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Measuring and interpreting oxygen consumption rates in whole fly head segment

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Corresponding Author:	Shahaf Peleg Ludwig-Maximilians-Universitat Munchen Medizinische Fakultat Planegg-Martinsried, GERMANY
Corresponding Author's Institution:	Ludwig-Maximilians-Universitat Munchen Medizinische Fakultat
Corresponding Author E-Mail:	shahaf.peleg3@googlemail.com
Order of Authors:	Louisa Jutta Dietz
	Anuroop Venkateswaran Venkatasubramani
	Martin Hrabe de Angelis
	Axel Imhof
	Lore Becker
	Shahaf Peleg
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TITLE:

Measuring and Interpreting Oxygen Consumption Rates in Whole Fly Head Segments

AUTHORS:

Louisa Jutta Dietz[1], Anuroop Venkateswaran Venkatasubramani[1], Martin Hrabe de Angelis[2,3,4], Axel Imhof[1], Lore Becker[2], and Shahaf Peleg[1,5,6*]

[1]Munich Center of Integrated Protein Science and Biomedical Center, Ludwig-Maximilians University of Munich, Planegg-Martinsried, Germany

[2]German Mouse Clinic, Helmholtz Zentrum Munich, German Research Center for Environment and Health (GmbH), Neuherberg, Germany

[3]German Center for Diabetes Research (DZD), Neuherberg, Germany

[4]Chair of Experimental Genetics, School of Life Science Weihenstephan, Technische Universität München, Freising, Germany

[5]Laboratory for metabolism and epigenetics in brain aging, Institute of Neuroregeneration & Neurorehabilitation, Qingdao, China

[6]Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Louisa Jutta Dietz
dietzlouisa@t-online.de

Anuroop Venkateswaran Venkatasubramani
Anuroop.Venkatasubramani@bmc.med.lmu.de

Martin Hrabe de Angelis
hrabe@helmholtz-muenchen.de

Axel Imhof
imhof@lmu.de

Lore Becker
lore.becker@helmholtz-muenchen.de

Shahaf Peleg
shahafpeleg3@gmail.com

CORRESPONDING AUTHOR:

Shahaf Peleg

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SUMMARY:

Measuring alterations in metabolic rates is central to understanding the progression of various diseases and aging. Here, we present a novel technique to measure whole head oxygen consumption that more closely resembles the physiological state and may aid in revealing novel drugs that modify mitochondrial activity.

ABSTRACT:

Regulated metabolic activity is essential for the normal functioning of living cells. Indeed, altered metabolic activity is causally linked with the progression of cancer, diabetes, neurodegeneration, and aging to name a few. For instance, changes in mitochondrial activity, the cell's metabolic powerhouse, have been characterized in many such diseases. Generally, the oxygen consumption rates of mitochondria were considered a reliable readout of mitochondrial activity and measurements in some of these studies were based on isolated mitochondria or cells. However, such conditions may not represent the complexity of a whole tissue. Recently, we have developed a novel method that enables the dynamic measurement of oxygen consumption rates from whole isolated fly heads. By utilizing this method, we have recorded lower oxygen consumption rates of the whole head segment in young *versus* aged flies. Secondly, we have discovered that lysine deacetylase inhibitors rapidly alter the oxygen consumption in the whole head. Our novel technique may therefore aid in uncovering new properties of various drugs, which may impact metabolic rates. Furthermore, our method may give a better understanding of metabolic behavior in an experimental setup that more closely resembles physiological states.

INTRODUCTION:

Regulated metabolic activity is essential for the survival of cells and healthy function of a tissue. Deregulated metabolic activity has been extensively shown to be linked to the onset and progression of various maladies[1]. For example, lower metabolic activity was previously described in neurodegenerative diseases such as Alzheimer's and age-associated memory impairment[2,3]. Furthermore, mitochondrial dysfunction is believed to be causally involved in the aging process[4,5]. On the other hand, higher mitochondrial and metabolic rates were described in cancer cells[6], where the use of mitochondrial inhibitors reduced tumorigenesis[7].

One readout of metabolic activity is the oxygen consumption rate (OCR) of mitochondria. Interestingly, this type of readout is primarily obtained from isolated mitochondria or cells, thus the majority of what is described in the literature is mainly based on a readout that does not resemble the physiological state. However, there are several drawbacks to this technique. First, the protocol of mitochondrial isolation can potentially damage its integrity[8], which may be a relevant artefact when comparing mitochondria isolated from young *versus* older tissues[9]. Furthermore, the isolation process is long and may result in loss of relevant protein posttranslational modifications which regulate mitochondrial function[9-11]. Moreover, it has been shown that isolated mitochondria do not consistently represent whole tissue metabolic rates[12,13]. Such cellular biological complexity could be viewed as, 'the whole is greater than the

sum of its parts', *i.e.,* mitochondria may display different metabolic rates inside a complex cell compared with their metabolic rate when isolated.

While cells may offer a better OCR readout than isolated mitochondria, cell to cell communication in the context of a whole tissue may be lost. For example, in the brain, the metabolic activity of neurons is highly dependent on the metabolic activity of neighboring glial cells[14]. As such, establishing new techniques to investigate OCR in whole tissue or whole organisms may prove more insightful for the onset and progression of various disorders.

Recently, new techniques have emerged to address these issues and enable the measurement of OCR from whole tissue, segment, or living organisms. For example, a recent work reported the oxygen measurement from a beetle flight muscle by using a permeabilized fiber approach with a respirometer[15]. New machines for micro-respirometry allow the measurement of OCR of pancreatic islets[16,17]. Consequently, it has been reported that this technology enables the measurement of OCR from whole worms[18] and Zebra fish[19]. However, the presence of the digestive barrier may pose a challenge for testing various drugs in the context of OCR alterations. Interestingly, recent reports by Neville and colleagues have shown a new technique for measuring single drosophila larva brain with the well plate[20,21].

In this study, we have used a similar setup to enable the measurement of whole OCR from whole living and non-mobile Drosophila[22]. This technique also offers a secondary advantage in measuring the impact of various drugs on metabolic activity in a whole segment, without having to pass through the digestive system barrier[13,22]. For example, it was previously demonstrated that direct injection of lysine deacetylase inhibitor (KDACi), a drug believed to alter epigenetic mechanism in the brain, resulted in an improved memories formation[23]. However, by using our novel technique, we discovered that KDAC inhibition resulted in a rapid increase of OCR, which may be a contributing factor by itself in the neuronal activity. Our protocol provides a simple and novel method to assess the impact of various drugs, genetic manipulation, or physiological states (disease, aging) on OCR in the context of a whole head.

PROTOCOL:

1. Instrument Preparation

Note: For this experiment, we have used a Seahorse XF24 device with "islet plates". The operation of the technique uses different cycles of mixing, waiting and measurements as well as the possibility to add substances to the measurement compartment.

1.1. Turn on the machine well before the start of the experiment so that there is adequate time to reach the desired temperature and remain stable.

1.2. In the software setup (administration mode), choose the length of the cartridge calibration (here, 20 min was chosen) and the desired temperature.

Note: While mitochondria or mammalian tissue measurements are typically carried out at 37 °C, fly head ambient temperature is 25 °C but results of measurements at 31 °C are published. We used 31 °C since this is the lowest temperature setting for the device at room temperature. To reach temperatures of 25 °C or lower, place the machine in a colder room or at 11 °C as recently published[21].

1.3. In the software, use the following protocol: 3 min mixing – 2 min waiting – 2 min measuring. Depending on the experimental design, add injection steps from ports A-D after a chosen measurement step.

1.3.1. For quality check, and determination of basal OCR, wait for at least three measurement cycles before injecting the first drug *via* port A. For a detailed timeline of the protocol, please see reference Becker *et al.*[13].

