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Predaceous *Toxorhynchites* mosquitoes require a living gut microbiota to develop

Kerri L. Coon, Luca Valzania, Mark R. Brown and Michael R. Strand

Article citation details
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Review timeline

Original submission:	1 July 2019
1st revised submission:	12 July 2019
2nd revised submission:	19 November 2019
3rd revised submission:	19 December 2019
Final acceptance:	20 December 2019

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

Review History

Decision letter (RSPB-2019-1552.R0)

02-Jul-2019

Dear Dr Coon:

Thank you for submitting your manuscript RSPB-2019-1552 entitled "Predaceous *Toxorhynchites amboinensis* mosquitoes require a living gut microbiota to develop despite dramatic differences in their life history" to Proceedings B.

All manuscripts are assessed by a specialist member of the Editorial Board, who decides whether the manuscript is suitable for Proceedings B.

Unfortunately, your manuscript has been rejected at this stage of the assessment process. Competition for space is currently extremely severe, and we receive many more manuscripts than we are able to publish. On this occasion it was felt that your manuscript was unlikely to be able to compete successfully for a space in the journal.

Please find below the specialist Board member's comments. I hope you may find these useful should you wish to submit your manuscript elsewhere.

Sincerely,
The Proceedings B Team
mailto:proceedingsb@royalsociety.org

Board Member
Comments to Author(s):
This manuscript addresses an interesting question that would be of broad interest. Unfortunately, the methodology of the study is flawed. The study is about gut microbiota, but the samples used for microbiota analyses are whole animals, rather than guts. This is acknowledged by the authors in the discussion, and they also acknowledge that they cannot prove that their results truly reflect only gut microbiota. As such, in my opinion, the results and conclusions drawn from them are not sufficiently robust for this journal.

RSPB-2019-1651.R0

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?
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?
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 manuscript entitled "Predaceous Toxorhynchites amboinensis mosquitoes require a living gut microbiota to develop despite dramatic differences in their life history" explores the necessity of bacteria in the prey for development of the predator. The authors have previously shown that living bacteria are indispensable for development of mosquitoes, although this has recently been questioned (Correa MA, Matusovsky B, Brackney DE, Steven B. 2018 Generation of axenic Aedes aegypti demonstrate live bacteria are not required for mosquito development. Nat. Commun. 9, 4464.). In this paper, the authors show convincingly that living bacteria in the prey are indeed indispensable for development also of this predatory species of mosquito.
The manuscript is well written and I only have a few comments that I give in order of appearance:
L2-3: the title is hard to understand, and even after reading the manuscript I cannot understand what the text "despite dramatic differences in their life history" relates to. It could explain better what the manuscript is about. What differences? Whose life history?

L309: perhaps there is a comma too many?

L344: larvae or embryos?

L381-383: either "A fundamental question of interest moving forward is: what features of mosquito life history have selected for this dependency?"
OR
"A fundamental question of interest moving forward is what features of mosquito life history have selected for this dependency."

Fig. 6. : I believe the bar for axenic larvae should be grey and not black.

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions 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?
Acceptable

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
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Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
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Is it accessible?
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Is it adequate?
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Do you have any ethical concerns with this paper?
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Comments to the Author
In this manuscript, the authors present a series of laboratory experiments on predator-microbiota interactions in which they demonstrate the importance of gut microbiota for the development of the predatory mosquito species Toxorhynchites amboinensis. The main findings of the study are that gut bacteria-free T. amboinensis larvae fail to develop to the second larval stage. In contrast, larvae containing only a single strain of gut bacteria develop as quickly to the last instar as larvae with the full set of gut bacteria. The authors come to the conclusion that mosquitoes need living bacteria for their development, but are not dependent on specific bacteria. Measurements of hypoxia-inducible transcription factors with antibodies indicate gut hypoxia as the underlying mechanism. Metabarcoding of the gut microbiome of T. amboinensis and its prey Aedes aegypti show that gut microbial communities of predators are likely to be acquired through feeding interactions.
Overall, I found the manuscript a pleasant read and learned a lot from it.

