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PROCEEDINGS B

Insect mitochondria as targets of freezing-induced injury

T. Štětina, L. E. Des Marteaux and V. Koštál

Article citation details
Proc. R. Soc. B **287**: 20201273.
http://dx.doi.org/10.1098/rspb.2020.1273

Review timeline
Original submission: 22 April 2020
1st revised submission: 3 June 2020
2nd revised submission: 23 June 2020
Final acceptance: 29 June 2020

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

Review History

RSPB-2020-0898.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
Summary/General Thoughts:

This paper nicely demonstrates the role of the mitochondria during freezing injury. The role of the mitochondria in freezing stress (and cold stress more generally) has long been discussed, and there have only been a few empirical studies that have demonstrated this principle. This work will set the stage for some important follow-up studies to determine how cold acclimation protects the mitochondria, and whether these findings are general across cold hardy insects. The study also uses a good series of methodologies that improve the robustness of the findings. Most of my comments are minor, although I have a few suggestions for organization and interpretation that will hopefully improve this nice paper.

Specific Comments:

1. I think it would be easier to follow some of the results if the figures were reorganized. The figures are aesthetically pleasing, but sometimes things that need to be compared together are in different figures. To properly interpret the morphology results, it would be nice to see all the groups together. For example, it would be useful to directly compare LD-30 with SDA-30, but these results are found in separate figures. Similarly, O2 consumption is also split between Figures 2 and 3. Perhaps you could consider organizing figures by the function/trait that is being measured? In other words, put all morphology results together to facilitate easy comparisons across conditions.

2. For interpretation of the cold acclimation results, you kind of have an unavoidable "chicken and egg" scenario, to use an English idiom. In other words, is mitochondrial function directly protected by cold acclimation, or is cold acclimation protecting other essential functions, and the mitochondria survive as a result? Is there anything in your transcriptomics studies, for examples, that point to specific protection of the mitochondria during cold acclimation? I think the PT hypotheses presented in the discussion are reasonable and highly likely, but it's also possible some other part of the cell is particularly freeze-sensitive, and when that function breaks down, it eventually leads to mitochondrial failure. This is particularly the case because measurements were taken after thawing, so a lot of other biological processes/injury pathways could be kickstarted. I realize this is one of those vague reviewer comments that is hard to address, so I don't expect a lot of changes, but perhaps a quick note in the discussion indicating that it's difficult to isolate direct effects on mitochondria vs. indirect effects.

3. I think the conclusion in lines 324-325 ("This result challenges the view of proteins as primary targets of freezing-induced injury") is perhaps a little too strong. Enzyme activity was severely reduced, so it still appears there is some damage to proteins. To me, your results could suggest that there are some factors (heat shock proteins, proline, etc.?) that protect proteins from freezing damage, but their capacity can be overwhelmed under extreme conditions. So, while some protein can be protected, much of it is no longer functional. We see similar results with cell viability measurements: even cold treatments that kill insects at the organismal level have some residual cell viability in their tissues. I still think it's an interesting finding that some enzyme activity survived, but you could consider dialing back your conclusions a bit, considering the results in Figure 4A.

The rest of my comments are quite minor:

1. The only other substantive work on mitochondrial physiology and cold stress in insects, to my knowledge, is Herve Colinet's work on cold acclimation in Drosophila (Colinet et al., 2017; Insect Biochemistry and Molecular Biology 80, 52-60). There are some interesting parallels to your work, so it's probably worth citing this work at some point.

2. Line 17: "phenotype" is vague. Perhaps change to "cold acclimation" for specificity.

3. Line 52: "Despite," by itself is somewhat awkward. Suggest changing to "Despite the challenges associated with freezing, freeze tolerance has evolved…"

4. It's unclear how the mitochondrial morphology data were analyzed. What is considered a replicate for this experiment? You mention numbers of mitochondria in line 165, but it wouldn't be appropriate to count these as independent replicates unless you had some sort of blocking term in your model (which is still difficult, because tissues can't be split across treatments).

5. Lines 181-185: Perhaps give a brief description of PreSens vs. Oxygraph, for those who aren't familiar with these technologies. Also, Figure S5 is nice, but you should probably include some more methodological details for these analyses, since they are technically challenging.

6. Line 184: Unclear what "basically after" is referring to. Is it pointing out that you followed the methods of refs 40-41?

7. Line 204-205: Perhaps you can elaborate this point a little. I think you are highlighting that there can be changes in enzyme abundance and regulation (e.g., allosteric regulation, posttranslational modifications, etc.) that could influence your results. Perhaps a specific example from your citations would help.

8. Line 227: Insert "and" before "their"

9. Line 371: With these hypotheses in mind, what are the normal benefits of having an IMM pore? Is it important in cell death regulation during normal development? Otherwise, if it leads to PT and cell death, why even have a pore?

10. Figure 1A: In lines 191-197, you discuss several potential reasons for the variation in mitotracker staining in fat body. To me, these images are typical of what you see in insect fat body, which due to its thickness and lipid content is difficult to stain evenly (even for simple stains that target the nucleus). So I'm not sure your descriptions of mitochondrial localization are particularly useful, although it is informative that staining pattern was consistent across groups. Did you try mitotracker with any other tissues that might stain better?

11. Figure 1E: These box plots are a little squished down to the bottom, which makes it harder to see differences. Perhaps have the micrographs outside of the graph so that you can have a narrower axis that better highlights the differences between groups.

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.
No

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
See my complete review attached. (See Appendix A)

Review form: Reviewer 3

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

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Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The paper "Insect mitochondria as targets of freezing-induced injury" by Štětina et al. reports major structural damage to mitochondria following freezing of non-acclimated larvae of the drosophilid fly, Chymomyza costata. In contrast, larvae that are acclimated to short days and low temperature retain "normal" morphological characteristics of the mitochondria. These data are somewhat supported by functional measurements of the mitochondria showing that respiratory function is preserved following freezing in acclimatized larvae. A major point made in this paper is also that protein function is retained although organelle function is impaired (to me this point is not as surprising as it is presented). Overall I find that the paper is very well written and it presents some new and interesting data. The question posed here is relevant and the discussion is generally relevant and well balanced in relation to the existing literature and future perspectives. I have no major concerns and my comments below should therefore mainly be seen as suggestions of aspects that could potentially improve an already good paper. My main suggestions can be summarised as follows. 1. The paper should present the acclimatization groups a little better to the reader that is not familiar with this study system. 2. The use of different tissues makes the coupling between morphological and functional characteristics unclear and it may be worth considering to only focus on the morphological data. 3. The speculation on the cause of mitochondrial collapse is long considering that the data here can not answer most of these questions. 4. I consider the presentation of enzymatic collapse as somewhat of a strawman. I believe that the general notion in the literature is that the cold sensitivity of the organism is higher than the organ which is higher than the organelle which is higher than the proteins (etc). This is not to say that protein function is not challenged by stressfull events, I just think the introduction and discussion is a little biased in terms of presenting loss of protein function as a principal problem – it would maybe be more fair to present the issue as if los of

protein function is an associated problem (In addition to the structural injury caused by freezing and osmotic shrinkage). Below I have listed a number of specific points that the authors could consider in a revision. Thank you once again for a very interesting and well written publication.

Specific points.
Line 16: It would be an idea to already early in the manuscript (abstract) to introduce the acclimation groups. Along the lines of "Here we investigate and compare the mitochondrial responses to freezing stress in the non-diapausing (freeze-sensitive) phenotype with that of the diapausing and cold acclimated (freeze tolerant) phenotype."I personally know of this study system from previous publications, but I would imagine that for the general reader it is important to emphasise these differences – particularly because the figures and data showing the difference in freeze tolerance are placed in supplements.

Line 29: As I read the paper there is no data in this study to either support of falsify the hypothesis of the involvement of a permeability transition of the inner mitochondrial membrane. It is fair to speculate about this, but in the absence of data I think this should have less space in the abstract. For example line 31-33 reads as if you have shown that the acclimation and proline is specifically targeting the inner mitochondrial membrane – but I don't see any data that proves this. There are other possibilities for the loss of mitochondrial integrity, so my suggestion is to tone the speculation down in the abstract (some of this space could then be used to introduce the different acclimation groups and the differences in freeze tolerance introduced by acclimation (and proline)).
Line 52: Write: "Despite this complexity" (at least to me it seems odd that "Despite" is all alone).
Line 54: I agree with the statement that it can be difficult to separate cause from consequence – after reading the paper I think this is also largely the case in regards to mitochondria. No need to make a change in the MS here – just a thought that it is quite difficult to discern if the mitochondria loose structural integrity because the cell dies or of the cell dies because the mitochondria loose structural integrity? (see comment further below)
Line 65: suggest you write "While the diapausing and cold acclimated phenotype can survive…"
Line 67: A small thing, but when I read the references I notised that there was inconsistency in how PNAS was mentioned in the refrences (PNAS or Proc. Nat. Ac. Sci.) – so be consistent.
Line 104: This could maybe be a good place to introduce some of the abbreviations for the treatment groups – (LD, SD and SDA) which are otherwise introduced a little too late and sporadic later in the MS.
Line 109 gives the (somewhat false) impression that function, morphology and count was made on all three tissues.
Line 110: hypothesised would probably be a better word than expected.
Line 126: Stick with latin name – malt fly is only used once elsewhere (the first time the species is mentioned in the intro).
Line 127 – explain the LD is an abbreviation of LongDay – not clear to all if you do not know the experimental system. Similar for SDA and SD.
Line 131: I like Fig S1 and think it is important. I understand that length constraints can make it impossible to fit a graph in, but if it turns out that there is space I think it would be great if you include it in the main MS. It is important for understanding the different phenotypes which is the premise for the question.
Line 139: Is it correct that at the time you selected the larvae it is inclear if the specific specimen is moribound (i.e. just above you show that survival was less than 100% in some groups). Maybe make a small sentence that this procedure means that some of the individuals are likely to be either dead or in the process of dying at the time of sampling. This is not a critism - I think it is a fine sampling protocol. In relation to this I think a great follow up study could be to look at the temporal recovery of mitochondrial integrity in surviving animals.'
Line 165: I am impressed with the enormous amount of mitochondria examined – must have been long days at the microscope . Great work and great patience…
Line 183: You could add that the main difference is that in the present you permeabilise the cells in the oroborus chamber, while the studies refenced to, do the permabilisation prior to