2. Cartridge Preparation

2.1. Pre-calibrate the cartridge a day (or at least 4 h) prior to testing. Add 1.0 mL of Calibrant (pH 7.4) to each well and place the sensor cartridge on top of the plate and store at 37 °C without CO_2 for overnight or up to 72 h. Prevent the evaporation of cartridge with parafilm if it is being hydrated for more than 24 h.

2.2. Ensure that the experimental drugs are well dissolved in the medium (fresh medium + 2.5% glucose) before the start of the experiment.

2.3. Measure and adjust the pH of the drug solution to the pH of the vehicle at the desired temperature to avoid any pH difference during drug injection.

2.4. Pipet the drug solution to its allocated injection port. For example, use 77 µL for port A to achieve a 1:10 dilution in a 770 µL solution and subsequently 85 µL for port B.

2.5. Load the cartridge into the machine and begin calibration.

3. Plate Preparation

Note: It is strongly recommended that two people prepare the plate simultaneously. The duration of one plate preparation per two people may require ~45-60 minutes.

3.1. Adjust the freshly prepared media + 2.5% glucose to the desired pH with 1 N HCl. Make sure that the pH is not affected by changes in temperature.

3.2. Prepare an ice box and place a metal plate on the ice.

3.3. Open the islet plate (the 24-well plate) package and immerse the nets in a Petri dish (92 mm x 16 mm) with media.

3.4. Collect one net with the inserter (a small instrument that places the net firmly in the well) and have the inserter stand up next to the microscope. Add a small drop of media to the net attached to the inserter.

3.5. Anesthetize the flies (one week or 4 weeks old canton males were used here) by placing the flies on the ice-cold metal plate.

3.6. Using forceps, grab the abdomen of a fly and immerse it in the media in a Petri dish under the microscope.

3.7. Using a second pair of forceps, gently remove the head of the fly. Place it in the middle of the net attached to the inserter and verify that the head is immersed in media.

3.8. Center the heads when there are 16 of them on the net. Remove superfluous fluid before centering the heads to prevent loss of heads while placing them in the well.

Note: 16 fly heads were used as this number gave sufficient and stable data within a reasonable time of plate preparation during method establishment.

3.9. Using the inserter, place the net in the well. Ensure that the heads are trapped under the net. Slowly add 700 µL of media + 2.5% glucose (**Figure 1**). Repeat the process for each of the wells.

Note: 20 wells of fly head samples and 4 empty wells for background calibration per plate is recommended. Make sure that empty wells also contain a net with 700 µL of the buffer + 2.5% glucose.

3.10. Check the wells for air bubbles under the nets *via* the microscope. Pipet gently up and down using a 1 mL pipet to remove any bubbles. Keep the heads centered for a reliable OCR reading.

3.11. Add the plate to the machine and start the measurement.

4. Analysis of the OCR Measurements

4.1. At the end of the protocol, remove the cartridge.

4.2. As a quality check, observe any visible leftovers in the port fillings. Discard the cartridge and plate (Option 1) if the heads are not to be used for protein extraction, *e.g.,* (see Option 2).

4.3. Extract the spreadsheet files and quality check each well for oxygen and pH levels. Make sure that the background wells show no OCR and that oxygen levels are stable.

4.3.1. Use an algorithm for data analysis, some of which can be chosen in the respective software. Use the AKOS algorithm for OCR values[2] if the range of oxygen levels during the entire measurement, between the first and last tick (= sub-measurement) are similar between two biological samples and the oxygen levels of the last ticks are not lower than 95 (mmHg) (the heads' OCR is markedly lower at this oxygen level), (**Figure 2**).

4.3.2. Some conditions will cause the sample to generate a rapid OCR and may display lower oxygen levels during the 1st tick and/or in the last tick (anoxia) (**Figure 3A**). In such a scenario, use an alternate measurement method such as the FIXED algorithm. In anoxia, the OCR is greatly reduced due to low levels of oxygen in the solution. As such, the AKOS algorithm yields misleading readings.

Note: The newer machine lacks the FIXED algorithm. Therefore, it is preferred to extract the total oxygen levels and plot the rate per time for the first 3-5 ticks (**Figure 3**).

5. (Option 2) Biochemical Analysis of the Head Segment

5.1. To measure the biochemical (metabolites, proteome, *etc.*) properties of a head segment, adjust the run time to the requirement; however, it is possible to abort the protocol at any time and remove the plate.

5.2. Once the plate is removed, use non-sharpened forceps to make a hole in the net and remove it thereby releasing the heads to float.

5.3. Using a 1 mL pipet with a cut tip and transfer the heads to a vial.

5.4. Quickly discard the buffer and snap-freeze the heads in liquid nitrogen. Store the heads at -80 °C for future analysis.

REPRESENTATIVE RESULTS:

The ability to record high quality OCR measurement relies on centering the head in the middle of the net (**Figure 1**). This is important for the XF24 machine, which has a rather small oxygen sensor spot compared to the newer XFe24 machine in which the sensor is larger. As previously shown, centering the heads display a steady OCR for at least 20 consecutive measurements in young flies[13].

One critical aspect of using the machines is to apply the correct analysis. It is recommended to check the oxygen levels during the experiments. Each 2 min measurement is subdivided into 10 sub-measurements (ticks). A well with 16 healthy heads usually displays an oxygen partial pressure (pO_2) of 140-170 (mmHg) for the first tick. In the first example, we compared young vs. midlife fly heads (**Figures 2A and 2B**). While the oxygen levels drop quicker in the middle-aged heads, the observed difference is small (**Figure 2A**). Moreover, the range of the oxygen levels is similar between the conditions, with 165 during the first tick to 120 during the last tick. In such a case, it is preferable to use the AKOS algorithm to automatically generate the OCR $(pmol/min)^2$, which reliably mirrors the oxygen level drop between young *versus* midlife heads (**Figure 2B**). Of note, the analysis program by the machine automatically chooses the AKOS algorithm.

However, based on our observations, automatically using the AKOS algorithm may give misleading, if not opposite results for the correct OCR. Such artefacts can be generated in conditions of a highly consuming sample which reaches anoxia[13,22]. For example, the addition of sodium butyrate (SB), a KDAC inhibitor, transiently changes the dynamics of the oxygen levels (**Figure 3A**). Whereas the vehicle controls display steady levels of oxygen during the first and last ticks, SB addition causes a considerable and transient drop of the oxygen levels in these ticks (**Figure 3A**). SB by itself does not alter the oxygen levels in the background wells, where no heads are added (data not shown). The data supports the notion that SB increases oxygen consumption. As the collection of first tick is delayed (12 seconds until the first tick is recorded in the measuring phase) the first data point is already lower in the SB treated wells. Therefore, it is difficult to capture the early changes in oxygen consumption following the addition of this HDAC inhibitor. Furthermore, the oxygen levels in the SB treated samples are reduced to already low levels (anoxia) as indicated by the collection of the last ticks. At anoxia, the heads slow down their oxygen consumption in the last ticks (**Figure 3A**). Because the AKOS calculation takes into account all ticks and ignores an anoxic state, it generates a misleading OCR. Indeed, the non-normalized AKOS based OCR levels show little change upon the injection (dashed line) of port A (Veh/SB) (**Figure 3B**).

Normalizing the OCR levels to the pre-injection measurement based on the AKOS reveals very similar levels of OCR before and after the injection of port A, which does not support the oxygen level changes (**Figure 3A**). Under these circumstances, the FIXED algorithm, which more closely models/resembles the OCR and oxygen level changes is recommended (**Figure 3C**).

Consequently, the FIXED algorithm based normalized measurement reveals an increased OCR upon SB treatment (**Figure 3C**).

A drawback with the new machine is the absence of the FIXED algorithm. Therefore, in experiments where highly consuming sample/treatment is used, it is recommended to calculate the OCR measurements manually, and calculating the decrease in oxygen level per time for the first 3-5 ticks in each measurement.

