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PROCEEDINGS B

Forest stratification shapes allometry and flight morphology of tropical butterflies

Sebastián Mena, Krzysztof M. Kozak, Rafael E. Cárdenas and María F. Checa

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Review timeline

Original submission:	11 May 2020
1st revised submission:	20 July 2020
2nd revised submission:	3 September 2020
Final acceptance:	25 September 2020

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

Review History

RSPB-2020-1071.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?
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
Please see attached file. (See Appendix A)

Review form: Reviewer 2

Recommendation
Reject – article is not of sufficient interest (we will consider a transfer to another journal)

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

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

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

Is the length of the paper justified?
Yes

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

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This paper documents the differences of the allometric relationships of various wing dimensions of tropical Nymphalid butterflies in relation to the vertical distributions of their flight activity in lowland neo-tropical forest. As such, this raises interesting questions about the evolutionary drivers of wing size, shape and wing loading in butterflies. However there are parts of this manuscript where greater clarity, explanation and justification is needed.

The introduction describes the hypotheses being studies, but these are not fully explained by the bulk of the introduction. Whilst light, humidity, wind and temperature do vary with height within forests, the allometric relationships and flight performance are hypothesised to be related to physiological constraints, but these constraints are not explained. Likewise wing area to thoracic volume is predicted to be higher in lower strata flying butterflies than canopy flying butterflies to accommodate optimal flight performance, but how is optimal defined here? Butterfly flight is not just constrained by environmental parameters and physical structure, wing shape and wing loading are also influenced by other aspects of biology, including predator avoidance and the type of mate locating mechanisms employed. The paper would benefit from a consideration of this in the introduction as well.

Sampling was by baited traps at two heights within the forest, and various wing and thoracic parameters obtained for the species occurring at these two heights. Some species were retained for identification and other released and marked. It is stated that mark-recapture data for the released butterflies were included in the analysis, but by not doing this for specimens that could not be easily recognised, the data for the later will be occurrence data for first (and only) capture but for the former might included re-occurrence. Greater explanation is needed as this is not a balanced design.

Digital images and thoracic measurements were used to obtain the primary morphometric data. Was a calibration rule used in this? Whilst wing area and centroid measurements were done with closed wing specimens more explanation is needed here for three reasons. First, it has to be assumed that all of the butterfly species here, from different subfamilies, actually fly with the angle between fore and hindwing constant in flight. This is not necessarily the case, some species fly with the leading wing edge of the forewings further forward than others, effectively giving a range of wing areas used in flight than would not be calculated here. Secondly some species decouple the fore and hindwings in flight, especially on 'power' strokes, again influencing wing area. The centroid position may thus be difficult to calculate as well. More explanation is needed in the methods section to explain why all species are therefore measured in exactly the same way.

Data analysis
Whilst the analysis is robust and appropriate, there is one aspect that is pertinent to the discussion. Flight ability, especially the ability to manouver and accelerate is also influenced by wing length to wing area ratio, especially of the forewing. Whilst this is in part covered by the distance to centroid measure, this ratio could be incorporated into this analysis as well, it might strengthen those parts of the discussion that consider flight 'agility'.

Figure 3 – it is not clear why just six species on the figure are named.
Figure 4c -with no significant relationship a 'best fit' slope should not be fitted as the slope is not significantly different to zero.

Discussion
Line311-315 The point is made that there is a relationship between wing beat frequency and wing size, with reference to within-species effects. Whether this argument is transferable across-species is debateable.

Line318-322 Wing beat frequencies of the studied species are not know, but more importantly arguments about gliding versus flapping flight and the energetics of either are also need to take into account the duration of flight. Short duration flapping flight is energetically expensive and in some species this is compensated for by adjustments of body temperature. When not in flight made by basking position and posture. Gliding may facilitate long duration flights after basking. Additionally, arguments made later about thermal requirements also need to take into account wing colouration, not just haemolymph flow through the wings.

Line 322-323 It would help the reader if the results also included a summary of data on wing area. A large wing area in relation to thorax size will necessarily mean that sustained flapping is not as efficient as in a butterfly with a larger musculature (i.e. bigger thorax), but a relatively small thorax size will enable more rapid heating at rest than would a large thorax. Therefore there needs to be analysis of flight durations and resting durations to fully explore this topic.

In summary, there is interesting data here, but there needs to be a more precise focus in the discussion and further relevant data, which is available, needs presenting in the results section.

Review form: Reviewer 3

Recommendation
Accept with minor revision (please list in comments)

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

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?
No

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

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors present an interesting and thorough study of wing morphology measurement from a group of butterflies captured during a long term study at a site in the Ecuadorian rainforest. The analysis uses appropriate statistical methods accounting for phylogenetic signal and yields highly significant results, likely due to the large sample size of individuals and species.

The authors conclude "that butterfly habitat preference for canopy and understorey is associated with a set of morphological flight traits. This highlights the importance of vertical dimensions in the generation of morphological and ecological diversity." These statements are well supported. However, there are large sections of the discussion that are more speculative and could be de-emphasized.

The introduction is quite expansive in its discussion of previous work showing morphological differences in features that might relate to flight performance. This could be reduced a little and I would recommend shifting those words to a more complete explanation of the hypotheses (Line 102 onward). Each hypothesis needs an explanatory sentence explaining why this is a likely relationship, since they do not all relate particularly well to the preceding text.
For example, "We hypothesized that butterflies in the canopy had a hyperallometric slope compared to understorey butterflies due to physiological constraints exerted by stratification." This is too vague. Hyperallometry of which relationship? Which physiological constraints, specifically, and how would they drive the change in slope? When variation in the gradients of the allometric relationship were found, the meaning of the slopes (an interesting finding) were not explored in the discussion.

Line 107-8. It is not immediately obvious how the butterflies could have higher aspect ratios AND a shorter distance to the wing centroid. This implies a more complex change in shape and needs greater explanation of i. what that might be and, ii. what the effect of that might be. This is important because there is a secondary question relating to how flight performance might change with respect to that shape change. i.e. is it due to the change in wing length, wing chord, aspect ratio, or the change in plan form.

"Allometry ensures that correct body proportion is maintained across taxa despite differences in body size (22–23)" I don't much care for the wording of this. One could argue that allometry means that body proportions are NOT maintained. I.e. parts are proportionally different. Also, "ensures" leads to the suggestion of purpose. Allometry is a result, rather than a design goal.

Line 155. Since the ipsilateral wing pairs are treated as one, I am not sure why the aspect ratio calculation is a 4 rather than a 2?

Line 176. Is it not possible to include trap site location in the statistical model to use more of the data?

Line 210. non-independence.

Line 262. It would be helpful to reiterate what negative and positive mean here with respect to stratification. I presume larger stratification means higher level?

Line 314. This is not a law that is set in stone; there are other Lepidopterans that have not shown a wing beat frequency response to wing wear. E.g. Hedrick, Fernandez papers on Manduca.

Line 311-313. This is incomplete. The compensation could also come from e.g. increased forward flight speed or greater wing sweep amplitude.

