THE ROYAL SOCIETY
PUBLISHING

PROCEEDINGS B

Pollen feeding in *Heliconius* butterflies: the singular evolution of an adaptive suite

Fletcher J. Young and Stephen H. Montgomery

Article citation details
Proc. R. Soc. B **287**: 20201304.
http://dx.doi.org/10.1098/rspb.2020.1304

Review timeline

Original submission:	5 June 2020
1st revised submission:	4 September 2020
2nd revised submission:	14 October 2020
Final acceptance:	15 October 2020

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2020-1304.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Acceptable

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
This paper seeks both to review and to question the adaptive "just-so stories" (used by SJ Gould to refer to the adaptive paradigm) for evolutionary changes in behavior, ecology, and life history of Heliconius butterflies that accompanied a unique adaptation, pollen feeding, among butterflies during the divergence of the group from sister taxa.

I know these stories well, and tend to believe them, although I do accept that the relevant hypotheses require testing better than they have been, and so I suppose this paper therefore could serve some useful function in this way. However, it does seem largely written like an introduction to a PhD thesis rather than answering any questions in any obviously detailed way.
.
One problem with answering the central question is that we really have an N=1 problem. As with the singular origin of life on earth, the singular origin of pollen feeding and a whole host of other traits that co-occur with it like longevity, shorter larval lifespan, lack of development of eggs or sperm by the emerging adult, adult provisional, greater longevity of adults, slower more hovering flight, use of Passiflora new shoots, greater unpalatability, and stronger and more diverse mimicry. It's almost unanswerable, but here the authors argue that we can at least look at the genetics and neural pathways that perhaps control some of these changes, and I agree.

I found the structure of the article rather weak. I would have preferred to see in the introduction, after a preamble about adaptive suites in general, a quick and general outline of the supposed pollen-feeding adaptive suites and story associated with the origin of Heliconius. Although some things have been further studied, this general story has changed little since the work of L.E. Gilbert, Keith Brown, and John R.G. Turner in the 1970s. If this is done, I think the reader will better understand the detailed treatments of each facet of the probably linked biology of Heliconius later in the article. For instance, you don't even really explain what pollen feeding is and how it works here, until line 161 onwards. There's good evidence for coevolution with Cucurbitaceae, particularly Gurania and Psiguria, and this might be best to mention in the introduction too. This makes it hard to know what's going on when you start to question some of the links in the rest of the paper. Another way you could write the story is to use the history of the story as a structure, beginning with the basic knowledge of the story in the 1970s, followed by updating in the more specialized sections with the newer work of Krenn and so on.

ll. 48-49: "pollen feeding is thought to have evolved only once, with a secondary loss". This may be true, but I'd say everything we do know about Heliconius is now up in the air with phylogenetic incongruence at many genes. Even if we do have the correct species tree, a simple phylogenetic analysis of the origins and loss of pollen feeding may be incorrect. See: Hahn, M.W., & Nakhleh, L. 2016. Irrational exuberance for resolved species trees. Evolution 70:7-17. http://dx.doi.org/10.1111/evo.12832

l. 72: The idea that butterflies feed predominantly on nectar is I believe a temperate biologist's error. In the tropics, very large clades are fruit feeding, and other clades like some of the big nymphalids feed on carrion.

ll. 81-82: ALL butterflies and moths transfer protein-rich spermatophores, of varying sizes. I don't think that Heliconius are so special in this respect, except that they mate significantly fewer times than many other butterflies.

l. 91: The Dunlap-Pianka et al. paper seems pretty clear, and there are other papers by Carol Boggs (esp. her PhD thesis). I don't think you need to cast so much doubt on this idea. Maybe just say it has not been well quantified?

ll. 115-123: Again, I think it is most unclear that either scenario is clear given the lack of certainty at the base of Heliconius. A third scenario involving Neruda as sister to the rest of Heliconius, but the phylogenetics fooled by introgression also seems possible, indeed likely. We now think we've identified inversions that are fixed between erato group and melpomene group species, but in which sometimes the inversion shows the erato arrangement, or sometimes the melpomene arrangement, is shared with H. burneyi and H. doris. This suggests good parsimony evidence for ancestral gene flow, or perhaps ancestral sorting of polymorphisms.

ll. 137-153: This section ignores the coevolution with cucurbits and male flowers (& sex switching to female).

Also, many temperate zone butterflies hatch with a full load of eggs, and the males are ready to mate as soon as the wings are hard. The protein provisioning for this is in the larval stage. In Heliconius and some other tropical butterflies (I believe Ithomiinae are the same way), the protein provisioning is done largely in the adult stage, and the adults eclose with no developed eggs in the female and an inability to mate in the male for around 5 days to 1 week post eclosion (and pollen feeding). Heliconius typically feed on new shoots which are perhaps low protein content, and take only ~21 days egg to adult, while larvae of Eueides, Agraulis, etc, take longer, around 30 days or more, and presumably must provision as larvae.

l. 164: "dissolves": does the pollen dissolve? Or is it eventually discarded. Certainly amino acids are released.

l. 168: "elongated proboscis". Compared with what? Where's the data?

l. 183: You say cocoonase may have been coopted, but you do not discuss what it might be used for. To digest exine components? It may be that there are proteins in the cell walls that enable the exine to become porous.

l. 210: here we have the first mention of coevolution, even though it's clearly a central part of the story, at least for the melpomene silvaniform clade!

l. 200-243: home range behaviour, gregarious roosting etc.: there are reports of many other nymphalid butterflies (e.g. Morpho, red admiral, Hamadryas, Agraulis etc.) doing similar things, although Heliconius do seem particularly well-developed in this respect. On the other hand, ithomiine butterflies seem to be much more pheromone/olfactory oriented and mark recapture studies suggests they just slowly drift around the place. I think the behavior is particularly noticeable in part in Heliconius because they're quite active and live a long time. But yes, they do seem better endowed than other butterflies in the memory department!

l. 258: no Dryas or Agraulis vanillae shown in Figs. 1. In general I thought the figures were lacking for explaining some of these points. Figs. 1 & 2 could be placed in the introductory section?

l. 281: maybe pheromones?

l. 299, 313: Work by Brower, Brower & Collins, and by Peng Chai tend to support the idea that Heliconius are more unpalatable than Eueides, Dryas etc. I don't think "toxicity" can be measured just in terms of cyanogenic glycoside content. In any case, the main effect is as a repellant, not as a toxin.

l. 347-355: You need to explain the phylogeny and also discuss what is known about the biology of Neruda (not much!) somewhere earlier in the more introductory section of the paper, to make this more clearly understandable.

l. 370-378: This seemed rather vague and a little uninformative.

l. 387: Yes, the N=1 problem!

l. 396: "cognitive senescence" seemed odd here! It's not clear that the senescence is cognitive; it could be just general whole-body senescence.

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

> **Is it accessible?**
> N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript reviews our understanding of the evolution of pollen feeding in Heliconius butterflies. Pollen feeding is highly unusual within the Lepidoptera and thus this group provides an interesting opportunity to study the evolutionary consequences of a major dietary shift.

Overall the manuscript is, in my opinion, an excellent piece of work. I enjoyed reading it. I have some comments that are minor and potentially easy to address.