Tables and Figures:

Figure 1. An example of a well containing 16 one-week old heads of male flies. The heads are centered below a net and floating in the media.

Figure 2. A representative example of OCR measurement comparison between one-week old fly heads (young) and four-week old fly heads (middle-age). (A) The oxygen levels are shown for three separate measurements; each 2 min measurement is subdivided into ten sub-measurements (ticks). (B) A quantification of (A). The levels of the first and last ticks are similar, although the levels of the middle-age sample are slightly lower. A quantification of the slope of the decrease in oxygen levels is used to generate the OCR levels. As previously described[22], the OCR of middle aged flies is 10%-15% higher than young flies.

Figure 3. An example of the alteration of OCR by sodium butyrate (SB) in young fly heads. (A) Oxygen levels recorded from seven measurements following the addition of 15 mM SB. The dashed line marks the injection of the drug (or vehicle) from port A. Of note, while the oxygen levels of ticks 1 and 10 remain stable in the control group (Blue), the levels of oxygen during these ticks are transiently (six measurements following the injection) reduced in the SB treated samples (orange). In addition, the decrease in oxygen levels is greatly reduced during the last ticks of SB treated samples. N=3 per group (B) [Left] Non-normalized OCR levels calculated from the AKOS algorithm. The calculation incorrectly shows similar levels of OCR before and after the injection of SB by port A. [Right] Normalization of the OCR to the measurement prior to the injection of port A. (C) [Left] 'Fixed' algorithm calculation of (A) showing the non-normalized OCR. Here, the OCR closely represents the transient increase in oxygen usage of the heads upon SB treatment; [Right] Normalization of the OCR to the measurement prior to the injection of port A. Error bars indicate the S.E.M. in all the graphs.

DISCUSSION:

Our new technique offers a novel approach to study metabolic changes in aging and disease in the context of whole fly head segments[22]. The method can also be suited to study the impact of KDAC sodium butyrate on oxygen consumption. As we have demonstrated, lysine deacetlyase inhibitors (HDACs/KDACs) result in OCR changes. Essentially, as the targets of such inhibitors are normally not localized in the mitochondria (these inhibitors do not impact the Class III

deacetylases, the Sirtuins)[24], such drugs could only be tested on an at least tissue level. Indeed, various drugs are injected directly to the brain, thus bypassing possible processing/modification/inactivation by the digestive system. As such, our technique offers novel insight into how such drugs directly impact the head segment.

There are several critical steps. First, as stated in the protocol, we highly recommend preparing a plate under one hour, with two pairs of hands preparing the plate. From our experience, the quality and stability of the OCR measurements are better when prepared in a timely manner. When taking too long, the occurrence of low OCR consuming wells is increasing, as well as shorter duration of stable OCR. Second, it is important to conduct a quality check and ensure that the experimental conditions between various samples are similar (pH, oxygen levels). Finally, a critical step is choosing the correct algorithm to analyze the samples. As we have demonstrated, the default AKOS algorithm yielded a misleading and sometimes opposing calculation in samples that consumed oxygen at high rates[13]. We therefore stress the importance of checking the raw data for oxygen levels and comparing the resulting OCR.

Currently, there are several limitations with this technique. At room temperature, the machine heats up to 31 °C (this is the minimal measuring temperature while the machine is at room temperature), which may represent a stress state for the fly heads[25]. This however can be overcome by placing the machine in a cold room, which will enable measurements at 25 °C and hence without a possible heat stress to the fly heads. Recent report has demonstrated placing the machine at 11 °C, thus enabling the OCR recording of flies at 25 °C[21]. Nevertheless, the fly head separation should be performed at room temperature. Furthermore, temperature fluctuations make it challenging to control pH changes and therefore it is highly recommended to test the impact of physiological conditions/drugs on OCR by using similar experimental setups. In addition, the contribution of oxygen consumption by non-mitochondrial-independent mechanisms has not yet been established[26]. By using various respiratory inhibitors that are efficient in fly heads, it would be possible to establish such non-mitochondrial oxygen consumption rates.

It is noteworthy that various mammalian maladies are characterized by alterations in energy metabolism. Among them are diseases that are characterized by either metabolic reduction such as Alzheimer's disease or metabolic rewiring such as cancer. Interestingly, KDAC inhibitors are used for both Alzheimer's disease and cancer treatment[27]. While the precise mechanisms by which KDAC inhibitors are achieving the therapeutic aspect remain unclear, the data from our technique supports the novel notion that such inhibitors may modulate metabolism.

In summary, this method is valuable for measuring overall oxygen consumption rates *in vivo* and more accurately displays drug effects on general metabolism, which may be overlooked in isolated mitochondria protocols[12]. For example, results obtained from this method, rather than previous techniques, have implicated novel insights for age-associated metabolic inflexibility

upon KDAC treatment. While additional work is necessary to optimize the experimental conditions for fly heads, the combination of our technique and suitable analysis may lead to further elucidation of the mitochondrial activity in the context of whole living tissues.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

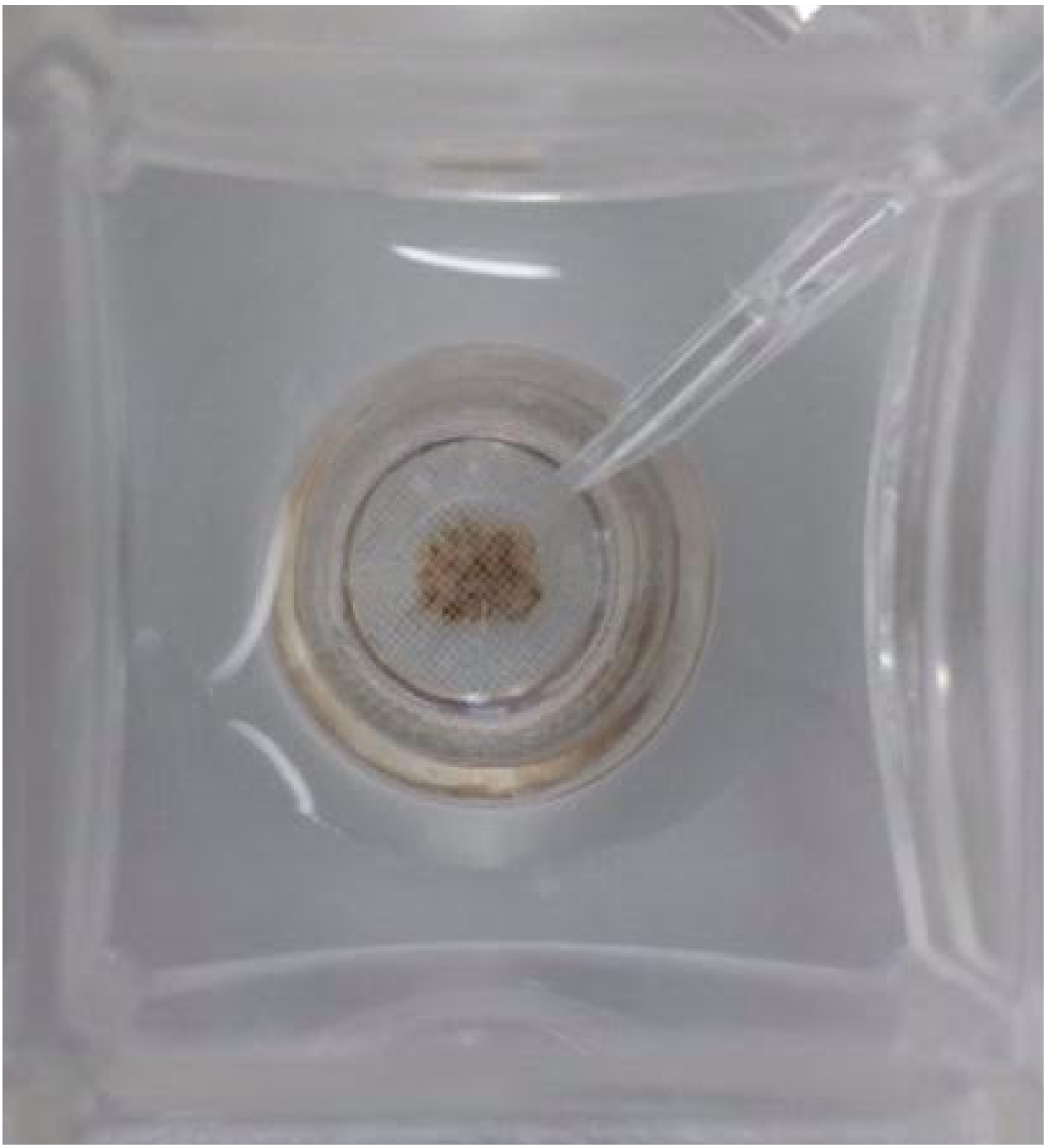
REFERENCES:

