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Major revisions

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Dr. Inês Fragata
Dr. Pedro Simões
Editors
PCI Evolutionary Biology

Thank you for the opportunity to submit a revised version of our manuscript entitled "***Evolutionary responses of energy metabolism, development, and reproduction to artificial selection for increasing heat tolerance in Drosophila subobscura***". Below you will find a detailed list of the actions we have taken to improve the manuscript following the suggestions from the editors and reviewers.

We hope the manuscript will now clarify all of their comments. Each comment is in Calabri bold followed by our reply in italic text. Additionally, we have made some changes in the text for a better explanation of our work.

Sincerely,
Luis Castañeda

Editors' comments

Thank you for submitting your study for recommendation in PCI Evol Biol. This is a very interesting paper on a quite relevant topic, the evolution of heat tolerance and correlated responses in metabolism and other relevant traits. A deeper understanding of the evolution of thermal responses is a fundamental question in the evolutionary biology of the XXI century. We have asked two reviewers to comment on your manuscript, that you can find attached below. While both are positive about the study, they ask for several revisions the most important of which include a better framing of the study within the existent literature, a re-structuring of the discussion as well as some relevant clarifications about the methodology used. Considering those comments, and well as our own assessment of the manuscript, we encourage you to resubmit a revised version of your manuscript considering the points raised by the reviewers.

We thank the comments of editors and reviewers on our manuscript. These comments definitively will improve the clarity and quality of the manuscript.

Below we also highlight some points that need addressing:

The introduction is lacking focus and is somewhat repetitive. While the literature on the topic appears to be well covered, it is hard to reconcile it in a coherent story, as it does not have a clear flow. For instance, there are several studies that are cited repeatedly in different paragraphs of the introduction, with apparently redundant purposes. Streamlining the reasoning throughout the introduction will also help in better framing the hypotheses and expectations presented in the end of the introduction.

We carefully worked in the Introduction to include the editors' suggestions. We have changed some sections of the Introduction to have fluent and clear text. I hope these changes have worked for this purpose.

The discussion is too long and redundant in several places. The findings of the study must be better synthetized and framed within the literature to convey a clearer overall message to the reader. This is also a point raised by reviewer 2. Our assessment is that this task will be facilitated by providing a better focus of the study in the introduction (see first comment above).

We edited the discussion and eliminated redundancy. The previous discussion had 1400 words and the new version has 1000 words.

Carefully consider the comments / questions of reviewer 1 about the methodology used. Providing a supplementary figure with a scheme of the traits and generations analyzed will also allow a better overall understanding of the study.

Excellent suggestion! We added the figure in the supplementary material.

Carefully revise the language of the manuscript as sometimes the wording is odd, and some sentences are not fully formed.

We apologize for this situation and now we carefully checked the manuscript's language.

Some other comments:

Line 29 – "Evolution of stress resistance is companied with a metabolic depression". This is a very bold statement, as there are several experimental studies that do not support such claim some of which you cite in the manuscript (e.g., Djawdan et al., 1997; Mallard et al., 2018).

We modified this point in the abstract.

Lines 82-84 – Why "on the other hand"? the Padfield et al., 2016 study appears to corroborate previous findings reported above. It makes more sense to cite studies that present contrasting results such as Djawdan et al. (1997), Mallard et al. (2018) that do not find such reduction in metabolism.

We changed this section. However, the work of Djawdan et al. (1997) is related to the consequences of selection for desiccation resistance (not heat tolerance) on metabolic rate.

Lines 109-110. Repetitive relative to lines 69-71.

Removed.

Lines 113-117. Please explain better this reasoning, it looks too speculative. For example, in the Porcelli et al. (2017) study the reduction of performance in reproductive traits was a consequence of direct exposure to heat stress, and no evidence was provided for an increased heat resistance.

This section was rewritten. Here, we propose that given the negative effects of warm temperatures on fitness, adaptation to warmer environments should involve metabolic readjustments to reduce the negative effects of warm temperatures on development and reproduction.

Lines 358-359 – this is a direct effect of temperature on enzyme activity. It is important to clarify that examples stated below represent an evolutionary response rather than a direct, plastic response.

You're right. We removed this sentence.

