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Hyperkalemia, not apoptosis, accurately predicts insect chilling injury

Jessica Carrington, Mads Kuhlmann Andersen, Kaylen Brzezinski and Heath A. MacMillan

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1st revised submission:	29 September 2020
2nd revised submission:	7 November 2020
Final acceptance:	21 November 2020

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

Review History

RSPB-2020-1663.R0 (Original submission)

Review form: Reviewer 1

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

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

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

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

Is the length of the paper justified?
Yes

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

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

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

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript describes an investigation into mechanisms of chilling injury in the migratory locust. The topic is important and the results are interesting. I was impressed by the sample sizes given that different measures were obtained from the same individuals allowing correlations between the measures. The manuscript is well written, the figures are clear and the supplementary files are useful.

The comparison between Fig 2C and Fig. 2E is certainly compelling. My main concern is about the focus on cell death (apoptosis or necrosis) and a potential mismatch between behavioral (neuromuscular) measures of "survival" and the choice of injury measurement. Whereas cell death certainly could be indicative of injury, I wonder whether there is a good rationale for suspecting that cell death underlies the behavioral impairments used to obtain the survival scores. The logic in the ms is from Lines 42-43 … "As such, cell death in the nerves and/or muscles is likely to directly underlie several common cold tolerance metrics" (this argument is weak) to Lines 91-93 … "the hypothesis that ionoregulatory collapse drives caspase-mediated cell death in both the nerves and muscles and is responsible for insect chilling injury". The fact that cell death occurs after chilling is not sufficient reason to attribute the behavioral impairment to cell death. Indeed, in Lines 381-385 the authors recognize that the behavioral impairments could be due to impairments other than cell death e.g. disruption of synaptic function. This possibility is not reflected in Fig 3 in which chilling injury is attributed to either apoptosis or other cell death pathways. My default assumption has been that injury (physiological impairment) would occur before cell death, so the flow chart seems odd to me (although I am aware that the chilling injury is an organismal measure). It makes more sense to me that apoptosis would be a consequence of injury caused by calcium overload. I also wonder about the solid arrow (cause and effect relationship supported by direct evidence) between apoptosis and chilling injury. This is presumably based on the results in Fig. 1C, but what is the "direct evidence" that muscle apoptosis underlies their measure of chilling injury? Is apoptosis a straw man? Does the flow chart in Fig. 3 need to be modified?

The behavioral measures underlying the survival scores are more likely, in my opinion, to be due to impaired neural communication and coordination and muscle activation than to cell death. I note in Fig. 2B that hemolymph [K+] does not recover completely before the behavioral assessments are made. Thus, the relationship in Fig. 2C is driven by behavioral assessments taken when hemolymph [K+] is more than twice its normal concentration. Couldn't this have affected neuromuscular function?

I recommend changing "Survival score" to "Recovery score". Survival implies to me a binary (dead or alive) whereas recovery can be complete (score 5) or variable on a scale down to death (score 0). I'm interested in the wing-specific score. In particular, it would be useful to have more information about how wing function was assessed. 0 and 1 are straightforward but I wonder what the stimulus was to assess reactivity for 2 and 3. How was range of motion assessed for 3 and 4? Does 5 imply that locust free flight was assessed?

Line 78 … insert 'of' after 'context'

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

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

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

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

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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
In their paper entitled "Hyperkalemia, not apoptosis, accurately predicts insect chilling injury" Carrington et al. have studied physiological responses of locusts to cold stress. The general question asked is whether hyperkalemia (an excess of blood potassium) or apoptosis is the major

driver of post-cold-stress related injuries. Using a very elegant experimental design they first measure cold-stress related apoptotic signals in three different tissues (of which one is a positive control) and subsequently link cold-stress on an individual level to various metrics of cold injury. Authors can show that different tissues react differently to cold stress, while muscles react by activating apoptotic mechanisms, no such signal can be detected in nervous tissue. Measuring chilling injury on an organismal level does not yield strong correlations between aptoptotic signals however, instead the correlation is very strong between chilling injury and hyperkalemia. These findings therefore suggest rather paradoxically that although cold stress leads to apoptosis, apoptosis is not the (main) mechanism leading to systemic injury.

The overall motivation for the study is sound and the language holds high quality. I have a few comments authors can consider when making their revisions.

1. In the abstract and elsewhere authors make a point of distinguishing between apoptotic and necrotic mechanisms leading to cell death and subsequent injury. But are they really mutually exclusive?

2. I find the design in general very elegant, but wonder how comparable the two levels of testing really are. For tissue specific tests, apoptotic mechanisms show clear patterns, and for systemic tests, hyperkalemia shows clear patterns. But how well do we expect apoptotic mechanisms to translate into systemic effects overall? I presume quite poorly. Therefore it is to me not surprising that hyperkalemia, a systemic trait, shows a better correlation with a whole organism stress measure. I think it would have been important to show local hyperkalemia in the target tissues (I know, technically very challenging) in addition to local caspase-3-like activity.

3. Please add “of” at the end of line 78.

4. Probably a dense question, but what does the “like” stand for in Caspase-3-like activity?

5. Given the overall very large variation in caspase-3-like activity, the obvious question is where it stems from? I think it is excellent that you run the tests both with and without the outliers, but would appreciate (1) some reasoning for whether these are true technical outliers or (2) if there is some biological reason for the variation. The fact that apoptosis becomes a significant explanatory variable (i.e. refutes the chosen hypothesis) with them removed suggests a bit more motivation for their inclusion is warranted.

6. Please replace “a” with “a” on line 298.

7. On line 317, please rewrite so it is clearer that the muscle depolarization result stems from another study.

8. On line 322 authors brush aside the decrease seen in midgut caspase-3-like activity (i.e. the control tissue) as something unclear and unimportant. I would appreciate a bit of caution here, since the effect size is similar to the increase seen in the ganglion (in the same panel). This effect gets a more direct treatise in the following paragraph, while the midgut does not. Why?

