ASSESSMENT OF MITOCHONDRIAL ENERGETICS IN A SKELETAL MUSCLE RESISTANT TO HYPOXIA.

by

Serena Mooney

A Senior Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

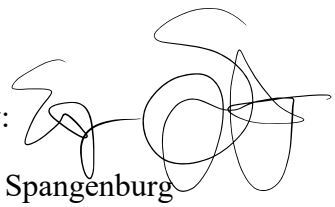
by

Serena Mooney

Greenville, NC

May 8, 2020

Approved by:



Dr. Espen E. Spangenburg

Department of Physiology, The Brody School of Medicine

Abstract

Skeletal muscle is dependent upon oxygen (O_2) to maintain normal physiological function by acting as the final electron acceptor in the mitochondria during ATP production. Loss of O_2 tension (i.e. hypoxia) leads to the collapse of the mitochondria resulting in myopathy development that is seen in various health conditions including peripheral arterial disease and dystrophy. Hypoxia induces a severe muscle pathology that can lead to complete loss of function ultimately contributing to increased mortality. We recently identified a mammalian muscle, the flexor digitorum brevis (FDB), that functions without O_2. No other muscle has the unique ability of the FDB, and it suggests that the FDB is not dependent upon mitochondria to function. To test this possibility, we compared mitochondrial bioenergetic function of two different mouse peripheral skeletal muscles, the extensor digitorum longus (EDL) and flexor digitorum brevis (FDB). Initially, we assessed mitochondrial energetics using permeabilized muscle fiber bundles (PMFB) from adult mice which allows us to measure mitochondrial function without disruption of the reticular organelle structure. We found that PMFBs from the FDB exhibit significantly lower mitochondrial O_2 respiration compared to the PMFBs EDL muscle. To confirm this finding, we sought to make these measures in isolated mitochondria. Due to the small size of the muscles, we developed a new approach for isolating mitochondria. EDL and FDB muscles were removed from adult mice and mitochondria were isolated from each muscle. Mitochondrial O_2 respiration and membrane potential were measured using an approach that mimics energetic conditions in a physiological manner. The data confirmed a lower mitochondrial respiratory capacity in the FDB compared to EDL even under normalized conditions. Further, when compared to the EDL muscle the data suggest that mitochondria within the FDB are poorly developed and are unlikely capable of preventing a significant energetic stress. These data

appear to confirm that unlike any other mammalian skeletal muscle, the FDB has evolved to operate without oxygen or mitochondria and is reliant on alternative metabolic pathways to provide ATP for the muscle. Future studies will seek to determine the metabolic pathways the FDB utilizes to provide energy for physiological function.

Introduction

Mitochondria are the principle site of energy conversion in eukaryotes and regulate many critical cellular processes for skeletal muscle physiology. They play central roles in muscle cell metabolism, energy supply, the regulation of energy-sensitive signaling pathways, reactive oxygen species (ROS) production/signaling, calcium homeostasis and the regulation of apoptosis (Brookes et al., 2004). Oxidative phosphorylation, one of the most important roles, is carried out by the electron transport chain. Electron transport is a series of oxidation-reduction (redox) reactions where electrons are passed rapidly from one complex to the next, to the endpoint of the chain where the electrons reduce oxygen, producing ATP and water. Three of the four protein complexes (Complexes I, III, IV) serve as proton pumps to maintain the electrochemical gradient that drives the redox reactions. Metabolic substrates (pyruvate, malate, succinate, etc.) are catabolized creating reducing equivalents (NADH or FADH) that are utilized by either complex I or II to create an electrochemical gradient resulting in a measurable membrane potential that is indicative of mitochondrial function.

