

## Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex

Jiangtao Liang and Igor V. Sharakhov

### Article citation details

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### Review timeline

Original submission: 6 February 2019  
1st revised submission: 10 May 2019  
2nd revised submission: 14 June 2019  
Final acceptance: 14 June 2019

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

## Review History

RSPB-2019-0318.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?**

Acceptable

**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**

Some notable sections in the result that require further clarification, consideration or editing:

Results

[Lines 141-142] backcrossing of F1 males is not a strong indicator of male sterility, as their might be behavioural isolation (or prezygotic isolation in F1 males)

[Lines 144] wording in incorrect: move "F1 females are fertile while F1 males are sterile" prior to describing the exception to Haldane's rule, otherwise this sentence reads as the exception.

[Lines 148] wording does not make sense. You found differences between testes morphology/size in one interspecies cross versus its reciprocal?

[Lines 156-158] Why was sperm only observed in 2 day old testes? Do sperm reach full maturation at 2 days? Could this be a sign of rapid aging in hybrid males instead of sterility?

## **Review form: Reviewer 2**

#### **Recommendation**

Accept with minor revision (please list in comments)

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

Excellent

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

Excellent

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

Good

**Is the length of the paper justified?**

Yes

**Should the paper be seen by a specialist statistical reviewer?**

No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**

No

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

**Is it accessible?**

Yes

**Is it clear?**

Yes

**Is it adequate?**

Yes

**Do you have any ethical concerns with this paper?**

No

### **Comments to the Author**

Postzygotic isolation in the form of hybrid male sterility (HMS) is a common isolation barrier between species. HMS is often identified by the inability of males to produce progeny and sometimes further described phenotypically by the observation of atrophied testes or more subtly morphologically normal testes but immotile sperm. Detail cytological characterizations of HMS are rather lacking. I find that the detail characterization at the cytological level of HMS is important to establish the order in which isolating mechanisms arise relative to one another and in providing us with phenotypic and mechanistic clues that can guide searches of speciation genes. This study provides a cytological characterization of defects in sterile male hybrids between species of Anopheles and highlights the possibility of previously unchecked defects in meiosis contributing to sperm abnormalities in sterile males. Overall I find the results of interest and novel considering that there have been very few attempts to conduct this type of work.

I have a few points that will need to be addressed:

My main questions relate to the choice of genes for tests of expression:

In line 216 you describe the phenotype of sterile male hybrids with normal testes as starting meiosis but then switching to a mitotic behavior in anaphase. I am not a cell biologist, but we always teach students that the second meiotic division is like mitosis. Is the phenotype you describe a rather premature entry into the second meiotic division? You test germline-specific genes expression and find nothing wrong, but there is no rationale as to why such genes were chosen. Are there genes known to control cell cycle transitions? If so, is their expression normal?

In figure 4, your cartoon implies the formation of extra spindles. Again, are there abnormalities in

the expression of genes that code for proteins that make the spindle? I think you have a more solid case here for testing beta2-tubulin, than the fact that it is a germline transcript.

I understand your focus is on HMS, but it is rather puzzling that an alteration of meiotic behavior leading to the formation of diploid sperm affects only males. Did you check meiosis in hybrid females? Is it normal? Why would meiosis progress normally in females but be aberrant in males? Something to check or at least to be discussed.

Some minor points:

Lines 13-14: I am not sure what you mean by "at the beginning stages of postzygotic isolation. I suggests deleting.

Lines 40-41: The citation is rather old. Recent work supports premating isolation evolves faster than postzygotic isolation (see for example Turissini et al. 2018). Often premating traits are first to be affected.

Line 119: I suggest you delete "the numbers of"

Lines 142-145: Why "except for crosses between gambiae and coluzii?" It follows Haldane's rule.

## Decision letter (RSPB-2019-0318.R0)

27-Feb-2019

Dear Dr Sharakhov:

I am writing to inform you that your manuscript RSPB-2019-0318 entitled "Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex" 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. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However 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. 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.

