Mitochondrial Respiration, Oxidative Capacity, and Oxidative Stress in Skeletal Muscle

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ABSTRACT

Biologists and physiologists have been interested in the differences between birds and mammals for many years. Part of this is due to the distinct differences observed in fat breakdown in these animals. Importantly, fat breakdown occurs in the mitochondria, which leads investigators to explore the mitochondrial differences between birds and mammals. Interestingly, when comparisons between birds and mammals have been done, overall mitochondrial function only seems to differ with fatty acid, and not carbohydrate oxidation. The purpose of this study was to continue to explore the differences between bird and mammalian mitochondrial biochemistry. We isolated mitochondria from the skeletal muscle of house finches (*Haemorhous mexicanus*), house mice (*Mus musculus*), and deer mice (*Peromyscus maniculatus*). Our results demonstrate higher maximal and basal mitochondrial respiration in house finches as compared to deer mice, but not in house finches compared to house mice. Although some differences were observed in state 3 and state 4 values, overall mitochondrial function was not different across species. Furthermore, when assaying mitochondrial complex activities, house finches present the highest activity of complex II, which supports prior data suggesting the heightened ability for birds to break down fats. Data on the normal oxidative stress of these three species revealed that house finches have significantly less protein oxidation, but similar levels of lipid oxidation compared to the house and deer mice. Moreover, antioxidant protein expression was higher in house mice compared to house finches for superoxide dismutase 2 (SOD2), whereas glutathione peroxidase (GPX1) had highest expression in house finches, and house mice had higher expression compared to deer mice. In conclusion, this study generally finds markers of mitochondrial performance (i.e. respiration and enzyme activity) of house finches and house mice being higher compared to deer mice. Furthermore, oxidative

stress measures determined an inconsistent pattern of antioxidants in the three species, with house finches having significantly less protein damaged compared to both mice species. This study continues to highlight the distinct differences between bird and mammalian physiology by highlighting the mitochondrial maximal and basal respiration differences, complex activity differences, oxidative damage differences, and antioxidant expression differences.

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LIST OF ABBREVIATIONS

4HNE → 4-hydroxyl-2-nonenal

8-OHdG → 8-Oxo-2'-deoxyguanosine

ADP → Adenosine diphosphate

ANT → Adenine nucleotide translocator

ATP → Adenosine triphosphate

ANOVA → A one-way analysis of variance

CS → Citrate Synthase

CAT → Catalase

CoQ → Coenzyme Q

DH → Dehydrogenase

DNA → Deoxyribonucleic acid

DNPH → 2,4-dinitrophenylhydrazine

ETC → Electron transport chain

FAD(H_2) → Flavin adenine dinucleotide

FCCP → Trifluoromethoxy carbonylcyanide phenylhdrazone

GPX → Glutathione peroxidase

GSR → Glutathione reductase

GRX → Glutaredoxin

GSH → Reduced glutathione

GSSG → Oxidized glutathione disulfide

H_2O_2 → Hydrogen peroxide

IMF → Intermyofibrillar mitochondria

IMM → Inner mitochondrial membrane

J_0 → oxygen flux across the IMM

MDA → Malondialdehyde

mRNA → Messenger ribonucleic acid

NAD(H) → Nicotinamide adenine dinucleotide

NADPH → Nicotinamide adenine dinucleotide phosphate

O_2 → Oxygen

$O_2^{\cdot -}$ → Superoxide

$OH^{\cdot}$ → Hydroxyl radical

OCR → Oxygen consumption rate

OMM → Outer mitochondrial membrane

Pi → Inorganic phosphate

PRX → Peroxiredoxin

PVM → Paravascular mitochondria

P/O → Phosphate/oxygen ratio

RCR → Respiratory control ratio

ROS → Reactive oxygen species

SS → Subsarcolemmal mitochondria

SOD → Superoxide dismutase

TEM → Transmission electron microscopy

TBARS → Thiobarbituric reactive species

TRX → Thioredoxin

XF → Extracellular flux

ΔG_{redox} → Mitochondrial matrix redox potential

ΔG_{H+} → Proton motive force

CHAPTER 1
INTRODUCTION

Bioenergetics and its link to exercise physiology

Bioenergetics has previously been defined as "the science that involves studies of energetic events in the biological world" [1]. The chemical energy, which is synthesized for any organism to live, is known as adenosine triphosphate (ATP) [2]. The chemical reactions which allow for synthesis of ATP involve the processes of glycolysis, β-oxidation, the Krebs cycle, and the electron transport system [2]. The Krebs cycle, β-oxidation, and the electron transport system all occur in the mitochondria [2]. This is why the mitochondria are commonly referred to as the "powerhouses of the cell". Since mitochondria are the location of energy production, it is not surprising there is a strong research interest in how the mitochondria are affected by exercise, aging, and disease.

Synthesis of ATP is required for life in all living organisms. Specifically, ATP synthesis occurs through processes known as anaerobic metabolism, when oxygen (O_2) is limited, or through aerobic metabolism, when O_2 is plentiful [1, 2]. As energy demand becomes higher, such as during exercise, both the anaerobic and aerobic metabolic reactions must work at an increased rate to produce enough ATP in skeletal muscle to meet the energy demand.

For example, if an individual goes from rest conditions to running, the metabolic demand increases. ATP powers neural signaling, muscle contraction, and associated cellular processes. Therefore, the necessity to produce ATP is higher, and reaction rates will increase in order to meet this demand.

Bioenergetics encompasses many facets of mitochondrial physiology. Specifically, it includes areas such as mitochondrial morphology, function, efficiency, and enzymatic activities of the enzymes located within the mitochondria. Importantly, these measures can be made from different types of biological samples and different scientific instruments. A combination of all these measurements can provide critical information about how the mitochondria may be changing to a given environment.

Oxidative stress and exercise physiology

Oxidative stress occurs when harmful chemicals known as reactive oxygen species (ROS), cause damage to proteins, lipids, and genetic material. Mitochondria are known as one source of ROS production, however current evidence suggests it is not the main source of ROS production [3]. A single exercise bout is known to increase ROS production [4-7]. This is partial due to the increase in ROS production that is seen with muscle contractions [8, 9]. An increase in ROS production can risk damage to proteins, lipids, and nucleic acids (genetic material). This is commonly seen with aging [10]. The exercise adaptation process, in which multiple bouts of exercise can result in a decrease of oxidative damage and an increase in antioxidant defenses, has previously been explained by mitohormesis [11]. Mitohormesis explains that mild perturbations in mitochondrial homeostasis will result in nuclear and cytosolic responses that leaves the whole cell, and mitochondria, less susceptible to future damage [11, 12]. Merry and Ristow [11] elegantly explain how ROS production, from both mitochondrial and non-mitochondrial forces, has recently been determined to be a signal to cause mitochondrial response. Part of the response to the mild ROS perturbations is an increase in antioxidant enzymes protein expression or activity in order to attenuate the damage as much as possible. Numerous resistance and endurance exercise training

studies have shown that exercise can decrease ROS production and increase the body's antioxidant defenses. Notably, the effects of exercise training can counter the increase in ROS seen with aging, which results in an increase in an individual's lifespan [13-16]. Repeated bouts of exercise therefore offer a solution to the increase in oxidative stress an individual encounters throughout their life in part due to mitohormesis.

CHAPTER 2
LITERATURE REVIEW

Mitochondria and mitochondrial respiration

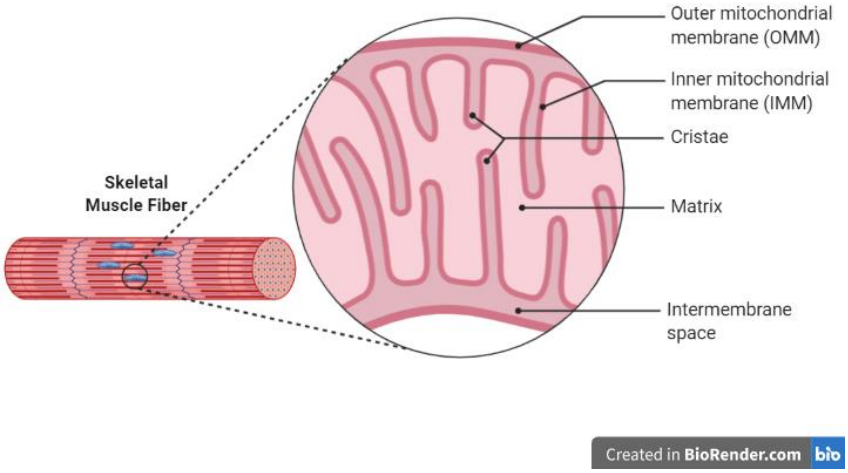

Figure 1. Mitochondrial Structure. Depicted are the common bean shaped mitochondria located within the skeletal muscle. Importantly, this image presents the general structure of a mitochondria. Mitochondria located in the skeletal muscle have previously been documented to form a reticulum. Mitochondria are located under the sarcolemma and between the myofibrils, shown in blue. A closer look at the mitochondria shows the important structures of the mitochondria, such as the outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), cristae, mitochondrial matrix, and the intermembrane space. Note that the size of the intermembrane space is greatly exaggerated. The figure was created in BioRender.com.

Mitochondria have been named the "powerhouse of the cell" (**Figure 1**). They have previously been described as "jellybean" structures, however, current literature suggests mitochondria consistent of a network throughout the skeletal muscle [17]. Notably, mitochondria have been documented to have tissue specific morphology such that mitochondria in the skeletal muscle are arranged in a network while mitochondria of the nervous system are separate tubules which facilitate communication between each mitochondrion [18]. In all forms of mitochondria, there are two cell membranes known as the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The space between the OMM and the IMM is known as the intermembrane space and has a relatively lower pH compared to the mitochondrial matrix. The space contained by the IMM is known as the mitochondrial matrix, which has a higher pH relative to the intermembrane

space. Importantly, the enzymes that make up the Krebs cycle are located within the mitochondrial matrix and the enzymes that construct the electron transport system are associated with the IMM. Furthermore, the IMM consist of many cristae, or folds. These folds greatly increase the surface area of the IMM, providing a larger surface area for membrane bound proteins.

Mitochondria are involved in a variety of functions. For the current literature review, the focus will be on the mitochondria's involvement in catabolism. Catabolism within the mitochondria occurs once Acetyl-CoA has been synthesized within the mitochondrial matrix [2]. Acetyl-CoA can come from either pyruvate formed from glycolysis, or through β-oxidation [2]. The Krebs cycle begins when acetyl-CoA and oxaloacetate are used as substrates by citrate synthase (CS) to produce citrate. A series of reactions follow in which the 6-carbon molecule of citrate is oxidized to form a 4-carbon molecule and is ultimately transformed back into oxaloacetate to be used in the CS reaction. The ultimate purpose of the Krebs cycle is to produce key electron transport carriers. These carriers include nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). Once reduced, NAD^+ becomes NADH and FAD becomes $FADH_2$.