9. Regarding lines 333-340 I wonder if one would not expect there to be counterbalancing forces in insect muscles that act to pump out Ca2+ for reestablishment of membrane potential (especially for high performance flight muscles)? Would these not counteract the influx? Or are these also shut down during the stress event

10. Line 366 please remove extra “s” in “desspite”

11. In figure 1 you can consider reordering the treatments so they always follow the same logic as outlined in the text, i.e. muscle - nerve - midgut.

12. In figure 2, panel A, figure legend, I do not understand what “recovered” refers to in the legend. Can you explain?

13. While I appreciate that authors put their findings in figure form (Figure 3) and build a logical argument, the novelty of the current manuscript is all in the hypothesized parts of the image, which makes one wonder how useful the image actually is, and how conclusive the findings are. Can you draw more direct conclusions?

While I think this is a well-written and elegantly performed experiment with clear questions. After reading the manuscript I am convinced that systemic hyperkalemia is a better predictor of systemic cold-injury than local apoptotic activity is. I am not sure how surprising that is, and the general conclusion (also in Figure 3) seems to be that many pathways regulate this complex phenotype. Also here I am not surprised. That hyperkalemia is a good metric of cold-stress has been shown before (albeit not on individual level, which is very nice), and the major novelty is the (very interesting!) tissue specific apoptotic signature, and that this signature correlates poorly with systemic cold stress (less surprising, and also not really something people have claimed).

Decision letter (RSPB-2020-1663.R0)

10-Sep-2020

Dear Dr MacMillan:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers’ comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them. All of the comments are detailed nicely below, so I will not repeat them here, however I do emphasize the AE's point to remember that Proceedings is a general biology journal, so as you revise your manuscript please keep this in mind and ensure that your paper is both accessible to and of interest to a broad biology readership, not just those specifically interested in insect physiology.

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:

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

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

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

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.

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

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

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

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

Best wishes,
Dr Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
The manuscript has been assessed by two expert reviewers. Both give very clear and constructive suggestions to improve the manuscript further, with insightful further probing of the results, so I do not repeat them in detail here, but highlight the main, general points and my own suggestions on careful reading of the paper.

While both reviewers agree that the paper is written clearly, Reviewer 1 in particular recommends providing a stronger rationale for why apoptosis should predict chilling injury, and Reviewer 2 points out that it may not be surprising that measures at a cellular level do not strongly correlate with those traits scored at a systemic level. I agree that aspects of the study design could be better justified in the Introduction, and encourage the authors to consider how useful the model schematic of Figure 3 is to illustrate the key findings as it is potentially too complex to be helpful. Moreover, given that one of the take-home results seems to be that apoptosis does not predict behavioural chilling injury, why the solid line linking these two boxes?

In revising this manuscript, I encourage the authors to keep in mind the broad readership of Proceedings B rather than appealing to those with an interest already in insect physiology. For example, the abstract could be more accessible if hyperkalemia is defined with a short phrase (Reviewer 2 gives an example) and stronger rationale is given on why it is interesting to consider apoptotic or necrotic cell death pathways underlying chill injury.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
This manuscript describes an investigation into mechanisms of chilling injury in the migratory locust. The topic is important and the results are interesting. I was impressed by the sample sizes given that different measures were obtained from the same individuals allowing correlations between the measures. The manuscript is well written, the figures are clear and the supplementary files are useful.

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Referee: 2

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1. In the abstract and elsewhere authors make a point of distinguishing between apoptotic and necrotic mechanisms leading to cell death and subsequent injury. But are they really mutually exclusive?

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3. Please add "of" at the end of line 78.

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5. Given the overall very large variation in caspase-3-like activity, the obvious question is where it stems from? I think it is excellent that you run the tests both with and without the outliers, but would appreciate (1) some reasoning for whether these are true technical outliers or (2) if there is some biological reason for the variation. The fact that apoptosis becomes a significant explanatory variable (i.e. refutes the chosen hypothesis) with them removed suggests a bit more motivation for their inclusion is warranted.

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7. On line 317, please rewrite so it is clearer that the muscle depolarization result stems from another study.

8. On line 322 authors brush aside the decrease seen in midgut caspase-3-like activity (i.e. the control tissue) as something unclear and unimportant. I would appreciate a bit of caution here, since the effect size is similar to the increase seen in the ganglion (in the same panel). This effect gets a more direct treatise in the following paragraph, while the midgut does not. Why?

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Author's Response to Decision Letter for (RSPB-2020-1663.R0)

See Appendix A.

RSPB-2020-1663.R1 (Revision)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

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

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

Quality of the paper: Is the overall quality of the paper suitable?
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
The results are very interesting and deserve to be published. I disagree with some of the interpretation and the other reviewer has similar reservations. The authors have come back with an extensive rebuttal but only very minor changes to the ms. I am not persuaded by some of their arguments.
I think the major concern is the mismatch between the measures of apoptosis (tissue-specific) and the measures of survival (behavioral, whole animal). In the previous papers referred to, the measures of injury were tissue-specific (muscle) e.g. cold acclimation reduces muscle tissue damage caused by cold hyperkalemia; muscle depolarization and calcium influx reduce cellular viability in muscle and ileum. I have no problem with linking cell death in muscle with a chilling injury to muscle. The current paper is linking cell death in muscle with whole animal chilling injury measured behaviorally. I'd like to know what the direct evidence for this is and, if there is