To upload a resubmitted manuscript, 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 Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,

Proceedings B  
mailto: proceedingsb@royalsociety.org

Associate Editor  
Board Member: 1

Comments to Author:

The manuscript has been reviewed by two reviewers and myself. While all have expressed interest in these findings, there are some areas for improvement that need to be addressed in order for this manuscript to be publishable in Proc B. In addition to the wording clarifications the reviewers suggest, I agree with the points raised by one reviewer regarding the choice of genes tested for mis-expression and the absence of any tests in females (overall fertile females could still have some gametes demonstrating failures in meiosis). Since addressing these concerns may require time for additional tests to be conducted, I have recommended to reject with the possibility for resubmission.

Additional minor comment:

Lines 279-283 are awkwardly worded. I recommend re-wording.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Some notable sections in the result that require further clarification, consideration or editing:

Results

[Lines 141-142] backcrossing of F1 males is not a strong indicator of male sterility, as their might be behavioural isolation (or prezygotic isolation in F1 males)

[Lines 144] wording in incorrect: move "F1 females are fertile while F1 males are sterile" prior to describing the exception to Haldane's rule, otherwise this sentence reads as the exception.

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[Lines 156-158] Why was sperm only observed in 2 day old testes? Do sperm reach full maturation at 2 days? Could this be a sign of rapid aging in hybrid males instead of sterility?

Referee: 2

Comments to the Author(s)

Postzygotic isolation in the form of hybrid male sterility (HMS) is a common isolation barrier between species. HMS is often identified by the inability of males to produce progeny and sometimes furthered described phenotypically by the observation of atrophied testes or more subtly morphologically normal testes but immotile sperm. Detail cytological characterizations of HMS are rather lacking. I find that the detail characterization at the cytological level of HMS is

important to establish the order in which isolating mechanisms arise relative to one another and in providing us with phenotypic and mechanistic clues that can guide searches of speciation genes. This study provides a cytological characterization of defects in sterile male hybrids between species of *Anopheles* and highlights the possibility of previously unchecked defects in meiosis contributing to sperm abnormalities in sterile males. Overall I find the results of interest and novel considering that there have been very few attempts to conduct this type of work.

I have a few points that will need to be addressed:

My main questions relate to the choice of genes for tests of expression:

In line 216 you describe the phenotype of sterile male hybrids with normal testes as starting meiosis but then switching to a mitotic behavior in anaphase. I am not a cell biologist, but we always teach students that the second meiotic division is like mitosis. Is the phenotype you describe a rather premature entry into the second meiotic division? You test germline-specific genes expression and find nothing wrong, but there is no rationale as to why such genes were chosen. Are there genes known to control cell cycle transitions? If so, is their expression normal?

In figure 4, your cartoon implies the formation of extra spindles. Again, are there abnormalities in the expression of genes that code for proteins that make the spindle? I think you have a more solid case here for testing beta2-tubulin, than the fact that it is a germline transcript.

I understand your focus is on HMS, but it is rather puzzling that an alteration of meiotic behavior leading to the formation of diploid sperm affects only males. Did you check meiosis in hybrid females? Is it normal? Why would meiosis progress normally in females but be aberrant in males? Something to check or at least to be discussed.

Some minor points:

Lines 13-14: I am not sure what you mean by “at the beginning stages of postzygotic isolation. I suggests deleting.

Lines 40-41: The citation is rather old. Recent work supports premating isolation evolves faster than postzygotic isolation (see for example Turissini et al. 2018). Often premating traits are first to be affected.

Line 119: I suggest you delete “the numbers of”

Lines 142-145: Why “except for crosses between *gambiae* and *coluzii*?” It follows Haldane’s rule.

## Author's Response to Decision Letter for (RSPB-2019-0318.R0)

See Appendix A.

## RSPB-2019-1080.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?**

Good

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

Good

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

Poor

**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**

Written communication of the data is still poor. I would suggest editing further, perhaps with a second editor, with an eye towards correcting grammar and syntax. This is needed throughout the entire paper. Some examples include:

Line 13: Detail cytological characterizations of...

Should be "detailed"

Lines 32-34: Premating isolation evolves faster than postmating isolation and hybrid male fertility is the first phenotype affected as the postzygotic isolation between species is being established between related taxa [1, 2].

- There are two completely different ideas in this one sentence
- alternative forms of terminology is present here (i.e. postmating versus postzygotic). Improper use of heavy jargon makes sentences difficult to read on the first pass.

Line 257: For example, our data show that such sperm abnormalities...