The destination for the electron carriers is the electron transport chain (ETC). Here they will deliver their electrons to specific complexes in the ETC. NADH will donate its electrons to complex I, also known as NADH:ubiquinone oxidoreductase. Two electrons will reduce coenzyme-Q (CoQ), and four hydrogen atoms will be "pumped" from the mitochondrial matrix into the intermembrane space (**Figure 2**). $FADH_2$ will donate its electrons to complex II (succinate dehydrogenase). Since this enzyme is a peripheral enzyme and not fully integrated into the membrane, no hydrogen atoms are transported to

the intermembrane space. Importantly, the two electrons donated by $FADH_2$ also reduce CoQ. CoQ then moves to complex III (coenzyme Q:cytochrome C oxidoreductase). Complex III will uptake two protons from the mitochondrial matrix and release four protons to the intermembrane space. Part of this discrepancy is during the Q-cycle, which occurs at complex III, two hydrogens taken up during the Q-cycle previous cycle are released to the intermembrane space along with the two hydrogens acquired in the current Q-cycle [19]. Electrons that have been used during the Q-cycle are accepted by cytochrome C. Cytochrome C will then transport these electrons to complex IV. At complex IV (cytochrome c oxidase), the electrons from cytochrome C participate in a reaction with O_2 and hydrogen to form water. Another two protons will also be pumped from the mitochondrial matrix to the intermembrane space at complex IV. Consequently, O_2 is the last/final electron acceptor of the ETC.

The synthesis of ATP occurs at complex V, commonly referred to as ATP synthase. This enzyme is unique in its ability to pair the proton motive force created by the movement of protons from the mitochondrial matrix to the intermembrane space and the natural energy movement of hydrogens from an area of high concentration and relatively low pH, to that of a lower concentration with a relatively higher pH, therefore overcoming the energy barrier needed to create a new covalent blond to synthesize ATP via the reaction:

$$ADP + Pi \rightarrow ATP$$

Once ATP is synthesized, it will be transported outside the mitochondria to be used in ATP demanding reactions throughout the cell. This is completed with the adenine nucleotide translocator (ANT). ANT is located within the IMM. The transporter exchanges an ATP molecule for an adenosine diphosphate (ADP) molecule while also allowing hydrogen atom to move down its electrochemical gradient from the intermembrane space to the mitochondrial matrix, similar to what is performed at ATP synthase.

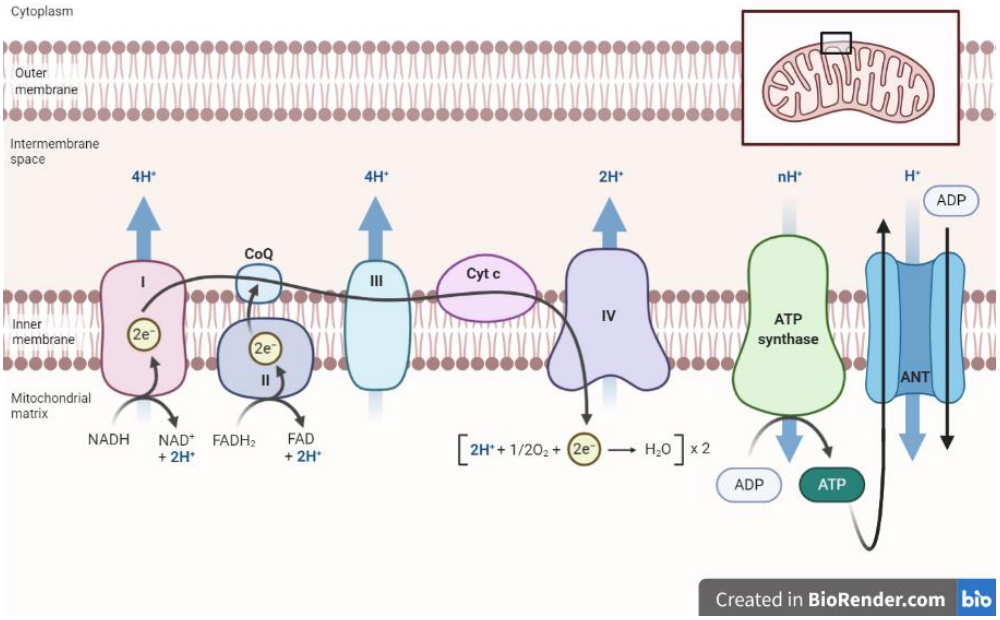

Figure 2. Mitochondrial Respiration. A simple summary of the mitochondrial respiration at the electron transport system with the ANT transporter. The figure was created in BioRender.com.

Biological samples to measure mitochondrial respiration

There are four main types of biological samples which can be used to measure mitochondrial function: cell types, crude homogenates, isolated mitochondria, and permeabilized cells. All four have advantages and disadvantages that will be discussed below.

Cells provide a unique scientific model to study bioenergetics. Brand and Nicholls provide a great comparison of intact cells with isolated mitochondria, and readers are guided

to their review for a more detailed comparison [20]. Briefly, intact cells allow for researchers to investigate whole-cell metabolism. However, there is still the limitation of intact cells lack a natural physiological environment in which physiologically relevant concentrations of substrates are naturally available. This requires investigators to choose what substrates to add to the cellular environment (e.g., hormones, growth factors, substrates, etc.). However, this could be considered a benefit, as it allows for significant control over the cell's environment. Although the physiological environment is not obtained through cells, mitochondria do remain in their natural morphology [17]. Additionally, one measurement of intact cell respiration provides data on basal respiration, ATP turnover, proton leak, coupling efficiency, maximum respiration rate, apparent respiration control ratio, spare respiratory capacity, and non-mitochondrial oxygen consumption [20]. These data can be obtained using sequential addition of inhibitors such as oligomycin, trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), rotenone, and antimycin A.

Nevertheless, cells offer unique challenges in terms of transport across the plasma membrane into the cell, given that cell membranes are semi-impermeable [20, 21]. This can pose some challenges in measuring mitochondrial responses since various substrates such as ADP or cytochrome c cannot cross into the cell, and therefore cannot be used during measurements with intact cells [21]. Another important limitation of intact cells is this biological material are typically normalized to cell number or cellular protein content. This provides the chance for changes in respiration due to changes in mitochondrial density to be overlooked [20]. However, the use of intact cells to measure mitochondrial respiration may have a simpler preparation compared to methods like isolated mitochondria and permeabilized fibers. Furthermore, mitochondrial respiration measurements with cell types

may be cleaner due to the lack of artifacts that may remain after mitochondrial isolation such as a disrupted OMM, damaged mitochondria due to shear stress, and bias in mitochondrial pools precipitated out during the isolation process. Respiration of intact cells can be measured using the Seahorse XF24 and XF96 extracellular oxygen flux analyzers. However, measurements of cells using Hansatech OxyTherms or the Oroboros has also been documented [22, 23].

Crude homogenates from tissues allow for the analysis of mitochondria with all the proteins that are found within the tissue. As stated by Pecinova et al., "The main advantage of tissue homogenate is the quick preparation..." [24]. However, despite the quick preparation, minimal studies have used crude homogenates to measure mitochondrial respiration. One study that did use crude homogenates to measure mitochondrial respiration found similar values for the respiratory control ratio (RCR) when compared to mitochondrial isolations [24]. The isolated mitochondria had higher maximal and basal respiration values, however they were not statistically different from the tissue homogenate. Another previous study documented mitochondrial respiration in nuclear fractions (crude homogenate centrifuged down to obtained components of only the nucleus) using a Seahorse instrument [25]. It is worth noting any O_2 consumption that is measured with crude homogenates would also be detecting non-mitochondrial O_2 consumption. Previous reports have documented non-mitochondria O_2 consumption to be as high as 10% the total O_2 consumption in cells [26-28], highlighting the importance of taking into consideration this measure in the context of mitochondrial O_2 uptake. Nonetheless, there is documentation in the literature that crude homogenates could be used for mitochondrial respiration measurements, but this is not a popular method.

Analysis of specific enzyme activities can also be measured with crude homogenates (e.g., citrate synthase activity, complex I – IV activities, ATP synthase activity, etc.). This method allows for indirect measurements of mitochondrial components. Importantly, these enzyme activity measurements can also be performed on isolated mitochondria. Ultimately enzyme activity assays measure the maximal rate of an enzyme given the amount of enzyme present in the sample. When using crude homogenates, the mitochondrial enzyme of interest will be "diluted" out by other cytosolic protein. This may impact measurements when normalizing to protein content, as a researcher would determine total protein content of the crude homogenate rather than only mitochondria protein content, as would be done with isolated mitochondria [29]. This may also require researchers to use more crude homogenate sample for a signal to be detected during assay measurements compared to isolated mitochondria.

Mitochondrial isolation through differential centrifugation allows for a direct analysis of overall mitochondrial function. Although this method has many steps, which increases the risk of technical errors, assaying isolated mitochondria enables the assessment of O_2 uptake during specific experimental conditions [20]. This is the major strength of the mitochondrial isolation method. Similar to intact cells, researchers need to specifically control for the types of substrates and inhibitors during solution, as the mitochondria are removed from their natural environment. This again provides strong control over the mitochondrial respiration measurements of isolated mitochondria. Additionally, with the cell membrane removed, isolated mitochondria can uptake compounds such as ADP that could not normally be used in intact cell measurement.

Many researchers have stated that part of the limitation of mitochondrial isolation is the process of isolating mitochondria removes the mitochondria from its cellular environment [21, 30]. Furthermore, one outcome of this procedure is the possibility of having a bias sample of mitochondria due to the isolation process [21, 30, 31]. Generally, it has been documented that the process of isolating mitochondria from skeletal muscle results in 20 – 40% of the mitochondria from the sample [32]. In skeletal muscle samples, it can also be difficult to select a heterogenous pool of mitochondria that represents all subsarcolemmal and intermyofibrillar mitochondria in muscle tissue, and healthy and diseased mitochondria in any tissue type [21]. During the process of isolating mitochondria there is a large risk of damaging the mitochondria. Although this is not unique to isolated mitochondria, as this is a similar limitation in permeabilized fibers, due to the nature of precipitating mitochondria out of tissue homogenates there can be more opportunity to damage the mitochondria. This has been documented with both electron micrograph images, and elevated cytochrome c response compared to permeabilized fibers [32, 33]. The last notable limitation of isolated mitochondria is the large amount of sample needed to obtain enough mitochondria to measure mitochondrial respiration [21, 34, 35].

An important regulator of mitochondrial respiration is adenine diphosphate (ADP). How sensitive ADP is to a particular cellular environment is determined through titrations which form a Michaelis-Menten curve. Using this curve, scientists can determine the concentration of sample, in this case ADP, that is needed to reach half of the maximal rate of a reaction (K_m). As discussed by Petrick & Holloway (2021), the original ADP Km determined by Chance and Williams using isolated mitochondria is 30 μM [36, 37]. Resting skeletal muscle has been reported to have a concentration of free ADP at 20 μM, suggesting

mitochondrial respiration capacity at rest would be at ~50% capacity [36]. Previous reports of *in vivo* respiration have determined mitochondrial respiration rates at rest are closer to 1 – 2% maximal respiration capacity [36]. Based off these data, the methodological challenges of using mitochondrial isolation to measure mitochondrial respiration must be taken into consideration when performing experiment.

Ultimately, mitochondrial isolation is currently considered the gold standard for mitochondrial measurements [35]. It is a well-established technique that provides important information about overall mitochondrial function. When used in conjunction with other methods, such as membrane potential and enzyme activities, mitochondrial isolations provide an established method to understand how and why mitochondria function the way they do.