1. Overall, the manuscript describes many potential consequences of a shift to pollen feeding, but the causes of that shift remain a mystery. There seem to be no clearly unique aspects of Heliconius' physiology or ecology that could (yet) explain why this shift occurred in this particular group. It would be helpful to make this point explicitly, rather than leaving it to the reader to surmise. For example, at line 157, you set up the next section by saying that identifying the adaptations necessary for the transition to pollen feeding is central to understanding why it is rare, but by the end of the section, there is still no indication of anything unique to Heliconius that could have brought the transition about.
2. It would have been helpful to include a clear description of the trait (i.e. pollen feeding) in the introduction. For example, at line 126 you mention "active" pollen feeding, but it wasn't clear until later in the manuscript what you meant by this (at lines 160-164). Likewise, until I got to the section beginning with line 210, I was wondering about whether pollen feeding is limited to specific plants, whether it co-occurs with nectar feeding, etc. A brief description of the natural history would be useful early on.
3. Lines 55-58: the order of the questions set out here differs from the order in which they are addressed below, where life-history comes first.
4. Line 134: It would indeed be uninformative to compare these groups with Heliconius but perhaps comparison with their own sister groups would be worthwhile?
5. Line 195: typo- should be "occur"
6. Section on foraging strategies: I think it is premature to say that "the cognitive demands of traplining are likely to be associated with elaborations in the Heliconius nervous system" (lines 254-255). It is clear that the mushroom bodies have expanded extensively in Heliconious, and that pollen feeding occurs here, but the link with traplining always seems a little tenuous. Yes, Heliconious trapline, but the critical evidence that other close relatives do not seems to be missing (as you point out at line 243- I just think this should be made more explicit). I can't see a specific reason why traplining should be particularly linked to pollen feeding per se rather than the fact that Psiguria and Guriana apparently flower for a very long time- which many other plants do to. And nectar feeders also trapline (hummingbirds come to mind). As you point out later, there are many other potential explanations (increased longevity perhaps leads to selection for increased long-term memory, etc).
7. Line 245: "Trapline foraging … rare among the Lepidoptera"- we don't really know that it's rare, as you point out at lines 242-3.
8. Section on "How quickly do behavioural innovations…." (line 347-355). It would be helpful to clarify what we would learn from understanding more about Neruda, given their lack of pollen feeding may apparently be derived- for example, I'm not sure that finding that they also had expanded MBs would really help? But of course, if they didn't, that would be useful information. It also seems important in this section to look at variation between-pollen feeding species. For example, if all of them possess the whole suite of adaptations, perhaps this would suggest rapid evolution right at the base of the group?

9. Line 374: “Pollen feeding likely involves considerable costs…. neural tissue”. As above, I don’t think there’s convincing evidence that pollen feeding per se involves these costs.

Overall, thank you for this interesting, well-written and informative piece of work, which I hope to see in print in the near future.

Decision letter (RSPB-2020-1304.R0)

17-Aug-2020

Dear Mr Young:

Your manuscript has now been peer reviewed by two reviewers. Both they and I find much to like about your review, and find it to be timely and potentially relevant to the readership of Proceedings B. However, both reviewers make a number of substantive comments that need to be addressed. Therefore, I invite you to revise your manuscript to address their concerns. I will not summarize their comments again as both reviewers do a very nice job of detailing them, but given Proceedings' broad readership, I do emphasize the importance of making sure that your paper is accessible to those who are not specialists by including more information about the system early on in the introduction. The reviewers’ comments (not including confidential comments to the Editor) are included at the end of this email for your reference.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions”, click on "Create a Revision”. Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers’ and Editors’ comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as ‘tracked changes’ to be included in the ‘response to referees’ document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:

If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr. Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
This paper seeks both to review and to question the adaptive "just-so stories" (used by SJ Gould to refer to the adaptive paradigm) for evolutionary changes in behavior, ecology, and life history of Heliconius butterflies that accompanied a unique adaptation, pollen feeding, among butterflies during the divergence of the group from sister taxa.

I know these stories well, and tend to believe them, although I do accept that the relevant hypotheses require testing better than they have been, and so I suppose this paper therefore could serve some useful function in this way. However, it does seem largely written like an introduction to a PhD thesis rather than answering any questions in any obviously detailed way.

One problem with answering the central question is that we really have an N=1 problem. As with the singular origin of life on earth, the singular origin of pollen feeding and a whole host of other traits that co-occur with it like longevity, shorter larval lifespan, lack of development of eggs or sperm by the emerging adult, adult provisional, greater longevity of adults, slower more hovering flight, use of Passiflora new shoots, greater unpalatability, and stronger and more diverse mimicry. It's almost unanswerable, but here the authors argue that we can at least look at the genetics and neural pathways that perhaps control some of these changes, and I agree.

I found the structure of the article rather weak. I would have preferred to see in the introduction, after a preamble about adaptive suites in general, a quick and general outline of the supposed pollen-feeding adaptive suites and story associated with the origin of Heliconius. Although some things have been further studied, this general story has changed little since the work of L.E. Gilbert, Keith Brown, and John R.G. Turner in the 1970s. If this is done, I think the reader will better understand the detailed treatments of each facet of the probably linked biology of Heliconius later in the article. For instance, you don't even really explain what pollen feeding is and how it works here, until line 161 onwards. There's good evidence for coevolution with Cucurbitaceae, particularly Gurania and Psiguria, and this might be best to mention in the introduction too. This makes it hard to know what's going on when you start to question some of the links in the rest of the paper. Another way you could write the story is to use the history of the story as a structure, beginning with the basic knowledge of the story in the 1970s, followed by updating in the more specialized sections with the newer work of Krenn and so on.

ll. 48-49: "pollen feeding is thought to have evolved only once, with a secondary loss". This may be true, but I'd say everything we do know about Heliconius is now up in the air with phylogenetic incongruence at many genes. Even if we do have the correct species tree, a simple phylogenetic analysis of the origins and loss of pollen feeding may be incorrect. See: Hahn, M.W., & Nakhleh, L. 2016. Irrational exuberance for resolved species trees. Evolution 70:7-17. http://dx.doi.org/10.1111/evo.12832

l. 72: The idea that butterflies feed predominantly on nectar is I believe a temperate biologist's error. In the tropics, very large clades are fruit feeding, and other clades like some of the big nymphalids feed on carrion.

ll. 81-82: ALL butterflies and moths transfer protein-rich spermatophores, of varying sizes. I don't think that Heliconius are so special in this respect, except that they mate significantly fewer times than many other butterflies.

l. 91: The Dunlap-Pianka et al. paper seems pretty clear, and there are other papers by Carol Boggs (esp. her PhD thesis). I don't think you need to cast so much doubt on this idea. Maybe just say it has not been well quantified?

ll. 115-123: Again, I think it is most unclear that either scenario is clear given the lack of certainty at the base of Heliconius. A third scenario involving Neruda as sister to the rest of Heliconius, but the phylogenetics fooled by introgression also seems possible, indeed likely. We now think we've identified inversions that are fixed between erato group and melpomene group species, but in which sometimes the inversion shows the erato arrangement, or sometimes the melpomene arrangement, is shared with H. burneyi and H. doris. This suggests good parsimony evidence for ancestral gene flow, or perhaps ancestral sorting of polymorphisms.

ll. 137-153: This section ignores the coevolution with cucurbits and male flowers (& sex switching to female).