The number of mitochondria per cell varies widely depending on the cell's energy requirements. Skeletal muscle cells need energy in the form of ATP to do mechanical work and maintain basal metabolic function, both of which are necessary for life of the organism. Thus, a higher number of mitochondria are present so that the muscle cells energy requirement is met allowing it to fulfill its specific function. Moreover, every muscle contains a mixture of distinct fiber types that allow the muscle to specialize based on the work they perform. Accordingly, each fiber type contains different mitochondrial volumes. Oxidative or "slow-twitch" fibers have high mitochondrial content and therefore increased reliance on oxidative phosphorylation. These are found in postural muscles such as the soleus (Mishra et al., 2015). In contrast, glycolytic or

"fast-twitch" fibers have lower mitochondrial content and therefore are thought to have decreased dependence on oxidative phosphorylation. They are found in larger muscle groups, such as the quadriceps, and are used for power and movement (Mishra et al., 2015). However, it is unclear how dependent the fast muscles are on mitochondria. We recently assessed this question in the lab and found that when comparing extensor digitorium brevis (EDL) muscle, a fast-twitch muscle, to the soleus muscle that both muscles were equally dependent upon their mitochondria. Specifically, inhibiting use of the mitochondria caused the muscle to die regardless of its fiber-type.

Dogma tells us that oxygen and mitochondria are an absolute requirement for mammalian muscle cell/tissue survival. We have recently discovered a mouse skeletal muscle that is remarkably and seemingly uniquely resistant to ischemia in that both force production and energy charge are maintained differently than all other tested peripheral skeletal muscles. Irrespective of the muscle cell type, mitochondria play a necessary role in meeting the energetic (i.e. ATP) demand of the cell. Even though the FDB does not depend upon O_2 to meet its energetic demand, the muscle still requires ATP for survival therefore it must have unique energetic systems that allow the muscle to function. We hypothesized that the ischemia resistant FDB has inherently unique mitochondria compared to other peripheral skeletal muscles in mice.

Methodology

Mitochondria Isolation

Differential centrifugation was used to isolate mitochondria from the FDB and EDL muscles of adult c57Bl/6NJ mice. Muscles were then minced in ice-cold PBS+EDTA (10mM) and added to trypsin (0.05%) for 5 minutes on ice. Samples were centrifuged at 300 x G for 5 minutes at 4°C. Muscle pellets were re-suspended in KMEM + BSA (KCl 100mM, MOPS

50mM, EGTA 1mM, MgSO4 5mM, BSA 0.2%) and homogenized via a Teflon pestle and borosilicate glass vessel. Muscle homogenates were then centrifuged at 600 x G for 10 minutes at 4°C. Supernatants were be filtered through a 40μm cell strainer and centrifuged at 10,000 x G for 10 minutes at 4°C. Mitochondrial pellets were then washed with KMEM (KCl 100mM, MOPS 50mM, EGTA 1mM, MgSO4 5mM), transferred to microcentrifuge tubes, and centrifuged at 10,000 x G for 10 minutes at 4°C. Supernatants were discarded and mitochondrial pellets were re-suspended in 100 μL of KMEM. Protein concentrations were determined via the Pierce BCA protein assay.

PMFB Preparation

The protocol for preparing permeabilized FDB and EDL muscle fiber bundles was adapted from previously described methods on the permeabilization of red gastrocnemius muscle bundles from Lark et al. and is outlined below (Lark et al., 2016). The FDB and EDL muscles were dissected and immediately added to ice-cold Buffer X (K_2EGTA 7.23mM, CaK_2EGTA 2.77mM, imidazole 20mM, taurine 20mM, ATP 5.7mM, 14.3 phosphocreatine 14.3mM, $MgCl_2 \cdot 6H_2O$ 6.56mM, MES 50mM, pH 7.1, 295 mosmol/kgH2O). Muscles were then subjected to hypoxic or normoxic conditions for one, two, or three hours. Using a dissecting microscope, connective tissue, fat, and blood vessels were removed carefully to avoid muscle loss. FDBs were cut into bundles and divided into groups of three to four bundles weighing 1.5-2.0 mg wet weight. EDLs were kept as a single bundle weighing 4.0-5.5 mg wet weight. FDB bundle groups were then permeabilized in Buffer X containing 22.5 μg/ml saponin with continuous rotation at 4 °C for 5 minutes. EDL bundle groups were permeabilized for longer at 30 minutes. Both muscle bundles were promptly transferred to ice-cold Buffer Z (k-MES 105mM, KCl 30mM, KH2PO4

10mM, MgCl2-6H2O 5mM, BSA 0.5mg/mL, pH 7.1) and washed with continuous rotation at 4 °C for 15 minutes.

