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Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertilizer

Rowan A. Lymbery, Jonathan P. Evans and W. Jason Kennington

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Proc. R. Soc. B **287**: 20202147.
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Review timeline
Original submission: 12 June 2020
1st revised submission: 5 September 2020
2nd revised submission: 15 October 2020
Final acceptance: 15 October 2020

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

Review History

RSPB-2020-1392.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?
Good

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
I enjoyed reading and thinking about this study. Human-induced environmental change is likely to have strong impacts on the reproduction of external fertilisers because, unlike internal fertilisers whose sperm will gain some shelter in the female reproductive tract, their sperm will be directly exposed the abiotic stressors of environmental change. Recent studies on external fertilisers have shown that stressful environmental change can affect sperm phenotypes, their fertilisation rates, and their subsequent embryos. Yet, the potential mechanisms behind these effects remain unclear. I agree with the authors that changes to sperm transcript profiles under stress (e.g. through cellular damage) could be one of the key mechanisms underlying such effects, and that seemingly no studies examined this in external fertilisers. Although I would have liked to see a slightly more direct test of how transcript profiles under stress influences the sperm (or embryo) performance/fitness, I believe that overall this study provides a valuable contribution to the literature. I have a several comments below that the authors should address, but I believe they will be able to do so without too many problems.

The introduction is generally well written, but I think the authors need to explain the link between experiment 1 and 2 more clearly. I presume the authors conducted experiment 1 and 2 together with the aims of linking some changes in sperm transcript levels to changes in sperm phenotype, but unless I have misses something, there seems to be little explanation of this link to the readers.

Line 150: Was there any possibility of sperm ageing during this 30-minute period or has this already been assessed in past/pilot studies? Any such ageing during the 30-minute period could have carry-over effects on sperm swimming behaviours and should at least be acknowledged.

Line 159: Seeing as this study does not appear to measure performance or fitness components (e.g., fertilisation or embryo success), it would be informative if the authors could provide some further information that shows 25C is high enough to impose thermal stress. For instance, are there any past studies that show 25C reduce any performance or fitness components in the study population?

Lines 159-162: Is ambient temperature the same as the average temperature? The phrasing here is a bit confusing.

Lines 164-166: While I don't want to understate substantial amount of work the authors carried out for experiment 1 and 2, was there a specific reason why the same males weren't used for

experiment 1 and 2? Doing so would have enabled the authors to more directly test (at least at the level of male) for covariation between gene expression and sperm phenotypic traits, which would provide further value information. But perhaps I'm missing something.

Line 183-187: The authors should provide more justification (or citations of their past work) to explain why these traits were chosen. I think this justification is particularly important given they found temperature had no significant effect of temperature on these traits.

Line 250: I am fine with an eigenvalue above 1 being used as a cut-off point here, but the authors should provide readers with a more detailed explanation of why this was done or provide a relevant citation (e.g., Reynolds et al. (2010). Evolution, doi.org/10.1111/j.1558-5646.2009.00874.x)

Similar to my comments on the introduction, I think the discussion needs to provide more information on the results of experimental 1, and also needs to set up the link between experiment 1 and 2. Also, I would like the authors to explore if there were any reasons, aside from gene expression, that may explain why there were no phenotypic differences? For instance, physiological reasons why sperm might be robust or potentially selection on sperm swimming is favouring the same phenotypes at both temperatures.

Finally, a recent paper (Hadlow et al. 2020; JEB) that included some of the same co-authors here argued that sperm motility traits should be measured when sperm are exposed to "egg water", as egg-derived chemicals can alter phenotypic expression of these traits. Here, sperm motility traits were measured on sperm that do not appear to be exposed to egg water. Was there a reason for not exposing sperm to egg water here?

Review form: Reviewer 2

Recommendation
Reject – article is scientifically unsound

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?
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?
No

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This study tackles an interesting and novel question about the possible impacts of changes I temperature on the RNA profile of sperm after ejaculation. The study uses a broadcast spawning mussel and exposes sperm to two different temperature, both within the natural range of the species, but one reflecting a heatwave. The authors report no effect of temperature on sperm swimming velocity parameters, but show a putative effect on the abundance of RNA transcripts in some of four genes tested by qPCR. The aim was to test heat shock gene RNAs specifically with the expectation that these would show an upregulation in the heatwave treatment, and two housekeeping genes as controls. Interestingly, three of the genes show a decrease in RNA abundance in sperm exposed to the high temperature, whereas one housekeeping gene transcript shows no effect of temperature on its abundance.