Permeabilized fibers offer a unique ability to maintain the mitochondrial morphology while also measuring mitochondrial respiration. The process of permeabilizing muscle fibers requires researchers to dissect skeletal muscle bundles in a specified buffer and then subject them to a compound (such as saponin or digitonin) to permeabilize the sarcolemma. This allows previously impermeable substrates, such as ADP, to now enter the cell. Although this method allows the mitochondria in the skeletal muscle to remain in its natural reticulum morphology, researchers must still create the appropriate physiologically relevant experimental conditions [30]. Authors have previously argued the superiority of the permeabilized fiber method, as mitochondrial isolation subjects the mitochondria to damage, loss of enzymes, and loss of morphology [30]. There are also contradictory reports of permeabilized fiber preparations having poor mitochondrial purity compared to isolated mitochondria [20]. Furthermore, there is a risk during the permeabilization procedure to

damage the OMM and release cytochrome c, which could lead to further cellular damage [20]. Other researchers have also reported additional limitations of permeabilized fibers such as not being able to distinct between different mitochondrial subpopulations, the impact of cytosolic factors cannot be studied, and factors that may cause rapid change within the mitochondria cannot be studied [21].

In comparison to mitochondrial isolations, some researchers have argued that permeabilized fibers provide a more biologically relevant sample to analyze. For example, mitochondrial respiration with permeabilized fibers have reported ADP Km values of 120 – 150 µM [36]. This value is much larger than the ADP Km of 30 µM predicted by isolated mitochondria. However, the ADP Km of permeabilized fibers is predicted to be 10-fold higher than estimates made *in vivo* [36], suggesting that this method may provide better data than mitochondrial isolations, but it isn't precisely reflective of the *in vivo* environment. A similar observation was made in which permeabilized fibers predicted a higher Km value for inorganic phosphate when compared to isolated mitochondria [34]. Other researchers have investigated if permeabilized fibers provide the same mitochondrial respiration values as isolated mitochondria in aging animals. These researchers did observe changes in mitochondrial respiration with aging, however the changes in permeabilized fibers were to a lesser extent than isolated mitochondria [31]. An interesting conclusion from these authors is that mitochondrial isolation should be used as a tool to facilitate mitochondrial stress and resistance whereas permeabilized fibers are better at providing day-to-day function of the mitochondria.

In conclusion, permeabilized fibers offer a unique opportunity to measure mitochondrial isolation in samples that have maintained their *in vivo* structure. Although

there are equally as many limitations with permeabilized fibers, it may provide more biologically relevant data to represent homeostatic measurements of mitochondrial respiration.

Methods to measure mitochondrial respiration

There are three pieces of equipment that are commonly used to measure mitochondrial respiration including the Hansatech OxyTherm, the Oroboros, and the Seahorse. The Hansatech OxyTherm and Oroboros are similar instruments such that they both use a Clark electrode to measure oxygen concentration within the system. Although both the Hanstech OxyTherm and the Oroboros are built to measure O_2 consumption, the Oroboros can also measure other variables at the same time as mitochondrial respiration measurements. The foundation of the Seahorse is to measure the extracellular flux of O_2 using the fluorescent properties of O_2. Importantly, fresh tissue/cells are needed for measurements with all instruments. However, a recent publication has shown successful measurement of mitochondrial respiration from mitochondria isolated from frozen liver samples when using a Seahorse instrument [25]. These three methods have different principles which are used to measure mitochondrial respiration and therefore cannot be directly compared. Nevertheless, the advantages and disadvantages to each method will be discussed below.

Each machine provides similar measurements of mitochondrial respiration. The three main measurements which will be discussed in detail are state 3, state 4, and the respiratory control ratio (RCR). State 3 is defined as the maximal ATP production rate [19, 20]. State 4 is defined as the basal ATP production rate [19, 20]. RCR is a ratio between state 3 and state 4, and provides an excellent measure of overall mitochondrial function [20].

The basis of measuring mitochondrial respiration using the Clark electrode is to assess O_2 concentrations within a sealed container with the isolate being assayed (e.g., isolated mitochondria, permeabilized fibers, etc.) along with the substrate of choice (e.g., pyruvate, succinate, etc.). During this measurement, O_2 concentrations provide an indirect measure of ATP production, as O_2 is the last electron acceptor of the mitochondria. Thus, as O_2 concentrations fall during the experiment, this inversely reflects amount of ATP being produced. Using isolated mitochondria or permeabilized fibers, state 3 can be induced by adding a limited bolus of ADP to the solution of mitochondria and substrates. This stimulates the mitochondria to uptake O_2. Once the limited amount of ADP has been used by the mitochondria to synthesize ATP, the rate of O_2 consumption will equilibrate with a smaller amount of O_2 remaining in the chamber. This period of respiration after exhausting the external bolus of ADP is known as state 4. When measuring extracellular O_2 flux, as such with the Seahorse instrument, the sample is exposed to a saturating amount of ADP [38]. Therefore, to determine maximal and basal respiration, mitochondrial inhibitors must be added to the solution to pharmacologically change the physiology of the mitochondria. As discussed above, one of the benefits of using cells for mitochondrial measurements, is the ability to obtain basal respiration, oligomycin-insensitive leak respiration, oligomycin-sensitive respiration, and maximal respiration [20].

The Clark electrode was used for over half a century in the critical work of Chance & Williams [37], Mitchell [39], and Williams [40]. Importantly, the Clark electrode can be used to measure O_2 consumption by cells, isolated mitochondria from tissues, or permeabilized skeletal muscle fibers. Use of the Clark electrode with the Hansatech OxyTherm is a well-established measurement of mitochondrial respiration. Therefore, it is

not surprising that the biggest benefit of the OxyTherm is the ability to produce reliable data. Measurements of mitochondrial isolation using the Clark electrode, either with the OxyTherm or the Oroboros, provides important information about overall mitochondrial function [20]. The Hanstech OxyTherm is a more accessible piece of equipment due to its low cost [41]. Although the OxyTherm can measure mitochondrial respiration from either cells, isolated mitochondria, or permeabilized fibers, for an appropriate reading a large amount of sample is needed. Furthermore, due to the semi-permeable cell membranes, cell types must be lysed prior to measurement to ensure all substrates, such as ADP, can have access to ETC. Regardless of the samples used during the O_2 consumption measurement, samples can also be damaged by the constant stirring with a stir bar. As previously described [41], further disadvantages of the Hansatech OxyTherm include a longer run time (15-20 minutes per sample) and potential error due to the decline in health of the mitochondria throughout the course of the data collection day.

The Oroboros is mechanistically similar instrument to the OxyTherm, as both instruments measure O_2 concentrations within a sealed chamber [42]. Measurements can be made with isolated mitochondria, permeabilized fibers, and cells types. The unique feature of the Oroboros is the high-resolution respirometry. What makes the measurements of respiration of high-resolution capacity is the ability for the Oroboros to combine many measurements to determine overall mitochondrial function. Specifically, in addition to measuring O_2 consumption, the Oroboros also has the capability to measure O_2 flux across the IMM (J_0), pH, and the cytochrome spectra within one measurement [35, 42]. Other benefits of the Oroboros are the small amount of sample needed to measure respiratory flux per volume and the slowly exhausted oxygen capacity of the system which allows sufficient

time to evaluate respiratory activity [35]. The throughput of the system is faster than the OxyTherm, due to its ability to use several instruments simultaneously. More impressive is the ability of the Oroboros to decrease any O_2 concentration from 4 pmol/s, as measured in the OxyTherm chamber, to 2 ± 1 pmol/s/cm^3 [35]. As previously stated, "The distinguishing features that make this instrument so well-suited for measurements in small amounts of tissue are high sensitivity, low instrumental background, precise temperature and stir control, all of which allow experiments to be performed at high dilutions (2mL)" [43].

Lastly, the Seahorse Bioscience Analyzer measures extracellular flux (XF) of oxygen. Importantly, this instrument is meant to investigate monolayers of cells [41], however as the technology has advanced it has been able to measure oxygen consumption in tissues as well [38]. This method is best used when there is limited sample, different cell types or substrates to be tested within one experiment, or the physiology of the cell would change dramatically if cells are suspended [41]. The Seahorse is capable of measuring state 3, state 4, and uncoupled rate of respiration, similar to the OxyTherm and Oroboros. Additionally, the Seahorse can measure glycolytic respiration and non-mitochondrial O_2 consumption. The XF method also has the benefit of a high through-put, allowing upwards of 24 or 96 samples to be measured at once [38]. Additionally, the Seahorse can measure respiration in isolated mitochondria, intact cells, and permeabilized cells [38, 44]. Although the Seahorse provides important data regarding cellular respiration, concerns about its calculation of the O_2 consumption rate (OCR) have been questioned recently [45]. Specifically, it has been posited that "the noise of OCR is multiplicative, that outlier data points can concern individual measurements to all measurements of a well, and that the inter-plate variation is greater than the intra-plate variation" [45]. These researchers even

developed different statistical methods to better estimate the OCR measured by a Seahorse. In particular, this paper demonstrates the importance in understanding the materials used by the Seahorse, which determine OCR. If different statistical methods are available to researchers, there is clearly discretion to the best way to determine OCR. Importantly, these differences will make it more complicated to directly compare studies.

Sub-fractioning of skeletal muscle mitochondria

The mitochondria within a skeletal muscle can be divided into two populations, subsarcolemmal mitochondria (SS) and intermyofibrillar mitochondria (IMF) [46]. As indicated by the name, IMF are located between the myofibrils, while SS are located just under the sarcolemma [46]. It has been proposed IMF make up about 80% of the mitochondrial volume within a skeletal muscle [46]. Additionally, these mitochondria are suggested to be responsible for ATP production during muscle contractions [46, 47]. The SS is estimated to compose of the other 20% of mitochondrial volume, and are suggested to be responsible for producing the ATP needed to maintain active membrane ion and substrate transporters [46]. The process of separating SS and IMF is similar to extracting mitochondria during the mitochondrial isolation process. However, after the first spin, the supernatant and pellet containing the SS and IMF respectively are separated and then washed in separate buffers to obtain two unique pools of mitochondria [48].

Researchers have investigated if the theoretical differences in these subpopulations are supported with experimental data. Mitochondria were isolated and separated into SS and IMF pools and each pool was compared to determine differences in morphology, ETC complex activities, and proteomics [47]. Results from this study did determine that IMF mitochondria composed a higher density of mitochondria than SS mitochondria (determined

via TEM). Furthermore, oxidative rates of IMF mitochondria were 1.4 – 2 times higher than those measured in the SS mitochondria. When researchers characterized these subfractions with proteomics, they observed 38 differentially expressed proteins [47]. Importantly, the IMF mitochondria were found to have a higher expression of proteins associated with oxidative phosphorylation compared to SS mitochondria [47]. These results confirm the proposed roles of the distinct mitochondrial pools.

Previous researchers have also investigated if other enzymes besides those that compose the ETC have different activities in IMF mitochondria versus SS mitochondria. Researchers have observed distinct differences between the subpopulations of mitochondria during fatty acid oxidation. In particular, it was documented that within red gastrocnemius the SS mitochondria had higher increases in fatty acid oxidation than IMF mitochondria and, not surprisingly, both of these subpopulations of mitochondria were higher in the red gastrocnemius fibers compared to the white gastrocnemius fibers [49]. Further, the increase in fatty acid oxidation rate in SS was accompanied by an increase in citrate synthase activity [49].

The different sub-fractions of mitochondria also react differently to stress. When exposed to hydrogen peroxide (H_2O_2), IMF has a greater release of cytochrome c, an apoptosis-inducing factor, compared to SS [50]. This also resulted in an increase in mitochondria permeability transition pore opening in the IMF compared to the SS. Other researchers have found that the SS is more affected by aging, and therefore oxidative stress, compared to the IMF [51, 52].