Line 317. While it is worth a passing comment in the discussion, I am not convinced of the available heat argument. Several other factors could be at play here. E.g. Larger thorax (absolute) might dissipate heat more slowly; a hairier thorax might retain heat longer; a darker coloured thorax might absorb heat more readily; flight itself generates heat by the muscle action. I can't see sufficient new data in this work to draw a firm conclusion. Certainly, the statement "understorey butterflies may be incapable of high-performance flight because of relatively low heat availability; having a large thorax would be impractical because it would add useless weight" seems to go way too far considering there are plenty of 'high performance' butterflies in temperate regions. Would a larger muscle mass be useless weight, especially given the self-generated heat?

Line 322 seems to neglect the additional force required by the flight motor to drive these larger wings. This line of reasoning is incomplete, at best.

Line 344. I'm not yet sure of the imperative for improved flight performance at higher altitudes. Why is lower flight performance acceptable lower down? Is this based on a notion of avian predation in the canopy? If so, how reliable is this as a selection pressure? What is the measure of flight performance (speed; tangential acceleration; hovering performance; manoeuvrability; agility)? I think there could be some clarification of this paragraph.

Decision letter (RSPB-2020-1071.R0)

Dear Mr Mena:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

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. 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 Sasha Dall
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Dear Dr. Mena,
Thank you for submitting your manuscript entitled "Forest stratification shapes allometry and flight morphology in tropical butterflies" to the Proceedings of the Royal Society. I have received three peer reviews, two of which are supportive of the manuscript, but all of which also raise some concerns about the manuscript.
I appreciate that your manuscript tests the central hypothesis that habitat complexity drives biodiversity by examining hypotheses about the flight morphology of butterflies and constraints imposed by different microhabitats within the tropical rainforest. The study finds that habitat stratification and the resulting differences in e.g. metabolic costs and flight demands can explain flight morphology in butterflies. The study is remarkable for its data set and the reviewers agree that the statistical analysis is sound. The reviewers, however, differ widely in their assessment of the quality of the manuscript. I would like to highlight three areas of concern, which I hope the authors will be able to address.
First, the reviewers find that the hypotheses stated in the Introduction need to be phrased more precisely. The authors should also include supplementary hypotheses that reflect the complexity of the form-function relations of butterfly wings.
Second, although the reviewers agree that the authors conducted their statistical analysis correctly, one reviewer expresses several concerns about the study design that have implications for the strength of the conclusions.
Third, the Discussion should discuss the study's limitations and broader implications in greater depth to increase the value of this manuscript to a broad readership.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
Please see attached file.

Referee: 2
Comments to the Author(s)
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Referee: 3

Comments to the Author(s)
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For example, "We hypothesized that butterflies in the canopy had a hyperallometric slope compared to understorey butterflies due to physiological constraints exerted by stratification." This is too vague. Hyperallometry of which relationship? Which physiological constraints, specifically, and how would they drive the change in slope? When variation in the gradients of the allometric relationship were found, the meaning of the slopes (an interesting finding) were not explored in the discussion.

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Author's Response to Decision Letter for (RSPB-2020-1071.R0)

See Appendix B.

RSPB-2020-1071.R1 (Revision)

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?
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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have addressed all of my main concerns from the first round of review and I think the manuscript has been significantly improved. I made some more stylistic comments on the paper, and some additional comments where some areas were still a bit unclear. Please see attached comments on the paper.

Review form: Reviewer 2

Recommendation
Accept as is

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

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

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?
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No

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
I thank the authors for the detailed responses to my previous comments, which has made the review process of the resubmission relatively straightforward. The manuscript is much improved, and the key arguments made are much clearer. In particular the introduction is more focused and the hypotheses more clearly expressed and the discussion (supported by the data is very focused and more evidence based than before. The manuscript warrants publication. There are some minor typographic issues that can be easily resolved, save for one comment on the abstract. These are given below. To aid the authors please note that I added continuous line numbers to the text supplied with the author responses and started the line numbering with the first line starting at 'Abstract'

Line 4 change 'steeper' to 'large'

Line 11 As the manuscript does not directly refer to life histories and metabolic rates in the data it might be better to change this sentence to 'We hypothesized that species show morphological adaptations related to differing micro-environments associated with the canopy and understorey.'

Line 31 '…better manoeuvring during flapping flight'

Line 47 it might be better to state 'increases inversely with organism size'

Line 64 'sensitivity' not 'sensibility'

Line 71 replace ' microhabitat they lived might' with 'microhabitats used'

Line 85 "…higher wingbeat frequency…'

Line 161 '2/3' not '2:3'

Line 175 add 'a' after 'finding'

Line 290 '..strata at the same location…'

Line342 '.. combined with behavioural..'

Line 343 '.. basking in sunlit gaps…'

Review form: Reviewer 3

Recommendation
Accept as is

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

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

Quality of the paper: Is the overall quality of the paper suitable?
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Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
.

Decision letter (RSPB-2020-1071.R1)

01-Sep-2020

Dear Mr Mena:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

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 Sasha Dall
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Dear Dr. Mena,
Thank you for submitting a thorough revision of your manuscript entitled "Forest stratification shapes allometry and flight morphology in tropical butterflies" to the Proceedings of the Royal Society. I have received two peer reviews, both of which are supportive of the manuscript and overall satisfied the revisions.
Reviewer 1 would like to share a few more suggestions to further improve the clarity and content of the manuscript. Please consider those comments and implement those suggestions that you feel improve the manuscript.

Reviewer(s)' Comments to Author:
Referee: 2

Comments to the Author(s)
I thank the authors for the detailed responses to my previous comments, which has made the review process of the resubmission relatively straightforward. The manuscript is much improved, and the key arguments made are much clearer. In particular the introduction is more focused and the hypotheses more clearly expressed and the discussion (supported by the data is very focused and more evidence based than before. The manuscript warrants publication. There are some minor typographic issues that can be easily resolved, save for one comment on the abstract. These are given below. To aid the authors please note that I added continuous line numbers to the text supplied with the author responses and started the line numbering with the first line starting at 'Abstract'

Line 4 change 'steeper' to 'large'

Line 11 As the manuscript does not directly refer to life histories and metabolic rates in the data it might be better to change this sentence to 'We hypothesized that species show morphological

adaptations related to differing micro-environments associated with the canopy and understorey.'

Line 31 '…better manoeuvring during flapping flight'

Line 47 it might be better to state 'increases inversely with organism size'

Line 64 'sensitivity' not 'sensibility'

Line 71 replace ' microhabitat they lived might' with 'microhabitats used'

Line 85 "…higher wingbeat frequency…'

Line 161 '2/3' not '2:3'

Line 175 add 'a' after 'finding'

Line 290 '..strata at the same location…'

Line342 '.. combined with behavioural..'

Line 343 '.. basking in sunlit gaps…'

Referee: 1

Comments to the Author(s)
The authors have addressed all of my main concerns from the first round of review and I think the manuscript has been significantly improved. I made some more stylistic comments on the paper, and some additional comments where some areas were still a bit unclear. Please see attached comments on the paper.

Referee: 3
Comments to the Author(s)
.

Author's Response to Decision Letter for (RSPB-2020-1071.R1)

See Appendix C.

RSPB-2020-1071.R2 (Revision)

Review form: Reviewer 1

Recommendation
Accept as is

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have addressed all the comments and suggestions that I made on the previous version, and I have no further comments. Congratulations on a great study!