Also, many temperate zone butterflies hatch with a full load of eggs, and the males are ready to mate as soon as the wings are hard. The protein provisioning for this is in the larval stage. In Heliconius and some other tropical butterflies (I believe Ithomiinae are the same way), the protein provisioning is done largely in the adult stage, and the adults eclose with no developed eggs in the female and an inability to mate in the male for around 5 days to 1 week post eclosion (and pollen feeding). Heliconius typically feed on new shoots which are perhaps low protein content, and take only ~21 days egg to adult, while larvae of Eueides, Agraulis, etc, take longer, around 30 days or more, and presumably must provision as larvae.

l. 164: "dissolves": does the pollen dissolve? Or is it eventually discarded. Certainly amino acids are released.

l. 168: "elongated proboscis". Compared with what? Where's the data?

l. 183: You say cocoonase may have been coopted, but you do not discuss what it might be used for. To digest exine components? It may be that there are proteins in the cell walls that enable the exine to become porous.

l. 210: here we have the first mention of coevolution, even though it's clearly a central part of the story, at least for the melpomene silvaniform clade!

l. 200-243: home range behaviour, gregarious roosting etc.: there are reports of many other nymphalid butterflies (e.g. Morpho, red admiral, Hamadryas, Agraulis etc.) doing similar things, although Heliconius do seem particularly well-developed in this respect. On the other hand, ithomiine butterflies seem to be much more pheromone/olfactory oriented and mark recapture studies suggests they just slowly drift around the place. I think the behavior is particularly noticeable in part in Heliconius because they're quite active and live a long time. But yes, they do seem better endowed than other butterflies in the memory department!

l. 258: no Dryas or Agraulis vanillae shown in Figs. 1. In general I thought the figures were lacking for explaining some of these points. Figs. 1 & 2 could be placed in the introductory section?

l. 281: maybe pheromones?

l. 299, 313: Work by Brower, Brower & Collins, and by Peng Chai tend to support the idea that Heliconius are more unpalatable than Eueides, Dryas etc. I don't think "toxicity" can be measured just in terms of cyanogenic glycoside content. In any case, the main effect is as a repellant, not as a toxin.

l. 347-355: You need to explain the phylogeny and also discuss what is known about the biology of Neruda (not much!) somewhere earlier in the more introductory section of the paper, to make this more clearly understandable.

l. 370-378: This seemed rather vague and a little uninformative.

l. 387: Yes, the N=1 problem!

l. 396: "cognitive senescence" seemed odd here! It's not clear that the senescence is cognitive; it could be just general whole-body senescence.

Referee: 2

Comments to the Author(s)
This manuscript reviews our understanding of the evolution of pollen feeding in Heliconius butterflies. Pollen feeding is highly unusual within the Lepidoptera and thus this group provides an interesting opportunity to study the evolutionary consequences of a major dietary shift.

Overall the manuscript is, in my opinion, an excellent piece of work. I enjoyed reading it. I have some comments that are minor and potentially easy to address.

1. Overall, the manuscript describes many potential consequences of a shift to pollen feeding, but the causes of that shift remain a mystery. There seem to be no clearly unique aspects of Heliconius' physiology or ecology that could (yet) explain why this shift occurred in this particular group. It would be helpful to make this point explicitly, rather than leaving it to the reader to surmise. For example, at line 157, you set up the next section by saying that identifying the adaptations necessary for the transition to pollen feeding is central to understanding why it is rare, but by the end of the section, there is still no indication of anything unique to Heliconius that could have brought the transition about.
2. It would have been helpful to include a clear description of the trait (i.e. pollen feeding) in the introduction. For example, at line 126 you mention "active" pollen feeding, but it wasn't clear until later in the manuscript what you meant by this (at lines 160-164). Likewise, until I got to the section beginning with line 210, I was wondering about whether pollen feeding is limited to specific plants, whether it co-occurs with nectar feeding, etc. A brief description of the natural history would be useful early on.
3. Lines 55-58: the order of the questions set out here differs from the order in which they are addressed below, where life-history comes first.
4. Line 134: It would indeed be uninformative to compare these groups with Heliconius but perhaps comparison with their own sister groups would be worthwhile?
5. Line 195: typo- should be "occur"
6. Section on foraging strategies: I think it is premature to say that "the cognitive demands of traplining are likely to be associated with elaborations in the Heliconius nervous system" (lines 254-255). It is clear that the mushroom bodies have expanded extensively in Heliconious, and that pollen feeding occurs here, but the link with traplining always seems a little tenuous. Yes, Heliconious trapline, but the critical evidence that other close relatives do not seems to be missing (as you point out at line 243- I just think this should be made more explicit). I can't see a specific reason why traplining should be particularly linked to pollen feeding per se rather than the fact that Psiguria and Guriana apparently flower for a very long time- which many other plants do to. And nectar feeders also trapline (hummingbirds come to mind). As you point out later, there are many other potential explanations (increased longevity perhaps leads to selection for increased long-term memory, etc).
7. Line 245: "Trapline foraging … rare among the Lepidoptera"- we don't really know that it's rare, as you point out at lines 242-3.
8. Section on "How quickly do behavioural innovations…." (line 347-355). It would be helpful to clarify what we would learn from understanding more about Neruda, given their lack of pollen feeding may apparently be derived- for example, I'm not sure that finding that they also had expanded MBs would really help? But of course, if they didn't, that would be useful information. It also seems important in this section to look at variation between-pollen feeding species. For example, if all of them possess the whole suite of adaptations, perhaps this would suggest rapid evolution right at the base of the group?
9. Line 374: "Pollen feeding likely involves considerable costs…. neural tissue". As above, I don't think there's convincing evidence that pollen feeding per se involves these costs.

Overall, thank you for this interesting, well-written and informative piece of work, which I hope to see in print in the near future.

Author's Response to Decision Letter for (RSPB-2020-1304.R0)

See Appendix A.

RSPB-2020-1304.R1 (Revision)

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

> **Is it accessible?**
> N/A
>
> **Is it clear?**
> N/A
>
> **Is it adequate?**
> N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have taken care to address my previous comments and I am happy with the edits that they have made. I have only two very minor comments on the revised version (below). I enjoyed reviewing this manuscript and I look forward to seeing it in print.

Line 212-213: This still seems a very certain statement- revise to "… and their pollen sources may have"?

Line 266-273: One further addition here. For completeness, I think it is important to mention that the Central Complex, rather than the Mushroom Bodies, is the neural region more typically associated with spatial task performance in insects (e.g. see refs within Varga et al. (2017) Front. Behav. Neuroscience 11: https://doi.org/10.3389/fnbeh.2017.00004). I do not question that there are many types of spatial task, and that the MBs may also be involved, particularly in the long-term retention of site-specific information.

Decision letter (RSPB-2020-1304.R1)

07-Oct-2020

Dear Mr Young

I am pleased to inform you that your manuscript RSPB-2020-1304.R1 entitled "Pollen feeding in *Heliconius* butterflies: the singular evolution of an adaptive suite" has been accepted for publication in Proceedings B.

The referee(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the referee(s)' comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into https://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Before uploading your revised files please make sure that you have:

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

5) Data accessibility section and data citation
It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository (https://royalsociety.org/journals/authors/author-guidelines/#data).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
- DNA sequences: Genbank accessions F234391-F234402
- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link http://datadryad.org/submit?journalID=RSPB&manu=(Document not available) which will take you to your unique entry in the Dryad repository. If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

6) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit https://royalsociety.org/journals/authors/author-guidelines/.

Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Dr. Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s)
The authors have taken care to address my previous comments and I am happy with the edits that they have made. I have only two very minor comments on the revised version (below). I enjoyed reviewing this manuscript and I look forward to seeing it in print.

Line 212-213: This still seems a very certain statement- revise to "… and their pollen sources may have"?

Line 266-273: One further addition here. For completeness, I think it is important to mention that the Central Complex, rather than the Mushroom Bodies, is the neural region more typically associated with spatial task performance in insects (e.g. see refs within Varga et al. (2017) Front. Behav. Neuroscience 11: https://doi.org/10.3389/fnbeh.2017.00004). I do not question that there are many types of spatial task, and that the MBs may also be involved, particularly in the long-term retention of site-specific information.

Author's Response to Decision Letter for (RSPB-2020-1304.R1)

See Appendix B.