As a whole, the structure of mitochondria is accepted to be a network/reticulum in the skeletal muscle rather than bean-shaped organelles. In particular, the mitochondrial

reticulum consists of different mitochondrial morphologies within the intrafibrillar network [17]. Notably, part of this network called the paravascular mitochondria (PVM) is part of SS. Interestingly, researchers have determined 20% of the PVM project into the contractile region and directly connect to mitochondria lining the I-band [17]. This research provides evidence that although these are thought to be two different pools of mitochondria, a fraction of them may be connected. Additionally, these researchers also determined a difference in the distribution of ETC enzymes throughout the reticulum. Specifically, PVM had more complex IV, whereas the intra-fibrillar region had a larger amount of complex V [17]. Complex IV and complex V have different roles within the ETC. Complex IV is where oxygen acts as the last electron acceptor to complete pumping of the hydrogen ions from the mitochondrial matrix to the intermembrane space, thus contributing the proton motive force. Complex V is responsible for the synthesis of ATP. The PVM had a higher proportion of complex IV on the periphery of the mitochondrial network which are closer to the capillaries to allow these mitochondria to use oxygen to generate the proton motive force. The higher proportion of complex V found in the intra-fibular region is indicative of higher ATP production in this region, which would be needed in times of muscle contraction. These results support the different functions previously observed in the IMF and SS mitochondria.

Mitochondria ROS Production

The process of metabolism is complex. During oxidative metabolism, electrons will be donated to the ETC to ultimately provide the energy needed to produce ATP. During these molecular events, molecules known as reactive oxygen species (ROS) are produced. The major ROS are superoxide and hydrogen peroxide, although other secondary ROS are

also found within the cell. Importantly, although mitochondria is considered a source of ROS, it is not the only source of ROS production during muscle contraction [3, 53].

Superoxide ($O_2^{\cdot -}$) is known as a free radical due to the extra electron found on the outer orbital of O_2. The unpaired electron makes the compound very unstable. To stabilize the radical, the extra electron will readily react with other molecules, and therefore donate the extra electron to another compound. This can result in damage to proteins, lipids, or genetic material. The consequential damage of a macromolecule due to its interaction with a free radical is known as oxidative damage. Superoxide is also commonly produced as an intermediate during many other biochemical reactions [54].

The other main ROS compound is hydrogen peroxide (H_2O_2). Hydrogen peroxide is formed by the combination of two superoxide molecules and two hydrogen ions.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Importantly, H_2O_2 is a non-radical ROS. It produces damage when it reacts with iron, copper, or a superoxide molecule. Any reaction with the previous compounds results in a hydroxyl radical ($OH^{\cdot -}$), which will donate its extra electron to any nearby compound that will accept it, thereby causing oxidative damage.

It is generally accepted that ROS production within the mitochondria mainly occurs at complex I and complex III [55-57]. In particular, the specific sites of the highest ROS production are at the IQ and IIIQo sites [58-61]. ROS produced from complex I is released exclusively into the mitochondrial matrix, whereas ROS released from complex III is documented to be directed into both the mitochondrial matrix and the intermembrane space [62]. Despite the general hypothesis that mitochondria produce most of the free radicals at these specific sites little research supports this claim. Rather, other researcher have

demonstrated mitochondria produce much less ROS than previously hypothesized [63]. Additionally, due to the duel function nature of mitochondria as both a producer and a sink for ROS, it is difficult to determine if the measured source of ROS is due to the production or absorption of ROS [3]. A recent study performed on different cell lines from humans, rats, and mice did determine that mitochondria produce 30 – 40% of the extracellular H_2O_2 under non-stressful conditions [61]. Importantly, the relative contribution of ROS from site IQ (70 ± 4%) versus site IIIQo (30% ± 4%) was similar in liver, cervix, skin, heart, lung, bone neuron, and skeletal muscle cell lines [61, 64, 65]. These studies only analyzed cells during non-stressful conditions, and therefore should not be extrapolated to stressful conditions such as exercise.

Mitochondrial endogenous antioxidants

Antioxidants are molecules that act to prevent oxidative damage to either proteins, lipids, or deoxyribonucleic acid (DNA). The main endogenous antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GSR), and catalase (CAT). It should be noted that there are also other enzymatic and non-enzymatic mitochondrial antioxidants which also act to maintain the redox balance within a cell. These minor antioxidants will be mentioned, but are discussed in more detail elsewhere [54].

SOD specifically provides defense against superoxide radicals. This enzyme has three isoforms: SOD1, SOD2, and SOD3. SOD1 is located in the cytosol and intermembrane space of the mitochondria and requires copper-zinc as a cofactor. SOD2 is located within the mitochondrial matrix and requires manganese as a cofactor. Lastly, SOD3 is located in the extracellular space, and uses copper-zinc as a cofactor. It has previously been reported that SOD activity is highest in type I muscle fibers compared to type IIx muscle fibers which

have low mitochondrial content [66, 67]. Of the SOD activity recorded in skeletal muscle, it is predicted that 15 – 35% of the activity is due to activity within the mitochondria, while the remaining 65 – 85% is due to activity within the cytosol [54, 67, 68]. Additionally, reports have documented regular endurance exercise increases SOD1 and SOD2 activity within the skeletal muscle [66, 67, 69]. Other authors have also reported an increase in SOD1 activity and mRNA content with resistance training in older adults and young rats [14, 70, 71].

There are five isoforms of GPX within the skeletal muscle. Depending on the isoform, GPX is located in the cytosol, mitochondria, and/or extracellular space [54, 72, 73]. GPX reduces H_2O_2 to water and alcohol using a reduced glutathione (GSH) [54]. This mechanism of action requires a reduced glutathione to donate hydrogen ions to H_2O_2, resulting in an oxidized glutathione disulfide (GSSG) [54].

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

In order to regenerate a GSH for this process to continue, GSSG is reduced via nicotinamide adenine dinucleotide phosphate (NADPH), primarily produced through isocitrate dehydrogenase in skeletal muscle [66, 74-76]. The main enzyme responsible for this reaction is GSR.

$$GSSG + NADPH \rightarrow GSH + NADP$$

GPX activity is also higher in oxidative skeletal muscle fibers, compared to glycolytic fibers, similar to SOD [67, 77]. Also similar to SOD, regular endurance training has been reported to increase GPX activity in both the cytosol and the mitochondria [68].

CAT is able to transform a H_2O_2 into water and a stable O_2 molecule.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

This enzyme is widely available throughout the cell. Similar to both SOD and GPX, CAT is highest in oxidative fibers compared to glycolytic fibers [67]. There is inconsistent evidence to the response of CAT with endurance training. Reports have documented increases [78, 79], decreases [80, 81], and no changes [67] in CAT enzyme activity with endurance training. Unilateral resistance training in older adults has been shown to increase CAT enzyme activity and mRNA content [14, 70]. However, data has also shown a decrease in CAT protein expression after resistance training in older adults [70].

Enzymes that assist in maintaining redox balance within a muscle cell are thioredoxin (TRX), glutaredoxin (GRX), peroxiredoxin (PRS). TRX and GRX control the redox state of thiol groups of cysteinyl side chains; however each enzyme has been reported to have different functions for the proteins with which they interact with [54, 82]. PRX differs from TRX and GRX. PRX enzymatic function reduces hydroperoxides and peroxynitrate using electrons provided by thiol groups [83, 84]. Other important non-enzymatic antioxidants include GSH, α-lipoic acid, uric acid, coenzyme Q, vitamin E and C, and carotenoids. A more in depth discussion about these antioxidants can be found elsewhere [54].

Methods to measure oxidative stress

Other researchers have provided an in-depth review on oxidative stress and methods to measure oxidative stress [54, 85]. I will provide a brief, but relevant review of the common measures of oxidative stress.

One way to measure oxidative stress is to measure the free radicals that causes oxidative damage. This can be done either in a direct or indirect manner. Direct approaches can only be performed by detection of spin resonance or through fluorescent assays [54,

86]. A limitation to spin resonance is this method may only measure relatively stable radicals, and therefore may not capture the totality of free radicals being produced. Fluorescent assays, such as Amplex® Red, detects the resorufin which is produced when hydrogen peroxide and a non-reactive ROS reacts with the Amplex® Red reagent [87]. A limitation to both fluorescent probes and spin traps is that these measures are known to disturb the biological system of the sample and may be toxic to cells [88]. Other indirect measures, known as fingerprinting methods, allow for measurement of the end-products after the interaction of a biological substance and the free radical [54, 85]. These end-products can be damaged DNA, proteins, and lipids. Mainly, these fingerprinting methods can be completed through immunohistochemical techniques [85].

More specifically, DNA damage can be detected by measurement of 8-Oxo-2'-deoxyguanosine (8-OHdG). 8-OHdG is the molecular end-product after hydrolysis of DNA. Lipid peroxidation can be measured through different procedures depending on the stage of peroxidation. For example, 4-hydroxyl-2-nonenal (4HNE) is a derivative of a lipid associated with a protein after a free radical attack. Notably, 4HNE is only found at the location of the free radical attack, whereas malondialdehyde (MDA) is detectable during the last stage of peroxidation and can provide a measure lipid peroxidation in the whole organism. The measure of damaged aldehydes is termed thiobarbituric reactive species (TBARS) and is also a measurement of lipid peroxidation [89]. Lastly, oxidative damage to proteins is done through free radicals interacting with amino acid residues. The resultant protein damage is measured through the carbonyl assay [90].

Another way to indirectly measure oxidative stress is to measure the antioxidants that work to neutralize free radicals. These procedures may involve measuring antioxidant

enzyme activity though spectroscopic measures or total antioxidant protein content through electrophoresis and blotting methods [85]. Measuring the ratio between oxidant and reductant may also provide a measure of oxidative stress. One way to do this is by measuring the ratio between GSH and GSSG [85]. Lastly, measuring total antioxidant activity provides a means to analyze overall oxidative stress of the tissue measured [85]. Total antioxidant activity has been argued to be a potentially better analysis of oxidative stress, as many antioxidants work together, and measurement of select antioxidants may misrepresent oxidative stress [85, 91]. However, when analyzing a select tissue, measurement of the activities and protein content of each antioxidant (i.e., SOD1, SOD2, CAT, GPX) can provide insight into the specific cellular mechanism(s) responding to the oxidative stress.

As with all techniques, there are limitations to each of these methods of measuring antioxidants. First, when measuring antioxidants from a tissue there is always risk of auto-oxidation during sample handling, possibly resulting in antioxidant depletion of tissues [88]. Additionally, oxidative molecules are only available within the cell for a limited period of time, which can make them difficult to measure [54]. Lastly, when measuring the GSH/GSSG ratio, researchers should be mindful that experimental artifacts may contaminate data. Such experimental artifacts may occur with mishandled tissue samples, such as increased shear stress imposed on the tissue during homogenization, resulting in auto-oxidation of GSH [54].

Exercise and mitochondrial volume, respiration, ROS, antioxidants, and oxidative stress

Exercise physiology have documented the positive benefits of exercise for many years. Most adaptations of the mitochondria from exercise result from endurance training. Exercise physiology first reported skeletal muscle mitochondrial adaptation in 1967.