1) While I do believe that this is a truly exciting question and a generally well written manuscript, I am less convinced of the actual results and their interpretation. I agree with the authors on one point: the observed differences in RNA abundance in three of the genes are probably not reflecting any change in gene expression but are rather a sign of increased rate of decay. However, the difference in abundance in the two heat shock genes seems marginal. It would be good to see some raw data of these, e.g. plots for each gene separately showing values in both treatments for each male linked by a colour coded line to show the consistent decrease across all tested males. Currently, the data in Figure 1 and significance of the result looks a little hard to believe given the large overlap in probability intervals between treatments 'A' and 'H'. The effect on one of the housekeeping genes seems much more pronounced.

2) In addition, it is not entirely clear why the authors decided to concentrate on only two heat shock genes and two housekeeping genes and did not take other genes such as genes involved in sperm metabolism into account? Heat shock is a very well specified reaction of cells to a very specific range of extreme temperatures. Do we know that 25°C induced a heat shock response in the blue mussel in the first place? If so, it would be great to see a reference and some explanation of this in the Introduction. Could other genes be tested in addition or could the temperature range be widened and the high temperature treatment go higher? Four genes seem very few given that we know so very little about what is going on in sperm after ejaculation.

3) Finally, it is surprising that no effects of temperature on sperm swimming velocity were found. Temperature usually has a rather pronounced effect on sperm metabolic rates in any species, and I am not sure what to make of this. Could it be that 25°C is not really such an unusual temperature and higher temperatures might need to be considered?

4) A minor comment is with respect to the statement on lines 47-49, claiming that the environmental changes and variation encountered by sperm in external fertilisers is likely to be higher than in internal fertilisers. I do think we still know very little about the environmental variation within female reproductive tracts, and factors such as pH, salinity, temperature (especially also in ectotherm species) etc. may vary just as much across females. I therefore would suggest phrasing this sentence more cautiously and expand it to become more general or remove

altogether.

So overall, I do think this is a potentially exciting study but it needs some more explanations and information in order to fully understand the results and whether they support the conclusions.

Minor comments

- It would be helpful to have the species mentioned in the abstract and not just referred to as a broadcast spawner.

- Overall, it would be better to stock to RNA profile or RNA abundance instead of RNA expression when describing the

- 220: To refer to 'expression of the heat shock genes' seems not entirely accurate here, as we don't know anything about the origin of these RNAs and whether the difference is actually caused by expression differences at all (or not by decay for example). Better stick to something like 'RNA abundance' of heat shock genes.

- 319/324: Maybe better describe the higher temperature treatment as 'high' is used in the Mat&Met and stick to the same term throughout the manuscript as 'heat shock' treatment seems not really appropriate here.

Decision letter (RSPB-2020-1392.R0)

@@date to be populated upon sending@@

Dear Dr Lymbery:

I am writing to inform you that your manuscript RSPB-2020-1392 entitled "Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser" 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.

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Sincerely,
Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Thank you for submitting your manuscript "Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser" to Proceedings B. I've now received two reviews of your manuscript and reviewed the paper myself. Both reviewers were positive about your manuscript but suggested revisions that need to be addressed. Both reviewers felt you should do more to explain the lack of effects of temperature on sperm motility and reconcile why your results differ from existing studies. Both reviewers also had concerns that the study lacked the depth needed to understand the implications of the effects on gene expression for sperm and subsequently embryo viability.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
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Author's Response to Decision Letter for (RSPB-2020-1392.R0)

See Appendix A.

RSPB-2020-2147.R0

Review form: Reviewer 1

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
As I commented with my previous review, I believe this manuscript makes a valuable contribution towards understanding the mechanistic basis of how ocean warming may impact sperm phenotypes, fertilisation rates, and subsequent embryos of external fertilising species. I was impressed by the authors' detailed and thoughtful response to each of my comments. I am happy to say that I now feel that all my concerns have been addressed, or at least suitably acknowledged. I have no further concerns that should prevent this manuscript from publication, and I congratulate the authors on a strong piece of work.

