

We would like to thank all people involved in the scientific and editorial review of our manuscript, especially for the patience as we faced critical moments of the current COVID-19 pandemic during the writing and revision of the work. We have revamped our manuscript along the lines proposed by the reviewers. In the new version, we indicate changes introduced in response to criticisms in red text, with accompanying marginal red boxes numbered according to specific reviewer or editorial comments, as below. We also would like to state that the revision of our manuscript required the intense participation of another member of our lab, Murilo F. Othonicar, whose name has now been removed from the Acknowledgements, and added as a coauthor in this work. We hope our manuscript is now suitable for publication in JoVE.

Editorial and production comments:

E1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

We believe we have proofread all document after revision, and that it should be ready for publication after the reviewers' reevaluation. We have also included a new author in the manuscript.

E2. *Please revise the title for conciseness.*

The title is now under JoVE's guidelines of no more than 150 characters: An affordable and efficient "homemade" platform for *Drosophila* behavioral studies, and an accompanying protocol for larval mitochondrial respirometry

E3. *Please do not provide URLs in the article text. You can cite these URLs as superscripted numbers similar to the references.*

URLs have been moved to the reference list and are now cited with superscripted numbers in the main text.

E4. *The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.*

We believe we have moved everything that is not essential for the protocol to the discussion section. We have, however, left the introductory paragraph of each protocol, as we consider this essential to contextualize the reader.

E5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

We have revised the Protocols section accordingly, and believe it now meets the requirements described here, except perhaps for Protocol 3. This protocol has a distinct nature, so we consider that its current format is very informative to the reader and that should not be altered.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript aims to introduce the use of 3D printing in drosophila research and provides examples of the types of equipment that can be produced. Further it shows how some of the equipment can be used to perform physiology studies on the flies.

We appreciate the comprehensive review provided by reviewer #1. We have edited the manuscript to address every single comment below to the best of our abilities. The 3D design and printing protocols have been improved significantly.

R1.1: My biggest concern is that while I wholeheartedly support the use and expanded use of 3D printing in all types of research, and agree that it can democratize access to equipment and research opportunities, this particular manuscript seems to fall in between the cracks. Its not enough of a guide in terms of the specifics needed for a complete novice that has never done any 3D printing or drosophila work so I worry that it will leave readers confused or not feeling capable of carrying out the presented work, and at the same time for those that do know how to do this and just need the files, all but the respirometry protocols are unnecessary.

In this revised version of the manuscript, we have significantly increased the details in Protocols 1 (3D model design) and 2 (3D printing), and Figures 1 and 2, in order to provide the reader enough guidance in conducting the design and printing of a fly vial rack. We realize that, even after these changes, the protocols might still be challenging for first time users, so we have included a sentence in the beginning of the Protocols and of the Results sections emphasizing that the 3D printing beginners should get acquainted with the different software and their help centers to ensure proper results. We are also counting on the video to provide the reader a more complete guide to the procedures.

R1.2: Making the files available on a common site like Thingiverse would handle the latter, and a lot more would be needed to support the former. That said, if this allows researchers that would otherwise not be thinking of this route to find out about this (and that's not something I convinced will happen), its great. I just wonder if a whole JOVE video is warranted without more basic "how-

to" and explanations of terms etc...(e.g. infill, supports, slicing etc...). Maybe that would be in the final video, it's hard for me to tell.

Please, see response to comment R1.1 above. In addition, we do agree with the reviewer that making our STL files available via Thingiverse would ultimately provide what more advanced 3D printing Drosophilist users need. However, we believe that there is no better way to disseminate scientific content than by providing our files via a thoroughly peer-reviewed scientific publication. Although not infallible, good peer review is the gold standard of science, so we believe the researcher audience we are aiming at will be more trustful if our files are available through the JoVE website.

R1.3) I still do not see where the authors come to the conclusion that its this misunderstanding that is holding back drosophila research. It may be, but it seems like its an unsubstiated guess. I would at least like to see them base it on pers. comm, or other first hand experience if they cannot point to studies or other empirical data.

The reviewer is right that this is pure speculation. To the best of our knowledge, there are no hard data on the matter, but we are planning on conducting an investigation in the future to provide reliable numbers to substantiate our statements. Meanwhile, for the purpose of this manuscript, we rewrote the referred passage of the text to clarify that this is indeed a speculation and that the issue of Drosophila research **in Brazil** being held back might **also** be due to the high costs of importing equipment, which is inherent to any scientific research area in the country. To circumvent the high costs and need for imports, we offer 3D printing protocols for new and established Drosophila researchers.

R1.4) What GMO are they referring to that would be a replacement for Dros.? Seems like a strawman and unnecessary.

We apologize for the confusion. Working with GMOs in Brazil requires authorizations, which can take time to be granted due to bureaucracy (please, see details in the response to comment R1.6), and is therefore a negative aspect of the work, considering that basically all work with Drosophila models of human biology/disease involve the use of transgenic lines. We have now clarified our statement in the manuscript.

R1.5) Could the authors approximate the cost of non-printed equipment to give the reader a feel for the limitation level? Is access to the 3D printing tech cheaper and easier to get (i.e. not imported etc...)

