I thank the author for his effort in replying to all the questions / comments raised in the first round of revision. My assessment is that the manuscript has improved. There are still some points raised by one reviewer - mostly concerning the statistical approach - that should be considered, to improve the clarity of the methodology used.
Best regards,
Pedro Simões

Dear editor,

Here you can find my replies to the reviewer's comments. I really appreciate the work of both of you to enhance the understanding of the manuscript

Review by Szymon Marian Drobniak, 15 Apr 2024 09:19
I have read the revision of the paper - it greatly improved, but I have some further suggestions that might improve the statistical presentation of results.

Dear reviewer,

Here you can find my replies to your comments. Thanks for them!

(1) I find it confusing at what stages the slow vs fast ramping regime was analysed in one analysis, and when these were analysed separately. From what I understand - the first analysis of knockdown times was done separately, and then at what stage/in which analyses the slow/fast selected lines were pooled? Does "Selection" fixed effect always mean slow vs fast, or does it mean selected vs control? It should be clearer and the naming of factors should leave no space for ambiguity here...
Only knockdown temperature was evaluated separately by ramping selection. This is because knockdown temperature measured in fast ramping assays is always higher than those measured in slow ramping assays. Additionally, the point of this analysis is to compare knockdown temperature (the train under selection) between selected and control lines within a same selection regimen.

(2) Random effects - from what I see you had 1 random effect (line) - how was it tested in lme4 using LRT? This package does not allow for fitting a model without random effects, which would be needed to compare it with the one with the line effect. Also - you report only 1 result of LRT - was it the only analysis where random effect were used?
I now clarify that the mixed linear model (MML) was fitted using the lmer packages, but fixed and random effects were tested using the lmerTest package. Specifically, the random effect (replicate line) was tested using a likelihood ratio test. Now, I included a table with the statistical results in the Table S2 of the Supplementary Materials.

The replicate line effect was only included for decay time because, for the analysis of CTmax and z, only one value of these parameters is obtained for each replicate line. So, there is no replication at the replicated line level. For the desiccation and starvation resistance, as far as I checked, the Cox regression analysis does not have the option to include nested random effects. However, this analysis has the option of including a random effect, which was done but the results did not change substantially and the message remained the same. Furthermore, the analysis presented in this manuscript, and previous published manuscripts using the same experimental design, did not find significant variation between replicate lines, which is likely true for the desiccation and starvation resistance analysis..

(3) I think analysing all static assay temperatures is superfluous (not to mention the multiple comparisons it generates, I think such results, if not corrected for false-positive discovery rate inflation (e.g., via p.adjust() in R), may be anticonservative).
I think it is important to analyze for each temperature separately because it exactly makes the point that studying thermal tolerance using only a static temperature can miss important effects when modeling thermal tolerance in ectotherms. On the other hand, as you suggested, I performed the posthoc analysis using FDR adjustment and the p-values changed a little, but the significances did not. I included the FDR results in Table S3 of the Supplementary Materials as a conservative approach to testing contrasts between levels of selection.

What I suggest is sticking to the knockdown time ~ temp relationship for each fly and line, and using a mixed effect ANCOVA to test hypotheses. In such analysis, the intercept differences would reflect changes in knockdown time, and changes in slope (interaction of temperature and selection regime) would reflect potential effects of selection on CT_max; such a model would also allow for random slopes (variation in t~temp slope variation across replicate lines).
Thanks for the suggestion. I did the analysis and only found a significant effect of exposure temperature and no significant interactions between selection (this result was included in the text). I think this approach does not invalidate the analysis of CTmax estimated from the TDT curves because the traits evaluated are not the same: knockdown time versus CTmax. On the other hand, the slope between knockdwon time and temperature did not change between selection regimes. Indeed, the slopes represent the thermal sensitivity, as does the parameter z estimated from the TDT curves. In both cases, the thermal sensitivity did not differ between the control and selected lines, which corroborates the previous results.

Minor
L18-20 There's something wrong with the word order in this sentence
Modified
L111-112 What are the "confounding effects" here? This prediction is not clear at all.
Modified
L143 at a rate, not TO a rate (similar typo in several other places)
Modified
L234 glmer, not glm?
glm is correct. Plesse see the "DS_Selection-Desiccation.R" or "DS_Selection-Starvation.R" scripts.
L346-347 In what species and/or study type?
Included: *D. subobscura*
L358 assay, not ASSAYED
Modified

I suggest one more read looking for other typos or leftovers from previous version.
The manuscript was reviewed by a translation service, which improved its clarity.