measurements.
Line 185: Although I understand that there were technical causes to the problem I find that one of the problems with this paper that different mitochondrial traits were measured in different tissues - and with different patterns in different tissues. Thus, the most detailed analysis of mitochondrial respiration was made in muscle tissue – which is characterised by a general better preservation of respiration rate following freezing and for this tissue there is no data on structural damage. In contrast there is convincing data on structural damage – which seemingly has a marked effect on overall respiration rate in fatbody. I personally think the study would be fine with only the structural data from the fatbody (and hindgut) because it is a little difficult to associate structural and functional data from different tissues when the responses seem to somewhat different. Alternatively one could suggest that the authors make a table where it is made even more clear what type of experiments that are made for which tissues.
Line 203 – should this be figure 1B – in general there are issues with a number of figure references, so please check this throughout the MS (see for example line 211 and 214).
Line 216: I struggled a little to understand what the background measurement represented. Is the background respiration rate (empty chamber?) as large as in the tissue with proline, or is this background subtracted from all measurement?. At least I think the figure legend could be a little clearer.
Line 258: Since this is a results and discussion section, you could maybe make a short comment on whether you can discern the order of the problems. My point is that it is difficult to know if the larvae are dead because the mitochondria are broken – or if the mitochondria are broken because the larvae are dead. I know it is difficult to add anything concrete to this, but just noticed that this paragraph had no discussion of the data (unlike the other paragraphs).
Line 269: I am not sure I understand how increased variability will lead to a greater chance of a significant result. Maybe this is not the point you are trying to make, but the power to detect a significant difference should decrease when variability increases – yet you still find a signinficant difference!. Maybe just let this slide and say that although you do not understand why there is this increase, the important point is that there is no decrease. Alternatively use a one-tailed test - because as I understand it you are really interested to investigate if respiration decreased.
Line 284: I do not disagree, but my understanding is that the paradigm of freeze injury ALSO include major structural damage at the organelle, cell and organ level due to the malformation caused by osmotic shrinking (and even from icecrystal formation). This section make it sound as if the MAIN explanation in the literature is related to loss of protein function and my understanding is more that this is also an associated problem. This is probably splitting of words – but consider to tone the arguments a little differently – or at least mention that these problems with protein function are in addition to simultaneous structural challenges at the organelle, cell and organ level (see also line 325 where it is said that proteins are the primary targets of freezing injury).
Line 299: I completely follow the argument of why you needed to move to a new tissue, but the study becomes a little decoupled at this point. It is very difficult to understand to what degree the ETC problem in muscles is linked to the structural changes seen in other tissues. Clearly muscle mitochondira have higher respiration rates after freezing (also in the LD group), but it is unclear if and how this is coupled to structural integrity of mitochondria. I am not convinced that this part of the study adds so much to the understanding of how the structural problems are linked to functional problems and I think most of the central points in this paper can be made without this muscle data (fig 4). One suggestion would be to include fig. S1 in the MS and exclude fig 4 (the muscle study could then be used in a different publication). This would maybe make the story here a little more narrow, but also more coherent and with room for a better introduction to the treatment groups. If you choose to keep it as it is, I think it is important to discuss explicitly how the detailed measurements of ETC are missing in the tissues where you have structural info, and vise versa.
Line 329: Can the data here show that mitochondrial swelling is the cause of injury, rather than a consequence of malfunctions in other physiological systems? It is difficult to separate cause and effect. (see initial comment on cause and consequence)
Line 331 and onwards: I think it is both appropriate and relevant to discuss a putative role of inner membrane barrier function, but I find that the section (particularly the section from line 346

to 354) to be somewhat lengthy in the absence of data to support or falsify the idea of involvement of specific pores/channels. Consider to shorten.
Page 10 in general: It would be interesting if the authors could speculate a little on the timing of when the osmotic problems occur in relation to freezing. For example, the authors suggest that loss of inner mitochondrial barrier function create an osmotic gradient towards the intermembrane space, and subsequently to the cytosol, and that this is the cause of swelling. However, during the actual freezing event ice formation is likely initiated in the extracellular fluid where it will draw water from the intracellular space and create and osmotic shrinkage of cells and obviously also organelles. I imagine that a swelling of mitochondria must then be a post-freeze phenomena? Is it possible that proline and trehalose have colligative effects (in addition to membrane stabilising effects) during this post freeze period?. I am aware that it is difficult to offer any answers on this aspect but to me it is at least a possibility that injury is initiated during the shrinking, and then this injury is later manifested in swollen mitochondria that have lost their ability to regulate across membranes. I don't think this idea is inconsistent with a role for proline/trehalose nor is this idea inconsistent with the notion that barrier function should be intact during recovery from shrinkage. You could write something along these lines (probably better to use your own words): During freezing cells and organelles shrink rapidly when water becomes locked to ice crystals in the animals extracellular compartments. This process involves rapid movement of water and possibly also osmolytes across cellular and organelle membranes and during the subsequent thawing this movement must be reversed in a controlled and regulated process… Maybe I am missing something in my understanding, but to me the injury could just as well occour during the shinking, and if you agree you could maybe mention this in some way?
Line 400: I guess this is only a paradox if the mitochondria of muscle cells are also ruptured and swollen in muscle cells, and this is not known?
Line 403: maybe write "biological membranes, including the inner mitochondrial membrane" – because the initiation of injury could originate from another membrane system not functioning and you do not know if it is specifically the inner mitochondrial membrane – I don't think there is strong evidence for pinpointing this membrane system specifically (this is also done in line 409 onwards). Consider to make the conclusions/perspectives a little more open – for example to suggest that it would be interesting to examine when during the freezing/thawing cascade injury is initiated, and (especially if you maintain the muscle data in the MS) to pinpoint that you need data from the same tissue to examine how loss of ETC and mitochondrial swelling are linked.
Again – thanks for an interesting paper.

Decision letter (RSPB-2020-0898.R0)

20-May-2020

Dear Dr Kostal:

I am writing to inform you that your manuscript RSPB-2020-0898 entitled "Insect mitochondria as targets of freezing-induced injury" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional

circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

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

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

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Thanks for your patience with the peer review process. We have now collected three expert reviews of your manuscript. All three were quite positive about the work, but gave extensive suggestions and comments. By responding to these suggestions I expect the work to be significantly improved. I agree with essentially all of the reviewers comments and since you have a good deal of comments to deal with I will refrain from adding to them myself :). I want to draw your attention particularly to comments from Reviewer 2 about quantifying mitochondria in the study as well as some over-reaching in the discussion section. Perhaps softening your statements on cause-and-effect relationships among cellular effects and suggesting some follow-up experiments would be prudent here. Reviewer 1 suggests some re-organization. This is a valid critique, but I suggest that you simply carefully review the order of ideas presented and make sure they are presented in an order that best tells your story.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
Summary/General Thoughts:

This paper nicely demonstrates the role of the mitochondria during freezing injury. The role of the mitochondria in freezing stress (and cold stress more generally) has long been discussed, and there have only been a few empirical studies that have demonstrated this principle. This work will set the stage for some important follow-up studies to determine how cold acclimation protects the mitochondria, and whether these findings are general across cold hardy insects. The study also uses a good series of methodologies that improve the robustness of the findings. Most of my comments are minor, although I have a few suggestions for organization and interpretation that will hopefully improve this nice paper.

Specific Comments:

1. I think it would be easier to follow some of the results if the figures were reorganized. The figures are aesthetically pleasing, but sometimes things that need to be compared together are in different figures. To properly interpret the morphology results, it would be nice to see all the groups together. For example, it would be useful to directly compare LD-30 with SDA-30, but these results are found in separate figures. Similarly, O2 consumption is also split between Figures 2 and 3. Perhaps you could consider organizing figures by the function/trait that is being measured? In other words, put all morphology results together to facilitate easy comparisons across conditions.

2. For interpretation of the cold acclimation results, you kind of have an unavoidable "chicken and egg" scenario, to use an English idiom. In other words, is mitochondrial function directly protected by cold acclimation, or is cold acclimation protecting other essential functions, and the mitochondria survive as a result? Is there anything in your transcriptomics studies, for examples, that point to specific protection of the mitochondria during cold acclimation? I think the PT hypotheses presented in the discussion are reasonable and highly likely, but it's also possible some other part of the cell is particularly freeze-sensitive, and when that function breaks down, it eventually leads to mitochondrial failure. This is particularly the case because measurements were taken after thawing, so a lot of other biological processes/injury pathways could be kickstarted. I realize this is one of those vague reviewer comments that is hard to address, so I don't expect a lot of changes, but perhaps a quick note in the discussion indicating that it's difficult to isolate direct effects on mitochondria vs. indirect effects.

3. I think the conclusion in lines 324-325 ("This result challenges the view of proteins as primary targets of freezing-induced injury") is perhaps a little too strong. Enzyme activity was severely reduced, so it still appears there is some damage to proteins. To me, your results could suggest that there are some factors (heat shock proteins, proline, etc.?) that protect proteins from freezing damage, but their capacity can be overwhelmed under extreme conditions. So, while some protein can be protected, much of it is no longer functional. We see similar results with cell viability measurements: even cold treatments that kill insects at the organismal level have some residual cell viability in their tissues. I still think it's an interesting finding that some enzyme activity survived, but you could consider dialing back your conclusions a bit, considering the results in Figure 4A.

The rest of my comments are quite minor:

1. The only other substantive work on mitochondrial physiology and cold stress in insects, to my knowledge, is Herve Colinet's work on cold acclimation in Drosophila (Colinet et al., 2017; Insect Biochemistry and Molecular Biology 80, 52-60). There are some interesting parallels to your work, so it's probably worth citing this work at some point.

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Referee: 2

Comments to the Author(s)
See my complete review attached.

Referee: 3

Comments to the Author(s)
The paper "Insect mitochondria as targets of freezing-induced injury" by Štětina et al. reports major structural damage to mitochondria following freezing of non-acclimated larvae of the drosophilid fly, Chymomyza costata. In contrast, larvae that are acclimated to short days and low temperature retain "normal" morphological characteristics of the mitochondria. These data are somewhat supported by functional measurements of the mitochondria showing that respiratory function is preserved following freezing in acclimatized larvae. A major point made in this paper is also that protein function is retained although organelle function is impaired (to me this point is not as surprising as it is presented). Overall I find that the paper is very well written and it presents some new and interesting data. The question posed here is relevant and the discussion is generally relevant and well balanced in relation to the existing literature and future perspectives. I have no major concerns and my comments below should therefore mainly be seen as suggestions of aspects that could potentially improve an already good paper. My main suggestions can be summarised as follows. 1. The paper should present the acclimatization groups a little better to the reader that is not familiar with this study system. 2. The use of different tissues makes the coupling between morphological and functional characteristics unclear and it may be worth considering to only focus on the morphological data. 3. The speculation on the cause of mitochondrial collapse is long considering that the data here can not answer most of these questions. 4. I consider the presentation of enzymatic collapse as somewhat of a strawman. I believe that the general notion in the literature is that the cold sensitivity of the organism is higher than the organ which is higher than the organelle which is higher than the proteins (etc). This is not to say that protein function is not challenged by stressfull events, I just think the introduction and discussion is a little biased in terms of presenting loss of protein function as a principal problem – it would maybe be more fair to present the issue as if los of

protein function is an associated problem (In addition to the structural injury caused by freezing and osmotic shrinkage). Below I have listed a number of specific points that the authors could consider in a revision. Thank you once again for a very interesting and well written publication.