Decision letter (RSPB-2020-1304.R2)

15-Oct-2020

Dear Mr Young

I am pleased to inform you that your manuscript entitled "Pollen feeding in *Heliconius* butterflies: the singular evolution of an adaptive suite" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact during this period, let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

Your article has been estimated as being 10 pages long. Our Production Office will be able to confirm the exact length at proof stage.

Open access
You are invited to opt for open access via our author pays publishing model. Payment of open access fees will enable your article to be made freely available via the Royal Society website as

soon as it is ready for publication. For more information about open access publishing please visit our website at http://royalsocietypublishing.org/site/authors/open_access.xhtml.

The open access fee is £1,700 per article (plus VAT for authors within the EU). If you wish to opt for open access then please let us know as soon as possible.

Paper charges
An e-mail request for payment of any related charges will be sent out shortly. The preferred payment method is by credit card; however, other payment options are available.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

You are allowed to post any version of your manuscript on a personal website, repository or preprint server. However, the work remains under media embargo and you should not discuss it with the press until the date of publication. Please visit https://royalsociety.org/journals/ethics-policies/media-embargo for more information.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Response to reviewers' comments

Referee: 1

We are grateful for the referee's critique and comments. We are largely in agreement with their position, with most comments on the biology of pollen feeding reflecting a lack of clarity in places, which we hope we have addressed, where we had previously sought to reduce the word count.

I know these stories well, and tend to believe them, although I do accept that the relevant hypotheses require testing better than they have been, and so I suppose this paper therefore could serve some useful function in this way. However, it does seem largely written like an introduction to a PhD thesis rather than answering any questions in any obviously detailed way.

> *We're glad the reviewer sees value in revisiting this topic. As they imply themselves, there is a risk that much of the discussion around pollen feeding has become a "just so story". We believe it can be much more useful, and our review aims to explain why, and how to go about tackling the outstanding questions. Given their interest in this topic we hope the reviewer agrees this is more constructive than leaving the limitations and challenges of this topic unstated. We believe the structure of the review follows a logical progression, but we detail edits we have made to improve the clarity below.*

One problem with answering the central question is that we really have an N=1 problem. As with the singular origin of life on earth, the singular origin of pollen feeding and a whole host of other traits that co-occur with it like longevity, shorter larval lifespan, lack of development of eggs or sperm by the emerging adult, adult provisional, greater longevity of adults, slower more hovering flight, use of Passiflora new shoots, greater unpalatability, and stronger and more diverse mimicry. It's almost unanswerable, but here the authors argue that we can at least look at the genetics and neural pathways that perhaps control some of these changes, and I agree.

> *We completely agree that the N=1 problem is a central challenge in this area. We have sought to make this clearer by raising this early on in the introduction [lines 55-57], where we write:*
>
> > *"the suite of traits associated with pollen feeding is assumed to have evolved only once, presenting difficulties in separating evolutionary cause and effect."*
>
> *As the reviewer points out this is singularity issue is likely to be the case for a whole suite of traits, but we would argue that this is an assumption, albeit a reasonable one, and without quantifying these traits properly and trying to understand the interactions between them it will be impossible to verify. Nevertheless, if it holds, in our manuscript we suggest there are still ways of disentangling these effects.*

I found the structure of the article rather weak. I would have preferred to see in the introduction, after a preamble about adaptive suites in general, a quick and general outline of the supposed pollen-feeding adaptive suites and story associated with the origin of Heliconius. Although some things have been further studied, this general story has changed little since the work of L.E. Gilbert, Keith Brown, and John R.G. Turner in the 1970s. If this is done, I think the reader will better understand the detailed treatments of each facet of the probably linked biology of Heliconius later in the article. For instance, you don't even really explain what pollen feeding is and how it works here, until line 161 onwards. There's good evidence for coevolution with Cucurbitaceae, particularly Gurania and Psiguria, and this might be best to mention in the introduction too. This makes it hard to know what's going on when you start to question some of the links in the rest of the paper. Another way you could write the story is to use the history of the story as a structure, beginning with the basic knowledge of the story in the 1970s, followed by updating in the more specialized sections with the newer work of Krenn and so on.

> *We agree with the reviewer that we should introduce the general biology earlier and have edited the introduction accordingly. The introduction is now structured as suggested, with paragraph 1 being a preamble on adaptive suites, and paragraphs 2-4 being a general outline of the pollen feeding story, including specifically, and clearly highlighting the n=1 problem.*
>
> *These edits also enable us to draw out the key questions we explore in the rest of the review more clearly early on, which hopefully clarifies the remaining structure of the article. We feel that this works well as a structure for a general reader, and also allows more focus on specific topics. We feel that a structure based on the history of the field would appeal more to the* Heliconius *community than the wide readership of this journal We also note that reviewer 2, who we assume does not work in this field, felt the writing was well written.*
>
> *On the role of co-evolution with cucurbits, as noted below, we do highlight this in the introduction [lines 43-44], writing:*
>
> > *"*Heliconius *from at least the* melpomene *clade show a particular preference for certain pollen-rich cucurbitaceous vines, with which they are hypothesised to have co-evolved."*
>
> *We then further discuss the topic in section 4i [beginning line 216]. However, we are perhaps less confident than the reviewer that this plays a major role in the **origin** of pollen feeding. This is because the specialisation on cucurbits is limited to the* melpomene/silvaniform *clade. To us, it seems equally plausible that pollen feeding evolved in a less specialised context, with the curcurbit co-evolution being a secondary adaptation within the* melpomene-silvaniform *clade, and we know of no clear test of either hypothesis. Hence, although we agree this aspect of the story should be flagged early in the introduction [as we do on lines 43-44] we think it is too disruptive to the flow of the text to go into this subject in detail early on.*

ll. 48-49: "pollen feeding is thought to have evolved only once, with a secondary loss". This may be true, but I'd say everything we do know about Heliconius is now up in the air with phylogenetic incongruence at many genes. Even if we do have the correct species tree, a simple phylogenetic analysis of the origins and loss of pollen feeding may be incorrect. See: Hahn, M.W., & Nakhleh, L. 2016. Irrational exuberance for resolved species trees. Evolution 70:7-17. http://dx.doi.org/10.1111/evo.12832

> *We totally agree with the reviewer that there is uncertainty in the timing of pollen feeding gains and losses. This is a central message of lines 118-134 and the main*

purpose of figure 1. Our point is not to say what happened, but rather to point out that we don't know, as the reviewer also implies is the case. As the reviewer notes below, the biology of the Neruda clade is likely to be very important in answering this question, and we highlight this on lines 353-363. We have edited the text in this section to make this clearer. We also now directly point this out in the caption to figure 1, which reads:

> *"Note the changing position of the Neruda clade, a group of four non-pollen feeding species."*

The reviewer also importantly points out that discordance between a single species tree and underlying gene trees can present difficulties in the phylogenetic analysis of traits. We now acknowledge this at lines 133-134 and cite the paper referred to above:

> *"discordance between any single Heliconiini species tree and underlying gene trees may present persistent difficulties in resolving this question."*

l. 72: The idea that butterflies feed predominantly on nectar is I believe a temperate biologist's error. In the tropics, very large clades are fruit feeding, and other clades like some of the big nymphalids feed on carrion.

We agree with the reviewer that for many Lepidoptera clades nectar is not the sole adult food source and have accordingly changed lines 80-84 to make this clearer. At line 83-85 we observe that although a number of alternate foraging strategies exist within the Lepidoptera, pollen feeding in Heliconius *is one of the clearest examples of a change in diet and foraging strategy associated with major life history changes among closely related genera.*

ll. 81-82: ALL butterflies and moths transfer protein-rich spermatophores, of varying sizes. I don't think that Heliconius are so special in this respect, except that they mate significantly fewer times than many other butterflies.