- shows not show

## Review form: Reviewer 2

### 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?**

Good

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

Good

**Is the length of the paper justified?**

Yes

**Should the paper be seen by a specialist statistical reviewer?**

No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**

No

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

**Is it accessible?**

Yes

**Is it clear?**

Yes

**Is it adequate?**

Yes

**Do you have any ethical concerns with this paper?**

No

### Comments to the Author

I am pleased with how my comments have been addressed in the revised version. I found the results interesting and novel.



## Decision letter (RSPB-2019-1080.R0)

07-Jun-2019

Dear Dr Sharakhov

I am pleased to inform you that your manuscript RSPB-2019-1080 entitled "Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex" has been accepted for publication in Proceedings B, subject to final editing for grammar.

The referees and the Associate Editor have recommended publication, but also request important revisions to your manuscript related to English grammar prior to publication. Therefore, I invite you to respond to the 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

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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=\(Document not available\)](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](mailto:proceedingsb@royalsociety.org)

Associate Editor  
Board Member  
Comments to Author:

The authors have addressed all of my primary concerns, and I find this study interesting and exciting. However, I agree with the reviewer that there are a large number of minor grammatical errors and sentences with poor wording throughout the manuscript that should be corrected prior to publication. I have detailed those for the Introduction, but these issues are present throughout the manuscript. I strongly suggest the authors enlist assistance (a well-versed colleague, or a professional service) to correct these minor errors throughout the manuscript prior to publication.

Conceptual:

It's stated in the introduction that the introgression of the Y chromosome from gambiae into arabiensis does not cause sterility (lines 59-60). This runs counter to your findings (and discussion) that X-Y pairing underlies sterility in this species pair. Which is fine, but should be mentioned in the Discussion.

Wording:

Line 25: change to "Thus, our study identified cytogenetic errors in"

Line 32-34: change to: "When species diverge, hybrid offspring that are produced can suffer from reduced fitness. Hybrid male fertility is usually one of the first of these postzygotic phenotypes affected [1,2]."

Line 34: change to "Therefore, the genetic factors, "

Line 47: "and to guide the identification of speciation genes."

Line 52: "genomic introgression is prevalent in autosomal regions"

Line 54-55: "F1 hybrid males, conforming to Haldane's rule"

Line 57: "degrees of testes atrophy and underdevelopment of sperm."

Line 72: "hybrid males stems from the"

Line 73: "study identifies cytogenetic errors"

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).

I am pleased with how my comments have been addressed in the revised version. I found the results interesting and novel.

Referee: 1

Comments to the Author(s).

Written communication of the data is still poor. I would suggest editing further, perhaps with a second editor, with an eye towards correcting grammar and syntax. This is needed throughout the entire paper. Some examples include:

Line 13: Detail cytological characterizations of...  
Should be "detailed"

Lines 32-34: Premating isolation evolves faster than postmating isolation and hybrid male fertility is the first phenotype affected as the postzygotic isolation between species is being established between related taxa [1, 2].

- There are two completely different ideas in this one sentence
- alternative forms of terminology is present here (i.e. postmating versus postzygotic). Improper use of heavy jargon makes sentences difficult to read on the first pass.

Line 257: For example, our data show that such sperm abnormalities...  
- shows not show

## Author's Response to Decision Letter for (RSPB-2019-1080.R0)

See Appendix B.

## Decision letter (RSPB-2019-1080.R1)

14-Jun-2019

Dear Dr Sharakhov

I am pleased to inform you that your manuscript entitled "Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex" 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](mailto:procb_proofs@royalsociety.org)

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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.

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



COLLEGE OF AGRICULTURE AND LIFE SCIENCES  
**ENTOMOLOGY**  
VIRGINIA TECH.

**Dr. Igor Sharakhov**  
Department of Entomology  
Fralin Life Science Institute, Room 203  
Blacksburg, Virginia 24061  
P: (540) 231-7316 F: (540) 231-7126  
e-mail: [igor@vt.edu](mailto:igor@vt.edu)

May 10, 2019

Dear Editors,

On behalf of the authors, I am resubmitting the manuscript “**Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex**” for consideration in *Proceedings B*. This version of the manuscript has been substantially revised in accordance with suggestions of the Board Member and Reviewers. We would like to thank the Board Member and Reviewers for expressing interest in our findings and for suggestions that improved the manuscripts. Below are our responses to their comments.

