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The Impact of Voluntary Running on Mitochondria in Liver and Skeletal Muscle

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The Impact of Voluntary Running on Mitochondria in Liver and Skeletal Muscle

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Microbiology, Immunology and Molecular Genetics

by

Claudia Adrienne Aliman

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ABSTRACT OF THE THESIS

The Impact of Voluntary Running on Mitochondria in Liver and Skeletal Muscle

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Claudia Adrienne Aliman

Master of Science in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles, 2020

Professor Aldons Lusis, Chair

Exercise is an effective means of preventing and treating metabolic disorders such as insulin resistance. The mechanism by which exercise prevents complex diseases, however, is not well understood. Mitochondria are intracellular organelles intimately linked with metabolic disorders. Mitochondria are unique in that they possess their own, albeit incomplete, genetic material termed mitochondrial DNA (mtDNA). The primary objectives of this thesis were to: (1) determine the impact of voluntary aerobic exercise upon mtDNA in various metabolic organs and (2) elucidate the possible mechanism(s) by which exercise influences mtDNA. To determine mitochondrial abundance, mtDNA is used as a surrogate readout. We subjected female mice from the Hybrid Mouse Diversity Panel (HMDP) to two separate lifestyles, with or without voluntary aerobic exercise. In skeletal muscle and liver, exercised animals showed a significant increase in mtDNA content compared to the unexercised sedentary group. Average running speed was positively correlated with mtDNA content in skeletal muscle. Candidate genes that may be involved in the increased mtDNA content have also been identified. Future research should focus on thorough in-depth studies examining the genes involved in the mechanism(s) by

which this increase occurs and if such increase confer the health benefits associated with exercise such as reductions in liver fats using genome-wide association studies.

The thesis of Claudia Adrienne Aliman is approved.

Robert Gunsalus

Steve Bensinger

Aldons Lusis, Committee Chair

University of California, Los Angeles

2020

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CHAPTER 1: Introduction

Exercise decreases the risk for developing obesity and Type 2 diabetes, complications of which are the leading causes of mortality worldwide (Zheng et al., 2018). There is mounting evidence that exercise is effective in reducing at least 26 diseases ranging from psychiatric, neurological, pulmonary, cancer, and cardiovascular diseases (Pedersen and Saltin, 2015). Recently, interest has grown in understanding the relationship between exercise and mitochondria because mitochondrial dysfunction has been associated with metabolic diseases (Pedersen and Saltin, 2015; Groennebaek et al., 2020; Wahwah et al., 2020; Konopka and Nair, 2013). Therefore, I investigated the impact of exercise on mitochondrial DNA (mtDNA) levels, a surrogate readout of mitochondrial abundance, in skeletal muscle and liver tissue, two metabolic organs with pivotal roles in metabolic diseases. I hypothesized that mtDNA levels increase following long-term voluntary exercise training in both metabolic organs due to changes in gene expressions related to mitochondrial biogenesis and dynamics.

Exercise elicits health benefits that most likely involve multiple biological systems, spanning from single cells to complex organ systems (Reugsegger and Booth, 2018). Exercise is generally characterized as a planned, structured and repetitive physical activity that expends energy at an active rate; it is a means to improve or maintain one or more aspects of the physical state (Bouca-Machado et al., 2020). Furthermore, exercise can be categorized accordingly to frequency, intensity, type and time (FITT) concepts (Bouca-Machado et al., 2020). Using the FITT concepts, there are four different types of exercise: endurance, resistance, flexibility, or a combination of balance and multi-component based exercise (Bouca-Machado et al., 2020). Endurance exercise focuses on moving large muscle groups rhythmically and repetitively over a set period of time. Resistance exercise focuses on pushing/pulling an applied force or weight so

as to improve skeletal muscle growth. Flexibility exercise focuses on recovering and extending of one's range of motion. Balance and multi-component exercise focuses on a multitude of different activities meant to increase lower body strength and stability (Bouca-Machado et al., 2020).

To investigate how exercise affects the mitochondria in diverse genetic backgrounds, I utilized the robust Hybrid Mouse Diversity Panel (HMDP). The HMDP is a collection of approximately 100 well-characterized inbred strains of mice that can be used to analyze the genetic and environmental factors underlying complex traits (Lusis et al., 2016). While not nearly as powerful for mapping genetic loci to connect traits as human genome-wide association studies, the HMDP has several important advantages compared to human GWAS studies: environmental factors can be controlled, all tissues are accessible for global molecular phenotype, both of which are not achievable in human studies and, inbred strains are renewable, allowing results from separate studies to be integrated (Flint and Eskin, 2012). Thus far, the HMDP has been studied for traits relevant to obesity, Type 2 diabetes, atherosclerosis, osteoporosis, heart failure, immune regulation, fatty liver disease, and host-gut microbiota interactions. High-throughput technologies have been used to examine the genomes, epigenomes, transcriptomes, proteomes, metabolomes, and microbiomes of the mice under various genotypic or environmental conditions. (Lusis et al., 2016).

Chapter 2: Literature Review

In the mitochondria, there are two major processes that occur: mitochondrial biogenesis and mitochondrial dynamics. Mitochondrial biogenesis is the growth and division of mitochondria (Ventura-Clapier et al., 2008). Mitochondrial dynamics include mitochondrial fusion and fission as well as the expression and regulation of genes that these processes (Liesa et al., 2009). Exercise can regulate and influence mitochondrial biogenesis and dynamics processes through mechanisms such as oxidative phosphorylation (Holloszy, 1967; Hood, 2001; Liesa et al., 2009; Tanaka et al. 2020; Perry and Hawley, 2018; Reugsegger and Booth, 2018; Bishop et al., 2019). Studies utilizing robust techniques such as transmission electron microscopy and biomarkers consisting of citrate synthase activity and cardiolipin, have confirmed that exercise impacts the mitochondria (Groennebaek et al., 2020; Bishop et al., 2019). However, these techniques are unable to address how exercise can specifically regulate and influence mitochondria in real time, which can elucidate how specific health benefits vary among different populations (Groennebaek et al., 2020; Bishop et al., 2019; Perry and Hawley, 2018). Thus, the use of the Hybrid Mouse Diversity Panel, a collection of approximately 100 inbred strains of mice of various traits, is a valuable resource to demonstrate how exercise may impact based on genetic variations (Lusis et al., 2016).

Exercise

Aerobic exercise is defined as repetitive, constant rhythmical movements (contraction and relaxation) of large muscle groups over a period of time (Yu, 2011; Dimeo et al., 1998; Mersy, 1991). It has been shown to be beneficial for the neuromuscular, metabolic,

cardiovascular and respiratory systems (Wilmore and Knuttgen, 2003). Exercises that would fit into the aerobic exercise category would be activities that are low intensity and done over a long period of time (Mersy, 1991; Vuori, 2020).

Exercise can improve cardiorespiratory fitness, with several studies showing that there is a decreased mortality rate amongst those who engage in regular physical activity (Ruegsegger and Booth, 2018). Aside from improving cardiorespiratory fitness, it can also improve mental health (Ruegsegger and Booth, 2018) and further studies have since then been conducted on how exercise could have an impact on many different biological systems in the body. Exercise has been shown to improve in cognition, depression, anxiety, neurodegenerative diseases and even responses to drug addictions (Ruegsegger and Booth, 2018). Several studies suggest a combination of diet control and exercise are able to reduce the occurrence of Type 2 diabetes (Pan et al., 1997; Tuomilehto et al., 2001; Knowler et al., 2002; Ruegsegger and Booth, 2018).

In addition to the studies highlighting exercise's positive effect on the body, there are many studies linking increased mitochondrial activity with exercise. Seminal work indicates that mitochondrial activity increases after a high level of aerobic exercise (Holloszy, 1967). Further studies have reported that skeletal muscle peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), a master regulator of mitochondrial biogenesis, could be activated during endurance exercise resulting in higher mitochondrial DNA (mtDNA) copy numbers and mRNA levels of genes associated with mitochondrial biogenesis (Bishop et al., 2019; Reugsegger and Booth, 2018). Other than PGC-1α, other small molecules such as transcription factor EB (TFEB) can regulate the rapid switch between glucose and fatty acid substrates in skeletal muscle tissue during endurance exercise, leading to increased ATP production (Reugsegger and Booth, 2018).Furthermore, there are many studies that show that the

manipulation of a single gene can alter mitochondrial levels and endurance exercise performance (Reugsegger and Booth, 2018).