Decision letter (RSPB-2020-1071.R2)

25-Sep-2020

Dear Mr Mena

I am pleased to inform you that your manuscript entitled "Forest vertical stratification shapes allometry and flight morphology in tropical butterflies" 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 please 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

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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,
Dr Sasha Dall
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

This paper documents the differences of the allometric relationships of various wing dimensions of tropical Nymphalid butterflies in relation to the vertical distributions of their flight activity in lowland neo-tropical forest. As such, this raises interesting questions about the evolutionary drivers of wing size, shape and wing loading in butterflies. However there are parts of this manuscript where greater clarity, explanation and justification is needed.

The introduction describes the hypotheses being studies, but these are not fully explained by the bulk of the introduction. Whilst light, humidity, wind and temperature do vary with height within forests, the allometric relationships and flight performance are hypothesised to be related to physiological constraints, but these constraints are not explained. Likewise wing area to thoracic volume is predicted to be higher in lower strata flying butterflies than canopy flying butterflies to accommodate optimal flight performance, but how is optimal defined here? Butterfly flight is not just constrained by environmental parameters and physical structure, wing shape and wing loading are also influenced by other aspects of biology, including predator avoidance and the type of mate locating mechanisms employed. The paper would benefit from a consideration of this in the introduction as well.

Sampling was by baited traps at two heights within the forest, and various wing and thoracic parameters obtained for the species occurring at these two heights. Some species were retained for identification and other released and marked. It is stated that mark-recapture data for the released butterflies were included in the analysis, but by not doing this for specimens that could not be easily recognised, the data for the later will be occurrence data for first (and only) capture but for the former might included re-occurrence. Greater explanation is needed as this is not a balanced design.

Digital images and thoracic measurements were used to obtain the primary morphometric data. Was a calibration rule used in this? Whilst wing area and centroid measurements were done with closed wing specimens more explanation is needed here for three reasons. First, it has to be assumed that all of the butterfly species here, from different subfamilies, actually fly with the angle between fore and hindwing constant in flight. This is not necessarily the case, some species fly with the leading wing edge of the forewings further forward than others, effectively giving a range of wing areas used in flight than would not be calculated here. Secondly some species decouple the fore and hindwings in flight, especially on ‘power’ strokes, again influencing wing area. The centroid position may thus be difficult to calculate as well. More explanation is needed in the methods section to explain why all species are therefore measured in exactly the same way.

Data analysis
Whilst the analysis is robust and appropriate, there is one aspect that is pertinent to the discussion. Flight ability, especially the ability to manouver and accelerate is also influenced by wing length to wing area ratio, especially of the forewing. Whilst this is in part covered by the distance to centroid measure, this ratio could be incorporated into this analysis as well, it might strengthen those parts of the discussion that consider flight ‘agility’.

Figure 3 – it is not clear why just six species on the figure are named.
Figure 4c -with no significant relationship a ‘best fit’ slope should not be fitted as the slope is not significantly different to zero.

Discussion

Line311-315 The point is made that there is a relationship between wing beat frequency and wing size, with reference to within-species effects. Whether this argument is transferable across-species is debateable.

Line318-322 Wing beat frequencies of the studied species are not know, but more importantly arguments about gliding versus flapping flight and the energetics of either are also need to take into account the duration of flight. Short duration flapping flight is energetically expensive and in some species this is compensated for by adjustments of body temperature. When not in flight made by basking position and posture. Gliding may facilitate long duration flights after basking. Additionally, arguments made later about thermal requirements also need to take into account wing colouration, not just haemolymph flow through the wings.

Line 322-323 It would help the reader if the results also included a summary of data on wing area. A large wing area in relation to thorax size will necessarily mean that sustained flapping is not as efficient as in a butterfly with a larger musculature (i.e. bigger thorax), but a relatively small thorax size will enable more rapid heating at rest than would a large thorax. Therefore there needs to be analysis of flight durations and resting durations to fully explore this topic.

In summary, there is interesting data here, but there needs to be a more precise focus in the discussion and further relevant data, which is available, needs presenting in the results section.

Appendix B

Dear Editor,
We would like to thank the reviewers and editor for their meticulous analyses and suggestions that helped us to greatly improve our manuscript. We made sure to reply all the queries and commentaries. Some sections of the text were modified in order to clarify the issues pointed by the reviewers. In particular we modified the introduction to include information relevant to the hypotheses which were expanded and explained in detail. We also clarified some points in the methods and expanded our discussion to touch some topics the reviewers suggested, while reducing speculative points.
Please find below our replies line by line in blue colour.

Editor:
First, the reviewers find that the hypotheses stated in the Introduction need to be phrased more precisely. The authors should also include supplementary hypotheses that reflect the complexity of the form-function relations of butterfly wings.
Done. Those new hypotheses are:
"We hypothesized that butterflies in the understorey have a hyperallometric slope of wing areas and thoracic volumes compared to canopy butterflies, due to a more restricted metabolic activity driven by lower temperatures at the understorey (65). Consequently, we predicted an evolutionary trend between microhabitat specialization and morphology: as conditions in the canopy are favourable for higher beat frequencies than in the understorey, an increasing preference for the canopy was hypothesized to be associated to butterflies with a decreasing wing area to thoracic volume (i.e. extended use of fast flapping-flight for the canopy butterflies and slower gliding for the understorey). Because this may restrict understorey butterflies in their performance in terms of speed and manoeuvrability, we also hypothesized that understorey butterflies may have shifted their wing shape as a means of compensation, towards higher aspect ratios and shorter distances to the wing centroid (wings more elongated, wider towards the tip) compared to canopy butterflies."

Second, although the reviewers agree that the authors conducted their statistical analysis correctly, one reviewer expresses several concerns about the study design that have implications for the strength of the conclusions.
Done. We have answered the second reviewer considering this important point. Basically, we have expanded our Methods and added figures to the Supplementary section to better explain how different wing coupling is accounted for. We have also clarified that re-occurrence data was not included.

Third, the Discussion should discuss the study's limitations and broader implications in greater depth to increase the value of this manuscript to a broad readership.
Done. We have de-emphasized certain sections to acknowledge our limitations, including other potential hypothesis for future research. We also expanded in topics suggested by referee 1.

Referee: 1
The vertical stratification of organisms in tropical forests has been studied in many groups, particularly in butterflies, but mostly from an ecological perspective. This study is novel in examining how flight height and microhabitat preference are associated with the evolution of
distinct morphologies linked to flight ability in Nymphalidae butterflies, a model group for studies of tropical community evolutionary ecology. The authors have assembled a robust dataset

based on multiple years of sampling. They use this to characterize flight preference, and explore
for relationships with morphological traits. The methods are appropriate for the questions being
addressed, although I didn't fully understand the integration of the 'backbone' and COI trees that
produced the final ultrametric tree – that needs a bit of clarification. I also felt the authors were
quite restrained in interpreting their results, and I feel a bit more discussion about the broader
implications of the results for the evolution of tropical communities and lineages might have been warranted. Overall, I found this a fascinating study that will likely be of broad interest, given ever-increasing interest in fine-scale partitioning of resources in tropical forests and the
popularity of bait-attracted nymphalid butterflies as a study group.
We agree. We have rewritten the cited paragraph (lines 207-224) to clarify the integration of published data in our tree. We have also expanded our discussion to mention these broader implications (lines 272-287).

