

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

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

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

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

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

## 73 **Methods**

### 74 Rearing conditions of *H. erato* stock population

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

### 86 Experimental Design and Diet treatments

87 Recently emerged adults (0-1 day after eclosion) were transferred to the experimental cages  
88 (60x60x90 cm). Only adults that had morphologically healthy, with uncrumpled dry wings were used  
89 in these experiments. In addition, only individuals with a forewings between 3.0 to 3.5 cm in length  
90 were used to control for potential size effects. One experimental cage was set up for each treatment

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

#### 106 Fertility assays

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

#### 113 Sample collection, metabolite extraction and HPLC-MS conditions

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

#### 127 Statistical analyses

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

134 used on variables that were not normally distributed: to analyse the effect of age and diet on laid eggs;  
135 and the effect of diet, age and sex on butterfly CG content.

## 136 Results

### 137 Females are more affected than males by the lack of pollen

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

Variables	Three-way ANOVA	
Diet	<b>F<sub>2-95</sub> = 4.597, p = 0.012*</b>	146
Age	<b>F<sub>1-95</sub> = 7.287, p = 0.008*</b>	150 151
Sex	<b>F<sub>1-95</sub> = 25.055, p = 2.5 x 10<sup>-6</sup>*</b>	
Diet:Age	F <sub>2-95</sub> = 2.016, p = 0.139	153
Diet:Sex	F <sub>2-95</sub> = 0.486, p = 0.617	
Age:Sex	<b>F<sub>1-95</sub> = 4.520, p = 0.036*</b>	155
Diet:Age:Sex:	F <sub>2-95</sub> = 1.791, p = 0.172	

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

157

### 158 Access to pollen only affects the chemical defences of females

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

165

### 166 Access to pollen delays reproductive senescence

167 Adult diet affected egg laying in mature butterflies of *H. erato* (Figure 3. Kruskal-Wallis,  $X^2 = 0.569$ , p =  
168 0.017\*), but not in young ones (Figure 3. Kruskal-Wallis,  $X^2 = 0.569$ , p = 0.752). Young females (14d) laid  
169 similar numbers of eggs regardless of their diet. In contrast, mature females (45d) that had access to  
170 pollen laid more eggs than butterflies that had access to sugar only, or sugar + supplement. This  
171 indicates that access to pollen delays reproductive senescence in *Heliconius*.

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

### 175 The nutritional uniqueness of pollen

176 Although the CCF supplement had far more proteins ( $651.70 \pm 19.97 \mu\text{g}$  per mg DW) than the *Lantana*  
177 flower extract (pollen and nectar) ( $1.71 \pm 0.45 \mu\text{g}$  per mg DW) (Table S3), it did not lead to  
178 improvements in the butterfly fitness traits. Thus, pollen might have an amino acid profile that fits  
179 better the nutritional needs of *Heliconius* and/or have them in a more accessible way (free amino acids  
180 instead of proteins/peptides).