My major concern with this manuscript is the missing information on statistical methods and number of replications. A final evaluation of the data analysis is therefore not possible at this time. In addition, the methods and results sections are poorly structured and coordinated, making it difficult to keep track of the individual studies. After a thorough revision of the methods and results section, especially a better and more easily comprehensible documentation of the statistical analysis, the manuscript could result in an interesting publication for Proceedings B - provided the data analysis turns out to be sound!

Let me list my major concerns below, followed by some more minor points.

1) Structure: The manuscript contains a series of different studies and measurements that build on each other and shed light on the different aspects of the interplay between food intake,

microbiome and the development of T. amboinensis. Restructuring the methods and results would make it easier for the reader to keep track.

A lot of information that belongs in the method section can be found at the beginning of each subsection of the results (examples are given below). I would suggest that these parts be moved to the methods. The methods should be further restructured to the order in which the results are described. Headings that clearly identify the different measurements/experiments would further facilitate the connection of both parts.

In the methods it would be helpful to distinguish between the preparations and the actual experiments/measurements. The preparations could include: the preservation of the colonies (a) and the preparation of the axenic prey (c) and T. amboinensis (first part of (d)). The actual experiments/measurements include: b, second part of d, e.

2) Replication level: Without looking at the R-script and the data stored in Dryad, it is completely unclear how many replications there are per treatment in each study/measurement. Again, the figures contain some information but often not really specify the exact number of replications which differs between treatments. The quality of the data and results cannot be estimated for the reader in this way. Please insert this information for each dataset!

True or pseudo-replications and repeated measurements of the same individuals: From the information in the manuscript and supplementary material I cannot tell whether all measurements are true replications or whether some data sets contain pseudo-replications (e.g. larvae of the same cohort) and whether this has been taken into account in the statistical analysis. Furthermore, in some data sets it is unclear whether the same individuals were measured several times or whether different larvae were measured.

3) Statistical analysis: The description of the statistical analyses is almost completely missing in the manuscript. The files in Dryad show that R and R-Studio were used, but this is not even mentioned in the manuscript. Which versions of R and R-Studio, which R packages (please also mention in the script!) and which functions were used for statistical analysis? For what reasons were the corresponding tests chosen (e.g. Wilcoxon, because the data is not normally distributed)? In the manuscript, information about the statistical tests can only be found in the captions. However, this does not replace a clear and structured description of the statistical methods used for the individual data.

4) Scope: It would be interesting to extend the scope in the discussion from mosquitoes at least to other insects. Are other taxonomic groups known to be dependent on living microbiota for larval development?

Line 105 – 110: Please move the first two sentences from the supplementary material here. Otherwise it is unclear what the "replicate trays" are and how many larvae were used. I first thought you meant the 20 trays mentioned in line 97, but in the supplements you mention six replicates (a subset of the 20?). I would prefer to find a little more, if not all, information from the additions here, unless you are already close to the word limit / length restriction of the manuscript. But at least quote the authors who developed the primers.

Line 114: Please provide any important information here or in the supplements. Readers may not have free access to your previous publications in Molecular Ecology.

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Line 322 to 323: The finding would indicate that microbiome-induced gut hypoxia is not the only mechanism by which bacteria influence development of mosquitos. It could be interesting to broaden the scope of your study at his point before you start with the second goal. Is it known if other insect orders require living gut bacteria for their development and, if yes, what is the assumed mechanism?

Line 326 to 330: "This information was needed to assess.." I don't think this justification is necessary at this point. You have already specified this in the methods/results and repeat it in lines 334 to 337.

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Figure 2 (b): Consider using box plots in both figures. The number of replications is specified in the text but it . It would be even easier for the reader to grasp this information if the number of

replications were written on or over the bars or box plots. Consider showing the number of replicates on or above each bar chart or boxplot.

Figure 3 (b): Boxplots or bar charts with a y-axis that only shows the range of about 5 to 7 days would be easier to read. The number of replicates vary widely between 30 and 127 (Information taken from R-Script). Why is that so? Consider showing the number of replicates on or above each bar / boxplot.

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Figure 4: For the results shown in Figure 4, a different presentation should be chosen or the figure should be moved to the Appendix. Perhaps you could show the number of individuals (y-axis) over the number of prey items as a continuous variable (x-axis) and indicate different larval stages and treatments with symbols and line types as in Figure 5. This figure does not contain any description replication levels or statistics, is it only for illustrative purpose and not for showing the results of statistical tests?