1. Wallace, D. C. Mitochondrial diseases in man and mouse. *Science (New York, N.Y.)* **283** (5407), 1482–1488 (1999).
2. Gerencser, A. A., *et al.* Quantitative microplate-based respirometry with correction for oxygen diffusion. *Analytical chemistry* **81** (16), 6868–6878, doi:10.1021/ac900881z (2009).
3. Cunnane, S., *et al.* Brain fuel metabolism, aging, and Alzheimer's disease. *Nutrition (Burbank, Los Angeles County, Calif.)* **27** (1), 3–20, doi:10.1016/j.nut.2010.07.021 (2011).
4. Wang, Y. & Hekimi, S. Mitochondrial dysfunction and longevity in animals: Untangling the knot. *Science (New York, N.Y.)* **350** (6265), 1204–1207, doi:10.1126/science.aac4357 (2015).
5. Wallace, D. C., Fan, W. & Procaccio, V. Mitochondrial energetics and therapeutics. *Annual review of pathology* **5**, 297–348, doi:10.1146/annurev.pathol.4.110807.092314 (2010).
6. Zhang, X., *et al.* Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. *Nature communications* **5**, 3295, doi:10.1038/ncomms4295 (2014).
7. Wheaton, W. W., *et al.* Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *eLife* **3**, e02242 (2014).
8. Picard, M., *et al.* Mitochondrial structure and function are disrupted by standard isolation methods. *PloS one* **6** (3), e18317, doi:10.1371/journal.pone.0018317 (2011).
9. Baker, D. J. & Peleg, S. Biphasic Modeling of Mitochondrial Metabolism Dysregulation during Aging. *Trends in biochemical sciences* **42** (9), 702–711, doi:10.1016/j.tibs.2017.06.005 (2017).
10. Zhao, S., *et al.* Regulation of cellular metabolism by protein lysine acetylation. *Science (New York, N.Y.)* **327** (5968), 1000–1004, doi:10.1126/science.1179689 (2010).

11. Baeza, J., Smallegan, M. J. & Denu, J. M. Mechanisms and Dynamics of Protein Acetylation in Mitochondria. *Trends in biochemical sciences* **41** (3), 231–244, doi:10.1016/j.tibs.2015.12.006 (2016).
12. Picard, M., *et al.* Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. *Aging cell* **9** (6), 1032–1046, doi:10.1111/j.1474-9726.2010.00628.x (2010).
13. Becker, L., Nogueira, M. S., Klima, C., de Angelis, M. H. & Peleg, S. Rapid and transient oxygen consumption increase following acute HDAC/KDAC inhibition in Drosophila tissue. *Scientific reports* **8** (1), 4199, doi:10.1038/s41598-018-22674-2 (2018).
14. Volkenhoff, A., *et al.* Glial Glycolysis Is Essential for Neuronal Survival in Drosophila. *Cell metabolism* **22** (3), 437–447, doi:10.1016/j.cmet.2015.07.006 (2015).
15. Newell, C. Kane, C. L., Kane, D. A., Mitochondrial substrate specificity in beetle flight muscle: assessing respiratory oxygen flux in small samples from Dermestes maculatus and Tenebrio molitor. *Physiological Entomology* **41**, 96–102 (2016).
16. Rogers, G. W., *et al.* High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PloS one* **6** (7), e21746, doi:10.1371/journal.pone.0021746 (2011).
17. Wikstrom, J. D., *et al.* A novel high-throughput assay for islet respiration reveals uncoupling of rodent and human islets. *PloS one* **7** (5), e33023, doi:10.1371/journal.pone.0033023 (2012).
18. Koopman, M., *et al.* A screening-based platform for the assessment of cellular respiration in Caenorhabditis elegans. *Nature protocols* **11** (10), 1798–1816, doi:10.1038/nprot.2016.106 (2016).
19. Kumar, M. G., *et al.* Altered Glycolysis and Mitochondrial Respiration in a Zebrafish Model of Dravet Syndrome. *eNeuro* **3** (2), doi:10.1523/ENEURO.0008-16.2016 (2016).
20. Neville, K. E., *et al.* A novel *ex vivo* method for measuring whole brain metabolism in model systems. *Journal of neuroscience methods* **296**, 32–43, doi:10.1016/j.jneumeth.2017.12.020 (2018).
21. Neville, K. E., *et al.* Metabolic Analysis of *Drosophila melanogaster* Larval and Adult Brains. *Journal of visualized experiments : JoVE* **138** (2018).
22. Peleg, S., *et al.* Life span extension by targeting a link between metabolism and histone acetylation in Drosophila. *EMBO reports* **17** (3), 455–469, doi:10.15252/embr.201541132 (2016).
23. Peleg, S., *et al.* Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science (New York, N.Y.)* **328** (5979), 753–756, doi:10.1126/science.1186088 (2010).
24. Drazic, A., Myklebust, L. M., Ree, R. & Arnesen, T. The world of protein acetylation. *Biochimica et biophysica acta* **1864** (10), 1372–1401, doi:10.1016/j.bbapap.2016.06.007 (2016).
25. Miquel, J., Lundgren, P. R., Bensch, K. G. & Atlan, H. Effects of temperature on the life span, vitality and fine structure of Drosophila melanogaster. *Mechanisms of ageing and development* **5** (5), 347–370 (1976).

26. Banh, R. S., *et al.* PTP1B controls non-mitochondrial oxygen consumption by regulating RNF213 to promote tumour survival during hypoxia. *Nature cell biology* **18** (7), 803–813, doi:10.1038/ncb3376 (2016).
27. Falkenberg, K. J. & Johnstone, R. W. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nature reviews. Drug discovery* **13** (9), 673–691, doi:10.1038/nrd4360 (2014).

