Peer Community In Evolutionary Biology

Impact of pollen-feeding on egg-laying and cyanogenic glucoside abundance in red postman butterflies

Adriana Briscoe *based on peer reviews by* ***Carol Boggs***, ***Caroline Mueller*** *and 1 anonymous reviewer*

Erika C. Pinheiro de Castro, Josie McPherson, Glennis Jullian, Anniina L. K. Mattila, Søren Bak, Stephen Montgomery, Chris Jiggins (2023) Pollen-feeding delays reproductive senescence and maintains toxicity of Heliconius erato. bioRxiv, ver. 5, peer-reviewed and recommended by Peer Community in Evolutionary Biology.
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Growth, development and reproduction in animals are all limited by dietary nutrients. Expansion of an organism's diet to sources not accessible to closely related species reduces food competition, and eases the constraints of nutrient-limited diets. Adult butterflies are herbivorous insects known to feed primarily on nectar from flowers, which is rich in sugars but poor in amino acids. Only certain species in the genus *Heliconius* are known to also feed on pollen, which is especially rich in amino acids, and is known to prolong their lives by several months. The ability to digest pollen in *Heliconius* has been linked to specialized feeding behaviors (Krenn et al. 2009) and extra-oral digestion using enzymes, possibly including duplicated copies of *cocoonase* (Harpel et al. 2016; Smith et al. 2016 and 2018), a protease used by some moths to digest silk upon eclosion from their cocoons. In this reprint, Pinheiro de Castro and colleagues investigated the impact of artificial and natural diets on egg-laying ability, body weight, and cyanogenic glucoside abundance in adult *Heliconius erato* butterflies of both sexes.

Previous studies (Dunlap-Pianka et al. 1981) in *H. charithonia* demonstrated that access to dietary pollen led to extended egg-laying ability among adult female butterflies compared to females deprived of pollen, and compared to Dryas iulia females which feed only on nectar. In the current study, Pinheiro de Castro et al. (2023) examine the impact of diet on both young and old *H. erato*, over a longer period of time than the earlier work, highlighting the importance of extending the time period over which effects are evaluated. In addition to

extending egg-laying ability in older females, the authors found that pollen in the diet appeared to maintain older female body weight, presumably because the pollen contained nutrients depleted during egg-laying.

The authors then investigated the effects of nutrition on the production of cyanogenic glycoside defenses. *Heliconius* are aposematic butterflies that sequester cyanide-forming defense chemicals from food plants as larvae or synthesize these compounds *de novo*. The authors found the abundance of cyanogenic glycosides to be significantly greater in butterflies with access to pollen, but again only in older females.

Curiously, field studies of male and female *H. charithonia* butterflies found that females in the wild collected more pollen than males (Mendoza-Cuenca and Macías-Ordóñez 2005). Taken together, these new findings raise the intriguing possibility that females collect more pollen than males, in part, because pollen has a bigger impact on female survival and reproduction. A small limitation of the study is the use of wing length, rather than body weight, at the zero time point. But the trend is clear in both males and females, and it adds supporting detail to the efficacy of pollen feeding as an unusual strategy for increasing fertility and survival in Heliconius butterflies.

References:

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Reviews

Evaluation round #2

DOI or URL of the preprint: https://doi.org/10.1101/2023.01.13.523799
Version of the preprint: 4

Authors' reply, 04 August 2023

Dear Editor,
Thank you very much for your comments and positive feedback.
We have made the requested minor edits and double-checked the reference list (bioRxiv version 5).
Best regards,
Erika de Castro & Co-authors
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Decision by Adriana Briscoe, posted 31 July 2023, validated 01 August 2023

Dear authors,
The article will be recommended once you have made the attached last minor edits on your manuscript.
Best wishes **Download recommender's annotations**

Evaluation round #1

DOI or URL of the preprint: https://doi.org/10.1101/2023.01.13.523799
Version of the preprint: 2