### **Board Member: 1**

The manuscript has been reviewed by two reviewers and myself. While all have expressed interest in these findings, there are some areas for improvement that need to be addressed in order for this manuscript to be publishable in Proc B. In addition to the wording clarifications the reviewers suggest, I agree with the points raised by one reviewer regarding the choice of genes tested for mis-expression and the absence of any tests in females (overall fertile females could still have some gametes demonstrating failures in meiosis). Since addressing these concerns may require time for additional tests to be conducted, I have recommended to reject with the possibility for resubmission.

**Response:** The following paragraph addresses the concerns about (i) the choice of genes tested for misexpression and (ii) the absence of tests in females.

(i) The choice of genes presented in the original manuscript allowed us to test if the premeiotic failure in degenerated gonads of the F1 hybrids from the cross ♀*An. coluzzii* × ♂*An. merus* is associated with the misexpression of the postmitotic germline transcripts. This was indeed the case: we found no visible transcripts for *Ams*, *mts*, *Dzip11*, and  $\beta 2$ -*tubulin* in degenerated testes of these F1 hybrid males. Expression of the premeiotic gene *vasa* indicated the development of germline stem cells even in degenerate testes of these interspecies hybrids. In the resubmitted manuscript, we followed the recommendation of the Board Member and Referee 2 to test expression of genes that could be associated with the errors in meiosis. We conducted additional

RT-PCR experiments for genes encoding for structural maintenance of chromosomes proteins, *SMC2*, *SMC4*, and *SMC3-like*. Higher expression levels of the premeiotic gene *vasa* and postmitotic genes *SMC3-like*, *SMC2*, and *SMC4* are consistent with our observation of extended premeiotic and spermatogenic stages in the normal-like reproductive organs of F1 hybrids from the ♀*An. merus* × ♂*An. coluzzii* cross.

(ii) We agree with the Board Member and Referee 2 that tests in hybrid females would be interesting to conduct because fertile female hybrids could still have some abnormal gametes. In the resubmitted manuscript we added females to our RT-PCR analysis. Our results show that all tested genes express at similar levels in ovaries of pure species and hybrids from the reciprocal crosses. We have also tried to conduct a cytogenetic study on female meiosis but were unsuccessful. Unlike *Drosophila*, the ovarian development in mosquitoes is triggered by the bloodfeeding and is highly synchronized. As a result, meiosis passes quickly in all follicles at the same time making difficult to capture the correct stage. Moreover, we don't have species-specific probes X chromosome probes to perform a similar cytogenetic analysis of female meiosis. For these reasons, we decided to focus our study only on sterility in hybrid males.

Additional minor comment:

Lines 279-283 are awkwardly worded. I recommend re-wording.

**Response:** Lines 279-283 were re-worded.

### **Reviewer(s)' Comments to Author:**

#### **Referee: 1**

Comments to the Author(s)

Some notable sections in the result that require further clarification, consideration or editing:

#### Results

[Lines 141-142] backcrossing of F1 males is not a strong indicator of male sterility, as their might be behavioural isolation (or prezygotic isolation in F1 males)

**Response:** We clarified the sentence: “Backcrossing of F1 males to parental females resulted in induction of laying of eggs that did not hatch, which confirmed mating of sterile F1 hybrid males since seminal fluids but not sperm are required to induce oviposition.”

[Lines 144] wording is incorrect: move "F1 females are fertile while F1 males are sterile" prior to describing the exception to Haldane's rule, otherwise this sentence reads as the exception.

**Response:** The wording has been corrected: "The observed sterility of hybrid males confirms Haldane's rule for the majority of interspecies crosses in the *An. gambiae* complex except for crosses between *An. gambiae* and *An. coluzzii*, which produce fertile hybrids of both sexes"

[Lines 148] wording does not make sense. You found differences between testes morphology/size in one interspecies cross versus its reciprocal?

**Response:** The wording has been corrected: "We found obvious differences between testes morphology/size in one interspecies cross versus its reciprocal."

[Lines 156-158] Why was sperm only observed in 2 day old testes? Do sperm reach full maturation at 2 days? Could this be a sign of rapid aging in hybrid males instead of sterility?

