

Dear Editor and Reviewers,

We are very grateful for feedback received on our paper. We are also pleased that it was evaluated by reviewers with different expertise that appreciated different aspects of our manuscript and could give us various suggestions. We addressed every received comment to the best of our knowledge and believe that they considerably improved our manuscript.

We completely agree that would be great to know the amino acid profile of *Lantana* pollen and CCF supplement and we tried to obtain this information, however this proved to be unfeasible (see detail in specific comments). However, we did quantify the protein abundance in both treatments to have a comparison between them. We would also like to emphasize that despite of our curiosity about the composition of pollen and supplement, our manuscript focuses on the fitness of the butterflies and the importance of amino acid in their adult diet, whether from a natural source or a supplement – not on the composition of these source of amino acid, which is an interesting but separate question.

Please find below our response to the received comments and a new version of our paper.

Thank you very much again.

Best regards,

Erika de Castro & Co-authors

Responses to reviewers' comments

Adriana Briscoe

1. All three reviewers wanted more details about the amino acid supplementation. What was the composition of amino acids in the supplement and in the pollen of offered flowers? One reviewer commented that the valine and isoleucine composition should be mentioned. Another observed that whey powder implies that the supplement is a peptide or protein supplement rather than an amino acid supplement. This should be clarified.

R= We contacted some local providers that could perform an amino acid profile analysis on pollen for us, but they requested 200 g per sample. We explained that it was challenge to collect pollen from *Lantana* flowers and their technical team said that they could try with a minimum amount of 2 g, but without guarantee that it would work. Collecting 2 g of *Lantana* pollen is completely unfeasible for us as ~20 flowers yielded 1-2 mg of pollen (we did try). We have enough flowers to feed our butterfly stock populations and some extras, but definitely not nearly enough for this.

About the CCF, the manufacturer's website has conflicting information about the composition of this supplement. The website says in the section 'DESCRIPTION': "The formulation is a mixture of selected short chain maltodextrins (derived from corn starch) and a protein concentrate supplemented with amino acids", but it says just "Dextrose, Whey protein powder (concentrate)" in the 'COMPOSITION' section, with no mention to free amino acids. We have contacted Vetark about the CCF formulation and amino acid composition, but we did not obtain an answer.

We did quantify the protein in CCF and *Lantana* flowers to at least have a comparison between these two treatments: CCF has far more protein than the *Lantana* flower extract. Yet, this higher protein content did not lead to an improvement in the measured fitness traits of *Heliconius erato* in comparison with pollen. This suggest that pollen has an amino acid profile that better meets the needs of *Heliconius* than CCF, or that the amino acids are more accessible in pollen (free amino acids instead of peptides). We added a small section about this in the *Results* and a table with the measurements in the Supplementary Material.

2. Related to this, one reviewer noted that the kind and composition of the sugar(s) used in the artificial nectar should be given.

R= We added this information in the *Methods*. The sugar used was sucrose.

3. Another reviewer stated that more details about which specific cyanogenic glycosides were analyzed are needed.

R= We added this information in the *Methods*. The CGs quantified were linamarin, lotaustralin and epilostauralin. We also added a brief explanation about how they were quantified.

4. The third reviewer had several comments on the introduction, including some relating to a few studies on the subject that are perhaps unknown to the authors (Boggs 1979, 1981, 1990).

R= We added the requested references, except for a PhD thesis which we did not find a digital copy of it. We did add a reference to the bioRxiv pre-print Boggs and Iyengar (2022) which has some of the unpublished results of this thesis.

To this I will add my own comment: From the introduction: "Nonetheless, comparative genomics has shown that they are duplicated in all heliconiines, even those that do not pollen feed (Cicconardi et al. 2022)." That cocoonase duplicates are duplicated in non-pollen feeding heliconiines was earlier

observed by Smith G, Macias-Muñoz A, Briscoe AD. 2016. Gene duplication and gene expression changes play a role in the evolution of candidate pollen-feeding genes in Heliconius butterflies. Genome Biology and Evolution, 8:2581-2596. This paper should be cited here if that sentence is retained.

R= This paragraph was removed to make the introduction shorter as suggested by another reviewer. The requested reference was added in the discussion.

5. Reviewer three was also curious about data not mentioned in the paper which might be useful for interpreting the results if available. I do not think it is necessary to include these data as a condition of publication but do think that it would be good if the authors addressed whether or not the amount of pollen collected by butterflies was recorded and whether the males used in the study had an opportunity to mate.