Authors' reply, 19 June 2023

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Decision by Adriana Briscoe, posted 31 March 2023, validated 31 March 2023

Revision

Three reviewers have responded with thoughtful and constructive comments on your manuscript. One reviewer noted the clarity with which the hypothesis was first stated, investigated, and illustrated. Two referees commented that the paper was well-written (with a number of specific comments about where the writing could be strengthened). I have read the manuscript myself and am in agreement with these comments. I enjoyed reading the paper, which investigates the impact of diet on body mass, fertility, and chemical defenses in *Heliconius erato*, a butterfly species which supplements its adult nectar diet with pollen. I especially appreciated the difficulty of trying to collect data from butterflies over a period of 45 days, as keeping them alive in the greenhouse is no trivial matter. The work, which illustrates the impact of diet on female defenses and fertility, should be of interest to the wider evolutionary biology community, pending revision.

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.

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

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

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).

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.

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.

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.

I look forward to reading your revised manuscript.

All the best,

Adriana Briscoe

Reviewed by Carol Boggs, 16 March 2023

Download the review

Reviewed by anonymous reviewer 1, 05 March 2023

The growth, development and reproduction of herbivorous insects is known to be limited by dietary nutrition, but it is not clear how this functions under natural conditions. The authors chose to investigate this question in a fascinating system where nutritional supplementation normally occurs. The butterflies of the genus Heliconius are the only butterflies that feed on pollen as adults. Although it is assumed that this behavior increases growth and development, much information is lacking about the magnitude of this effect and the differences between butterflies of different ages and sexes.

Here the authors found that pollen and sugar feeding significantly increased the growth of H. erato butterflies compared to those fed on just sugar, but this was true only in older females. The effect of feeding on diet supplemented with sugar and amino acids was the same as on sugar and pollen. They also pursued another exciting aspect by investigating the effects of nutrition on production of cyanogenic glycoside defenses. These aposematic butterflies synthesize cyanide-forming defense compounds de novo or sequester than from food plants as larvae. The content of cyanogenic glycosides was found to significantly increase by feeding on pollen or sugar plus amino acids versus sugar only, but again only in older females. The results make a significant contribution in showing the actual effects of nutritional supplementation on this insect.

This is an exciting scientific study that is very well introduced in the context of previous research, with a clear statement of the simple hypothesis to be tested. The methods lay out the experimental design very clearly, but could have been more comprehensive in some areas. For instance, I would have liked more detail on the analysis of the butterfly defensive compounds. Also, it would be nice to know more about the amino acid supplementation used in the diet to determine the concentration of nitrogen and the composition of

amino acids. In addition, I am somewhat concerned that the laboratory culture has been running for seven years. Is there any possibility that butterflies might have adapted to the controlled conditions, and so be responding in ways different than butterflies in nature?

The results are succinctly reported in very easy-to-grasp figures, and I could detect no flaws in the data analysis and statistical tests. The well-referenced discussion section presents the results in the context of the important prior data, without pushing the interpretation too far.

There is little else to criticize in this well-written, well-illustrated paper. One hopes that future studies will conduct experiments running for a longer time, since the life span of H. erato can last up to six months. Maybe males will respond too if given long enough on the different treatments. The authors might additionally investigate whether the pollen contains high levels of valine and isoleucine, essential amino acids for insects that are also the precursors for the cyanogenic glycosides made. It would also be interesting to know if the pollen supplies other valuable nutrients, such as sterols, fatty acids and vitamins. Hopefully, these topics will be covered in future research. In the meantime, I congratulate the authors on an excellent study and wish them further success.

Reviewed by Caroline Mueller, 28 February 2023

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.

Detailed comments:

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.

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.

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, 46-50, 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.

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

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

Line 77-79: Check sentence.

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.

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

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.

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

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.

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"?

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.

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?

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

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?

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)…

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

Line 259: check spelling: "is" tightly regulated

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.

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

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

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

Figure 1: Is it dry weight or fresh weight?

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