We agree and it was not our intention to suggest this was peculiar to Heliconius*. However, for reasons of space, we have now removed discussion of the spermatophore, as there is no evidence regarding the effects of pollen feeding on spermatophore production. On reflection, the points raised here were not central to the purpose of this section.*

l. 91: The Dunlap-Pianka et al. paper seems pretty clear, and there are other papers by Carol Boggs (esp. her PhD thesis). I don't think you need to cast so much doubt on this idea. Maybe just say it has not been well quantified?

We agree with the reviewer that an indirect or direct connection between pollen feeding and longevity is likely. However, currently this link is not well documented as empirical evidence supporting this claim is limited. Dunlap-Pianka et al (1977) [4] *only considered one* Heliconius *species and one non-pollen feeding outgroup* (Dryas), *which is not the closest relative to* Heliconius. *The main focus of the data in this paper was female reproductive lifespan. Total longevity is not well-quantified and there is no statistical comparison between the diet groups.*

Regarding longevity, Dunlap-Pianka et al (1977) simply write:

- *“*H. charitonius *can live more than 3 months in our greenhouses, and up to 4 ½ months in nature (20)” [ref 20 estimates lifespan for H. charithonia using mark release recapture data]*
- *“Pollen-deprived* H. charitonius *have lived from 21 to 38 days”*
- *“Dryas have lived from 15 to 40 days in our cages”*

While we don’t doubt these figures, which are consistent with our own experience, they do not provide quantitative data or much basis for formal analyses across Heliconiini.

Prof. Boggs has also investigated relationships between lifespan and pollen-load size, but didn’t conduct any pollen deprivation experiments to test for a direct, causative effect of pollen provision on longevity. In communications with Prof. Boggs during these revisions, in which she kindly provided a copy of her unpublished thesis, she also agreed that the links between pollen feeding and lifespan do need to be better quantified.

As suggested, we have edited the text to clarify that the association is likely, in some form, but not well quantified or understood. At lines 94-96 we now write:

> *“the causal relationship between pollen feeding and longevity is not well quantified. One study reports that pollen-deprived H. charithonia are shorter-lived, but this difference was not tested statistically”*

ll. 115-123: Again, I think it is most unclear that either scenario is clear given the lack of certainty at the base of Heliconius. A third scenario involving Neruda as sister to the rest of Heliconius, but the phylogenetics fooled by introgression also seems possible, indeed likely. We now think we've identified inversions that are fixed between erato group and melpomene group species, but in which sometimes the inversion shows the erato arrangement, or sometimes the melpomene arrangement, is shared with H. burneyi and H. doris. This suggests good parsimony evidence for ancestral gene flow, or perhaps ancestral sorting of polymorphisms.

We agree with the reviewer that this is a potential scenario. We intended to imply this in the previous manuscript when we wrote:

> “However, lingering uncertainty concerning the phylogenetic position and ecology of the Neruda makes it difficult to resolve this question.*”*

However, we appreciate this was a bit too vague to be widely understood. We have now raised the possibility of Neruda being a sister group to Heliconius, noting this is reflected in the older phylogenies based on morphology as presented in figure 1a. At lines 129-131 where we write:

> *“given evidence of widespread introgression throughout the evolution of the genus* [5]*, which could mislead the species tree, it also remains possible that the Neruda are, after all, a sister clade to Heliconius, as suggested by morphological data”*

ll. 137-153: This section ignores the coevolution with cucurbits and male flowers (& sex switching to female).

We agree with the reviewer that coevolution with cucurbits is an important element of the pollen-feeding story and discuss it in detail at lines 216-229. However, this section *is primarily concerned with reconstructing how pollen feeding could arise in a nectar-feeding butterfly and therefore considers the effects of adult amino acid intake in non-pollen-feeding butterflies.*

We appreciate the interesting point about sex switching in these plants, and have included it in our discussion of the coevolution with cucurbits at lines 221-222, however, given space constraints, we are not able to go into this specific point at length.

Also, many temperate zone butterflies hatch with a full load of eggs, and the males are ready to mate as soon as the wings are hard. The protein provisioning for this is in the larval stage. In Heliconius and some other tropical butterflies (I believe Ithomiinae are the same way), the protein provisioning is done largely in the adult stage, and the adults eclose with no developed eggs in the female and an inability to mate in the male for around 5 days to 1 week post eclosion (and pollen feeding). Heliconius typically feed on new shoots which are perhaps low protein content, and take only ~21 days egg to adult, while larvae of Eueides, Agraulis, etc, take longer, around 30 days or more, and presumably must provision as larvae.

Earlier in the review, at lines 79-90 we explain that for most butterflies reproductive output is constrained by larval nutrition, and that Heliconius *incorporate pollen-derived proteins into eggs. The reviewer raises the important point that female* Heliconius *eclose without developed eggs. Dunlap-Pianka et al (1977) report that* both D. iulia *and* H. charitonia *emerge without fully developed eggs, suggesting this trait is not wholly linked to pollen feeding. However,* H. charitonia do *emerge with smaller ovaries and less oocytes, suggestive of reduced larval investment in vitellogenesis. We now state this at lines 313-314 where we discuss the knock-on effects of pollen feeding:*

> *"compared with D. iulia, H. charithonia females emerge with smaller ovaries containing fewer oocytes, further suggesting a reduction in larval investment in reproduction"*

Mallet (1980) [6] *does describe a "callow" period of 5 days in* Heliconius in his Masters thesis*, however, we cannot find any comparable data on male reproductive state in other Heliconiini to infer whether* Helicoinus *are in some way unique in this trait so prefer not to speculate on this point.*

We do discuss previous speculation that pollen feeding may lead to shorter larval periods in Heliconius *(also shown in figure 3), writing at lines 308-310:*

> *"Pollen feeding has also been suggested to allow Heliconius to reduce time spent in the vulnerable larval stage by trading off increased growth rates against investment in nitrogen reserves."*

However, to the best of our knowledge there is currently is no published evidence to suggest that Heliconius *have a reduced larval development time. In fact, our lab has recently collected data on Heliconiini development times, which is currently being prepared for submission. Our data suggests that, counter to these expectations,* Heliconius *larvae do not develop faster than non-pollen feeding Heliconiini.*

l. 164: "dissolves": does the pollen dissolve? Or is it eventually discarded. Certainly amino acids are released.

We agree with the reviewer that this description of the process was somewhat inaccurate and have changed our wording of that sentence (now placed in the introduction) at lines 40-42:

> *"The pollen load is mixed with saliva and externally digested to release amino acids that are drawn up the proboscis"*

l. 168: "elongated proboscis". Compared with what? Where's the data?

The probosces of Heliconius *are elongated relative to non-pollen-feeding Heliconiini and comes from Krenn & Penz (1998)* [7]*. Here the authors compared proboscis morphology across many Heliconiini species. We have edited lines 172-173 to read:*

> *"Heliconius do have elongated proboscises compared to non-pollen-feeding Heliconiini"*

l. 183: You say cocoonase may have been coopted, but you do not discuss what it might be used for. To digest exine components? It may be that there are proteins in the cell walls that enable the exine to become porous.

This is a very good point and we agree. The papers discussing cocoonase in Heliconius [8–10] *propose that cocoonase may assist in the digestion of pollen proteins, but do not speculate in detail how this is achieved.*

For example, Smith et al 2018 write:

> *"heliconiine butterflies may have coopted cocoonase for the digestion of peptides found in their natural diet, and* Heliconius *butterflies may use these specifically for feeding on pollen"*

However, even though this is an enticing story, a role for cocoonase in pollen digestion has not been directly demonstrated, and its links to pollen feeding remain speculative. This is our main conclusion here, and we are hesitant to speculate too much on this point ourselves in the absence of further data. However, we agree with the reviewer that it may be possible that cocoonase functions to breakdown proteins in the pollen intine, helping to compromise the pollen wall, which we now indicate at line 190-191.

l. 210: here we have the first mention of coevolution, even though it's clearly a central part of the story, at least for the melpomene silvaniform clade!