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Figure 6: This figure could be shown in the appendix.

Decision letter (RSPB-2019-1651.R0)

15-Aug-2019

I am writing to inform you that this version of your manuscript RSPB-2019-1651 entitled "Predaceous *Toxorhynchites amboinensis* mosquitoes require a living gut microbiota to develop despite dramatic differences in their life history" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. Based on their comment's, however, we are willing to consider a further resubmission, provided the points of the referees and the Associate Editor are fully addressed. Please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A "response to referees" document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your "response to referees" comments document.
3) Line numbers in your main document.

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Sincerely,
Victoria Braithwaite

==========
Professor V A Braithwaite
mailto: proceedingsb@royalsociety.org
==========

Associate Editor Board Member, Comments to Author:

You manuscript has received two reviews from experts in the field. While one review is supportive, the other has identified a number of significant issues with your manuscript and analyses as it stands. I have gone through your manuscript again with this review in hand, and find that their questions and points are valid and need consideration and action. In a revised version, you need to have addressed all their points, both in the manuscript itself, and in a detailed cover letter. It is particularly important that you are clear about how your experimental design maps on to sample sizes and to statistical analyses in the methods section. To pre-empt a standard error that authors frequently make in the revision process, if you have to explain something in your cover letter in response to a comment, then that explanation (or a version of it) needs to appear in the manuscript or supplementary files too - if the reviewer queried something, then readers will likely do the same.

=====
Reviewer's Comments to Author:

Referee: 1

The manuscript entitled "Predaceous Toxorhynchites amboinensis mosquitoes require a living gut microbiota to develop despite dramatic differences in their life history" explores the necessity of bacteria in the prey for development of the predator. The authors have previously shown that living bacteria are indispensable for development of mosquitoes, although this has recently been questioned (Correa MA, Matusovsky B, Brackney DE, Steven B. 2018 Generation of axenic Aedes aegypti demonstrate live bacteria are not required for mosquito development. Nat. Commun. 9, 4464.). In this paper, the authors show convincingly that living bacteria in the prey are indeed indispensable for development also of this predatory species of mosquito.
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Figure 6: This figure could be shown in the appendix.

Author's Response to Decision Letter for (RSPB-2019-1651.R0)

See Appendix A.

RSPB-2019-2705.R0

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?
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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?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have adequately addressed the critique and comments raised in the previous round of review. They have restructured the material, methods and results so that the reader can easily link the two parts. It is now more obvious that the experiments were conducted rigorously, with appropriate controls, and replication. The statistical analysis is adequate and the conclusions are drawn appropriately on the basis of the data presented.

I have only one more comment:

Line 26: Since the journal targets a wider audience, you should consider explaining the terms "axenic" and "gnotobiotic" in the summary to readers unfamiliar with microbial research, as you do in lines 85-86.

Decision letter (RSPB-2019-2705.R0)

16-Dec-2019

Dear Dr Coon

I am pleased to inform you that your manuscript RSPB-2019-2705 entitled "Predaceous *Toxorhynchites* mosquitoes require a living gut microbiota to develop" has been accepted for publication in Proceedings B.

The referee has recommended publication, but also suggests 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.

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,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member
Comments to Author:
Thanks for your work on this manuscript, which has been read and approved by the reviewer. I am happy to accept this, and look forward to seeing it come out!

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
The authors have adequately addressed the critique and comments raised in the previous round of review. They have restructured the material, methods and results so that the reader can easily link the two parts. It is now more obvious that the experiments were conducted rigorously, with appropriate controls, and replication. The statistical analysis is adequate and the conclusions are drawn appropriately on the basis of the data presented.

I have only one more comment:

Line 26: Since the journal targets a wider audience, you should consider explaining the terms "axenic" and "gnotobiotic" in the summary to readers unfamiliar with microbial research, as you do in lines 85-86.

Author's Response to Decision Letter for (RSPB-2019-2705.R0)

See Appendix B.

