluating the importance of pollen-feeding during adulthood for *Heliconius*
209 butterflies likely need to be performed for periods longer than a month. Cardoso and Gilbert (2013)
210 did not observed differences between the cyanide concentration from 20 day old *Heliconius*
211 butterflies (*H. ethila*, *H. hecale* and *H. charithonia*) fed only sugar and supplemented with amino acids,
212 as we observed here for females. The authors discussed the importance of larval diet shaping the
213 chemical defences of young *Heliconius* butterflies. Additionally, the pheromone bouquet of 14 day old
214 *Heliconius* males (genital and androconia) was also not affected by access to pollen during adulthood,
215 only by the hostplant species used during larval feeding (Darragh et al., 2019). Indeed, the results seen
216 here would probably be more striking if we have maintained the experiment for more than 45 days.
217 Combined, these studies emphasize that the importance of the resources accumulated during larval
218 feeding for young butterflies and the latter importance of pollen during adulthood.

219 It is a common knowledge among researchers breeding *Heliconius* under insectary conditions that
220 they die sooner without access to pollen/amino acids in their adult diet. In this study, 45 days was not
221 enough to observe differences in mortality between diet treatments (1-3 butterflies died in each
222 treatment, mostly within the first week of eclosion), contrary to previous findings (Dunlap-Pianka et
223 al. 1977). As previous experiments used *H. charithonia* (Dunlap-Pianka et al. 1977) while we used *H.*
224 *erato*, this could indicate that different *Heliconius* species might rely on the amino acids acquired
225 during adult-feeding sooner than others. Nevertheless, the previous work had few replicates (N=8 in
226 total, N=3 after 40 days.) and might have underestimated how long *H. charithonia* can live on average
227 without pollen. How different *Heliconius* species respond to the lack of pollen is an interesting
228 question for the future.

229 Do females pay a higher cost for reproduction than males when resources are scarce?

230 This study demonstrates that access to amino acids delays reproductive senescence in *H. erato*
231 females, as old females (45 d) supplemented with pollen lay as many eggs as young females (14d),
232 whereas females fed only sugar lose fertility throughout adulthood (Fig. 3). Boggs (1990) observed
233 that females of *H. charithonia* and *H. cydno* drastically increase pollen collection between 15-20 days
234 old, possibly to maintain their fertility and chemical defences. This corroborates with the earlier work
235 of Dunlap-Pianka et al. (1977) demonstrating that *H. charithonia* that have access to pollen can keep
236 daily egg-laying rates until their natural death (up to 72d), however they continuously decrease their
237 egg-production and reach ovarian depletion when pollen is absent.

238 The disparity between how females and males of the same species alter their life-history dynamics in
239 response to resource availability has intrigued evolutionary ecologists. Without pollen-feeding, *H.*
240 *erato* females lose weight (Fig. 1) and decrease their chemical defences (Fig. 2) as they get older,
241 whereas males do not. As females collect significantly more pollen than males in the wild (Boggs et
242 al., 1981), it could be that females feed more than males and therefore their fitness is more impacted
243 by diet. Regardless, only females of *H. erato* were strongly affected by adult diet and this was reflected
244 in their fertility, which might suggest that females are paying a higher energetic cost for reproduction
245 than males when access to amino acids is limited. Even though diet did not affect male weight or CG
246 content, we cannot discard the possibility that the effect of diet on fertility might be associated with
247 other male fitness traits, such as sperm viability and quality of nuptial gifts (Boggs & Gilbert, 1979;
248 Boggs, 1990).

249 Some of the old female butterflies in the cage supplemented with pollen had a strong smell of anti-
250 aphrodisiac (personal observations) suggesting that they recently re-mated. Although re-mating was
251 not expected in this experiment, since *H. erato* belongs to the monoandrous clade of *Heliconius*
252 (Beltrán, Jiggins, Brower, Bermingham, & Mallet, 2007) and rarely re-mates in the wild (Cardoso et al.
253 2009; Walters et al. 2012), the insectary conditions might have induced them to re-mate. Re-mating
254 would allow the transference of more nuptial gifts, which includes CGs, from the male to the female
255 (Cardoso and Silva 2015), diluting the effect of pollen supplementation on male chemical defences
256 and body weight. Further studies of spermatophore quality will be necessary to unravel the effect of
257 pollen deprivation on the fitness of *Heliconius* males.

258 Do high condition adults lay better protected eggs?

259 Many insects protect their eggs by transferring defensive compounds to them, which can improve
260 offspring establishment. Thus, we hypothesized that butterflies with access to pollen would produce
261 eggs with more CGs, as these compounds are not toxic when intact and can be stored in high
262 concentrations. Old females of *H. erato* that had access to pollen are heavier (Fig. 1), had more CGs
263 (Fig. 2) and laid more eggs (Fig. 3) than old females that had access to sugar only. Contrary to our
264 predictions, this does not translate into a higher investment in the chemical defences of their offspring
265 (Fig. 3). Eggs of butterflies from all ages and diets have similar CG content which suggests that this
266 process is tightly regulated - butterflies might lay less or more eggs depending on their diet, but all
267 eggs have a similar level of chemical defences. The amount of CG per egg observed here is similar to
268 other heliconiines (Nahrstedt and Davis 1983; Nahrstedt and Davis 1985; Castro et al. 2020).