We recognise that coevolution with certain cucurbitaceous species is important for at least the melpomene-silvaniform *clade and have accordingly mentioned it in the introduction at lines 43-44. However, we are also careful not to overemphasise its importance to the origin of pollen feeding, as specialisation on cucurbitaceous vines is restricted to one clade in* Heliconius*. Nevertheless, this likely coevolution is important to discuss and assess, and we do so here from lines 216-229.*

l. 200-243: home range behaviour, gregarious roosting etc.: there are reports of many other nymphalid butterflies (e.g. Morpho, red admiral, Hamadryas, Agraulis etc.) doing similar things, although Heliconius do seem particularly well-developed in this respect. On the other hand, ithomiine butterflies seem to be much more pheromone/olfactory oriented and mark recapture studies suggests they just slowly drift around the place. I think the behavior is particularly noticeable in part in Heliconius because they're quite active and live a long time. But yes, they do seem better endowed than other butterflies in the memory department!

> *While home-range like behaviour has been described in other butterflies, we are unaware of other species that have been reported to display similar long-term roost-site fidelity to* Heliconius*.* Agraulis vanillae *are briefly reported to establish temporary home ranges in Mallet (1987)* [11]*, but unfortunately, to the best of our knowledge, these data remain unpublished. We would certainly be interested in these data, and more generally, this aligns with our call for more field studies of non-*Heliconius *Heliconiini. We have added a sentence at line 232-234 to clarify our point which reads:*
>
> > *"Although other butterfly species, including the heliconiine* Agraulis vanillae*, are reported to temporarily establish home ranges,* Heliconius *seem peculiar in maintaining long-term, stable home ranges with high roost-site fidelity."*
>
> *However, we would, of course, appreciate any specific studies the reviewer suggests may contain data we have missed.*

l. 258: no Dryas or Agraulis vanillae shown in Figs. 1. In general I thought the figures were lacking for explaining some of these points. Figs. 1 & 2 could be placed in the introductory section?

> *We have followed the reviewer's suggestion and referred to figures 1 and 2 in the introduction at lines 40 and 48. We understand the reviewer as suggesting that figure 1 should also include images of* D. iulia *and* A. vanillae *showing the mushroom bodies. However, while there is data on mushroom body volumes for one or both species from Sivinski (1989)* [12] *and Snell-Rood et al (2020)* [13]*, the methods used to collect these data are not comparable with our own, and no images are currently published of the brains of these species. Although we have collected these data and images, they are currently part of a large, on-going comparative study, which we are reluctant to pre-empt here without the context of statistical analyses. This is particularly so as the main purpose of the brain image in figure 1 is to simply present readers unfamiliar with insect neuroanatomy with an example of a mushroom body and its relationship with the rest of the brain.*

l. 281: maybe pheromones?

> *We agree that pollen feeding may have some effect on pheromone production. However, Darragh et al (2019)* [14] *found that although male pheromone composition was affected by larval diet, pollen consumption had no effect. We are hesitant to speculate further in the absent of additional data.*

l. 299, 313: Work by Brower, Brower & Collins, and by Peng Chai tend to support the idea that Heliconius are more unpalatable than Eueides, Dryas etc. I don't think "toxicity" can be measured just in terms of cyanogenic glycoside content. In any case, the main effect is as a repellant, not as a toxin.

> *This is a good point and we agree that cyanogenic glycoside concentration is an imperfect proxy for distastefulness or toxicity and have edited lines 307-308 to reflect this:*

> *“Moreover, cyanogen concentration is an imperfect proxy for distastefulness as there is considerable interspecific variation in chemical profile.”*

We also appreciate that cyanogens are mainly acting as a repellent rather than a toxin, and have according replaced instances of “toxicity” with “distastefulness” at lines 300 and 307.

l. 347-355: You need to explain the phylogeny and also discuss what is known about the biology of Neruda (not much!) somewhere earlier in the more introductory section of the paper, to make this more clearly understandable.

We agree and discuss the Heliconiini phylogeny in detail earlier at lines 118-134, which has now been expanded based on the helpful points the reviewer raises above. We also present a series of phylogenies in figure 1, which we discuss in the figure legend. We have also expanded our discussion of Neruda specifically here, pointing out that the clade offers a potentially decoupling of pollen feeding from associated traits.

We also note in that section at lines 123-124, and here in section 6.i that very little is known about the biology of the Neruda. At lines 358-363 we write:

> *“little is known about the biology of the Neruda, particularly their foraging behaviour… Unresolved questions include whether the Neruda trapline or possess expanded mushroom bodies, and the level of proteolytic activity in their saliva.”*

l. 370-378: This seemed rather vague and a little uninformative.

We have edited this section to strengthen our message.

l. 387: Yes, the N=1 problem!

As noted above we highlight this in the introduction and discuss it in detail elsewhere.

l. 396: "cognitive senescence" seemed odd here! It's not clear that the senescence is cognitive; it could be just general whole-body senescence.

We agree that this sentence may have been confusing and have edited lines 403-407 to better distinguish between Heliconius’ *apparent ability to mitigate somatic senescence effects, from their potential delay of cognitive senescence. It now reads:*

> *“The greatly extended lifespans of Heliconius indicate a remarkable ability to mitigate bodily senescence. Additionally, the ability to maintain long-term memories of foraging routes suggest that Heliconius may mitigate cognitive senescence observed in other insects”*

Referee: 2

Overall the manuscript is, in my opinion, an excellent piece of work. I enjoyed reading it. I have some comments that are minor and potentially easy to address.

> *We are pleased that the reviewer enjoyed our manuscript and thank them for their positive and constructive comments.*

1. Overall, the manuscript describes many potential consequences of a shift to pollen feeding, but the causes of that shift remain a mystery. There seem to be no clearly unique aspects of Heliconius' physiology or ecology that could (yet) explain why this shift occurred in this particular group. It would be helpful to make this point explicitly, rather than leaving it to the reader to surmise. For example, at line 157, you set up the next section by saying that identifying the adaptations necessary for the transition to pollen feeding is central to understanding why it is rare, but by the end of the section, there is still no indication of anything unique to Heliconius that could have brought the transition about.

> *As the reviewer correctly notes, there is not yet a clear explanation for why pollen feeding arose in* Heliconius *but not other butterflies. We agree that the manuscript would be improved by making this uncertainty more explicit and have edited lines 24-25 in the abstract, and lines 58-59 in the introduction, which now reads:*
>
> > *"Moreover, no physiological, morphological or molecular traits have been specifically linked to the origin of pollen feeding in this genus"*
>
> *Later at lines 204-205 we also now write:*
>
> > *"Although it remains unclear why pollen feeding arose in Heliconius but not other butterflies, this question can potentially be answered by combining functional genetics, physiology and anatomy."*

2. It would have been helpful to include a clear description of the trait (i.e. pollen feeding) in the introduction. For example, at line 126 you mention "active" pollen feeding, but it wasn't clear until later in the manuscript what you meant by this (at lines 160-164). Likewise, until I got to the section beginning with line 210, I was wondering about whether pollen feeding is limited to specific plants, whether it co-occurs with nectar feeding, etc. A brief description of the natural history would be useful early on.