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## 185 Discussion

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

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

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

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

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

### 227 Do females pay a higher cost for reproduction than males when recourses scarce?



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

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

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

#### 256 Do high condition adults lay better protected eggs?

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

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

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

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

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

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



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463

464 **DATA ACCESSIBILITY**

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

468

469 **COMPETING INTERESTS**

470 The authors declare that they have no competing interests.

471

472 **FIGURE LEGENDS**

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

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

487

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

494

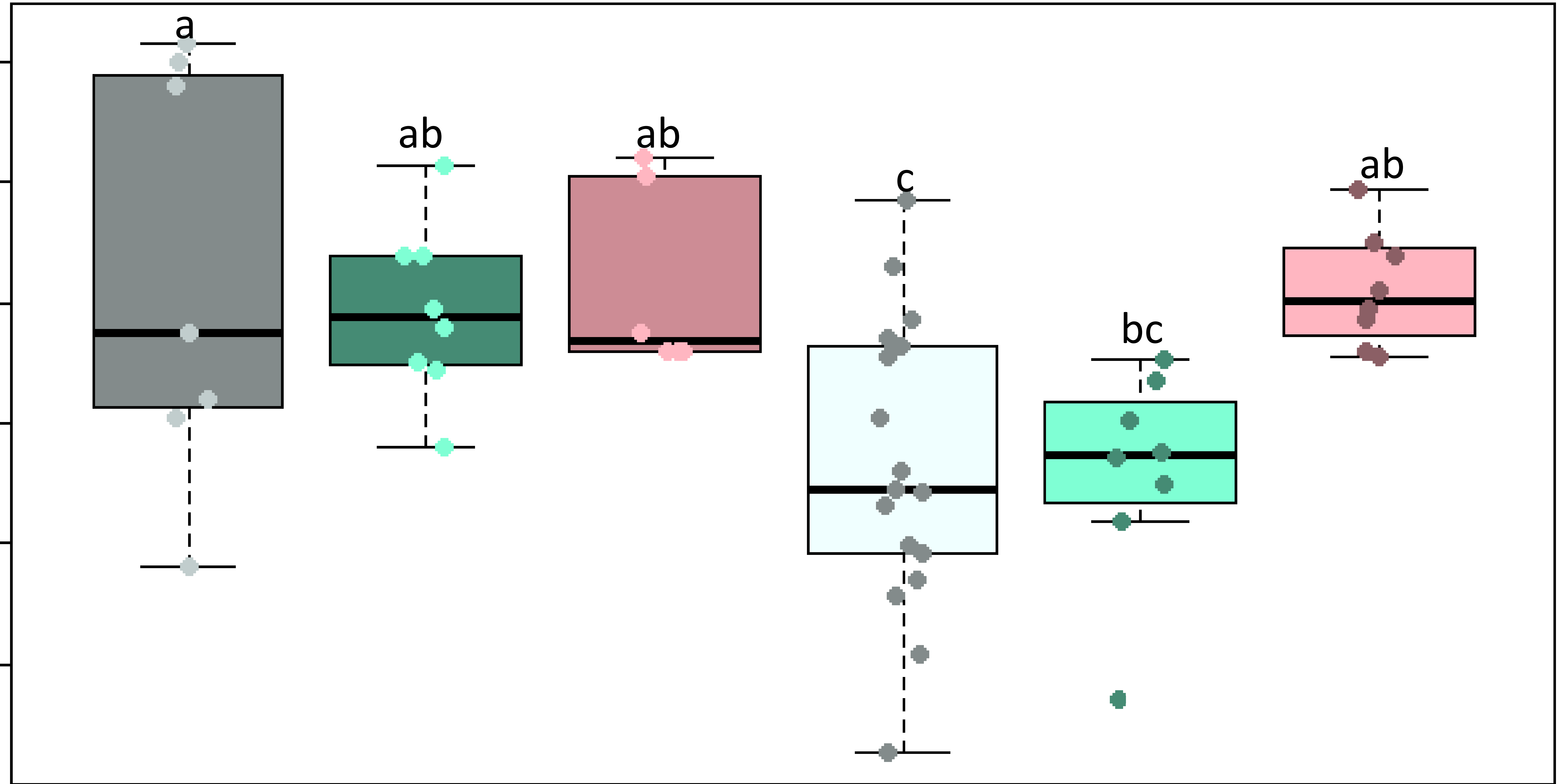


Young butterflies (14d)

Mature butterflies (45d)

Female weight (mg)