In skeletal muscle, there are high concentrations of mitofusin 1 and 2 (MFN1/MFN2), both of which increase in response to exercise (Liesa et al., 2009). MFN1/MFN2 are transmembrane GTPase proteins found on the outer mitochondrial membrane; MFN2 is also found on the endoplasmic reticulum (Liesa et al., 2009). Both proteins play a role in mitochondrial fusion which is a two-step process where the outer and inner mitochondria fuse (Liesa et al., 2009). In obese patients, skeletal muscle mitochondria have reduced expression levels of MFN2 and the size of the organelle is reduced (Liesa et al., 2009). Deletion of MFN1/MFN2 can also result in decreased performance in exercise and training (Bell et al., 2019). Mitochondria in the liver and the skeletal muscle, however, are independently regulated with exercise impacting mitochondrial biogenesis in the liver in a selective manner reducing transcription factor A (TFAM) expression, cyclooxygenase 2 (COX2) expression and mtDNA content (Lezi et al., 2013).

Mitochondria

The mitochondria is an important intracellular organelle that contains its own DNA (mtDNA) and is the major source of ATP produced by oxidative phosphorylation (OXPHOS) pathway (Krasich and Copeland, 2017; Smeitink et al., 2006). Human and mouse mtDNA are circular genomes that consists of 16,569 base pairs and encoding 37 genes that are necessary for ATP production (Young and Copeland, 2016). Of these 37 genes, 13 genes encode for proteins necessary for the mitochondrial electron transport chain (Complex I, Complex III, and Complex

IV) with the remaining 24 genes encoding for 22 transfer RNAs and two ribosomal RNAs that are needed for synthesizing the 13 mitochondrial polypeptides (Kolesar et al., 2014; Young and Copeland, 2016). A single cell can contain anywhere from a few dozen to thousands of mtDNA copy numbers and are often distributed in an interconnected network of reticular mitochondria (Miller et al., 2003; Young and Copeland, 2016). Proteins such as TFAM can interact with mtDNA to form structures called nucleoids giving protection and allow for the replication, transcription and repair of the mtDNA in a secure environment (Hensen et al., 2014). Mitochondrial DNA encodes for less than 2,000 proteins and all other proteins must be imported into the mitochondria (Wahwah et al., 2020). This complex transportation system allows the mitochondria to control its own proteome and its function (Wahwah et al., 2020). Furthermore, sub-populations of mitochondria within a single tissue or in other different tissues could also exhibit differences in morphology, biochemical, function, biogenesis, and dynamics (Wahwah et al., 2020; Little et al., 2011).

The mitochondria reduces electrons and consumes oxygen through the electron transport chain resulting in the byproduct reactive oxygen species (ROS) (Kolesar et al., 2014; Ajith, 2018). Low ROS levels can positively affect cell differentiation, immune system activation, and metabolism (Ajith, 2018). Excessive ROS can damage mtDNA, mitochondrial lipids and proteins may occur (Ajith, 2018). Furthermore, high ROS levels can detrimentally affect the OXPHOS pathway resulting in further ROS production and a disastrous feedback loop (Ajith, 2018).

Mitochondria also play a very important role in fatty acid oxidation and glycolysis (Ajith, 2018). Lipids can change the composition of the phospholipids such as cardiolipin in the organelle affecting fatty acid oxidation (Ajith, 2018). When lipids exceed normal levels, it can

negatively affect fatty acid oxidation, altering the sensitivity of carnitine acyltransferase 1, preventing binding to its inhibitor malonyl-CoA (Ajith, 2018). Decreased sensitivity of carnitine acyltransferase 1 can lead to poor transport of long-chain fatty acids into the mitochondrial matrix and to oxidative lipid degradation (Ajith, 2018). Abnormally high lipid levels can affect the OXPHOS pathway by producing excess ROS, activating nuclear factor-kappa β and causing cytokine production which ultimately results in inflammation (Ajith, 2018). Aging, Type 2 diabetes and a sedentary lifestyle can also affect the OXPHOS pathway detrimentally leading to the mitochondria performing poorly (Menshikova et al., 2006; Lanza and Nair, 2009).

ATP production in the mitochondria occurs by conversion of metabolites accumulated at a specific tissue site by enzymes involved in the tricarboxylic (TCA) cycle (Wahwah et al., 2020). The conversion of these metabolites powers of the electron transport chain (ETC) by which protons are pumped across the mitochondrial membrane (Wahwah et al., 2020). The ETC involves electrons being transported across a series of protein complexes embedded in the mitochondria ultimately driving the phosphorylation of ADP to ATP (Wahwah et al., 2020).

Mitochondrial disorders can result from mutations in mtDNA or from mtDNA depletion (Wallace, 1999; Copeland and Longley, 2014). mtDNA depletion is defined as a decrease in mtDNA content in affected tissues but does not harbor any mutations or rearrangements in the mtDNA itself (Mandel et al., 2001). Such mtDNA depletions can be inherited through an autosomal recessive mode of inheritance (Mandel et al., 2001). mtDNA is used as a marker to diagnose these dysfunctions (Kolesar et al., 2014). To deal with mitochondrial disorders, the mitochondria utilizes the ubiquitin-proteasome pathway, which maintains mitochondrial function by removing damaged organelles (Wahwah et al., 2020).

Although mitochondria and its proteins have been studied extensively, many questions remain to be answered. The utilization of transmission electron microscopy as the current standard does not explain how mitochondrial dynamics respond in real-time to certain external factors such as exercise (Groennebaek et al., 2020). Furthermore, long sample preparation time, massive costs, and difficult accessibility can make transmission electron microscopy an ineffective means of analyzing mitochondrial content (Groennebaek et al., 2020). The use of biomarkers of mitochondria, while seemingly effective, also does not explain how mitochondria changes in response to exercise in real-time (Groennebaek et al., 2020). The development of "omics" particularly proteomics does help to overcome these two barriers allowing observation of what sets of proteins are being produced by the mitochondria in real-time, but this still requires the entire mitochondrial content library and imaging of the organelle so as to be able to observe the proteins being secreted by the mitochondria. Thus, the need for more effective assays is in now much greater demand (Groennebaek et al., 2020).

Mitochondrial Dynamics and Biogenesis

Mitochondrial dynamics are defined as the heterogeneity of mitochondria morphology, only including mitochondrial networks and mega-mitochondrial formation (Liesa et al., 2009). It is also involved in the expression and post-translational regulation of genes that control mitochondrial fusion and fission (Liesa et al., 2009). The dynamism of mitochondria play a role in how the cell responds to exercise to meet the cell's demands of homeostasis (Bell et al., 2019; Wahwah et al., 2020).

Mitochondria are constantly in a state of turnover that is regulated by both mitochondrial fusion and fission enabling proper mitochondria function (Wahwah et al., 2020). These two processes enable the exchange of mitochondrial DNA and oxidative enzymes as well as the removal of any dysfunctional mitochondria (Bell et al., 2019). Mitochondrial fusion is the joining of two mitochondria into a single large mitochondrion allowing for matrix contents to intermix (Wahwah et al., 2020). Mitochondrial fission is the process in which a mitochondria are split apart often separating damaged portions for eventually degradation (Wahwah et al., 2020). Despite many advances in the study of mitochondrial dynamics, it is still unknown how mitochondrial dynamics are regulated across different tissues (Liesa et al., 2009). Thus, there have been many studies investigating the genes involved in these two processes considering the importance of their roles in the mitochondria. One such study found that when MFN1/MFN2 are removed, there is a significantly reduced exercise performance due to a reduction in the electron transport chain in the mitochondria (Bell et al., 2019). Another study also found that when the mitochondrial protein OPA1, a mitochondrial protein known to play a role in in the fusion of the mitochondrial inner membrane, is deleted, exercise training-induced mitochondrial biogenesis is impaired (Caffin et al. 2013).

Previous studies of mitochondrial biogenesis have mostly focused on skeletal muscle. Mitochondrial biogenesis is defined by the production of new components of the mitochondrial reticulum relying on the coordinated synthesis and import of about 1,000 proteins encoded by the nuclear genome (Ventura-Clapier et al., 2008; Bishop et al., 2019). Some of these proteins are used to assemble with other proteins encoded by the mitochondrial genome within the synthesized phospholipid membranes of the inner and outer mitochondrial membranes (Ventura-Clapier et al., 2008; Bishop et al., 2019). Proteins such as PGC-1α along with nuclear respiratory

factor 1 (NRF-1) and mitochondrial TFAM also play a role in mitochondrial biogenesis along a series of pathways during exercise (Lezi et al., 2013; Granata et al., 2018). During exercise, signals are transported along these pathways, activating sensor proteins such as AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinases (p38 MAPK) and Sirtuin 1 (Granata et al., 2018). Once these sensor proteins are activated, co-activators such as PCG-1α, the central regulator of mitochondrial biogenesis, and other transcription factors such as p53, NRF1, and NRF2 are expressed and transcribe both the nuclear genome and the mitochondrial genome to express mitochondrial genes in a controlled manner (Wahwah et al., 2020; Granata et al., 2018). Exercise can increase other transcriptional regulators such as estrogen-related receptor (ESRR) and can even activate pathways such as Sirtuin 1 (SIRT1) increasing mitochondrial biogenesis (Granata et al., 2018; Ballmann et al., 2016). Mitochondrial biogenesis is further regulated by miRNAs and epigenetic modifications increasing mRNA content which will be translated and imported into the mitochondria (Granata et al., 2018).