direct evidence, it needs to be added to the ms.
The problem is not with the solid arrows from cold stress through to apoptosis; it's with the solid arrow from apoptosis to chilling injury. On the one hand, this is accurate with respect to muscle injury. However, on the other hand, the survival measures in this manuscript are all behavioral and the support for this connection in the legend is "resulting apoptotic cell death <u>likely</u> contributes to injury at the organismal level" (emphasis added). In the context of this paper, this doesn't sound worthy of a solid arrow to me (though it may well be true).
Neither Figure 3, nor the last sentence in the intro ("Thus, other cell death pathways are likely responsible for chilling injury.") reflect the possibility, recognized by the authors, that impairments other than cell death, apoptotic or otherwise, could underlie their behavioral measures of survival. In their rebuttal the authors also recognize that this is a working hypothesis ("Our working hypothesis is that cell death in the muscles underlies chilling injury measured at the whole animal level.") rather than a cause and effect relationship supported by direct evidence.
The work from Mel Robertson's group shows that interstitial [K+]o within the CNS can surge and recover very rapidly and this is associated with coma induced by acute stress. I don't think it says anything about how hemolymph [K+], increased by prolonged stress, could impact neural function. The CNS is protected from variation in the content and composition of the hemolymph by an effective BBB, however, this is not perfect. Indeed, work by Schofield and Treherne in the 1980s (on cockroaches) showed that increased hemolymph [K+] does affect transperineurial potential and action potential amplitude in the connectives and can induce SD at the ganglion. Moreover, SD is a phenomenon of integrating centers (the ganglia) not of connectives or nerve roots. In addition, the neuromuscular junction is not similarly protected from hemolymph [K+] by the BBB.
I have no objection to "survival score" if this is the accepted term in the field. The additional text to describe wing-specific scores was not particularly informative. What is a "full range of motion", how is it evaluated and how is it limited? What is the external stimulus – is it also wing-specific? What does "able to fly" indicate"? Wing beating or maintained flight that generates sufficient lift to support the body weight? For how long? This is a minor point and I'm not recommending changes. It's just that I was interested in how a wing-specific score might give a different value to a whole animal score and how it is possible to make the distinction. It doesn't affect the conclusions.

Review form: Reviewer 2

Recommendation
Accept as is

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

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

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

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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
The authors have done a stellar job with their revisions and I am happy to recommend acceptance of the manuscript.

PS. Please apologize my mistake in the previous revision regarding comment #6, which lacked the underline typo I was referring to (to comical effect…). The correct comment reads:

Please replace the underlined "a" with "a" on line 309 in the revised ms. As far as I can see, the typo persists also in the revised version.

Decision letter (RSPB-2020-1663.R1)

04-Nov-2020

Dear Dr MacMillan:

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. The reviewers, Associate Editor, and I all appreciates your careful revision, however one substantive concern remains regarding the interpretation of your results. As discussed in detail, below, by reviewer 1, it is not clear that there is direct evidence linking cell death in muscle with whole animal chilling injury measured behaviorally. If there is not, then the conclusions drawn in your manuscript need to be tempered accordingly. Note that I do not think that direct evidence is needed for the paper to be interesting and important, but there may need to be more careful wording to ensure that the data and interpretations match. I encourage you to consider the AE's and Reviewer 1's comments carefully in this regard.

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.

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

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

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,
Dr Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
The revised manuscript has been assessed by two expert reviewers. The second reviewer is very positive about the manuscript, and notes only one typo. The first reviewer remains unsatisfied, however, with some of the interpretation of the results. The authors should consider their comments carefully and revise the manuscript where appropriate, or explain why they choose not to do so. The solid arrow in Figure 3 linking apoptosis and chilling injury could be replaced with a dashed one, for example, and there could be acknowledgement that pathways other than cell death might be involved in organismal chilling injury.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
The results are very interesting and deserve to be published. I disagree with some of the interpretation and the other reviewer has similar reservations. The authors have come back with an extensive rebuttal but only very minor changes to the ms. I am not persuaded by some of their arguments.
I think the major concern is the mismatch between the measures of apoptosis (tissue-specific) and the measures of survival (behavioral, whole animal). In the previous papers referred to, the measures of injury were tissue-specific (muscle) e.g. cold acclimation reduces muscle tissue damage caused by cold hyperkalemia; muscle depolarization and calcium influx reduce cellular viability in muscle and ileum. I have no problem with linking cell death in muscle with a chilling injury to muscle. The current paper is linking cell death in muscle with whole animal chilling injury measured behaviorally. I'd like to know what the direct evidence for this is and, if there is direct evidence, it needs to be added to the ms.
The problem is not with the solid arrows from cold stress through to apoptosis; it's with the solid arrow from apoptosis to chilling injury. On the one hand, this is accurate with respect to muscle injury. However, on the other hand, the survival measures in this manuscript are all behavioral and the support for this connection in the legend is "resulting apoptotic cell death likely

contributes to injury at the organismal level" (emphasis added). In the context of this paper, this doesn't sound worthy of a solid arrow to me (though it may well be true).
Neither Figure 3, nor the last sentence in the intro ("Thus, other cell death pathways are likely responsible for chilling injury.") reflect the possibility, recognized by the authors, that impairments other than cell death, apoptotic or otherwise, could underlie their behavioral measures of survival. In their rebuttal the authors also recognize that this is a working hypothesis ("Our working hypothesis is that cell death in the muscles underlies chilling injury measured at the whole animal level.") rather than a cause and effect relationship supported by direct evidence.
The work from Mel Robertson's group shows that interstitial [K+]o within the CNS can surge and recover very rapidly and this is associated with coma induced by acute stress. I don't think it says anything about how hemolymph [K+], increased by prolonged stress, could impact neural function. The CNS is protected from variation in the content and composition of the hemolymph by an effective BBB, however, this is not perfect. Indeed, work by Schofield and Treherne in the 1980s (on cockroaches) showed that increased hemolymph [K+] does affect transperineurial potential and action potential amplitude in the connectives and can induce SD at the ganglion. Moreover, SD is a phenomenon of integrating centers (the ganglia) not of connectives or nerve roots. In addition, the neuromuscular junction is not similarly protected from hemolymph [K+] by the BBB.
I have no objection to "survival score" if this is the accepted term in the field. The additional text to describe wing-specific scores was not particularly informative. What is a "full range of motion", how is it evaluated and how is it limited? What is the external stimulus – is it also wing-specific? What does "able to fly" indicate"? Wing beating or maintained flight that generates sufficient lift to support the body weight? For how long? This is a minor point and I'm not recommending changes. It's just that I was interested in how a wing-specific score might give a different value to a whole animal score and how it is possible to make the distinction. It doesn't affect the conclusions.