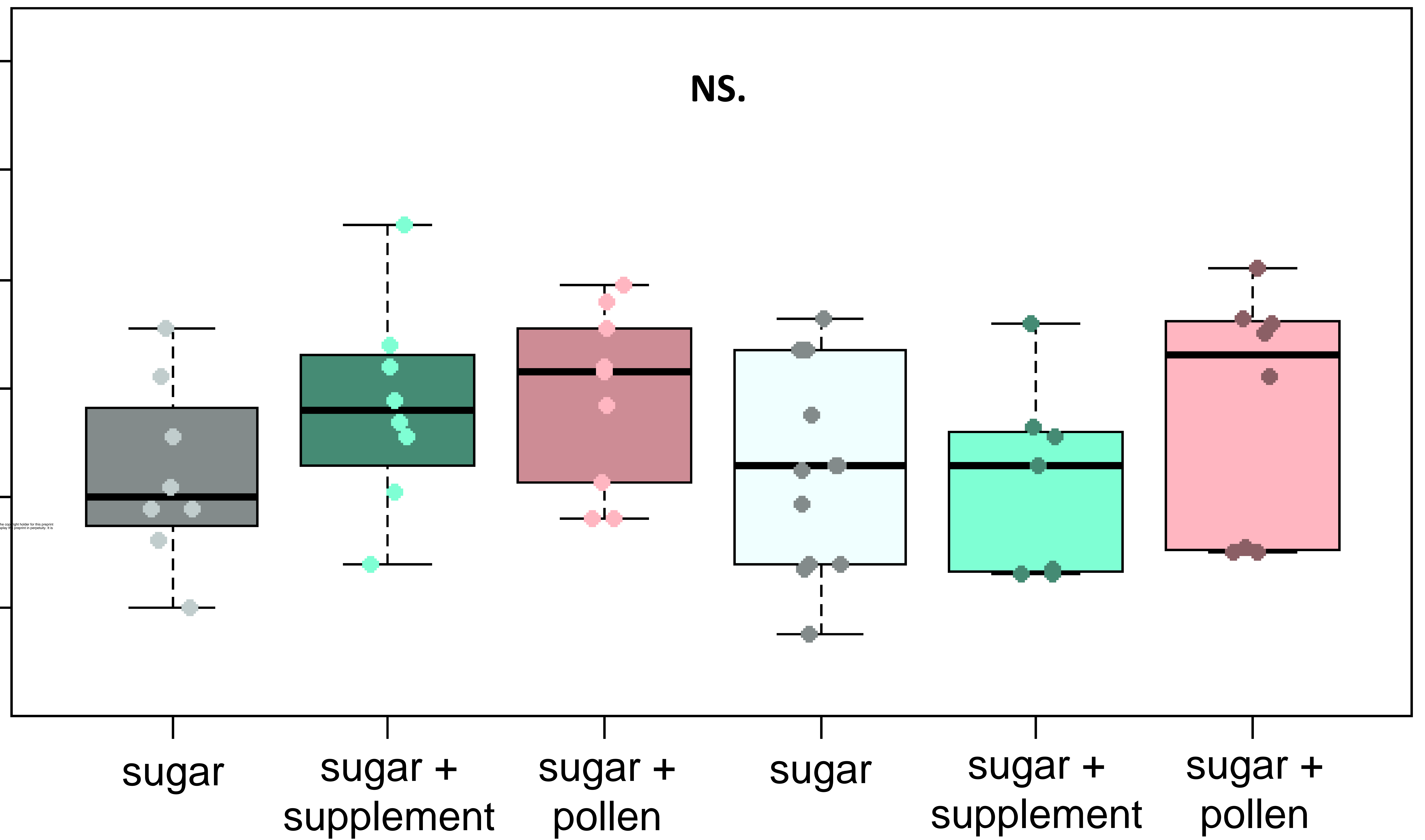
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Male weight (mg)

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120  
100  
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NS.