Dysregulation of the mitochondrial biogenesis pathway can result in skeletal muscle tissue fiber deterioration. But exactly how this dysregulation is altering mitochondrial biogenesis is not well-known (DiMauro and Davidzon, 2005; Joseph et al., 2016). There are many factors that could play a part in the dysregulation of the mitochondrial biogenesis pathway. Age is one of several factors that affects mitochondria (Katayama et al., 1991; Cortopassi et al., 1992; Zhang et al., 1992). Age can induce rearrangements in the mitochondrial DNA post-thirty years of age and continues to induce greater rearrangements for the remainder of life (Katayama et al., 1991; Cortopassi et al., 1992; Zhang et al., 1992). As humans age, subpopulations of mitochondria in varying tissues may have different responses to the increasing mitochondrial dysfunction. The reduction in oxidative capacity and proton leakage would lead to a decrease in

energy however only subsarcolemmal mitochondria is observed to have oxidative damage. (Crescenzo et al., 2014). Another study also showed that age-related muscle loss results in lower expressions of PGC-1α which results in reduced number of mitochondria, increased ROS production, and increased levels of oxidative damage resulting (Trifunovic et al., 2004; Kujoth et al., 2005; Hiona et al., 2010; Joseph et al., 2013; Kolesar et al., 2014; Chabi et al., 2008). Aerobic exercise could reduce these detrimental effects of mitochondrial dysfunction by increasing the mitochondria's oxidative capacity in aging adults (Lanza and Nair, 2009).

Genetics/HMDP

Since genome-wide association studies (GWAS) were first introduced, hundreds of loci have been discovered for many different human diseases (Flint and Eskin, 2012). Linkage analysis is a common technique that utilizes two inbred strains to produce offspring that are then either be mated to each other or to a progenitor strain (Flint and Eskin, 2012). Such a technique was used in initial studies in order to identify single-nucleotide polymorphisms (SNPs) that caused disease but this was a difficult and cost-inefficient process as there are hundreds of genes in one quantitative trait loci (Flint and Eskin, 2012). With the utilization of association analysis among different mouse strains, it is easier to locate the causal variant due to greater statistical power, mapping resolution, coverage, and reproducibility (Flint and Eskin, 2012). Of the many types of GWAS designs using mice, there is the classic cross, the classic inbred strain association, the heterogenous stock, the outbred stock which are commercially available, the Collaborative Cross and finally the Hybrid Mouse Diversity Panel (Flint and Eskin, 2012).

Each GWAS design differs in terms of genotyping, breeding, genetic diversity, genomic-wide power, resolution and references. With the classic cross, the heterogenous stock, and commercial outbred stock, they do require genotyping whereas the classic inbred strain association, the Collaborative Cross and the Hybrid Mouse Diversity Panel do not. Only the classic cross requires breeding and only the Collaborative Cross has a diverse range of genetics but the inbred strain association could have high genetic diversity if included with wild-derived inbred strains. The classic inbred strain association also does not have genome-wide power compared to the other panels. As for resolution, the commercial outbred stock has the highest resolution; the Hybrid Mouse Diversity Panel, the classic inbred strain association, and the heterogenous stock has a high resolution; the Collaborative Cross has a medium level of resolution; and the classic cross has the lowest resolution. Furthermore, the reference strains used in each GWAS design differ with the classic cross having the highest number of reference strains with the heterogenous stock and the commercial outbred stock having also a high number of reference strains. The classic inbred stock still requires a high number of reference strains but not as high as the previous three designs with the Hybrid Mouse Diversity Panel and the Collaborative Cross having the lowest number of reference strains (Flint and Eskin, 2012).

The Hybrid Mouse Diversity Panel is a collection of approximately 100 inbred strains of mice which have been extensively studied on a variety of traits over the past decade (Lusis et al., 2016). The diversity panel is composed of some variation of approximately 30 classic inbred strains and 70 or more recombinant inbred strains (Lusis et al., 2016; Gini and Hager, 2012). These recombinant inbred strains consist of a crosses between progenitor strains A/J and C57BL/6J (AXB and BXA), C57BL/6J and DBA/2J (BXD), and C57BL/6J and C3H/HeJ (BXH). The inbred and recombinant inbred strains play two separate genetic roles. The inbred

strains aid in mapping resolution measuring the size of the interval of a gene map and allowing for identification of a number of candidate genes while the recombinant inbred strains give statistical power which measures the likelihood of detection of a locus that may have a genetic effect on a specific trait to the whole data set (Flint and Eskin, 2012; Lusis et al., 2016). With the usage of this panel, one can recreate natural variations under a set of controlled environmental factors and obtain any desired tissue samples (Lusis et al., 2016; Civelek and Lusis, 2014).

Chapter 3: Methods and Materials

Animals

A total of 100 strains of female mice from the HMDP panel were used in this experiment (Table 1). Mice randomly divided into groups (SED = Sedentary or no exercise; EX or TRN = Exercise trained) at three months of age ensuring equal body weight between groups within a strain. TRN mice were singly housed for the duration of the experiment. SED mice were housed one to four per cage.

List of Strains from HMDP			
129S1/SvlmJ	BXD16/TyJ	BXD42/TyJ	DBA/2J
129X1/SvJ	BXD161/RwwJ	BXD44/RwwJ	FVB/NJ
A/J	BXD171/RwwJ	BXD48a/RwwJ	I/LnJ
AKR/J	BXD172/RwwJ	BXD5/TyJ	LP/J
AXB12/PgnJ	BXD18/TyJ	BXD50/RwwJ	MRL/MpJ
AXB19/PgnJ	BXD19/TyJ	BXD56/RwwJ	NOD/ShiLtJ
AXB5/PgnJ	BXD191/RwwJ	BXD6/TyJ	NOR/LtJ
BALB/cByJ	BXD194/RwwJ	BXD66/RwwJ	NZB/BINJ
BALB/cJ	BXD195/RwwJ	BXD68/RwwJ	NZW/LacJ
BTBR T<+> tf/J	BXD2/TyJ	BXD70/RwwJ	PL/J
BXA1/PgnJ	BXD20/TyJ	BXD71/RwwJ	RIIIS/J
BXD1/TyJ	BXD205/RwwJ	BXD73/RwwJ	SEA/GnJ
BXD102/RwwJ	BXD21/TyJ	BXD75/RwwJ	SJL/J
BXD11/TyJ	BXD216/RwwJ	BXD79/RwwJ	SM/J
BXD113/RwwJ	BXD22/TyJ	BXD87/RwwJ	SWR/J
BXD12/TyJ	BXD24/TyJ-Cep290<rd16>/J	C3H/HeJ	TALLYHO/JngJ
BXD124/RwwJ	BXD27/TyJ	C3HeB/FeJ	
BXD125/RwwJ	BXD28/TyJ	C57BL/10J	
BXD128a/RwwJ	BXD31/TyJ	C57BL/6J	
BXD13/TyJ	BXD32/TyJ	C57BLKS/J	
BXD14/TyJ	BXD33/TyJ	C57L/J	
BXD15/TyJ	BXD34/TyJ	CAST/EiJ	
BXD151/RwwJ	BXD38/TyJ	CBA/J	
BXD152/RwwJ	BXD39/TyJ	CXB7/ByJ	
BXD154/RwwJ	BXD40/TyJ	DBA/1J	

Table 1. List of strains from the HMDP Panel used in this study.

Exercise Design

Mice were raised until 3 months of age whilst fed with a normal chow diet and weighed before the exercise regime. Mice were randomly separated into two groups, sedentary and exercise such that the average mouse weights were similar each experimental group. Mice in the exercise group ran on the wheel voluntarily for 30 days. An exercise program software called Vital View STARR monitored the daily running distance, time and speed for each mouse in the exercise group. Immediately following the completion of the 30 days, the running wheels were locked in place for 30 hours to ensure there was no carryover effect from the exercise. Mice from both groups were also fasted for 6 hours to prevent any carryover effects from diet before the mice were sacrificed and harvested for their tissues.

Tissue harvest

Mice were weighed post-exercise before being euthanized by anesthesia and cardiac puncture. Liver and quadriceps muscle tissue was harvested from the sacrificed mice and all collected tissues were weighed and stored at -80℃ temperature.