Decision letter (RSPB-2020-2147.R0)

14-Oct-2020

Dear Dr Lymbery

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

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

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

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

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

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

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

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

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

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
• DNA sequences: Genbank accessions F234391-F234402
• Phylogenetic data: TreeBASE accession number S9123
• Final DNA sequence assembly uploaded as online supplemental material
• Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311
NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

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

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

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

Sincerely,
Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member
Comments to Author:
Thank you for submitting your manuscript "Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser" to Proceedings B. I've now received a reviews from one reviewer who is pleased with the revisions made to your manuscript, and I agree with this assessment.

Reviewer(s)' Comments to Author:

Referee: 1
Comments to the Author(s).
As I commented with my previous review, I believe this manuscript makes a valuable contribution towards understanding the mechanistic basis of how ocean warming may impact sperm phenotypes, fertilisation rates, and subsequent embryos of external fertilising species. I was impressed by the authors' detailed and thoughtful response to each of my comments. I am happy to say that I now feel that all my concerns have been addressed, or at least suitably acknowledged. I have no further concerns that should prevent this manuscript from publication, and I congratulate the authors on a strong piece of work.

Author's Response to Decision Letter for (RSPB-2020-2147.R0)

See Appendix B.

Decision letter (RSPB-2020-2147.R1)

15-Oct-2020

Dear Dr Lymbery

I am pleased to inform you that your manuscript entitled "Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser" 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.

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

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

THE UNIVERSITY OF WESTERN AUSTRALIA

Centre for Evolutionary Biology
School of Biological Sciences (M092)

Appendix A

31 August 2020

Dr Maurine Neiman

Preprint Editor, *Proceedings of the Royal Society B*

Re: Manuscript RSPB-2020-1392 Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser

Dear Dr Neiman,

We are delighted to accept the invitation to provide a re-submission of our revised manuscript to *Proceedings of the Royal Society B*. We believe we have fully addressed the helpful and constructive comments from both reviewers and the Associate Editor in our revisions, as detailed in the point-by-point responses to the referee comments provided below.

We are grateful for your time in considering our manuscript, and the invitation to re-submit to *Proceedings of the Royal Society B*. We look forward to hearing your decision.

Yours sincerely,

Dr Rowan Lymbery (corresponding author)

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M092 Perth WA 6009 Australia

T +61 8 6488 5824
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E rowan.lymbery@uwa.edu.au
CRICOS Provider Code 00126G

Associate Editor

Comments to Author:

Thank you for submitting your manuscript "Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser" to Proceedings B. I've now received two reviews of your manuscript and reviewed the paper myself. Both reviewers were positive about your manuscript but suggested revisions that need to be addressed. Both reviewers felt you should do more to explain the lack of effects of temperature on sperm motility and reconcile why your results differ from existing studies. Both reviewers also had concerns that the study lacked the depth needed to understand the implications of the effects on gene expression for sperm and subsequently embryo viability.

Response: We are grateful to the Associate Editor for their time in assessing our manuscript. We have provided responses, and associated revisions to the manuscript, for each of the comments from the referees below.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

I enjoyed reading and thinking about this study. Human-induced environmental change is likely to have strong impacts on the reproduction of external fertilisers because, unlike internal fertilisers whose sperm will gain some shelter in the female reproductive tract, their sperm will be directly exposed the abiotic stressors of environmental change. Recent studies on external fertilisers have shown that stressful environmental change can affect sperm phenotypes, their fertilisation rates, and their subsequent embryos. Yet, the potential mechanisms behind these effects remain unclear. I agree with the authors that changes to sperm transcript profiles under stress (e.g. through cellular damage) could be one of the key mechanisms underlying such effects, and that seemingly no studies examined this in external fertilisers. Although I would have liked to see a slightly more direct test of how transcript profiles under stress influences the sperm (or embryo) performance/fitness, I believe that overall this study provides a valuable contribution to the literature. I have a several comments below that the authors should address, but I believe they will be able to do so without too many problems.

Response: We are delighted that the referee considers our manuscript to be a valuable contribution to the literature overall, and thank them for taking the time to assess the manuscript and provide valuable comments. We believe we have addressed the referee's comments as outlined in our responses below.

The introduction is generally well written, but I think the authors need to explain the link between experiment 1 and 2 more clearly. I presume the authors conducted experiment 1 and 2 together with the aims of linking some changes in sperm transcript levels to changes in sperm phenotype, but unless I have misses something, there seems to be little explanation of this link to the readers.

