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Whole-organism responses to constant temperatures do not predict responses to variable temperatures in the ecosystem engineer *Mytilus trossulus*

Katie E. Marshall, Kathryn M. Anderson, Norah E. M. Brown, James K. Dytnerski, Kelsey L. Flynn, Joey R. Bernhardt, Cassandra A. Konecny, Helen Gurney-Smith and Christopher D. G. Harley

Article citation details
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Review timeline

Original submission:	30 November 2020	
Revised submission:	19 February 2021	
Final acceptance:	26 February 2021	

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

Review History

RSPB-2020-2968.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
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
MAJOR POINTS.
This is a very fine study, one that examines an underappreciated aspect of thermal relationships, the relative effects of stable versus variable temperatures on organismal physiological performance. Jensen's Inequality is a critically important issue in thermal relationships yet is only infrequently considered by experimenters. This paper provides a clear demonstration of why this Inequality can matter so much. The experiments appear to have been well designed and carefully performed. My main criticisms concern aspects of the writing that could use some attention in order to improve clarity.

MINOR ISSUES.
1. I have some issues with the title. The phrase, "Thermal sensitivity at constant temperatures…" sounds almost oxymoronic. Might there be a better way to phrase the title? I suggest giving the name of the species in the title. I suggest replacing, "…an ecosystem engineer" with "the ecosystem engineer, Mytilus trossulus. Lines 46-47: I note that the species' name is not in the keywords either. It should be.
2. 34: "stable conditions" and "variable conditions" of what sort? Temperature, diet, or ?? Please be more specific here as to what "conditions" are referred to.
3. 39-40: why not replace "conditions" with "temperatures?"
4. Line 74: Again, it sounds rather oxymoronic to speak of a TPC being determined "under stable temperature conditions." Can this point be restated?
5. When I teach Jensen's Inequality, I find it helpful to give a quantitative example based on temperature coefficient (Q10) relationships. For example, if a biological process like respiration has a Q10 of 2.0 and a rate at 10C of 10 units/hour, then it will exhibit a rate of 40 units/hour at 30C. If the process runs for one hour at each temperature, then a total of 50 units of activity will occur and the average value over the two hours is 25 units/hour. The mean temperature is 20C in this study. If the process runs at a constant 20C for two hours, then 40 units of activity will occur. Thus, the acceleration in rate due to Q10 effects gives a higher average rate under variable temperature conditions. It might be helpful to some readers to give an example of this sort (perhaps in the legend to Figure 1) to illustrate Jensen's Inequality. I'm not convinced that the text in lines 65-68 presents the Inequality in an especially clear manner. We've all taken calculus, but the expression, "predictable from the direction of the second derivative" is not all that intuitively clear in my estimation.

6. Line 141: “different measures of thermal performance” might be better stated as “thermal performances of different traits”.
7. Line 224: Place an “at” before “three.”
8. Line 314: “led”
9. Line 353: “data” “indicate”
10. Line 422: different “traits” instead of “measures”

Review form: Reviewer 2

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

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

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

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

Is the length of the paper justified?
Yes

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

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
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> **Is it clear?**
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>
> **Is it adequate?**
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Do you have any ethical concerns with this paper?
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Comments to the Author
Summary/General Thoughts:

This paper addresses important hypotheses regarding the extent to which thermal performance at constant acclimation temperatures predicts performance at fluctuating temperatures. The thermal performance curve is a gold standard in the field of thermal biology, but its applicability to natural, variable environments is uncertain. Here, the authors make three important findings regarding thermal performance: 1) measurements at constant conditions rarely predict measurements at variable conditions, 2) distinct traits have different thermal sensitivities, and 3) the relationship between static and fluctuating measurements is population dependent. Overall, the paper is well-conceived and well-presented, although the limited ecological relevance of the treatments, and the limited number of conditions, somewhat limits the scope of the work. Also, some of the conclusions are perhaps too strongly worded, considering the actual results. That said, I do think the paper will stimulate discussion and follow-up studies in other systems to test the generalizability of these findings. See comments below.