Tissue Digestion and Isolation of MtDNA

A small homogenous portion of tissue approximately 25 mg in weight was added into 500 ul of extraction buffer consisting of 100 mM NaCl, 10 mM Tris-Cl, pH 8, 25 mM EDTA, pH 8 and 0.5% SDS and 4 µl of 20 mg/ml Proteinase K and incubated/digested for 5 – 18 hours at a temperature of 55℃. After tissue digestion, mtDNA was extracted through a series of

processes. First, 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the digested solution and following removal of the first aqueous phase content and transfer, 400 ul of chloroform/isoamyl alcohol (24:1) was added. Second, following the transfer of second aqueous phase, 150 µl of 7.5 M ammonium acetate and 2 volumes of ice-cold 100% ethanol of approximately 750 µl was added into the 1.5 mL tube that contained the mtDNA. After the final collection, the samples were left to incubate for 30 minutes at -20℃.

Following incubation, the content was centrifuged at 4℃ at maximum speed for 15 minutes to retrieve the DNA pellet. Once observed, the supernatant was removed leaving behind 250 µl and 700 µl of room temperature 75% ethanol was then added before being centrifuged. After centrifuging for 1 minute at 10,000 RPM at room temperature, the supernatant was removed. Another cycle of centrifuging for 30 seconds at 5,000 RPM is done to remove any remaining supernatant. DNA pellets were then left to dry for 10 minutes at room temperature. After drying, Tris-EDTA buffer pH 8.0 was added. The mixture was then vortexed thoroughly and centrifuged at a low speed followed by incubation at 50℃ for 1 hour while being shaken at 300 RPM. Mixture was then centrifuged again for 30 seconds at 5,000 RPM after incubation and was then immediately stored at < -20℃.

qPCR Amplification

Tert and *D-loop* were used in qPCR of the extracted DNA. TERT is consistently expressed across cell types making it a good housekeeping (reference) gene (Ghr.nlm.nih.gov, 2020). The D-Loop (otherwise known as ‘displacement loop’) is a non-coding region within

mtDNA and is conserved across many species making it a viable marker for mtDNA (Sciencedirect.com, 2020).

Further rigorous steps such as cross spot-checking and replicates were taken to ensure errors were at a minimum and that data was as accurate as possible for each qPCR plates for every run. Such errors that could affect the data may include: errors in pipetting, contaminants that may end up in the samples, appropriate controls used, non-validated primers, or handling and treatment of the sample (Taylor et al., 2019). Errors from the qPCR run could be from samples being too dilute, the qPCR calculations that stem from the quantitative cycles, raising PCR efficiency, and obtaining the normalized relative expression for statistical analysis (Taylor et al., 2019). Most importantly, errors can occur from the poor selection of a randomized set of individual samples from the experimental group in the study which may result in differences between samples (Taylor et al., 2019). Subsampling errors may also occur if concentrations of DNA are too low and when quantitative cycles are too high (Taylor et al., 2019). Even the handling of large data sets can result in manipulation of certain data that would propagate errors and lead to a different conclusion (Taylor et al., 2019). For these reasons, normalization of the data is necessary to true expression differences between samples.

RNA Sequencing

Muscle and liver tissues were crushed and homogenized in 800 µL of Trizol using a metal tissue homogenizer for 15 seconds in a 5 mL volume glass test tube.

RNA was isolated using the Qiagen RNA Isoaltion kit following the manufacturer's instructions without deviation. Sample concentration were measured using the NanoDrop

Spectrophotometer. After quantification, RNA quality control was measured by RNA integrity number (RIN) to determine how much of RNA was degraded. Sample with low concentrations or quality (RIN < 7) were not used.

Libraries were made using the KAPA mRNA HyperPrep Kit and KAPA Dual Index Adapter Kits for Illumina platforms follow the manufacturer's instructions. The resultant libraries were quality checked, quantified, pooled, and then sequenced using a Illumina Hi-Seq 3000 within the UCLA TCGB core.

Statistical Analysis

Sequencing output files were aligned and counted using Rsubread. Differential expression and normalization was completed using DeSeq2.

Changes in between mtDNA copy numbers in the exercise vs. sedentary lifestyle were captured using the unpaired t-test on GraphPad Prism 8.4.2 (GraphPad.com, 2020). Observations of the mtDNA copy numbers between the two groups in each different strain were analyzed in Microsoft Excel using t-Test: Two-Sample Assuming Unequal Variances and t-Test: Two-Sample Assuming Equal Variances taken from the Data Analysis packet. Correlations between mtDNA and running traits (average distance run per day, average running speed, and percentage of time running), running pattern analysis, body weight pre-exercise and post-exercise, and tissue weight of liver and quadriceps liver tissues were captured using GraphPad Prism 8.4.2 (GraphPad.com, 2020). Volcano plots of differential expression of liver and quadriceps skeletal muscle were completed using the online software application, VolcaNoseR (Goedhart and Luijsterburg, 2020 PREPRINT).

Chapter 4: Results

In this study, I investigated how voluntary aerobic exercise influenced mtDNA levels in metabolic tissues with the ultimate goal of identifying the mechanisms by which exercise modulates mitochondria levels. I first sought to determine if there was a difference in the levels of mtDNA between groups in metabolic tissues, namely liver and quadriceps muscle. Exercised animals had significantly increased mtDNA levels of about 4% compared to their sedentary counterparts in the liver tissue after the 30-day voluntary aerobic exercise ($P = 0.0004$; Fig. 1). I also observed a significant increase of about 4% in mtDNA levels in quadriceps muscle after a period of 30 days ($P = 0.0001$; Fig. 2).

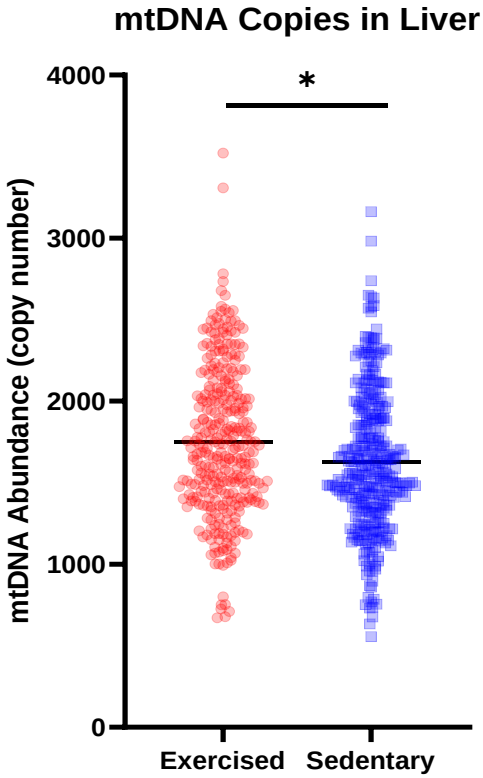

Figure 1. Copy numbers of mtDNA in liver between sedentary lifestyle vs. exercise lifestyle mice. Each bar represents the mean number of mtDNA copy numbers taken from across various strains exposed to a certain lifestyle. Significant difference between strains of individual mice taken from sedentary vs. exercise lifestyle ($p < 0.05$).

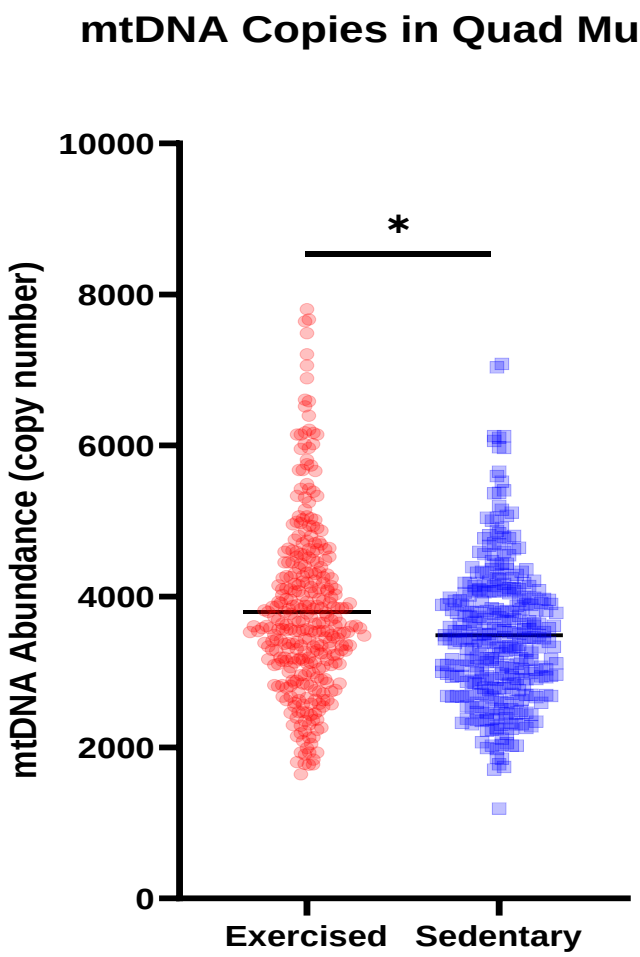

Figure 2. Copy numbers of mtDNA in quadriceps muscle between sedentary vs. exercise lifestyle mice. Each bar represents the mean number of mtDNA copy numbers across taken from the various strains exposed to a certain lifestyle. Significant difference between the strains of individual mice from sedentary vs. exercise lifestyle ($p < 0.05$).