Referee: 2
Comments to the Author(s)
The authors have done a stellar job with their revisions and I am happy to recommend acceptance of the manuscript.

PS. Please apologize my mistake in the previous revision regarding comment #6, which lacked the underline typo I was referring to (to comical effect…). The correct comment reads:

Please replace the underlined "a" with "a" on line 309 in the revised ms. As far as I can see, the typo persists also in the revised version.

Author's Response to Decision Letter for (RSPB-2020-1663.R1)

See Appendix B.

RSPB-2020-1663.R2 (Revision)

Review form: Reviewer 1

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?
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 thank the authors for their attention to these details. I have no further concerns and I look forward to seeing this paper published.

Decision letter (RSPB-2020-1663.R2)

21-Nov-2020

Dear Dr MacMillan

I am pleased to inform you that your manuscript entitled "Hyperkalemia, not apoptosis, accurately predicts insect chilling 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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All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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

Sincerely,

Dr Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Response to Reviewers
Proceedings B - Manuscript ID RSPB-2020-1663

Comments from the associate editor and reviewers appear in black
Responses from the authors appear in blue

Line numbers refer to the updated manuscript with changes tracked.

Preamble: We want to thank the editor for handling the manuscript and for pointing out what they see as the most pressing criticisms to deal with in our revision. Below, we have responded to the reviewer comments individually. Some concerns voiced by the reviewers were challenging to address through changes because they lack specificity or a clear suggestion, but we have done our best to tackle them as best we can in this document and have reviewed how the relevant information was expressed in the manuscript to try and avoid similar confusion for future readers. If in doing so we have misinterpreted the reviewers' intention or advice on how to remedy these issues in the text, we would be happy to revisit any of these topics. We think that the review process has strongly improved the manuscript and we sincerely thank the editor and reviewers for their time and effort.

Associate Editor
Comments to Author:
The manuscript has been assessed by two expert reviewers. Both give very clear and constructive suggestions to improve the manuscript further, with insightful further probing of the results, so I do not repeat them in detail here, but highlight the main, general points and my own suggestions on careful reading of the paper.

While both reviewers agree that the paper is written clearly, Reviewer 1 in particular recommends providing a stronger rationale for why apoptosis should predict chilling injury, and Reviewer 2 points out that it may not be surprising that measures at a cellular level do not strongly correlate with those traits scored at a systemic level. I agree that aspects of the study design could be better justified in the Introduction, and encourage the authors to consider how useful the model schematic of Figure 3 is to illustrate the key findings as it is potentially too complex to be helpful. Moreover, given that one of the take-home results seems to be that apoptosis does not predict behavioural chilling injury, why the solid line linking these two boxes?

In revising this manuscript, I encourage the authors to keep in mind the broad readership of Proceedings B rather than appealing to those with an interest already in insect physiology. For example, the abstract could be more accessible if hyperkalemia is defined with a short phrase (Reviewer 2 gives an example) and stronger rationale is given on why it is interesting to consider apoptotic or necrotic cell death pathways underlying chill injury.

We have addressed the points mentioned here in our responses to the reviewers below. In regard to the point about broad readership, this is well taken, and we have worked to broaden our target audience through revisions to the abstract and introduction using the advice provided.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
This manuscript describes an investigation into mechanisms of chilling injury in the migratory locust. The topic is important and the results are interesting. I was impressed by the sample sizes given that different measures were obtained from the same individuals allowing correlations between the measures. The manuscript is well written, the figures are clear and the supplementary files are useful.

Thank you for your very positive words. We are glad you liked it!

The comparison between Fig 2C and Fig. 2E is certainly compelling. My main concern is about the focus on cell death (apoptosis or necrosis) and a potential mismatch between behavioral (neuromuscular) measures

of "survival" and the choice of injury measurement. Whereas cell death certainly could be indicative of injury, I wonder whether there is a good rationale for suspecting that cell death underlies the behavioral impairments used to obtain the survival scores. The logic in the ms is from Lines 42-43 … "As such, cell death in the nerves and/or muscles is likely to directly underlie several common cold tolerance metrics" (this argument is weak) to Lines 91-93 … "the hypothesis that ionoregulatory collapse drives caspase-mediated cell death in both the nerves and muscles and is responsible for insect chilling injury". The fact that cell death occurs after chilling is not sufficient reason to attribute the behavioral impairment to cell death. Indeed, in Lines 381-385 the authors recognize that the behavioral impairments could be due to impairments other than cell death e.g. disruption of synaptic function. This possibility is not reflected in Fig 3 in which chilling injury is attributed to either apoptosis or other cell death pathways. My default assumption has been that injury (physiological impairment) would occur before cell death, so the flow chart seems odd to me (although I am aware that the chilling injury is an organismal measure). It makes more sense to me that apoptosis would be a consequence of injury caused by calcium overload. I also wonder about the solid arrow (cause and effect relationship supported by direct evidence) between apoptosis and chilling injury. This is presumably based on the results in Fig. 1C, but what is the "direct evidence" that muscle apoptosis underlies their measure of chilling injury? Is apoptosis a straw man? Does the flow chart in Fig. 3 need to be modified?

