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

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Reviewer 3: Alba Timon-Gomez

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Manuscript reviewed 2023-04-25: *Only major points included.*

Reviewer 3

In this study, Rodriguez and colleagues reviewed the literature on *Drosophila* studies using Fluorespirometry and concluded that there is a lack of harmonization of protocols and methodologies in this model organism when investigating mitochondrial function. The authors compared different mitochondrial preparations (imt, thom and pfi), determining that thom preparations are not suitable for these analyses. They also assessed the effect of different oxygen concentrations on the results to conclude that oxygen diffusion, contrary to mammalian pfi, is not a problem in this organism. Finally, they showed that some fluorescent probes used in Fluorespirometry might affect mitochondrial function, pointing out the necessity to optimize their concentration before any assay.

The study is well-written and organized, following logical reasoning. The methodology is appropriate, and enough details are described in order to be reproducible; and the conclusions match the results. Therefore, I only have minor comments to be addressed by the authors:

In methods section 2.6, in line 206, it is only specified "followed by pfi". Please, clarify whether the same protocol was followed for thom or not.

Authors

Thank you, we clarified that we indeed did the same for thom.

Reviewer 3

In Figure 3C, it is not clear whether the authors are plotting cytochrome *c* efficiency (Mitopedia link: https://wiki.orooboros.at/index.php/Cytochrome_c_control_efficiency). Please, either clarify or explain how those results were obtained (and in case it was not calculated, consider to use the cytochrome *c* control efficiency).

Authors

Yes, this is akin to cytochrome *c* efficiency, we used the same calculations and multiplied by 100 to present it as a % increase in respiration after cytochrome *c* addition (as done in multiple studies in the past). This is briefly explained in methods section 2.5.,

but we can further explain the calculation in the figure legend or in the methods if this is critical.

Reviewer 3

In Table 1, RCR parameter is used. It will be very informative that the authors discuss why they use it instead of E-L coupling efficiency (Mitopedia link: [https://wiki.oroboros.at/index.php/Respiratory acceptor control ratio](https://wiki.oroboros.at/index.php/Respiratory_acceptor_control_ratio)).

Authors

We used the RCR, as it is the classical parameter reported by most studies in the mitochondrial field. This is fortunately changing as the push is being made by the Oroboros community, and we also encourage this shift. However, it would've been difficult or impossible for us to convert these RCRs into E-L coupling efficiency values for the sake of the table. We've added an explanation at the end of section 3.1., before the table.