Specific points (line numbers)
2: I suggest using a more specific phrase than 'allometries' for a general audience
There is no use of the word "allometries" in this line.

25-28: I suggest something like 'We used phylogenetic methods to control for similarity resulting from common ancestry, to explore for relationships between species stratification and
flight morphology. We hypothesized that species might show morphological adaptations related
to differing life histories and metabolic rates, associated with different micro-enviroments'.
Agreed. Changed accordingly.

33: 'microclimate' rather than 'climate'?
Agreed. Changed accordingly.

53: why 'mean wing chord' and not just 'wing chord'?
This is because the width is not constant along the butterfly wing. Therefore, a mean chord must be estimated using the wing area.

54: Do you mean a 'shorter distance' between wing centroid and base?
We mean a larger distance. Changed accordingly.

74: 'explain', or contribute?
Agreed. Changed to 'explain'.

80-81: you might also note that there is evidence for partioning at much finer scales too, between
closely related species and even within species, e.g. recent paper by Nice et al. (2019, Biology
Letters) which showed genetic differentiation within A. demophon between canopy and understorey individuals. These examples emphasize that shifts between strata could be associated
with speciation and diversification.

Good point. We have included key information of the above-mentioned references.

97: bit unclear what you mean by 'high organization level'
Citations in the previous line were examples of trends across species of a genera or tribe, but we were aiming to find a pattern at the community level. We changed it to "community level" to be more precise.

102: 1. not clear what you mean by 'hyperallometric', this is the first time this term has been used.

2. Also, I would say that the predictions are that 'butterflies have a hyperallometric slope...', rather
than 'had'.
1. Bonduriansky and Day's paper (Evolution, 57(11), 2003, pp. 2450–2458) state: "*Isometry occurs when b=1* [eq is: log(Y)=log(a)+blog(X), therefore b meaning the slope], *so that the ratio of trait to body size remains constant across the range of body sizes. In contrast, negative (hypo-)allometry occurs when b,1, so that larger individuals have relatively smaller traits, whereas positive (hyper-) allometry occurs when b.1, so that larger individuals have relatively larger traits.*". Shingleton et al. (Proc. R. Soc. B (2008) 275, 1875–1885) use this term similarly (see figure). We preferred not to use 'positive/negative allometry', since it may be confusing.

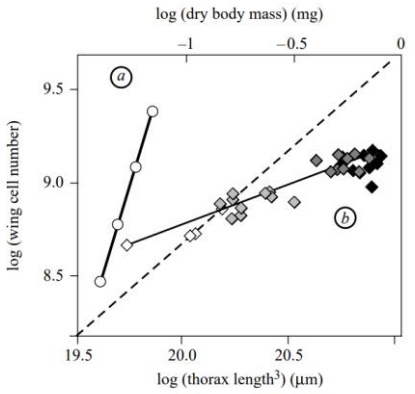


Figure 4. Modelled and observed allometric relationship between wing size and body size in *Drosophila*. (*a*) When both the wing and the body have no intrinsic growth rate (c_b, c'_b, c_d and $c'_d = 0$) the model predicts a highly hyperallometric relationship between wing cell number and body size (values as in table 1). (*b*) In fact, the nutritional static allometry between wing cell number and body volume (thorax length^3) is linear but hypoallometric, such that $\alpha = 0.43$. Flies reared on diets of increasing nutritional quality (2–100% of standard diet). White diamonds, 2% diet; grey diamonds, 5% diet; dark grey diamonds, 10% diet; black diamonds, 100% diet. Dashed line is isometry. See electronic supplementary material for methods.

2 Agreed. Changed accordingly.

99: Might be worth including a sentence or two as to why Nymphalidae are a particularly suitable group – diversity, ecological and morphological variability, ease of sampling, good taxonomic and phylogenetic knowledge etc.
Agreed. We added: "due to its diversity and ease of sampling across strata". We already mentioned that butterflies are diverse and ecologically and morphologically variable.

98-110: overall, I suggest explaining in more general terms what you are testing here, for the
benefit of non-expert readers; rather than using terms such as aspect ratio, wing centroid, etc., I
would suggest phrases like 'relatively long, narrow wings' or 'shorter, more compact wings' etc.

Agreed. We added: “wings more elongated, wider towards the tip”. Additionally, the first and last sections of the introduction were modified to address this point.

109: by ‘presumed shortage in speed’ do you mean ‘presumably reduced speed’?
Yes, changed accordingly.

110: suggest replacing ‘led’ with ‘in association with’; led imples selection on morphology that
results in a change in flight speed, but there might be selection on flight speed (e.g., reducing energy consumption) that results in a change in morphology (reducing resource allocation to
flight muscles while maintaining the same flight speed).
Agreed. Changed accordingly.

119: ‘eight sampling sites were established, with each 40 m apart’.
Agreed. Changed accordingly.

122: what does 10% inclination mean? Is the landscape almost completely flat, or were traps set
along ridges?
The traps were entirely set along ridges since valleys were mostly flooded. However, the landscape was fairly flat, hence the 10%. We have clarified this in line 120.

139: not sure what you mean by ‘full names’?
We meant the inclusion of the author and year. Changed to “see Lamas (69) for a list of taxonomic authorities for these species” for clarity.

168: different in what, taxonomic composition presumably?
Agreed, changed to: “different in their structure and composition”.

170: ‘occurrence’ (which suggests presence/absence) or abundance?
We meant ’abundance’. Changed accordingly.

180: ‘To test whether Nymphalidae species had a relationship between flight height and the ratio
of wing area (WA) to thoracic volume (TV), as hypothesized by...’
Agreed. Changed accordingly.

183: what do you mean by ‘visually classified’?
Since we observed two clear distinct trends when plotting wing area vs. thoracic volume, we decided to test stratification as a discrete binary variable for this analysis, to ask whether a preference for canopy or understorey was associated to each trend.

210: ‘We analysed our data in a phylogenetic context to account for the influence of phylogeny
on traits and resulting potential loss of statistical independence among our species data points
(77-78)’
Agreed. Changed accordingly.

211-212: Not clear what you mean by using published data as the backbone. Do you mean you

constructed a tree topology at the generic level, for example, based on published data, and then
used this to constrain the COI tree?
Correct. We rewrote the "Accounting for phylogenetic bias" section to better explain our approach. Here we present novel nucleotide data that make it possible to expand hugely the number of species under consideration. However, we need to account for sometimes introduced with the use of fast-evolving mitochondrial sequences. Thus, we use a backbone topology, which constrains 21 out of 65 splits, to reflect the relatively well understood higher level systematic relationships. The fact that our phylogenetic estimates are based only on the mitochondrial sequence is an obvious limitation of the study: however, no nuclear marker sequences (e.g. Wg, EF1a) are presently available for the newly included species.

Additionally, we have added a file in the Supplementary section to describe the calibration points used in the tree ultrametrization.

213: should provide evidence that the sequences are deposited in a public database, e.g. BOLD
or Genbank, with accession numbers, and sample voucher data.
Agreed. The sequences (with their respective metadata) were deposited in GenBank (SUB7771288, we are awaiting confirmation of individual sequence numbers) and the phylogeny was stored in TreeBase (Submission 26598). This information has been included in the Data Accessibility section.