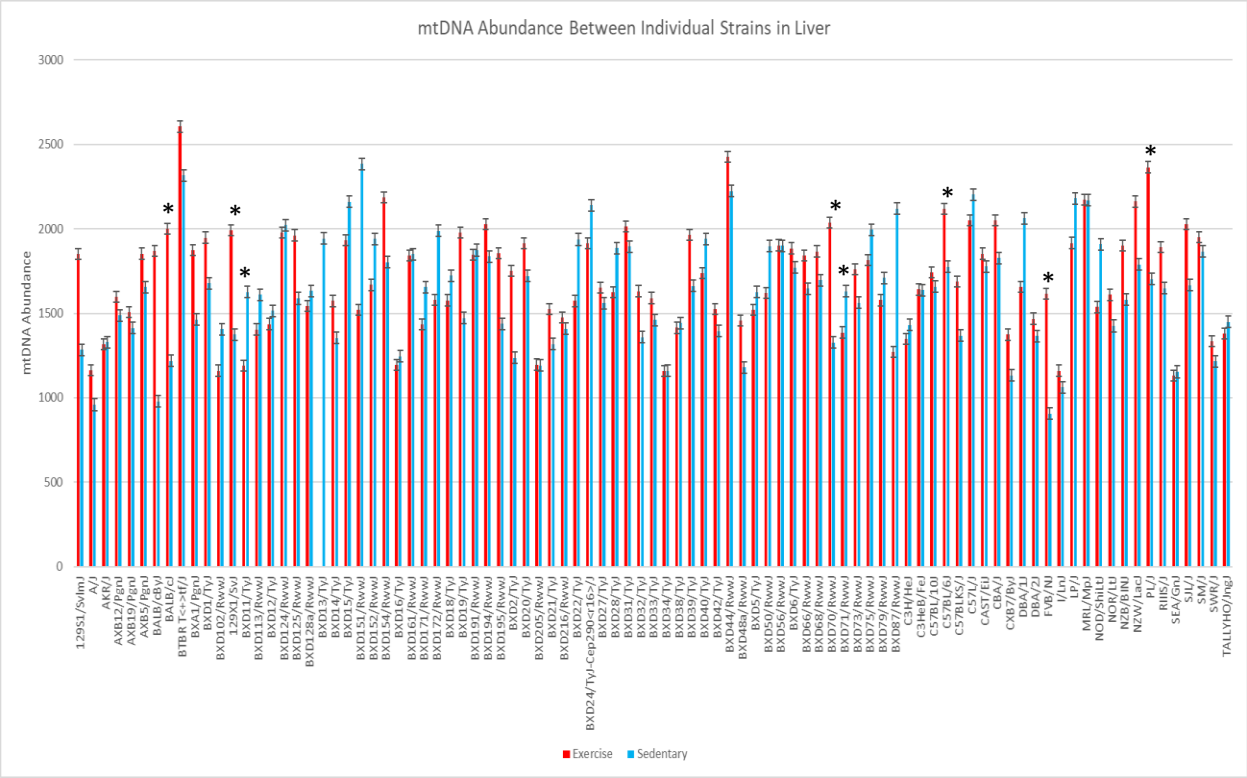

Figure 3. Copy numbers of mtDNA in each of the 91 strains in the liver tissue between sedentary and exercise groups (n = 4). Each bar represents the mean number of mtDNA copy numbers between the two lifestyle groups. Significant differences between mice in each strain exposed to either exercise or sedentary were marked by an asterisk ($p < 0.05$).

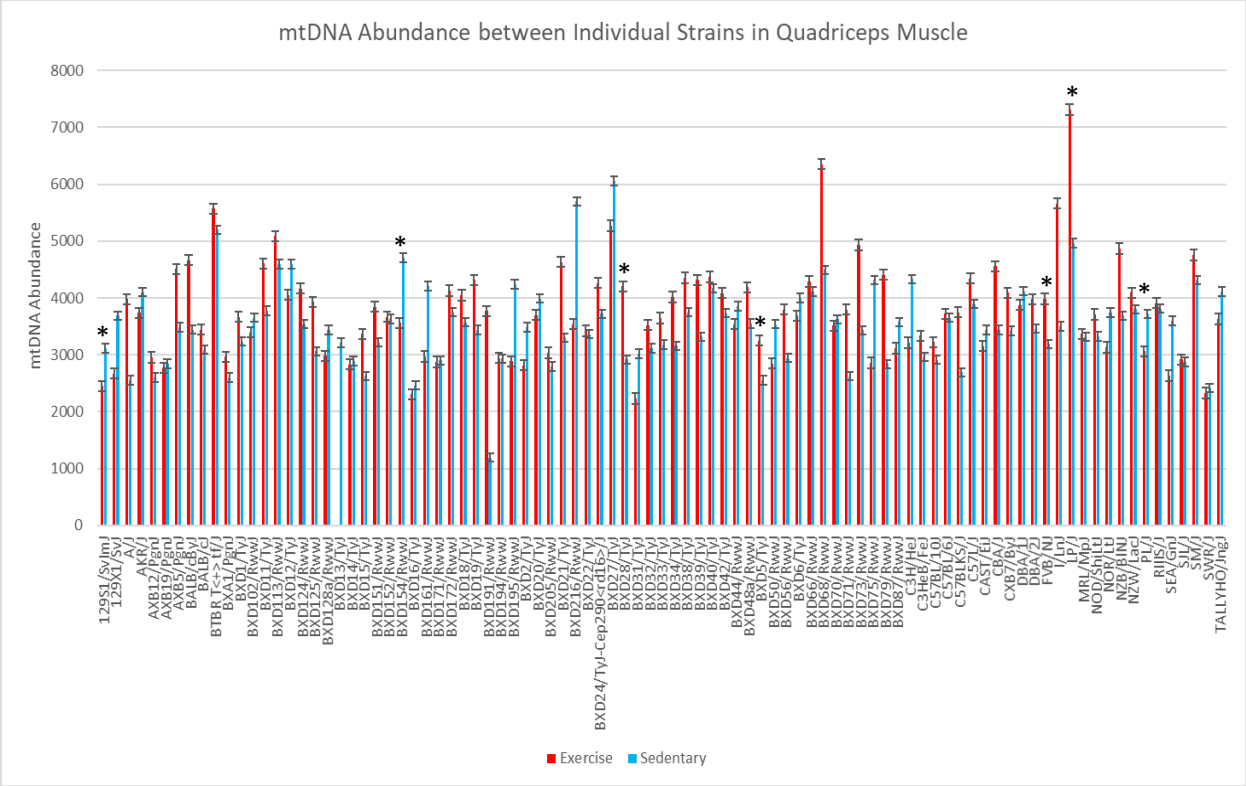

Figure 4. Copy numbers of mtDNA in each of the 91 strains in the quadriceps muscle between sedentary and exercise groups (n = 4). Each bar represents the mean number of mtDNA copy numbers between the two lifestyle groups. Significant differences between mice in each strain exposed to either exercise or sedentary ($p < 0.05$) were marked as by an asterisk.

With clear differences of mtDNA levels between exercised and sedentary mice in both liver and quadriceps muscle post-exercise, I then looked for differences in individual strains to

determine the impact of genetics upon the exercise response. Of the 91 strains from the HMDP panel, only the strains BALB/cJ (P = 0.007383; Figure 3), 129X1/SvJ (P = 0.005783; Figure 3), BXD11/TyJ (P = 0.006013; Figure 3), BXD70/RwwJ (P = 0.006138; Figure 3), BXD71/RwwJ (P = 0.029885; Figure 3), C57BL/6J (P = 0.024242; Figure 3), FVB/NJ (P = 0.001243; Figure 3), and PL/J (P = 0.008739; Figure 3) had significantly increased levels of mtDNA between the exercise and sedentary groups in the liver tissue ($P < 0.05$; Figure 3). In the quadriceps muscle, only the strains FVB/NJ (P = 0.018770; Figure 4), LP/J (P = 0.009376; Figure 4), PL/J (P = 0.027508; Figure 4), 129S1/SvlmJ (P = 0.033361; Figure 4), BXD154/RwwJ (P = 0.008967; Figure 4), BXD28/TyJ (P = 0.011855; Figure 4) and BXD5/TyJ (P = 0.007061; Figure 4) were found to have significantly increased levels of mtDNA between the exercise and sedentary groups ($P < 0.05$; Figure 4).