Major Comments:

1. While the authors acknowledge that their conditions are not particularly ecologically relevant, I would like to see additional justification for the selection of temperatures. According to the data in Figure S1, 18 and 6°C are rarely experienced in Reed Point and never experienced Tofino. In both populations, survival decreased in the 18°C treatment, suggesting this is close to the thermal limits for this species. Thus, while your results are fairly straightforward, I wonder whether some of the effects are inflated by the "sledgehammer" approach of using such extreme temperatures (not to mention the dramatic, instantaneous shift between 6 and 18 in the fluctuating group). Also, in some sense, you have an unreplicated experiment, since there is N=1 fluctuating condition. I would expect that the magnitude and timing of fluctuation would have some bearing on the relationship between static and fluctuating thermal performance. While I don't expect you to conduct more experiments (especially in a pandemic!), I would caution against strongly worded statements like "…these assumptions do not hold in an ecologically important marine species" (line 424) and that "significant caution must be taken in the use of thermal performance curves" (line 42). You nicely show that the assumptions break down under the limited set of extreme conditions used in this study, but I think it is premature to conclude that all constant temperature thermal performance curves have limited utility in wild settings (although it wouldn't surprise me if that ultimately ends up being true).

2. As someone who is unfamiliar with the mathematical aspects of a thermal performance curve, this could be a naïve comment, so apologies in advance. However, with the limited number of conditions, it is impossible to truly estimate a thermal performance curve, at least as presented in Figure 1, and indeed, very few measurements seem to be following a trajectory consistent with the classic shape of a thermal performance curve. For example, some traits monotonically increase or decrease (see Fig. 2, 3, 5) or they show little to no response to temperature change (Figure 4). Thus, is it fair to test assumptions of thermal performance curves, when many of your results do not match with the expected shape of a performance curve (or there is limited resolution to say for sure)? I still think you can clearly say that responses to these fluctuating conditions cannot be predicted from constant conditions, but I'm not sure the link to thermal performance curves sensu stricto is as direct as you imply in the Introduction. But again, I'm not as familiar with the mathematics that underlie construction of thermal performance curves, so I could be off base.

3. While the results clearly show that some assumptions of thermal performance curves might need revisiting, it is unclear what can be done about it. Perhaps there is some opportunity in the Discussion or Conclusions section to make recommendations for how to overcome these problems. There is a bit of that in lines 430-432, but these ideas could be expanded. In particular, what can be done to make this information useful for predictive modeling, since that is the stated goal for many thermal biology studies?

Minor Comments:

1. Line 69: You mention a "point A" in Figure 1, but I don't see any points labeled. Also, the caption of Figure 1 could be expanded a bit. I assume the purple lines are reflecting predictions

based on the red lines, but there is no explanation of the colors in the caption.

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3. Line 159-160 and elsewhere: Parts of the paper suggest that all mussels were identified to species level, while the supplemental methods indicates that only a subset were identified. Perhaps the subsample was large enough to conclude that all mussels were the same species, but make sure you're clear about whether all mussels were identified with PCR.

4. Figure S2: The boxplots are probably unnecessary, because some groups appear to only have N=3 (a boxplot summarizes a minimum of five data points).

5. All figures: when possible, I would recommend indicating statistical significance, or a lack thereof, directly on the figure. That way readers looking at figures can tell if a difference is significant without having to look back through the results section.

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7. Statistical analyses: were any random effects included in the models to account for mussels being grouped together and for tank effects? Form looking at the raw data, it seems some data do not include any sort of grouping variable.

8. Lines 326-330: Can you provide any quantitative support for these claims? You don't report thermal optima for any of the traits in the paper, and some traits were not particularly sensitive to temperature change. I agree with this statement qualitatively, but this conclusion could be strengthened with some empirical support.

9. While many of the measured results differed from the predicted results, there are some cases where the measured values are at least in the expected direction. For example, that appears to be the case for Figure 2, partially for Figure 3, and Figure 5. So while the values may not be 100% accurate, it might be fair to point out that in at least a few cases, the directionality was predicted.

Decision letter (RSPB-2020-2968.R0)

15-Jan-2021

Dear Dr Marshall:

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

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

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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.

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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. Please see our Data Sharing Policies (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

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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.