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Line 52: Write: "Despite this complexity" (at least to me it seems odd that "Despite" is all alone).
Line 54: I agree with the statement that it can be difficult to separate cause from consequence – after reading the paper I think this is also largely the case in regards to mitochondria. No need to make a change in the MS here – just a thought that it is quite difficult to discern if the mitochondria loose structural integrity because the cell dies or of the cell dies because the mitochondria loose structural integrity? (see comment further below)
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Line 126: Stick with latin name – malt fly is only used once elsewhere (the first time the species is mentioned in the intro).
Line 127 – explain the LD is an abbreviation of LongDay – not clear to all if you do not know the experimental system. Similar for SDA and SD.
Line 131: I like Fig S1 and think it is important. I understand that length constraints can make it impossible to fit a graph in, but if it turns out that there is space I think it would be great if you include it in the main MS. It is important for understanding the different phenotypes which is the premise for the question.
Line 139: Is it correct that at the time you selected the larvae it is inclear if the specific specimen is moribound (i.e. just above you show that survival was less than 100% in some groups). Maybe make a small sentence that this procedure means that some of the individuals are likely to be either dead or in the process of dying at the time of sampling. This is not a critism – I think it is a fine sampling protocol. In relation to this I think a great follow up study could be to look at the temporal recovery of mitochondrial integrity in surviving animals.'
Line 165: I am impressed with the enormous amount of mitochondria examined – must have been long days at the microscope . Great work and great patience…

Line 183: You could add that the main difference is that in the present you permeabilise the cells in the oroborus chamber, while the studies refenced to, do the permabilisation prior to measurements.
Line 185: Although I understand that there were technical causes to the problem I find that one of the problems with this paper that different mitochondrial traits were measured in different tissues – and with different patterns in different tissues. Thus, the most detailed analysis of mitochondrial respiration was made in muscle tissue – which is characterised by a general better preservation of respiration rate following freezing and for this tissue there is no data on structural damage. In contrast there is convincing data on structural damage – which seemingly has a marked effect on overall respiration rate in fatbody. I personally think the study would be fine with only the structural data from the fatbody (and hindgut) because it is a little difficult to associate structural and functional data from different tissues when the responses seem to somewhat different. Alternatively one could suggest that the authors make a table where it is made even more clear what type of experiments that are made for which tissues.
Line 203 – should this be figure 1B – in general there are issues with a number of figure references, so please check this throughout the MS (see for example line 211 and 214).
Line 216: I struggled a little to understand what the background measurement represented. Is the background respiration rate (empty chamber?) as large as in the tissue with proline, or is this background subtracted from all measurement?. At least I think the figure legend could be a little clearer.
Line 258: Since this is a results and discussion section, you could maybe make a short comment on whether you can discern the order of the problems. My point is that it is difficult to know if the larvae are dead because the mitochondria are broken – or if the mitochondria are broken because the larvae are dead. I know it is difficult to add anything concrete to this, but just noticed that this paragraph had no discussion of the data (unlike the other paragraphs).
Line 269: I am not sure I understand how increased variability will lead to a greater chance of a significant result. Maybe this is not the point you are trying to make, but the power to detect a significant difference should decrease when variability increases – yet you still find a signinficant difference!. Maybe just let this slide and say that although you do not understand why there is this increase, the important point is that there is no decrease. Alternatively use a one-tailed test - because as I understand it you are really interested to investigate if respiration decreased.
Line 284: I do not disagree, but my understanding is that the paradigm of freeze injury ALSO include major structural damage at the organelle, cell and organ level due to the malformation caused by osmotic shrinking (and even from icecrystal formation). This section make it sound as if the MAIN explanation in the literature is related to loss of protein function and my understanding is more that this is also an associated problem. This is probably splitting of words – but consider to tone the arguments a little differently – or at least mention that these problems with protein function are in addition to simultaneous structural challenges at the organelle, cell and organ level (see also line 325 where it is said that proteins are the primary targets of freezing injury).
Line 299: I completely follow the argument of why you needed to move to a new tissue, but the study becomes a little decoupled at this point. It is very difficult to understand to what degree the ETC problem in muscles is linked to the structural changes seen in other tissues. Clearly muscle mitochondira have higher respiration rates after freezing (also in the LD group), but it is unclear if and how this is coupled to structural integrity of mitochondria. I am not convinced that this part of the study adds so much to the understanding of how the structural problems are linked to functional problems and I think most of the central points in this paper can be made without this muscle data (fig 4). One suggestion would be to include fig. S1 in the MS and exclude fig 4 (the muscle study could then be used in a different publication). This would maybe make the story here a little more narrow, but also more coherent and with room for a better introduction to the treatment groups. If you choose to keep it as it is, I think it is important to discuss explicitly how the detailed measurements of ETC are missing in the tissues where you have structural info, and vise versa.
Line 329: Can the data here show that mitochondrial swelling is the cause of injury, rather than a consequence of malfunctions in other physiological systems? It is difficult to separate cause and effect. (see initial comment on cause and consequence)

Line 331 and onwards: I think it is both appropriate and relevant to discuss a putative role of inner membrane barrier function, but I find that the section (particularly the section from line 346 to 354) to be somewhat lengthy in the absence of data to support or falsify the idea of involvement of specific pores/channels. Consider to shorten.
Page 10 in general: It would be interesting if the authors could speculate a little on the timing of when the osmotic problems occur in relation to freezing. For example, the authors suggest that loss of inner mitochondrial barrier function create an osmotic gradient towards the intermembrane space, and subsequently to the cytosol, and that this is the cause of swelling. However, during the actual freezing event ice formation is likely initiated in the extracellular fluid where it will draw water from the intracellular space and create and osmotic shrinkage of cells and obviously also organelles. I imagine that a swelling of mitochondria must then be a post-freeze phenomena? Is it possible that proline and trehalose have colligative effects (in addition to membrane stabilising effects) during this post freeze period?. I am aware that it is difficult to offer any answers on this aspect but to me it is at least a possibility that injury is initiated during the shrinking, and then this injury is later manifested in swollen mitochondria that have lost their ability to regulate across membranes. I don't think this idea is inconsistent with a role for proline/trehalose nor is this idea inconsistent with the notion that barrier function should be intact during recovery from shrinkage. You could write something along these lines (probably better to use your own words): During freezing cells and organelles shrink rapidly when water becomes locked to ice crystals in the animals extracellular compartments. This process involves rapid movement of water and possibly also osmolytes across cellular and organelle membranes and during the subsequent thawing this movement must be reversed in a controlled and regulated process… Maybe I am missing something in my understanding, but to me the injury could just as well occour during the shinking, and if you agree you could maybe mention this in some way?
Line 400: I guess this is only a paradox if the mitochondria of muscle cells are also ruptured and swollen in muscle cells, and this is not known?
Line 403: maybe write "biological membranes, including the inner mitochondrial membrane" – because the initiation of injury could originate from another membrane system not functioning and you do not know if it is specifically the inner mitochondrial membrane – I don't think there is strong evidence for pinpointing this membrane system specifically (this is also done in line 409 onwards). Consider to make the conclusions/perspectives a little more open – for example to suggest that it would be interesting to examine when during the freezing/thawing cascade injury is initiated, and (especially if you maintain the muscle data in the MS) to pinpoint that you need data from the same tissue to examine how loss of ETC and mitochondrial swelling are linked.
Again – thanks for an interesting paper.

Author's Response to Decision Letter for (RSPB-2020-0898.R0)

See Appendix B.

RSPB-2020-1273.R0

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Summary/General Thoughts:

I think the authors did a nice job addressing review comments to the best of their ability. The paper is more focused, and the conclusions are properly couched. In my first review, I requested some reorganization of the figures, and while my personal preference is to reorganize based on methodology, I can see the authors' logic in keeping it as is. I'm not qualified to judge whether Reviewer #2's concerns on methodology were adequately addressed, but my primary concerns have been addressed. I just have a few minor comments to consider.

Specific Comments:

1. In first reading the sentence "No loss of activity…" it wasn't clear to me at first that you were discussing the same species as the previous sentence, since they are different references. So perhaps add a transition word/phrase to the beginning, like "However," for example

2. Line 104: Change "effect" to "effects" for singular plural agreement.

3. I apologize for not noticing before, but there seems to be some discrepancy between the mitochondrial counts and tissue metabolic rate for the acclimation treatments. Specifically, diapause appears to reduce mitochondrial content, but the metabolic rate of fat body tissue is

unaffected. It would be good to briefly address this discrepancy and perhaps speculated what could be causing it.

4. The purpose of the two sentences starting in line 283 might not be obvious for someone who didn't read the first draft of the paper. Instead, I would just simply conclude that you don't have strong evidence that citrate synthase is damaged by freezing (although you did see a significant reduction in LD larvae in Figure 3B) and transition to the mitochondrial information.

5. Similarly, the sentence in line 292 sounds more like a review rebuttal than something you read in a Discussion. Perhaps just state this problem without claiming you are in fact aware of it.