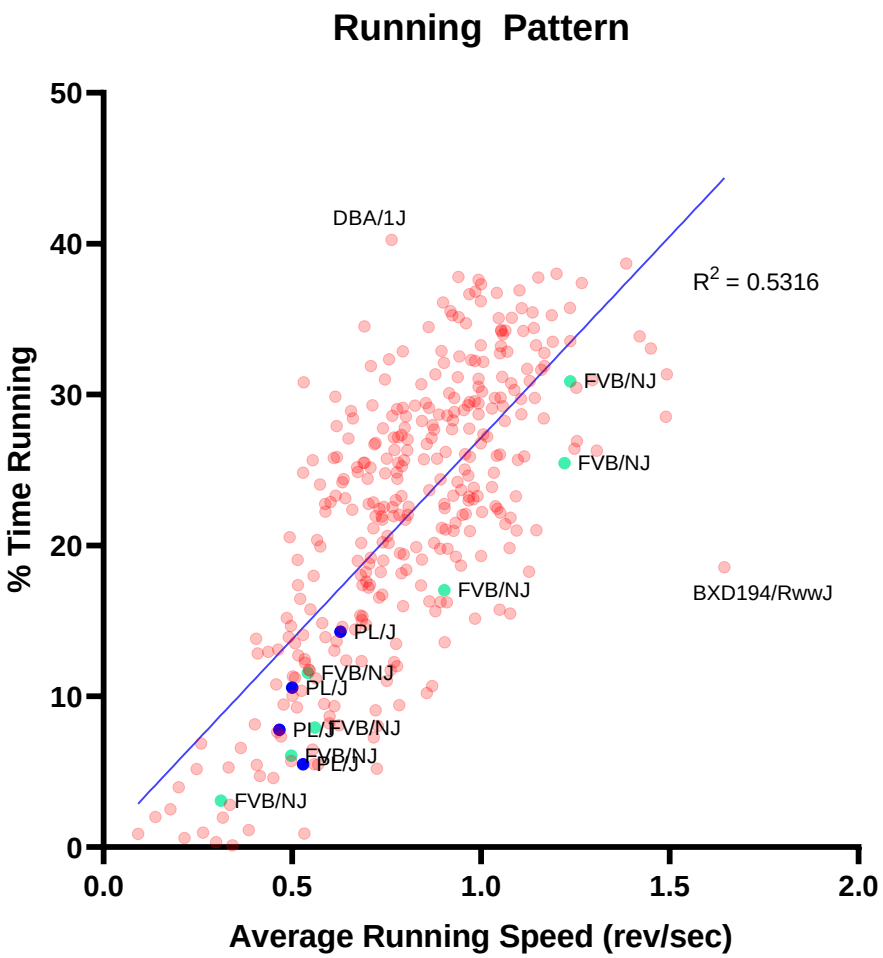

Figure 5a. Relationship of average running speed against the percentage of time running of each mouse from the exercise group ($p < 0.0001$).

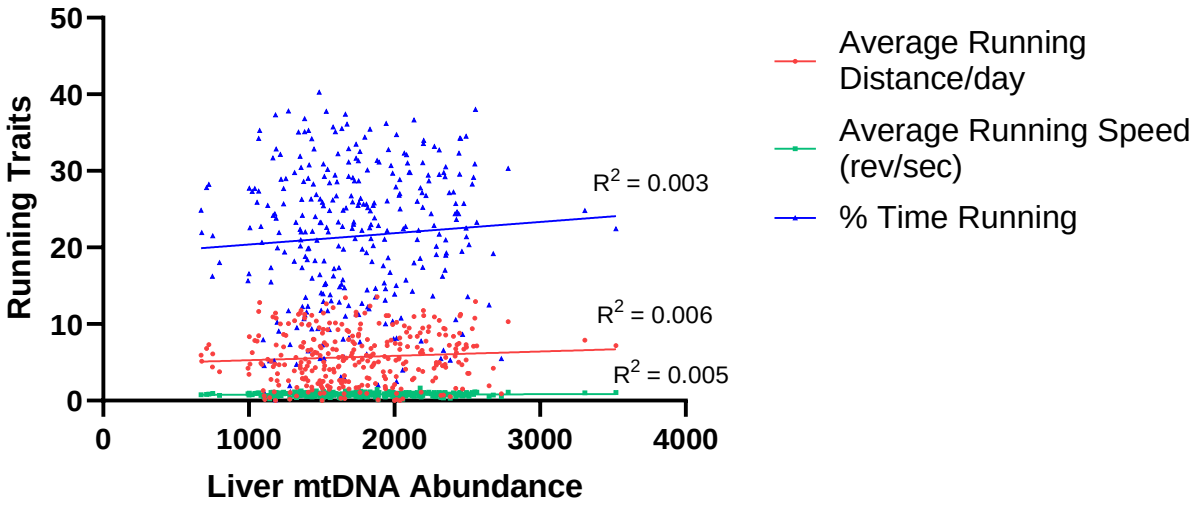

Figure 5b. Relationship between running parameters and copy numbers of liver mtDNA in mice on an exercise lifestyle under the categories average running distance per day (red), average running speed (green) and percentage of time running (blue).

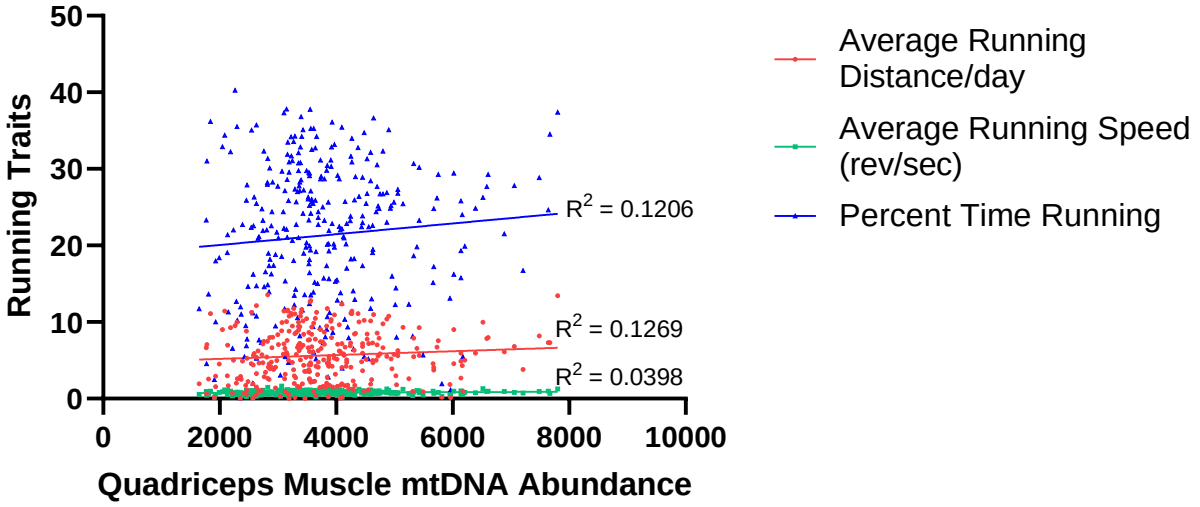

Figure 5c. Relationship between running parameters and copy numbers of quadriceps skeletal muscle mtDNA in mice on an exercise lifestyle between the categories average running distance per day (red), average running speed (green) and percentage of time running (blue).