Mitochondrial Respiration

Mitochondrial respiration was measured via high-resolution respirometry using the OROBOROS Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria). Mitochondrial membrane potential and redox status was measured using a QuantaMaster Spectrofluorometer (QM-400; Horiba Scientific). All experiments were conducted at 37°C using isolated mitochondria suspended in Buffer Z, containing 5mM creatine monohydrate, 5mM ATP, 20U/mL creatine kinase, and 1mM phosphocreatine.

Redox and Membrane Potential

Fluorometer experiments were supplemented with 0.2uM TMRM. All experiments were assessed under four substrate conditions 1) Pyruvate 5mM/Malate 2.5mM, 2) Glutamate 10mM/Malate 2.5mM, 3) Palmitoyl carnitine 16uM/Malate 2.5mM, 4) Rotenone 5mM/Succinate 10mM. Sequential additions of phosphocreatine (6, 11, 16, 21mM) were made to slow respiratory activity. Mitochondrial membrane potential was measured using the ratio of the following excitation/emission parameters [Ex/Em, (572/590)/(548/590)]. Redox state was assessed via detection of NAD(P)H using excitation/emission parameters of 340/450.

PMFB Respiration

PMFBs were used to test mitochondria in their inherent structure using the OROBOROS Oxygraph-2K as previously described. Experiments were conducted at 37°C with a starting oxygen concentration of ~300-350μM in Buffer Z, containing 5mM creatine monohydrate and 25μM blebbistatin. Mitochondrial respiration was assessed using the same four substrate conditions as previously described. Mitochondrial membrane integrity was confirmed by the

addition of exogenous cytochrome c. Muscle fiber bundles were then rinsed in distilled H_2O, freeze-dried (Labconco, Kansas City, MO) and weighed (Orion Cahn C-35, Thermo Electron, Beverly, MA). O_2 consumption rates were normalized to dry weight and converted to pmol/sec/mg dry wt O_2. All chemicals and reagents were purchased from Sigma Aldrich.

Results

Comparison of Isolated Mitochondrial Respiration and Membrane Potential between the FDB and EDL

Data show consistent trends for both respiration and membrane potential across all three substrate conditions. Using isolated PMFBs, the FDB has a lower mitochondrial respiratory capacity compared to the EDL when assessing oxygen consumption across a range of ATP-free energy (ΔG_{ATP}) (Fig. 1).

Collectively, when isolated mitochondrial fractions were assessed, the FDB exhibited lower O_2 consumption across all the ΔG_{ATP} tested when compared to the EDL (Fig. 2 A, D, G). In addition, the FDB membrane potential (ψ) was significantly lower in the FDB compared to the EDL across all substrates tested (Fig 2 B, E, H). Thus, regardless of the metabolic substrate delivered to the mitochondria the O_2 consumption and membrane potential were reduced.

The lower respiratory conductance in the FDB, while in the presence of a depolarized mitochondrial membrane potential suggests that the FDB has a lower capacity of proton pump activity in the electron transport system and/or lower capacity of dehydrogenase enzyme activity responsible for generating the reducing charge when compared to the EDL (Fig 2 B, E, H). This relationship is visualized when plotting the mitochondrial oxygen consumption against the membrane potential where it is clear that the FDB exhibits a lower oxidative phosphorylation potential compared to the EDL regardless of the metabolic substrate (Fig. 2 C, F, I).

Comparison of PMFB Mitochondrial Respiration between the FDB and EDL

Mitochondrial respiration rates under the glutamate/malate substrate condition were consistently higher in EDL compared to FDB in PMFBs as well (Fig. 1). The magnitude of the difference in respiration between and FDB muscle was dependent on the corrective method employed. Correcting to muscle fiber freeze dried weight demonstrated the greatest relative difference in respiration between the two muscles. This confirms the respiratory data shown from the isolated mitochondria.