Line 382 – This recent paper appears to be relevant in this context: Tüzün N, Stoks R. (2022). A fast pace-of-life is traded off against a high thermal performance. Proc. R. Soc. B 289:20212414 https://doi.org/10.1098/rspb.2021.2414

Thanks! Added it.

Lines 412-413 – this response is likely dependent on the environmental challenges imposed. Also, in *D. subobscura*, Santos et al. (2022) https://doi.org/10.1111/evo.14366 found no short-term adaptive response in populations evolving under a warming environment.

Thanks! Added it.

REVIEWER 1

The study uses *Drosophila subobscura* as a model to study the metabolic rate responses to thermal stress and does so using slow- and fast-ramping temperatures and compares them to a control treatment. These designs are important and give us a snapshot into how species will respond to future climate change. However, there are a few points to clarify and a major revision of the text so that the study is received well and is consistent in its presentation. The language needs to be checked thoroughly as in places they are not fully formed sentences. I have highlighted a few and did not want to go through all of them, please could you take some time and look at the entire manuscript for consistency.

Thanks for your comments. The manuscript's language was checked.

The other issue with this is that it is not clearly defined in terms of the importance of the context of this work. For example, why not compare thermal plasticity and its impacts on insects within fluctuating and constant environments, providing a solid basis for the framework of this design (like for e.g., https://www.annualreviews.org/doi/abs/10.1146/annurev-ento-010814-021017).

We include this reference in the context of thermal selection beyond static and constant conditions.

Title:

Typo- change heat tolerant to "heat tolerance"

Changed.

Abstract:

Typo- # 2 change which allows to organisms to "allows organisms to"

Changed.

Reword- #5 sentence needs rewording (L44-46). For example: 'should be taken into account for future studies to understand and predict adaptive responses to continued climate change'

Done.

Introduction:

Changed.

Typo- L49 change led to 'lead'

Changed.

Reword- L52 change organisms cause that fitness to 'causes fitness declines, their abundance and distribution is likely to be affected exposing them to current and future increases of temperature'

Changed by similar sentence.

L54 change to understand the capacity of…'to explore how ectotherms will withstand global warming'

Changed by similar sentence.

L58 change responses of to 'responses at'

Changed.

Throughout the manuscript, please check the use of replicated lines and change it to 'replicate' lines.

Changed.

L96 'an' emergent property

Changed.

L114-L115, the references provided are quite limited and recent systematic empirical work shows the extent of damage of reproduction from thermal/heat stress (Sales et al. 2018; https://www.nature.com/articles/s41467-018-07273-z Parratt et al. (2021); https://www.nature.com/articles/s41558-021-01047-0 Van Heerwarden and Sgro (2021); https://www.nature.com/articles/s41467-02122546-w)

This section was rewritten and some proposed references were included.

Methodology:

L138 change feed to 'fed' (make changes throughout the manuscript for grammatical inconsistencies).

Changed.

L139 population, mention 'population size like you do in L136'

Changed.

L143 and elsewhere throughout the manuscript, replicated = 'replicate'

Changed.

L145 what is 'positive oviposition?'

We mean to the presence of eggs and larvae in the vial. This information was included.

L149 delete 'flies belong to the' and follow this style elsewhere in the article

Changed.

L165 change it to 'four-day old virgin' maintain a consistent style!

Changed.

L170 size of the cotton mesh? Is this for ventilation?

For clarification, we used a 5 mm^2 fabric mesh to avoid flies passing from the metabolic chambers to the respirometry system.

L184 change from 27th generation to generation 27 (keep it consistent throughout)

Changed.

L237-238 poisson (link=?) also mention it everywhere you have used a distribution family!

Changed. We used a log link function.

Results

L257-262, why is it important to run tests on the mean ramping of the temperatures- to me this makes little sense, but perhaps you have a better idea? Make it clearer for the reader here please.

We did not compare the mean ramping temperature. As it was written, we compare the knockdown temperature between control and selected lines. For clarification, we included an explication of these results.

L285 and throughout the results P=0.04 is only a marginal significance, so be careful on over emphasis of low P values and report it responsibly!

We agree that this P-value is close to significance threshold but significant anyway. Actually, we verified the P-value for this test and the exact P-value is 0.03515 (expressed in the text as 0.035)

L285 significatively? A typo?

Changed.

L288-291 is hard to follow, please simplify this

Changed.