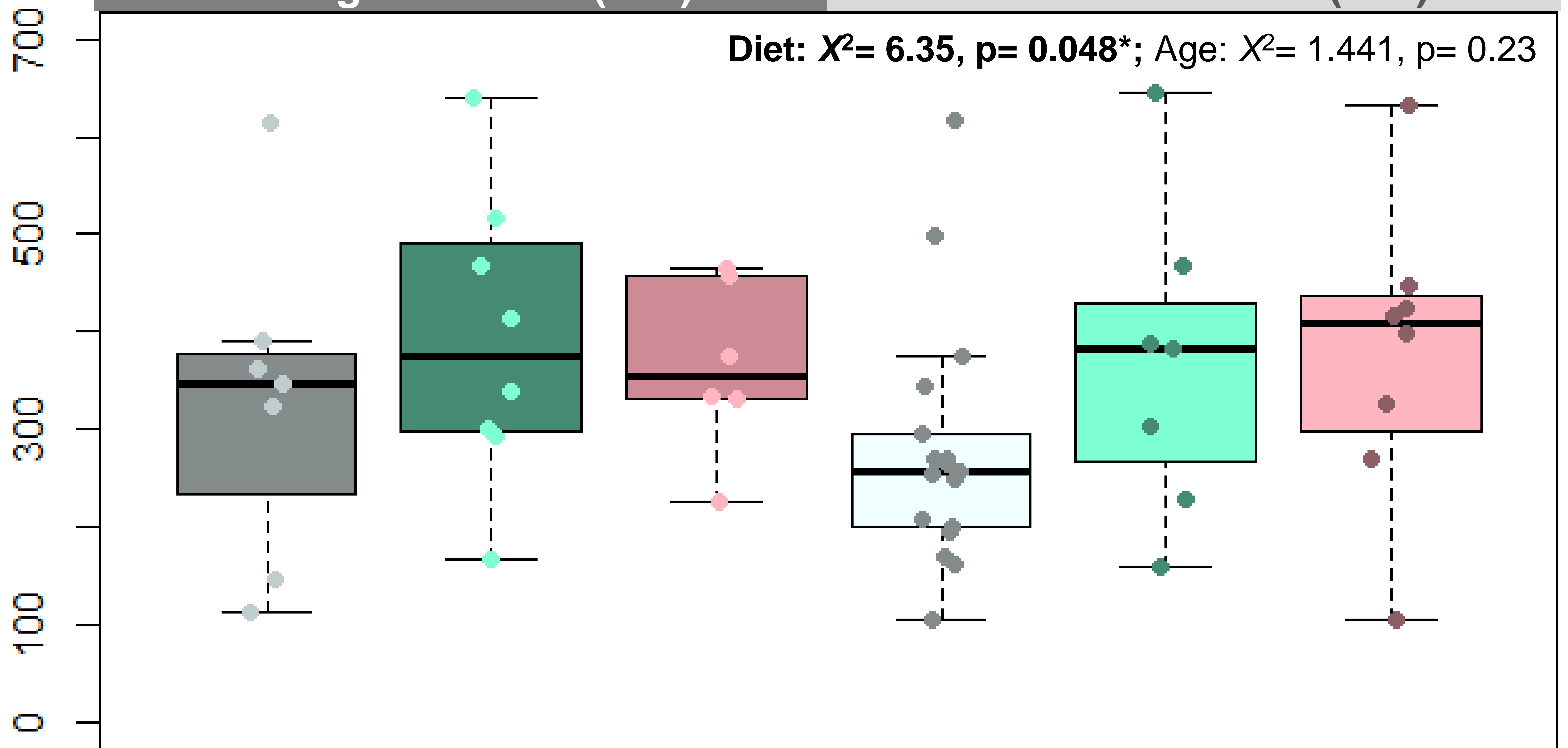
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Young butterflies (14d)

Mature butterflies (45d)