Figure 1

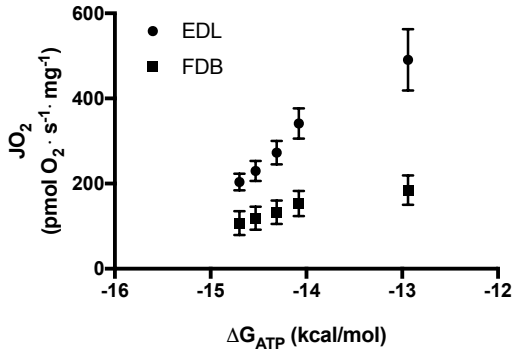


Figure 1. Comparison of PMFB Mitochondrial Respiration between the FDB and EDL. Relationship between mitochondrial oxygen consumption and ATP-free energy under the glutamate and malate substrate condition.

Figure 2

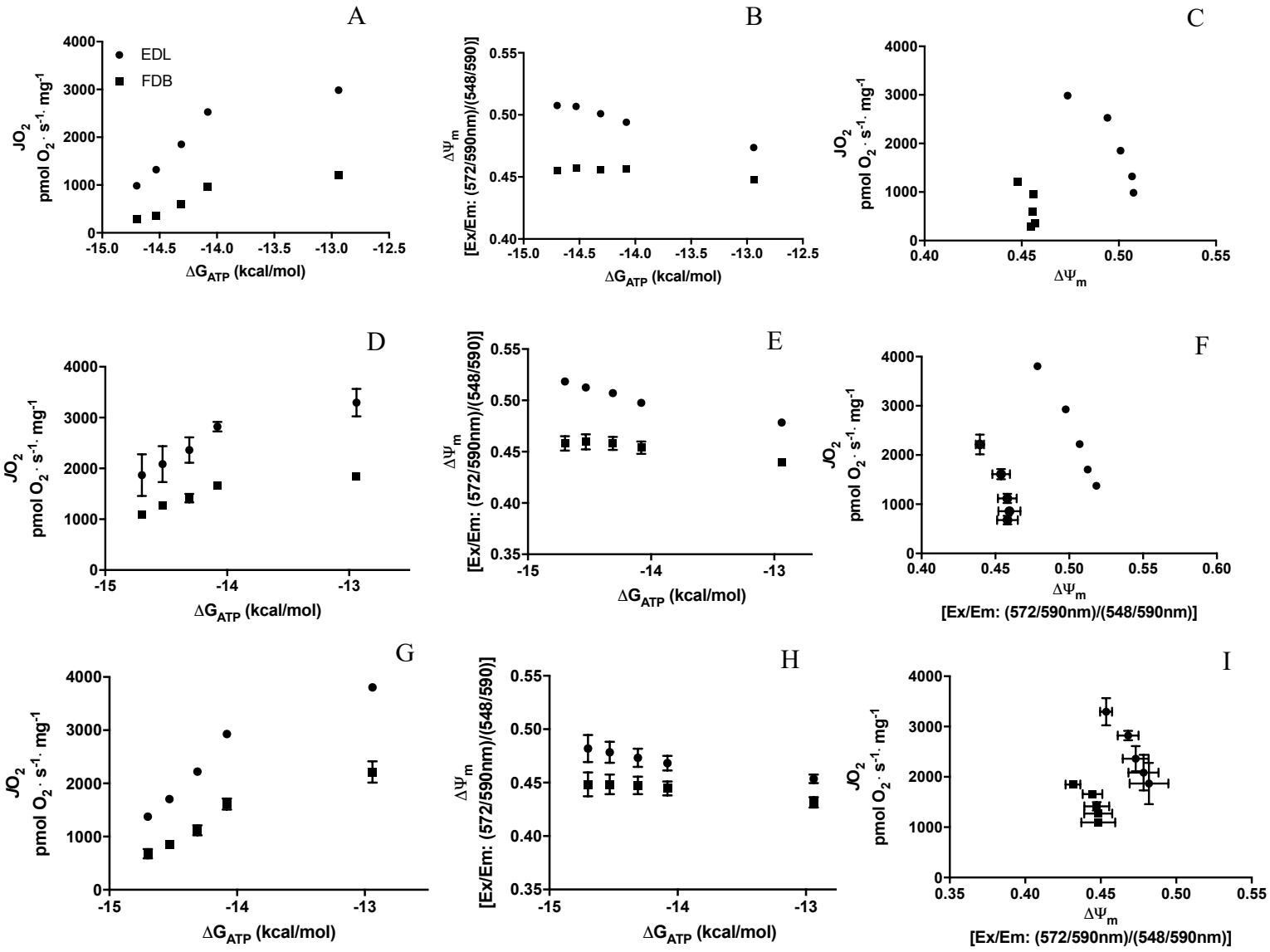


Figure 2. Comparison of Isolated Mitochondrial Respiration and Membrane Potential between the FDB and EDL. (A) Relationship between mitochondrial oxygen consumption and ATP-free energy under the pyruvate and malate substrate condition. (B) Relationship between mitochondrial membrane potential and ATP-free energy under the pyruvate and malate substrate condition. (C) Relationship between mitochondrial oxygen consumption and membrane potential under the pyruvate and malate substrate condition. (D) Relationship between mitochondrial

oxygen consumption and ATP-free energy under the glutamate and malate substrate condition. (E) Relationship between mitochondrial membrane potential and ATP-free energy under the glutamate and malate substrate condition. (F) Relationship between mitochondrial oxygen consumption and membrane potential under the glutamate and malate substrate condition. (G) Relationship between mitochondrial oxygen consumption and ATP-free energy under the rotenone and succinate substrate condition. (H) Relationship between mitochondrial membrane potential and ATP-free energy under the rotenone and succinate substrate condition. (I) Relationship between mitochondrial oxygen consumption and membrane potential under the rotenone and succinate substrate condition.

Discussion

The results of our experiments provide rationale for how the FDB both functions without O_2 and can survive acute hypoxic insults. Studies from our lab have shown that FDB muscles retain contractile function after exposure to the acute hypoxic conditions for more than 3 hours. This is unlike our comparative muscle, the EDL, or any other phenotypically similar peripheral murine hindlimb muscles which is functionally dead within 60 mins of hypoxia exposure. This suggests that the FDB has unique mitochondria and/or relies on alternative metabolic pathways in order to function.