215-216: were there non-protein coding sequenes among your COI sequences? Otherwise, why
do you need to say that you used protein-coding COI sequences?
Agreed. There were not. Changed accordingly.

262-263: would be nice to clarify here what these results mean, e.g. a negative association between stratification and WA:TV means that species with relatively small wings and large thoraces tended to occur in the canopy.
Agreed. We added "In other words, butterflies in the canopy have relatively larger thoraxes and smaller, more elongated wings than understorey butterflies".

271: 'strong vertical stratification'
Agreed. Changed accordingly.

272-274: This sentence is unclear. 1. Are you saying that the phylogenetic influence on stratification was lower for species and subfamilies in Ecuador than it was for genera in Costa
Rica? Or that it was similar in Ecuador and Costa Rica for species and subfamilies, but lower for
genera? Needs clarification to understand the next few sentences explaining these results.

2. I can see how there would be less phylogenetic influence for species than genera, but not how the
result would switch with subfamilies, i.e. one would expect a general trend from lower to higher
taxonomic levels.

1. Indeed, we meant the phylogenetic influence on stratification was lower for species and subfamilies in Ecuador than it was for genera in Costa Rica. To better clarify this, we

modified the sentence as follows: "the phylogenetic signal of stratification was lower than reported by Fordyce & DeVries (50) in Costa Rican Nymphalidae, in a study done at the genera level".

2. Yes, one would expect a trend from lower to higher taxonomical levels. The lower the taxonomical level (i.e. subspecies/species), the more similar they are. We deleted the phrase "and subfamilies" to avoid confusion.

We think another problem is that we are tackling two different ideas in the same paragraph (i.e. phylogenetic signal of stratification only, and the relation stratification-morphology). We have separated these sentences in distinct paragraphs. We have also changed 'among' to 'within' in the second paragraph for greater clarity as follows: "Within Morphini and Haeterini butterflies, a relation between sex-specific behaviours, flight height and wing morphology".

282-283: 1. Alternatively, selection on morphology (e.g., for interactions among territorial males,
foraging, avoiding predation) may lead to preferences for microhabitats that enable, for example,
faster flight. Also discussed later, lines 299-300.

2. Throughout, I think it's clearer to talk about associations among flight height, resource availability, life style and morphology, which doesn't imply which of these traits is driving the others – I think one can make an argument that selection on any of these traits may have an impact on the other traits.
1 We agree that there are probably many factors, different to each species, that contribute to shape morphology (e.g. territorial behaviour, mimicry, foraging preferences, etc.). We switched the order to ensure these other factors are discussed first (lines 287-294). But our point after (lines 294-300) is that we found a pattern that is common to a whole community, which suggests that there is another layer on top of the specific factors. We have also changed "This suggests that microhabitat preference may drive, at least partially, the evolution of flight morphology" to "This suggests that microhabitat preference may be an important factor contributing to the evolution..." We think this explains better our idea.

2. We agree. We have changed the words 'drive' and similar to 'associated' throughout the discussion.

316: 'high performance in environments'
Agreed. Changed accordingly.

319: a large thorax also involves allocation of resources for growth during larval stage and presumably a greater supply of nutrients as an adult.
We agree, and as we cited in the introduction, about 96% of the thorax is flight muscle. We measured 10 individuals of each species to account for intraspecific differences.

320: 'less energetically expensive'
Agreed. Changed accordingly.

330-335: Did this study control for phylogeny? If not these results could reflect major differences among subfamilies, for example.
Agreed. Yes, they controlled for the phylogeny. We have clarified this (line 335-336).

350: instead of 'sprightly' (an adjective), you could perhaps use 'nimbly' or 'agilely'.

Agreed. Changed accordingly.

357-360: Again, I think you could perhaps expand on the potential role of shifts in the vertical
dimension in terms of speciation – ranging from the Nice et al. (2019) paper which shows possible incipient speciation, to examples of closely related species that differ in flight height,
morphology and resource use – Colobura dirce/annulata was hypothesized to be an example,
although your data do not support differences between these species. But you could also highlight some interesting examples in your own dataset, for example Heliconius (and discussions about vertical stratification of mimicry rings), Pyrrhogyra (amphiro/crameri and
otolais occur in differing strata and are morphologically distinct, as predicted by your overall
models), Archaeoprepona – any of these could be the focus of a future study of the selective pressures that lead to flight height and morphological divergence. Furthermore, although there
are obviously some interesting examples of shifts between closely related species, there is also a
strong phylogenetic influence on both stratification and morphology, which suggests that in general it's relatively difficult to shift in flight height (or speed) because of associated other traits. Thus, those lineages that do manage to move into a new vertical stratum may find opportunities for adaptive radiation.
Thanks a lot for walking us through these interesting papers and ideas. We have modified our second paragraph in the discussion to include these points and expand a little in the potential role of stratification in adaptive radiation.

Appendix S1
Hermeuptychia hermes is almost certainly a complex of species in the study area and 'hermes' is
unlikely to be the correct name. I would suggest excluding the genus from the analysis.
Good point. Thank you for this information. We have changed the name to *Hermeuptychia sp*. to account for the mentioned taxonomical caveat. However, we do not agree with the exclusion. Even if it is not the correct name, we think it is still convenient having a representative of this genus in the analysis, since it's the only one we have. The hierarchical manner in which PGLS models work may well account for the inclusion of a species complex.

Referee: 2

Comments to the Author(s)
This paper documents the differences of the allometric relationships of various wing dimensions of tropical Nymphalid butterflies in relation to the vertical distributions of their flight activity in lowland neo-tropical forest. As such, this raises interesting questions about the evolutionary drivers of wing size, shape and wing loading in butterflies. However there are parts of this manuscript where greater clarity, explanation and justification is needed.

The introduction describes the hypotheses being studies, but these are not fully explained by the bulk of the introduction. 1 Whilst light, humidity, wind and temperature do vary with height within forests, the allometric relationships and flight performance are hypothesised to be related to physiological constraints, but these constraints are not explained. Likewise wing area to thoracic volume is predicted to be higher in lower strata flying butterflies than canopy flying butterflies to accommodate optimal flight performance, but how is optimal

defined here? 2 Butterfly flight is not just constrained by environmental parameters and physical structure, wing shape and wing loading are also influenced by other aspects of biology, including predator avoidance and the type of mate locating mechanisms employed. The paper would benefit from a consideration of this in the introduction as well.
We agree. We have changed the introduction to address better these points.
1. It is explained that light and temperature influence insect metabolism, affecting wingbeat frequency. Therefore, a relation between stratification and wing-area/thoracic-volume is hypothesized.
2. Yes, we discuss that there are aspects of biology that influence butterfly flight in different manners depending on the species. However, we also note that the fact that we found a community-level trend suggests that microhabitat preference is an important factor (among others) shaping flight morphology.