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

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

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr Sasha Dall
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Both reviewers are generally positive, while also coming up with a range of questions or suggestions which, if acted on, will result in a clearer and more accessible ms. While the two reviewers raise generally different specific points, they do not appear to me to contradict, and do share an aspect of asking for clearer explanations (for instance explaining Jensen's Inequality in a more accessible way). I would advise following the suggestions of one reviewer to moderate or be more balanced in some of the concluding statements made.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)

MAJOR POINTS. This is a very fine study, one that examines an underappreciated aspect of thermal relationships, the relative effects of stable versus variable temperatures on organismal physiological performance. Jensen's Inequality is a critically important issue in thermal relationships yet is only infrequently considered by experimenters. This paper provides a clear demonstration of why this Inequality can matter so much. The experiments appear to have been well designed and carefully performed. My main criticisms concern aspects of the writing that could use some attention in order to improve clarity.
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3. While the results clearly show that some assumptions of thermal performance curves might need revisiting, it is unclear what can be done about it. Perhaps there is some opportunity in the Discussion or Conclusions section to make recommendations for how to overcome these problems. There is a bit of that in lines 430-432, but these ideas could be expanded. In particular, what can be done to make this information useful for predictive modeling, since that is the stated goal for many thermal biology studies?

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8. Lines 326-330: Can you provide any quantitative support for these claims? You don't report thermal optima for any of the traits in the paper, and some traits were not particularly sensitive to temperature change. I agree with this statement qualitatively, but this conclusion could be strengthened with some empirical support.

9. While many of the measured results differed from the predicted results, there are some cases where the measured values are at least in the expected direction. For example, that appears to be the case for Figure 2, partially for Figure 3, and Figure 5. So while the values may not be 100% accurate, it might be fair to point out that in at least a few cases, the directionality was predicted.

Author's Response to Decision Letter for (RSPB-2020-2968.R0)

See Appendix A.

Decision letter (RSPB-2020-2968.R1)

26-Feb-2021

Dear Dr Marshall

I am pleased to inform you that your manuscript entitled "Whole-organism responses to constant temperatures do not predict responses to variable temperatures in the ecosystem engineer Mytilus trossulus " 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,
Dr Sasha Dall
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Comments to Author:
Thank you for the detailed attention to the points raised by the reviewers, and for the clear explanations given of the revisions made

Appendix A

Response to Referees

Associate Editor

Board Member: 1

Comments to Author:

Both reviewers are generally positive, while also coming up with a range of questions or suggestions which, if acted on, will result in a clearer and more accessible ms. While the two reviewers raise generally different specific points, they do not appear to me to contradict, and do share an aspect of asking for clearer explanations (for instance explaining Jensen's Inequality in a more accessible way). I would advise following the suggestions of one reviewer to moderate or be more balanced in some of the concluding statements made.

We thank the editor for their positive comments on our work and have endeavoured to clarify our writing and moderate our concluding statements.

Referee: 1

MAJOR POINTS. This is a very fine study, one that examines an underappreciated aspect of thermal relationships, the relative effects of stable versus variable temperatures on organismal physiological performance. Jensen's Inequality is a critically important issue in thermal relationships yet is only infrequently considered by experimenters. This paper provides a clear demonstration of why this Inequality can matter so much. The experiments appear to have been well designed and carefully performed. My main criticisms concern aspects of the writing that could use some attention in order to improve clarity.

We thank the reviewer for their kind words and appreciate the time they have taken to improve our manuscript.

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We have rephrased the title to “Whole-organism responses to constant temperatures do not predict responses to variable temperature in the ecosystem engineer *Mytilus trossulus*”

Lines 46-47: I note that the species’ name is not in the keywords either. It should be.

Completed as requested.

2. 34: “stable conditions” and “variable conditions” of what sort? Temperature, diet, or ?? Please be more specific here as to what “conditions” are referred to.

We have added “temperature” here to clarify.

3. 39-40: why not replace “conditions” with “temperatures?”

Completed as requested.

4. Line 74: Again, it sounds rather oxymoronic to speak of a TPC being determined “under stable temperature conditions.” Can this point be restated?