Our data confirm this suggestion by the results of two different experimental approaches that assessed mitochondrial respiration. PMFBs allow the mitochondria to remain in their native state – within the muscle fiber. In this condition, we found that the FDB consumes less O_2 when compared to the EDL across all the different free energy states. Thus, when the mitochondria in the FDB are allowed to retain their native structure, less O_2 is utilized than the EDL fibers. This is significant because of the fiber types of both muscles suggest otherwise. The EDL is

considered a glycolytic muscle composed of mostly type IIb fibers (Soukup et al., 2002), which have a low mitochondrial content and do not rely heavily on oxidative phosphorylation. Contrarily, the FDB exhibits a mixed muscle fiber type comprising of predominantly type IIa fibers and type IIx fibers (Tarpey et al., 2018). Although these muscle fibers are categorized as "fast-twitch," they are considered more oxidative in nature than type IIb fibers. This suggests that the FDB should also function and exhibit characteristics similar to oxidative muscles, but according to our PMFB data it does not.

While PMFBs allow the mitochondria to retain its inherent structure, the mitochondrial volume for each fiber bundle can be variable. Thus, the reduced mitochondrial respiration by the FDB PMFBs may be result of few mitochondria compared to the EDL rather than an inherent difference in the mitochondria. To test this possibility, we developed a novel approach to isolated mitochondria. A new approach was necessary due to the small size of the EDL and FDB muscles. This technique allows the same amount of mitochondria to be tested from each muscle group ensuring no differences in amounts. Using the isolated mitochondria, we found that FDB mitochondria still had reduced O_2 consumption when compared to the EDL under all substrate conditions and across energetic states. Additionally, the membrane potential of the FDB mitochondria was much lower than that of the EDL independent of substrate condition. Both the lower respiratory conductance in the FDB and the presence of a depolarized mitochondrial membrane potential implies that the FDB has reduced proton pump activity in the electron transport system and/or lower capacity of dehydrogenase enzyme activity when compared to the EDL. Overall, this suggests that the FDB mitochondria are of poor quality and may be underdeveloped. Furthermore, this explains why the FDB does not depend on O_2 to function. Its

mitochondria have developed to work independent of oxidative phosphorylation – making the muscle unique to mammals.