R= All analysed data from this project is available within the paper or in the supplementary material. The supplementary tables and figures are also briefly mentioned in the text, so readers could check them if they want to know more about that. Unfortunately, we did not record behaviour or the amount of pollen collected per individual. 8 males and 8 females were kept in each cage until the end of the trial and they were allowed to mate at their choice and feed at libitum. We tried to improve clarity about this in the text.

6. The title was commented on as being overly-broad for a study that investigates a single species. I am in agreement with this comment. Perhaps changing Heliconius to Heliconius erato would be the simplest fix.

R= We changed the title as suggested.

Carol Boggs

Title: The title is a bit over-broad, given that only one species was examined!

R= We replaced “*Heliconius butterflies*” with “*Heliconius erato*”

Introduction: Lines 18-28. Earlier studies (1960s-70s) showed that pollen can start germinating in water or sugar solutions and release free amino acids. I have seen pollen tubes growing in pollen from loads removed from *Heliconius*. Cocoonase or any other salivary enzyme would likely alter or speed that process. If kept, this paragraph needs to be a bit more nuanced as to digestion.

We agree that pollen germination would indeed be triggered just by the contact with water/saliva. Yet, this is a complex process, and several reactions need to happen until free amino acids could be released and used by pollinators. Because of this complexity, most pollinators cannot use the nutrients in pollen, although all pollen germinates in solution and some point release its nutrients. As discussed by Young and Montgomery (2020), there are mixed evidence of passive uptake of nutrients from pollen by pollinators. We rephrased the paragraph to discuss pollen germination, but our focus here is on the adaptations that allow *Heliconius* to effectively access and use the amino acids from pollen which is absent in other butterflies.

Line 43. For those lepidopterans that feed as adults, carbohydrates are obtained in the adult stage. Please re-phrase.

R= That is true, however even Lepidoptera that feed as adults acquire most of their nutrients during larval stage, which enables them to go through metamorphosis (non-feeding stage), so we feel this is an important point.

Line 47, but also elsewhere: Boggs, CL 1979 Resource allocation and reproductive strategies in several heliconiine butterfly species. PhD dissertation, U Texas. Chapters 3 and 6 contain mostly otherwise unpublished data on lifespan, mating, and pollen feeding in greenhouse and field for H. charithonia, H. cydno and D. julia

R= Unfortunately, we could not find a digital copy of this thesis. We would be grateful if the reviewer is able to provide a copy. We did find a pre-print with some of the unpublished results of this thesis, which was added as a reference (Boggs & Iyengar, 2022).

Line 52: "maintaining their...structural morphology..." I don't know of any evidence that Heliconius are any better at repairing, e.g., chitin, than are non-pollen feeding butterflies. Rather, it's a difference in allocation in the pupal stage to a more durable morphology (body, wings etc). This is also a trade-off balance that can be understood in terms of nitrogen. See Boggs, C.L. 1981. Nutritional and life history determinants of resource allocation in holometabolous insects. American Naturalist 117:692-709. Note: this paper is also relevant elsewhere in the introduction and discussion.

R= We removed this part to shorten the introduction and improve its structure, as requested by other reviewer. Requested reference was added to the discussion.

Line 54: Mating multiple times isn't restricted to butterflies that feed on pollen. Please re-phrase.

R= We removed "males mate multiple times".

Methods: Line 106: what sugar was used? Glucose? Sucrose? A mix?

R=Sucrose. We added this information to the text.

Line 107: The vetark website indicates that this is whey powder. So presumably it is not an amino acid supplement, but a protein or peptide supplement. This makes a difference for whether it's a mimic of what the butterflies get out of pollen.

R= We strongly agree that supplementation with free amino acids is different from supplementation with proteins. However, as the Vetark website has conflicting information about the CCF composition, we decided to state that supplement is a source of (sugar and) amino acids without mentioning in which form (free or as proteins), as we lack this information. The website says in the 'DESCRIPTION' section: "The formulation is a mixture of selected short chain maltodextrins (derived from corn starch) and a protein concentrate supplemented with amino acids", but it says just "Dextrose, Whey protein powder (concentrate)" in the 'COMPOSITION' section, with no mention of free amino acids.

Line 113: Did you record whether the butterflies collected pollen, and how much? This makes a big difference in interpreting the results. For example, males could've been outcompeted by females for pollen, which would then be reflected in your results. Also, females may not have started collecting much pollen until they had used larval + male-derived reserves, which again would influence the interpretation of your results for 14 vs 45 days old females (see Boggs, C. L. 1990. A general model of the role of male-donated nutrients in female insects' reproduction. American Naturalist 136:598-617, as well as the O'Brien paper that you cite already).