**Response:** Yes, sperm reach maturity within 2 days after emergence of adult males from pupae. We observed sperm in 5 day old males as well and found no difference with 2 day old males. We added: "Sperm reach maturity within two days after emergence of *Anopheles* adult males."

## **Referee: 2**

Comments to the Author(s)

Postzygotic isolation in the form of hybrid male sterility (HMS) is a common isolation barrier between species. HMS is often identified by the inability of males to produce progeny and sometimes further described phenotypically by the observation of atrophied testes or more subtly morphologically normal testes but immotile sperm. Detail cytological characterizations of HMS are rather lacking. I find that the detail characterization at the cytological level of HMS is important to establish the order in which isolating mechanisms arise relative to one another and in providing us with phenotypic and mechanistic clues that can guide searches of speciation genes. This study provides a cytological characterization of defects in sterile male hybrids between species of *Anopheles* and highlights the possibility of previously unchecked defects in meiosis contributing to sperm abnormalities in sterile males. Overall I find the results of interest and novel considering that there have been very few attempts to conduct this type of work.

I have a few points that will need to be addressed:

My main questions relate to the choice of genes for tests of expression:

In line 216 you describe the phenotype of sterile male hybrids with normal testes as starting meiosis but then switching to a mitotic behavior in anaphase. I am not a cell biologist, but we always teach students that the second meiotic division is like mitosis. Is the phenotype you describe a rather premature entry into the second meiotic division?

**Response:** Indeed, the phenotype we describe is a premature entry into the second meiotic division by skipping the reductional division, in which homologous chromosomes must segregate. We clarified this in the text.

You test germline-specific genes expression and find nothing wrong, but there is no rationale as to why such genes were chosen. Are there genes known to control cell cycle transitions? If so, is their expression normal?

**Response:** The choice of genes presented in the original manuscript allowed us to demonstrate strong suppression of postmitotic genes in degenerate gonads of the F1 hybrids from the cross ♀*An. coluzzii* × ♂*An. merus*. In contrast, transcripts for the same genes are present in hybrid testes from the reciprocal cross. In the resubmitted manuscript, we identified *vasa* and three chromosomal protein genes, *SMC2*, *SMC4*, and *SMC3-like*, that are upregulated in the normal-like reproductive organs of F1 hybrids from the ♀*An. merus* × ♂*An. coluzzii* cross. Higher expression levels of the premeiotic and postmitotic genes are consistent with our observation of extended premeiotic and spermatogenic stages in these hybrids. It is also possible that the observed overexpression of *SMC3-like*, *SMC2*, and *SMC4* in the hybrid males may contribute to increased sister-chromatid cohesion, recombination, and segregation causing abortion of the homologous chromosome segregation.

In figure 4, your cartoon implies the formation of extra spindles. Again, are there abnormalities in the expression of genes that code for proteins that make the spindle? I think you have a more solid case here for testing beta2-tubulin, than the fact that it is a germline transcript.

**Response:** In figure 4, the cartoon may give impression of the formation of extra spindles because it shows a premature entry into the second meiotic division by skipping the reductional



division. As a result, a diploid cell instead of a haploid cell undergoes anaphase. However, this process does not produce extra spindles, and we don't see changes in expression of beta2-tubulin.

I understand your focus is on HMS, but it is rather puzzling that an alteration of meiotic behavior leading to the formation of diploid sperm affects only males. Did you check meiosis in hybrid females? Is it normal?

**Response:** In the resubmitted manuscript we added females to our RT-PCR analysis. We found no obvious problems with gene expression in ovaries of F1 hybrids. It has been very challenging to check meiosis in hybrid females. This is because in synchronously developed follicles meiosis passes quickly making it difficult to capture the correct stage. Also the lack of species-specific probes X chromosome probes would not allow us to perform a cytogenetic analysis of female meiosis.

Why would meiosis progress normally in females but be aberrant in males? Something to check or at least to be discussed.

**Response:** We added the discussion of why would meiosis progress normally in females but be aberrant in males. We think this is because X and Y chromosomes may pair through specific pairing sites located in heterochromatin, which could be easily altered during evolution. In contrast, the X-X and autosome-autosome pairing capacity is widely distributed along chromosome arm and is more robust to evolutionary changes.