> *Both reviewers have raised this point and we are in complete agreement that it would improve the manuscript. We have edited the introduction at lines 38-51 to provide a brief description of pollen feeding to provide better context for readers unfamiliar with* Heliconius*.*

3. Lines 55-58: the order of the questions set out here differs from the order in which they are addressed below, where life-history comes first.

> *This is a good suggestion and we have re-ordered the points listed at lines 67-69 to match the structure of the review.*

4. Line 134: It would indeed be uninformative to compare these groups with Heliconius but perhaps comparison with their own sister groups would be worthwhile?

> *The reviewer raises a good point. However, these comparisons are difficult for several reasons. The Heterobathmiidae and Micropterigidae are very ‘basal’ moths, branching off near the root of Lepidoptera, and are both separated from other lineages by very large phylogenetic distances (spanning approximately 150 million years). Regarding pollen feeding in the Gelechiidae, it has only been described in two species for which very little other information is known about their biology. It is uncertain how widespread pollen feeding may be in the Gelechiidae. It would certainly be interesting to explore the possibility of comparative studies in this group, but currently the scarcity of basic data on these species is very limiting. At lines 143-145 we now briefly explain this:*
>
> > *“However, the ecology and life history of these species, and the prevalence of pollen feeding across Gelechiidae, are poorly understood, making it difficult to assess the feasibility of comparative analyses within this group.”*

5. Line 195: typo- should be “occur”

> *This has been fixed, thank you.*

6. Section on foraging strategies: I think it is premature to say that “the cognitive demands of traplining are likely to be associated with elaborations in the Heliconius nervous system” (lines 254-255). It is clear that the mushroom bodies have expanded extensively in Heliconious, and that pollen feeding occurs here, but the link with traplining always seems a little tenuous. Yes, Heliconious trapline, but the critical evidence that other close relatives do not seems to be missing (as you point out at line 243- I just think this should be made more explicit). I can’t see a specific reason why traplining should be particularly linked to pollen feeding per se rather than the fact that Psiguria and Guriana apparently flower for a very long time- which many other plants do to. And nectar feeders also trapline (hummingbirds come to mind). As you point out later, there are many other potential explanations (increased longevity perhaps leads to selection for increased long-term memory, etc).

> *We agree with the reviewer that a connection between mushroom body expansion and traplining has not yet been directly demonstrated. One of the purposes of writing this review is to point out that many of these trait relationships are not well tested, and this is a good example. We have therefore edited the line quoted above, changing “likely” to “predicted to be” at line 259, referencing Sivinski (1989)* [12] *and Montgomery et al (2016)* [15].
>
> *However, all the current evidence for traplining in* Heliconius *is in the context of pollen feeding and it seems highly probable that these two traits are linked in some way. Although* Heliconius *do also take nectar from pollen resources, they are most motivated by pollen, their foraging routes are based around the distribution of pollen rich plants, and these routes are updated to incorporate changes to this distribution. However, we do not consider pollen feeding* per se *as necessarily being the driving force behind the evolution of traplining, but rather the spatial distribution of pollen resources. Unlike general nectar resources, the preferred pollen sources tend to be at low densities but highly reliable across long periods of time. This may favour a switch in foraging behaviour towards learning the spatial distribution of these resources to improve foraging efficiency, especially given competition for these resources with other*

Heliconius *and, indeed, hummingbirds* [16]. *Longevity is no doubt part of this story, as the benefits of learning the location of resources probably increase the longer those resources are used. Increased lifespan, traplining, mushroom body size and pollen feeding are therefore all linked in ways we do not currently fully understand. We hope this is clear in the review, and that our edits have removed any ambiguity.*

Although there are no formal, published studies on foraging on non-Heliconius *Heliconiini, we do know that they do not collect pollen and do not appear to form long term, stable home ranges, instead dispersing over larger distances (e.g.* [11,17]*). The clear assumption is therefore that they also do not trapline as both pollen feeding and roost site fidelity are linked to traplining in* Heliconius*. While it is possible that they have some spatial fidelity, traplining has only been reported in one other butterfly (a myrmecophilous lycaenid,* Jalmenus evagorus*, in which males trapline between ant nests* [18]*) so we believe that, until further data is available, this hypothesis is fairly reasonable.*

Turning to the mushroom bodies directly, at lines 267-270 we point out that, across all insects, direct evidence for a functional link between the mushroom bodies and visually-oriented spatial memory is currently limited and comes from only two studies in a cockroach and an ant [19,20]*. Indeed, we are currently testing this hypothesis ourselves. However, a comparative study in Hymenoptera also suggests that spatially foraging drives mushroom body expansion* [21]*, which provides further reason to expect that traplining requires neural enhancement in Heliconius.*

As an aside, the reviewer is of course correct that hummingbirds, for example, are nectar feeders, but we would hesitate to extrapolate between the behaviours of birds and butterflies in that sense. Interestingly, however, hummingbirds do have enlarged hippocampi [22]*, again supporting the general idea that spatially faithful foraging (regardless of the nature of the resource) requires investment in neural structures that underpin long term spatial memory.*

In sum, we wholly agree that better data on the foraging behaviours of non-pollen-feeding Heliconiini are necessary for answering this question, and now state at lines 235-238:

> *"Site fidelity is presumably a pre-requisite for trap lining, which, together with the central role of pollen resources in Heliconius trap lines, suggests that these behaviours are linked to the acquisition of pollen feeding. However, the lack of data on whether non-pollen feeding Heliconiini use spatial information during foraging, means this hypothesis is yet to be formally tested."*

Similarly, at lines 283-284, we write:

> *"Likewise, a better understanding of the foraging behaviours of non-pollen-feeding Heliconiini is crucial to understanding the drivers behind mushroom body expansion in Heliconius."*

7. Line 245: "Trapline foraging … rare among the Lepidoptera"- we don't really know that it's rare, as you point out at lines 242-3.

The reviewer correctly points out that we do not know for certain that this behaviour is rare in Lepidoptera. However, similar traplining behaviour has only been tentatively *described in one other Lepidoptera species,* Jalmenus evagoras [18]*. We have therefore changed “rare” to “rarely reported” at line 256.*

8. Section on “How quickly do behavioural innovations….” (line 347-355). It would be helpful to clarify what we would learn from understanding more about Neruda, given their lack of pollen feeding may apparently be derived- for example, I’m not sure that finding that they also had expanded MBs would really help? But of course, if they didn’t, that would be useful information. It also seems important in this section to look at variation between-pollen feeding species. For example, if all of them possess the whole suite of adaptations, perhaps this would suggest rapid evolution right at the base of the group?

All Heliconius*, apart from the Neruda clade, appear to possess the full pollen-feeding suite, which raises a problem of evolutionary singularity. We now state this more clearly at lines 118-120:*

> *“Except for the four species of the ‘Neruda’ clade (figure 1e-g), all Heliconius species feed on pollen and appear to possess the complete suite of associated traits, presenting a challenge to reconstructing the origin of pollen feeding.”*

The Neruda clade therefore offers the only opportunity within Heliconius *to examine a decoupling between pollen feeding and its associated traits, which could help to resolve the timing of these shifts and some of their relationships. Currently, we have so little data on them we do not know what to expect. We now explain this much earlier in the review at lines 120-122 to provide clarity on the points raised in this section, writing:*

> *“As the only non-pollen-feeding Heliconius, the four Neruda species may offer the possibility of decoupling pollen feeding and its associated adaptations, helping to resolve the timing of these shifts, and the relationships between traits.”*

We have also edited lines 355-357 to explain that a better understanding of variation in foraging behaviour between the two main Heliconius *clades could also prove helpful:*

> *“Variation in the degree of specialisation on Cucurbit vines across Heliconius may provide one avenue to investigate ties between pollen feeding and foraging behaviours.”*

However, as we note above, this intra generic variation is likely to be considerably smaller than between genera.