R- Unfortunately, we did not do any behavioural analyses, but food resources were plentiful and competition for it was unlikely. Cages had 8 males and 8 females, and the pollen treatment was

supplied with 3 feeders and 5 *Lantana* bouquets (with 30-40 small flowers each). Females in our stock population start visiting flowers as soon as their wings are properly dried, but we have no data on how food consumption change with age., which has been studied already (Boggs and Iyengar, 2022)

Line 117 and elsewhere. Did males have the opportunity to mate? Who mated with the females initially? The discussion indicates that females might have mated multiply (I've seen the system break down in greenhouse populations for *H. charithonia*). It seems like knowing the male reproductive history is key to understanding age-specific data for males.

R= Each treatment (diet/age) correspond to one experimental cage containing 8 males and 8 females, as described in Line 103. They were kept in this arrangement until their sampling date and were free to attempt to mate freely. We agree that having the mating history of males would improve our discussion about the results, but when we planned this experiment we did not anticipate that this could have such an effect on their toxicity and body weight. This is an interesting result that can be built upon in future, with more targeted experiments.

Results: Table 1: Isn't this a 3-way ANOVA?

R= Indeed, this is a Three-way ANOVA and table title has been corrected.

Do you have initial body mass for females and males that were assayed at 14 and 45 days? That would be helpful to put into your analysis as a covariate, and might help reduce some of the unexplained variance in the results.

R= No, we do not have the initial body mass of the butterflies, as we did not anticipate that this would be strongly affected by adult diet. In fact, we only measured their weight at the end of the experiment to be able to calculate their cyanogenic glucoside concentration (normalized by their weight). We did however control for size to set the experiment (as smaller butterflies tend to die sooner in our stock population), selecting only butterflies that have 3-3.2 cm forewing size axis.

As far as I can tell, the statistical analysis section doesn't match the stats given in the figures, especially for figure 2 (chi-square???)

R= We double-checked the statistical analyses section and the descriptions match the statistics provided in the figures. The data on CG content and number of eggs laid were not normally distributed and we therefore analysed this data with non-parametric test Kruskal-Wallis, which gives a chi-square value as well as a p value. Out-put of the tests for the normality and heterogeneity assumptions are also provided in supplementary tables.

Discussion: Line 195, 238-9: Also Boggs 1981 American Naturalist.

R=Requested reference added.

Generally: The authors should check for typographical errors (several look like auto-correct induced mistakes).

R= We double-checked.

In addition, Nahrstedt & Davis 1983 is duplicated in the reference list.

R= Removed

Caroline Muller

*The authors investigated the impacts of pollen feeding on fertility, body mass and chemical defences of adult *Heliconius erato* butterflies as well as chemical defences of their offspring. They found that an addition of amino acids or pollen to the usual nectar diet can lead to a longer-lasting high fertility. Effects on CG contents differed between females and males, whereas the offspring CG levels were not affected. Overall, the manuscript is written well in most parts. However, some more details are needed in parts, some parts need clarification and some parts could improve by rewriting.*

Abstract: “used for target metabolomics to quantify cyanogenic glucosides (CG)” could be shortened to “used for quantification of cyanogenic glucosides (CG)”. As only total CG contents are presented, there is no need to drop the term “targeted metabolomics”, which raises other expectations, i.e. that authors would show a detailed list of various CGs

R= Changed

The own findings could be reported consistently in past tense in the abstract (at the moment mix between present and past tense) and also in the discussion.

R=Verbs related with our own results were changed to the past sentence.

*The introduction could be somewhat clearer structured and condensed to five paragraphs. All reported aspects are definitely very interesting, but several facts are not relevant for the present study, e.g., lines 22-28 (coconase), 46-50 (life spam), etc. In line 14 one wonders already if all *Heliconius* can feed on pollen, but this is then only elaborated two paragraphs later. Thus, information from the second and fourth paragraph could be fused, just as one example for re-sorting.*

R= We shortened and re-structured the introduction following the reviewer’s suggestions.

*Line 66: Please specify here: which exact cyanogenic gluosides are found in *Heliconius*?*

R= Both sequestered (deidaclin, tetraphyllyn A, tetraphyllyn B, epivolkenin, dehydrogynocardin, gynocardin) and biosynthesized (linamarin ,lotaustralin, epilotasutralin) cyanogenic glucosides. We added now “total concentration of cyanogenic glucoside”, to clarify that we are talking about their total concentration and not specific compounds.