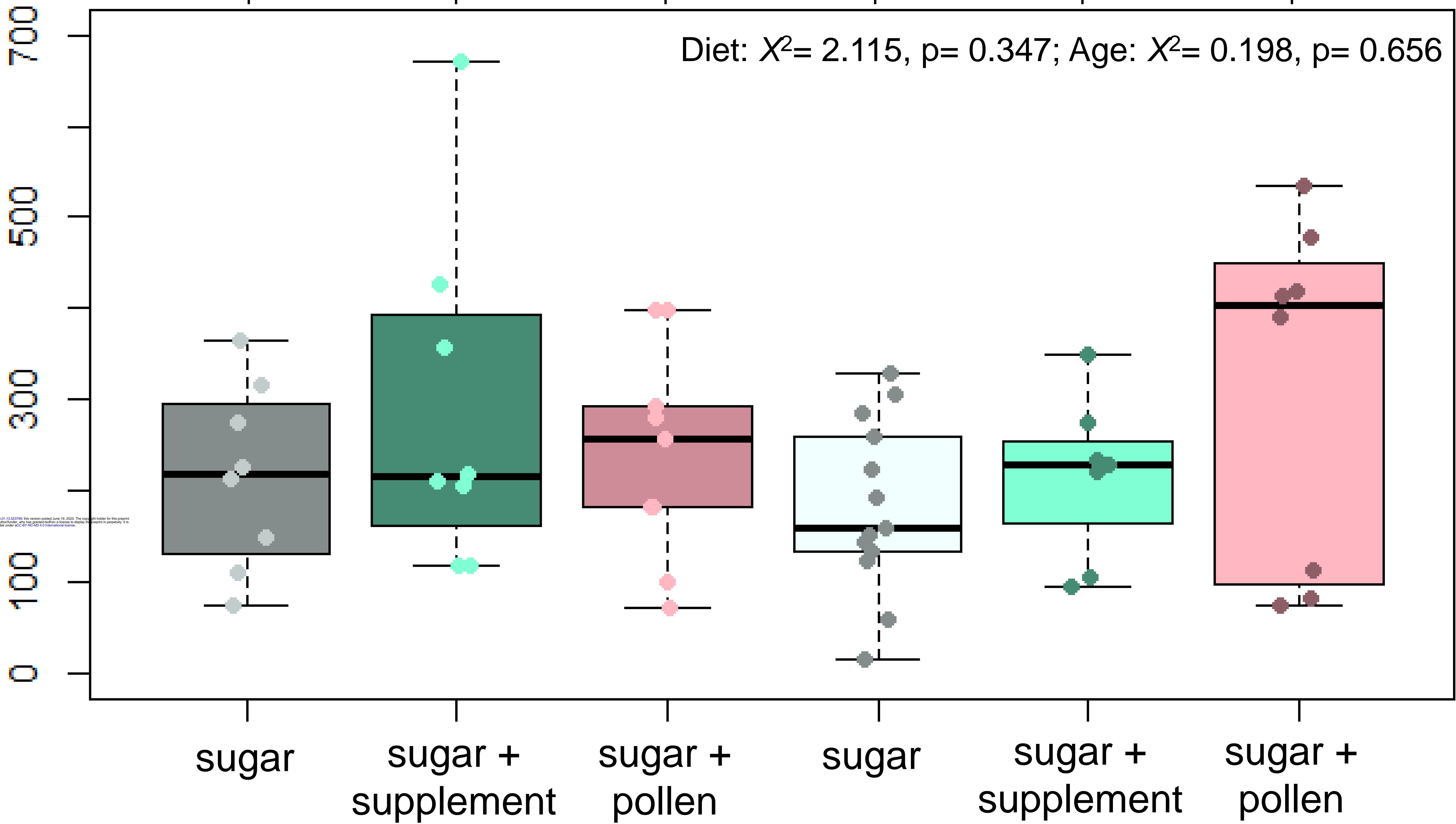
Diet:  $X^2= 6.35$ ,  $p= 0.048^*$ ; Age:  $X^2= 1.441$ ,  $p= 0.23$