Sampling was by baited traps at two heights within the forest, and various wing and thoracic parameters obtained for the species occurring at these two heights. Some species were retained for identification and other released and marked. It is stated that mark-recapture data for the released butterflies were included in the analysis, but by not doing this for specimens that could not be easily recognised, the data for the later will be occurrence data for first (and only) capture but for the former might included re-occurrence. Greater explanation is needed as this is not a balanced design.
For those species of which we had a large number of specimens in our collection and their taxonomy is stable we stopped collecting and started to mark and release them. The mark-recapture data were included but only once, i.e. the first time a specimen was captured and marked. Therefore, re-occurrences were not included (that was considered for our own). We thought it was important to mention that not all specimens were need to be collected. However, we have deleted those two sentences since they seem to confuse more than what they help.

Digital images and thoracic measurements were used to obtain the primary morphometric data. Was a calibration rule used in this? Whilst wing area and centroid measurements were done with closed wing specimens more explanation is needed here for three reasons. First, it has to be assumed that all of the butterfly species here, from different subfamilies, actually fly with the angle between fore and hindwing constant in flight. This is not necessarily the case, some species fly with the leading wing edge of the forewings further forward than others, effectively giving a range of wing areas used in flight than would not be calculated here. Secondly some species decouple the fore and hindwings in flight, especially on 'power' strokes, again influencing wing area. The centroid position may thus be difficult to calculate as well. More explanation is needed in the methods section to explain why all species are therefore measured in exactly the same way.
These are good points indeed. However, all the specimens need to be photographed with a reference to be able to be measured by the software. Otherwise it is impossible to obtain such measurements.

To control for different wing coupling at flight we separated the wings until the scale melanisation marks. Thus our statement "with the wings closed and the forewing and hindwing overlapped in a position similar to that seen during flight". To clarify we have included: "using scale melanisation as proxies" in line 143. Additionally, we changed our figure in Appendix 3 to include a photo of wing couplings and the mentioned scale markings, and included a more specific description.

Regarding the argument of possible changes on wing coupling at flight, we agree, and acknowledge that we assumed a constant wing coupling at flight in our approach. We have also modified our discussion to avoid stating strong conclusions on flight ability of

butterflies and include the considerations of alternative mechanisms to flight performance. For example, we included in lines 354-360: "Nevertheless, other morphological traits, such as having a dark, hairy and large absolute thorax (to dissipate heat more slowly), combined behavioural strategies, such as a short flight duration, shifts in wing coupling, or basking at gaps, may support high flight performances in understorey butterflies too (124-125)."

Unfortunately, it is extremely difficult, if not impossible, to know the exact wing placement at flight for several reasons: first, different species may use different types of flight behaviours at different degrees, which may imply different wing placements. Such behavioural information is not available at all for neotropical and Chocó butterflies. We agree some species might have slight changes in wing coupling at each moment of wingbeat that could only be correctly measured using a set of high-speed cameras and computer modelling, species by species. Even if we could go to the field with such an equipment it would be impossible to capture footage of 99% of the species in free flight. We need to consider that half of them fly almost exclusively at the canopy. It took us 5 years to gather our full dataset. An alternative might be using the captured butterflies (not free flight), but handling the butterflies to a studio would produce a larger set of problems (unnatural or escape flight, different microclimatic conditions, etc).

The whole idea of using morphological parameters to describe flight is certainly a simplification, in the sense that it is likely that different species may have details in their flight modes that are not accounted by the model. Some details probably remain to be discovered. However, the parameters we used are standards for the flight-morphology studies that we are aware of, and do represent general principles of aerodynamics. Therefore they tell us something about the way butterflies flight. This is noted by many of our references, some of which tested the validity of using such parameters across a wide set of taxa (birds, bats, aphids, dragonflies, flies, butterflies -please see references therein-). For butterflies in particular, there are two recent reviews that broadly support the use of morphology to access ecologically functional traits: Le Roy et al. 2019 (Adaptive evolution of butterfly wing shape: from morphology to behaviour. Biological Reviews of Cambridge Philosophical society 94(4),1261-1281) and Rossato et al. 2018 (More than colours: An Eco-Evolutionary Framework for Wing Shape Diversity in Butterflies. Adv.Ins.Phys. 54: 55-84).

Data analysis

Whilst the analysis is robust and appropriate, there is one aspect that is pertinent to the discussion. Flight ability, especially the ability to manouver and accelerate is also influenced by wing length to wing area ratio, especially of the forewing. Whilst this is in part covered by the distance to centroid measure, this ratio could be incorporated into this analysis as well, it might strengthen those parts of the discussion that consider flight 'agility'.

Yes, we agree. However, the parameter you are describing has been already included in the analyses. It is called the Aspect Ratio (AR) and is considered in the Introduction and Discussion sections.

Figure 3 – it is not clear why just six species on the figure are named.

Agreed. These are the six species with the strongest preference for canopy or understorey according to the Bayesian analysis. They were mentioned in the Results section, therefore we decided noting these names, so readers can quickly have an idea of what their morphology looks like. We added a note in the figure to clarify this.

Figure 4c -with no significant relationship a 'best fit' slope should not be fitted as the slope is not significantly different to zero.

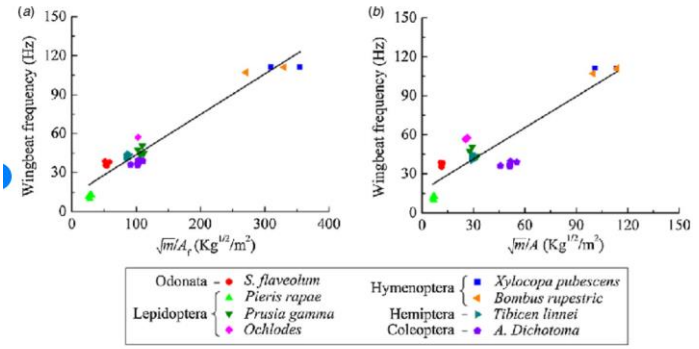
Agreed. Modified accordingly.

Discussion

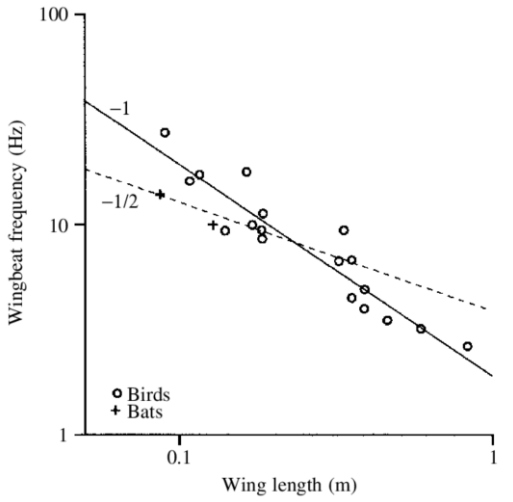
Line311-315 The point is made that there is a relationship between wing beat frequency and wing size, with reference to within-species effects. Whether this argument is transferable across-species is debateable.

This is a good point, however we have found a number of papers supporting the relation between wing size/mass and wingbeat frequency within and across species and across different taxa. Here are three examples from [Ha NS, Truong TGN, & Park H. (2013). Relationship between wingbeat frequency and resonant frequency of the wing in insects. Bioinspiration & biomimetics. 8. 046008. 10.1088/1748-3182/8/4/046008.], [Berg, C. & Rayner, Jeremy. (1995). Moment of inertia of bird wings and the inertial power requirement for flapping flight. The Journal of experimental biology. 198. 1655-64.] and [Van Roy, J., De Baerdemaeker, J., Saeys, W., & De Ketelaere, B. (2014). Optical identification of bumblebee species: effect of morphology on wingbeat frequency. Computers and Electronics in Agriculture, 109, 94–100. doi:10.1016/j.compag.2014.09.014].

