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PROCEEDINGS B

Sperm cryopreservation reduces offspring growth

David Nusbaumer, Lucas Marques da Cunha and Claus Wedekind

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Review timeline

Original submission:	6 May 2019
1st revised submission:	12 July 2019
2nd revised submission:	12 August 2019
3rd revised submission:	4 September 2019
Final acceptance:	5 September 2019

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

Review History

RSPB-2019-1041.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions in comments)

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

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

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

Is the length of the paper justified?
No

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

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?
No

Is it clear?
No

Is it adequate?
No

Do you have any ethical concerns with this paper?
Yes

Comments to the Author
Authors declared that the Article does not present research with ethical considerations. However, animal experimentation was carried out. Please, check that this aspect is clear.

Introduction and discussion are too long, having content review-like, including too many bibliographic references. Moreover, the information of invertebrates, fish, mammals in general and humans is mixed and results in a group of species are used to justify general conclusions to be applied in other animals. Evolution caused differences, and not all the information in a group of animals can be immediately applicable in others. Comparative studies are required before arriving to certain conclusions.

As the authors said through the manuscript, many aspects can condition the effect of sperm cryopreservation on the offspring phenotype, including survival at different times. This is complex enough to study, without the need of introducing stress factors as bacterial infections effects. The inclusion of a challenge test: testing the effect of a bacterial infection, showed negative results, and in my opinion is scarcely conclusive, masking the main results.

Authors MUST report the number of larvae measured per experimental group (L183 and next). The main (and only positive) result of the work are the differences in growth theoretically caused as a consequence of the sperm cryopreservation effect (a reduction of near 5% length). So, a high number of measures is the only way to guarantee the strength of this conclusion. In especial, if we consider that the range of length measurements is very small, can your method really discriminate between these small differences?, what is the error of your measurement method?

Tables 1 and 2 seems too cryptic to me. Is there alternative ways to show these results?, Something more graphic maybe?

Considering the results shown by the study, some of the conclusions are extremely speculative (L377-379, L387-388, L393 and next).

M+m

L168 Were water changes carried out during the embryo incubation?
L173 Bacteria were commercialized “frozen and dried”? If not, add the method to do it.

Minor comments and corrections
L16 stripped
L66 ... spp.), ...
L89 Alencoao(n?)
L136, 138, 144, 154, 167, 174, 176, 187 (2x), 206, 208 Separate digit and unit (i.e. 2 mL)
L162 Add the composition of the “standardized water”.
L178 Reference 55 (mentioned before 45-54) or 45?
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Figure 1 and 2. Add symbols evidencing the significant differences.
L278 ...effects of sperm cryopreservation...
L285 the effect of the pathogen?
L330 and next. “Sperm subpopulations” could be a concept to be included here. Check doi: 10.1071/RD13198, for instance.
References. Check when to use “et al.” as part of the references.
L484 Use italics for species name
L494 Silurus
L532 (Salmo trutta)
L584, 605 Herráez
L658 0 > 72, 2478–2490

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?
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
Comments to the authors
Sperm cryopreservation is a widely used technique in animal production, conservation and human medicine, however, the potential effects of cryopreservation of the gametes on the produced offspring are not well understood, and the results are often confounded with other techniques. This paper studies effects of sperm cryopreservation on the embryos and hatchlings of the brown trout. While several earlier studies assessed the effect of sperm cryopreservation in fish and other animals (including humans) with varying results, the present study differs in the rigorous experimental design with a large number of individually reared embryos and hatchlings. The authors also included pathogen as an additional factor in the design in order to study if environmental stress interacts with cryopreservation.
The authors found relatively mild and expected negative effects on sperm motility and fertilization success and very little effects on most of the embryonic and hatchling traits. However, hatchling growth is significantly negatively affected by sperm cryopreservation suggesting that cryopreservation may have significant fitness effects on trout fry. The pathogen had strong independent effects on fish performance but did not interact with cryopreservation. I find this study interesting and well-conducted and the results potentially important. The experimental design is rigorous, as already noted above, the data well analyzed and the paper is well written. I have only a few comments.
My main comment relates to the strength of the observed fitness effect. How important is early growth performance for later fitness? Is this early growth performance correlated with juvenile survival or size at or timing of maturation in salmonids or fish in general? If I understood correctly growth at this stage is supported by the maternal yolk. Is it possible that the slow-growing fry can later compensate for the reduced growth once they started independent feeding? Continuing this experiment to the stages where the fry feed independently would have considerably increased the strength of the present results.
Lines 151-153. Why do you call these breeding blocks rather than effects of individual females? Is it because offspring of each female are spatially close to each other in the rearing array?
Lines 164-170. Related to the previous comment: how were the eggs distributed to the plates – were all eggs at one plate offspring of one female or of mixed origin? Please explain.
The text is unnecessarily detailed in some parts off the introduction and discussion, these sections could be cut by some 10-15%. For example, the fish species list in lines 66-71 and the information on male skin color in lines 322-324 seem unnecessary.