Decision letter (RSPB-2019-2705.R1)

20-Dec-2019

Dear Dr Coon

I am pleased to inform you that your manuscript entitled "Predaceous *Toxorhynchites* mosquitoes require a living gut microbiota to develop" 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

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

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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.

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

Appendix A

RSPB-2019-1651
Response to Referees

Below we write out verbatim the points raised by the two referees. For each referee, we first write out the point they raised (regular font) and then follow with our response and how we have revised our manuscript (bold font).

=====
Reviewer's Comments to Author:

Referee: 1

The manuscript entitled "Predaceous Toxorhynchites amboinensis mosquitoes require a living gut microbiota to develop despite dramatic differences in their life history" explores the necessity of bacteria in the prey for development of the predator. The authors have previously shown that living bacteria are indispensable for development of mosquitoes, although this has recently been questioned (Correa MA, Matusovsky B, Brackney DE, Steven B. 2018 Generation of axenic Aedes aegypti demonstrate live bacteria are not required for mosquito development. Nat. Commun. 9, 4464.). In this paper, the authors show convincingly that living bacteria in the prey are indeed indispensable for development also of this predatory species of mosquito.
The manuscript is well written and I only have a few comments that I give in order of appearance:

We appreciate the referee noting that our results were convincing and that our manuscript was well written.

L2-3: the title is hard to understand, and even after reading the manuscript I cannot understand what the text ?despite dramatic differences in their life history? relates to. It could explain better what the manuscript is about. What differences? Whose life history?

We agree with the referee and have modified the title accordingly.

L309: perhaps there is a comma too many?

We have reviewed this sentence and believe that the use of commas is grammatically correct.

L344: larvae or embryos?

We understand why the referee made this comment, however upon reconsideration we believe that “larvae” is still the most appropriate term given that development of embryos into larvae is completed *prior* to egg hatching.

L381-383: either ?A fundamental question of interest moving forward is: what features of mosquito life history have selected for this dependency??
OR
?A fundamental question of interest moving forward is what features of mosquito life history have selected for this dependency.?

We have modified this sentence to include a colon as suggested by the referee.

Fig. 6. : I believe the bar for axenic larvae should be grey and not black.

The "bar" referenced by the referee was intended to represent a line at the base of the y-axis (since no HIF-α was detected in axenic larvae). We have modified the figure in two ways to avoid confusion: (a) we have decreased the weight of the line so that it does not look like a bar; and (b) we have included labels along the x-axis to distinguish "Axenic" from "Conventional" instead of using different colors.

=====

Referee: 2

In this manuscript, the authors present a series of laboratory experiments on predator-microbiota interactions in which they demonstrate the importance of gut microbiota for the development of the predatory mosquito species Toxorhynchites amboinensis. The main findings of the study are that gut bacteria-free T. amboinensis larvae fail to develop to the second larval stage. In contrast, larvae containing only a single strain of gut bacteria develop as quickly to the last instar as larvae with the full set of gut bacteria. The authors come to the conclusion that mosquitoes need living bacteria for their development, but are not dependent on specific bacteria. Measurements of hypoxia-inducible transcription factors with antibodies indicate gut hypoxia as the underlying mechanism. Metabarcoding of the gut microbiome of T. amboinensis and its prey Aedes aegypti show that gut microbial communities of predators are likely to be acquired through feeding interactions.
Overall, I found the manuscript a pleasant read and learned a lot from it.

We thank the referee for their comprehensive review and are glad that they found our manuscript to be both pleasant to read and informative.

My major concern with this manuscript is the missing information on statistical methods and number of replications. A final evaluation of the data analysis is therefore not possible at this time. In addition, the methods and results sections are poorly structured and coordinated, making it difficult to keep track of the individual studies. After a thorough revision of the methods and results section, especially a better and more easily comprehensible documentation of the statistical analysis, the manuscript could result in an interesting publication for Proceedings B - provided the data analysis turns out to be sound!

Let me list my major concerns below, followed by some more minor points.

1) Structure: The manuscript contains a series of different studies and measurements that build on each other and shed light on the different aspects of the interplay between food intake, microbiome and the development of T. amboinensis. Restructuring the methods and results would make it easier for the reader to keep track.