We have added these references in the introduction to highlight this relation.



(a) Relationship between wingbeat frequency and both body mass and forewing area (y = 0.3139x + 12.282, r 2 = 92.8%. (b) Relationship between wingbeat frequency and both body mass and total wing area (y = 0.8043x + 17.147, r 2 = 79.8%).



Wingbeat frequency versus wing length. For comparison, the slopes of two predicted relationships (Hill, 1950; Pennycuick, 1975) between frequency and wing length (1 and 1/2) are indicated. Note the double logarithmic scale. Frequency data from Rayner (1988) and J. M. V. Rayner (unpublished results).

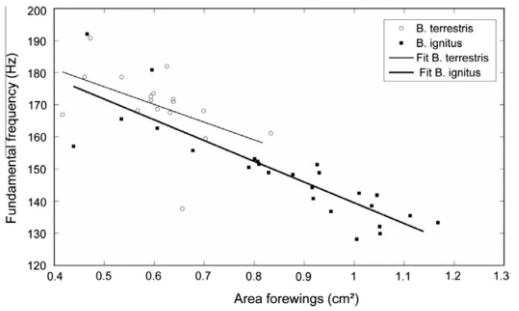


Fig. 5. Scatter plot of the fundamental frequencies vs. total area of the forewings measured for the *B. ignitus* (squares) and *B. terrestris* (circles) species together with the linear trend line.

Line318-322 Wing beat frequencies of the studied species are not know, but more importantly arguments about gliding versus flapping flight and the energetics of either are also need to take into account the duration of flight. Short duration flapping flight is energetically expensive and in some species this is compensated for by adjustments of body temperature. When not in flight made by basking position and posture. Gliding may facilitate long duration flights after basking. Additionally, arguments made later about thermal requirements also need to take into account wing colouration, not just haemolymph flow through the wings.

We acknowledge that the wingbeat frequency of our species was not tested. However, as noted in the previous point, there is strong evidence of a correlation between the wing area to thoracic volume ratio and wingbeat frequency. We have modified our introduction and discussion to better address this.

As mentioned before, such a behavioural dataset would be very much appreciated, but unfortunately, this information is not yet available. Apart from *Morpho* and maybe *Heliconius*, Nymphalidae butterflies in rainforests are very hard to observe. Again, half of our species are canopy specialists. This lack of knowledge is highlighted in the reviews cited above (LeRoy et al. 2019 and Rossato et al. 2018). However, we thank you for this great suggestion, and have mentioned the potential advantages of a switch in flight duration in the second-to-last paragraph.

We agree that colouration is a remarkable and interesting topic. We have included considerations of its potential role in lines 357-361. Unfortunately, fully addressing coloration would require specific analyses and careful considerations, since coloration accomplishes other functions apart from thermoregulation, such as interspecific and interspecific communication (sex attraction, predator avoidance via mimicry or camouflage, etc). Furthermore, it is still unclear how different colours and the overall colour patterning affects thermoregulation. Therefore, colour analysis was out of the scope of our paper.

Line 322-323 1. It would help the reader if the results also included a summary of data on wing area.

2. A large wing area in relation to thorax size will necessarily mean that sustained flapping is not as efficient as in a butterfly with a larger musculature (i.e. bigger thorax), but a relatively small thorax size will enable more rapid heating at rest than would a large thorax.

3. Therefore there needs to be analysis of flight durations and resting durations to fully explore this topic.

1. The data on wing area calculations are available in figure 2 (along with the phylogeny) and in the supplementary material.

2. Yes, a low WA:TV helps flapping flight. A relatively small thorax size will enable more rapid heating at rest than would a large thorax if heat availability was constant, but it is not. Understorey butterflies have small thoraxes and also low heat availability, which means less metabolic capacity.

3. As mentioned above, flight/rest durations data is unfortunately not available. However, we understand its importance and have mentioned it in the Discussion. We agree that we have not fully explored this topic. However, we have extensively reviewed available literature and expanded our discussion to mention these limitations.

In summary, there is interesting data here, but there needs to be a more precise focus in the discussion and further relevant data, which is available, needs presenting in the results section.

Referee: 3

Comments to the Author(s)
The authors present an interesting and thorough study of wing morphology measurement from a group of butterflies captured during a long term study at a site in the Ecuadorian rainforest. The analysis uses appropriate statistical methods accounting for phylogenetic signal and yields highly significant results, likely due to the large sample size of individuals and species.
The authors conclude "that butterfly habitat preference for canopy and understorey is associated with a set of morphological flight traits. This highlights the importance of vertical dimensions in the generation of morphological and ecological diversity." These statements are well supported. However, there are large sections of the discussion that are more speculative and could be de-emphasized.

The introduction is quite expansive in its discussion of previous work showing morphological differences in features that might relate to flight performance. This could be reduced a little and I would recommend shifting those words to a more complete explanation of the hypotheses (Line 102 onward). Each hypothesis needs an explanatory sentence explaining why this is a likely relationship, since they do not all relate particularly well to the preceding text.
For example, "We hypothesized that butterflies in the canopy had a hyperallometric slope compared to understorey butterflies due to physiological constraints exerted by stratification." This is too vague. Hyperallometry of which relationship? Which physiological constraints, specifically, and how would they drive the change in slope?
When variation in the gradients of the allometric relationship were found, the meaning of the slopes (an interesting finding) were not explored in the discussion.
Agreed, we have reduced accordingly, and expanded in the explanation of hypotheses: "We hypothesized that butterflies in the understorey have a hyperallometric slope of wing areas and thoracic volumes compared to canopy butterflies, due to a more restricted metabolic activity driven by lower temperatures at the understorey (65). Consequently, we predicted an evolutionary trend between microhabitat specialization and morphology: as conditions in the canopy are favourable for higher beat frequencies than in the understorey, an increasing preference for the canopy was hypothesized to be associated to butterflies with a decreasing wing area to thoracic volume (i.e. extended use of fast flapping-flight for the canopy butterflies and slower gliding for the understorey). Because this may restrict

understorey butterflies in their performance in terms of speed and manoeuvrability, we also hypothesized that understorey butterflies may have shifted their wing shape as a means of compensation, towards higher aspect ratios and shorter distances to the wing centroid (wings more elongated, wider towards the tip) compared to canopy butterflies."

Line 107-8. It is not immediately obvious how the butterflies could have higher aspect ratios AND a shorter distance to the wing centroid. This implies a more complex change in shape and needs greater explanation of i. what that might be and, ii. what the effect of that might be. This is important because there is a secondary question relating to how flight performance might change with respect to that shape change. i.e. is it due to the change in wing length, wing chord, aspect ratio, or the change in plan form.
Agreed. The aspect ratio describes wing length with respect to an area, while the centroid describes the distribution of this area along the wing length. For example, two wings that have the same AR (same wing length and wing area) can differ in their RDC if the shape changes (trapezoidal vs. rectangular). We added "wings more elongated, wider towards the tip" to illustrate concern i.
ii. We have expanded on how these morphologies affect flight performance in the first two paragraphs of the Introduction. The effect of a high aspect ratio is creating less induced drag, therefore economizing power at gliding. A high RDC minimizes power consumption in flapping flight, and aids agility. Powerful thoraxes can help performing complex manoeuvres that rely on the use of unsteady flow induced by flapping flight.