Decision letter (RSPB-2019-1041.R0)

07-Jun-2019

Dear Dr Wedekind:

I am writing to inform you that your manuscript RSPB-2019-1041 entitled "Sperm cryopreservation reduces offspring growth" 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,

Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor

Comments to Author:

Dear Claus et al.

Sorry for the delay getting back to you, but I was waiting a third reviewer whose report is now 6 days over due, so I decided to proceed without it. As you can see from the reports, both reviewers find your work very interesting (as do I), but raised some concerns. Given nature of these concerns as well as the demand towards publishing only the best papers, I will

recommending rejection with possibility to resubmit. Of course, I hope that you will be able to revise the ms in light of the referee comments and make a resubmission.

Best wishes
Juha Merilä

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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References. Check when to use “et al.” as part of the references.
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L494 Silurus
L532 (Salmo trutta)
L584, 605 Herráez
L658 0 > 72, 2478–2490

Referee: 2

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The text is unnecessarily detailed in some parts off the introduction and discussion, these sections could be cut by some 10-15%. For example, the fish species list in lines 66-71 and the information on male skin color in lines 322-324 seem unnecessary.

Author's Response to Decision Letter for (RSPB-2019-1041.R0)

See Appendix A.

RSPB-2019-1644.R0 (Revision)

Review form: Reviewer 1

Recommendation
Reject – article is scientifically unsound

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

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

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

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

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

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors corrected some of the major aspects found in the first version (as introduction and discussion lengths, or the unjustified use of not fish species) and apparently all the minor mistakes identified.
However, in my opinion they failed in the description of experimental design and ns used in each trial. Apparently (page 5), they used 40 males and 10 females. For fertilization tests they took 96 eggs/female (96x10=960) that were distributed in 8(?) Petri dishes (960/8=120 eggs/dish). Then, eggs were fertilized "with either cryopreserved or non-cryopreserved sperm" (following the method described in page 6). Each mix of eggs (from 10 females) was fertilized with sperm from 4 males, creating 8x4= 32 families. Nevertheless, that was done with fresh and cryopreserved sperm, meaning 64 experimental groups (of 120 potentially fertilized eggs). This means 64 groups x 120 eggs = 7680 eggs, being far of the number indicated in the manuscript. Are these numbers right? What do you call "breeding block"?,
Later, L140-141 you said that you added 6 ml of activating solution to each Petri dish (same than mentioned before?) meaning 500 microl per egg. So, each dish had 12 eggs??
Similar doubts reach with the description of the pathogen challenge experiment (page 7). What was the number of infected eggs/plate?
Finally, in the figure 1, you indicated a n=15. Are they independent sperm samples? Pools?...?
Resuming, even with the number shown in the figure 2 (ranging between 214 and 362), the reader cannot understand your experimental design.
Moreover, there was a loss of samples at later stages of the experiment that is only mentioned in the legend of figure S1. What happened? Can this jeopardize the results?
As was indicated in my previous evaluation, the main (and only positive) result of the work are the differences in growth theoretically caused as a consequence of the sperm cryopreservation effect (a reduction of, now, a bit over 4% length). So, a high number of measures is the only way to guarantee the strength of this conclusion.
Without having a clear information on the experimental design and the number of evaluated groups and samples, is difficult to evaluate the results of this work. However, I must insist in my comment regarding the complexity of this type of experiment without the need of introducing stress factors as bacterial infections effects. The challenge test showed negative results, and it was scarcely conclusive, masking the main results.