Review form: Reviewer 2 (Nicolas Pichaud)

Recommendation
Accept with minor revision (please list in comments)

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
I would like to congratule the authors for their extensive work on this second version of the manuscript. The thorough and quality revision, and the authors' attention to detail, have satisfied my initial concerns that prompted my recommendation of revision prior to acceptance. In particular, the restructured discussion has yielded, in my opinion, a manuscript that will be much more valuable to others both to stimulate additional studies and also as a useful resource. I just have one minor comment for this second version: I think that the last section ((d) Potential linkages between mitochondrial swelling and larval mortality) is a bit long. I suggest to shorten some parts (for example, the link between mercury exposure and mPTP in Artemia), so it will be easier to follow.
Thanks for a stimulating paper!
Best,
Nicolas Pichaud

Review form: Reviewer 3

Recommendation
Accept as is

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
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
Congratulations on a great paper - I think all my concerns have been dealt with in an appropriate manner and I enjoyed reading the paper again.
I have a few very Minor suggestions:

Line 27: I suggest you change the sentence "The phenotypic... To "We therefore suggest that the phenotypic transition to diapause and cold acclimation could be associated with" (In other words I suggest to introduce the words "suggest" and "could be" because it is important in an abstract to be cautions with suggestions that are not founded in real data but only in intelligent speculation :-).
Line 45: You could mention cellular dehydration as one of the stressors

Decision letter (RSPB-2020-1273.R0)

22-Jun-2020

Dear Dr Kostal:

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 Associate Editor have raised some issues 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.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

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

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

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

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

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

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

Best wishes,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Thank you for submitting your revision and for so effectively responding to the comments from the reviewers. As you will see, all three reviewers were positive about the considerable effort you have clearly put into revising the manuscript. In particular, the discussion section is now a pleasure to read and raises a number of new and exciting ideas for further exploration in the field.

Some minor issues remain that the reviewers and I would like to see you address. In particular, one of the reviewers has commented on an apparent discrepancy between mitochondrial counts and fat body tissue metabolic rate that does deserve some consideration in the discussion section, and I encourage you to add a sentence or two explaining potential causes of this discrepancy. Another reviewer points out that the last section discussing the mPTP is a bit long, so consider ways to get to your point more efficiently. I hope that you can address these issues and look forward to seeing a revised manuscript.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
I would like to congratule the authors for their extensive work on this second version of the manuscript. The thorough and quality revision, and the authors' attention to detail, have satisfied my initial concerns that prompted my recommendation of revision prior to acceptance. In particular, the restructured discussion has yielded, in my opinion, a manuscript that will be much more valuable to others both to stimulate additional studies and also as a useful resource. I just have one minor comment for this second version: I think that the last section ((d) Potential linkages between mitochondrial swelling and larval mortality) is a bit long. I suggest to shorten some parts (for example, the link between mercury exposure and mPTP in Artemia), so it will be easier to follow.
Thanks for a stimulating paper!
Best,
Nicolas Pichaud

Referee: 1

Comments to the Author(s).
Summary/General Thoughts:

I think the authors did a nice job addressing review comments to the best of their ability. The paper is more focused, and the conclusions are properly couched. In my first review, I requested some reorganization of the figures, and while my personal preference is to reorganize based on methodology, I can see the authors' logic in keeping it as is. I'm not qualified to judge whether Reviewer #2's concerns on methodology were adequately addressed, but my primary concerns have been addressed. I just have a few minor comments to consider.

Specific Comments:

1. In first reading the sentence "No loss of activity…" it wasn't clear to me at first that you were discussing the same species as the previous sentence, since they are different references. So perhaps add a transition word/phrase to the beginning, like "However," for example

2. Line 104: Change "effect" to "effects" for singular plural agreement.

3. I apologize for not noticing before, but there seems to be some discrepancy between the mitochondrial counts and tissue metabolic rate for the acclimation treatments. Specifically,

diapause appears to reduce mitochondrial content, but the metabolic rate of fat body tissue is unaffected. It would be good to briefly address this discrepancy and perhaps speculated what could be causing it.

4. The purpose of the two sentences starting in line 283 might not be obvious for someone who didn't read the first draft of the paper. Instead, I would just simply conclude that you don't have strong evidence that citrate synthase is damaged by freezing (although you did see a significant reduction in LD larvae in Figure 3B) and transition to the mitochondrial information.

5. Similarly, the sentence in line 292 sounds more like a review rebuttal than something you read in a Discussion. Perhaps just state this problem without claiming you are in fact aware of it.

Referee: 3

Comments to the Author(s).
Congratulations on a great paper - I think all my concerns have been dealt with in an appropriate manner and I enjoyed reading the paper again.
I have a few very Minor suggestions:

Line 27: I suggest you change the sentence "The phenotypic... To "We therefore suggest that the phenotypic transition to diapause and cold acclimation could be associated with" (In other words I suggest to introduce the words "suggest" and "could be" because it is important in an abstract to be cautions with suggestions that are not founded in real data but only in intelligent speculation :-).
Line 45: You could mention cellular dehydration as one of the stressors

Author's Response to Decision Letter for (RSPB-2020-1273.R0)

See Appendix C.

Decision letter (RSPB-2020-1273.R1)

29-Jun-2020

Dear Dr Kostal

I am pleased to inform you that your manuscript entitled "Insect mitochondria as targets of freezing-induced injury" 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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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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

Sincerely,

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

Associate Editor:
Board Member
Comments to Author:
(There are no comments.)

Appendix A

In this study, the authors examine the effects of phenotype on mitochondrial responses to freezing stress using larvae of the fly *Chymomyza costata*. Specifically, they evaluated mitochondrial counts, morphology and functions in different tissues (fat bodies, hindgut and muscle) of the relatively freeze-susceptible (non-diapause, warm-acclimated) and freeze-tolerant (non-diapause, proline-fed; and diapause, cold acclimated) larvae exposed to cold (-5°C) and freezing stresses (-5°C and -30°C). The authors show that mitochondria in freeze sensitive larvae (non-diapause) swell, become rounded and sometimes burst upon exposure to lethal freezing stress, while sublethal freezing stress and supercooling stress were not associated with mitochondrial swelling. Moreover, the swollen mitochondria have decreased respiration rates, but maintain citrate synthase activity and functional oxidative phosphorylation. They suggest that this swelling is caused by an increased sensitivity of the inner mitochondrial membrane (IMM) to freezing stress, leading to permeability transition, loss of barrier function and osmotic influx of cytosolic water into the matrix. On the other hand, freeze-tolerant larvae (diapause and cold-acclimated) do not show any signs of mitochondrial swelling and mitochondrial dysfunctions. They suggest that adaptive changes associated with the diapause and cold-acclimation might be due to downregulation of critical components involved in the formation of permeability transition pore (or other non-characterized unregulated pores in the IMM), high concentrations of cryoprotective substances (proline, trehalose) that stabilize the IMM's liquid-crystalline phase, and/or restructuration of the IMM's phospholipid bilayer. This is an interesting study that provides new insights on the mitochondrial responses to cold and freezing in insects, which has been barely studied in these organisms. The manuscript is generally well-written, and the results are clearly expressed and mostly put in context with relevant statistics. There are however several aspects of this study that concern me, and I hope the comments below will help the authors to improve their manuscript.

Major comments:

1. The citrate synthase activity, which is used to evaluate both mitochondrial counts and mitochondrial functions, was measured using acetyl-CoA and DTNB. However, no concentrations of these compounds are given (supplementary methods). Moreover, there is no mention of oxaloacetate being used in the reaction, which is absolutely required to start the reaction.
2. The different samples were collected after cold or freezing challenges and a return to 5°C. Overall, this protocol is well executed. However, some experiments are then done at different temperatures. For example, the oxygen consumption in dissected fat body tissues using the PreSens system was performed after an incubation of 2 hours at 23°C (while for example the whole -5°C stress was performed in around 2.5 hours). Thus, the results obtained might not be due to the induced stress *per se*, but rather from the incubation used in the experiments. There is no mention of the temperature used for mitochondrial respiration in muscle tissues using the Oroboros oxygraphy though. Moreover, an incubation of 30 min at 30°C was performed for the Mitotracker experiment before visualizing mitochondria, which also raise questions about the effect of cold/freezing stress *per se*.
3. The authors are commended for using three different separate techniques to evaluate mitochondrial counts. However, as they say in the text, the MitoTracker method did not allow to have a relevant quantitative analysis of mitochondrial counts. Moreover, they also mentioned that while CS activity might be a good proxy for mitochondrial counts, in case of complex phenotypic transitions this might not be the case, and I fully agree with the authors on this. Finally, the authors stipulate that the third method, mtDNA copy number, might be the most reliable indicator of mitochondrial counts (although not in insects). However, in one of the studies cited (reference 37) mtDNA copy number has been demonstrated to be a poor marker of mitochondrial content. Overall, this raise the question about the relevance of the results obtained on mitochondrial counts.
4. The muscle preparations used for the mitochondrial respiration experiments were permeabilized with digitonin. The authors titrated the digitonin in control LD larvae to optimize the method and concluded that a concentration of 55 mM was optimal for the permeabilization, which was then the concentration used for all the other groups. However, as the authors mentioned, a restructuration of the IMM might occur in the larvae exposed to different temperature stress. Considering that digitonin interacts with cholesterol to allow permeabilization, the titration of digitonin should have been done for all individual groups to ensure that the digitonin concentration was optimal whatever the treatment used.
5. The authors used a combination of proline, glutamate, pyruvate and malate to trigger electron transport into the ETS at the level of complex I. However, they stipulate in the results (lines 311-312) that 'this result shows that proline dehydrogenase can supply electrons to ETS even in lethally-frozen LD larvae'. First, they use state 2 (which is not the state 2 as defined in the literature, see minor comments) to conclude that. However, state 2 might not be a relevant state in this case, as oxygen consumption in this state is limited by the absence of ADP and thus no conclusions about proline dehydrogenase can me made here. Second, their state 3 for complex I might actually be a combination of the electron being

supplied to both complex I and proline dehydrogenase (and not only complex I). They also used succinate to trigger electron transport into complex II after inhibition of complex I by rotenone. However, to evaluate complex II individually, substrates for complex I should not be used. This could lead to overestimation of complex II due to reverse electron flux. Finally, the respiration rates are normalized with the number of muscle tissue used. Did the authors verify if muscle mass was the same between control and frozen tissues?

6. The paragraph in the discussion about the potential linkages between IMM permeability, mitochondrial functions, and larval mortality (lines 327-391) is based on a lot of extrapolations. Several tests could have been done to verify that such as injection of cytochrome c during mitochondrial respiration to verify mitochondrial membrane integrity or apoptosis tests. I suggest to shorten this paragraph and focus on the results obtained in the context of the scientific literature.

Minor comments:

- ETS refers to either electron transport chain or electron transport system throughout the manuscript. Please correct.

- State 2 as measured by the authors is not the state 2 defined in the literature. State 2 represents oxygen consumption in mitochondrial preparations with ADP but no substrates.

- While the groups are well defined in the supplementary methods, there are not mention of what are LD, LDpro, SD and SDA in the main text which makes the manuscript sometimes confusing.

- A ‘Satistical analysis’ paragraph should be added at the end of the ‘Materials and methods’ section.