A lot of information that belongs in the method section can be found at the beginning of each subsection of the results (examples are given below). I would suggest that these parts be moved to the methods. The methods should be further restructured to the order in which the results are described. Headings that clearly identify the different measurements/experiments would further facilitate the connection of both parts.

In the methods it would be helpful to distinguish between the preparations and the actual experiments/measurements. The preparations could include: the preservation of the colonies (a) and the preparation of the axenic prey (c) and T. amboinensis (first part of (d)). The actual experiments/measurements include: b, second part of d, e.

In response to the referee's comments above, we have done the following:

1. **We have restructured the Methods section to explicitly parallel the order of the Results section.**

2. **We have modified the subheadings in both the Methods and Results sections so that: (a) methods related to preparation of experimental materials vs. experimental measurements are clearly distinguished; and (b) the subheadings in each section match.**

3. **We have moved nearly all contextual information provided in the Results section to the Methods.**

2) Replication level: Without looking at the R-script and the data stored in Dryad, it is completely unclear how many replications there are per treatment in each study/measurement. Again, the figures contain some information but often not really specify the exact number of replications which differs between treatments. The quality of the data and results cannot be estimated for the reader in this way. Please insert this information for each dataset!

True or pseudo-replications and repeated measurements of the same individuals: From the information in the manuscript and supplementary material I cannot tell whether all measurements are true replications or whether some data sets contain pseudo-replications (e.g. larvae of the same cohort) and whether this has been taken into account in the statistical analysis. Furthermore, in some data sets it is unclear whether the same individuals were measured several times or whether different larvae were measured.

We agree with the referee that it would be beneficial to include more detailed information regarding replication. We also appreciate that our original manuscript did not clearly describe how we measured larval growth at different time points. In response to the referee's comments, we have done the following:

1. **We have now included a "Statistical Analyses" section in our Methods that provides an outline of the statistical tests used to analyze all of the data produced in the study.**

2. **All figures and tables included in the manuscript and supplementary material now report the exact sample sizes for each treatment group.**

3. **We have modified the Methods section to clearly state that larval growth over time was measured by destructively sampling larvae at random every 2 h. This approach was necessary to obtain accurate body measurements (which requires immobilization or killing of larvae) and to prevent contamination that could influence results at later time points.**

It is unclear what the referee means by pseudo-replication in the context of the current study. As outlined in each section of the Methods, all *T. amboinensis* larvae used in the study were

individually assayed. While it is true that the eggs used for our experiments were derived from adults from multiple larval cohorts (i.e. pupae from multiple rearing trays would have been combined in the same cage for adult emergence and egg laying), we would expect that any cohort effects would be uniformly distributed across our treatment groups and controls and therefore inconsequential to downstream data analyses.

3) Statistical analysis: The description of the statistical analyses is almost completely missing in the manuscript. The files in Dryad show that R and R-Studio were used, but this is not even mentioned in the manuscript. Which versions of R and R-Studio, which R packages (please also mention in the script!) and which functions were used for statistical analysis? For what reasons were the corresponding tests chosen (e.g. Wilcoxon, because the data is not normally distributed)? In the manuscript, information about the statistical tests can only be found in the captions. However, this does not replace a clear and structured description of the statistical methods used for the individual data.

As stated above, we now include a "Statistical Analyses" section in our Methods that includes what version of R we used. We have also modified the R script uploaded to Dryad to include any specific R packages used for our analyses. The statistical tests implemented in this study are standard in the field and we believe that a comprehensive discussion of the statistical theory underlying why these tests are appropriate for the data generated in this study is beyond the scope of this manuscript and the space constraints of Proceedings B.

4) Scope: It would be interesting to extend the scope in the discussion from mosquitoes at least to other insects. Are other taxonomic groups known to be dependent on living microbiota for larval development?

A discussion of our results in the context of several other taxonomic groups is included in the final paragraph of the Discussion.

Line 105 ? 110: Please move the first two sentences from the supplementary material here. Otherwise it is unclear what the "replicate trays" are and how many larvae were used. I first thought you meant the 20 trays mentioned in line 97, but in the supplements you mention six replicates (a subset of the 20?). I would prefer to find a little more, if not all, information from the additions here, unless you are already close to the word limit / length restriction of the manuscript. But at least quote the authors who developed the primers.