269 Our data demonstrate how strongly *H. erato* biosynthesize CG during adulthood to maintain their
270 defences while also investing in the protection of their offspring, corroborating previous findings
271 (Castro et al. 2020; Mattila et al. 2022). Considering that a *Heliconius* female lays ca. 10 eggs per day
272 (Dunlap-Pianka et al. 1977), each egg has on average 3 µg of CG (Fig. 3) and they can live for 45 days.
273 Egg-laying would therefore result in a depletion of over 1000 µg of CG from a female butterfly, which
274 can be more than their whole reservoir of chemical defences at any one time (Fig. 3). In contrast, male

275 contribuitions for offspring chemical defences seems minimal (Cardoso & Gilbert, 2007). Pedigree
276 experiments with *H. erato* also found strong maternal effects on offspring toxicity, but no paternal
277 effects (Mattila et al., 2021).

278 Mattila et al. (2022) demonstrated that *Heliconius* butterflies keep their CG concentration at high
279 levels during adulthood until their natural death. Indeed, if these aposematic butterflies lost their
280 toxicity as they age, this would dilute the protection signal of their colour pattern. Thus, there is
281 probably strong selection for *Heliconius* to maintain toxicity as they age, but it is likely challenging to
282 maintain these levels while reproduction depletes their chemical reservoir (Fig. 2 and 3).

283 Moreover, valine and isoleucine are used as substrate for the biosynthesis of aliphatic CGs (Nahrstedt
284 and Davis 1983). These are essential amino acids that have to be acquired by diet (not produced by
285 animals) (O'Brien et al., 2002) and they tend to be abundant in pollen (Gilbert 1972). This suggests a
286 strong effect of pollen-feeding on chemical defences in *Heliconius*. Yet, a lack of pollen/amino acids
287 during adult-feeding does not affect the chemical defences of young *Heliconius* butterflies. As already
288 discussed, access to pollen would become crucial at later stages of adulthood, but the remaining
289 question is: where did the valine and isoleucine used for CG biosynthesis come from during the first
290 weeks of *Heliconius* adulthood in the control group (sugar only)? A recent comparative genomic study
291 has found that two hexamerins, storage proteins, have been duplicated multiple times in heliconiines
292 (Cicconardi et al., 2022). Hexamerins might provide valine and isoleucine for CG biosynthesis during
293 the beginning of their adulthood, if pollen is not available. Moreover, valine and isoleucine might be
294 produced by bacteria in the microbiome of these butterflies, as happens for other insects (Jing, Qi, &
295 Wang, 2020), a hypothesis that can be investigated in the future.

296 In summary, although the link between pollen-feeding, fertility and chemical defences in *Heliconius*
297 butterflies is clear, these interactions are more complex than initially predicted. We demonstrated
298 that there is an age-specific and sex-specific effect of pollen-feeding on life-history traits. Older
299 females supplemented with pollen were heavier, more toxic and laid more eggs than those in the
300 control diets, suggesting that this dietary innovation has eased energetic constraints and led to
301 optimization of multiple life-history traits.

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472

473 **DATA ACCESSIBILITY**

474 The metadata and coding script associated with this publication are available as supplementary
475 material. These files and the raw chemical data are available in the Apollo repository from University
476 of Cambridge at <https://doi.org/10.17863/CAM.92867>.

477

478 **COMPETING INTERESTS**

479 The authors declare that they have no competing interests.

480

481 **FIGURE LEGENDS**

482 **Figure 1.** Effect of diet and age on the fresh weight of females (top) and males (bottom) of *H. erato*.
483 Butterflies were fed either sugar or sugar + supplement (Critical Care Formula) or sugar + pollen (from
484 Lantana flowers). Young butterflies were collected after 14d of trial while mature butterflies after 45d.
485 Legend: Different letters over the boxplots correspond to statistically significant differences (Two-
486 ways ANOVA, Tukey HSD). NS = not statistically significant ($p > 0.05$). Lines in the middle of boxplots
487 correspond to the median and boxes to the lower and upper quartile. Dots correspond to values of
488 each analysed replicate/individual butterfly.

489 **Figure 2.** Effect of diet and age on cyanogenic glucosides content of females (top) and males
490 (bottom) of *H. erato*. Butterflies were fed either sugar or sugar + supplement (Critical Care Formula)
491 or sugar + pollen (from Lantana flowers). Young butterflies were collected after 14d of trial while
492 mature butterflies after 45d. Statistical analyses on the top of the plots correspond to Kruskal-Willis
493 on Diet and Age for the subsets. Lines in the middle of boxplots correspond to the median and
494 boxes to the lower and upper quartile. Dots correspond to values of each analysed
495 replicate/individual butterfly.

496

497 **Figure 3.** Number of laid eggs per female during fertility test (top) and cyanogenic glucose content per
498 egg (bottom). Butterflies were fed either sugar or sugar + supplement (Critical Care Formula) or sugar
499 + pollen (from *Lantana* flowers). Young butterflies were collected after 14d of trial while mature
500 butterflies after 45d. Lines in the middle of boxplots correspond to the median and boxes to the lower
501 and upper quartile. Dots correspond to values of each analysed replicate/total eggs laid by each
502 butterfly.

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