- The term ‘Mitochondrial counts’ should be replaced by ‘mitochondrial content’ as the authors did not measure specific mitochondrial counts but rather estimated the mitochondrial content.

- Lines 214-217: as the oxygen consumption measured with the PreSens system is not an indicator of mitochondrial coupling, the comparison with E. solidaginis does not make sense.

Appendix B

Rebuttal

We would like to thank all referees for their constructive criticism. We accepted most of referees' recommendations and feel that the manuscript was significantly improved thanks to their effort.

Because all referees expressed their concerns with the organization of our manuscript, we decided to accept the suggestion by referee 3 and have excluded all data concerning the muscle tissue (Oroboros respirometry). This single change resulted in profound restructuring of the rest of paper. The revised text is shorter and, as we believe, much more concise and easy to follow. We would like to keep the Oroboros respirometry data for our next paper.

All referees also suggested that we tone down some of our claims on causality of relationships between damage to proteins, IMM permeability transition, mitochondrial swelling, freezing-induced injury, and organismal mortality. We apologize for not making this absolutely clear in our first version, but it was not our intention to push any unsupported interpretations of our results. On the other side, we wished to offer at least hypothetical explanations for our observations. In the revised text, we have changed the text accordingly (to referees' suggestions) at many places and also have added explicit sentences: "*... we will focus on the major observation of this study – freezing stress-induced mitochondrial swelling in freeze-sensitive larvae – and offer at least speculative explanation of this phenomenon. Our ambition is not to make categorical conclusions but rather to provide hypotheses for further research*" (L288-291 of the revised version).

Our point to point response to all comments by three referees follows:

Referee: 1

Summary/General Thoughts:

This paper nicely demonstrates the role of the mitochondria during freezing injury. The role of the mitochondria in freezing stress (and cold stress more generally) has long been discussed, and there have only been a few empirical studies that have demonstrated this principle. This work will set the stage for some important follow-up studies to determine how cold acclimation protects the mitochondria, and whether these findings are general across cold hardy insects. The study also uses a good series of methodologies that improve the robustness of the findings. Most of my comments are minor, although I have a few suggestions for organization and interpretation that will hopefully improve this nice paper.

We thank the referee 1 for overall positive evaluation and for concrete suggestions how to improve the manuscript.

Specific Comments:

1. I think it would be easier to follow some of the results if the figures were reorganized. The figures are aesthetically pleasing, but sometimes things that need to be compared together are in different figures. To properly interpret the morphology results, it would be nice to see all the groups together. For example, it would be useful to directly compare LD-30 with SDA-30, but these results are found in separate figures. Similarly, O2 consumption is also split between Figures 2 and 3. Perhaps you could consider organizing figures by the function/trait that is being measured? In other words, put all morphology results together to facilitate easy comparisons across conditions.

We have reorganized the Figures (and the text as a whole) by removing all parts on muscle tissue. We understand, however, that this is not exactly the sort of reorganization suggested by referee. Indeed, we were considering two

possible ways of the data organization: (i) according to methods as suggested by referee (i.e. compare the data obtained using a single method, for instance citrate synthase assay, for all treatments directly in a single figure) or, (ii) according to treatments (i.e. show all data obtained by different methods for each treatment in a single figure). Finally, after very careful assessment of pros and cons, we decided for the organization (ii) and would like to stick with it also in the revised version. This organization better allows us to 'tell the story' in its logical sequence: 1. different phenotypes/acclimations per se have relatively little effect on mitochondria; 2. lethal cold stress has drastic effects on mitochondria of freeze-sensitive phenotype (while sublethal stresses do not have such effects); 3. cold stress has negligible effect on mitochondria of freeze-tolerant phenotypes.

2. For interpretation of the cold acclimation results, you kind of have an unavoidable “chicken and egg” scenario, to use an English idiom. In other words, is mitochondrial function directly protected by cold acclimation, or is cold acclimation protecting other essential functions, and the mitochondria survive as a result? Is there anything in your transcriptomics studies, for examples, that point to specific protection of the mitochondria during cold acclimation? I think the PT hypotheses presented in the discussion are reasonable and highly likely, but it’s also possible some other part of the cell is particularly freeze-sensitive, and when that function breaks down, it eventually leads to mitochondrial failure. This is particularly the case because measurements were taken after thawing, so a lot of other biological processes/injury pathways could be kickstarted. I realize this is one of those vague reviewer comments that is hard to address, so I don’t expect a lot of changes, but perhaps a quick note in the discussion indicating that it’s difficult to isolate direct effects on mitochondria vs. indirect effects.
We agree with the referee's recommendation to stay maximally careful and conservative in interpreting the data. Accordingly to this recommendation, we have changed the text at many places trying to be more explicit in that we are fully aware of the 'chicken and egg' problem (including new sentences in L286-297 of the revised version).

3. I think the conclusion in lines 324-325 (“This result challenges the view of proteins as primary targets of freezing-induced injury”) is perhaps a little too strong. Enzyme activity was severely reduced, so it still appears there is some damage to proteins. To me, your results could suggest that there are some factors (heat shock proteins, proline, etc.?) that protect proteins from freezing damage, but their capacity can be overwhelmed under extreme conditions. So, while some protein can be protected, much of it is no longer functional. We see similar results with cell viability measurements: even cold treatments that kill insects at the organismal level have some residual cell viability in their tissues. I still think it’s an interesting finding that some enzyme activity survived, but you could consider dialing back your conclusions a bit, considering the results in Figure 4A.
Most of our speculations on 'proteins as primary targets of freezing-induced injury' were removed from the revised version (together with removing all data on muscle tissue). We agree with the referee that this part was formulated 'too strong' in original version and we have changed this by adding a sentence (L283-286 of the revised version) including a clear statement: "... *we are careful to challenge the paradigm of freezing-induced injury to protein molecular structure in general*".

The rest of my comments are quite minor:

1. The only other substantive work on mitochondrial physiology and cold stress in insects, to my knowledge, is Herve Colinet’s work on cold acclimation in Drosophila (Colinet et al., 2017; Insect Biochemistry and Molecular Biology 80, 52-60). There are some interesting parallels to your work, so it’s probably worth citing this work at some point.

Thank you for reminding us the paper by Colinet et al. We know this work and consider it very important. Nevertheless, the Colinet's study differed from ours in focusing on effects of relatively mild cold stress (+4*C) in non-adapted species (*D. melanogaster*), where the main problem is to 'maintain function' at low temperature. We were dealing with much harsher conditions of freezing stress (-30*C) in well adapted species, where the main problem is to "maintain integrity and protect structures" at extremely low temperature.

2. Line 17: “phenotype” is vague. Perhaps change to “cold acclimation” for specificity.
We tried to be more precise and clear in usage these terms throughout the paper (including this particular place in Abstract). In biological terms, we are dealing with different 'phenotypes' of larvae, which we generate by different 'acclimations', while, technically, they represent different experimental 'variants' or 'groups'. For instance, using the term 'cold acclimation' instead of freeze-tolerant phenotype would not be appropriate in all places since we had two different freeze-tolerant phenotypes, the first induced by cold acclimation and the second induced by proline-augmented diet.

3. Line 52: “Despite,” by itself is somewhat awkward. Suggest changing to “Despite the challenges associated with freezing, freeze tolerance has evolved…”
Thank you for this suggestion, we accepted it literally.

4. It’s unclear how the mitochondrial morphology data were analyzed. What is considered a replicate for this experiment? You mention numbers of mitochondria in line 165, but it wouldn’t be appropriate to count these as independent replicates unless you had some sort of blocking term in your model (which is still difficult, because tissues can’t be split across treatments).
We apologize for unclear descriptions. Each larva was taken as biological replicate. We added this explanation to the Legend of Figure S2 and also to the text (L166-167 of the revised version).

5. Lines 181-185: Perhaps give a brief description of PreSens vs. Oxygraph, for those who aren’t familiar with these technologies. Also, Figure S5 is nice, but you should probably include some more methodological details for these analyses, since they are technically challenging.
We believe that situation is now much easier as we removed all data on muscle tissue (Oxygraph respiration). The details of PreSens system are illustrated and described in detail in Figure S3 of the revised version.

6. Line 184: Unclear what “basically after” is referring to. Is it pointing out that you followed the methods of refs 40-41?
Sorry for wrong term. The intended meaning was "according to". Nevertheless, this sentence was removed from the revised version together with references [40] and [41] and all data on muscle tissue.

7. Line 204-205: Perhaps you can elaborate this point a little. I think you are highlighting that there can be changes in enzyme abundance and regulation (e.g., allosteric regulation, posttranslational modifications, etc.) that could influence your results. Perhaps a specific example from your citations would help.
Yes, we are speaking about allosteric regulations, posttranslational modifications, etc. and have added this into brackets (L200-201 of the revised version).

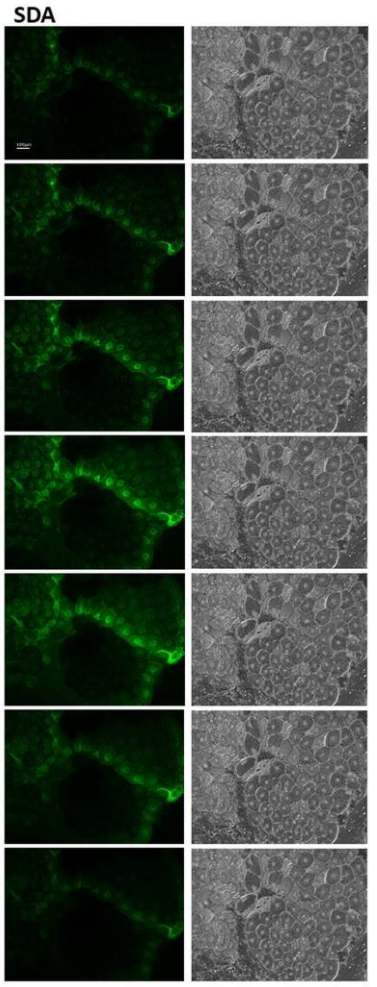
8. Line 227: Insert “and” before “their”
Added.

9. Line 371: With these hypotheses in mind, what are the normal benefits of having an IMM pore? Is it important in cell death regulation during normal development? Otherwise, if it leads to PT and cell death, why even have a pore?

We feel that discussions on possible roles of regulated mPTP pore opening are beyond the scope of this paper. Literature on mPTP is enormously rich (also because the role of mPTP in number of human pathologies). We had to reduce the discussions on IMM pores to minimum and, even like that, the referees felt that we devote too much space to this topic...