Response: We agree that this link was not made sufficiently clear in the introduction. Previous findings for sperm motility phenotypes in this species suggest they might maintain normal function under elevated temperatures (Eads et al. 2016 *Mar. Ecol. Prog. Ser.* 562:101–111), although decreases in fertilisation rate have also been reported (Eads et al. 2016 *Ecol. Evol.* 6:6578–6585). **We now explicitly refer to these previous findings on L 98-101.** Importantly, molecular effects of heat shock have never been explored; as previously noted on L 73-76, changes to components such as RNA profiles could underlie the maintenance of normal phenotypic function in sperm. Moreover, molecular changes can be unlinked to sperm phenotypic responses, yet can still have important effects on fertilisation and embryo development (Siklenka K et al. 2015 *Science* 350: aab2006; see L 76-79). **We now explicitly refer to back to these arguments when explaining the rationale for exploring molecular changes to sperm in *M. galloprovincialis* (L 102-104). In the final paragraph of the Introduction, we have also explicitly clarified the links between Experiment 1 and Experiment 2; i.e. clarifying whether sperm can indeed maintain normal phenotypic function at elevated temperatures, and then exploring the putative underlying molecular responses to heat stress (L 111-115).**

Line 150: Was there any possibility of sperm ageing during this 30-minute period or has this already been assessed in past/pilot studies? Any such ageing during the 30-minute period could have carry-over effects on sperm swimming behaviours and should at least be acknowledged.

Response: Sperm in *Mytilus* spp. can remain fertilization-competent for longer than 11 hours (Sprung and Bayne 1984 *ICES Journal of Marine Science* 41: 125-128), and studies in *M. galloprovincialis* have found sperm remain fully motile after 3-hour trials (Evans et al. 2012 *Proc. R. Soc. B* 279: 20120181; Oliver and Evans 2013 *Proc R. Soc. B* 281: 20140148). Therefore, sperm ageing effects are likely to be negligible over the 30-minute window of gamete collection. Moreover, the procedures we employ here for gamete collection, quantification and preparation have been applied with no detrimental effects on sperm motility in a number of other studies in this species (e.g. Fitzpatrick et al. 2012 *Evolution* 66:2451–2460; Lymbery et al. 2018 *Am. Nat.* 192: 94-104, in addition to the two studies mentioned in the previous sentence). **We have added pertinent information on this to the manuscript (see L 155-158).**

Line 159: Seeing as this study does not appear to measure performance or fitness components (e.g., fertilisation or embryo success), it would be informative if the authors could provide some further information that shows 25C is high enough to impose thermal

stress. For instance, are there any past studies that show 25C reduce any performance or fitness components in the study population?

Response: A previous study on this population of *M. galloprovincialis* has reported that elevated temperatures of 24°C cause significant reductions in fertilisation rate (Eads et al. 2016 *Ecol. Evol.* 6:6578–6585), and further work on this species has found that larval survival and size are reduced at temperatures 3-4°C above average (Vihtakari et al. 2013 *Water* 5:1890–1915). In fact, even adult mortality can be substantial at temperatures of 3°C above average (Gazeau et al. 2014 *Front. Mar. Sci.* 1:62.), and a heatwave event off the Western Australian coastline in 2011, where temperatures regularly reached 5-6°C above average, caused mass mortality of marine invertebrates (Pearce and Feng. 2013 *J. Mar. Syst*. 111–112:139–156**). We have added a sentence to the introduction referring to these fitness implications of temperature anomalies in *M. galloprovincialis* (L 94-96).**

Lines 159-162: Is ambient temperature the same as the average temperature? The phrasing here is a bit confusing.

Response: We agree that the wording here was not clear; **we have added "average temperature during the spawning season" after "ambient" (L 166-167).**

Lines 164-166: While I don't want to understate substantial amount of work the authors carried out for experiment 1 and 2, was there a specific reason why the same males weren't used for experiment 1 and 2? Doing so would have enabled the authors to more directly test (at least at the level of male) for covariation between gene expression and sperm phenotypic traits, which would provide further value information. But perhaps I'm missing something.

Response: The major reason for not always using the same males for collection of both motility and RNA data was, as alluded to in the referee's comment, logistical. Due to the location of the respective experimental set-ups and equipment, the RNA sample collection had to be performed in a separate lab (indeed, a different building) to the mussel spawning and CASA analysis. Therefore, there was typically not enough time to perform both sets of sample collection on the same day.