Line 67: “more of these toxic compounds”: Do you mean more different compounds or a higher amount of CGs?

R= Replaced with “higher concentrations”

Line 77-79: Check sentence.

R= Rephrased as “This suggests that *Heliconius* butterflies might biosynthesize CGs initially using amino acids acquired during the larval stage, with resources from pollen-feeding only used later in adulthood”.

Line 80: Maybe state rather exactly which traits were tested, as trade-offs were not directly examined. The traits that were investigated should be listed in consistent order throughout the manuscript.

R= The specific traits are described in the sentence that follows (Line 81). We reordered the traits to match the order that they appear in the manuscript, as “We investigate the effect of pollen-feeding

on *H. erato* body weight, chemical defences, and fertility, controlling for sex and age, and specifically comparing young adults (14d) with mature adults (45d).”

What was the expectation/hypotheses for young versus mature adults and for females versus males?

R= This is also explained in the same paragraph (Line 83): “We therefore tested the hypothesis that mature butterflies that only had access to sugar during adulthood would have lower fertility, body weight and depleted chemical defences.” The rationale for this hypothesis is that previous studies with young butterflies did not find an effect of pollen deprivation on *Heliconius* chemistry (defence and pheromones), and we hypothesized that this was because the tested butterflies were too young (14-20 days). We did not expect to see a difference between males and females, but we controlled for sex to check if this could explain the lack of signal in previous studies.

Breeding colony: did adults of the breeding colony ever access pollen, i.e., were the plants offered for pollination offered at a flowering stage and do they provide pollen for Heliconius? This would be interesting in terms of initial experience.

R= Yes, adults of the breeding colony have access to pollen ad libitum as well. The breeding colony has in their cages feeders containing the artificial nectar and *Lantana* plants with flowers. We rotate the *Lantana* plants in the butterfly cages so they always have flowers. Some cages also have *Psiguria* and Mexican vines, but the first shift-between male and female (no pollen) flowers through time and the later does not bloom all the time. This information was already provided in the session Rearing condition of *H. erato* stock population Line 90-93, so we added the word ‘flowering’ to improve clarity. “Adults were kept in breeding cages (60x60x90 cm) containing plants of *Passiflora biflora* for oviposition, as well as flowering *Lantana sp.* and few *Psiguria sp.* for adult feeding. Cages were provided with artificial nectar made from 10% sucrose solution (m/v) with 1.5% (m/v) Vetark Critical Care Formula (CCF).

Line 100: how many experimental cages were set up per treatment?

R= One per diet/age. To make it clear for the readers, we rephrased as “One experimental cage was set for each treatment (diet/age) and each had initially 8 males and 8 females (N=16)”

Line 107: Which amino acids were present in the artificial supplement, does it also contain essential amino acids (to which you refer in the introduction)? What is the amino acid composition in the pollen of the offered flowers? And doesn't the pollen also contain lipids and vitamins etc? In other words, the use of the amino acid supplement versus pollen could be a bit more elaborated and different effects of these two amino-acid diets also discussed.

R= Unfortunately, we do not know the amino acid composition of *Lantana* pollen and CCF supplement. We contacted some providers that could perform amino acid profile analyses of pollen, but they require a standard 200 g of sample for this analysis. They said that the bare minimum amount would be 2 g (without certainty that this would work), which is still completely unfeasible. We did try, but we collected ~1 mg of pollen per flower bud and we do not have extra 2000 flowers. We produce enough *Lantana* flowers to feed our stock population and have a few extras, but not nearly enough to get 2 g of pollen. Nevertheless, we have now quantified the amount a protein in CCF and *Lantana* pollen, and added this info into our paper

Line 112-113: I do not fully understand this sentence. What is the role of the other heliconiids here, to which heliconid did you refer in line 56, where you state that they can live for many months and why is 45 days then a sign for “mature heliconids”?

R= We are explaining the rationale behind the tested butterfly age groups: “All other heliconiines live for ~1 month, therefore 45 days is the beginning of an adulthood period that is specific of mature *Heliconius* butterflies”. In line 56, the mentioned study used *Heliconius charithonia* and *Dryas iulia* and the organisms used in the study was previously explained in line 46-47. In order to simplify, we are only mentioning species names when there is something particular about the species to be discussed and using instead “other heliconines” versus “*Heliconius*” for traits well-known within these two groups.