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?
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

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Is it accessible?
No

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors addressed my comments, mainly by adding points to the Discussion, which is fine. I have no further comments.

Decision letter (RSPB-2019-1644.R0)

05-Aug-2019

Dear Dr Wedekind:

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, one reviewer has raised some issues 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,

Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor Board Member
Comments to Author:
Dear Authors,

Thank you for the resubmission of you work. Two of the the original reviewers of your manuscript have now provided feedback to the revised version. One of the reviewers is satisfied with the revision, but the other one asks for clarification for the experimental design. I hope you can respond to these criticisms and revise the ms in way that the issues raised by the reviewer are not of concern.

Best wishes

Juha Merilä

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The authors corrected some of the major aspects found in the first version (as introduction and discussion lengths, or the unjustified use of not fish species) and apparently all the minor mistakes identified.
However, in my opinion they failed in the description of experimental design and ns used in each trial. Apparently (page 5), they used 40 males and 10 females. For fertilization tests they took 96 eggs/female (96x10=960) that were distributed in 8(?) Petri dishes (960/8=120 eggs/dish). Then, eggs were fertilized “with either cryopreserved or non-cryopreserved sperm” (following the method described in page 6). Each mix of eggs (from 10 females) was fertilized with sperm from 4 males, creating 8x4= 32 families. Nevertheless, that was done with fresh and cryopreserved sperm, meaning 64 experimental groups (of 120 potentially fertilized eggs). This means 64 groups x 120 eggs = 7680 eggs, being far of the number indicated in the manuscript. Are these numbers right? What do you call “breeding block”?,
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Finally, in the figure 1, you indicated a n=15. Are they independent sperm samples? Pools?...? Resuming, even with the number shown in the figure 2 (ranging between 214 and 362), the reader cannot understand your experimental design.
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Without having a clear information on the experimental design and the number of evaluated groups and samples, is difficult to evaluate the results of this work. However, I must insist in my comment regarding the complexity of this type of experiment without the need of introducing stress factors as bacterial infections effects. The challenge test showed negative results, and it was scarcely conclusive, masking the main results.

Referee: 3

Comments to the Author(s).
The authors addressed my comments, mainly by adding points to the Discussion, which is fine. I have no further comments.

Author's Response to Decision Letter for (RSPB-2019-1644.R0)

See Appendix B.

RSPB-2019-1644.R1 (Revision)

Review form: Reviewer 1

Recommendation
Reject – article is scientifically unsound

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?
Yes

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?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors were not able to describe the experiments properly. With the answers given to my previous questions it gets evident the low nuber of eggs (12!) considered in each treatment (male x female x fresh/cryopr. sperm). This is very low in fish fertilization tests.
The figures (1 and 2 A,B,C) show ns of 15 (when only 10 males were used, apparently only once...???) or 200-300 (apparently considering all the lengths of the measured fish, but without considering wich male+female were their parents, and probably creating slants).
Impossible to understand the bacterial challenge test. n??

Decision letter (RSPB-2019-1644.R1)

30-Aug-2019

Dear Dr Wedekind:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewer's 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, there remain some issues with your manuscript and we would like to invite you to revise your manuscript to address them.

It is unusual at Proceedings B to allow an additional round of revision so we urge you to make every effort to fully address all of the comments at this stage. 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.

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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
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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,

Professor Hans Heesterbeek
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Dear Authors,

The reviewer who was critical in the last round has provided an additional review and being in opinion that some unclarity about the study design remains. He is also in opinion that the sample sizes are sometimes quite low. I had a critical look on the ms again, and to my reading, the methods are quite clearly described. For sure, sample sizes could be larger, but since the data has been analysed using statistical methods which take sample sizes into account, I do not consider this to be a major issue here. Nevertheless, I would like to ask you, once again, have a look on the reviewer's comments and make the modifications to the ms which you deem appropriate.