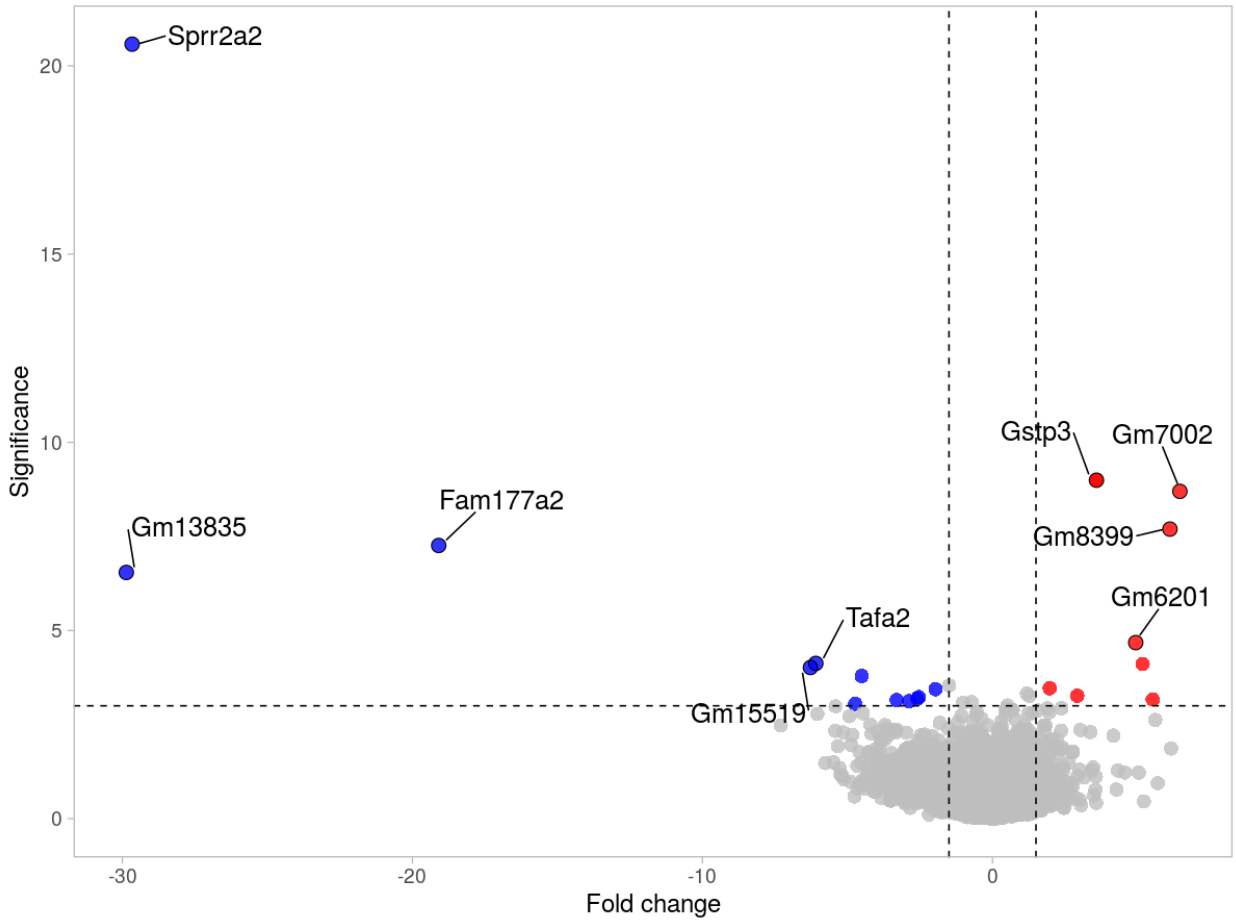

Figure 6a. Differential expression of genes within liver after exercise. Volcano Plot. This volcano plot gives a visualization of the differential expression of 20,513 genes in a liver cell. The figure plots the x-axis as the $\log_2$ fold change versus the y-axis as the $-\log_{10}$ p-values of the same genes. A horizontal line intersecting at 3.0 on the y-axis separating above and below of the genes meeting at the threshold of $p < 0.001$. Genes of low p-value are highlighted as red dots. Genes that are upregulated are highlighted as red dots and genes that are downregulated are highlighted as blue dots.

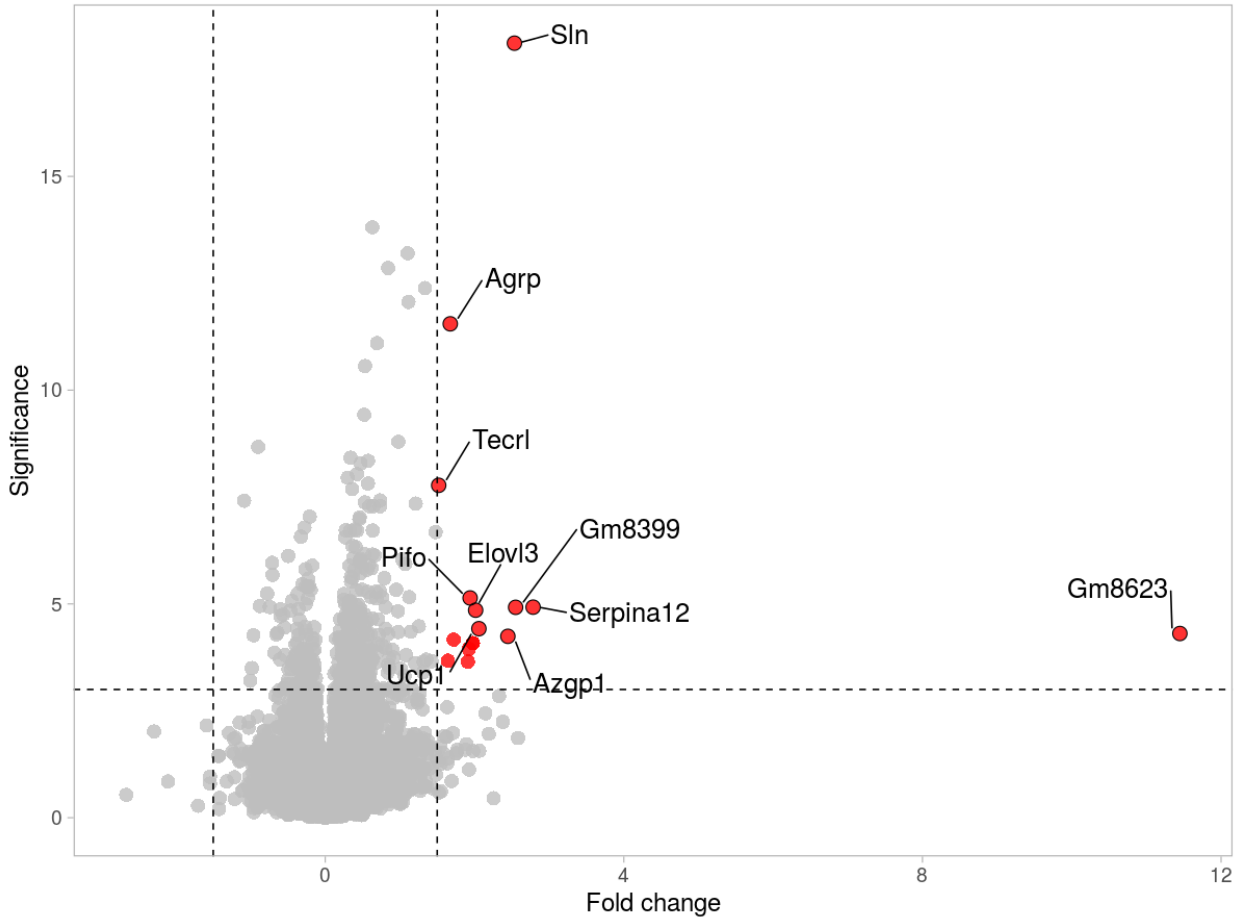

Figure 6b. Differential expression of genes within quadriceps skeletal muscle after exercise. Volcano Plot. This volcano plot gives a visualization of the differential expression of 20,247 genes in a quadriceps skeletal muscle cell. The figure plots the x-axis as the $\log_2$ fold change versus the y-axis as the $-\log_{10}$ p-values of the same genes. A horizontal line intersecting at 3.0 on the y-axis separating above and below of genes meeting at the threshold of $p < 0.001$. Genes that are upregulated are highlighted as red dots and genes that are downregulated are highlighted as blue dots.

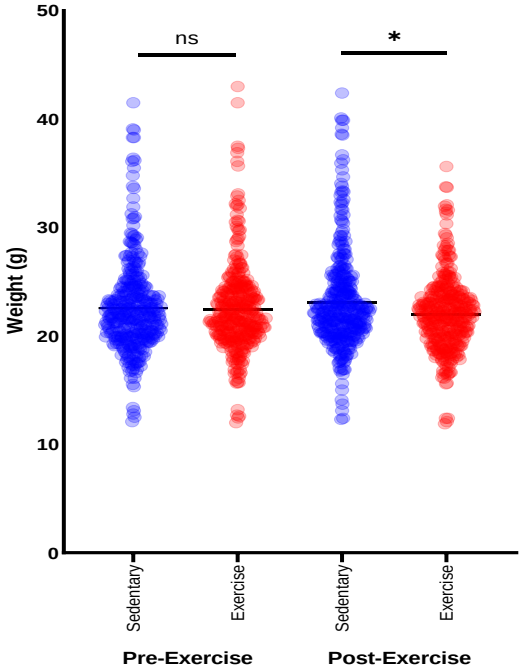

Figure 7. Body weight pre-exercise and post-exercise between the assigned exercised and sedentary groups of mice (n = 4). Each line represents the mean body weight of the group assigned. Significance is at $p < 0.05$.