Some minor points:

Lines 13-14: I am not sure what you mean by "at the beginning stages of postzygotic isolation. I suggests deleting.

**Response:** We deleted the phrase.

Lines 40-41: The citation is rather old. Recent work supports premating isolation evolves faster than postzygotic isolation (see for example Turissini et al. 2018). Often premating traits are first to be affected.

**Response:** We added the citation and modified the sentence accordingly.

Line 119: I suggest you delete “the numbers of”

**Response:** Done.

Lines 142-145: Why “except for crosses between *gambiae* and *coluzzii*?” It follows Haldane’s rule.

**Response:** *An. gambiae* and *An. coluzzii* produce fertile hybrids of both sexes. We clarified this in the text.

Thank you very much for your time and consideration. We look forward to hearing from you.

Sincerely yours,



Igor V. Sharakhov, Ph.D.

Professor

## Appendix B



COLLEGE OF AGRICULTURE AND LIFE SCIENCES  
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June 12, 2019

Dear *Proceedings B* Editor,

On behalf of the authors, I am resubmitting the revised manuscript “**Premeiotic and meiotic failures lead to hybrid male sterility in the *Anopheles gambiae* complex.**” Below are our responses to the comments from the Board Member and Reviewers.

### **Board Member**

Comments to Author:

The authors have addressed all of my primary concerns, and I find this study interesting and exciting. However, I agree with the reviewer that there are a large number of minor grammatical errors and sentences with poor wording throughout the manuscript that should be corrected prior to publication. I have detailed those for the Introduction, but these issues are present throughout the manuscript. I strongly suggest the authors enlist assistance (a well-versed colleague, or a professional service) to correct these minor errors throughout the manuscript prior to publication.

**Response:** Thank you for the comments and suggestions. We asked well-versed colleagues to correct grammatical errors and sentences with poor wording throughout the manuscript.

Conceptual:

It's stated in the introduction that the introgression of the Y chromosome from *gambiae* into *arabensis* does not cause sterility (lines 59-60). This runs counter to your findings (and discussion) that X-Y pairing underlies sterility in this species pair. Which is fine, but should be mentioned in the Discussion.

**Response:** We added several sentences to the Discussion: “A study of introgression of the Y chromosome from *An. gambiae* into *An. arabensis* suggests that the Y chromosome does not cause sterility [17]. This runs counter to our findings that X-Y unpairing underlies sterility in male hybrids. Identification and analysis of X-Y pairing sites in species of the *An. gambiae* complex may shed light on this problem.”

Wording:

Line 25: change to "Thus, our study identified cytogenetic errors in"

Line 32-34: change to: "When species diverge, hybrid offspring that are produced can suffer from reduced fitness. Hybrid male fertility is usually one of the first of these postzygotic phenotypes affected [1,2]."

Line 34: change to "Therefore, the genetic factors, "

Line 47: "and to guide the identification of speciation genes."

Line 52: "genomic introgression is prevalent in autosomal regions"

Line 54-55: "F1 hybrid males, conforming to Haldane's rule"

Line 57: "degrees of testes atrophy and underdevelopment of sperm."

Line 72: "hybrid males stems from the"

Line 73: "study identifies cytogenetic errors"

**Response:** We corrected the wording as suggested by the Board Member.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).

I am pleased with how my comments have been addressed in the revised version. I found the results interesting and novel.

**Response:** Thank you for the comments.

Referee: 1

Comments to the Author(s).

Written communication of the data is still poor. I would suggest editing further, perhaps with a second editor, with an eye towards correcting grammar and syntax. This is needed throughout the

entire paper. Some examples include:

Line 13: Detail cytological characterizations of...

Should be "detailed"

Lines 32-34: Premating isolation evolves faster than postmating isolation and hybrid male fertility is the first phenotype affected as the postzygotic isolation between species is being established between related taxa [1, 2].

- There are two completely different ideas in this one sentence
- alternative forms of terminology is present here (i.e. postmating versus postzygotic). Improper use of heavy jargon makes sentences difficult to read on the first pass.

Line 257: For example, our data show that such sperm abnormalities...

- shows not show

**Response:** We corrected the poor wording, grammar, and syntax.

Thank you very much for your time and consideration.

Sincerely yours,



Igor V. Sharakhov, Ph.D.

Professor