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

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Reviewer 2: Adam Chicco
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Manuscript reviewed 2023-05-04: *Only major points included.*

Reviewer 2

The authors provide a well-written account of the relevant issues surrounding the appropriate sample preparation, chamber oxygen concentration, and impacts of common fluorescent probes for mitochondrial fluorespirometry experiments in drosophila, and provide data-driven recommendations for addressing these to scientists in the field. In addition, they provide a comprehensive review of experimental parameters used in drosophila respirometry studies, and examined the frequently neglected impacts of sex on these parameters. Therefore, the paper is ideally suited to BEC and an important contribution to researchers working in fields of drosophila biology and comparative bioenergetics. Below are few comments and suggestions for improvement.

The authors added 10 uM cytochrome prior to titrating fluorophores. While perhaps not relevant for respirometry measurements, the authors are probably aware that cytochrome c is strictly incompatible with the Amplex Red assay, and likely interferes with fluorescence measurements using the other probes as well. This should at least be noted in the paper to prevent inexperienced readers from following the same protocol without consideration of the fluorescence data. It might also be prudent to repeat at least a few experiments without cytochrome c to confirm there is no interaction with the probes on J_{O_2}.

Authors

The reviewer is absolutely correct in pointing out the incompatibility of cytochrome c with fluorescent probes (among which AmR), and in fact we never use it when measuring fluorescence in our analyses of ROS flux, membrane potential and ATP production. Because we were specifically interested in the effects of those probes on the O_2 flux respiration, and not on fluorescence signal (in fact, we didn't even attach the Fluo-sensors to the O2ks), we wanted to make sure that we were working with intact preparations, hence why we added cytochrome c after ADP. The reviewer is right to point out that it might lead to confusion for some inexperienced users (although it is well explained in several pages of the MitoPedia), hence **we added a sentence in the methods section 2.7.** I think repeating the experiments with cytochrome c might not be very useful, as potentially damaged tissues will not be filtered out of the analysis. Moreover, all the probes and the ethanol controls were done in the same conditions, hence any effect of cytochrome *c* might be evenly distributed between the groups.

Reviewer 2

If possible, it would be informative to present both the LEAK and OXPHOS state J_{O_2} data (pmol/sec) along with the FCRs for each of the 5 fluorophores in Figure 4. While impacts on FCRs are clearly important and provide strong support for the authors' study and conclusions, it would be useful to present how the probes impact L vs. P respiration in the main manuscript if room allows.

Authors

This would be a good suggestion if we had indeed looked at the impact of the probes on LEAK respiration, however we only looked at their impact on coupled respiration with N-pathway substrates… The FCRs allow us to normalise for maximal respiration rates before addition of the probes and make it clearer to see how much respiration was impacted on a percent basis. We are not sure what more information the O_2 flux in pmol/mg/sec would provide, but we would be happy to show them if the reviewer thinks it adds value?

Reviewer 2

The authors provide good evidence for a minimal O_2 diffusion limitation in thorax pfi, so recommend that future studies do not hyperoxygenate at the start of a SUIT protocol. While this is certainly reasonable and appropriate based on their data (e.g, to avoid impacts on ROS production, etc.), they should consider noting that it might still be advantageous to hyperoxygenate the chamber prior to long SUIT protocols to avoid the need for reoxygenation – at least when ROS is not being examined.

Authors

This is a fair point raised by the reviewer, however the issue of reoxygenating can also be circumvented by adding fewer amounts of tissue/mitochondrial isolates to the chambers to account for very high rates of respiration. We believe hyperoxygenating might not represent a true physiological state of the thoracic tissue and is best to be avoided.