10. Figure 1A: In lines 191-197, you discuss several potential reasons for the variation in mitotracker staining in fat body. To me, these images are typical of what you see in insect fat body, which due to its thickness and lipid content is difficult to stain evenly (even for simple stains that target the nucleus). So I'm not sure your descriptions of mitochondrial localization are particularly useful, although it is informative that staining pattern was consistent across groups. Did you try mitotracker with any other tissues that might stain better?

Yes, partially. The fat body tissue thickness and lipid content are definitely two obstacles for proper staining. But not exclusively. We are pretty sure that the differences in staining can be observed also in two adjacent cells, both situated in the center of tissue, both having similar content of fat droplets ... we did not wish to overload the paper with additional figures to document this fact (as it is not in focus of our study). Just as an example, please, look at the figure showing depth series of confocal images for SDA tissue. Note the band of brightly stained cells in the center ...



11. Figure 1E: These box plots are a little squished down to the bottom, which makes it harder to see differences. Perhaps have the micrographs outside of the graph so that you can have a narrower axis that better highlights the differences between groups.

We were facing the problem how to present micrographs AND diagrams together in a SMALL space. The compromise solution was to leave a bit more space for micrographs (to make all important details still visible), and to reduce the space for diagrams, while presenting ALL diagrams with the SAME y axis. Although we tried other formats, they all had some troubles (too small micrographs, too much empty space, uneasy comparisons among figures). In fact, the differences between larvae (biological replicates) are not something we would like to stress ... (these differences can be appreciated in full in the supplementary Excel datasets where all results for each single larva are graphically presented). We are primarily interested in differences among treatments.

Referee: 2

In this study, the authors examine the effects of phenotype on mitochondrial responses to freezing stress using larvae of the fly *Chymomyza costata*. Specifically, they evaluated mitochondrial counts, morphology and functions in different tissues (fat bodies, hindgut and muscle) of the relatively freeze-susceptible (non-diapause, warm-acclimated) and freeze-tolerant (non-diapause, proline-fed; and diapause, cold acclimated) larvae exposed to cold (-5°C) and freezing stresses (-5°C and -30°C). The authors show that mitochondria in freeze sensitive larvae (non-diapause) swell, become rounded and sometimes burst upon exposure to lethal freezing stress, while sublethal freezing stress and supercooling stress were not associated with mitochondrial swelling. Moreover, the swollen mitochondria have decreased respiration rates, but maintain citrate synthase activity and functional oxidative phosphorylation. They suggest that this swelling is caused by an increased sensitivity of the inner mitochondrial membrane (IMM) to freezing stress, leading to permeability transition, loss of barrier function and osmotic influx of cytosolic water into the matrix. On the other hand, freeze-tolerant larvae (diapause and cold-acclimated) do not show any signs of mitochondrial swelling and mitochondrial dysfunctions. They suggest that adaptive changes associated with the diapause and cold acclimation might be due to downregulation of critical components involved in the formation of permeability transition pore (or other non-characterized unregulated pores in the IMM), high concentrations of cryoprotective substances (proline, trehalose) that stabilize the IMM's liquid-crystalline phase, and/or restructuration of the IMM's phospholipid bilayer. This is an interesting study that provides new insights on the mitochondrial responses to cold and freezing in insects, which has been barely studied in these organisms. The manuscript is generally well-written, and the results are clearly expressed and mostly put in context with relevant statistics. There are however several aspects of this study that concern me, and I hope the comments below will help the authors to improve their manuscript.

We thank the referee 2 for kind words as well as for critical comments to which we provide our answers below.

Major comments:

1. The citrate synthase activity, which is used to evaluate both mitochondrial counts and mitochondrial functions, was measured using acetyl-CoA and DTNB. However, no concentrations of these compounds are given (supplementary methods). Moreover, there is no mention of oxaloacetate being used in the reaction, which is absolutely required to start the reaction.

We apologize for insufficient descriptions. The information on concentrations of both CS substrates (acetyl CoA and oxaloacetate) was added into ESM 1.

2. The different samples were collected after cold or freezing challenges and a return to 5°C. Overall, this protocol is well executed. However, some experiments are then done at different temperatures. For example, the oxygen consumption in dissected fat body tissues using the PreSens system was performed after an incubation of 2 hours at 23°C (while for example the whole -5°C stress was performed in around 2.5 hours). Thus, the results obtained might not be due to the induced stress *per*

se, but rather from the incubation used in the experiments. There is no mention of the temperature used for mitochondrial respiration in muscle tissues using the Oroboros oxygraphy though. Moreover, an incubation of 30 min at 30°C was performed for the Mitotracker experiment before visualizing mitochondria, which also raise questions about the effect of cold/freezing stress *per se*.

We generally agree that each method has some technical limitations. Using a combination of different techniques, we hoped to minimize the influence of unwanted effects specific for each single method. What concerns the temperatures and timings:

Oxygen consumption in PreSens system was measured at 23*C in order to assess the capacity for function (functionality) at optimal temperature. It is true, however, that we had to allow the tissues to equilibrate for 2.5 hours prior to measurement ... which could have some effect. On the other side, all tissues were treated equally.

The cold stresses differed in timing (as shown in Fig. 1). The time at low temperature was much shorter for the -5*C stresses that for the -30*C stress. This is because we aimed to maintain constant and slow (optimal) rate of cooling, which was found as critically important in one of our earlier studies (Rozsypal et al., 2018 [15]).

We apologize for not giving the information on temperature used for Oroboros analysis. It was 23*C (according to literature). Nevertheless, all data on muscle tissue were removed from the revised version.

The incubation of 30 min at 30*C for MitoTracker experiment is a technical step which aims to shortening the time for diffusion. Again, all tissues were treated equally. But it is true that we can hardly exclude that different tissues have different sensitivity to relatively high temperature of 30*C and, therefore, that this treatment could have some effect on results.

3. The authors are commended for using three different separate techniques to evaluate mitochondrial counts. However, as they say in the text, the MitoTracker method did not allow to have a relevant quantitative analysis of mitochondrial counts. Moreover, they also mentioned that while CS activity might be a good proxy for mitochondrial counts, in case of complex phenotypic transitions this might not be the case, and I fully agree with the authors on this. Finally, the authors stipulate that the third method, mtDNA copy number, might be the most reliable indicator of mitochondrial counts (although not in insects). However, in one of the studies cited (reference 37) mtDNA copy number has been demonstrated to be a poor marker of mitochondrial content. Overall, this raise the question about the relevance of the results obtained on mitochondrial counts.

We are very open in describing the limitations we encountered using three different techniques to quantify mitochondria. None of them provided absolutely reliable results. Despite these limitations, all techniques are used (though seldom in combination) to quantify mitochondria in different organisms ... Hence, we would like to keep these data as relevant for the paper as they at least show convincingly that there is no drastic reduction of mitochondrial counts associated with phenotypic transition to diapause and cold acclimation in C. costata fat body tissue.

4. The muscle preparations used for the mitochondrial respiration experiments were permeabilized with digitonin. The authors titrated the digitonin in control LD larvae to optimize the method and concluded that a concentration of 55 mM was optimal for the permeabilization, which was then the concentration used for all the other groups. However, as the authors mentioned, a restructuration of the IMM might occur in the larvae exposed to different temperature stress. Considering that digitonin interacts with cholesterol to allow permeabilization, the titration of digitonin should have been done for all individual groups to ensure that the digitonin concentration was optimal whatever the treatment used.

Indeed, we performed separate titration of digitonin for SDA tissue. Based on result of this, we used a different, 30 mM, concentration for permeabilization of SDA tissues (this information was given, though somewhat hidden, in the Legend of our former Figure S5). Nevertheless, all data on muscle tissue were removed from the revised version.

5. The authors used a combination of proline, glutamate, pyruvate and malate to trigger electron transport into the ETS at the level of complex I. However, they stipulate in the results (lines 311-312) that 'this result shows that proline dehydrogenase can supply electrons to ETS even in lethally-frozen LD larvae'. First, they use state 2 (which is not the state 2 as defined in the literature, see minor comments) to

conclude that. However, state 2 might not be a relevant state in this case, as oxygen consumption in this state is limited by the absence of ADP and thus no conclusions about proline dehydrogenase can me made here. Second, their state 3 for complex I might actually be a combination of the electron being supplied to both complex I and proline dehydrogenase (and not only complex I). They also used succinate to trigger electron transport into complex II after inhibition of complex I by rotenone. However, to evaluate complex II individually, substrates for complex I should not be used. This could lead to overestimation of complex II due to reverse electron flux. Finally, the respiration rates are normalized with the number of muscle tissue used. Did the authors verify if muscle mass was the same between control and frozen tissues?

We accept these critical comments and agree that our way of microrespiration analysis was not optimally performed to separate the roles of different substrates and different ETS complexes. In fact, this was a secondary reason why we decided to exclude Oroboros data from the paper. The primary reason was to make the paper more concise and focused. The major purpose for adding the respiration analysis into the original version was to document that a tissue dissected from dead larva STILL shows highly organized responses to both stimulations and inhibitions of the complex respiratory system. This result was rather surprising for us ... and led us to extensively discuss on 'proteins as primary targets of freezing-induced injury'. In the revised version, responding to critical comments of all referees, we have removed most of our discussions on proteins (together with data on muscle respiration), and made the paper focused on single major observation – mitochondrial swelling.

6. The paragraph in the discussion about the potential linkages between IMM permeability, mitochondrial functions, and larval mortality (lines 327-391) is based on a lot of extrapolations. Several tests could have been done to verify that such as injection of cytochrome c during mitochondrial respiration to verify mitochondrial membrane integrity or apoptosis tests. I suggest to shorten this paragraph and focus on the results obtained in the context of the scientific literature.

We are sorry to say that most of the literature on mechanisms of insect freeze tolerance is based on extrapolations. Our text is no exception and we openly confess this. In fact, the first sentence in the Abstract identifies one of the major sources of such uncertainty – it is the enormous complexity of the freezing stress which hits all molecules, structures and processes simultaneously by at least three different disasters: low temperature, absent liquid water, and ice crystals. In this situation, it is quite difficult to distinguish between 'chicken and egg', using the words of the referee 1.

We have changed our text at many places to make clear that our aim is to provide hypotheses for further research rather than to make categorical conclusions. That is also why we would like to keep our (shortened) discussion on potential linkages between ...

Minor comments:

- ETS refers to either electron transport chain or electron transport system throughout the manuscript. Please correct.
- State 2 as measured by the authors is not the state 2 defined in the literature. State 2 represents oxygen consumption in mitochondrial preparations with ADP but no substrates.