Line 130-131: Please give here at least some more information. Which CGs were in the end analysed and in particular, how were they quantified? This is very important for the present study, as you argue with amounts of CGs.

R= We added the requested info to the text

Statistics: Again it would be important to know how many replicate cages were set up per treatment and whether cage ID was then considered in the statistics. Otherwise, do the adults interact, thus, in other words, can they be really seen as independent replicates?

R= We set one cage per treatment (diet/age) and the individual butterflies in each cage are the replicates (n=16 per treatment, n= 124 in total). It is not because the individuals in the same treatment interacted with one another that they were not independent replicates, as the concepts of independence and interaction in statistics are not the same of in biology. We are measuring the effect of diet on the body weight, CG content and fertility of each individual, controlling for sex and age. Each individual is a replicate and the replicates are statistically independent, as each individual belonged to a single treatment group (diet-age) and was not re-assayed for the measured variables. Some variables need to be measured at the group/population level, such as % mortality, yields, hybridization, etc. (and for those, each group is a replicate), but that is not the case of the variables that we studied. Moreover, It has been demonstrated that social interactions do not affect foraging choices of *Heliconius erato* (Moura, Cardoso and Montgomery, 2023). Finally, we ran our treatments simultaneously. Heliconiine butterflies are relatively big and they need to be in big cages for mating, otherwise they do not feed well and perform courtship behaviour. If we have set up many cages per treatments, we would have had to run some treatments at different times, which would create a statistical/feasibility issue.

Results: All the sudden you talk about the absence of nitrogen, I would stick to amino acids.

R= We did as suggested by the reviewer.

Line 140 vs. 179: In the statistics section you state that you used CG content (this would be the total amount per individual), in the results you state that you used concentration (which would be relative to 1 mg). This could potentially give very different information. So what exactly was done?

R= Sorry, we did not find the sentence where it was stated that we used CG concentration for the butterflies. Maybe we missed that, but we double-checked and we said CG content in the Figure 2 legend, Results and Methods. Since there was an effect of diet on butterfly weight, we only used CG content and not concentration for the butterflies. CG concentration was used only for the eggs, but normalized per egg (for which each batch of eggs laid per female was considered a replicate).

*Line 240: Is it known that CG content is directly correlated with toxicity in this *Heliconius* species? If not, rather say CG content here. In line 254 you state that CGs are not toxic (at least when intact)..*

R= We replaced “toxicity” with “CG content” in the specified sentence. Although we are unaware of a study assessing correlations between CG concentrations with toxicity, we do know that *Heliconius*

have higher CG concentration and are less palatable than other heliconiines Based on these findings, we argue that higher CG content can be translated as higher toxicity.

Line 242ff: So indeed, it should be noted that the data points are not independent in this experiment.

R= Each datapoint is a single-assayed individual and it is an independent replicate.

Line 259: check spelling: "is" tightly regulated

R= "in" replaced with "is"

Line 273: Is there a correlation between colour of the wings and CG content? And towards which predators is the signal acting? The discussion may be a bit more focused on the points that were really studied.

R= We do not know yet if the colour of *Heliconius* wings are an honest signal about their CG content/concentration, for mating peers and predators, but we have a project about this in progress.

Line 276ff: So did the supplement and the pollen contain valine and isoleucine? At least for the supplement this information should be given.

R= We unsuccessfully tried several times to get this information from the manufacturer. We do not know if the supplement and the pollen contain valine and isoleucine. We tried to get an amino acid profile analyses of *Lantana* pollen, but local providers request 5-200g of sample for this analysis and collecting this amount of pollen in our facilities is unfeasible.

Line 454: Add "amino acid" before "supplement" here and throughout the manuscript.

R- We were reluctant to use the word "amino acid" to describe this supplement because we are unsure if they are composed of proteins or a mix of proteins and free amino acids. The manufacturer website has confronting information about its composition. We are now calling it amino acid supplement, but added a small paragraph in the results to discuss about whether these amino acid are free or as proteins.

Legends of figures: Please explain what exactly the box plots show – they can be plotted in different ways.

R= We added the requested explanation

Figure 1: Is it dry weight or fresh weight?

R= Fresh weight

Figure 2 top and Fig 3 top right: Could you indicate the significant differences among the groups here as well with letters?

R= Unfortunately, we cannot run a Tukey HSD test for these variables, as they were not normally distributed.