Best wishes

Juha Merilä

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
The authors were not able to describe the experiments properly. With the answers given to my previous questions it gets evident the low nuber of eggs (12!) considered in each treatment (male x female x fresh/cryopr. sperm). This is very low in fish fertilization tests.

The figures (1 and 2 A,B,C) show ns of 15 (when only 10 males were used, apparently only once...???) or 200-300 (apparently considering all the lengths of the measured fish, but without considering wich male+female were their parents, and probably creating slants).
Impossible to understand the bacterial challenge test. n??

Author's Response to Decision Letter for (RSPB-2019-1644.R1)

See Appendix C.

Decision letter (RSPB-2019-1644.R2)

05-Sep-2019

Dear Dr Wedekind

I am pleased to inform you that your manuscript entitled "Sperm cryopreservation reduces offspring growth" 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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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,

Professor Hans Heesterbeek
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Comments to Author:
Dear Authors,

Thank you for patience with these repeated requests for revisions, and thank for attending the last round of comments and clarifying the the points raised by the reviewer. I am glad to see - as I assumed once requesting the revision - that the issues raised by the reviewer very easily addressed. My apologies for the hurdles it took get in this point - but I am glad to see that everything looks great at the end!

Best wishes
Juha Merilä

Appendix A

Response to Editorial Board Member Prof. Juha Merilä

Sorry for the delay getting back to you, but I was waiting a third reviewer whose report is now 6 days over due, so I decided to proceed without it. As you can see from the reports, both reviewers find your work very interesting (as do I), but raised some concerns. Given nature of these concerns as well as the demand towards publishing only the best papers, I will recommending rejection with possibility to resubmit. Of course, I hope that you will be able to revise the ms in light of the referee comments and make a resubmission.

>>>> Thanks for your feedback and for your interest in our work! We could respond to the comments of all three reviewers.

Response to Reviewer #1

>>>> We would like to thank reviewer #1 for the detailed and constructive comments that helped us to improve our MS.

Comments to the Author(s)
Authors declared that the Article does not present research with ethical considerations. However, animal experimentation was carried out. Please, check that this aspect is clear.

>>>> We added a paragraph “Ethics approval” in the declarations at the end of the MS.

Introduction and discussion are too long, having content review-like, including too many bibliographic references. Moreover, the information of invertebrates, fish, mammals in general and humans is mixed and results in a group of species are used to justify general conclusions to be applied in other animals. Evolution caused differences, and not all the information in a group of animals can be immediately applicable in others. Comparative studies are required before arriving to certain conclusions.

>>>> We rewrote parts of the Introduction and the Discussion in response to this comment, reducing these chapter by 20% and 10%, respectively.

As the authors said through the manuscript, many aspects can condition the effect of sperm cryopreservation on the offspring phenotype, including survival at different times. This is complex enough to study, without the need of introducing stress factors as bacterial infections effects. The inclusion of a challenge test: testing the effect of a bacterial infection, showed negative results, and in my opinion is scarcely conclusive, masking the main results.

>>>> We prefer to keep the stress test in the MS because it allowed us to test whether cryopreservation would increase susceptibility to environmental stress. This has, to our knowledge, never been tested before.

Authors MUST report the number of larvae measured per experimental group (L183 and next). The main (and only positive) result of the work are the differences in growth theoretically caused as a consequence of the sperm cryopreservation effect (a reduction of near 5% length). So, a high number of measures is the only way to guarantee the strength of this conclusion. In especial, if we consider that the range of length measurements is very small, can your method really discriminate between these small differences?, what is the error of your measurement method?

>>>> We are now more explicit about the fact that we started the experiment with 960 eggs, and we rearranged the Result section to stress that fertilization success was high (ca. 95%). The numbers of embryos and larvae that could be measured are now given in all the figure panels. More detailed information is now given regarding the loss of samples at later stages of the experiment (legend in Figure S1).

In response to this comment, we determined again all length measurements to calculate the repeatability of our measurements (r^2) and to illustrate the precision of the measurements from enlarged photos. These r^2s turned out to be very high (89% and 95%), and we now use the means of these two sets of independent measurements. This information is added to the Methods. We also determined again all yolk sac volumes directly from enlarged photos (determining the long and the short axes of an ellipsoid that would describe the yolk sac best) and found that, in this case, the r^2 to the previous measurements that had been done with a macro in ImageJ were only 75% and 41%, respectively. We therefore used only the second set of measurements for the yolk volumes. While determining all dependent variables again, a few accidentally missed values could be replaced with observations.