However, on two of the experimental days, we did have sufficient research assistance to collect both motility and RNA data from the same males, meaning a subset of $n = 11$ males had both sets of data recorded. In response to the referee's comment, we compared correlation tests of the change in each motility PC across treatments, with the change in RNA counts of *hsp70, hsp90* and *gapdh*. As expected, there were no significant correlations between the (lack of) change in either motility PC and the changes in RNA counts. **We have added these correlation tests to the supplementary materials (Table S4), with reference to them in the main text Results (L 341-345), and also now mention in the Methods that a subset of males had both sets of data recorded (L 168-170)**

Line 183-187: The authors should provide more justification (or citations of their past work) to explain why these traits were chosen. I think this justification is particularly important given they found temperature had no significant effect of temperature on these traits.

Response: We have previously reported these traits to predict fertilisation success (both competitive and non-competitive) in a series of studies on this species; **we have added this justification and the relevant citations to the manuscript (L 199-200).**

Line 250: I am fine with an eigenvalue above 1 being used as a cut-off point here, but the authors should provide readers with a more detailed explanation of why this was done or provide a relevant citation (e.g., Reynolds et al. (2010). Evolution, doi.org/10.1111/j.1558-5646.2009.00874.x)

Response: We have added the citation as suggested by the referee (L 263). We also agree that the eigenvalue > 1 rule can be somewhat arbitrary; in practice, our choice of the first two PCs was based on applying this criterion *and* examining the cumulative variance in original traits explained by the PCs (> 90% in our case). **We now mention in the methods the percentage of variance explained by the first two PCs (L 263-264), and in the results we have de-emphasised the 'eigenvalue greater than one' criterion (L 309).**

Similar to my comments on the introduction, I think the discussion needs to provide more information on the results of experimental 1, and also needs to set up the link between experiment 1 and 2. Also, I would like the authors to explore if there were any reasons, aside from gene expression, that may explain why there were no phenotypic differences? For instance, physiological reasons why sperm might be robust or potentially selection on sperm swimming is favouring the same phenotypes at both temperatures.

Response: We agree with the referee that, as for the introduction, these links were not made clear enough in the discussion. We have adjusted the first paragraph of the discussion to rectify this; **specifically, we now mention that the changes to sperm RNAs such as heat shock genes may represent a functional response, in which proteins involved in stress response are translated to maintain normal (unchanged) swimming patterns (L 355-357). We also now note on L 357-360 that maintaining unchanged sperm motility phenotypes (i.e. not swimming faster at higher temperatures) might be important for fitness in broadcast spawners, where swimming in slow patterns is critical for tracking eggs** (Fitzpatrick et al. 2012. *Evolution* 66: 2451–2460; Lymbery et al. 2018. *Am. Nat.* 192: 94-104). **Finally, we state explicitly that regardless of whether the molecular changes to sperm are related to phenotypic function, they could have fitness implications for early embryos; therefore, we may miss important effects of factors such as heat stress when we only examine sperm phenotypes (L 360-362; 365-366).**

Finally, a recent paper (Hadlow et al. 2020; JEB) that included some of the same co-authors here argued that sperm motility traits should be measured when sperm are exposed to "egg

water", as egg-derived chemicals can alter phenotypic expression of these traits. Here, sperm motility traits were measured on sperm that do not appear to be exposed to egg water. Was there a reason for not exposing sperm to egg water here?

Response: There were two important reasons for not exposing sperm to egg water in the current study. First, we would not have able to separate the effects of high temperature treatments on the sperm themselves from effects on components of the egg water. For example, if sperm motility in egg water was different at high temperature, would that be due to phenotypic effects on heat shock on sperm, or degradation of egg water signals? While this might be an interesting question, it is beyond the scope of the current study. It should also be noted that in Hadlow et al. 2020 (JEB), we argue that both swimming in sea water and swimming in egg water are relevant to overall sperm function and fitness. **We have added a line in the methods to explain the rationale for not measuring sperm motility traits in egg water in this study (L 189-193)**. Second, if we had measured sperm motility in egg water, we would then have needed to include egg water in the sperm RNA assays to maintain comparability of the two experiments. In that scenario, there would have been a strong likelihood of contamination by non-sperm RNAs, i.e. egg- or egg water-borne RNAs, which could have confounded the target sperm RNA profiles.