There is a lot to unpack here! First, the reviewer is right to point out the possibility that cell death is not required for injury to occur and may instead result from a disruption of synaptic function. Indeed this is a reasonable hypothesis that warrants testing in our field, but has not yet been addressed in the context of low temperature except for one case that noted that muscles could still be electrically stimulated through the nerve following severe chilling stress (Findsen et al. 2016; http://jeb.biologists.org/lookup/doi/10.1242/jeb.137604). We will explain our reasoning here briefly but have also examined the manuscript carefully to identify where the confusion arises on the mechanisms at play. Our work here is based off of a series of studies linking low temperature hyperkalemia to cell death in this and other model species, and these papers are cited here. Some work is correlational, for sure, but others are direct manipulative experiments that identify cause and effect relationships. While low temperature alone causes some cell death, extensive cell death occurs only when cold and hyperkalemia co-occur (MacMillan et al. 2015; http://rspb.royalsocietypublishing.org/lookup/doi/10.1098/rspb.2015.1483). This has been replicated and an ability to block this affect is associated with cold tolerance plasticity (https://jeb.biologists.org/content/220/3/487.abstract). Importantly, this causal role effect of hyperkalemia has been directly linked to Ca2+ overload (which the reviewer seems more convinced by; Bayley et al. 2018; http://www.pnas.org/lookup/doi/10.1073/pnas.1813532115). The current model argues that hyperkalemia depolarizes cells, triggering calcium overload that causes cell death (so hyperkalemia and calcium are linked), but in these earlier studies, cell death was not adequately attributed to any particular cell death pathway. This is a key gap we are trying to fill with the present study.

In regard to Figure 3, we left this arrow solid because indeed cold does lead to apoptosis (based on the results in Fig. 1 and previously published work on Drosophila cited in the text), and so it is likely playing some role in chilling injury. In fact, as ours is the first study of chilling injury to measure a mechanism specifically involved in apoptosis (caspase activity) rather than mechanisms linked to apoptosis but also other forms of cell death (e.g. membrane permeability or DNA fragmentation; see lines 78-93). As we see it that role is likely minor because it cannot explain injury at the whole animal level, but we think there is enough evidence to warrant its inclusion in a holistic view of the mechanisms of injury. We have adjusted the wording in both the conclusion and the caption for figure 3 to better explain this reasoning.

The behavioral measures underlying the survival scores are more likely, in my opinion, to be due to impaired neural communication and coordination and muscle activation than to cell death. I note in Fig. 2B that hemolymph [K+] does not recover completely before the behavioral assessments are made. Thus, the relationship in Fig. 2C is driven by behavioral assessments taken when hemolymph [K+] is more than twice its normal concentration. Couldn't this have affected neuromuscular function?

This is a valid question and remains an area that deserves further attention. It has been repeatedly observed that hemolymph [K^+] does not return quite to baseline in the hours following a cold stress, but to our knowledge no one has examined the effect of this on indices of injury. In the present study we did want to get a measure of chilling injury relatively early, so that we could sample the animals. This was because we wanted to distinguish between activation of apoptosis during the cold period and that happening several hours after removal from the cold. Additional cell death happening later might lead to progressive injury as we recently identified in Drosophila (El-Saadi et al. 2020; https://doi.org/10.1016/j.jinsphys.2020.104055). Our working hypothesis is that cell death in the muscles underlies chilling injury measured at the whole animal level. While the unstoppable march of time may show this to be wrong with more evidence, it seems reasonable given what we know now; Previous results support this hypothesis with strong correlational data using the live/dead cell viability assays and evidence from the same species of locust suggests that signal

transduction is intact (albeit when measured in vitro; Findsen study referenced above). Ultimately, establishing a stronger case for a cause-and-effect relationship between cell death and a given measure of injury is a future direction we hope to explore. Lastly, note that the neural environment is protected from the hemolymph by the blood brain barrier. While the hemolymph takes some time to re-establish balance (as this is dependent largely on renal function), hemolymph in the local environment of the nerves is re-established very quickly after removal from a cold stress (plenty of work on this from Mel Robertson's group at Queens University), so we are doubtful that this lag observed in the hemolymph would directly affect the nervous system. Neural ion balance is lost and recovered on a different timescale and the effects of this on systemic performance measures remain poorly understood.

I recommend changing "Survival score" to "Recovery score". Survival implies to me a binary (dead or alive) whereas recovery can be complete (score 5) or variable on a scale down to death (score 0). I'm interested in the wing-specific score. In particular, it would be useful to have more information about how wing function was assessed. 0 and 1 are straightforward but I wonder what the stimulus was to assess reactivity for 2 and 3. How was range of motion assessed for 3 and 4? Does 5 imply that locust free flight was assessed?

This criticism is valid, but we respectfully disagree, mainly because making this change runs into another issue. Many studies measure chill coma recovery time (CCRT; as in figure 1), and recovery score could be easily confused with this measure for those in our field. While survival can be considered as binary life and death it is rarely that simple in practice. For example, we would consider an insect with a score of 3 or 4 to be "ecologically dead" in that it is unlikely to avoid predators or reproduce. We and others often use the term survival score and have never been asked to change it by another reviewer so we can only assume this is a very minor issue.
Regarding wing score assessment, we have elaborated on this in the Materials and Methods (now lines 144-151 and lines 206-218). Briefly, locusts were encouraged to jump and/or fly by simulation a predator (moving a hand over them and trying to grab a hold of them) and by gently prodding them to observe their 'ability' to respond to external stimulus.

Line 78 … insert 'of' after 'context'
Done.

Referee: 2

Comments to the Author(s)
In their paper entitled "Hyperkalemia, not apoptosis, accurately predicts insect chilling injury" Carrington et al. have studied physiological responses of locusts to cold stress. The general question asked is whether hyperkalemia (an excess of blood potassium) or apoptosis is the major driver of post-cold-stress related injuries. Using a very elegant experimental design they first measure cold-stress related apoptotic signals in three different tissues (of which one is a positive control) and subsequently link cold-stress on an individual level to various metrics of cold injury. Authors can show that different tissues react differently to cold stress, while muscles react by activating apoptotic mechanisms, no such signal can be detected in nervous tissue. Measuring chilling injury on an organismal level does not yield strong correlations between aptoptotic signals however, instead the correlation is very strong between chilling injury and hyperkalemia. These findings therefore suggest rather paradoxically that although cold stress leads to apoptosis, apoptosis is not the (main) mechanism leading to systemic injury.

