BEC BIOENERGETICS COMMUNICATIONS
Bioenerg Commun 2023.4
https://doi.org/10.26124/bec:2023-0004
Open Access

BEC Spotlight

Cite
Oliveira MF, Aveiro Y, Couto-Lima CA (2023) A navigation chart to avoid turbulence in *Drosophila* mitochondrial research. Bioenerg Commun 2023.4. https://doi.org/10.26124/bec:2023-0004

Author contributions
All authors wrote and designed the framework of the manuscript.

Conflicts of interest
The authors declare that no conflicts of interest exist.

Received 2023-08-22
Reviewed 2023-10-31
Accepted 2023-11-02
Published 2023-11-06

Open peer review
Lisa Chakrabarti (editor, reviewer)
Nicoleta Moisoi (reviewer)

Keywords:
Drosophila; method; mitochondria; standard; permeabilization; oxygen diffusion; probe

A navigation chart to avoid turbulence in *Drosophila* mitochondrial research

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Summary

Drosophila **fruit flies have been used as a valuable, cheap, and powerful model organism to understand fundamental biological processes for many years. However, standardized methodologies specifically designed to assess mitochondrial physiology in this model are not available. Rodríguez and colleagues provided a detailed analysis of publicly available protocols to assess mitochondrial physiology in** ***Drosophila melanogaster*** **and performed experiments in flight muscles to address three technical parameters to define the optimal conditions for respirometry. The authors show that oxygen diffusion is not limited to sustaining respiratory capacity in either isolated mitochondria or chemically permeabilized fibers. In addition, chemical permeabilization was revealed as the best approach to assess mitochondrial physiology in fruit flies. Finally, the authors demonstrate that magnesium green is the only fluorescent probe that caused no effects on respiratory rates. Methodological standardization to study** ***Drosophila*** **mitochondrial physiology, as presented by Rodríguez and colleagues, represents a critical step towards more reproducible and comparative metabolic research in this important model organism.**

Fruit flies of the genus *Drosophila* (Insecta: Diptera) have been used for more than a century as one of the most powerful model organisms in biomedical research (Morgan 1910). The enormous success and popularity of *Drosophila* in the scientific community stem from a combination of factors, including the ease of culture and short life cycle with a low financial cost compared to mammalian models. Importantly, *Drosophila* researchers have a powerful and ever-growing portfolio of genetic tools available to address the biological functions of specific genes (Richhariya et al 2023, Wangler et al 2015, "Why funding fruit fly research is essential for the biomedical sciences," n.d.). Technologies such as CRISPR-Cas9 and the UAS-Gal4 system using *Drosophila* have directly contributed to expanding our understanding of key cellular and molecular processes in biomedical sciences (Kohsaka, Nose 2021). This suitability allowed the remarkable output of nearly 3000 original papers per year on *Drosophila* in the last 20 years (Figure 1A). However, the share of mitochondrial metabolism studies in *Drosophila* represents about 5 % (~150 papers/year) of total publications (Figure 1A). This strikingly contrasts with the explosion in mitochondrial metabolism research observed from the late 1990´s to nowadays (Figure 1B), reflecting the key importance of this organelle to cell and molecular biology and its involvement in the pathogenesis of human diseases. However, one of the critical factors that limit the strong pace of biomedical research using mammalian models is its high financial cost and alternatives to overcome this scenario have been considered over the years (Abkowitz, Hromas 2018; Wangler et al 2015).

Conceivably, the low success rate of NIH and other funding agencies grant applications (Abkowitz, Hromas 2018) have potentially pushed many scientists to seek affordable alternative model organisms (yeast, worms, and flies) to foster basic biomedical research. In this sense, estimates indicate that average cost of a NIH R01 grant is ~ 20 % lower when *Drosophila* is used as a model organism (Wangler et al 2015). One might think that the scientific output of *Drosophila* studies in a given area would be boosted by financial and practical reasons as pointed out above. Analyzing the field of mitochondrial metabolism, we observed a parallel steady increase in studies using *Drosophila* since the late 1990´s (Figure 1B, blue line), perfectly matching with the studies carried out with all model organisms (Figure 1B, red line). Indeed, we think that *Drosophila* research enjoyed the groundbreaking discovery of cytochrome *c* and apoptosis-inducing factor as key drivers of programmed cell death (Liu et al 1996, Susin et al 1996), which placed mitochondria at the center of modern cell biology, boosting mitochondrial research for any organism since then. However, the huge increase observed in *Drosophila* mitochondrial metabolism from the late 1990´s represents only ~ 1.3 % of all mitochondrial metabolism papers (Figure 1B, blue line). The question is: why is the scientific community so reluctant to embrace the use of *Drosophila* for mitochondrial physiology investigation?