Total CGs ( $\mu\text{g}$ ) in **females**

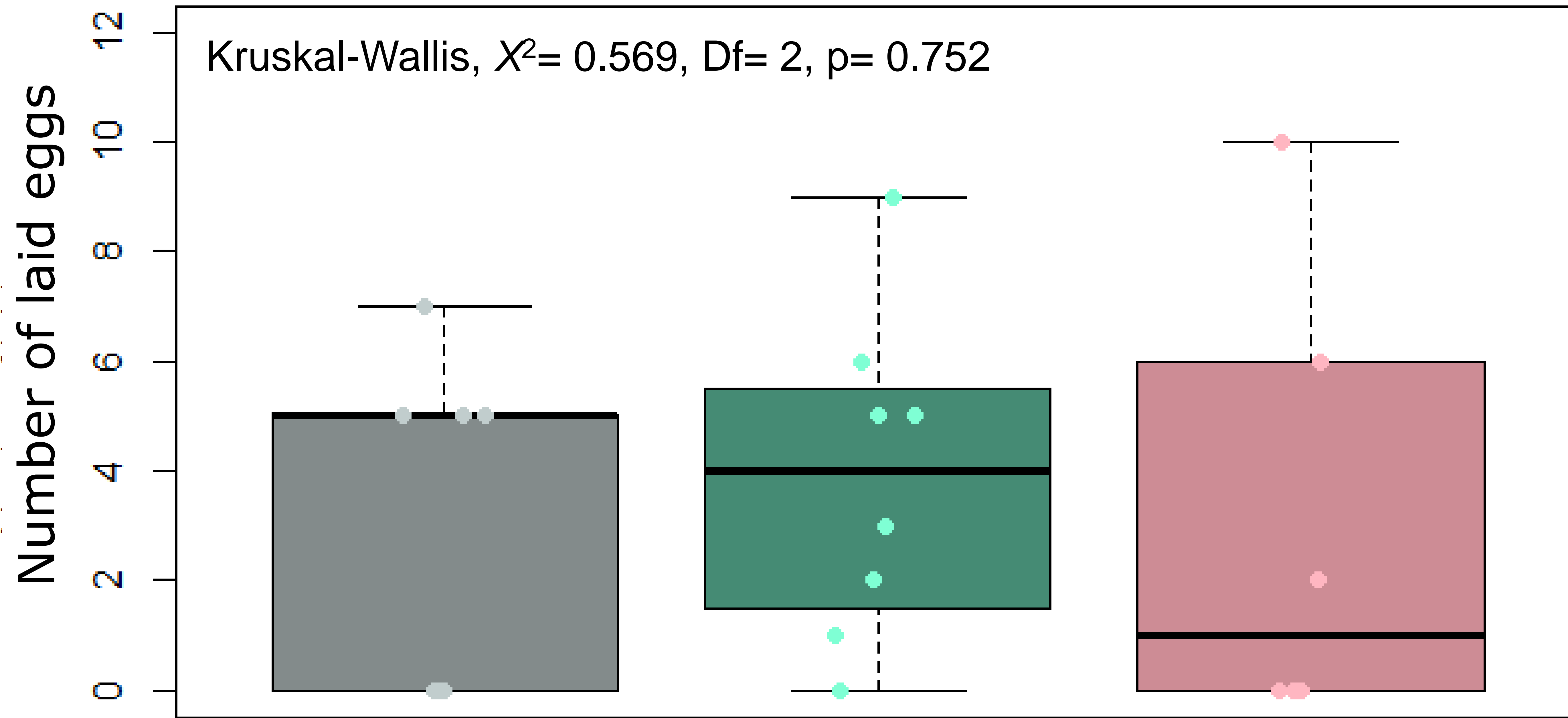


Diet:  $X^2= 2.115$ ,  $p= 0.347$ ; Age:  $X^2= 0.198$ ,  $p= 0.656$

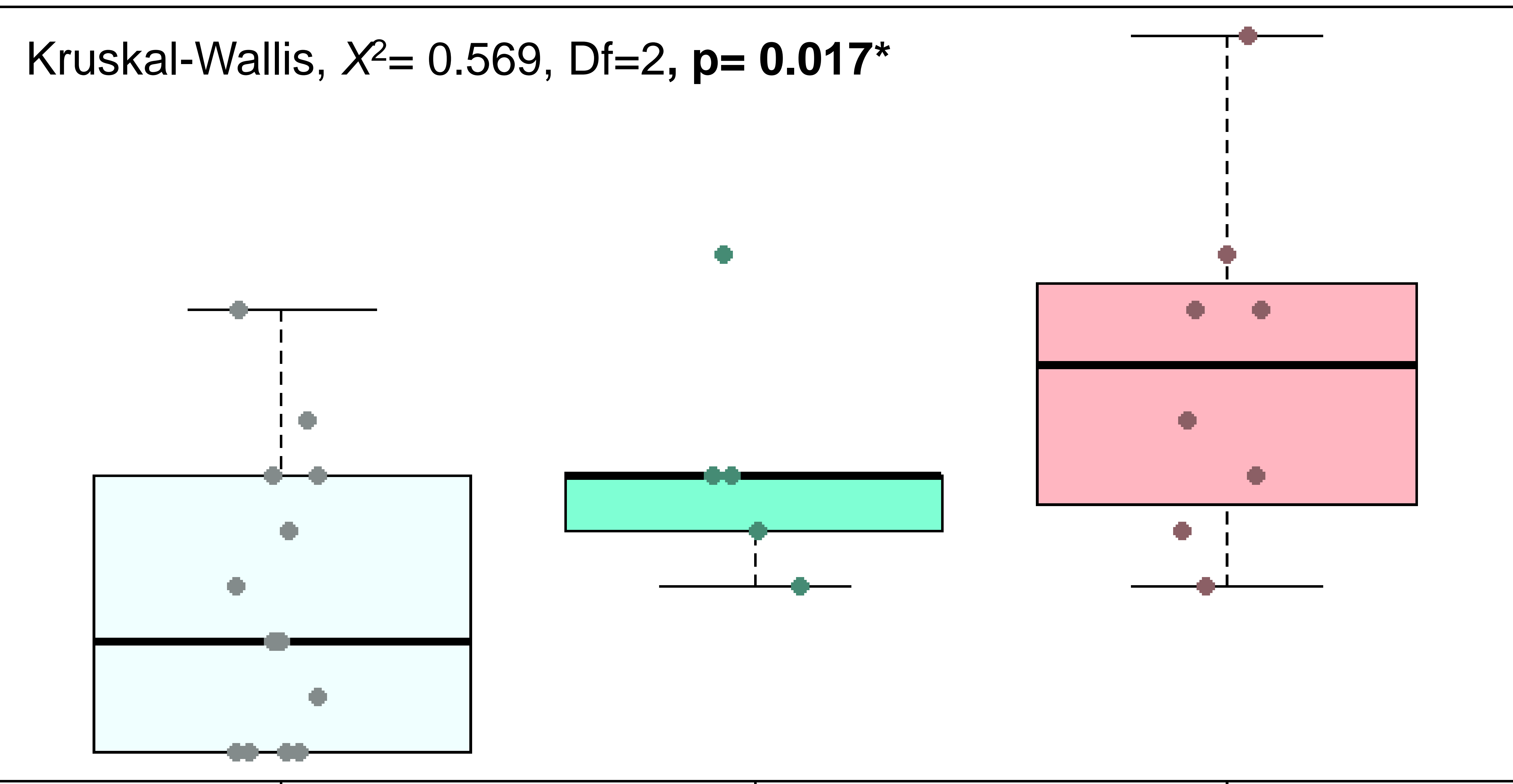
Total CGs ( $\mu\text{g}$ ) in **males**