We were attempting to highlight that TPCs are generally determined by subjecting organisms to a temperature exposure once but appreciate that in our attempt at brevity we oversimplified. We have restated this point as:

In addition, the vast majority of TPC thermal performance studies have been conducted in the laboratory where organisms only experience test temperatures a single time, and often there is little information about source population history [1,2].

5. When I teach Jensen’s Inequality, I find it helpful to give a quantitative example based on temperature coefficient (Q10) relationships. For example, if a biological process like respiration has a Q10 of 2.0 and a rate at 10C of 10 units/hour, then it will exhibit a rate of 40 units/hour at 30C. If the process runs for one hour at each temperature, then a total of 50 units of activity will occur and the average value over the two hours is 25 units/hour. The mean temperature is 20C in this study. If the process runs at a constant 20C for two hours, then 40 units of activity will occur. Thus, the acceleration in rate due to Q10 effects gives a higher average rate under variable temperature conditions. It might be helpful to some readers to give an example of this sort (perhaps in the legend to Figure 1) to illustrate Jensen’s Inequality. I’m not convinced that the text in lines 65-68 presents the Inequality in an especially clear manner. We’ve all taken calculus, but the expression, “predictable from the direction of the second derivative” is not all that intuitively clear in my estimation.

We have added to the figure caption in Figure 1 to illustrate this point more carefully. While Q10 effects are a very useful example of the effect of the degree of curvature, many TPCs contain both accelerating and decelerating portions, and so we wanted to express how these impact the predicted response:

In general, Jensen's Inequality calculates that the mean of the response under fluctuating temperatures is elevated relative to the mean under constant temperatures in areas of the curve that are accelerating, while the converse is true in areas of the curve that are decelerating. The magnitude of this effect is directly related to both the amplitude of temperature variation and the degree of curvature of the TPC.

6. Line 141: "different measures of thermal performance" might be better stated as "thermal performances of different traits".

Completed as requested

7. Line 224: Place an "at" before "three."

Completed as requested

8. Line 314: "led"

Completed as requested

9. Line 353: "data" "indicate"

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10. Line 422: different "traits" instead of "measures"

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Referee: 2

Summary/General Thoughts:

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We thank the reviewer for their support and appreciate the limitations they pointed out. We have endeavoured to moderate our language as described below.

Major Comments:

1. While the authors acknowledge that their conditions are not particularly ecologically relevant, I would like to see additional justification for the selection of temperatures. According to the data in Figure S1, 18 and 6°C are rarely experienced in Reed Point and never experienced Tofino. In both populations, survival decreased in the 18°C treatment, suggesting this is close to the thermal limits for this species. Thus, while your results are fairly straightforward, I wonder whether some of the effects are inflated by the "sledgehammer" approach of using such extreme temperatures (not to mention the dramatic, instantaneous shift between 6 and 18 in the fluctuating group). Also, in some sense, you have an unreplicated experiment, since there is N=1 fluctuating condition. I would expect that the magnitude and timing of fluctuation would have some bearing on the relationship between static and fluctuating thermal performance. While I don't expect you to conduct more experiments (especially in a pandemic!), I would caution against strongly worded statements like "…these assumptions do not hold in an ecologically important marine species" (line 424) and that "significant caution must be taken in the use of thermal performance curves" (line 42). You nicely show that the assumptions break down under the limited set of extreme conditions used in this study, but I think it is premature to conclude that all constant temperature thermal performance curves have limited utility in wild settings (although it wouldn't surprise me if that ultimately ends up being true).

We had decided to use the "sledgehammer" approach because we expected our animals to be quite tolerant of temperature fluctuations—*M. trossulus* is frequently found in the intertidal zone, and so our temperature conditions were designed to be less extreme than the aerial exposures in the intertidal, but a little more extreme than surface water conditions. However even seawater temperatures can vary quickly and by more than one might expect in mussel habitats; we have measured water temperature changes of up to 10°C on a single rising tide in Burrard Inlet due to the presence of a thermally stratified water column, so laboratory fluctuations of 12°C are not as unrealistic as they may seem at first glance. We have added more to this justification in the text, and a new Figure S2 that contains temperature logger data illustrating this.