Determining the exact reasons behind this is hard but we can point out some factors. First, the feeling of complacency that affects the vast majority of researchers who have historically worked with mammalian organisms and do not feel comfortable taking the leap to use an "exotic" model such as a fly. Second, there is a general perception that flies are too distinct from mammals, which makes it hard to imagine that complex biological events would be evolutionarily conserved. However, many critical biological events were first identified in *Drosophila* and later in mammalian models (Wangler et al 2015). Surely, not all mammalian biological processes can be studied using the fly, but we argue that there is no reason to avoid using such a powerful and cheap model in biomedical sciences. Although this reluctance seems to be slowly vanishing (Figure 1B, blue line), a third factor

must also be considered: the lack of standardized methods to use *Drosophila* to allow reproducible and comparative assessment of specific biological processes such as mitochondrial metabolism.

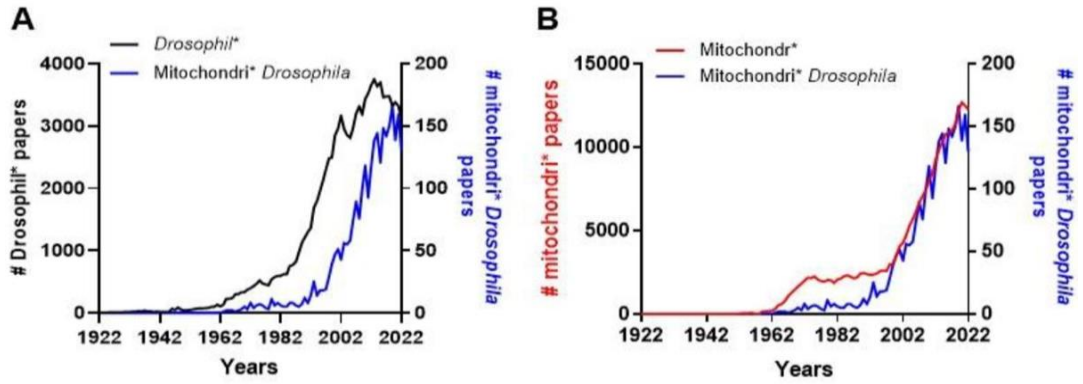

Figure 1: A century of *Drosophila* and mitochondrial metabolism research. (A) Number of original publications using *Drosophila* in all areas (black line) and on *Drosophila* and mitochondria (blue line) from 1922-2022. **(B)** Number of original publications in mitochondria in all areas (red line) and on *Drosophila* and mitochondria (blue line) from 1922-2022.

In this regard, the study of Rodríguez and colleagues provides a solution for those aiming to use *Drosophila* as an model organism for mitochondrial physiology studies (Rodríguez et al 2023). The authors established the technical grounds toward the standardization of methodologies routinely used for assessment of mitochondrial physiology of *Drosophila* by fluorespirometry. This is a pioneering and remarkable achievement considering the vast diversity and heterogeneity of protocols available in the literature using *Drosophila* in mitochondrial studies which hampers reproducibility and comparative analyses between different laboratories.