(Figure 1)



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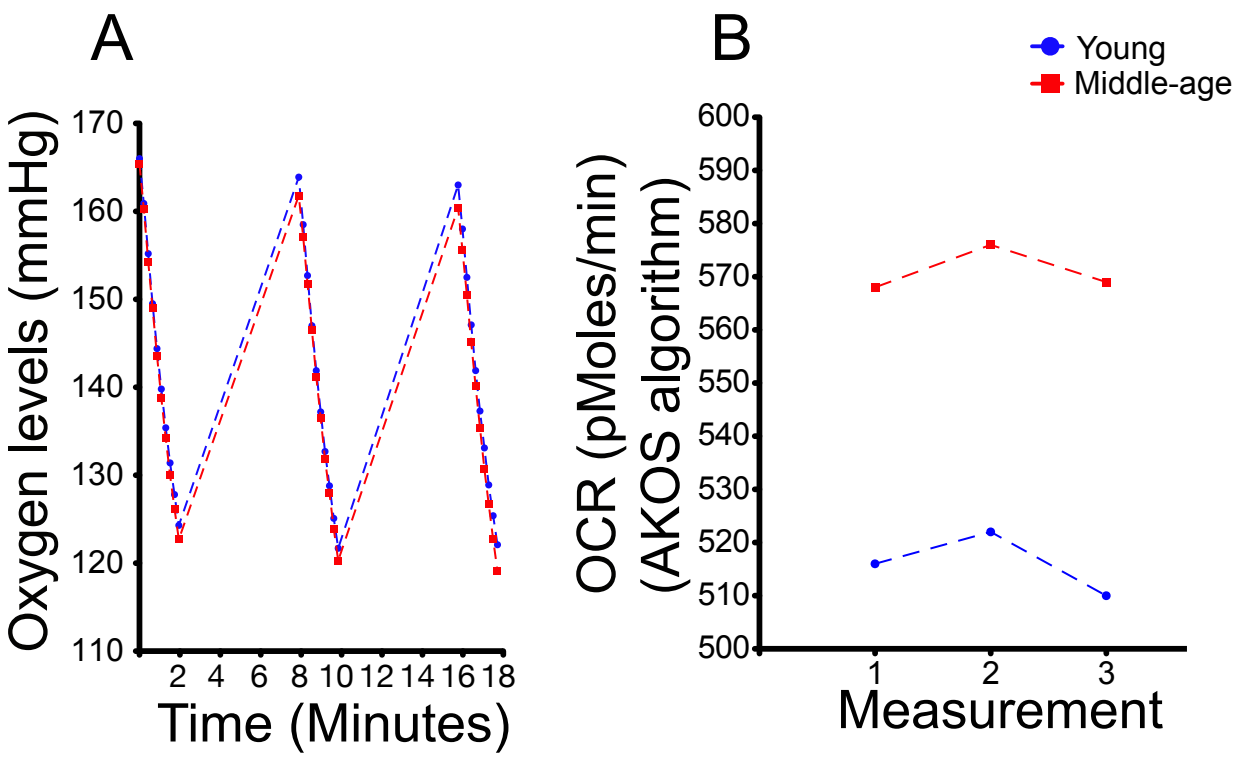

Figure 3

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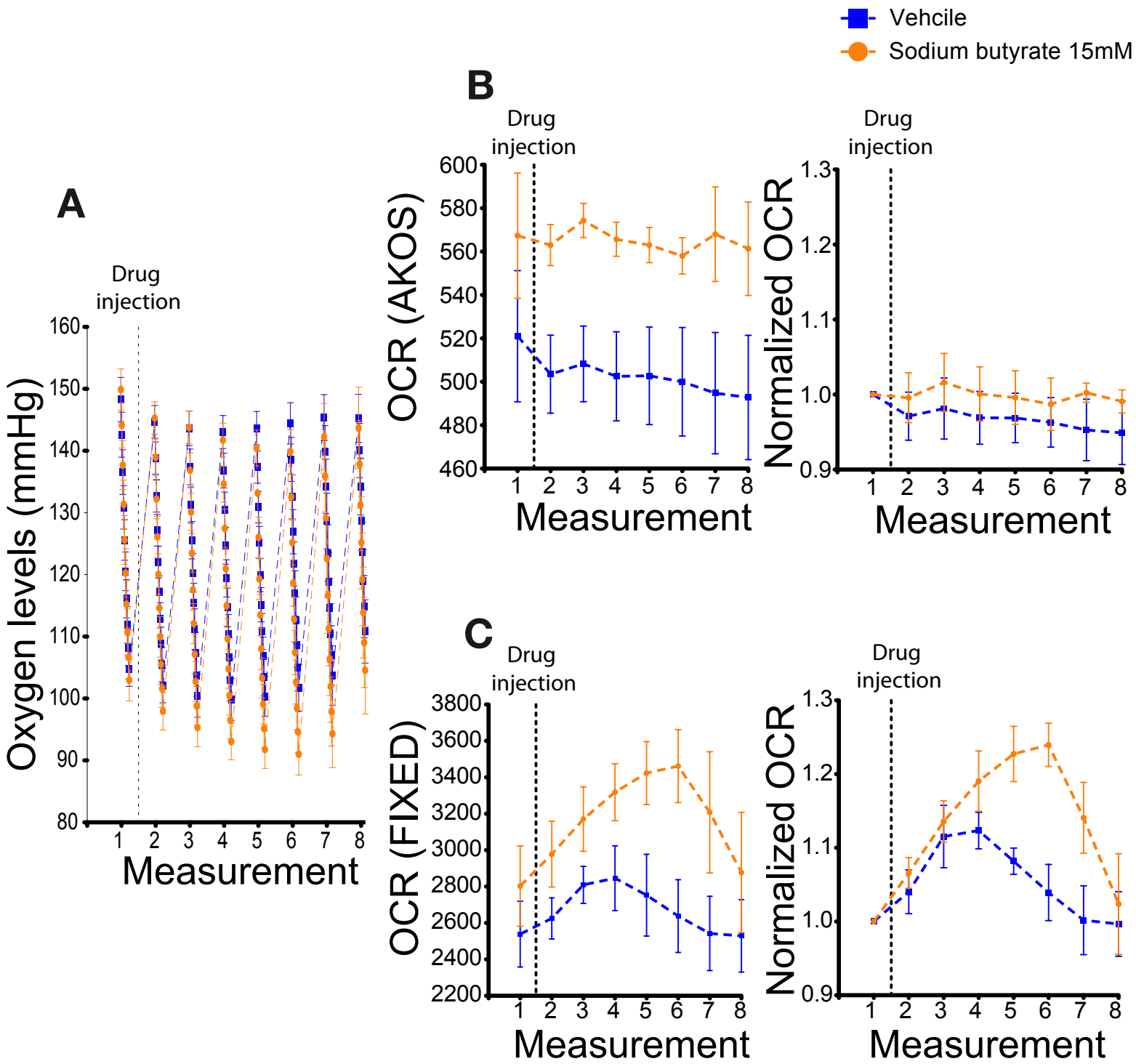

Table of Materials

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Glucose	Sigma-Aldrich	G8644	D-(+)-Glucose solution 100 g/L in H2O, sterile-filtered
XF assay Medium	Agilent	103575-100	Seahorse XF DMEM Medium, pH 7.4
Sodium butyrate	Merck	817500	Dissolved in XF assay buffer
Seahorse XF24/e24 analyzer	Agilent		
XF24/e24 Extracellular Assay Kit	Agilent	100850-001	Cartridge
XF24/e24 Islet Capture Microplates	Agilent	101122-100	Plate
Seahorse Capture Screen Insert Tool	Agilent	101135-10	Insertor
Petri dish	Sarstedt	821,472	Petri dish 92 x 16 mm

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CORRESPONDING AUTHOR

Name:	Shahaf Peleg
Department:	Metabolism and Epigenetics in Brain ageing
Institution:	Institute of Neuroregeneration and Neurohabilitation, QDU
Title:	Prof.
Signature:	
Date:	15.6.18

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Point to point response

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

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

The manuscript has been thoroughly checked for spelling and grammar issues.

2. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

We have uploaded the signed ALA.

3. Keywords: Please provide at least 6 keywords or phrases.

We have now added the keyword Drosophila, and changed ‘tissue’ to ‘head’.

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have rephrased the short abstract accordingly.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

We have gone throughout the manuscript and changed it accordingly.

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

We have gone throughout the manuscript and changed it accordingly.

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We have gone throughout the manuscript and reduced the frequency we used the term, “seahorse” accordingly.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The protocol has been revised accordingly.

9. Please revise the protocol to contain only action items that direct th.e reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

The sentences have been changed to the imperative tense.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

We have made adjustments to the protocol without dropping essential steps.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. For example:

3.2: What is used to adjust pH?