In our experimental design we traded-off replicating tank conditions for more experimental conditions, and as a result can only make conclusions from one level of fluctuation. Experiments including more than one amplitude of temperature fluctuation are relatively rare, but we agree are an important next step and so have both moderated the language above (removing "significant" from the abstract text, added "do not always hold") and included a sentence to this effect in our conclusions:

While the limited set of experimental conditions we were able to perform necessarily limits the conclusions we can draw from this experiment, we encourage further studies that both increase the number of fluctuating experimental groups and use ecologically relevant exposure events.

2. As someone who is unfamiliar with the mathematical aspects of a thermal performance curve, this could be a naïve comment, so apologies in advance. However, with the limited number of conditions, it is impossible to truly estimate a thermal performance curve, at least as presented in Figure 1, and indeed, very few measurements seem to be following a trajectory consistent with the classic shape of a thermal performance curve. For example, some traits monotonically increase or decrease (see Fig. 2, 3, 5) or they show little to no response to temperature change (Figure 4). Thus, is it fair to test assumptions of thermal performance curves, when many of your results do not match with the expected shape of a performance curve (or there is limited resolution to say for sure)? I still think you can clearly say that responses to these fluctuating conditions cannot be predicted from constant conditions, but I'm not sure the link to thermal performance curves sensu stricto is as direct as you imply in the Introduction. But again, I'm not as familiar with the mathematics that underlie construction of thermal performance curves, so I could be off base.

Interestingly, thermal performance curves often come in a wide variety of shapes that depend on the trait of interest and the temperature range over which that trait was measured (see examples in Sinclair et. al. 2016 *Ecol. Lett.*). The curves themselves are strictly empirically defined and therefore do not have a formal mathematical formulation. We have added language to this effect in the introduction:

While TPCs often occur in these canonical shapes, many shapes are possible depending both on the particular biological rate being measured and the range of temperatures over which it is measured (see examples in [4]), and therefore TPCs are a strictly empirical phenomenon that only describes the relationship between body temperature and biological rates.

We have also removed a few references to TPCs throughout where "thermal sensitivity" can be used instead.

Our bootstrapping approach made no assumptions about the underlying shape of the TPC, so we believe it is valid to contrast the effects under variable vs. constant temperatures. This method is outlined graphically in Denny J. Exp. Biol. 2017 (Fig 1). Thus, we believe it is valid to contrast the effects under variable vs. constant temperatures.

3. While the results clearly show that some assumptions of thermal performance curves might need revisiting, it is unclear what can be done about it. Perhaps there is some opportunity in the Discussion or Conclusions section to make recommendations for how to overcome these problems. There is a bit of that in lines 430-432, but these ideas could be expanded. In particular, what can be done to make this information useful for predictive modeling, since that is the stated goal for many thermal biology studies?

We strongly agree with this point, and suggest this study (and others) indicate there are two important gaps in our understanding of thermal biology: first that we need a much better empirical understanding of the physiology and biochemistry of acclimation to thermal variation, and second that we need good theory rooted in this data to allow for better predictions. We have expanded on this in the conclusion, and also discussed these points more throughout the discussion:

We suggest several ways forward from this impasse. First, by making the assumptions of the thermal performance curve model explicit, and including rate summation effects as a mathematical null hypothesis in physiological and ecological studies, we can move forward on this complex problem in a more rigorous way. Second, we suggest that progress on understanding the physiological and biochemical mechanisms of responses to temperature fluctuation will accelerate the construction of more mechanistic models of responses to temperature variation that can incorporate a wide range of effects and drive theoretical understanding. Finally, ecological studies conducted in the laboratory should re-evaluate "control" conditions and include natural temperature variation whenever possible.

Minor Comments:

1. Line 69: You mention a "point A" in Figure 1, but I don't see any points labeled. Also, the caption of Figure 1 could be expanded a bit. I assume the purple lines are reflecting predictions based on the red lines, but there is no explanation of the colors in the caption.

