February 13, 2024

Dr. Pedro Simões
Editors
PCI Evolutionary Biology

Thank you for the opportunity to submit a revised version of our manuscript entitled "**Cross-tolerance evolution is driven by selection on 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 their comments. Each comment is in bold text followed by our reply in plain text. Additionally, we have made some changes in the manuscript for a better explanation of our work.

Sincerely,
Luis Castañeda

Editor's comments

Thank you for submitting your study for recommendation in PCI Evol Biol. The study of how heat tolerance evolves as a function of stress intensity is a very relevant question. It is also important to understand how changes in heat tolerance affect other relevant traits in a correlated manner. I have asked two reviewers to comment on your manuscript, that you can find attached below. Both reviewers are generally positive about the manuscript although asking for several revisions to be made. Based on the reviewers comments I highlight the need for a flow-chart/image associated with each key stage of your experiment, the accessibility of data as well as to consider their comments on the interpretation / discussion of your results. I would also include the need for further discussion concerning the starvation resistance patterns, which I found particularly surprising as well as some additional clarifications (see also my comments below).
We thank the editors and reviewers for their comments on the manuscript. These comments will improve the clarity and quality of the manuscript.

Considering the above, I encourage you to resubmit a revised version of your manuscript carefully considering all points raised.

Below I include some additional comments:
Lne 42 – "..these traits" – heat tolerance? Please be more specific.
Replaced by "heat tolerance".

Line 58 – ref. 28 is incorrectly attributed.
Corrected.

Lines 81-83 - the work by Bubliy and Loeschcke 2005 is an important reference in this context.
This reference was included as well aso the classic work of Lande & Arnold 1982.

Lines 84-87 – briefly explain the selection lines which are the basis of this study and/or connect them with refs. 26 and 27. The selection lines are not explained in the context of the present study (only mentioned in the expectations) and are not integrated with previous studies (refs 26 and 26).
I added a section explaining the selection protocol used in the context of the referred papers
"The effects of heat stress intensity have been previously studied on the heat knockdown temperature in D. subobscura and its correlated responses on the thermal performance curves (Mesas et al. 2021), and energy metabolism and fitness-related traits (Mesas and Castañeda 2023). The evolutionary responses of these traits were evaluated using two thermal selection protocols that differed in the rate of temperature increase (hereafter, ramping rate) used to measure the heat knockdown temperature: slow ramping selection (0.08ºC min^{-1}) and fast ramping selection (0.4ºC min^{-1})."

Line 101. Italicize "D. subobscura"
Done.

Line 119. "…four population cages assigned to different selection regimes"
This section will read "After three generations, each replicate cage was divided into four population cages, which were assigned to each artificial selection protocol in triplicate: fast-ramping selection, fast-ramping control, slow-ramping selection, and slow-ramping control lines".

Line 159. 5 flies from each sex? how many vials were used per replicate?? this is needed to obtain the total sample size
Yes, five flies of each sex were placed separately in a vial.
For desiccation and starvation resistance, 126 vials each were used (7 vials $\times$ 2 sexes $\times$ 3 selection treatments $\times$ 3 replicate lines).

Line 172. "response of the knockdown temperature" seems counterintuitive as this is not a trait that can evolve/respond …consider changing to "knockdown resistance"?
I used "heat tolerance" as the title. I changed this as the manuscript progressed

Line 228 – This reference is incorrectly attributed, please carefully check the reference list.
Modified.

Line 309-310. This sentence has a weird structure, please correct.
Modified.

Line 314. I would be more cautious here as for starvation resistance you do not have such a positive cross-resistance pattern (only for males in slow ramping)...
I was trying to be more cautious now.

Lines 330-331. Because the assay is longer right? Please clarify
Done and I added a few references.

Line 373. This sentence has a weird structure, please correct.
This sentence has been rewritten.

Line 381-382. I do not follow this reasoning...wouldn't this mean that selection would then be stronger in males than in females?
No, it means than the lower desiccation resistance in Drosophila males is explained by the fact that males consume resources more quickly than female flies.

Line 387-390. The decline in starvation resistance in the selected lines is not intuitive and should be more clearly discussed. Could it be related with changes in other traits, namely with the previous reported changes in fecundity (ref 27)? In this context, I believe Rogell et al (2014) https://doi.org/10.1111/1365-2435.12179 is a study to consider, as the authors discuss the processes underlying sex-specific differences in populations under thermal selection.
Discussion of the relationship between starvation resistance and fecundity was included as suggested. Information provided by Rogell et al. (2014) was included in the conclusion of the manuscript.

Please cite Table 2 in the main text.
Table 2 has been removed.

Line 627-629. "Average value for each replicated population" – wouldn't this mean three data points for each thermal regime (considering that each has 3 replicate lines)? Or are you plotting the average of the three replicates? Please clarify.
This sentence has been clarified.

Reviews

Reviewed by anonymous reviewer 1, 22 Nov 2023 23:33
I read the paper with great interest - it is an nice experiment that targets the artificial (co)evolution of traits associated with thermal sensitivity in the fruit fly. However, I have some comments and questions that may be valid for the interpretation of the results.

Thank you very much for your comments on the manuscript. I hope that my responses to your comments will help for a better understand of the manuscript.

1) So you created 100 isofemale lines - but then their identiy is largely lost, where they maintained (i.e., 100 lines, then each split into 3 reps and 4 groups) throughout the experiment? It is confusing as it is very difficult to find ones way through the maze of N values, and how different sample sizes come together to describe the power of the study. Please provide: a flow-chart/image of your experiemnt, where at each key stage you specify the number of enclosures (bottles/cages/vials), number of lines and number of individual flies breeding/participating in assays.
I provide a figure of the experimental design in the supplementary material. This should clarify the design.