Tissue that can be hypoxia resistant is practically unheard of in mammals. However, several studies have been conducted on freshwater turtles that retain function in anoxic environments during hibernation. *Chrysemys picta*, more commonly known as the painted turtle, spends long periods during the winter in ice-covered ponds without access to the surface, often in water or mud with little or no O_2 (Ultsch & Jackson, 1982). One of its physiological adaptive responses is a synchronized depression of metabolism within the cells. This includes both the glycolysis pathway that produces ATP and the ion transporting that consumes ATP. As a result, both the rate of substrate depletion and the rate of lactic acid production due to anaerobic respiration are slowed greatly (Jackson, 2002). Since turtles are ectotherms, their energy metabolism is only 10–20% that of a mammal of similar size and at the same body temperature (Bennett & Ruben, 1979). Moreover, the second physiological adaptation that allows them to survive anoxic environments is a lactic acid buffering system by the turtle's shell (Jackson, 2002). While these differences make it difficult to compare turtle skeletal muscle physiology to that of a human, the knowledge of hypoxia resistant tissue can help guide future studies about the function of the FDB.

The FDB is a unique muscle because it functions without O_2. No other muscle has this capability, and it suggests that the FDB is not dependent upon mitochondria to function. Our data appear to confirm that unlike any other mammalian skeletal muscle, the FDB has evolved to operate without oxidative phosphorylation and is reliant on alternative metabolic pathways to provide ATP for the muscle. This has a broader implication for the field of skeletal muscle physiology in terms of muscle atrophy and muscle fatigue. Determining how this hypoxia

resistant muscle functions could shed light on possible preventative measures for muscle degeneration. Future studies will seek to determine the metabolic pathways that the FDB utilizes to provide energy for physiological function.

Acknowledgements

Thanks for the help from Michael D. Tarpey, PhD with this project. Funding for this project came from an ECU URCA award and NIH RO1AR066660.

References

Bennett, A. F., & Ruben, J. A. (1979). Endothermy and activity in vertebrates. *Science (New York, N.Y.)*, *206*(4419), 649–654. https://doi.org/10.1126/science.493968

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *American journal of physiology. Cell physiology, 287(4)*, C817–C833. https://doi.org/10.1152/ajpcell.00139.2004

Jackson D. C. (2002). Hibernating without oxygen: physiological adaptations of the painted turtle. *The Journal of physiology*, *543*(Pt 3), 731–737. https://doi.org/10.1113/jphysiol.2002.024729

Lark, D. S., Torres, M. J., Lin, C.-T., Ryan, T. E., Anderson, E. J., & Neufer, P. D. (2016). Direct real-time quantification of mitochondrial oxidative phosphorylation efficiency in permeabilized skeletal muscle myofibers. *American Journal of Physiology-Cell Physiology, 311(2)*. doi: 10.1152/ajpcell.00124.2016

Mishra, P., Varuzhanyan, G., Pham, A. H., & Chan, D. C. (2015). Mitochondrial Dynamics is a Distinguishing Feature of Skeletal Muscle Fiber Types and Regulates Organellar Compartmentalization. *Cell metabolism*, *22*(6), 1033–1044. https://doi.org/10.1016/j.cmet.2015.09.027

Soukup, T., Zacharová, G., & Smerdu, V. (2002). Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta histochemica*, *104*(4), 399–405. https://doi.org/10.1078/0065-1281-00660

Tarpey, M. D., Amorese, A. J., Balestrieri, N. P., Ryan, T. E., Schmidt, C. A., McClung, J. M., & Spangenburg, E. E. (2018). Characterization and utilization of the flexor digitorum brevis for assessing skeletal muscle function. *Skeletal muscle*, *8*(1), 14. https://doi.org/10.1186/s13395-018-0160-3

Ultsch, G. R., & Jackson, D. C. (1982). Long-term submergence at 3 degrees C of the turtle Chrysemys picta bellii in normoxic and severely hypoxic water. III. Effects of changes in ambient PO2 and subsequent air breathing. *The Journal of experimental biology*, *97*, 87–99.