Specifically, Holloszy et al. recorded a two-fold increase in mitochondrial enzyme activity in the hind limb muscles of rats that ran on a treadmill for 12 weeks versus sedentary controls [92]. Since then, numerous studies have shown that either continuous endurance training (e.g., cycling 70% VO_{2max} for one hour three days a week) or high intensity training (e.g., one minute sprint at 90% VO_{2max} followed by 2 minutes of rest for 30 minutes three days a week) is sufficient to cause positive mitochondrial adaptations [93-96].

The term "mitochondrial adaptations" is broad, and this literature review covers the changes in mitochondrial volume, respiration, and redox balance. In particular, mitochondrial volume, either measured via transmission electron microscopy (TEM) or citrate synthase activity, has been shown to increase significantly after endurance training [97-99]. Lundby et al. reported that 6 weeks of endurance training increased citrate synthase activity (44±12%) and mitochondrial volume (assessed via TEM, 55±9%) in healthy males [96]. Additionally, these authors reported that the increase in mitochondria volume occurred through the enlargement rather than *de novo* biogenesis [96]. Investigations on high-intensity interval training in older adults also results in a large increase in citrate synthase activity (55%) and mitochondrial respiratory chain complexes (70 – 100%) [100].

Mitochondrial dynamics encompass the fusion (two mitochondria into one) and fission (one mitochondrion into two) of mitochondria. Previous research has observed one acute bout of low-volume speed endurance and high-volume continuous moderate-intensity exercise results in post-exercise increases in the messenger ribonucleic acid (mRNA) of Mfn2 (fusion) and Drp1 (fission) in humans [101]. However, a single treadmill exercise for 60 minutes in rats did not result in any changes to mitochondrial dynamics markers [102].

Older adults respond remarkably well to exercise training, and therefore it is not surprising that exercise is used as an intervention to combat sarcopenia. Previous investigations have observed increases in the mitochondrial fusion marker OPA1 and the size of the mitochondria (as determined via TEM) from the skeletal muscle of older adults the underwent electrical stimulation of the quadricep muscles [103]. Chronic exercise training for 16 weeks resulted in increased mitochondrial fusion in both males and females [104]. The authors of this study also reported that lifelong trained individuals had increased mitochondrial fusion and mitophagy (mitochondrial breakdown) and decreased mitochondrial fission, indicative of increased mitochondrial turnover. Similar results have been shown after 12-weeks of aerobic training in sedentary adults. Specifically, the sedentary individuals presented decreased mitochondrial fission, which resulted in a higher ratio of fusion to fission proteins [105].

Changes in mitochondrial dynamics can also accompany changes in mitochondrial function. Often researchers use measures such as state 3, state 4, RCR, and P/O ratio to determine mitochondrial respiration and efficiency. When determining how endurance training changes mitochondrial respiration and efficiency, researchers studied individuals that performed 6 weeks of endurance training. Their results demonstrated maximal mitochondrial respiration (state 3) significantly increased after 6 weeks of endurance training, however no other changes in basal respiration (state 4) or overall mitochondrial function (RCR) was observed [106]. While these researchers also observed an increase in maximal ATP production, there was no change in the mitochondrial P/O ratio, a measurement of mitochondrial efficiency, after 6 weeks of endurance training. Previous investigators have reported an increase in mitochondrial function in both male and female

rats after treadmill training for 6 weeks [107]. Other investigators have found improvement in mitochondrial function (via RCR) after 3 weeks of two-a-day endurance training [108]. Additionally, there is plenty of evidence to show that there is an increase in mitochondrial respiration after an endurance training intervention in individuals that are obese and/or have type 2 diabetes [109-111].

It has also been documented that an acute bout of exercise increases ROS in the skeletal muscle [112]. The increase in free radicals is known to cause an increase in lipid oxidation, DNA oxidation, and protein oxidation [113-115]. Exercise training has been reported to decrease ROS production through either increased coupling of the ETC, or through greater ROS scavenging capacity [116, 117]. Furthermore, increase in antioxidants is also observed with training to combat the free radicals [95, 116, 118]. Significant negative associations between total antioxidant status and protein carbonyl content ($r = -0.7$, $p<0.0010$) and total antioxidant status and total protein content of an endurance trained individual's serum ($r=-0.4$, $p=0.005$) have been previously reported [119]. These data suggest that training results in an increase antioxidant capacity to challenge the ROS produced during muscular contraction. Further supporting this notion, 6-weeks of high-intensity discontinuous training in swimmers resulted in decreased ROS production at rest and after incremental arm-ergometer exercise while also increasing antioxidant capacity by 13% [120]. Contrary to these data, other researchers have reported a decrease in antioxidant activity when normalized to citrate synthase activity with six weeks of endurance training [106]. Other researchers have also observed no differences in mitochondrial H_2O_2 emission after two weeks of limb immobilization and four weeks of recovery and training [121].

Previous investigations have found that single bouts of exercise can increase, decrease, or cause no change on markers of oxidative stress in rats, mice or humans. For example, authors have found an increase in TBARS and DNA damage with exhaustive treadmill running in both rats and humans [112, 122]. However, other reports document decreases in the GSH/GSSG ratio in rats with either exhaustive treadmill running or an acute swimming bout [123-125]. The MDA marker of oxidative damage has been reported to increase or have no change with an acute bout of swimming or exhaustive treadmill running in rats [68, 123, 124, 126-128]. Training has shown to cause little changes in oxidative stress markers. In particular, 10-week swim training in young and adult rats resulted in no change in MDA levels [129, 130]. However, a whole-body resistance training program for 14 weeks in male humans resulted in decreases in 8-OHdG [13].

Both endurance and resistance training has also resulted in mix results of antioxidant enzyme adaptations. An increase in GPX and GSR enzyme activity was observed in young and adult rats after a 10-week swimming program [129-131]. The same results were observed in young mice after 6-weeks of swim training [132]. Additionally, this training program resulted in no changes in SOD2 enzyme activity, and reports of increased SOD1 enzyme activity or no changes to SOD1 enzyme activity in young or older mice [132]. Treadmill training in rats have shown an array of results. In regards to CAT, scientists have reported decreases in enzyme activity [107, 133], increases in enzyme activity [134, 135], or no changes in enzyme activity and mRNA content [134, 135] with treadmill training. Additionally, treadmill training in rats resulted in increases in enzyme activity [134-137] and no changes in mRNA content [134-136] in antioxidant enzyme GPX. Furthermore, general increases in SOD enzyme activity have been observed [136], as well as no changes

in enzyme activity with treadmill training [80, 136]. More specifically, increases in enzyme activity and protein content [137] and no changes in enzyme activity, protein content, or mRNA content [134, 135] have been observed for SOD1. Increases in enzyme activity and protein content [134, 135], decreases in enzyme activity and mRNA content [134], and no changes in enzyme activity and protein content [135, 137] have been observed for SOD2. Research into the antioxidant changes with exercise have also been performed in humans. After 6-weeks of maximal cycling sprint training, no changes were observed for GPX, GSR, or SOD enzyme activity [138]. When these individuals continued training for another week, increases in GPX and GSR enzyme activity were observed.

Another way to discuss exercise adaptations within the mitochondria is to use the Hydraulic Analog Model described by Willis et al. [139]. As shown in **Figure 3**, mitochondrial function can be thought of as water moving through a series of pipes. For a brief context, "Fuel" in the figure demonstrates the carbon sources that being oxidized for mitochondrial respiration. "DH" represents dehydrogenases, or the enzymes that work in functioning to oxidize the carbon compounds. The oxidation of the carbon molecules creates mitochondrial matrix redox potential (ΔG_{redox}) which is then converted into the proton motive force (ΔG_{H+}) by the complexes that make up the electron transport chain. The energy gradient generated by proton motive force is then used to synthesize ATP via ATP Synthase. ATP is subsequentially transported out of the mitochondria where it provides energy for cytosolic ATPases.

In an untrained state, if an individual were to participate in exercise the ATPase valve would open. Realistically this involves an increase in activity of ATPases throughout the cell, such as the myosin ATPase, which would be active and using any immediate supply

of ATP. This would cause an increase in activity in metabolic enzymes, therefore creating the flow of water described to move through the "pipes". However, due to the untrained state, there is a finite number of mitochondria available to produce the necessary ATP. This is analogous to the pipes having a small diameter. This results in a back-up of flux through the pipes, and results in production of ROS through electron leak. However, when an individual participates in regular exercise and is therefore in a trained state, these "pipes" have a larger diameter, allowing for an increase flux through the mitochondrial processes, and no back-up (i.e., little to no ROS production). Ultimately, the "increase in diameter" is facilitated through an increase in mitochondrial volume. It is this increase in mitochondrial volume that results in a larger free energy level, allowing the mitochondria to produce more ATP at a given rate of O_2 consumption [139].

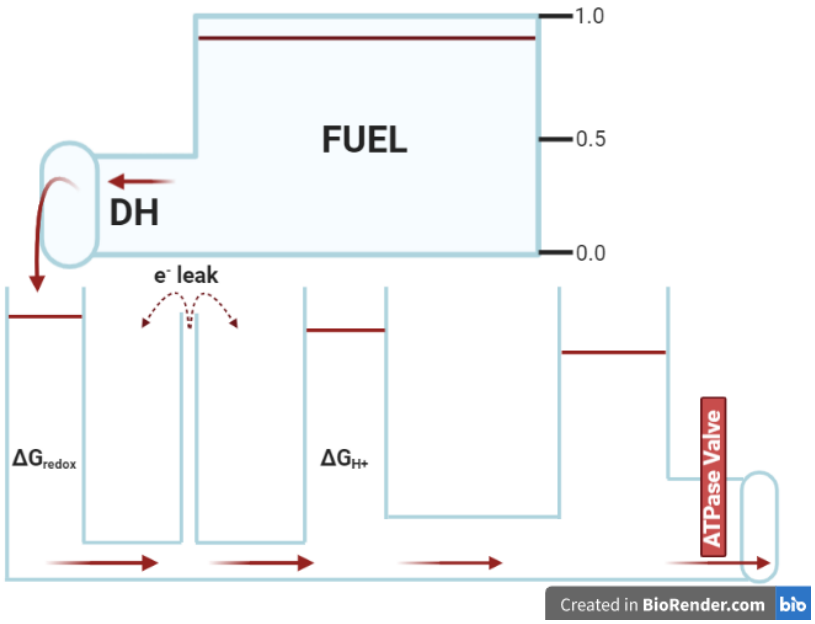

Figure 3. Adopted Hydraulic Model. The figure above is adopted from Willis et al. 2016 manuscript (113). This provides a simplified model of oxidative phosphorylation to help understand how exercise changes ATP synthesis. The figure was created in BioRender.com.

CHAPTER 3
MANUSCRIPT

Comparing the mitochondrial physiology between house finches, house mice, and deer mice

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Abstract

Birds are primarily dependent on fats as a fuel source, relying on 90% fats and 10% protein during flight. At the same energy intensity (~85% VO_2max) mammals will break down 85% carbohydrates, 10% fats, and 5% protein. These animals clearly have opposite metabolic fuel use during periods of heightened activity. Interestingly, when the overall mitochondrial function between these two species have been compared, differences are only seen with fatty acids substrates, and no differences are observed with carbohydrate substrates. The purpose of this study was to continue to explore the mitochondrial differences between bird and mammalian physiology. Mitochondria were isolated from the skeletal muscle of house finches (*Haemorhous mexicanus*), house mice (*Mus musculus*), and deer mice (*Peromyscus maniculatus*). The results of the current study show that maximal and basal mitochondrial respiration with carbohydrate substrates was higher in house finches compared to deer mice, but was not different between house finches and house mice. Despite these differences, overall mitochondrial function was not different across species. Furthermore, mitochondrial complex activities from isolated mitochondria determined that house finches have higher complex II activity, which supports the previous data on the heightened ability for birds to break down fats. Additionally, complex III and IV showed significant less activity in house finches and house mice compared to deer mice. Data on the oxidative stress of these animals revealed that house finches have significantly less protein damage, but similar levels of lipid damage compared to the house and deer mice. Antioxidant expression was higher in house mice compared to house finches for SOD2, whereas GPX presented the highest expression in house finches, and house mice had higher expression of GPX compared to deer mice in the isolated mitochondria. This study continues to highlight the distinct differences between bird and mammalian physiology by highlighting the mitochondrial maximal

and basal respiration differences, complex activity differences, oxidative damage differences, and antioxidant expression differences.