2) Also on the question of lines and replicates: what where the results for random effects?
Results of replicate lines nested within selection regimes (the random effect) for heat knockdown time were reported in the manuscript: "On the other hand, replicate lines had no significant effect on knockdown time, indicating consistent evolutionary responses within each selection and control treatment (variance of replicate lines= 0, $\chi^2_1 = 0$, and P =1 for all static assays)."

Variance estimates etc - this information is entirely missing from the paper.
I included this information in the Result section: "On the other hand, replicate lines had no significant effect on knockdown time, indicating consistent evolutionary responses within each selection and control treatment (variance of replicate lines= 0, $\chi^2_1 = 0$, and P =1 for all static assays)."

Can you provide open data and code (I strongly suggest thi is available before next review)?
I'm sorry, but I failed to include this important information in the manuscript. Now, you can find a Figshare link in the Data Availability Statement in the main text.

Where there changes in variance components between selection regimes?
I don't understand this question. Selection is a fixed effect in our design, so it's not possible to estimate the variance components.

3) Presentation of results: please provide CIs for all relevant estimates (instead of SE, it emphasizes interval-based conclusions).
Done

Also - what are LTR_5 etc in the desication results section? Why are some presented as just LTR and some with a chi-squared statistic?
It was a typo and I corrected for "LTR: $\chi^2_5 = 83.55$, $P < 2\times10^{-16}$"

4) Is it possible that actually selection you imposed was also selecting for desication resistance (so dessication resistance would not evolve in correlation but under direct selection?) I mean - your selection procedure could target both individual's ability to resist thermal stress but also to survive dessication. Would that be a valid explanation?
Yes, it is possible. In fact, this is exactly the hypothesis that I wanted to test. The problem with slow-ramping assays is that these assays can be so long that knockdown can be influenced both thermal tolerance and desiccation resistance. If this is true, then it's expected that both traits to have evolved correlatedly in the selection experiments, which is exactly what I found, but only for females.

5) Since it is a selection experiment: I miss basic estimates (in the founding population) of standing genetic variance in traits that were then studied as evolving under selection. Do you have such estimates? Would isofemale lines be inbred enough to provide such estimates? Would genetic variance in base population be enough to justify the observed response to directional selection?
I don't have these estimates, but I have published data in D. subobscura in a different population (Castañeda et al. 2019). This study shows that heat tolerance has a narrow-sense heritability of 0.13 when flies are assayed in a fast-ramping assay.

6) What is the interpretation of the polygon are in Fig 2B? You refer to it as "area occupied in the thermal sensitivity landscape" - but is it just a measure of error in linear relationship between z and CT_max? Are CT_max and z expected to be thermally related? If so - this area measure would largely be a statistical byproduct of uncertain values...
The idea was to represent the "thermal niche" of the selected and control lines evaluated in this study. However, each polygon only connects the mean value of CTmax and z of each replicate line. To avoid confusion, I remove these polygons from the plot.
On the other hand, CTmax and z are expected to be thermally related simply because thermal tolerance decreases with higher temperature. However, it is interesting to evaluate how the relationship between the two parameters(e.g., the regression slope) might change in response to thermal selection.

The text needs some typo fixing and English editing.
Thanks for the advice.

Reviewed by Marina Stamenkovic-Radak, 11 Nov 2023 12:15
Adaptive response to global warming, although species specific, generally depends on the population genetic variability and thermal stress intensity. The Author of this paper clearly emphasize the significance and background of research within that topic in introduction. Natural populations are exposed to multiple environmental stressors, and it is known that increased tolerance to one stressor can boost tolerance to another. The major controbution of the present study is that focus is on the effect of variable heat stress intensity on the correlated responses of resistance traits, such as the desiccation

and starvation resistance in a Chilean population of D. subobscura. The experimental design is given clearly, with sufficient details, including descripiton of methods performed.
Statistical analyses are appropriate and I do not find any missing interpeatation in the results. All Tables and Figures are readable and clear. The conclusions of this study are adequately supported by the results. The obtained results show the correlated response to thermal stress selection for the studied resistance traits in D.subobscura under the given experimental design, which demonstrates that the evolutionary response to tolerate higher temperatures also confers the capacity to tolerate other stress such as desiccation and starvation.
Thanks for your positive comments.

As Author correctly states, these correlated responses depended on the intensity of thermal selection and sex, which could limit the capacities to transfer these findings to natural scenarios. However, it does not downplay the the value of experimental evolutionary approach to explore and to understand the adaptive responses of natural populations to global warming.
I included a sentence explaining the value of the experimental evolutionary approach to understanding the adaptive responses to global warming.

The chosen species D. subobscura has been proven to be ideal to study "contemporary evolution" as natural experiment, since it has adapted to New world from Palearctic and spreaded quickly by adapting to new enviroments. In that respect, my only remark (suggestion) for this paper is that discussion should take account the results of the genetic variabilty background and possible causes of evolutionary and cross evolution response obtained in this particular species. In D. subobacura, the inversion coadaptation studies related to thermal selection have been studied throughly in the light of climatic change. Thus, some of the results, relevant for this research I think should be commented. The Author says that further evidence is needed such as quantitative genetic or genome-wide analysis studies to elucidate the genetic basis of the cross-tolerance evolution in D. subobscura, but I think that they already exist and could shed some light to the results based on the hypothesis in this paper. This species has unique inversion polymorphism, and some of the gene arrangements within individual chromosomes have been proved as useful and informative markers for adaptaion under thermal stess.
I completely agree with the reviewer. The results should be placed in the context of the current knowledge of thermal adaptation *in D. subobscura.*