We rerun the statistical models with these improved measurements. The main finding regarding the effects of cryopreservation remained qualitatively the same. We also added a new Figure S2 to illustrate how larval length and yolk sac volume were determined.

Tables 1 and 2 seems too cryptic to me. Is there alternative ways to show these results?, Something more graphic maybe?

>>>> We added 3 new panels in the main MS (Figure 1D, Figure 2A-D), produced the new Figures S3 and S7, and added 2 new panels to Fig. S4 (previous Fig. S2) to further illustrate the results of Tables 1 and 2.

Considering the results shown by the study, some of the conclusions are extremely speculative (L377-379, L387-388, L393 and next).

>>>> We removed the most speculative sentences (former L377-379, L387-388) and reworded the corresponding paragraphs in response to this comment. We also reworded to the final paragraph to avoid speculation while summarizing the main conclusions.

M+m
L168 Were water changes carried out during the embryo incubation?

>>>> We now specify that there was no water exchange during the incubation.

L173 Bacteria were commercialized “frozen and dried”? If not, add the method to do it.

>>>> Yes, they were commercialized in this form.

Minor comments and corrections
L16 stripped

>>>> corrected

L66 ... spp.), ...

>>>> This part has been cut from the MS.

L89 Alencoao(n?)

>>>> It's indeed Alencoão.

L136, 138, 144, 154, 167, 174, 176, 187 (2x), 206, 208 Separate digit and unit (i.e. 2 mL)

>>>> Corrected.

L162 Add the composition of the "standardized water".

>>>> Added.

L178 Reference 55 (mentioned before 45-54) or 45?

>>>> Both references (45 and 55) provide this method. We now cite reference 45 only.

L195 16.6%

>>>> corrected

Tables 1 and 2. Explain what does it means AIC

>>>> We reworded the last sentence of the Method section to be clearer about the AIC.

L242 : > .

>>>> Changed.

L253 ...cryopreserved sperm showed...

>>>> Corrected.

Figure 1 and 2. Add symbols evidencing the significant differences.

>>>> Done.

L278 ...effects of sperm cryopreservation...

>>>> Rephrased.

L285 the effect of the pathogen?

>>>> Corrected.

L330 and next. "Sperm subpopulations" could be a concept to be included here. Check doi: 10.1071/RD13198, for instance.

>>>> Yes, this is what was meant by "types of spermatozoa". We are now clearer about the concept of sperm sub-subpopulations and added a further reference.

References. Check when to use “et al.” as part of the references.

>>>> We now give all authors in the reference list.

L484 Use italics for species name

>>>> Corrected.

L494 Silurus

>>>> Corrected.

L532 (Salmo trutta)

>>>> Corrected.

L584, 605 Herráez

>>>> Corrected.

L658 0 > 72, 2478–2490

>>>> Corrected.

Response to Reviewer #2

Comments to the Author(s)
Comments to the authors
Sperm cryopreservation is a widely used technique in animal production, conservation and human medicine, however, the potential effects of cryopreservation of the gametes on the produced offspring are not well understood, and the results are often confounded with other techniques. This paper studies effects of sperm cryopreservation on the embryos and hatchlings of the brown trout. While several earlier studies assessed the effect of sperm cryopreservation in fish and other animals (including humans) with varying results, the present study differs in the rigorous experimental design with a large number of individually reared embryos and hatchlings. The authors also included pathogen as an additional factor in the design in order to study if environmental stress interacts with cryopreservation.
The authors found relatively mild and expected negative effects on sperm motility and fertilization success and very little effects on most of the embryonic and hatchling traits. However, hatchling growth is significantly negatively affected by sperm cryopreservation suggesting that cryopreservation may have significant fitness effects on trout fry. The pathogen had strong independent effects on fish performance but did not interact with cryopreservation. I find this study interesting and well-conducted and the results potentially important. The experimental design is rigorous, as already noted above, the data well analyzed and the paper is well written. I have only a few comments.