Introduction

Increased amount of mitochondrial volume, fatty-acid transporters, and activity of β-oxidation enzymes all contribute to birds' superior ability at oxidizing fatty acids compared to mammals [140-145]. However, few studies have investigated how mitochondrial respiration itself differs between these taxa. Of the research that has been performed, it has been reported bird mitochondria have higher respiratory capacity to when using fatty acid substrates, but mitochondrial respiration is not different between species when using carbohydrate substrates [146]. These comparisons provide an interesting starting point to understand the extent to which the mitochondrial physiology between birds and mammals contrast.

Following the oxidative stress theory of aging, most of the studies evaluating oxidative stress within and between birds and mammals has emphasized differences in ROS production, oxidative damage, and antioxidant levels. For example, the longer-lived *Peromyscus maniculatus* (deer mice) have been reported to have lower reactive oxygen species (ROS) production than *Mus musculus* (house mice) [147-149]. The oxidative stress theory of aging suggests individuals and species that experience higher oxidative stress will have reduced lifespans relative to those that have lower levels of oxidative stress [150-152]. Researchers have documented the longevity of *P. maniculatus* to be as high as 8.3 years whereas the documented longevity of *M. musculus* has been as high as 4 years [153]. Due to this distinct longevity difference, previous researchers have suggested the higher levels of ROS production observed in *M. musculus* may be responsible for the difference in longevity of these species [147, 149].

Scientists have previously proposed that the oxidative stress theory of aging does not apply to birds partly due to the lack of correlation between oxidative stress parameters and increasing age [154]. Researchers have previously reported the susceptibility of membranes in numerous tissues to damage by ROS is higher in rat than pigeon [155]. Based off these data, the researchers suggested that the oxidative theory of aging may not apply in birds. However, the same authors later reported that differences in fatty acid composition of cellular membranes does not explain the difference in lifespan between birds and mammals [156]. More consistent data have documented a decrease in oxidative damage to DNA, a decrease in ROS, and differences in antioxidant activity in birds compared to mammals [157, 158]. Regarding the differences observed in antioxidant activity, researchers have observed increases in SOD activity and decreases in catalase activity in pigeons compared to rats [157]. These data suggest that birds are better able to maintain a positive redox balance. These aging studies which compare birds and mammals provide valuable insight into basic physiology. Nevertheless, more research is needed to determine where the differences in ROS production and oxidative damage is in birds versus mammals.

The purpose of this investigation was to explore the mitochondrial differences between wild birds and mice. Animals used in this investigation include caught house finches (*Haemorhous mexicanus*), deer mice (*Peromyscus maniculatus*) that were maintained in semi-natural enclosures, and wild-derived house mice (*Mus musculus*) that were maintained in standard rodent boxes.

Methods

Animal Collection

Three species were collected for this study: house finch (*Haemorhous mexicanus*), house mouse (*Mus musculus*), and deer mouse (*Peromyscus maniculatus*). House finches (n=13, male=4, female=9) were caught in Lee County, AL during September 2019. House mice (n=11, male=9,

female=2) and deer mice (n=9, male=4, female=5) were obtained from populations maintained on the Auburn University campus. All animals were brought to one of two laboratories. One was a stationary laboratory located at the School of Kinesiology at Auburn University. The other was a mobile laboratory located just outside the School of Kinesiology. Wild house finches were caught at a nearby location and brought to the mobile laboratory (n=6) or the stationary laboratory (n=7). House mice were also collected from brought to the mobile laboratory (n=5) or the stationary laboratory (n=6). These animals were kept in standard rodent boxes with multiple mice per box, and all mice had access to a running wheel. These animals were exposure to outdoor ambient temperatures. Deer mice were collected from outdoor semi-natural enclosures on the Auburn University campus and brought to the mobile laboratory (n=4) or the stationary laboratory (n=5). These animals were kept in semi-natural enclosures which did not restrict their daily movement. Animals were euthanized via decapitation at the respective location, and fresh tissue of the skeletal muscle was dissected out for immediate mitochondrial isolation. Collection of wild house finches was approved under the Alabama state permit #2019128472668680 and federal migratory permit #MB42176A-0. Collection of University animals was approved under Auburn University IACUC #2019-3582.

Mitochondrial Isolation and Respiration

All mitochondria were isolated by the same individual. Immediately after euthanasia, the skeletal muscle was immediately dissected out and place in 10 volumes of ice-cold buffer (buffer 1; 100 mM KCl, 40 mM Tris-HCl, 10 mM Tris-Base, 1mM $MgSO_4$, 0.1 mM EDTA, 0.2 mM ATP, and 0.2% (wt/vol) free fatty acid bovine serum albumin (BSA), pH 7.40). Pectoralis muscle (~0.5 g) was used in the house finches and whole limb muscle (~1 g) was used for the mice. Skeletal muscle was minced with scissors in the buffer, and then homogenized for 5 seconds at 50% power

with a polytron (Thomas Scientific, Swedesboro, NJ,USA). Trypsin (5mg/g wet muscle) was then added to the homogenized solution and digested for 7 minutes. Solution was mixed by hand every 30 seconds. Termination of reaction completed by adding equal volume of buffer 1. The total homogenized solution was then centrifuged at *500g* for 10 minutes at 4° C. The supernatant was then transferred through a double-layered cheesecloth, and the decanted solution was then centrifuged again at *3500g* for 10 minutes at 4°C. The supernatant was discarded, and the remaining mitochondrial pellet was resuspended in 10 volumes of buffer 1. Again, the solution was centrifuged at *3500g* for 10 minutes at 4°C. The supernatant was discarded, and now the mitochondrial pellet was resuspended in 10 volumes of buffer 2 (the same as buffer1 but without BSA). The solution was centrifuged at *3500g* for 10 minutes at 4°C. The final mitochondrial pellet was resuspended in a mannitol-sucrose solution (220 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EGTA, pH 7.40). This is the final solution to be used for mitochondrial respiration measurements.

Mitochondrial oxygen consumption was measured in a OxyTherm respiration chamber (Hansatech Instruments, Norfolk, England) maintained at 40°C for house finches and 38°C for deer and house mice. These are the physiological temperatures of these animals, and therefore justify the different temperatures used. Isolated mitochondria were incubated with 1 mL of respiration buffer (100 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 50 mM MOPS, 10 mM $MgCl_2$, and 0.2% (wt/vol) BSA) in a water-jacketed OxyTherm respiratory chamber with continuous stirring. Flux through complex I was measured using 2 mM pyruvate, 2 mM malate, and 10 mM glutamate. Flux through complex II was measured using 5 µM rotenone to inhibit electron back flow to complex I, and then the addition of 5 mM succinate. The maximal respiration (state 3) was defined as the rate of respiration in the presence of ADP, initiated by adding 5.0 µL of a 100 mM

solution of ADP in the respiration chamber to raise the known concentration of ADP to 0.25 mM. State 4 respiration was recorded following the phosphorylation of ADP. The respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 respiration.

Enzymatic Assays for Electron Transport Chain Complex Activity

Methods for electron transport chain (ETC) complex activity were performed based off previous investigations [159]. Briefly, complex I (NADH dehydrogenase) enzyme activity was measured as a function of the decrease in absorbance from NADH oxidation by decylubiquinone before and after rotenone addition. Complex II (succinate dehydrogenase) activity was measured as a function of the decrease in absorbance from 2,6-dichloroindophenol reduction. Complex III (ubiquinol cytochrome *c* oxidoreductase) activity was determined as a function of the increase in absorbance from cytochrome *c* reduction. Complex IV (cytochrome c oxidoreductase) activity was determined as a function of the decrease in absorbance from cytochrome *c* oxidation in the presence of KCN. Citrate Synthase (CS) was measured as a function of the increase in absorbance from 5,5'-dithiobis-2-nitrobenzoic acid reduction. All measurements were performed using the BioTek Synergy H1 spectrophotometer (BioTek, Winooski, VT, USA). Each enzyme activity was normalized to citrate synthase. Due to limited sample, only a subsample of individuals for *M. musculus* and *P. maniulatus* were measured for complex II, complex III, and complex IV activity.

Protein Content Determination

To determine protein expression via Western blot analysis, total protein content of the isolated mitochondria from pectoralis skeletal muscle in house finches and hindlimb muscle in deer and house mice was determined via Bradford [160]. Isolated mitochondria from house finches were diluted with a 1:40 dilution factor and isolated mitochondria from both house mice and deer

mice were diluted with a 1:20 dilution factor. Samples were run in triplicate. Protein concentration was determined with use of a standard curve.

Western Blot Analysis

Homogenates were prepared for Western blotting using 4x Laemmli buffer at 1 µg/µL. Subsequently, 15 µl of prepped sample were separated using polyacrylamide gel electrophoresis via pre-casted 4-15% polyacrylamide gels (BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Amresco, Solon, OH, USA) for 2 hours at 200 mA. Nonspecific sites were blocked for 1 hour at room temperature in TBS solution containing 0.05% Tween and 5% nonfat milk. Membranes were then incubated for 1 hour with primary antibodies directed against the proteins of interest. The primary antibodies used were superoxide dismutase 2 (SOD2; # GTX116093; GeneTex), glutathione peroxidase 1 (GPX1; # GTX116040; GeneTex), and 4-hydroxynonenal-conjugated proteins (4-HNE, # ab46545; Abcam, Cambridge, MA, USA). Following incubation with primary antibodies, membranes were washed extensively with TBS–Tween and then incubated with secondary antibodies for 1 hour. Membranes were then developed using an enhanced chemiluminescent reagent (Amersham, Pittsburgh, PA), and imaged using UVP Machine (UVP, LLC, Upland, CA, USA). Band densitometry was performed with ImageJ software (NIH, Bethesda, MD, USA). Ponceau staining was used as the normalizing control. Due to limited sample, only a subsample of individuals were measured for all species.

Oxidative Damage Measures

Lipid and protein oxidation were measured in the pectoralis skeletal muscle and the liver tissue. Lipid peroxidation was determined using 4-hydroxynoneal (4HNE) antibody for a Western blot (described above). Protein oxidation was determined by measuring protein carbonyls with the

Oxyblot kit (EMD Millipore; Belliricia, MA, USA), as outlined by the manufacture instructions. Briefly, tissue homogenates were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by a reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incubated with the primary and secondary antibodies provided in the kit. Membranes were imaged with UVP Machine, and band densitometry was determined with ImageJ software. Due to limited sample, only a subsample of individuals were measured for all species.

Statistics

All statistics were performed using Prism (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed to determine statistical significance ($p<0.05$). If significance was determined, a Tukey's Post-Hoc test was performed. All data are presented mean±standard deviation values.

