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Harmonizing protocols to measure *Drosophila* respiratory function in mitochondrial preparations

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Reviewer 1: Marcos Oliveira
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Manuscript reviewed 2023-05-04: *Only major points included.*

Reviewer 1

The manuscript by Rodriguez et al. is a formidable document that address a central problem in Drosophila mitochondrial biology, especially considering that this is one of the most versatile model organisms in science. Ever since I have begun to study Drosophila at the mitochondrial physiology level, and especially after starting my own lab, I myself have stumbled upon different preparation and respirometry protocols and have often chosen the ones that were just handed to me by a lab mate. It has been taking my lab years to explore what Drosophila mitochondria has to offer and show us, just because our minds were stuck in previous protocols that were likely adapted from the work with mammals. Although I think there are many more areas of mitochondrial respirometry to be optimized in Drosophila, such as tissue- and developmental stage-specific substrate preferences, I believe the current manuscript will be a "must read" reference paper for all Drosophila mitochondrial biologists. I do have very few comments that I'd like the authors to address, though.

We have also noticed how "fragile" Drosophila tissues and mitochondria are. In fact, we are now using a protocol that I wish the authors had tested as well. Obviously, the authors do not need to test it for this manuscript, but it would be extremely important for them to mention and discuss it in their paper. I guess this protocol can be called "mechanical permeabilization" of the thorax muscle tissues, and consists of simply allowing the dissected adult thorax to get permeabilized in the assay buffer inside the O2k chambers by the action of the magnetic stirring bars. It turns out that at least the adult thoraces and heads can be completely permeabilized this way, without the need for digitonin. This was first brought to our attention by Dr. Marcus F. Oliveira from the Federal University of Rio de Janeiro and was described along with a respirometry protocol for Aedes aegypti by his group in a chapter of a Methods in Molecular Biology issue (https://pubmed.ncbi.nlm.nih.gov/34060033/). It works and the Drosophila community deserves to know more about it. Because I have opted in to publish my name along with my review of this manuscript, I must clarify that I am not asking the authors to cite one of my own papers. Dr. Marcus Oliveira and I coincidently have almost the same name and are from the same country, so people get us confused all the time. Please, check Dr. Oliveira's work, not mine, and scrutinize it appropriately.

Authors

Thanks to the reviewer for pointing out this study, which we were not familiar with at the time of running the experiments for this part of the manuscript. We did read the methods paper on Aedes aegypti while preparing this manuscript, however failed to mention this as a potentially useful method to apply to Drosophila tissues. I think the reviewer is right in pointing it out, a**nd we've now added it page 17 lines 464-467**.

Reviewer 1

Since one of my lab's work has been listed in Table 1, I sort of feel compelled to comment on the fact that we did not separate males from females in our analyses of larval mitochondrial respiration. I do not want to justify why we did this or that, I just want to point out that in some circumstances it is just not that easy to sex the animals before the experiments, especially in developing stages. I recognize this is not ideal and wish we had done many of the experiments differently, but am also confident they were done correctly to the best of our abilities at the time. We were working with very severe mitochondrial mutants, which died at the third instar larva stage. To avoid getting dead or dying larvae for our analyses, we chose an earlier time of development, at which sexing the sick mutant larvae was very difficult; so, we ended up ignoring this. Nevertheless, the differences of males and females in this case was irrelevant, since they all had the same lethal phenotype; the differences (or lack thereof) between the controls and the mutants were much more important in this case. The authors wrote their manuscript almost "crucifying" other researchers for not separating males and females in their analyses, but this does not seem totally fair to me, as the specific context of the cited papers were not evaluated. Although I am a ferocious advocate of Drosophila research, I have to accept the fact that some things are just not as easy when working with this tiny insect. Sexing sick larvae is just one of them.

Authors

These are fair points raised by the reviewer and we totally agree with them. We also find it very difficult to sex the larvae (and have published research in the past without sexing them), and we totally understand that we often can only chose to control for as many variables as the study design allows us to. We also didn't mean to "crucify" other researchers for not separating the two sexes, we listed the studies where this was done or not, however we didn't mean to judge or dismiss studies for omitting this. Rather, we want to push the field in general to try to tease apart the two sexes in order to account for the differences in responses between the two. We have added some nuance to the text for this, **see page 15 lines 423-429.**