>>>> Thanks also to Reviewer #2 for his/her encouraging and constructive comments.

My main comment relates to the strength of the observed fitness effect. How important is early growth performance for later fitness? Is this early growth performance correlated with juvenile survival or size at or timing of maturation in salmonids or fish in general? If I understood correctly growth at this stage is supported by the maternal yolk. Is it possible that the slow-growing fry can later compensate for the reduced growth once they started independent feeding? Continuing this experiment to the stages where the fry feed independently would have considerably increased the strength of the present results.

>>>> We added further information about the importance of early growth in salmonid fry (first paragraph of Discussion). We also re-determined all dependent variables to calculate the repeatability of our measurements and to illustrate the precision of the measurements from enlarged photos (see chapter "Measurements of embryo traits" in the Methods)

Lines 151-153. Why do you call these breeding blocks rather than effects of individual females? Is it because offspring of each female are spatially close to each other in the rearing array?

>>>> We prefer using the term "breeding block" instead of "dam effect" because we usually determine dam effects in full-factorial breeding designs (e.g. Marques da Cunha et al. Evol Appl 2019, cited in the MS), i.e. crossing several males and females within individual blocks. Here, the breeding design was not full-factorial but 1 female x 4 males each, and our focus was on the within-male comparisons.

Lines 164-170. Related to the previous comment: how were the eggs distributed to the plates – were all eggs at one plate offspring of one female or of mixed origin? Please explain.

>>>> The new Supplementary Figure S1B explains the distribution scheme.

The text is unnecessarily detailed in some parts off the introduction and discussion, these sections could be cut by some 10-15%. For example, the fish species list in lines 66-71 and the information on male skin color in lines 322-324 seem unnecessary.

>>>> We rewrote parts of the Introduction and the Discussion in response to this comment, reducing these chapter by 20% and 10%, respectively.

Response to Reviewer #3

>>>> Thanks also to Reviewer #3 for the detailed and constructive comments.

The treatment of sperm sub-samples differ in several aspects. The cryopreserved sperm are kept in a straw, warmed up to 25°C and then kept on ice for 1 minute, whereas control sperm presumably were kept on ice in minitubes until use (no details are given on this). The transfer into a straw may damage sperm (mechanically and lack of oxygen), the sudden changes in temperature (from frozen o 25 and then back on ice may affect sperm in various ways. The present experiment does not really allow the firm conclusion that the difference observed in offspring size is caused by the cryopreservation as such, or rather my the the different procedures before and after freezing. It would have been great to see control samples being kept in straws, and additional sample being warmed to 25°C and then put on ice to see how these different steps affect sperm.

>>>> We added to the Discussion "… *Because sperm cryopreservation is a procedure that includes dilution of sperm in an extender, equilibration, freezing, storage usually over long periods, and thawing for final use, it remains to be shown which step(s) in the protocol is/are responsible for the observed effects on offspring growth*." (4th paragraph)

Cryopreservation is usually also used to store sperm for much longer, so the time frame used here of storage (33 minutes on average) may not be fully representing the potential impact. I realise that testing the effects of long-term cryopreservation in a male would require a different experimental design and repeated sampling of the same male over time. The effects described here may therefore be a substantial underestimation of the true effects.

>>>> We added to the Discussion "… *If storage time creates such negative effects, the short time frame used in our study would lead to an underestimation of the effects that would be relevant in medicine and population management."* (4th paragraph)

The authors discuss to quite some extent the possibility of cryopreservation damaging the sperm genome. It would have been ideal to test this directly using DNA integrity assays on the two different sperm samples.

>>>> We added to the Discussion "… *Future studies could explore the possible links between an induced loss of DNA integrity, offspring growth, and tolerance to infection in brown tout."* (8th paragraph)

Appendix B

Response to Editorial Board Member Prof. Juha Merilä

Thank you for the resubmission of you work. Two of the the original reviewers of your manuscript have now provided feedback to the revised version. One of the reviewers is satisfied with the revision, but the other one asks for clarification for the experimental design. I hope you can respond to these criticisms and revise the ms in way that the issues raised by the reviewer are not of concern.