We have now added this information to the manuscript.

3.3: How large is the petri dish?

We have now added this information to the manuscript.

3.6: Please specify the age, gender and strain of the flies. How many flies are used?

We have now added this information to the manuscript.

12. **After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of**

the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have now highlighted the protocol part.

13. Figure 3: Please define error bars in the figure legend.

We have now added this information to the manuscript.

14. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

We have added the essential materials in xls file.

Reviewers' comments:

We would like to thank the referees for their encouraging and positive criticism. Below, we outline our responses to each individual point and the respective revisions that we have made to the manuscript.

Reviewer #1:

Manuscript Summary:

Dietz et al. describe a novel method to measure oxygen consumption rates in whole fly heads using the Seahorse XF24/XFe24. The authors compare aged to young flies and examine the use of the HDAC inhibitor sodium butyrate (SB) to alter oxygen consumption rates. This research method is interesting as it provides an alternate method of assessing oxygen consumption rates compared to the conventional isolated mitochondria techniques, and the Authors speculate that this method may help identify novel drug targets. However, there are some questions and suggestions that should be tended to prior to publication.

We thank the author for his/her positive overview of our manuscript. Reading throughout all the reviewer's comments, we wish to thank the reviewer for the critical reading and constructive suggestions to improve clarity.

Major Concerns:
The authors state directly in their introduction that they have the "first recorded opposing results of oxygen consumption rates in isolated mitochondria versus whole head tissue in young versus aged flies". This statement and comparison is however very confusing and

misleading since it appears to the Reviewer that there are no measurements comparing isolated mitochondria to whole head tissue in this manuscript.

We have removed this sentence from the abstract. Indeed, we only mentioned that we have previously compared the readouts obtained from isolated mitochondria protocols to whole head tissue protocols. We apologize for this misleading statement.

A primary aspect of the present manuscript is concluding that using whole fly head tissue enables "dynamic measurement of oxygen consumption rates". This Reviewer finds this statement difficult to conclude since the only measurement of oxygen consumption is the single OCR measure when using only the Seahorse Media and glucose.

In our statement, ‘dynamic measurement of oxygen consumption rates’, we have suggested that because the heads display stable OCR for several hours, such setup may be suitable for dynamic OCR changes upon drug injection. That is especially true, as demonstrated, when testing the impact of KDAC, which is transient by nature.

In order to convince researchers that this method can be used to measure mitochondrial and non-mitochondrial oxygen consumption, to the degree stated, it is suggested that a protocol examining mitochondrial respiration (at least) be completed and shown. Ideally comparing the whole head method to isolated mitochondria to validate that this method is sound.

Furthermore, it is directly stated that "By using various respiratory inhibitors that are efficient in fly heads, it is possible to establish such non-mitochondrial oxygen consumption rates." This is however unsubstantiated by the results/data presented, and as proposed in the comment above, should be completed to demonstrate the validity of the proposed method.

One drawback of this technique is that it is difficult to measure and determine the non-mitochondrial oxygen consumption. Apparently, the fly heads are resilient to some of the classical respiratory chain inhibitors. An additional concern is that some of the inhibitors do not readily diffuse efficiently to the cells in the whole head in a timely manner.

We have previously shown the rotenone treatment induced a 30% reduction after 20 measurements. However, a mixture of several inhibitors could only achieved a 60% OCR reduction after 30 measurements, which is very different from classical experiments in isolated mitochondria where almost all the OCR is rapidly abolished. Significantly more work is required to accurately determine the non-mitochondrial OCR, which is beyond the scope of this

manuscript. We have expressed our concern and referred to this potential drawback in our manuscript:

'Currently there are several limitations with this technique…, the contribution of oxygen consumption by non-mitochondrial-independent mechanisms has not yet been established[20]'

Minor Concerns:

Line 93 - Please explain why this is interesting.

We have now clarified that it is interesting because a lot of scientific evidence is based on readouts which are non-physiological. We have now added an explanation:

'.., thus making the majority of what is described in the literature based on a readout that does not resemble physiological state.'

Lines 103-107 - The authors fail to mention the permeabilized fibre technique as another method which examines oxygen consumption. Furthermore, this technique has been used in small samples from insects in the past (Physiological Etymology 2016).

We thank the reviewer for pointing out a relevant work that we were not aware of. We have now included and cited this work accordingly.

'Interestingly, a recent work reported the oxygen measurement from a beetle flight muscle by using a permeabilized fibre approach with a Oxygraph-2k (Oroboros Instrument)'

Lines 96-98 - A reference is required here, otherwise this statement is speculation.

We have added suitable citations discussing the reversible nature of protein acetylation.

Lines 99-102 - Language is colloquial, should be concise and scientific.

This has been modified.

Line 134 - Please explain why 31 degrees was selected.

Typically ambient temperature for Drosophila would be 25 °C. The machine we used is placed at room temperature without air condition. However, the machine itself generates heat. As such, the lowest temperature we could

measure in our setup was 31 degrees. We agree that this temperature may impact OCR as it may induce a heat shock-like response. We recommend, if possible, to place the machine in a colder room and set the temperature to 25 degrees. We address this point in the text:

'While mitochondria or tissue measurements are typically measured at 37 °C, we have used 31 °C for fly heads 31 °C was the lowest temperature setting for the XF24 at room temperature). To reach lower temperatures of 25 °C or lower, place the machine in a cooled room. '

Line 183 - Please explain why the fly heads are required to be face down.

We apologize for the mistake in this sentence. We have now changed it:

'Using the inserter, place the net in the well. The heads should be trapped under the net, thus ensuring the heads cannot freely move in the rest of the well during the measurements.'

Is it required to have 16 heads per well? How was this determined? Can you investigate individual insects? How do you account for pooling effects?

We thank the reviewer for bringing up this point. The number 16 was chosen after optimization and as 16 heads cover the majority of the net area (Figure 1), thus ensuring good coverage of the whole net area. With the older XF24 machine, it was important to cover the whole net area to get reliable results. We suspect that this is because of the small O2 sensor in the older machine version – thus, 16 heads ensures that the heads are located directly below the sensor. However, it is possible that fewer fly heads are needed in the new XFe24 machine, because of its larger O2 sensor. In our experimental setup, heads are pooled together.

Line 205 - Please be more specific (1st and 10th ticks).

We have rearranged this line for better clarity:

'4.2.1 We recommend using the OCR values of the AKOS algorithm{Gerencser:2009hn} if:

The range of oxygen levels (During the entire measurement, between the first and last tick) are similar between two biological samples

The oxygen levels of the last ticks are not lower than 95 (mmHg) (the heads' OCR is markedly slower at this oxygen level), (Figure 2). '

Lines 250-258 - This section is very difficult to read, and the readers

could benefit if it were re-written to highlight that SB increases oxygen consumption and that there is a delay in the time to collect the first data point. Therefore, it is difficult to capture the change in oxygen consumption following the addition of this HDAC inhibitor.

We agree that this was a difficult to follow section. We strove to clarify and have made the following changes:

'The data supports the notion that SB increases oxygen consumption. As the collection of first tick is delayed (12 seconds) the first data point is already lower in the SB treated wells. Therefore, it is difficult to capture the early changes in oxygen consumption following the addition of this HDAC inhibitor. Furthermore, the oxygen levels in the SB treated samples are reduced to such low levels (anoxia) as indicated by the collection of the last ticks. At anoxia, the heads slow down their oxygen consumption in the last ticks (Figure 3A). Because the AKOS calculation takes into account all ticks and ignores an anoxic state, it generates a misleading OCR. Indeed, the non-normalized AKOS based OCR levels show little change upon the injection (dashed line) of port A (Veh/SB) (Figure 3B). '

Lines 278-279 - Where is the previous mention of the change in OCR between groups?