Results

Overall mitochondrial function does not differ between house finches, house mice, or deer mice

Mitochondrial respiration measurements were performed with mitochondria isolation from house finch pectoralis and deer and house mice hindlimb skeletal muscle. Using complex I substrates, state 3 (maximal ATP production) was significantly different across species ($p<0.001$; Figure 1A). A post-hoc analysis determined there was a higher state 3 in house finches compared to deer mice ($p<0.001$), and higher state 3 in house mice compared to deer mice ($p=0.001$). Likewise, state 4 (basal ATP production) using complex I substrates was statistically significant across species ($p=0.0016$; Figure 1B). Further analysis determined higher state 4 values for house finches compared to deer mice ($p=0.0021$), and higher state 4 in house mice compared to deer

mice (p=0.007). Overall mitochondrial function, determined by the respiratory control ratio (RCR), was not statistically significant between species (p=0.0651; Figure 1C).

Using complex II substrates, state 3 was significantly different across species (p<0.0001; Figure 1D). A post-hoc analysis determined significantly higher state 3 in house finches compared to deer mice (p<0.0001), and higher state 3 in house mice compared to deer mice (p<0.001). Similarly, complex II state 4 was also significantly different across species (p<0.0001; Figure 1E). Significantly higher state 4 was observed in house finches compared to deer mice (p<0.0001) and higher in house mice compared to deer mice (p<0.0001). No significant differences were observed for the complex II RCR between species (p=0.6427).

Complex I – IV activities differ between house finches, house mice, and deer mice

Complex I – IV activities were determined in the skeletal muscle mitochondria. Complex I activity was significantly different across species (p<0.0001; Figure 2A). Specifically, house finches and house mice were observed to have higher complex I activity compared to deer mice mitochondria (p<0.0001 and p=0.0038, respectively). Complex II activity was also significantly different across species (p=0.0035; Figure 2B). Further analysis revealed that house finches had higher complex II activity compared to both house mice and deer mice (p=0.0349 and p=0.0059, respectively). There was no significant difference between species of mice (p=0.8603). Complex III activity was statistically significantly different across species (p=0.0002; Figure 2C). Interestingly, house finches and house mice had significantly less complex III activity compared to deer mice (p=0.0003 and p=0.0004, respectively). Finally, complex IV activity was significantly different across species (p<0.0001; Figure 2D). House finches had lower complex IV activity compared to house mice (p=0.0034) and deer mice (p<0.0001), and house mice had lower complex IV activity compared to deer mice (p<0.0001).

Antioxidants have different expression between avian and mammalian species

Protein expression of antioxidants SOD2 and GPX1 were determined in the skeletal muscle mitochondria. SOD2 protein expression was significantly different across species ($p=0.0158$; Figure 3A). Post-hoc analysis determined house mice had significantly more SOD2 than the house finches ($p=0.0123$) and were not significantly different from deer mice ($p=0.1391$). Furthermore, house mice and house finches presented similar amounts of SOD2 expression ($p=0.3774$).

GPX1 protein expression was also significantly different across species ($p=0.0002$; Figure 3B). House finches expressed significantly more GPX1 compared to both house mice ($p=0.0264$) and deer mice ($p=0.0001$). Additionally, house mice had more GPX1 expression than deer mice ($p=0.0467$).

House finches showed no protein oxidation, but similar levels of lipid oxidation compared to mammalian species

Lipid oxidation was determined via the 4HNE marker. The three species measured had similar levels of lipid oxidation ($p=0.1401$; Figure 4A). Interestingly, the Western blot images show house finches had different sized proteins with lipid oxidation compared to the house mice and deer mice. Visual analysis of the bands would suggest house finches had smaller proteins with lipid oxidation whereas mice had an even size distribution of proteins with lipid oxidation.

Protein damage was determined by measuring total protein carbonyls of the skeletal muscle mitochondria. OxyBlot showed a significant difference across species ($p=0.0002$; Figure 4B). Post-hoc analysis determined that house finches had significantly less protein carbonyls compared to both house mice ($p=0.0315$) and deer mice ($p=0.0001$). Additionally, house mice had significantly less protein carbonyls compared to deer mice ($p=0.0303$).

Discussion

Birds have a superior capability of fat oxidation compared to mammals [141, 142, 146, 161, 162]. In part, this difference is due to the differences in mitochondrial physiology between these animals. The purpose of this study was to compare wild house finches, deer mice, and house mice to further investigate how mitochondrial physiology differs across these animals.

To determine mitochondrial respiration differences, mitochondria were isolated from the skeletal muscle of animals and carbohydrate substrates were used to determine respiration driven by complex I and complex II. The current study reports state 3 and state 4 rates being significantly lower for the deer mouse (*P. maniculatus*) compared to the house mice (*M. musculus*) and the wild house finch (*H. mexicanus*). No difference was observed between the house mice and house finch. This data is consistent with previous reports have found that ATP production via ATP Synthase is lower in deer mice compared to house mice [149]. Moreover, other researchers reported ATP synthesis rates to be higher in a pigeon muscle compared to rat soleus or biceps muscles of rats [146]. Additionally, the current data is supported by reports on mitochondria respiration between a rat and a pigeon in which no difference in state 3 respiration, higher state 4 respiration in the pigeon, and no difference in RCR values when using complex II substrates were reported [155]. These previous literature data are generally consistent with the pattern collected in the current study where house finches had higher state 3 and state 4 values compared to deer mice. The current study also reports no differences in overall mitochondrial function, as determined by the RCR, across the three species. An earlier study has reported no differences in state 3, state 4, or RCR between sparrow pectoralis muscle and rat hindlimb muscle when using carbohydrate substrates [161]. This data is consistent with the current report of no differences in state 3 and state 4 values between the house mice and the house finch, and no differences in the overall mitochondrial function across species. Contrary to the current findings, previous investigators have observed

birds have a lower oxygen consumption rate compared to mammals in isolated fibroblasts [154]. The methodological differences between the aforementioned study, which used cells, and the current investigation, which measured isolated mitochondria from tissue, may explain the contradicting results. It is particularly interesting that the current study observed no significant differences between the wild house finches and the house mice for state 3 and state 4 measurements. More research should be done to replicate and confirm these results, and possibly explain why such a phenomenon occurs.

We also investigated the enzyme activities of each complex along the electron transport chain. Notably, significant differences were presented for each complex across species. Deer mice had significantly lower complex I activity compared to both the house finches and the house mice. These data follow the consistent pattern observed for complex I state 3 mitochondrial respiration measurements. This is not surprising since state 3 represents maximal respiration rates, and enzyme activity assays determine enzyme rates in a saturated environment to produce maximal enzyme rates. Therefore, complex I activity, an enzyme that contributes greatly to complex I state 3 respiration, should follow similar patter to complex I state 3 rates observed. Additionally, the current study reports complex II activity being higher in the house finches compared to the house mice or the deer mice. No differences were observed between the two species of mice. This is consistent with the notion that complex II is involved in fat oxidation, which is more abundant in birds than mammals [141, 142]. Additionally, these data follow similar patterns of house finches having higher complex II state 3 mitochondrial respiration compared to deer mice. Following the same logic for the complex I activity results, since complex II enzyme activity contributes greatly to complex II maximal respiration, it is not surprising these data follow similar patterns. Complex III activity is currently reported as highest in the deer mice compared to the house finches or the

house mice. Lastly, complex IV activity was observed to have a step wise increase, such that house mice had higher complex IV activity compared to the wild house finches, and deer mice had the highest complex IV activity.

Interestingly, high-altitude deer mice have previously been shown to have significantly higher mitochondrial volume compared to low-altitude deer mice, mainly driven by the increase in subsarcolemmal mitochondria [163, 164]. Previous authors have hypothesized that the similar distribution of intermyofibrillar mitochondria between high-altitude and low-altitude deer mice suggest a similar capacity to produce ATP [163]. However, the increase in subsarcolemmal mitochondrial increases their ability to take up and use oxygen, thereby generating the proton motive-force for ATP production [163]. Importantly, complex III and complex IV are both involved with generating the proton motive force and oxygen consumption. Given these functions of the proteins, the proposed hypothesis of the previous authors is consistent with the current data which observes similar overall mitochondrial function across species (RCR) but higher complex III and IV activity in deer mice. Additionally, the same researchers have also noticed high-altitude bar-headed geese having higher subsarcolemmal mitochondria compared to low flying geese [163]. Considering that house finches also fly at low altitude, the lower complex III and complex IV activity may reflect the difference in subsarcolemmal mitochondrial content between house finches and deer mice. Future research should continue to investigate the differences in mitochondrial morphology and complex activity in birds and mammals.

Importantly, the complex activity data in the mice species reported in the present study is mostly consistent with what has been previously published in the literature [149]. However, it is worth highlighting these previous authors reported deer mice having lower complex III activity compared to the house mice [149]. This previous investigation used *P. leucopus* whereas the

current investigation used *P. maniculatus*. The difference in species may account for the difference in complex III activity results. Seeing as no previous data, to our knowledge, has investigated complex activities in birds versus mammals, future research should continue to investigate how each complex may be aiding in the overall mitochondrial respiration in these animals.

The current data on SOD2 and GPX1 expression is inconsistent with previously reported data. Montgomery et al. reported total muscle mitochondria SOD activity was higher in pigeons than rats and GPX activity was higher in rats than pigeons [155]. This study reports less SOD2 expression in house finches compared to house mice and higher amounts of GPX1 expression in house finches than both the species of mice. Differences in measurements and species may be able to account for the inconsistent results between studies. We also reported no significant differences in SOD2 content between mice species, which has previously been reported [149]. However, these authors also reported deer mice having higher amounts of GPX enzyme activity, whereas we report house mice having higher expression of GPX1 than deer mice. Importantly, these authors reported enzyme activity in skeletal muscle homogenates whereas the current study determined enzyme protein expression in isolated mitochondria [149]. These methodological differences could explain the inconclusive results.

Previous literature has reported deer mice to have less ROS production and higher resistance to oxidative damage than house mice [147-149]. Our data on lipid and protein damage does not support these studies. We found there was no significant difference in lipid oxidation across species, and that deer mice had more protein damage compared to house mice and house finches. Birds have previously shown lower 8-OHgG values, a marker of DNA damage, in both the heart and brain compared to mammals [158]. Our data of minimal protein oxidation in the skeletal muscle mitochondria of house finches would be consistent with birds having less oxidative

damage compared to mammals. Other scientists hypothesize that the decrease in damage observed in birds is due to a higher antioxidant amount and activity, which is supported with data from rat versus pigeon comparison studies [155, 157]. It is worth noting that the house finches used in the current study are within their hatch year and the mice species had a wide variation in ages (25.5% maximal lifespan – 39.9% maximal lifespan). Therefore, the young age of the house finches may contribute to the low protein damaged whereas the variation in age will increase the amount of protein damage observed in the house and deer mice.