This is a matter of demand and supply. Even though a personal 3D printer is also an imported piece of equipment, it can be purchased in any tech website for anyone in the country to use in a variety of domestic needs (our latest acquisition costed ~R\$1,900.00 – i.e., ~US\$380.00), whereas specialized fly apparatuses are only needed by few lab researchers, aiming at conducting behavioral or other analyses. How reduced the costs of printing and assembling homemade lab

equipment are is classically represented by the FlyPi, which can be built for less than €100.00 and serves as a light and fluorescence microscope able to use opto- and thermogenetic stimulation of the genetically tractable zebrafish, *Drosophila* and nematodes. We have recently received a quote of a basic Zeiss fluorescence microscope for the price of €41,449.11, an amount of money sufficient to pay the salary of two PhD students (or one post-doc) for 4 years. We provide the quote attached to this rebuttal letter and apologize it is only in Portuguese. However, the equipment specifications are easily translatable even without basic knowledge of the language, and the price is clearly shown. To illustrate to the reader of our manuscript how cheap printed equipment can be, we have now included the example of the FlyPi in the introduction.

R1.6) Out of curiosity, since you are using a transgene for the OXPHOS work, does that need permission, or is any fly work is exempt? (since you bring it up earlier).

According to the Brazilian law, all work with GMOs must be authorized by an expert committee, after an evaluation that will determine environmental, animal and human health safety. In the case of GM *Drosophila*, the Local Biosafety Committee of each institution can provide such authorization, given that the National Technical Committee on Biosafety has previously approved the use of particular lab spaces for activities with GMOs. Work with non-GM flies does not require a biosafety authorization. We have now included our authorization numbers in the Acknowledgements section.

R1.7) Line 162: worth noting that access is free with a simple registration

The information has been included.

R1.8) Line 167-: if you are going to go into this amount of detail, do you need to provide info on how to move, align the boxes? i.e., click and drag, and setting the movement scale (i.e. to mm or 0.1mm etc...)?

As part of the response to the reviewer's comment R1.1 (see above), we have included more procedural details in Protocol 1, especially in the new steps 1.6 and 1.7, which should also address this comment sufficiently.

R1.9) If this is really to be an intro to 3D printing, which it appears to be (for people that have done this before) an explanation of what infill or supports are and when they are necessary or not seems to be in order. The use of 100% infill seems unnecessary, just adding to time, materials used and chances of things going wrong. If this is needed, please explain why.

We thank the reviewer for pointing out our mistake. We corrected the manuscript to say "20% infill", as this is standard for our 3D printed pieces. We have also included explanations about infill, supports and other printing parameters in the Discussion section and in the legend to Figure 2.

R1.10) *Line 255-270 I do not think the way "modular" is being used is correct.*

We have excluded this term from steps 3.4 and 3.5 of Protocol 3.

R1.11) *Line 460: I think this is sort of an overstatement in terms of what this manuscript provides.*

Although we have improved Protocols 1 and 2 substantially, we have also rewritten the beginning of the Results section, the Abstract, the Summary and the Introduction to ensure this comment of the reviewer is properly addressed, and that we are not giving the reader the false impression that we are providing solutions for all lab problems with our protocols.

Reviewer #2:

Manuscript Summary:

The manuscript aims to describe simple and affordable protocols for rearing and behavioral studies in Drosophila melanogaster. Secondly, this manuscript describes methods for measuring mitochondrial respiration in Drosophila larvae. Mutant Drosophila strains are used to assess the role of mitochondrial alternative oxidase on larval locomotion and mitochondrial respiration. Overall, this manuscript is well written and very thorough.

The AOX portion of this manuscript is very interesting. I look forward to seeing this work continue in the future.

We truly appreciate the positive comments, and can assure more interesting work using AOX-expressing flies will be available in the near future.

R2.1) *There are some grammatical and type-editing that needs to be done.*

We ourselves have revised the manuscript thoroughly for these errors, and the manuscript has gone through professional scientific English revision, so we hope everything is fixed now.

Reviewer #3:

Manuscript Summary:

The authors described in detail the methods of 3D-printing various instruments used in fly behavior experiments and the method of measuring oxygen consumption in the mitochondria of fruit fly larvae. The article describes step-by-step steps how to use 3D model design software to design models, 3D-printing, at the same time, considering that it is very time-consuming for beginners to use 3D modeling software to design models from beginning, the authors generously provided readers with several designed models files they created for downloading.

The method described in this article is not only suitable for developing countries where materials are relatively scarce, even the simplest experimental instruments (such as funnels) need to be imported, but also suitable for developing countries or developed countries with relatively better economic conditions and relatively abundant resources, as even in these countries, not all research groups have sufficient funds to afford expensive instruments. In addition, the instruments provided by biotechnology companies are not always suitable for the needs of researchers. Researchers sometimes need to improve existing experimental instruments or design and manufacture instruments suitable for their own experiments. The 3D printing described in the article is a better choice.

We are glad to hear the reviewer foresee our protocols being useful in labs in developing and developed countries alike.

R3.1) *It is advisable to use the symbol Φ to indicate the diameter, such as line 294, "90 X 15 mm Petri dishes". Although there are such expressions in the literature, I think it is more reasonable to use $\Phi 90 \times 15$ mm.*

We have included the symbol Φ in every instance needed to indicate diameter measurements.

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