Discussion:

The entire discussion needs restructuring and having a better context on the findings of the study, then link it to the findings of other studies that support or fail to support what you find.

Thanks for the suggestion. We worked again in the Discussion for clarifications, following your comments.

Then, also speculate on the reasons why this might be because of the less harsh temperatures that were used in this experiment and that metabolic rate could be evolutionarily conserved as changing it means serious consequences for an organism like an ectotherm. Use the Colinet et al. (2015) paper to construct some of the ideas! As

metabolic rates at higher temperatures remain constant for maintenance purposes etc. (pg. 129)

Sorry but we don't understand your point. Metabolic rate was measured only at 21ºC and, unfortunately, we don't know if the relationship between metabolic rate and temperature could be different between selected and control lines (for instance changes in Q_{10}).

Within the area where you discuss adaptive responses, you could refer to a study that looked at adaptive thermal plasticity in reproduction in an insect, especially when temperatures were drastically changes, sperm and reproductive output changed too in order to improve male fitness (see, Vasudeva et al. (2019))

The point here is that we did not test phenotypic plasticity for reproductive traits. Tested females never experienced thermal stressful conditions. Their responses are consequences of evolutionary changes due to evolution for higher thermal tolerance. We try to clarify this point in the Discussion.

Your conclusion is good and provides an importantly open-ended question on how organisms will respond in the future to climate change!

Thanks for your comments and suggestions. We hope that the changes we made throughout the manuscript help to have a better impression of the manuscript.

REVIEWER 2

The manuscript entitled "Correlated responses of energy metabolism, development and reproduction to evolution for increasing heat tolerant in *Drosophila subobscura*" was designed to evaluate the effects of different heat intensity selection regimens (using slow- and fast- ramping protocols) on metabolic rate, activities of four chosen enzymes of G6P branch included in metabolic pathway and life-history traits – fecundity and egg-to-adult viability. The authors hypothesized, according to literature data, that in conditions of high temperature environmental stress traits will evolve in direction of heat tolerance over metabolic depression and increase of some fitness traits, in order to allocate energy necessary for population adaptation. Metabolic rate was not decreased in selected experimental lines, but activity of two enzymes was decreased in slow-ramping in comparison to control (HEX) or fast-ramping selection in comparison to slow-ramping selection (G6PD), only in non-stressed conditions. Fertility was increased in both selected lines, compared to control lines, and viability was increased in fast-ramping selected compared to slow-ramping lines. This research contributes significantly in understanding of the population responses to temperature-changing environment, indicating complexity of adaptation responses as long-term and short-term environmental conditions can make it difficult to distinguish evolutionary and plastic responses. Generally, manuscript is well written and organized, some suggestions and corrections are listed below.

Thanks for your comments and suggestions. We really appreciate them.

Title

Consider the title change, it is not necessary, as "correlated responses" implies a statistical correlation between traits. Maybe to exclude "correlated". If authors accept this change, they should correct in the manuscript.

Thanks for the suggestion. Now the title is "Evolutionary responses of energy metabolism, development and reproduction to artificial selection for increasing heat tolerance in Drosophila subobscura."

Towards this, in the manuscript is sometimes confusing terminology – correlated responses, evolutionary responses, positive responses....

We consider that "correlated responses" and "evolutionary responses" are the right concepts used in the manuscript. Checking some literature we can look that "correlated response" does not imply statistical correlation but also imply a concert response between two or more traits in response to selection (https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/correlated-responses). However, in order to uniform the use of both concepts, we use "evolutionary responses" to explain the changes in heat tolerance (the target trait under selection) to artificial selection and "correlated responses" to explain the changes of other traits (e.g., metabolism, fecundity) in response to selection for increased heat tolerance.

Introduction

Raw 89: Reference is already cited at the beginning of the sentence.

Changed.

Methodology

Heat knockdown temperature selection

Raws 139-141: As there were 4 experimental groups and each group was done in triplicate, I recommend to better explain this step in the experimental procedure. At the first glance it is confusing. In the paper from 2021 is more understandable.

We modified this section and included a figure of the experimental design in the Supplementary Material.

Raws 143-144: Does this mean that female originated from one IF lines, and males from other IF lines? How it was done as females and males from different IFL were mixed to gain experimental lines 6 generations before the selection?

IFLs were crossed to establish population cages (replicate lines). Population cages were maintained for 6 generations before the selection. We modified this section for clarity.