We have moved all of the information previously included in the supplementary material back into the manuscript as requested by the referee.

Line 114: Please provide any important information here or in the supplements. Readers may not have free access to your previous publications in Molecular Ecology.

We have now included a section in the supplementary material that describes the egg surface sterilization protocol. We have also included a section in the supplementary material that describes how we: (a) verified the sterility of axenic larvae; and (b) tested for the presence of particular bacterial isolates in gnotobiotic larvae.

Line 124: How many larvae (= number of replicates) were used?

As stated above, all figures and tables included in the manuscript and supplementary material now report the exact sample sizes for each treatment group.

Line 129- 130: Of how many larvae (= number of replications)? Are these the same primers you mentioned in line 107?

As stated above, all figures and tables included in the manuscript and supplementary material now report the exact sample sizes for each treatment group.

It is unclear what primers the referee is referring to in Line 107 (none were listed in the original version of the manuscript). However, this is no longer a potential point of confusion as this section now contains all of the information previously included in the supplementary material (including the primers used for sequencing library preparation).

Line 131-132: How many larvae (= number of replicates) were used?

As stated above, all figures and tables included in the manuscript and supplementary material now report the exact sample sizes for each treatment group.

Line 134-136: How many larvae (= number of replicates) were used?

As stated above, all figures and tables included in the manuscript and supplementary material now report the exact sample sizes for each treatment group.

Line 139: Again, how many larvae in total? How big was the cohort size ? five as described in the supplemental material? Doe the different cohorts have to be accounted for as ?blocks ? in the statistical analysis because they were e.g. measured at different time points (see Festing 2014; Randomized Block Experimental Designs Can Increase the Power and Reproducibility of Laboratory Animal Experiments. ILAR Journal).

As stated above, all figures and tables included in the manuscript and supplementary material now report the exact sample sizes for each treatment group. The term “cohorts” was intended to mean “groups”. All larvae used in the experiment were derived from the same set of surface-sterilized eggs.

Line 151, 187, 226, 261: Species names are not cursive in sub headings of the results part (but in the methods part).

Consistent with the formatting style of Proceedings B, all subheadings are no longer italicized (with the exception of species names).

Line 151-161: That sounds more like methods than results. Consider moving this part to the methods.

As suggested, this section has been moved to the Methods.

Line 139-143: Were the same individuals measured at different times or were always different individuals measured? Perhaps one would then have to consider temporal autocorrelation in the statistical analysis. The data "tox_growth.csv" (from Dryad) show that the number of individuals measured at each point in time is constant in the axenic treatment (always n= 4) and higher, but also

variable in the conventional treatment (n = 4 to 14). What is the reason for the different number of measurements? Why do so many replication measures have exactly the same number? For example, the width of the head capsule is 61 times 378.7878788 ?m and 14 times the same number for the width of the prothorax. This makes it highly unlikely that it is a real measurement!

As stated above, different individuals were measured for each time point. A consideration of temporal autocorrelation is therefore not necessary. There are more conventional measurements because conventional larvae are easier to maintain while axenic larvae are periodically discarded due to well contamination. Similar measurements between replicates can be explained by the following:

1. **Our use of an ocular micrometer, where measurements are recorded as ocular micrometer units (omu) then multiplied by a calibration factor (mm omu^{-1}) to obtain the actual size of an object. This can limit the ability to detect very fine scale differences in length between objects;**

2. **Our experimental design, which standardized prey availability and environmental conditions between individual larvae to minimize inter-individual variation; and/or**

3. **In many holometabolous insects, head capsule width serves as an instar indicator (i.e. we would expect head capsule width to be highly similar, if not the same, between larvae of the same instar). While this has not been exhaustively examined in mosquitoes, our results are consistent with this expectation and our own previous results in *A. aegypti* using the same methods.**

Line 188 to 211: This whole section describes methods, not results. Consider moving this part to the methods.

As suggested, this section has been moved to the Methods.

Line 242 to 249: Also this part does rather not belong in the result section.

As suggested, this section has been moved to the Methods.

Line 262 to 266: Here, too, I find this introduction somewhat too long for the results.

As suggested, this section has been moved to the Methods.