All data on muscle tissue, including ETS and its different states, were removed from the revised version.

- While the groups are well defined in the supplementary methods, there are not mention of what are LD, LDpro, SD and SDA in the main text which makes the manuscript sometimes confusing.

We have moved the description of experimental groups from supplementary material to the main text.

- A 'Satistical analysis' paragraph should be added at the end of the 'Materials and methods' section.

We would prefer to keep the descriptions of statistical methods at relevant places of the text. The main reason for this is that we were not using any sophisticated way of statistical analysis that would deserve its own chapter. Relatively simple statistical treatments (ANOVAs, t-tests) of our data can easily be mentioned directly in text. Moreover, the (statistical) analysis of mitochondrial shapes in TEM micrographs has its own section both in the text and in the supplementary materials.

- The term ‘Mitochondrial counts’ should be replaced by ‘mitochondrial content’ as the authors did not measure specific mitochondrial counts but rather estimated the mitochondrial content.
Despite we clearly see the problem with mitochondrial counting in absolute terms, we are afraid that the alternative term 'mitochondrial content' is also ambiguous. We hope that the revised section 3(a) now describes very clearly what we did and what are the limits of our 'counting'.

- Lines 214-217: as the oxygen consumption measured with the PreSens system is not an indicator of mitochondrial coupling, the comparison with E. solidaginis does not make sense.
We have changed the sentence in revised version (L210-211) to remove the (misleading) linkage to coupling: "*Similarly, the mitochondria of winter-acclimated E. solidaginis retained capacity to function at 20°C [33]*".

Referee: 3

Comments to the Author(s)

The paper “Insect mitochondria as targets of freezing-induced injury” by Štětina et al. reports major structural damage to mitochondria following freezing of non-acclimated larvae of the drosophilid fly, Chymomyza costata. In contrast, larvae that are acclimated to short days and low temperature retain “normal” morphological characteristics of the mitochondria. These data are somewhat supported by functional measurements of the mitochondria showing that respiratory function is preserved following freezing in acclimatized larvae. A major point made in this paper is also that protein function is retained although organelle function is impaired (to me this point is not as surprising as it is presented). Overall I find that the paper is very well written and it presents some new and interesting data. The question posed here is relevant and the discussion is generally relevant and well balanced in relation to the existing literature and future perspectives. I have no major concerns and my comments below should therefore mainly be seen as suggestions of aspects that could potentially improve an already good paper. My main suggestions can be summarised as follows.
We thank the referee 3 for overall positive evaluation of our paper.

1. The paper should present the acclimatization groups a little better to the reader that is not familiar with this study system.
We have moved the descriptions of acclimatization groups from supplementary material to the main text (Figure 1).

2. The use of different tissues makes the coupling between morphological and functional characteristics unclear and it may be worth considering to only focus on the morphological data.
We accepted this recommendation and have removed all data on muscle tissue (Oroboros respiration) while focusing on morphological data (mitochondrial swelling) taken in fat body and hindgut tissues. As a result of this single change, the manuscript was profoundly reorganized.

3. The speculation on the cause of mitochondrial collapse is long considering that the data here can not answer most of these questions.
We have shortened text and made clear that we are presenting hypotheses (speculations).

4. I consider the presentation of enzymatic collapse as somewhat of a strawman. I believe that the general notion in the literature is that the cold sensitivity

of the organism is higher than the organ which is higher than the organelle which is higher than the proteins (etc). This is not to say that protein function is not challenged by stressfull events, I just think the introduction and discussion is a little biased in terms of presenting loss of protein function as a principal problem – it would maybe be more fair to present the issue as if los of protein function is an associated problem (In addition to the structural injury caused by freezing and osmotic shrinkage). Below I have listed a number of specific points that the authors could consider in a revision. Thank you once again for a very interesting and well written publication.
The discussion on 'enzymatic collapse' was significantly shortened in the revised version. The removal of muscle respiration data from the paper allowed us much better focusing on major observation (mitochondrial swelling). We still consider the results of Oroboros muscle respiration analysis rather unexpected or surprising (tissue of dead larva shows highly organized enzymatic response of ETS complexes to substrates and inhibitors). Nevertheless, we agree with the referee that presenting these results in this particular study was ambiguous and diverting from the major topic (mitochondrial morphological changes).

Specific points.

Line 16: It would be an idea to already early in the manuscript (abstract) to introduce the acclimation groups. Along the lines of "Here we investigate and compare the mitochondrial responses to freezing stress in the non-diapausing (freeze-sensitive) phenotype with that of the diapausing and cold acclimated (freeze tolerant) phenotype."I personally know of this study system from previous publications, but I would imagine that for the general reader it is important to emphasise these differences – particularly because the figures and data showing the difference in freeze tolerance are placed in supplements.
The acclimation groups are presented in the revised version very briefly in Abstract (L18-20), briefly (and indirectly) in Introduction (L60-74), and in detail in M&M section 2(a) to which we have added a novel Figure 1.

Line 29: As I read the paper there is no data in this study to either support of falsify the hypothesis of the involvement of a permeability transition of the inner mitochondrial membrane. It is fair to speculate about this, but in the absence of data I think this should have less space in the abstract. For example line 31-33 reads as if you have shown that the acclimation and proline is specifically targeting the inner mitochondrial membrane – but I don't see any data that proves this. There are other possibilities for the loss of mitochondrial integrity, so my suggestion is to tone the speculation down in the abstract (some of this space could then be used to introduce the different acclimation groups and the differences in freeze tolerance introduced by acclimation (and proline)).
Yes, the IMM permeability transition is a hypothesis (speculation). Nevertheless, we feel that the main controversy is not about the IMM permeability transition per se (it was almost inevitably the cause of mitochondrial swelling as we have stated in the L304-306). The controversy most likely consists in that our explanations of potential *mechanistic causes* of the IMM permeability transition are highly speculative. We further toned down these speculations or, at least, clearly marked them as 'hypotheses' (see, for instance, L25 or L330 of the revised version).

Line 52: Write: "Despite this complexity" (at least to me it seems odd that "Despite" is all alone ☹).
Changed to: "*Despite the challenges associated with freezing, freeze tolerance has evolved*…"

Line 54: I agree with the statement that it can be difficult to separate cause from consequence – after reading the paper I think this is also largely the case in regards to mitochondria. No need to make a change in the MS here – just a

thought that it is quite difficult to discern if the mitochondria loose structural integrity because the cell dies or of the cell dies because the mitochondria loose structural integrity? (see comment further below)
Yes, other two referees made a similar point, and we agree. In order to be even more explicit on that, we have added a sentence (L292-295) in the revised version.

Line 65: suggest you write "While the diapausing and cold acclimated phenotype can survive…"
Accepted.

Line 67: A small thing, but when I read the references I notised that there was inconsistency in how PNAS was mentioned in the refrences (PNAS or Proc. Nat. Ac. Sci.) – so be consistent.
Done.

Line 104: This could maybe be a good place to introduce some of the abbreviations for the treatment groups – (LD, SD and SDA) which are otherwise introduced a little too late and sporadic later in the MS.
A novel Figure 1 added.

Line 109 gives the (somewhat false) impression that function, morphology and count was made on all three tissues.
All data on muscle removed.

Line 110: hypothesised would probably be a better word than expected.
Changed.

Line 126: Stick with latin name – malt fly is only used once elsewhere (the first time the species is mentioned in the intro).
Accepted, malt fly removed.

Line 127 – explain the LD is an abbreviation of LongDay – not clear to all if you do not know the experimental system. Similar for SDA and SD.
All abbreviations clarified in novel Figure 1.

Line 131: I like Fig S1 and think it is important. I understand that length constraints can make it impossible to fit a graph in, but if it turns out that there is space I think it would be great if you include it in the main MS. It is important for understanding the different phenotypes which is the premise for the question.
Figure S1 moved from original ESM1 to the main text (a novel Figure 1).

Line 139: Is it correct that at the time you selected the larvae it is inclear if the specific specimen is moribound (i.e. just above you show that survival was less than 100% in some groups). Maybe make a small sentence that this procedure means that some of the individuals are likely to be either dead or in the process of dying at the time of sampling. This is not a critism – I think it is a fine sampling protocol. In relation to this I think a great follow up study could be to look at the temporal recovery of mitochondrial integrity in surviving animals.'
Yes, the referee is right in that we were unable to assess the survival status or destiny of larva at the time of sampling. We tried to sample the larvae AS EARLY AS possible after melting the ice (practically at the moment of melting the ice) when the larvae were still immobilized by preceding cold stress. Later on (say, after 30 min), we

could rather easily distinguish dead from (still) alive larvae (as many larvae start crawling within this short time). Nevertheless, we still would have no chance to predict the destiny of the larva (some larvae are moribund but will die only days or even weeks later ...). That is why we supply, in the Figure 1, data on survival to adult stage.

Line 165: I am impressed with the enormous amount of mitochondria examined – must have been long days at the microscope ☺. Great work and great patience…
We are grateful to Tomas Stetina who made this effort.

Line 183: You could add that the main difference is that in the present you permeabilise the cells in the oroborus chamber, while the studies refenced to, do the permabilisation prior to measurements.
True, but we have removed these data from the paper ...

Line 185: Although I understand that there were technical causes to the problem I find that one of the problems with this paper that different mitochondrial traits were measured in different tissues – and with different patterns in different tissues. Thus, the most detailed analysis of mitochondrial respiration was made in muscle tissue – which is characterised by a general better preservation of respiration rate following freezing and for this tissue there is no data on structural damage. In contrast there is convincing data on structural damage – which seemingly has a marked effect on overall respiration rate in fatbody. I personally think the study would be fine with only the structural data from the fatbody (and hindgut) because it is a little difficult to associate structural and functional data from different tissues when the responses seem to somewhat different. Alternatively one could suggest that the authors make a table where it is made even more clear what type of experiments that are made for which tissues.
We gratefully accepted this recommendation and agree that it solved number of problems at once.

Line 203 – should this be figure 1B – in general there are issues with a number of figure references, so please check this throughout the MS (see for example line 211 and 214).
We apologize for wrong numbering. Checked and changed.

Line 216: I struggled a little to understand what the background measurement represented. Is the background respiration rate (empty chamber?) as large as in the tissue with proline, or is this background subtracted from all measurement?. At least I think the figure legend could be a little clearer.
The background respiration rate (empty chamber) was subtracted from all measurements. We have added the explanation to Figure 2 and Figure S3.