>>>> Thanks! We could respond to all comments of Reviewer #1.

Response to Reviewer #1

Comments to the Author(s).
The authors corrected some of the major aspects found in the first version (as introduction and discussion lengths, or the unjustified use of not fish species) and apparently all the minor mistakes identified.

>>>> Thanks for critically reading our MS again and providing further constructive comments!

However, in my opinion they failed in the description of experimental design and ns used in each trial. Apparently (page 5), they used 40 males and 10 females. For fertilization tests they took 96 eggs/female (96x10=960) that were distributed in 8(?) Petri dishes (960/8=120 eggs/dish). Then, eggs were fertilized “with either cryopreserved or non-cryopreserved sperm” (following the method described in page 6). Each mix of eggs (from 10 females) was fertilized with sperm from 4 males, creating 8x4= 32 families. Nevertheless, that was done with fresh and cryopreserved sperm, meaning 64 experimental groups (of 120 potentially fertilized eggs). This means 64 groups x 120 eggs = 7680 eggs, being far of the number indicated in the manuscript. Are these numbers right?

>>>> We amended the descriptions in lines 117-120 and rewrote the first paragraph of the chapter “Fertilisation and incubation of embryos” to avoid further confusion.

What do you call “breeding block”?,

>>>> We are now more explicit about this, see lines 134 – 135.

Later, L140-141 you said that you added 6 ml of activating solution to each Petri dish (same than mentioned before?) meaning 500 microl per egg. So, each dish had 12 eggs??

>>>> Yes. We are now more explicit about this, see lines 136 – 137.

Similar doubts reach with the description of the pathogen challenge experiment (page 7). What was the number of infected eggs/plate?

>>>> We amended the description of the pathogen challenge (lines 177-182), and we cite Supplementary Fig. S1 for a graphical representation.

Finally, in the figure 1, you indicated a n=15. Are they independent sperm samples? Pools?...?

>>>> Yes, the sperm motility measurements were done on new samples. See chapter “Effect of cryopreservation on sperm motility” (Methods).

Resuming, even with the number shown in the figure 2 (ranging between 214 and 362), the reader cannot understand your experimental design. Moreover, there was a loss of samples at later stages of the experiment that is only mentioned in the legend of figure S1. What happened? Can this jeopardize the results?

>>>> Figure 2 gives the number of hatchlings that could be measured per treatment. This number does not fully reflect the experimental design because some eggs were classified as not fertilized, some embryos died before hatching (e.g. because of the bacterial stress treatment), and some larvae could not be measured twice (to determine growth) because of larval mortality, low photo

quality, or accidents during handling (see lines 203-205). Loss of samples due to low photo quality or accidents were about equally spread over the experimental treatments (see new statistical analyses in the amended legend of Figure S2), and variation in fertilization success and in embryo or larval mortality was not dependent on sperm treatment (as shown in Table 1, Table S1). The main results of the study are therefore not jeopardized by the loss of samples over the course of the experiment.

As was indicated in my previous evaluation, the main (and only positive) result of the work are the differences in growth theoretically caused as a consequence of the sperm cryopreservation effect (a reduction of, now, a bit over 4% length). So, a high number of measures is the only way to guarantee the strength of this conclusion. Without having a clear information on the experimental design and the number of evaluated groups and samples, is difficult to evaluate the results of this work.

>>>> A loss of samples cannot be avoided in a study like ours. However, the number of independent replicates that our main conclusions are based on still range from N = 512 (Figure 2C,D) to N = 960 (Table 1A). Moreover, our results are based on powerful within-subject comparisons, full-factorial treatments within breeding blocks, an experimental control of all possibly confounding effects (i.e. controlling environmental effects in the laboratory), and a high number of sperm donors (40 wild-caught males).

However, I must insist in my comment regarding the complexity of this type of experiment without the need of introducing stress factors as bacterial infections effects. The challenge test showed negative results, and it was scarcely conclusive, masking the main results.

>>>> We explain in the Introduction (line 78-86) and at various locations in the Discussion why we believe that the stress test we performed is important. We therefore prefer to report it.