"Allometry ensures that correct body proportion is maintained across taxa despite differences in body size (22–23)" I don't much care for the wording of this. One could argue that allometry means that body proportions are NOT maintained. I.e. parts are proportionally different. Also, "ensures" leads to the suggestion of purpose. Allometry is a result, rather than a design goal.
We agree. To clarify, we changed the text to: "It can describe how different spatial niches can exert distinct selective pressures on flight behaviour, leading to changes in body proportions".

Line 155. Since the ipsilateral wing pairs are treated as one, I am not sure why the aspect ratio calculation is a 4 rather than a 2?
Agreed. This is a typing mistake. We had originally doubled the wing area for this calculation (therefore $AR=4fwl^2/2wa$) following Betts & Wooton (1988), effectively, the same as not doubling the area and doubling the fwl. Corrected accordingly.

Line 176. Is it not possible to include trap site location in the statistical model to use more of the data?
Unfortunately, not for this analysis. However, there is abundant evidence of vertical stratification in tropical rainforests, and our analysis helps illustrating this for our study site. This choice has no implication in the subsequent analyses.

Line 210. non-independence.
Agreed. Changed accordingly.

Line 262. It would be helpful to reiterate what negative and positive mean here with respect to stratification. I presume larger stratification means higher level?
Agreed. We included "In other words, butterflies in the canopy have relatively larger thoraxes and smaller, more elongated wings than understorey butterflies".

Line 314. This is not a law that is set in stone; there are other Lepidopterans that have not shown a wing beat frequency response to wing wear. E.g. Hedrick, Fernandez papers on Manduca.
Agreed. Thank you for recommending these papers. From what we have read, Fernandez et al. (2017) do find a significant effect in both maximum load lifting and hovering efficiency in *Manduca*. However, we found two citations in one of his papers about not significant effects (bee and hummingbirds). We therefore changed to "A reduction in wing area is typically compensated with an increase in wingbeat frequency (116)".

Line 311-313. This is incomplete. The compensation could also come from e.g. increased forward flight speed or greater wing sweep amplitude.
Agreed. We changed this section to acknowledge that there may be other mechanisms such as different wing coupling, larger total thoracic mass, with hair or dark colorations.

Line 317. 1. While it is worth a passing comment in the discussion, I am not convinced of the available heat argument. Several other factors could be at play here. E.g. Larger thorax (absolute) might dissipate heat more slowly; a hairier thorax might retain heat longer; a darker coloured thorax might absorb heat more readily; flight itself generates heat by the muscle action.

2. I can't see sufficient new data in this work to draw a firm conclusion.

3. Certainly, the statement "understorey butterflies may be incapable of high-performance flight because of relatively low heat availability; having a large thorax would be impractical because it would add useless weight" seems to go way too far considering there are plenty of 'high performance' butterflies in temperate regions. Would a larger muscle mass be useless weight, especially given the self-generated heat?

1. We agree. we added a mention to the factors you suggest in the second-to-last paragraph.

2. We agree. We acknowledge in lines 357-362 that further research will help drawing a firm conclusion.

3. We agree. We deleted the cited phrase.

Line 322 seems to neglect the additional force required by the flight motor to drive these larger wings. This line of reasoning is incomplete, at best.
We actually took into account your consideration. Precisely because there is need of additional force, the wingbeat frequency must be reduced, therefore, the incremented use of gliding flight. In addition, larger wings may also help increasing body temperature (lines 329-332).

Line 344. 1. I'm not yet sure of the imperative for improved flight performance at higher altitudes. Why is lower flight performance acceptable lower down?

2. Is this based on a notion of avian predation in the canopy? If so, how reliable is this as a selection pressure? What is the measure of flight performance (speed; tangential acceleration; hovering performance; manoeuvrability; agility)? I think there could be some clarification of this paragraph.

1. We agree indeed. We have changed this line to avoid such conclusion (lines 351-353) and added other possibilities through which understorey butterflies may reach high performance flight: "other morphological traits, such as having a dark, hairy and large

absolute thorax (to dissipate heat more slowly), combined behavioural strategies, such as a short flight duration, shifts in wing coupling, or basking at gaps, may support high flight performances in understorey butterflies too (124-125)".

2. We stated what we meant by high flight performance in line 350-351: "high performance in terms of speed and manoeuvrability". This is based on the predictions of flight morphology in flight performance. The notion of higher selection pressure towards the canopy is only one explanation for the inferred pattern.

Appendix C

Dear Editor,

We would like to thank the reviewers and editor for their suggestions. We have implemented almost all of the reviewers' proposed changes. These helped us to improve the clarity of our manuscript.

A 'tracked changes' version of our manuscript is included below.

Associate Editor
Comments to Author:
Dear Dr. Mena,
Thank you for submitting a thorough revision of your manuscript entitled "Forest stratification shapes allometry and flight morphology in tropical butterflies" to the Proceedings of the Royal Society. I have received two peer reviews, both of which are supportive of the manuscript and overall satisfied the revisions.
Reviewer 1 would like to share a few more suggestions to further improve the clarity and content of the manuscript. Please consider those comments and implement those suggestions that you feel improve the manuscript.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s)
I thank the authors for the detailed responses to my previous comments, which has made the review process of the resubmission relatively straightforward. The manuscript is much improved, and the key arguments made are much clearer. In particular the introduction is more focused and the hypotheses more clearly expressed and the discussion (supported by the data is very focused and more evidence based than before. The manuscript warrants publication. There are some minor typographic issues that can be easily resolved, save for one comment on the abstract. These are given below. To aid the authors please note that I added continuous line numbers to the text supplied with the author responses and started the line numbering with the first line starting at 'Abstract'

Line 4 change 'steeper' to 'large'

Line 11 As the manuscript does not directly refer to life histories and metabolic rates in the data it might be better to change this sentence to 'We hypothesized that species show morphological adaptations related to differing micro-environments associated with the canopy and understorey.'
done

Line 31 '…better manoeuvring during flapping flight'
done

Line 47 it might be better to state 'increases inversely with organism size'
done

Line 64 ‘sensitivity’ not ‘sensibility’
done

Line 71 replace ‘ microhabitat they lived might’ with ‘microhabitats used’
done

Line 85 “…higher wingbeat frequency…’
done

Line 161 ‘2/3’ not ‘2:3’
done

Line 175 add ‘a’ after ‘finding’
done

Line 290 ‘..strata at the same location…’
done

Line342 ‘.. combined with behavioural..’
done

Line 343 ‘.. basking in sunlit gaps…’
done

Referee: 1

Comments to the Author(s)
The authors have addressed all of my main concerns from the first round of review and I think the manuscript has been significantly improved. I made some more stylistic comments on the paper, and some additional comments where some areas were still a bit unclear. Please see attached comments on the paper.
done

Referee: 3

Comments to the Author(s)