Raw 145: These 120 females are chosen from former 160 females?

Yes. We modified this section for clarity.

Raws 155-157: These groups are also treated every generation but flies are randomly chosen? You choose same number of flies as in selected experimental groups for next generation?

No. We measured the heat tolerance of 40 females as mentioned above and randomly select the offspring of 10 of them to found the next generation. Now, we included this information.

Raws 160-161: I suppose that logistic reason is justified, but authors should comment in Discussion if this choice of control line could influence the results of statistical analyses. The influences of short-term exposures can be visible in next generations with no intensive selection pressures.

According to our experimental design, founder flies were never exposed to heat stress. Females were crossed and lay eggs before measuring the knockdown temperature. This allowed us to have the offspring of the selected females.

Early fecundity and egg-to-adult viability

Raws 213-214: Same comment as previous - Does this mean that female originated from one IF lines, and males from other IF lines?

No. Females and males were collected from the same cage.

Raw 218: "by five days-old females (age of maximal activity of oviposition in D. subobscura)" Is this data from some reference? Authors should add it.

We included the reference Foucaud et al. (2016) published in Ecology and Evolution.

Results

The statistics for knockdown temperature analysis is not listed in the Material and methods.

Thanks for the comment. Now, this information is included.

RMR and body mass

Raw 266: Add Fig 1. after the number 0.79

Done.

Raw 267: Add Fig1. after the number 0.0007

Done.

Or maybe instead of these suggestions, add "The results are presented in Figure 1" after last sentence of this part of the Results.

Enzyme activity

In Figure 2 (page 33) results for each analyzed enzyme are not labeled as A, B, C and D as it is specified in this part of the Results, and in the legend of Figure 2.

Labels are now included.

Rows 276, 280, 282, 286: instead of Fig 1A, 1B, 1C and 1D should write Fig 2A, 2B, 2C and 2D

Text was modified.

Raw 278: "whereas fast-ramping selected lines showed similar HEX basal activity than control lines" – did the authors mean "as control lines" instead of "than control lines"

Changed.

Early fecundity and egg-to-adult viability

Are there any differences by day in fecundity between two selected regimes? It should be mentioned

It was included in the text, but we included some changes for clarity.

Raw 301: (Fig. 3A) – "A" should be deleted as in this figure there are no parts A, B...

Labels in the text were removed.

Raw 305: (Fig. A) – it should write Fig. 4

Changed.

Raw 306: delete comma and Fig.4, it is already written at the beginning of the sentence

We removed the "Fig. 4" from the beginning of the sentence.

Discussion

Raws 321-322: "we detected evolutionary correlated responses in a specific enzyme related to energy metabolism and positive correlated responses of fitness-related traits" This sentence must be reorganized, as I suggested - it is confusing with correlation terms without statistics, sometimes authors mention positive correlation, sometimes evolutionary correlated responses.

As mentioned above, we decided to use "evolutionary responses" to explain the changes in heat tolerance (the target trait under selection) to artificial selection, and "correlated responses" to explain the changes in other traits (e.g., metabolism, fecundity) in response to selection for increasing heat tolerance. We agree that "positive correlated responses" is not appropriate.

Raws 341-342: delete Alton et al (2017) – instead: "they"

Removed but we also changed this section for clarity.

Raw 354: add "the study on" the moth.

We changed this section for clarity.

Raws 368: Did the authors mean the same enzyme (not enzymes) – did they mean HEX enzyme?

We changed this section for clarity.

At the end of the Discussion, the authors consider the local adaptations, temporal and spatial variability in understanding of adaptive response to ongoing and future climate change. Maybe they include in Discussion some papers with experiments on populations of different altitude or origin, and different temperature selection regimens, especially for the species D.subobscura.

Thanks for the suggestion. We included more general literature about the impact of thermal variability and selection on ectotherm species.

Also, the paper below can be included in order to explain results of enzyme activities: JM Flowers, E Sezgin, S Kumagai, DD Duvernell, LM Matzkin, PS Schmidt, WF Eanes, Adaptive Evolution of Metabolic Pathways in Drosophila, Molecular Biology and Evolution, Volume 24, Issue 6, June 2007, Pages 1347–1354, https://doi.org/10.1093/molbev/msm057

Thanks for the suggestion. This work was included in the discussion.