Referee: 2

Comments to the Author(s)

This study tackles an interesting and novel question about the possible impacts of changes I temperature on the RNA profile of sperm after ejaculation. The study uses a broadcast spawning mussel and exposes sperm to two different temperature, both within the natural range of the species, but one reflecting a heatwave. The authors report no effect of temperature on sperm swimming velocity parameters, but show a putative effect on the abundance of RNA transcripts in some of four genes tested by qPCR. The aim was to test heat shock gene RNAs specifically with the expectation that these would show an upregulation in the heatwave treatment, and two housekeeping genes as controls. Interestingly, three of the genes show a decrease in RNA abundance in sperm exposed to the high temperature, whereas one housekeeping gene transcript shows no effect of temperature on its abundance.

Response: We are very grateful to the referee for their time in assessing our manuscript, and for providing important and helpful comments. Below, we outline our responses and associated revisions for each of the comments.

1) While I do believe that this is a truly exciting question and a generally well written manuscript, I am less convinced of the actual results and their interpretation. I agree with the authors on one point: the observed differences in RNA abundance in three of the genes are probably not reflecting any change in gene expression but are rather a sign of increased rate of decay. However, the difference in abundance in the two heat shock genes seems marginal.

It would be good to see some raw data of these, e.g. plots for each gene separately showing values in both treatments for each male linked by a colour coded line to show the consistent decrease across all tested males. Currently, the data in Figure 1 and significance of the result looks a little hard to believe given the large overlap in probability intervals between treatments 'A' and 'H'. The effect on one of the housekeeping genes seems much more pronounced.

Response: We appreciate the referee's thoroughness in wishing to interrogate the biological significance (in addition to the statistical significance) of the results. We acknowledge that our original Figure 1 did not make this immediately clear. We should, however, emphasise two features of the original figure: (1) the y-axis is on a log scale, with estimates extracted from a Poisson model; and (2) the whiskers are 95% credible intervals from the posterior distributions. Thus, overlap in the intervals does not necessarily cast doubt on the size or significance of differences, but the referee is correct in pointing out that it is difficult to clearly judge this from the original figure alone.

We have therefore adjusted Figure 1 to better visualise the results based on the referee's suggestions. We have separated the plot by gene into four panels and have added individual lines of difference colours for each male's transcript count in the two treatments. As expected, the majority of males experience a decrease in expression for the two genes that had statistically significant changes (*hsp90* and *gapdh*; Fig. 1B and 1C), although intriguingly there were some males (approximately 4-5) in each gene that experienced the opposite effects. This raises the possibility that males might vary in their plastic responses to temperature, although our experiment was not designed to statistically test among-male variation in responses. However, these apparent among-male variations are insightful, as they provide additional evidence that transcript count does not universally decrease, as might be expected if the response was due solely to heat-induced mRNA decay. Indeed, among-individual variation in plasticity is a hallmark of biological responses to temperature changes, including for sperm cells (e.g. Purchase et al. 2010. *Can. J. Fish. Aquat. Sci.* 67:498–510; Eads et al. 2016 *Ecol. Evol.* 6:6578–6585). **We have added these points and their potential interpretation to the discussion paragraph that deals with the possibilities of global vs. specific and functional vs. non-functional RNA changes (L 398-403).**

For *hsp70* (Fig. 1B), we avoided interpreting the trend of a negative fold change in our manuscript given its non-significance, focussing on the significant changes in *hsp90* and *gapdh* (see L 387-388; 394-397; 405-419). The stable housekeeping gene, *actin*, has no obvious pattern of change across males (Fig. 1D).

Regarding the size of the effect for *hsp90* and *gapdh*, as noted above the plot visualisations are on a log scale; the fold-changes that we reported in the manuscript were calculated by back-transforming from the posterior results of the analyses. This revealed that the transcript counts were on average approximately 1.5 times and 3 times higher in the ambient treatment than the high temperature treatment for *hsp90* and *gapdh* respectively. As noted by the reviewer, the effect on *gapdh* is more pronounced; however, a 50% reduction in transcript number as reported for *hsp90* could certainly be biologically meaningful for cells (Vallejos et

al. 2016 *Genome Biology* 17:70). **We now explicitly note these fold changes and their potential to be biologically meaningful in the discussion (L 351-354).**

2) In addition, it is not entirely clear why the authors decided to concentrate on only two heat shock genes and two housekeeping genes and did not take other genes such as genes involved in sperm metabolism into account? Heat shock is a very well specified reaction of cells to a very specific range of extreme temperatures. Do we know that 25°C induced a heat shock response in the blue mussel in the first place? If so, it would be great to see a reference and some explanation of this in the Introduction. Could other genes be tested in addition or could the temperature range be widened and the high temperature treatment go higher? Four genes seem very few given that we know so very little about what is going on in sperm after ejaculation.