We have edited the figure to include Point A, and rewritten the caption in response to this comment as well as Reviewer 1's to read:

A generalized thermal performance curve for M. trossulus relating heart rate to body temperature (redrawn using data from [3]*) and illustrating predictions for mean responses over time under fluctuating conditions from Jensen's Inequality. The red lines indicate rates derived from the extremes at 6 and 18 °C while the purple lines indicate mean rates derived from a constant 12 °C (dashed line) as well as variable temperatures between 6 and 18 °C (dotted line). Point A indicates the inflection point of the curve (where it switches between acceleration and deceleration). In general, Jensen's Inequality calculates that the mean of the response under fluctuating temperatures is elevated relative to the mean under constant temperatures in areas of the curve that are accelerating, while the converse is true in areas of the curve that are decelerating. The magnitude of this effect is directly related to both the amplitude of temperature variation and the degree of curvature of the TPC.*

2. I had a hard time following the rainbow trout example in line 99. It is unclear which part of the previous sentence (stress resistance vs. repair) the example is relating to.

We have decided to substitute a different example to make for a clearer illustration.

3. Line 159-160 and elsewhere: Parts of the paper suggest that all mussels were identified to species level, while the supplemental methods indicates that only a subset were identified. Perhaps the subsample was large enough to conclude that all mussels were the same species, but make sure you're clear about whether all mussels were identified with PCR.

We apologize for the error and have updated the manuscript to reflect this.

4. Figure S2: The boxplots are probably unnecessary, because some groups appear to only have N=3 (a boxplot summarizes a minimum of five data points).

We have redrawn this figure to display only dots.

5. All figures: when possible, I would recommend indicating statistical significance, or a lack thereof, directly on the figure. That way readers looking at figures can tell if a difference is significant without having to look back through the results section.

Because we used mixed effect models with covariates, we tested only for fixed effects and did not feel that post-hoc tests were appropriate. We have indicated significant fixed effects on plots with text and have also indicated where our bootstrap tests indicated a significant difference.

6. Line 168-169: While this may be true, I would assume that intertidal mussels only briefly experience daily highs and lows, rather than four continuous days. Also, since these mussels were collected underwater, would local adaptation make them less tolerant of extreme fluctuations?

We do not think local adaptation would impact temperature tolerance as mussels have aquatic larvae and therefore there is gene flow between subtidal and intertidal populations. It is possible that the mussels we used were acclimatized to the subtidal conditions from which they were collected; the degree to which their responses may differ from intertidal mussels would make for an interesting future research project. It is also true that the time scale of variation in our study does not precisely equal the time scale of daily or tidal variation. Regardless, our main aim was to establish a defensible range of temperatures in the laboratory based on realistic mussel body temperatures in field conditions, not a precisely realistic thermal timecourse which - while desirable - was beyond our logistical capabilities. We have included some relevant high-resolution temperature data in a new Figure S2 to illustrate the scale of relevant ecological variation.

7. Statistical analyses: were any random effects included in the models to account for mussels being grouped together and for tank effects? Form looking at the raw data, it seems some data do not include any sort of grouping variable.

We have rebuilt our statistical models, where appropriate, to include mesocosm as a random effect nested within the population and treatment fixed effects using linear and generalized linear mixed effect models. The code posted on OSF has been updated, as have the methods and results section to reflect the updated models. While this has resulted in small quantitative changes to our reported results, the direction and significance of tests has not changed.

8. Lines 326-330: Can you provide any quantitative support for these claims? You don't report thermal optima for any of the traits in the paper, and some traits were not particularly sensitive to temperature change. I agree with this statement qualitatively, but this conclusion could be strengthened with some empirical support.

We have removed "optima" from this sentence and believe the differing thermal sensitivities in our data makes our point.

9. While many of the measured results differed from the predicted results, there are some cases where the measured values are at least in the expected direction. For example, that appears to be the case for Figure 2, partially for Figure 3, and Figure 5. So while the values may not be 100% accurate, it might be fair to point out that in at least a few cases, the directionality was predicted.

We have added a caveat to line 354 here addressing this:

None of these effects were explainable based on rate summation—the measured effects demonstrated differing acclimation to fluctuating and constant temperature conditions that differed occasionally in direction but more often in magnitude of effect.

Please find a copy of our manuscript with revisions tracked below.