We apologize for omitting the correct citation and have added it accordingly.

Lines 297-298 - Only one inhibitor was measured, please change this.

We have changed it accordingly.

It also enables examination of impact of the KDAC sodium butyrate on oxygen consumption

Lines 306-307 - Please explain what "quality of plates" and "measurements are better" means here.

We intended to state that making a plate in a timely manner generates more stable OCR reads from the well during the experimental run. We apologize for this mistake:

'From our experience, the quality and stability of the OCR measurements are better when prepared in a timely manner. If too much time is taken, the occurrence of low OCR consuming wells increases, as well as the time duration of stable OCR.'

Line 315 - Please provide a citation corroborating this.

We have now included the following citation to show how temperature impacts oxygen consumption and longevity:

Effects of temperature on the life span, vitality and fine structure of Drosophila melanogaster (Miquel1976)

Lines 315-316 - Did the Authors perform measurements with the XF24 in a "cold room"? Data should be presented to make this statement true.

We apologize for the lack of clarity. We did not perform any measurements in a cold room. However, we suggest that placing the XF24 in a colder room will enable one to measure OCR at 25 degrees. That was recently demonstrated for the XF 96 (Neville, 2018 JOVE)

'Currently there are several limitations with this technique. First, at room temperature the XF24 heats up to above 30 C (this is the minimal measuring temperature while the machine is at room temperature), which may represent a stress state for the fly heads. We propose that placing the XF24 machine in a colder room will enable measurements at 25 C decreasing the possibility of heat stress to the fly heads.'

Lines 324-326 - Please let the Reviewer understand what you are trying to convey here.

We are trying to convey that by using this novel technique, insight into physiological changes in metabolism, as observed during the aging process – in particular, the reduced response in OCR flexibility in aged tissue upon KDAC inhibition can be gained. We have now changed these lines accordingly:

'For example, results obtained from this method, rather than previous techniques, has implicated novel insights for age-associated metabolic inflexibility upon KDAC treatment.'

Figures 2 and 3 - Both sets of figures are misleading by not having a 0 value on the y axis. Secondly, the axes for the graphs showing 'oxygen levels' are different between figures 2 and 3 - please make the same.

We thank the reviewer for point out the difficulties with this graph. Indeed, the graph only focuses on specific values, rather than starting at 0, as we wished to convey the differences between young and old (Figure 2) or AKSO vs FIXED algorithm analysis (Figure 3). We believe, that focusing on the relevant graph area is sufficient to illustrate the differences between the analysis type. Unlike our previous work, which focused on differences in OCR between age/drug treatment, here we wished to focus on determining the correct analysis type and their drawbacks. Moreover, we believe that the normalized

analysis on the right panels of Figure 3 are sufficient to give the reader a clear idea as to the real percentage difference between the vehicle and KDACi treatment.

Reviewer #2:

Manuscript Summary:

The authors describe an interesting method for measuring oxygen consumption in Drosophila head tissue using a Seahorse XF24 or XFe24. They illustrate differences between the Seahorse's AKOS and FIXED algorithms and indicate that the AKOS method is misleading when oxygen consumption rates are high. This method is valuable because it provides a way to examine drug effects on primarily brain tissue metabolism in vivo. However, it does not differentiate between mitochondrial oxygen consumption and non-mitochondrial oxygen consumption. The paper was well written, although I have provided some points requiring clarification under "minor concerns".

We thank the reviewer for his/her comments. We completely agree that we are yet to differentiate between mitochondrial and non-mitochondrial oxygen consumption, which is a major drawback for the current technique.

Major Concerns:

1. It is not accurate for the authors to state that this method is an alternative to isolated mitochondria experiments (line 324) since there are several respiring tissues and non-mitochondrial oxygen consumption occurring in this model. Rather, this method is valuable for measuring overall oxygen consumption rates in vivo and more accurately displays drug effects on general metabolism.

We agree that without determining the non-mitochondrial respiration, it is difficult to state that this new approach is an alternative to isolated mitochondria experiments. We therefore changed it accordingly:

In summary, this method is valuable for measuring overall oxygen consumption rates in vivo and more accurately displays drug effects on general metabolism, which may be overlooked in isolated mitochondria protocols

2. What are the implications of stored air in air sacs of the Drosophila head for this method? Since the trachea and air sacs are hydrophobic, they likely remain air-filled during the procedure. See WRITTEN, J.M. (2006). The Post-embryonic Development of the Tracheal System in Drosophila melanogaster.

This is an interesting point raised by the reviewer. We assume the reviewer meant the following work:
The Post-embryonic Development of the Tracheal System in *Drosophila melanogaster,* JOAN M. WHITTEN. Journal of Cell Science 1957 s3-98: 123-150;

We are not sure what the implications of such possible air in the air sacs are. However, our data supports the notion that the oxygen levels markedly decrease during a measurement (micro chamber) and are only restored to the starting levels when the wells are mixed. As such, we assume that the impact of possibly stored air is minimal.

Minor Concerns:

Line 134: Why was 31 degrees chosen? Please indicate the reasoning since this is higher than typical rearing temperatures for adult Drosophila and therefore can impact OCR.

We thank the reviewer for addressing this point. The machine we use is placed at room temperature. However, the machine itself generates heat. As such, the lowest temperature we could measure in this setup was 31 degrees. We do agree that this may impact OCR as it may induce a heat shock-like response. We recommend, if possible, to place the machine in a cold room and then set the temperature to 25 degrees. We address this point in the text:

'While mitochondria or tissue measurements are typically measured at 37 °C *, we have used 31* °C *for fly heads (31* °C *is the lowest temperature setting for the XF24 at room temperature). To reach lower temperatures of 25* °C *or lower, place the machine in a cooled room. '*

Line 174: Are flies placed in a refrigerator beforehand. If so, for how long? How is the fly maintained on the plate?

The flies are kept at room temperature before the experiment. In order to handle them, we anesthetize them by placing the flies on an ice-cold metal plate. We believe a video visualization will clarify this procedure further.

Line 236: What is meant by 140-170 "levels" of oxygen (mmHg)? I think here and on the axis of the figures the authors mean PO2 (mmHg)

The levels are indeed the oxygen partial pressure in mmHg.

Figure 2 legend, are these three measurements for the young and middle-age fly heads means of all 20 wells? If so, can error bars be included as in figure 3? Also, can the authors verify the cause of the increased O2 between measurements? Is the chamber opened to the atmosphere at this point?

The data in Figure 2 is a representative data from n=1. A much larger cohort comparing young vs middle was previously described (Peleg et al 2016). Here we wished to focus on determining the correct analysis, rather than emphasizing the aging phenotype. Hence there are no error bars.

We cannot verify the cause of the increase between measurements. We would like to point out that the increase is very minor, and thus we speculate that such an increase may result from the machine sensitivity range. The chamber is not opened to the atmosphere during the procedure. Rather, the solution is mixed between the measurements. It is possible, however, that some amount of oxygen leaks. In fact, the AKOS algorithm was first developed to take into account possible oxygen leakage (Gerencser et al 2009)

Figure 3: It is difficult to see the control data below the SB treated samples in orange. One solution might be to expand panel A horizontally and reduce the thickness of the orange line.

Indeed, it was difficult to detect the control data. As suggested by the reviewer, we have reduced the thickness of both lines. The resulting graph is clearer now and the control data is more visible.

Figure 3, line 288: "The calculation incorrectly shows similar levels of OCR before and after the injection of port A (veh vs. SB)." This statement is confusing to me. First, can the authors please verify if the dotted vertical line is the timepoint at which injection took place? This was not described in the legends. Second, I do not understand what this statement is comparing. Do you mean the OCR for each treatment is the same after injection as it was before injection? This does appear to be true. Or, do you mean that the two treatments are the same compared to one another (which does not appear to be true)? Can the authors please add more detail about which levels of OCR are the same (which points are you referring to)?