9. Line 374: “Pollen feeding likely involves considerable costs…. neural tissue”. As above, I don’t think there’s convincing evidence that pollen feeding per se involves these costs.

This is a fair point. As explained above, we do not think pollen feeding itself involves these costs, but that because dependable pollen sources are at low densities, traplining may have evolved for efficient collection of pollen, and this behaviour may itself depend on costly neural elaborations. We have edited lines 380-381 to clarify that Heliconius *exhibit certain costly adaptations, which we argue may be indirect costs of efficient pollen feeding. That part now reads:*

> "It is possible that reliable collection of pollen can only be achieved through increased investment in neural tissue and learning, both of which can be costly."

Overall, thank you for this interesting, well-written and informative piece of work, which I hope to see in print in the near future.

> Thank you for your comments and suggestions!

References

1. Cardoso MZ. 2001 Patterns of pollen collection and flower visitation by *Heliconius* butterflies in southeastern Mexico. *J. Trop. Ecol.* **17**, 763–768. (doi:10.1017/S0266467401001572)

2. Boggs CL, Smiley JT, Gilbert LE. 1981 Patterns of pollen exploitation by *Heliconius* butterflies. *Oecologia* **48**, 284–289. (doi:10.1007/BF00347978)

3. Estrada C, Jiggins CD. 2002 Patterns of pollen feeding and habitat preference among *Heliconius* species. *Ecol. Entomol.* **27**, 448–456. (doi:10.1046/j.1365-2311.2002.00434.x)

4. Dunlap-Pianka H, Boggs CL, Gilbert LE. 1977 Ovarian dynamics in Heliconiine butterflies: programmed senescence versus eternal youth. *Science* **197**, 487–90. (doi:10.1126/science.197.4302.487)

5. Edelman NB *et al.* 2019 Genomic architecture and introgression shape a butterfly radiation. *Science* **366**, 594–599. (doi:10.1126/science.aaw2090)

6. Mallet J. 1980 A laboratory study of gregarious roosting in the butterfly *Heliconius melpomene*. University of Newcastle-upon-Tyne, Newcastle-upon-Tyne.

7. Krenn HW, Penz CM. 1998 Mouthparts of *Heliconius* butterflies (Lepidoptera: Nymphalidae): a search for anatomical adaptation to pollen-feeding behavior. *Int. J. Insect Morphol. Embryol.* **27**, 301–309. (doi:10.1016/S0020-7322(98)00022-1)

8. Harpel D, Cullen DA, Ott SR, Jiggins CD, Walters JR. 2015 Pollen feeding proteomics: salivary proteins of the passion flower butterfly, *Heliconius melpomene*. *Insect Biochem. Mol. Biol.* **63**, 7–13. (doi:10.1016/j.ibmb.2015.04.004)

9. 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 Biol. Evol.* **8**, 2581–2596. (doi:10.1093/gbe/evw180)

10. Smith G, Kelly JE, Macias-Muñoz A, Butts CT, Martin RW, Briscoe AD. 2018 Evolutionary and structural analyses uncover a role for solvent interactions in the diversification of cocoonases in butterflies. *Proc. R. Soc. B Biol. Sci.* **285**, 20172037. (doi:10.1098/rspb.2017.2037)

11. Mallet J, Longino JT, Murawski D, Murawski A, Gamboa ASD, Journal T, Jun N. 1987 Handling effects in Heliconius: Where do all the butterflies go? *J. Anim. Ecol.* **56**, 377–386.

12. Sivinski J. 1989 Mushroom body development in Nymphalid butterflies: a correlate of learning? *J. Insect Behav.* **2**, 277–283. (doi:10.1007/BF01053299)

13. Snell-Rood EC, Davidowitz G, Papaj DR. 2011 Reproductive tradeoffs of learning in a butterfly. *Behav. Ecol.* **22**, 291–302. (doi:10.1093/beheco/arq169)

14. Darragh K, Byers KJRP, Merrill RM, McMillan WO, Schulz S, Jiggins CD. 2019 Male pheromone composition depends on larval but not adult diet in *Heliconius melpomene*. *Ecol. Entomol.* **44**, 397–405. (doi:10.1111/een.12716)

15. Montgomery SH, Merrill RM, Ott SR. 2016 Brain composition in *Heliconius butterflies*, posteclosion growth and experience-dependent neuropil plasticity. *J. Comp. Neurol.* **524**, 1747–1769. (doi:10.1002/cne.23993)

16. Murawski DA, Gilbert LE. 1986 Pollen flow in *Psiguria warscewiczii*: a comparison of *Heliconius* butterflies and hummingbirds. *Oecologia* **68**, 161–167. (doi:10.1007/BF00384782)

17. Benson WW. 1971 Evidence for the Evolution of Unpalatability Through Kin Selection in the Heliconinae (Lepidoptera). *Am. Nat.* **105**, 213–226. (doi:10.1086/282719)

18. Pierce NE, Elgar MA. 1985 The influence of ants on host plant selection by Jalmenus evagoras, a myrmecophilous lycaenid butterfly. *Behav. Ecol. Sociobiol.* **16**, 209–222. (doi:10.1007/BF00310983)

19. Mizunami M, Weibrecht JM, Strausfeld NJ. 1998 Mushroom bodies of the cockroach: their participation in place memory. *J. Comp. Neurol.* **402**, 520–537. (doi:10.1002/(SICI)1096-9861(19981228)402:4<520::AID-CNE6>3.0.CO;2-K)

20. Kamhi JF, Barron AB, Narendra A. 2020 Vertical Lobes of the Mushroom Bodies Are Essential for View-Based Navigation in Australian Myrmecia Ants. *Curr. Biol.* **30**, 1–6. (doi:10.1016/j.cub.2020.06.030)

21. Farris SM, Schulmeister S. 2011 Parasitoidism, not sociality, is associated with the evolution of elaborate mushroom bodies in the brains of hymenopteran insects. *Proc R Soc B* **278**, 940–51. (doi:10.1098/rspb.2010.2161)

22. Ward BJ, Day LB, Wilkening SR, Wylie DR, Saucier DM, Iwaniuk AN. 2012 Hummingbirds have a greatly enlarged hippocampal formation. *Biol. Lett.* **8**, 657–659. (doi:10.1098/rsbl.2011.1180)

Appendix B

Response to reviewers' comments

Referee: 2

We thank the reviewer for their previous suggestions and are pleased that they are satisfied with the changes we have made. We are in complete agreement with their two additional suggestions.

Line 212-213: This still seems a very certain statement- revise to "… and their pollen sources may have"?

> *We agree that this statement was too definitive and followed the reviewers suggestion. Lines 209-211 now read:*
>
> > *"In Heliconius, the interactions between butterflies and their pollen sources may have led to notable refinements in both the brain and behaviour."*

Line 266-273: One further addition here. For completeness, I think it is important to mention that the Central Complex, rather than the Mushroom Bodies, is the neural region more typically associated with spatial task performance in insects (e.g. see refs within Varga et al. (2017) Front. Behav. Neuroscience 11: https://doi.org/10.3389/fnbeh.2017.00004). I do not question that there are many types of spatial task, and that the MBs may also be involved, particularly in the long-term retention of site-specific information.

> *The reviewer correctly points out that there is a substantial body of evidence implicating the central complex in spatial learning and orientation in several insect groups. We agree that this is important to acknowledge and now explain this at lines 271-274:*
>
> > *"Though suggestive, these data are relatively impoverished compared to our understanding of the role of the central complex, another sensory-motor integration structure in the central brain, in spatial learning and orientation in insects."*