This study is not without limitations. First, mitochondrial respiration was not measured using Palmitoyl-L-carnitine substrate. Therefore, the data collected does not provide a direct comparison of mitochondrial function during β-oxidation. Comparison of mitochondrial respiration was performed with carbohydrates as the metabolic substrates. This is not always physiologically relevant to birds, given their high reliance on fatty acids for metabolic fuel [140-142, 146, 161, 162, 165, 166]. Second, the different muscles used to isolate mitochondria have inherently different fiber types. The bird pectoralis is more oxidative than mammalian type I muscle fibers, which allow them to have a much higher content of mitochondria. Hindlimb skeletal muscle of mice are much more mix muscle fiber types, therefore providing less mitochondria due to a higher content of type II muscle fiber types that exist in the hind limb skeletal muscles. Third, we did not investigate possible differences between bird and mammal uric acid system. Uric acid is a non-enzymatic antioxidant that is heavily used by avian species [167-170]. It has previously been documented that in migrating Hudsonian godwits, uric acid and total antioxidant capacity increases to counter the amount of ROS which may be produced during long migrations [171]. Thus, uric acid could have contributed to the decreased amount of protein damaged observed in the current study, and future studies are needed to further explore this potential mechanism.

Additionally, the animals all came from different living conditions. The birds were captured from the wild, and therefore represent natural living mitochondrial functioning measurements. The deer mice were maintained in a semi-natural enclosure while the house mice were maintained in standard boxes. Although each animal had access to either a wheel or enough area to be active, these differences in housing may affect overall energy expenditure, and therefore effect the mitochondrial measurements made in this study. Lastly, antibodies used for the western blots were not different for the birds and the mammal samples. It is possible that there could be a difference in cross-reactivity of the antibodies used with each species that could also impact the results reported.

The results from this study demonstrate the vast differences between birds and mammals. The novelty of the study reports data on skeletal muscle mitochondria, rather than whole tissue homogenate, allowing for a unique look into the different properties of the mitochondria across species. Importantly, this work highlights the increased ability of birds to break down fatty acids, and the heightened antioxidant defense to decrease protein damage. Further studies should continue to investigate the mitochondrial differences between birds and mammals to gain a better understanding to the metabolic and oxidative differences between these animals.

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MANUSCRIPT FIGURES

Figure 1. ***Mitochondrial respiration in house finches, house mice, and deer mice.***

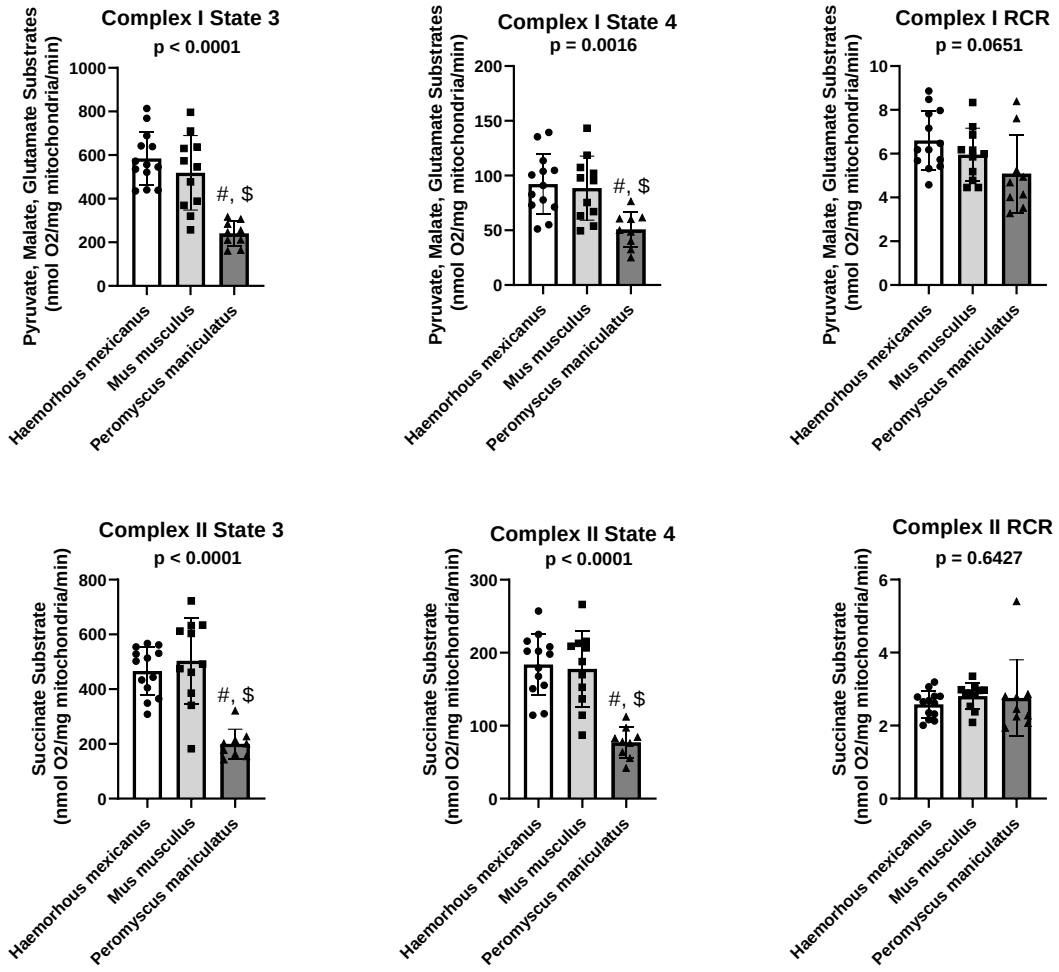


Figure 2. ***Electron transport chain complex activities in house finches, house mice, and deer mice.***

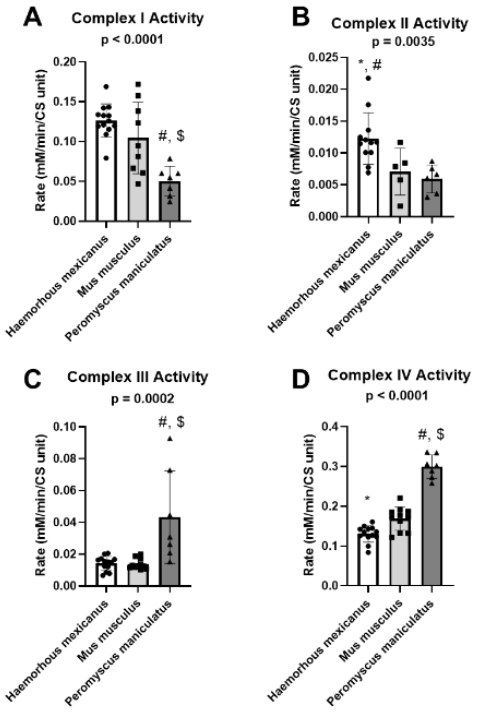


Figure 3. ***Antioxidant expression in house finches, house mice, and deer mice.***

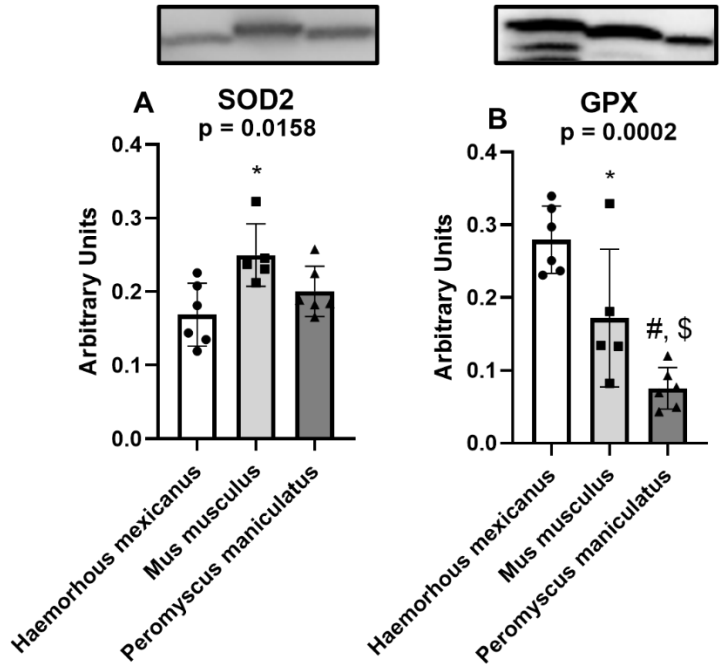
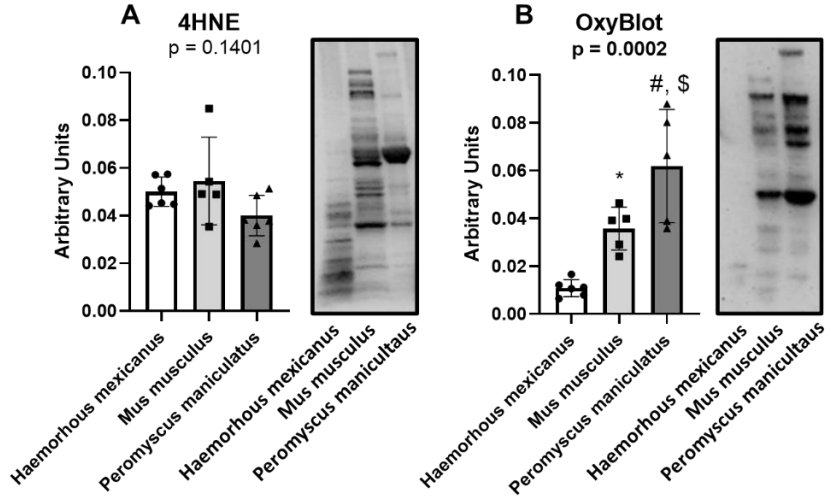


Figure 4. ***Oxidative damage in isolated mitochondria from house finches, house mice, and deer mice.***



MANUSCRIPT FIGURE LEGENDS

Figure 1. ***Mitochondrial respiration in house finches, house mice, and deer mice.*** Mitochondria from skeletal muscle was isolated to determine mitochondrial respiration using carbohydrate substrates to determine oxygen consumption driven by complex I and complex II. Significance was defined as $p < 0.05$. * represents significance between house finches (*H. mexicanus*) and house mice (*M. musculus*), # represents significance between house finches (*H. mexicanus*) and deer mice (*P. maniculatus*), and $ represents significance between house mice (*M. musculus*) and deer mice (*P. maniculatus*).

Figure 2. ***Electron transport chain complex activities in house finches, house mice, and deer mice.*** Enzyme activity for complex I – IV was determined using isolated mitochondria from the skeletal muscle. Significance was defined as $p < 0.05$. * represents significance between house finches (*H. mexicanus*) and house mice (*M. musculus*), # represents significance between house finches (*H. mexicanus*) and deer mice (*P. maniculatus*), and $ represents significance between house mice (*M. musculus*) and deer mice (*P. maniculatus*).

Figure 3. ***Antioxidant expression in house finches, house mice, and deer mice.*** Western blots were performed on the isolated mitochondria from the skeletal muscle to determine protein expression of the antioxidants SOD2 and GPX. Significance was defined as $p < 0.05$. * represents significance between house finches (*H. mexicanus*) and house mice (*M. musculus*), # represents significance between house finches (*H. mexicanus*) and deer mice (*P. maniculatus*), and $ represents significance between house mice (*M. musculus*) and deer mice (*P. maniculatus*).

Figure 4. ***Oxidative damage in isolated mitochondria from house finches, house mice, and deer mice.*** Using the isolated mitochondria from the skeletal muscle, lipid (4HNE) and protein (OxyBlot) damage was determined via Western blot. Significance was defined as $p < 0.05$. * represents significance between house finches (*H. mexicanus*) and house mice (*M. musculus*), # represents significance between house finches (*H. mexicanus*) and deer mice (*P. maniculatus*), and $ represents significance between house mice (*M. musculus*) and deer mice (*P. maniculatus*).