Firstly, the authors have performed a meta-analysis investigation of studies available on the Oroboros Ecosystem O2k-Publications database, as well as a Google Scholar search for "Drosophila" AND "Oroboros" to assess the available methodologies on mitochondrial physiology in *Drosophila*. As expected, they found out an enormous diversity of protocols where most studies use adult, male flies of *Drosophila melanogaster* species. As a multi-cellular organism, adult *Drosophila* flies have distinct tissues with functions quite similar to those found in mammals (Leader et al 2018, Li et al 2022). However, the vast majority of *Drosophila* mitochondrial studies do not address the molecular and functional tissue heterogeneity as the thorax/flight muscle was the most represented tissue. Importantly, the authors also found a significant share of studies that used the whole fly to assess mitochondrial metabolism, overlooking the critical tissue heterogeneity aspect of metabolism. This underscores the urgent need for benchmark development of tissue-specific assessment of mitochondrial metabolism in fruit flies. Although this survey represents a valuable source of the available mitochondrial protocols for *Drosophila*, it lacks some critical points including: *i)* the inclusion of alternative methods of tissue processing (Ebanks et al 2023, Gaviraghi et al 2021); *ii)* the effect of potentially different diet composition (Bonfini et al 2021), *iii)* temperatures and fly strains (Huda et al 2022, McGraw et al 2009) would affect mitochondrial metabolism.

The authors next investigated whether oxygen diffusion would be limited in *Drosophila* tissues and investigated the potential beneficial effect of oxygen supplementation in respirometry experiments. They found that increasing oxygen supply caused no apparent effects on the pattern of respiratory flux, when comparing experiments carried out in normoxia and hyperoxia. However, a careful analysis of these experiments revealed that maximal coupled respiratory rates under hyperoxia (~290 pmols $O_2 \cdot s^{-1} \cdot mg^{-1}$) were remarkably lower than in normoxia (~420 pmols $O_2 \cdot s^{-1} \cdot mg^{-1}$), suggesting a detrimental effect of increased oxygen supply for *Drosophila* mitochondria. Despite this aspect not being addressed by the authors, it is conceivable that under hyperoxic conditions (even short term) might oxidize mitochondrial proteins and compromise the electron flux and respiratory rates (Walker, Benzer 2004). In any case, a final assessment revealed that increasing oxygen supply caused no apparent effects on the cytochrome *c* oxidase affinity suggesting that oxygen diffusion is not a limiting barrier to assess mitochondrial physiology in *Drosophila* tissues.

A third aspect addressed by the authors was the tissue preparation to assess mitochondrial physiology in *Drosophila*. Given the diversity of protocols to accomplish this task, there is a general lack of standardization of methodologies and a thorough assessment of potential interferents should be considered when analyzing mitochondrial physiology in this model organism. The authors observed that tissue homogenization is not a suitable procedure to assess respiratory rates in *Drosophila* when compared to isolated mitochondria or chemically permeabilized tissue. An interesting possibility would be a comparison of these tissue processing techniques with mechanical permeabilization of flight muscle as recently described (Gaviraghi et al 2021).

Finally, the authors investigated the potential side-effects of fluorescent probes on respirometry. Indeed, previous studies demonstrated that exposure to several fluorescent probes designed to assess redox balance and a variety of mitochondrial processes have detrimental effects on mitochondrial electron flux (Cheng et al 2018, Roelofs et al 2015). Indeed, the authors found that is also the case when assessing mitochondrial physiology in *Drosophila* since only one (magnesium green) out of the five fluorescent probes investigated had no effects on flux control ratios. The cautionary note provided by these studies emphasizes the need for careful consideration of which probes and conditions can be used when assessing mitochondrial physiology in *Drosophila*.

As shown by Rodríguez and colleagues, the way to hell is full of shortcuts when assessing mitochondrial physiology in *Drosophila*. As such, one must resist the temptation of simply applying protocols designed for mammalian models without proper validation and standardization in *Drosophila*. Also, we should exert caution by assessing the potential artifacts generated from reagents and methods regularly applied to mammalian models, even if these were certified and validated for these organisms. In summary, the development and optimization of novel methods to specifically assess mitochondrial metabolism in *Drosophila* strengthen the importance of this organism as an easy, cheap, and powerful model for metabolic investigations. We envisage a brave new world emerging for fruit flies as genuine model organisms to be used in mitochondrial physiology studies in the next future.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) through the Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT-EM). M.F.O. is a CNPq fellow [#308629/2021-3]. C.A.C.-L is a Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [#2022/05632-4]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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