The overall motivation for the study is sound and the language holds high quality. I have a few comments authors can consider when making their revisions.
Thank you for your clear description of the work and positive impression of the writing in the manuscript. We agree that the ultimate conclusion is a bit paradoxical. Our original expectation was that apoptosis would be rampant in both the nerves and muscles, and we are glad that additional experiments help to clarify why that might be.

1. In the abstract and elsewhere authors make a point of distinguishing between apoptotic and necrotic mechanisms leading to cell death and subsequent injury. But are they really mutually exclusive?
Yes, but also no. ☺ The diversity of cell death pathways is a large and active area of research. Much of this work is conducted on mammalian cell lines and so it is challenging to extrapolate this diversity to insects (which are studied much less in the context of cell death mechanisms). Because of this paucity of information, most work on cell death in insects focuses on these two traditionally described pathways (while acknowledging it is likely much more complicated). We have tried to do this in the present manuscript. We

are confident that we can make a reasonable distinction between caspase-mediated (typically called apoptotic) cell death and everything else, since this is an ancient path to cell death that is well studied in Drosophila and reliant on caspase-3-like proteins. In the introduction (now at lines 82-83) we refer to necrotic and apoptotic pathways in the context of live/dead cell viability assays, but also tried to point out what we mean by these terms in parentheses. We also (at line 89) note that there are many forms of cell death and that DNA fragmentation cannot adequately distinguish among these forms. What we have tried to do is acknowledge and create awareness for this complexity without spending a large portion of the introduction on the nuance of cell death pathways. We have made edits to the text here to try and further improve this section while working to preserve the brevity and clarity of the introduction as a whole.

2. I find the design in general very elegant, but wonder how comparable the two levels of testing really are. For tissue specific tests, apoptotic mechanisms show clear patterns, and for systemic tests, hyperkalemia shows clear patterns. But how well do we expect apoptotic mechanisms to translate into systemic effects overall? I presume quite poorly. Therefore it is to me not surprising that hyperkalemia, a systemic trait, shows a better correlation with a whole organism stress measure. I think it would have been important to show local hyperkalemia in the target tissues (I know, technically very challenging) in addition to local caspase-3-like activity.
Indeed, that would be technically very challenging! The key to our expectations here is that repeatedly cell death has been associated with damage observed in a single muscle (the exact same muscle, in fact). This has been done with the same species as used in this manuscript at least three times previously and live/dead cell viability assays consistently show a strong association between organismal injury and the proportion of "dead" cells in a single muscle. While we would not have been surprised to find that hemolymph $[K^+]$ correlates "better" with organismal injury measures than caspase activity does, we were indeed surprised that caspase activity did not correlate at all (unless the data are thoroughly massaged as in the supplementary material). In part, this is why the "wing score" was used. As we expected that a wing muscle would best be associated with wing movement. That was not the case. If both hyperkalemia and caspase activation lie in a series of causally linked events, we expect them to correlate, and they simply do not. Thus, while the reviewer makes a valid point, our conclusion seems reasonable to us given the data at hand. To test this more thoroughly we would have to know for certain that cell death underlies chilling injury at all (as discussed in responses to reviewer 1 - strongly supported through correlational studies but hard to test) and measure caspase activity in all muscles. While this could be worthwhile, we argue it goes well beyond the goals of the present work.

3. Please add "of" at the end of line 78.
Done.

4. Probably a dense question, but what does the "like" stand for in Caspase-3-like activity?
Not dense at all! There is more than one described caspase that cleaves the same amino acid sequence. Because of this you cannot be certain that the activity measured is caspase 3 or another executioner caspase in the same family. It is simply a caveat. Also, naming conventions mean that insect caspases do not have the same names as those described in mammals (on which the kit is based).

5. Given the overall very large variation in caspase-3-like activity, the obvious question is where it stems from? I think it is excellent that you run the tests both with and without the outliers, but would appreciate (1) some reasoning for whether these are true technical outliers or (2) if there is some biological reason for the variation. The fact that apoptosis becomes a significant explanatory variable (i.e. refutes the chosen hypothesis) with them removed suggests a bit more motivation for their inclusion is warranted.
We have also had several discussions among us about this choice. We can see the merit in both removing the outliers or keeping them in. However, we have no reason to suspect these particular data points are biological or technical outliers; the samples don't come from particularly injured locusts (i.e. survival scores close to 0), and both hemolymph and tissue sampling went smoothly (a few muscle samples were difficult to dissect out, but these turned out to have relatively 'normal' caspase-3-like activities except for one – and this sample has a modest value among the 'outliers').
In all of our experiments, apoptosis is clearly activated during cold exposure, no doubt about it, however, the predictive value of caspase-3-like activity (values listed in lines 311-316) is either poor (removing outliers, Fig. S2) or very poor (keeping outliers, Fig. 2) compared to that of hemolymph $[K^+]$, so no matter if they are removed or not our overall conclusion remains unchanged: cold exposure activates apoptotic cell death, but it predicts very little of the variance, indicating that another cell death pathway likely are stronger predictors (i.e. more directly involved in the observed cell/organismal death).

6. Please replace "a" with "a" on line 298.

Um…done… ☺

7. On line 317, please rewrite so it is clearer that the muscle depolarization result stems from another study.
Good point. This has been reworded such that it now reads: "*The exposure used to induce cell death in the present study causes hemolymph hyperkalemia and this hyperkalemia is already known to cause muscle membrane depolarization (Fig. 2D; [16])…*"

8. On line 322 authors brush aside the decrease seen in midgut caspase-3-like activity (i.e. the control tissue) as something unclear and unimportant. I would appreciate a bit of caution here, since the effect size is similar to the increase seen in the ganglion (in the same panel). This effect gets a more direct treatise in the following paragraph, while the midgut does not. Why?
We agree that the decrease in caspase-3-like activity in the midgut is interesting in its' own regard. However, our reason for discarding (or ignoring) this finding is that caspase-3-like enzymes are known to not be associated with cell death in the insect midgut (Denton et al. 2009, https://www.sciencedirect.com/science/article/pii/S0960982209016182), meaning that this finding is largely irrelevant to the questions we seeks to answer. We could go into details about possible mechanisms to explain this, however, we feel that this would take away from the overall message we wish to relay. Nonetheless, we have briefly elaborated on how this trend may come about (now lines 349-352).