Response: While we agree with the referee that building on our findings with a broad range of sperm genes could be interesting and informative, we chose this specific set of genes in our current study for several reasons. First, very little is known about the sperm RNA content of external fertilisers generally; the vast majority of sperm transcriptomic analyses have focused on internal fertilisers such as mammals and fruit flies (Jodar et al. 2013 *Hum. Reprod. Update* 19:604–624; Evans et al. 2019 *Reproduction* 157:R109–R126; Immler 2018. *Heredity* 121:239–247). Further, mussels (including *M. galloprovincialis*) are under-represented in terms of genomic resources, making identification of genes and transcripts in these species challenging (Gomes-dos-Santos et al. 2020. *Hydrobiologia* 847:1705–1726). While there have been some RNA-seq and proteomic investigations into the gonads and sperm of *M. galloprovincialis*, annotation rates are typically very low, particularly for RNA transcripts (e.g. Romero et al. 2019 *J. Proteomics* 192:169–187). As we outline in the introduction (L 67-84), we were specifically interested in RNAs, given their potential sensitivity to environmental change and relevance for both sperm and embryos. We chose here to focus on a small number of genes that had *a priori* biological relevance to our question, and that we knew occurred in sperm of *M. galloprovincialis*, rather than fish for transcripts that we could not be certain were present.

Second, our heat shock genes met our above criteria: they had previously been identified as present in *M. galloprovincialis* sperm, and were specifically relevant to the temperature treatments in our study (see L 115-118; 120-123). Indeed, in answer to one of the referee's specific points above, temperature increases in the range we employ here (i.e. 5-6°C above ambient seawater temperature) have previously been shown to induce the heat shock reaction in *M. galloprovincialis*, including upregulation of *hsp70* and *hsp90* expression (e.g. Dutton, and Hoffman 2009 *J. Exp. Mar. Bio. Ecol.* 376:37–42; Ioannou et al. 2009 *J. Exp. Mar. Bio. Ecol.* 381:136–144). **As suggested by the reviewer, we have added this explanation to the introduction with the relevant citations (L 118-120).**

While it is entirely possible that pushing the high temperature treatment to more extreme levels would have led to an even stronger response, we do not feel this would be biologically relevant. As described in the manuscript (L 92-94; 167-168), the 25°C treatment reflects the upper maxima of extreme heatwave events at 6°C above average ambient conditions. In

comparison, *average* changes in sea surface temperature under high emissions scenarios by the end of the century are predicted to be at most 3°C (IPCC. 2013. Climate Change 2013: The Physical Science Basis). Thus, our treatment represents the upper level of heat shock that mussel sperm would be expected to experience in nature.

3) Finally, it is surprising that no effects of temperature on sperm swimming velocity were found. Temperature usually has a rather pronounced effect on sperm metabolic rates in any species, and I am not sure what to make of this. Could it be that 25°C is not really such an unusual temperature and higher temperatures might need to be considered?

Response: Previous findings regarding the effects of temperature on sperm motility and function in *M. galloprovincialis*, and broadcast spawners generally, have been inconsistent. For example, sperm velocity, linearity or proportion of motile sperm have been reported to increase, decrease or be unaffected by elevated spawning temperatures in *M. galloprovincialis*, in some cases depending on the time of exposure (Vihtakari et al. 2013 *Water* 5:1890–1915; Eads et al. 2016 *Mar. Ecol. Prog. Ser.* 562:101–111). Other studies have examined the effects of heat exposure in adults prior to spawning, but not the post-spawning temperature experienced by sperm themselves (e.g. Boni, R. et al. 2016 *Mol. Reprod. Dev.* 83:162–173). For broadcast spawners more generally, sperm performance appears to be relatively robust to temperature increases (see e.g. review by Byrne *2011 Oceanogr. Mar. Biol. Annu. Rev*. 49: 1–42).

In our study, we aimed to clarify the effects of temperature on spawned sperm during a biologically relevant period of exposure prior to fertilisation; the dense, competitive spawning environments in this species means that an acute short-term exposure is likely to be most relevant to reproductive success (as outlined on L 173-175). We were also interested in the effects of extreme temperature fluctuations such as heatwaves in the current environment, i.e. situations relevant to contemporary populations. As we note in our response to the referee's previous comment, our treatment represents the upper level of heat shock that mussel sperm would be expected to experience in nature, and therefore higher temperatures are unlikely to be biologically relevant.