Response to Reviewer #3

Comments to the Author(s).
The authors addressed my comments, mainly by adding points to the Discussion, which is fine. I have no further comments.

>>>> Thanks again for critically reading our MS!

Appendix C

Response to Editorial Board Member Prof. Juha Merilä

The reviewer who was critical in the last round has provided an additional review and being in opinion that some unclarity about the study design remains. He is also in opinion that the sample sizes are sometimes quite low. I had a critical look on the ms again, and to my reading, the methods are quite clearly described. For sure, sample sizes could be larger, but since the data has been analysed using statistical methods which take sample sizes into account, I do not consider this to be a major issue here. Nevertheless, I would like to ask you, once again, have a look on the reviewer's comments and make the modifications to the ms which you deem appropriate.

>>>> Thanks for allowing us to respond to the last comments of reviewer 1. As explained below, we believe that we had been clear about the points the reviewer now raised. However, we revised the MS again in order to amend the descriptions further and to avoid further misunderstandings.

Response to Reviewer #1

Comments to the Author(s)
The authors were not able to describe the experiments properly. With the answers given to my previous questions it gets evident the low nuber of eggs (12!) considered in each treatment (male x female x fresh/cryopr. sperm). This is very low in fish fertilization tests.

>>>> Half of the 960 eggs we started with, i.e. N = 480 (!), were treated with cryopreserved sperm, the other 480 eggs were treated with non-cryopreserved sperm (as described in lines 131-138 and illustrated in Fig. S1A). The cryopreservation treatment was fully replicated 40 times (!) in the most powerful within-subject experimental design. This is, to the best of our knowledge, a level of replication that exceeds by far all previous experimental studies on the effects of cryopreservation.

It is clearly a strength of our experimental design that we used different males and different females to produce the 480 embryos per treatment group. Of course, the number of independent replicates per experimental cell declines with increasing number of dams and sires. We chose to work with many dams and many sires in order to get good estimates of parental effects and to properly control for them when testing for treatment effects. However, we kept the number of embryos per experimental cell high enough to allow for the type of statistical analyses we present.

The figures (1 and 2 A,B,C) show ns of 15 (when only 10 males were used, apparently only once...???) …

>>>> We had stated the number of males that were used in Figure 1A-C, see lines 192-193: "*In order to investigate potential effects of the cryopreservation protocol on sperm characteristics, 15 further brown trout males were sampled from the same populations*." The breeding experiment was performed with 40 males, as stated in lines 131-132. It is unclear to us how the reviewer could conclude that "… only 10 males were used". However, in order to possibly avoid further misunderstandings, we amended the description of the breeding blocks in the Methods (lines 131-133, lines 156-157) and the legend of Figure 1.

… or 200-300 (apparently considering all the lengths of the measured fish, but without considering wich male+female were their parents, and probably creating slants).

>>>> We had stated that we did "… *10 breeding blocks*" and that a breeding block was *"… 1 female crossed with 4 males*" (lines 131-132), i.e. each male was crossed with only one female. Therefore, when Figure 2 shows the "… *means and 95% CI (based on family means)"* (line 679), each of the 40 families, i.e. each of the 40 males, received equal weight in the figure.

Impossible to understand the bacterial challenge test. n??

>>>> We had stated that "*Half of the eggs of each combination of male x female x sperm treatment were exposed to bacteria, the other half was sham exposed, i.e. the treatments were full-factorial within each breeding block (see Figure S1)"* (line 168-171). Supplementary Figure S1A and B

illustrates this. As explained in the first paragraph of the Results, around 5% of the eggs in both treatment groups had to be classified as not fertilized. Therefore, the numbers of independent replicates were slightly reduced at the time we added the pathogen treatment. The statistical models in Tables 1 and 2 take this into account because they are based on embryos (except Table 1A that was based on eggs because this part is about the fertilization rates). In order to possibly avoid further misunderstandings, we now state that "*The calculation of embryo mortality was based on fertilized eggs*" (line 156-157).

All figures give the sample sizes per treatment group and dependent variable. Figures S4 and S5 give the respective numbers for the pathogen treatment. And all data are published on the Dryad repository.