9. Regarding lines 333-340 I wonder if one would not expect there to be counterbalancing forces in insect muscles that act to pump out Ca2+ for reestablishment of membrane potential (especially for high performance flight muscles)? Would these not counteract the influx? Or are these also shut down during the stress event
Indeed, this is the job of Ca^{2+}-ATPase. Unfortunately for the insect chilling supresses the activity of ion-transporting ATPases. This is a general effect on all enzymes. In the context of ion transport and chilling injury much of this work has focused on temperature effects on Na^+/K^+-ATPase (to understand the causes of hyperkalemia), rather than Ca^{2+}-ATPase (as calcium overload was only identified under these conditions in 2018), but it is a safe bet that it is operating at a small fraction of its usual activity level when the insect is in the cold.

10. Line 366 please remove extra "s" in "desspite"
Done.

11. In figure 1 you can consider reordering the treatments so they always follow the same logic as outlined in the text, i.e. muscle - nerve - midgut.
Done.

12. In figure 2, panel A, figure legend, I do not understand what "recovered" refers to in the legend. Can you explain?
We agree that this part of the legend is confusing. "Recovered" was meant to refer to the measurement of hemolymph K^+ concentration in the cold exposed locusts after the 2 h recovery period. However, we realize that this is also shown in the legend specific to panel B in the same figure and we have therefore removed form the legend in panel A.
For the same figure there was an error in the caption. Specifically, one of the parameters in the sigmoidal model fit should be 34.811 and not 38.811.

13. While I appreciate that authors put their findings in figure form (Figure 3) and build a logical argument, the novelty of the current manuscript is all in the hypothesized parts of the image, which makes one wonder how useful the image actually is, and how conclusive the findings are. Can you draw more direct conclusions?
We can appreciate this perspective. The novelty, as we see it, is that our work is the first to provide evidence that these "other pathways" are at play in cold stress at all, and this is an important shift in perspective. While we are confident that they exist, we would not argue that we have yet confirmed cause and effect (if we had we would be able to draw solid lines). The fact that solid cause-and-effect has not been established does not, in our impression, undermine the importance of this work. As we make clear in the introduction if this paper, although apoptosis is commonly stated to be a consequence of cold stress, in actuality all measures of it in the past have been flawed in that they have measured apoptosis-related events (e.g. membrane permeability, DNA fragmentation) that are not exclusive to apoptosis. Importantly, this model also distinguishes between what is occurring in the muscles and nerves. We think this conceptual model provides clarity on the complex links among all of these physiological phenomena. Regardless of our specific contribution to the model in this work, including the model will help readers understand the paper

and the state of these questions at this time. The associate editor's request for us to consider the broad readership of the journal leads us to think it is best to keep this model in (even when putting aside how the model has changed based on this work).

While I think this is a well-written and elegantly performed experiment with clear questions. After reading the manuscript I am convinced that systemic hyperkalemia is a better predictor of systemic cold-injury than local apoptotic activity is. I am not sure how surprising that is, and the general conclusion (also in Figure 3) seems to be that many pathways regulate this complex phenotype. Also here I am not surprised. That hyperkalemia is a good metric of cold-stress has been shown before (albeit not on individual level, which is very nice), and the major novelty is the (very interesting!) tissue specific apoptotic signature, and that this signature correlates poorly with systemic cold stress (less surprising, and also not really something people have claimed).

We appreciate the kind words of the reviewer on the novel aspects of the study and the fair criticism on what is perhaps less novel or surprising. For the reasons described above, we politely disagree that apoptotic activity in a representative muscle would be unlikely to correlate with performance measures (it must correlate to some degree, no?). We agree that we were surprised and encouraged that hyperkalemia so strongly correlated with performance detriments and the individual level and that this is very novel and strong support for the ionoregulatory collapse model. This is a major strength of this paper. If nothing else, it reinforces that we are either on the right track or at least dancing close to it ☺.

Appendix B

Responses to reviewers

Hyperkalemia, not apoptosis, accurately predicts insect chilling injury

Reviewer comments appear in black

Author responses appear in blue

Comments to Author:

The revised manuscript has been assessed by two expert reviewers. The second reviewer is very positive about the manuscript, and notes only one typo. The first reviewer remains unsatisfied, however, with some of the interpretation of the results. The authors should consider their comments carefully and revise the manuscript where appropriate, or explain why they choose not to do so. The solid arrow in Figure 3 linking apoptosis and chilling injury could be replaced with a dashed one, for example, and there could be acknowledgement that pathways other than cell death might be involved in organismal chilling injury.

We thank the editorial board for a second chance at revising our manuscript. Our reply to the reviewer comments can be found below. Additionally, we have made a few other, very minor, amendments to the manuscript.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

The results are very interesting and deserve to be published. I disagree with some of the interpretation and the other reviewer has similar reservations. The authors have come back with an extensive rebuttal but only very minor changes to the ms. I am not persuaded by some of their arguments.

We thank the referee for the positive comment and interest in our work and have done our best to respond to their comments.

I think the major concern is the mismatch between the measures of apoptosis (tissue-specific) and the measures of survival (behavioral, whole animal). In the previous papers referred to, the measures of injury were tissue-specific (muscle) e.g. cold acclimation reduces muscle tissue damage caused by cold hyperkalemia; muscle depolarization and calcium influx reduce cellular viability in muscle and ileum. I have no problem with linking cell death in muscle with a chilling injury to muscle. The current paper is linking cell death in muscle with whole animal chilling injury

measured behaviorally. I'd like to know what the direct evidence for this is and, if there is direct evidence, it needs to be added to the ms.