### Young butterflies (14d)

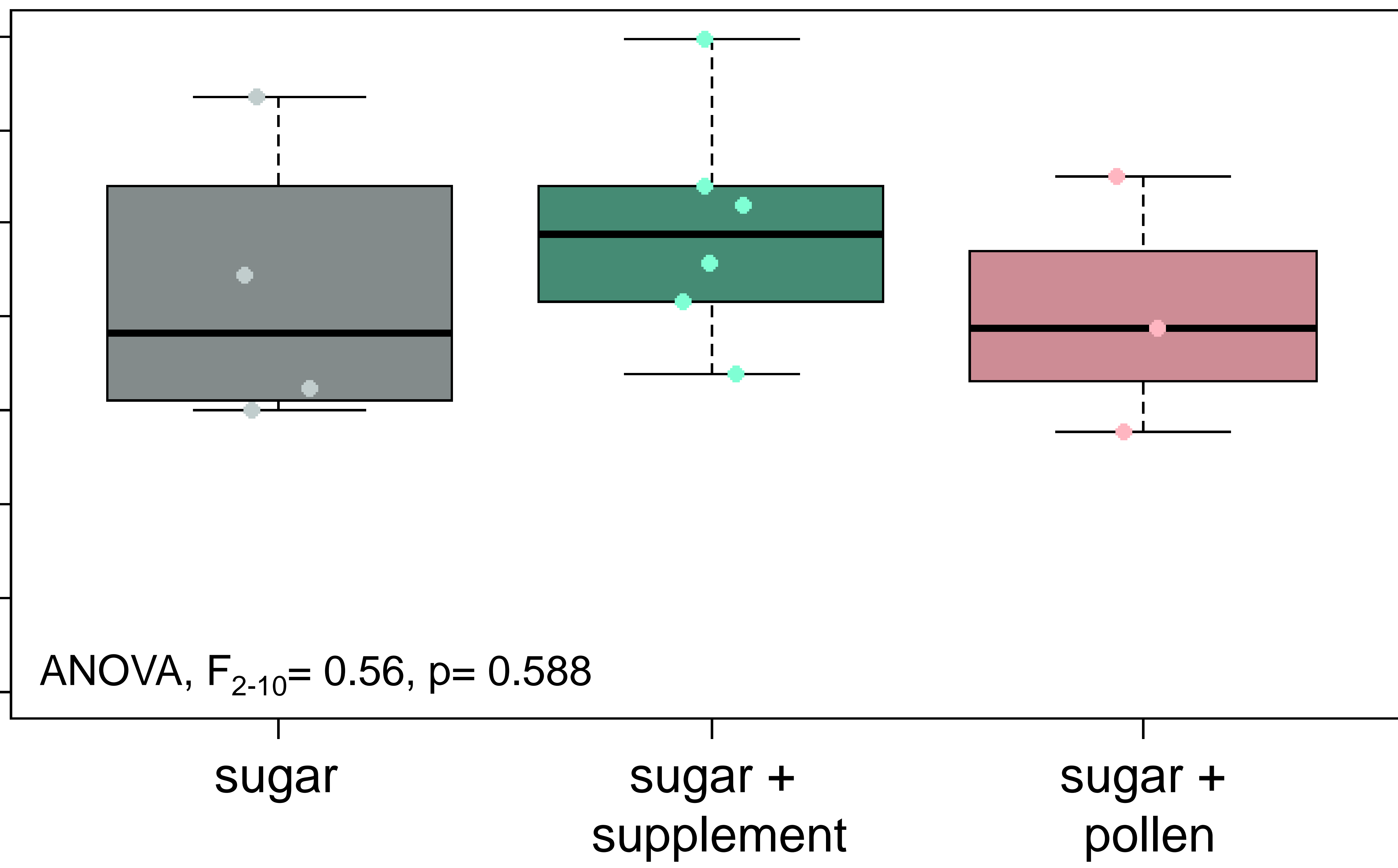


### Mature butterflies (45d)



Total CGs ( $\mu\text{g}$ ) per egg

ANOVA,  $F_{2-10}= 0.56$ , p= 0.588



ANOVA,  $F_{2-17}= 0.09$ , p= 0.914