Line 322 to 323: The finding would indicate that microbiome-induced gut hypoxia is not the only mechanism by which bacteria influence development of mosquitos. It could be interesting to broaden the scope of your study at his point before you start with the second goal. Is it known if other insect orders require living gut bacteria for their development and, if yes, what is the assumed mechanism?

As noted above, a brief discussion of how our results compare to what is known several other taxonomic groups of animals is included in the final paragraph of the Discussion. A more comprehensive overview of what is known regarding microbiota function in insects and other animals is beyond the scope of this manuscript and the space constraints of Proceedings B.

Line 326 to 330: ?This information was needed to assess..? I don't think this justification is necessary at this point. You have already specified this in the methods/results and repeat it in lines 334 to 337.

We agree and have removed both sentences.

Line to 351 to 367: You could consider to also include Pekas et al. 2017 (Comparison of bacterial microbiota of the predatory mite Neoseiulus cucumeris (Acari: Phytoseiidae) and its factitious prey Tyrophagus putrescentiae (Acari: Acaridae). Scientific Reports)

We appreciate the referee's suggestion to include this interesting study. However, due to space constraints we have limited our discussion to include only comparisons with other insect predators. A comprehensive discussion of microbiota diversity in arthropod predators more broadly is beyond the scope of this manuscript and the space constraints of Proceedings B.

Figure 2 (b): Consider using box plots in both figures. The number of replications is specified in the text but it . It would be even easier for the reader to grasp this information if the number of replications were written on or over the bars or box plots. Consider showing the number of replicates on or above each bar chart or boxplot.

We have modified Figure 2*(b)* so that the data in both panels are presented as boxplots. The number of replicates is also now reported in the figure.

Figure 3 (b): Boxplots or bar charts with a y-axis that only shows the range of about 5 to 7 days would be easier to read. The number of replicates vary widely between 30 and 127 (Information taken from R-Script). Why is that so? Consider showing the number of replicates on or above each bar / boxplot.

We have modified the range of the y-axis of Figure 3*(b)* as suggested by the referee and the number of replicates is now shown above each bar. Differences in the number of replicates between treatment groups and controls can be explained by: (a) differences in survivorship to the fourth instar, which impacted the number of individuals for which development time was measured; (b) random loss of replicates due to contamination or other factors; and/or (c) differences in the number of plates set up at the start of the experiment.

Figure 4: For the results shown in Figure 4, a different presentation should be chosen or the figure should be moved to the Appendix. Perhaps you could show the number of individuals (y-axis) over the number of prey items as a continuous variable (x-axis) and indicate different larval stages and treatments with symbols and line types as in Figure 5. This figure does not contain any description replication levels or statistics, is it only for illustrative purpose and not for showing the results of statistical tests?

As suggested by the referee, we have moved this figure to the supplementary material.

Figure 5 (a) and (b): Why not use linear regression models to describe the growth over time?

Our primary goal was to compare larval body size between treatments over time (not to compare growth rates between treatments). More specifically, we were interested in assessing: (a) when conventional larvae achieved critical size (i.e. when cessation of growth occurred); and (b) whether axenic larvae achieved critical size. With this in mind, we believe our original approach for analyzing these data was appropriate.

Why are there no standard errors for the last time point? Are all these larvae still L1 or have larvae that molted into L2 also been included in the analysis? If L2 larvae were included, please indicate the timepoint of molting in the figure and /or caption.

Three L2 larvae were included in the analysis. In order to restrict our analysis to larval growth over a single instar, we have removed these individuals and re-made Figure 5. Omission of standard error bars for the last time point was unintended in the original submission and has also been fixed.

Figure 6: This figure could be shown in the appendix.

As suggested by the referee, we have moved this figure to the supplementary material.

Appendix B

RSPB-2019-2705
Response to Referees

We thank the referee for their review and are glad that they found our revised manuscript to have adequately addressed the critique and comments raised in the previous round of review. We are also glad that the referee and Associate Editor have recommended publication of our manuscript in *Proceedings B.*

As suggested by the referee, we have defined the terms ‘axenic’ and ‘gnotobiotic’ in the Abstract of the final manuscript. These changes, along with additional minor edits, are marked as tracked changes in the document below.