The problem is not with the solid arrows from cold stress through to apoptosis; it's with the solid arrow from apoptosis to chilling injury. On the one hand, this is accurate with respect to muscle injury. However, on the other hand, the survival measures in this manuscript are all behavioral and the support for this connection in the legend is "resulting apoptotic cell death likely contributes to injury at the organismal level" (emphasis added). In the context of this paper, this doesn't sound worthy of a solid arrow to me (though it may well be true).

Neither Figure 3, nor the last sentence in the intro ("Thus, other cell death pathways are likely responsible for chilling injury.") reflect the possibility, recognized by the authors, that impairments other than cell death, apoptotic or otherwise, could underlie their behavioral measures of survival. In their rebuttal the authors also recognize that this is a working hypothesis ("Our working hypothesis is that cell death in the muscles underlies chilling injury measured at the whole animal level.") rather than a cause and effect relationship supported by direct evidence.

We thank the reviewer for being persistent. It seems we did not fully understand the issue as originally described. We agree that we may have been a little overzealous with how "direct" the link between cellular chilling injury and behavioural injury really is. A direct link between muscle injury and behavioural injury is elusive; most of it is correlative (despite the correlation being very strong). Our argument follows the logic that if the muscles are more or less dead after a cold exposure, it would negatively impact the locusts' ability to behave (move, walk, jump, fly, etc.). Nonetheless, we see the point raised by the reviewer clearly now and have made several adjustments to correct this issue. We now acknowledge the potential importance of other neuromuscular impairments in the Introduction, Discussion, and Conclusion, as well as in Fig. 3.

The work from Mel Robertson's group shows that interstitial [K+]o within the CNS can surge and recover very rapidly and this is associated with coma induced by acute stress. I don't think it says anything about how hemolymph [K+], increased by prolonged stress, could impact neural function. The CNS is protected from variation in the content and composition of the hemolymph by an effective BBB, however, this is not perfect. Indeed, work by Schofield and Treherne in the 1980s (on cockroaches) showed that increased hemolymph [K+] does affect transperineurial potential and action potential amplitude in the connectives and can induce SD at the ganglion. Moreover, SD is a phenomenon of integrating centers (the ganglia) not of connectives or nerve roots. In addition, the neuromuscular junction is not similarly protected from hemolymph [K+] by the BBB.

The referee makes an excellent point and we agree that muscle cell death alone (no matter through which pathway) is unlikely to be the sole contributor to the organismal/behavioural chilling injury. We have updated our Fig. 3 (the conceptual model) to on one hand link muscle apoptosis to muscle cell death and on the other hand include other hypothesized neuromuscular impairments. Moreover, we acknowledge the important role of other neuromuscular impairments at the end of the introduction (where the referee mentioned that we left it out), at the end of our discussion (new lines 400-408), and more importantly in our concluding paragraph.

I have no objection to “survival score” if this is the accepted term in the field. The additional text to describe wing-specific scores was not particularly informative. What is a “full range of motion”, how is it evaluated and how is it limited? What is the external stimulus – is it also wing-specific? What does “able to fly” indicate”? Wing beating or maintained flight that generates sufficient lift to support the body weight? For how long? This is a minor point and I’m not recommending changes. It’s just that I was interested in how a wing-specific score might give a different value to a whole animal score and how it is possible to make the distinction. It doesn’t affect the conclusions.

We agree with the referee that we should have explained our approach further with regard to how the wing score was assessed. Our reasoning for keeping it this short was that its’ role in our overall conclusion is relatively minor. However, we realize that we may have shortened it too much and we have therefore added more information to the section regarding this methodology.

The methods section relating to wing score assessment (new lines 192 to 206) now reads:

“After 2 h of recovery, the locusts were scored for survival (0-5 as described above) and an additional wing-specific score was estimated (also 0-5) to rank motor function defects and injury to the wing muscles by observing range of motion and reaction time during predator escape simulations (hovering a hand over the locust and trying to pick them up), by gently prodding them, during free flight attempts, and by manually moving the wings while the locusts were handled after the flight attempts (0 = wing motionless, 1 = unresponsive but twitching, 2 = barely reactive, 3 = definite reaction to external stimulus but limited range of motion (e.g. wings not fully opening or closing), 4 = full range of motion, but uncoordinated wing beats (wings used during jumps to attempt flight, unsuccessfully), or with delayed reaction (still unable maintain flight), 5 = fully functional (i.e. maintained flight over several meters)).”

With regards to the referee’s interest in the relation between the wing score and the organismal score, there actually is a fairly tight correlation between the two (slope = 0.91, P = 0.009, R2 = 0.64). So the difference between the two measures is actually quite small, which is likely why the use of the wing score only slightly improves the correlation with muscle apoptosis (Fig. S1). Looking at the data itself, the wing score also contains more “extreme” values, that is, the wings of more injured locusts hardly ever did anything other than twitching or reacting ever so slightly to the stimulus, whereas healthy locusts always were able to at least jump and use their wing to attempt to fly. Thus, the improved relation with apoptosis is likely driven by extremes at both end of the survival score “spectrum”.

Referee: 2

Comments to the Author(s)

The authors have done a stellar job with their revisions and I am happy to recommend acceptance of the manuscript.

We thank the referee for the positive comment and appreciate them taking their time to help improve our manuscript.

PS. Please apologize my mistake in the previous revision regarding comment #6, which lacked the underline typo I was referring to (to comical effect…). The correct comment reads:

Please replace the underlined “a” with “a” on line 309 in the revised ms. As far as I can see, the typo persists also in the revised version.

Thank you for noticing this oddly underlined ”a” (which we can now also see and have changed to a regular ”a”). Thanks also for being a good sport about it ☺