We would like to thank the reviewer for pointing this issue out.
Firstly, the vertical dotted line is indeed the addition of Veh vs. SB. We have now added this information to the figure and the legend:

'The dashed line marks the injection of the drug (or vehicle) from port A.'

Secondly, we always compare the OCR of each individual well, just before the drug/vehicle addition (always normalized as '1'), to the OCR measurements following the drug addition (after the dashed line) in that very same well. Based on Figure 3A, it is apparent that the levels of OCR do not change in the vehicle group. However, the OCR appears to transiently increase in the SB treated group (before and after the dashed line). We wished to illustrate that the AKOS analysis does not represent the change in the SB treated samples – probably due to the artifact of anoxia during the final ticks.

Please clarify why the absolute OCR values in Figure 3 B and C (left) are so different from one another (5X larger in C compared to B)? Is this a function of the AKOS v. FIXED algorithm calculations or are there more, or larger, fly heads in the wells in C?

We thank the reviewer for asking for this clarification. The heads and the experiment is identical, but AKOS and FIXED are two different algorithms used to analyze the identical data.
We asked the company about the discrepancy in the algorithms and their outputs and we received the following answer:

The AKOS algorithm takes into account back diffusion of oxygen through plastic, and the dimensions of the measurement chamber. It is the most accurate way to determine O2 consumption in the XF system, which is why we recommended you use this.
The Fixed algorithm (not supported) assumes a linear O2 depletion during the measurement time, works well when there is a lot of oxygen consumed. The Fixed algorithm dont take into account any backdiffusion or non-linearity in the measurement chamber. It assumes the oxygen consumption is linear all the time over 3 min, no plateau. Therefore much higher.

Because of the back diffusion calculations the OCR values will be very different between the two. Not a problem as long as your experiments are looking at comparing 2 conditions, and not trying to report actual, quantitative OCR.

The AKOS paper:

https://www.ncbi.nlm.nih.gov/pubmed/19555051

More description in the introduction or discussion about the relevance of Drosophila head tissue compared to other mammalian model tissues would strengthen the paper.

We thank the reviewer for his/her suggestion. We have now added the following to our discussion:

It is noteworthy that various mammalian maladies are characterized by metabolic alterations. Among them are diseases characterized by either metabolic reduction such as Alzheimer's diease or metabolic rewiring such as as cancer. Interestingly, KDAC inhibitors are used in both Alzheimer's diease and cancer treatment{Falkenberg:2014eu}. While the previous mechanisms through which KDAC inhibitors achieve their therapeutic benefits remain unclear, the data from our technique supports the novel notion that such inhibitors may modulate metabolism.

Lines 298-301: Can the authors elaborate on this statement: "As we

have demonstrated, lysine deacetlylase inhibitors (HDACs/KDACs) result in OCR changes. Essentially, as the targets of such inhibitors are normally not localized in the mitochondria (these inhibitors do not impact the Class III detacetylases, the Sirtuins)19, such drugs could only be tested with our method." Why is this finding new and how does it compare to other studies? Why can this drug only be tested with your method?

Isolated mitochondria lack Class I and II KDACs as they are washed away during the mitochondria isolation process. These KDACs are the target of the sodium butyrate inhibitor. Thus, the role of these KDAC or the KDAC inhibitors in modulating OCR cannot be evaluated in isolated mitochondria. Our approach allows for maintenance of intact cells/head and therefore provides insight into the role of non-mitochondrial KDAC in modulating OCR.

Reviewer #3:

Manuscript Summary:

This manuscript provides some additional detail on the methods used by this group to measure the oxygen consumption of whole Drosophila heads published in Becker, L., et al. (2018). "Rapid and transient oxygen consumption increase following acute HDAC/KDAC inhibition in Drosophila tissue." Scientific Reports 8(1): 4199 and in Peleg et al. (2016). "Life span extension by targeting a link between metabolism and histone acetylation in Drosophila." EMBO Reports 17(3): 455-469. Specific additional details not previously published include the pH's used for the incubation media, the use of the "islet plate package" to contain the heads, the position of the heads, and the need to eliminate air bubbles.

It is somewhat debatable whether there is additional new information added here justifies a publication in JOVE. The additional detail provided does not appreciably add to the ability of a researcher to utilize the technique. Fig. 1 is not particularly informative, and Fig. 2 is simply the raw data from Fig. 2A and B in the Becker 2018 paper. Also, step 3.5 is difficult to follow. That being said, the step-by-step description here will speed the efforts of new labs to adopt this technique, so this publication will be useful in that regard.

We would like to thank the reviewer for expressing his/her concerns. We do agree that the previous publications have already presented the results from this technique. Nonetheless, we believe that this manuscript will make it easier for fellow researchers to follow the protocol and assist them in establishing the protocol themselves. We believe that the protocol, accompanied by the video, will facilitate other labs to learn and implement the techniques portrayed in our protocol. Furthermore, this manuscript helps clarify considerable concerns regarding the automatic analysis algorithm AKOS which calculates the OCR. We believe it is crucial to choose the right OCR algorithm/analysis to accurately interpret the experimental outcome. We

believe that our previous publications did not sufficiently highlight the algorithm issue properly and believe that reading our JOVE manuscript will provide a better guide to the correct analysis.

Regarding step 3.5, and perhaps several other steps, we think that JOVE provides an important opportunity to observe these steps via video, as they are not commonly used biochemical methods. From our own personal experience, we had required demonstrations by company representatives (Seahorse/Agilent) in order to successfully carry out this protocol and conduct experiments.

Major Concerns:

Lines 61-3 (and other places within the manuscript): The abstract and paper refers to measurements of the metabolic rates of tissues, but the head is not a tissue, it is body segment which contains multiple tissues including the brain, muscles, salivary glands, etc.

We are grateful for this comment, which we realized was a mistake we made not only in this manuscript, but also in previous ones. Indeed, we have now corrected this throughout the manuscript and referred to the head as a body segment, or just as the head. We have removed the word ‘tissue’ when inappropriate.

Lines 66-74: This is excessive, somewhat dubious rationalization for the abstract of this study. Condense.

This has been modified accordingly.

Lines 76-77: Give the result of the comparison of the mitochondria and whole heads; don't just say you made the measurement.

We have changed it accordingly.

Lines 92-102: In addition to the technical issues discussed here, metabolic rates in vivo depend on what the organism is doing. Relevant to the fly head, metabolic rates likely depend on the degree of neuronal activity and the activity of the mandibular muscles.

We thank the reviewer for this comment. We would appreciate a concrete suggestion on how to incorporate this aspect to the introduction.

Line 167: What is the islet plate package?

The islet plate package is the seahorse 24 well plate that we used for measuring OCR for the heads. We have added this information to the text.

Line 171, step 3.5. This step is difficult to understand and follow.

We have now clarified this step and believe that the JOVE video will clarify this part of the method:

3.3 Open the islet plate (the 24-well plate) package and immerse the nets in a petri dish (92 x 16 mm) with media .

3.4 Collect one one net with the inserter (a small instrument that places the net firmly in the well) and have the inserter stand up next to the microscope. Add a small drop of media to the net attached to the inserter.

Minor Concerns:

Line 71: diseases is mis-spelled

We apologize for the spelling mistake. We have reviewed themanuscript and corrected the spelling errors.

Line 109: Capitalize Agilent. Also, since the Seahorse company has been purchased by Agilent, it would be good to revise this sentence. Perhaps something like, "For example, new machines for micro-respirometry (Seahorse instruments, Agilent, Inc.) allow the measurement of…"

As suggested by the reviewer, we have corrected it accordingly.

Line 115: Capitalize Drosophila

We have made the change.