Given the inconsistencies in previous findings, the absence of an effect of our acute high temperature treatment on sperm motility is not entirely surprising. While cellular metabolic rate might be expected to be temperature-dependent, the apparent robustness of broadcast spawner sperm to high temperatures suggests they may have mechanisms to maintain normal functionality. For example, prior to reaching critical thresholds, metabolic rate might be expected to increase with temperature, leading to faster sperm swimming. However, in broadcast spawners such as *M. galloprovincialis*, faster swimming might actually reduce relative fitness (e.g. Fitzpatrick et al. 2012. *Evolution* 66: 2451–2460; Lymbery et al. 2018. *Am. Nat.* 192: 94-104); therefore, cellular mechanisms to help maintain normal swimming velocity may be adaptive. Indeed, this is a key component of our rationale in examining molecular changes that may be involved in maintaining cellular function under heat stress, alongside sperm motility phenotypes (see also responses to comments by referee 1). **We have altered components of the introduction and discussion to make these links, and the expectations regarding sperm motility, clearer (L 98-104; 111-115; 355-362; 365-366).**

4) A minor comment is with respect to the statement on lines 47-49, claiming that the environmental changes and variation encountered by sperm in external fertilisers is likely to be higher than in internal fertilisers. I do think we still know very little about the environmental variation within female reproductive tracts, and factors such as pH, salinity, temperature (especially also in ectotherm species) etc. may vary just as much across females. I therefore would suggest phrasing this sentence more cautiously and expand it to become more general or remove altogether.

Response: We agree with the referee that this statement was too strong in comparing the environmental variation experienced by externally and internally fertilising sperm. **We have therefore reduced this sentence; it now reads: "We would therefore also expect highly variable post-ejaculatory modifications in these taxa" (L 46-47).**

So overall, I do think this is a potentially exciting study but it needs some more explanations and information in order to fully understand the results and whether they support the conclusions.

Response: We are pleased that the referee considers this to be a potentially exciting study. **Please see our responses to the comments above and associated changes to the manuscript, which provide further explanation and information as requested by the referee.**

Minor comments

- It would be helpful to have the species mentioned in the abstract and not just referred to as a broadcast spawner.

Response: We have changed the phrase as requested to "the mussel *Mytilus galloprovincialis*" (L 20).

- Overall, it would be better to stock to RNA profile or RNA abundance instead of RNA expression when describing the

Response: This is an excellent point; we have changed instances of "RNA/gene expression" to "RNA abundance", "transcript abundance" or "RNA profile" throughout the manuscript.

- 220: To refer to 'expression of the heat shock genes' seems not entirely accurate here, as we don't know anything about the origin of these RNAs and whether the difference is actually

caused by expression differences at all (or not by decay for example). Better stick to something like 'RNA abundance' of heat shock genes.

Response: **We agree completely and have changed the wording to "RNA abundance" as requested (L 233).**

- *319/324: Maybe better describe the higher temperature treatment as 'high' is used in the Mat&Met and stick to the same term throughout the manuscript as 'heat shock' treatment seems not really appropriate here.*

Response: We have changed the wording as requested to "the high temperature treatment" (L327-328; 330-331; 335-336).

Appendix B

Associate Editor

Board Member

Comments to Author:

Thank you for submitting your manuscript "Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertiliser" to Proceedings B. I've now received a review from one reviewer who is pleased with the revisions made to your manuscript, and I agree with this assessment.

Response: We thank the Associate Editor, and both anonymous reviewers, very much for their time in assessing our manuscript.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).

As I commented with my previous review, I believe this manuscript makes a valuable contribution towards understanding the mechanistic basis of how ocean warming may impact sperm phenotypes, fertilisation rates, and subsequent embryos of external fertilising species. I was impressed by the authors' detailed and thoughtful response to each of my comments. I am happy to say that I now feel that all my concerns have been addressed, or at least suitably acknowledged. I have no further concerns that should prevent this manuscript from publication, and I congratulate the authors on a strong piece of work.

Response: We are pleased the reviewer considers that our revision has addressed all their concerns, and we thank them very much again for their time in assessing our manuscript and providing valuable comments.