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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 organism model 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*** **while 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 revealed 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 organism model.**

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 scientific community lie from a combination of factors including the easiness of culture, 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 expand our understanding of key cellular and molecular processes in biomedical sciences (Kohsaka and 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 contrast with the explosion in mitochondrial metabolism research observed from 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 and Hromas, 2018; Wangler et al., 2015).

Conceivably, the low success rate of NIH and other funding agencies grant applications (Abkowitz and Hromas, 2018) have potentially pushed many scientists to seek affordable alternative organism models (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 late-1990´s represent only ~ 1.3 % of all mitochondrial metabolism papers (Figure 1B, blue line). The question is: why the scientific community is 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 so complex biological events would be evolutionarily conserved. However, many critical biological events were firstly 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.

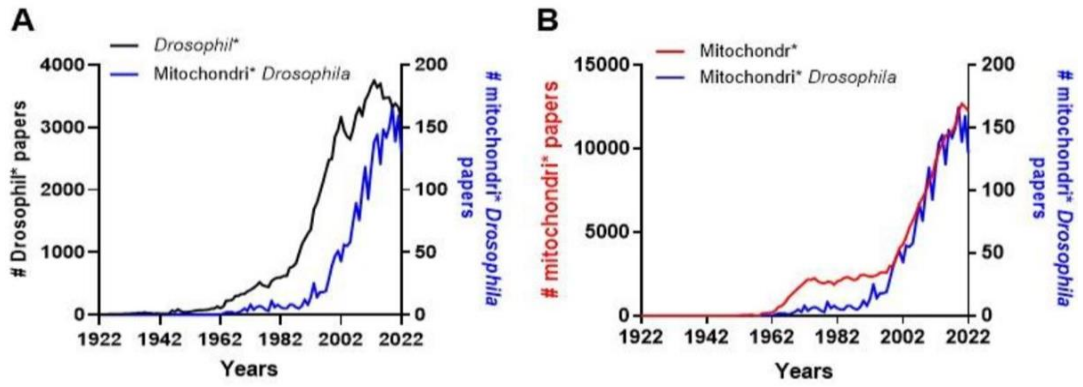

Figure 1: A century of *Drosophila* and mitochondrial metabolism research. (A) Number of original publications using *Drosophila* in all areas (black line) and on *Drosophila* and mitochondria (blue line) from 1922-2022. **(B)** Number of original publications in mitochondria in all areas (red line) 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 organism model for mitochondrial physiology studies ("Rodriguez 2023 BEC - Bioblast," n.d.). The authors established the technical grounds toward the standardization of methodologies routinely used for assessment of mitochondrial physiology of *Drosophila* by fluororespirometry. This is a pioneer 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 Mitopedia database to assess the available methodologies on mitochondrial physiology in *Drosophila*. As expected, they have 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/s/mg) were remarkably lower than in normoxia (~420 pmols O_2/s/mg), suggesting a detrimental effect of increased oxygen supply for *Drosophila* mitochondria. Despite this aspect was not addressed by the authors, it is conceivable that under hyperoxic conditions (even at a short term) might oxidize mitochondrial proteins and compromise the electron flux and respiratory rates (Walker and 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 throughout assessment of potential interferents should be considered when analyzing mitochondrial physiology in this organism model. 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 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 emphasize the need for a 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 organism models to be used in mitochondrial physiology studies in the next future.

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