Line 258: Since this is a results and discussion section, you could maybe make a short comment on whether you can discern the order of the problems. My point is that it is difficult to know if the larvae are dead because the mitochondria are broken – or if the mitochondria are broken because the larvae are dead. I know it is difficult to add anything concrete to this, but just noticed that this paragraph had no discussion of the data (unlike the other paragraphs).
Yes, other two referees made a similar point. We have added sentences (L292-295) in the revised version: " *We are fully aware of the problem in distinguishing between whether the damage to mitochondria is a cause or a consequence of freezing-induced mortality. Nevertheless, this is a general problem stemming from scientific reduction of a whole complex (organismal death) to isolated pieces (e.g. mitochondrial swelling)*".

Line 269: I am not sure I understand how increased variability will lead to a

greater chance of a significant result. Maybe this is not the point you are trying to make, but the power to detect a significant difference should decrease when variability increases – yet you still find a signinficant difference!.
Maybe just let this slide and say that although you do not understand why there is this increase, the important point is that there is no decrease.
Alternatively use a one-tailed test - because as I understand it you are really interested to investigate if respiration decreased.
We apologize for awkward wording. We wished to say that it is problematic, due to toxicity of proline-augmented diet, to perfectly 'standardize' the LDPro larvae. The control LDPro larvae in one set of tubes might have different mortality rates than the 'frozen' LDPro larvae in other set of tubes ... they all have big problems, many of them die, but not always the SAME proportion, which is probably reflected also in the difference in respiration rates. We understand that this is difficult to explain (and defend) in scientific text. Nevertheless, we were not able to tackle this problem until now and we need to keep the LDPro variant as the proline-toxified larvae (paradoxically) survive freezing and cryopreservation (while none LD larvae can survive – will be dead already at the time of melting!).

Line 284: I do not disagree, but my understanding is that the paradigm of freeze injury ALSO include major structural damage at the organelle, cell and organ level due to the malformation caused by osmotic shrinking (and even from ice crystal formation). This section make it sound as if the MAIN explanation in the literature is related to loss of protein function and my understanding is more that this is also an associated problem. This is probably splitting of words – but consider to tone the arguments a little differently – or at least mention that these problems with protein function are in addition to simultaneous structural challenges at the organelle, cell and organ level (see also line 325 where it is said that proteins are the primary targets of freezing injury).
We say 'one of the ...' (L276 in the revised version). Nevertheless, we agree that we devoted too much space to this topic in the original version (being under influence of 'surprising' results of the Oroboros respiration analysis). Since the Oroboros respiration analysis was removed from the revised version, our discussion on protein injury has been significantly shortened.

Line 299: I completely follow the argument of why you needed to move to a new tissue, but the study becomes a little decoupled at this point. It is very difficult to understand to what degree the ETC problem in muscles is linked to the structural changes seen in other tissues. Clearly muscle mitochondira have higher respiration rates after freezing (also in the LD group), but it is unclear if and how this is coupled to structural integrity of mitochondria. I am not convinced that this part of the study adds so much to the understanding of how the structural problems are linked to functional problems and I think most of the central points in this paper can be made without this muscle data (fig 4). One suggestion would be to include fig. S1 in the MS and exclude fig 4 (the muscle study could then be used in a different publication). This would maybe make the story here a little more narrow, but also more coherent and with room for a better introduction to the treatment groups. If you choose to keep it as it is, I think it is important to discuss explicitly how the detailed measurements of ETC are missing in the tissues where you have structural info, and vise versa.
We fully accepted this complex recommendation. We have included former Figure S1 in the MS and excluded former Figure 4 (and, indeed, we would like to use the muscle study in a different publication).

Line 329: Can the data here show that mitochondrial swelling is the cause of injury, rather than a consequence of malfunctions in other physiological

systems? It is difficult to separate cause and effect. (see initial comment on cause and consequence)
Yes, see above.

Line 331 and onwards: I think it is both appropriate and relevant to discuss a putative role of inner membrane barrier function, but I find that the section (particularly the section from line 346 to 354) to be somewhat lengthy in the absence of data to support or falsify the idea of involvement of specific pores/channels. Consider to shorten.
Shortened.

Page 10 in general: It would be interesting if the authors could speculate a little on the timing of when the osmotic problems occur in relation to freezing. For example, the authors suggest that loss of inner mitochondrial barrier function create an osmotic gradient towards the intermembrane space, and subsequently to the cytosol, and that this is the cause of swelling. However, during the actual freezing event ice formation is likely initiated in the extracellular fluid where it will draw water from the intracellular space and create and osmotic shrinkage of cells and obviously also organelles. I imagine that a swelling of mitochondria must then be a post-freeze phenomena? Is it possible that proline and trehalose have colligative effects (in addition to membrane stabilising effects) during this post freeze period?. I am aware that it is difficult to offer any answers on this aspect but to me it is at least a possibility that injury is initiated during the shrinking, and then this injury is later manifested in swollen mitochondria that have lost their ability to regulate across membranes. I don't think this idea is inconsistent with a role for proline/trehalose nor is this idea inconsistent with the notion that barrier function should be intact during recovery from shrinkage. You could write something along these lines (probably better to use your own words ☺): During freezing cells and organelles shrink rapidly when water becomes locked to ice crystals in the animals extracellular compartments. This process involves rapid movement of water and possibly also osmolytes across cellular and organelle membranes and during the subsequent thawing this movement must be reversed in a controlled and regulated process… Maybe I am missing something in my understanding, but to me the injury could just as well occour during the shinking, and if you agree you could maybe mention this in some way?
We have added a sentence on timing (L295-297). We agree with the referee that mitochondrial swelling is most likely associated with melting phase (for reasons specified by the referee). However, we hesitate to add another speculative explanation into the paper that is criticized for being 'too speculative'.

Line 400: I guess this is only a paradox if the mitochondria of muscle cells are also ruptured and swollen in muscle cells, and this is not known?
No, not known right now (we continue the study). Another reason to remove data on muscle.

Line 403: maybe write "biological membranes, including the inner mitochondrial membrane" – because the initiation of injury could originate from another membrane system not functioning and you do not know if it is specifically the inner mitochondrial membrane – I don't think there is strong evidence for pinpointing this membrane system specifically (this is also done in line 409 onwards). Consider to make the conclusions/perspectives a little more open – for example to suggest that it would be interesting to examine when during the freezing/thawing cascade injury is initiated, and (especially if you maintain the muscle data in the MS) to pinpoint that you need data from the same tissue

to examine how loss of ETC and mitochondrial swelling are linked.
The suggested wording "*biological membranes, including the inner mitochondrial membrane*" used (L376 of the revised version).
Data on muscle removed, which solves the other points (?).

Again – thanks for an interesting paper.
We thank you for fair and stimulating comments.

Appendix C

Dear editor,

We are happy that you give us another opportunity to improve our manuscript.

We are grateful to all referees for their encouraging words and, mainly, for their extremely helpful comments to earlier versions. We feel that all three referees have significantly contributed to the amendment of our manuscript. Here we address the remaining minor issues identified by referees in the 2nd round of revision.

Referee 1 raised five specific points:

1. In first reading the sentence "No loss of activity…" it wasn't clear to me at first that you were discussing the same species as the previous sentence, since they are different references. So perhaps add a transition word/phrase to the beginning, like "However," for example

Thank you for this suggestion. We hope that by adding the transition phrase "In addition," (Line 89) it becomes more clear that we continuously speak about the same species in this paragraph.

2. Line 104: Change "effect" to "effects" for singular plural agreement.

Done.

3. I apologize for not noticing before, but there seems to be some discrepancy between the mitochondrial counts and tissue metabolic rate for the acclimation treatments. Specifically, diapause appears to reduce mitochondrial content, but the metabolic rate of fat body tissue is unaffected. It would be good to briefly address this discrepancy and perhaps speculated what could be causing it.

The apparent discrepancy most likely reflects the technical limits of both, analysis of mitochondrial counts and analysis of tissue respiration. Nevertheless, we hope that the main message is not affected by these limits: the fat body mitochondria of two contrasting phenotypes (LD vs. SDA) do not *dramatically* differ in their counts and major (respiratory) function at optimal temperature.

In response to you comment, we formulated an explanatory extension of the sentence (L211-214): "... which shows that the capacity for respiratory function was influenced neither by entry into diapause and subsequent cold acclimation nor by any potential diapause-linked reduction in mitochondrial counts (see discussion above)."

4. The purpose of the two sentences starting in line 283 might not be obvious for someone who didn't read the first draft of the paper. Instead, I would just simply conclude that you don't have strong evidence that citrate synthase is damaged by freezing (although you did see a significant reduction in LD larvae in Figure 3B) and transition to the mitochondrial information.

Thank you for this comment. We further shortened this paragraph by 50 words (L280-293).

5. Similarly, the sentence in line 292 sounds more like a review rebuttal than something you read in a Discussion. Perhaps just state this problem without claiming you are in fact aware of it.

This part (L294-296) was shortened by 46 words in comparison to earlier version and the phrases stylistically resembling a rebuttal were removed.

Referee 2 had one general suggestion:

I think that the last section ((d) Potential linkages between mitochondrial swelling and larval mortality) is a bit long. I suggest to shorten some parts (for example, the link between mercury exposure and mPTP in Artemia), so it will be easier to follow.

We understand the concern, expressed in some form by all referees, about the speculative character of the paragraph (d). Working on earlier versions of manuscript (and also thanks to referees' suggestions), we have reduced our speculations (hypothesizing) by at least 50%.

In the 2nd revision, we shortened the section (d) by 96 words (see responses to ref. 1). Nevertheless, if possible, we would like to keep the specific part on the link between mercury and PTP in *Artemia*. This part (53 words) presents a concrete example explaining the hypothesis '(a)' and the size of this part is approximately in balance with the explanation for hypothesis '(b)' (71 words).

Referee 3 had two minor suggestions:

Line 27: I suggest you change the sentence "The phenotypic... To "We therefore suggest that the phenotypic transition to diapause and cold acclimation could be associated with" (In other words I suggest to introduce the words "suggest" and "could be" because it is important in an abstract to be cautions with suggestions that are not founded in real data but only in intelligent speculation .

Thank you for this suggestion. We changed the text accordingly.

Line 45: You could mention cellular dehydration as one of the stressors

The cellular dehydration is mentioned (even if indirectly) as the 'absence of bulk liquid water'. We wished to distinguish between 'bulk' and 'free' water as only the osmotically active, 'free' water usually solidifies during extracellular freezing. We also speak about cellular dehydration more directly in other parts of text (for instance L286, 295, 341).