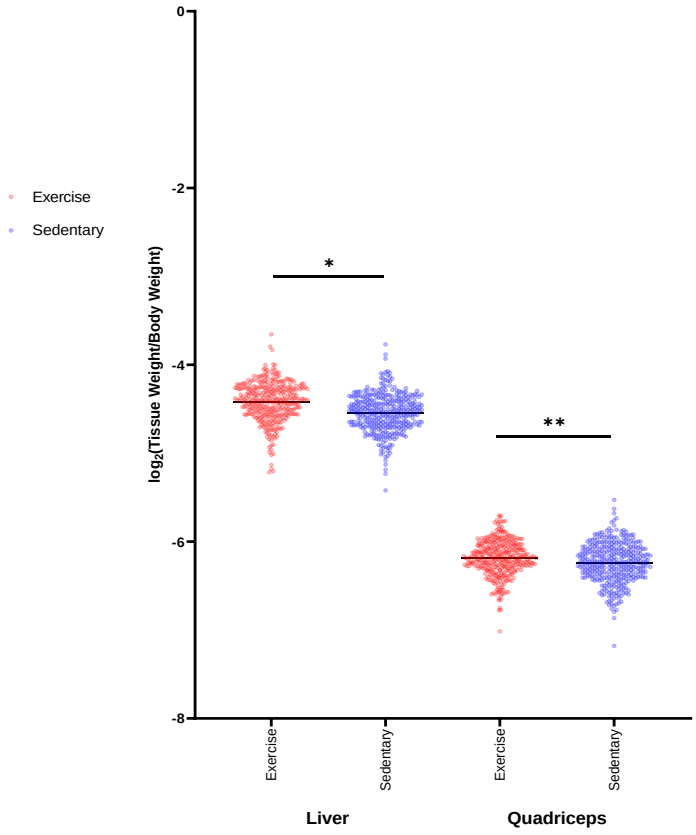

Figure 8.Tissue weight of liver and quadriceps skeletal muscle tissues between the assigned exercise and sedentary groups of mice (n = 4). Each line represents the mean weight of the group assigned. Significance value is at $p < 0.05$.

My previous results established exercise as an effective means to increase mtDNA abundance in both liver and quadriceps muscle tissues. Furthermore, certain genetic differences between each strain of mice can also affect the levels of mtDNA and the response to exercise in both metabolic tissues (Figure 1-4). I then sought to determine which specific exercise parameter influenced this increase in the levels of mtDNA in both metabolic tissues. Running traits used for analysis include: average running distance per day, average running speed per second in each revolution and percentage of time spent running. Average running speed and the percentage of time each mouse spent running were strongly correlated (Figure 5a). Further correlations were performed between all exercise parameters and the levels of mtDNA in both liver and quadriceps skeletal muscle (Figures 5b and 5c). There were no significant correlations between the levels of liver mtDNA with the average running distance of each mouse that ran per day, the average running speed and the percentage of time each mouse ran (Figure 5b; $P = 0.1634$, r = 0.07809; P = 0.3127, r = 0.05661; $P = 0.1926$, r = 0.07302). However, there was a significant correlation between the level of quadriceps skeletal muscle mtDNA of the exercised mice and the average running speed of these mice (revolutions per second) (Figure 5c, $P = 0.0398$, r = 0.1155). The remainder exercise parameters, average distance ran per day and the percentage of time each mouse ran, did not have any relationships with the level of quadriceps skeletal muscle mtDNA (Figure 5c, $P = 0.1269$; r = 0.08591; $P = 0.1206$, r = 0.08737).

Differentially expressed genes post-exercise in both liver and quadriceps skeletal muscle tissues were then examined. Of the 20,513 genes expressed in the liver, 971 genes were significantly differentially expression (Figure 6a, $P < 0.001$). Of the 971 genes, eight genes were found to be most differentially expressed (Figure 6a). Genes *Sprr2a2*, *Gm13835*, *Tafa2*, *Gm15519*, and *Fam177a2* were significantly downregulated and genes *Gstp3*, *Gm8399*,

Gm6201, and *Gm7002* were upregulated (Figure 6a, $P < 0.001$). Gene *Sprr2a2* was the most significantly and highly downregulated gene among all of the downregulated genes (Figure 6a, $P < 0.001$). Gene *Gm7002* was the most significantly and highly upregulated gene among all of the upregulated genes (Figure 6a, $P < 0.001$). In the quadriceps skeletal muscle, it was observed that out of the 20,247 genes only 3,085 genes were found to be significantly expressed (Figure 6b, $P < 0.001$). Out of the 3,085 genes, 9 genes were found to be differentially expressed at significant levels (Figure 6b, $P < 0.001$). Genes *Sln*, *Gm8623*, *Agrp*, *Tecrl*, *Elovl3*, *Pifo*, *Ucp1*, *Azgp1*, *Serpina12*, and *Gm8399* were found to be significantly expressed and highly upregulated and the gene *Gm8623* to be the most upregulated with the gene *Sln* to be the most significantly expressed among all the upregulated genes (Figure 6b, $P < 0.001$).

A comparison was conducted between body weights of both sedentary and exercised groups pre-exercise and post-exercise. There was a significant difference between the exercise and sedentary groups after exercise but not before exercise ($P = 0.0003$; Figure 8). There was also a significant difference in liver tissue weight ($P = < 0.0001$, Figure 9) and quadriceps skeletal tissue weight between the exercise and sedentary groups ($P = 0.0009$; Figure 9).

Chapter 5: Discussion

This study aims to investigate and determine the impact of voluntary aerobic exercise on levels of mtDNA and gene expression in various metabolic tissues. From the findings of this study, it is clear that levels of mtDNA do increase significantly in the exercise group after 30 days across the HMDP but that the degree of change varies among strains.

One explanation for these differing levels of mtDNA in the strains could be due to their genetic variation. Of all the strains, only two were found to have significantly increased mtDNA levels between the exercise and sedentary groups for both quadricep skeletal muscle and liver tissues: FVB/NJ and PL/J (Figure 3 and Figure 4). The inbred strain FVB/NJ is very vulnerable to asthma and particularly sensitive to chemically induced squamous cell carcinomas, as these mice tend to have a high rate of malignant conversion (Jax.org, 2020). The second strain, PL/J, has moderate susceptibility to late onset allergic encephalitis (EAE), high mortality, and has a high incidence of gender-dependent leukemia (Jax.org, 2020).

Genetic variations in the strains could intensify mitochondrial dysfunction leading to greater risk of diseases such as neurological diseases, pulmonary diseases and cancer (Pedersen and Saltin, 2015; Groennebaek et al., 2020; Wahwah et al., 2020; Konopka and Nair, 2013). With exercise, the risk of such diseases is reduced due to indirect effects that occur in the quadriceps skeletal muscle. For example, exercise influences myokine production in skeletal muscle mediating beneficial cross-talk with specific organs such as the liver and the brain (Reugsegger and Booth, 2018). Furthermore, myokine production may be disrupted when PGC-1α transcription is reduced leading to lower mitochondrial biogenesis (Pedersen and Saltin, 2015; Arnold et al., 2011). Physical variations between the strains could also be another explanation.

The mice of the strain FVB/NJ can run at much higher workloads and have greater exercise capacity before exhaustion which may lead to increased levels of mtDNA (Gibb et al., 2016). The strain PL/J appears to have an average running speed pattern similar to the mouse strain FVB/NJ indicating that this strain of mice may have similar levels of mtDNA content (Figure 5a) as the strain PL/J do exhibit an significant increase in exercise capacity as well (Avila et al., 2017).

As a result, both strains during exercise may have an increased in the expression of PGC-1α and Tfam content. This would lead to higher expression of downstream targets in the skeletal muscle and increased expression of mitochondrial biogenesis, mitochondrial electron transport chain complexes, and COX activity (Safdar et al., 2011). In the liver tissue, this increase in levels of mtDNA could be attributed to exercise's effect in preventing a decreased activation of the electron transport chain complex and COX activity by reducing point mutations in the mtDNA-encoded subunits of the COX complex (Safdar et al., 2011). Although increased ROS production can result in decreased mtDNA levels, voluntary exercise instead of forced exercise without rest for a prolonged period would not elicit the negative effects because corticosterone was not induced (Marcuello et al., 2005; Maclaine et al., 2020).

Running parameters could also affect levels of mtDNA particularly in the quadricep skeletal muscle (Figure 5c). This is because a higher running speed could also affect muscle growth, as evident by the increase of muscle weight and increased expression of mitochondrial biogenesis (Figure 8). Although the levels of mtDNA in the liver seem to be unaffected by the running parameters, mtDNA abundance could be linked indirectly to other pathways that induce mitochondrial biogenesis, which would explain the increase in tissue weight of liver tissue (Figure 8). Such a mechanism may be through the expression of pathways Foxo1, p38 MAPK

and EGFR resulting in activation of PGC-1α (Rasbach and Schnellmann, 2007; Hoene and Weigert, 2010; Hoene et al., 2010; Ropelle et al., 2009). Body weight of exercised mice after exercise were lower than the sedentary mice (Figure 7) indicating that the mice had improved mitochondrial metabolism and increased mitochondrial dynamics. A mechanism of such would be the expression of genes such as MFN2 in specific localization mitochondrial populations in specific tissues (Bach et al., 2003; Wahwah et al., 2020).

Genes of Interest
Gm7002
Gstp3
Sprr2a2
Sln

Table 2. Genes of interest that were upregulated or downregulated in each specific metabolic organ.

Differential expression of several specific genes may also explain the underlying mechanisms of how exercise may influence the levels of mtDNA. In the liver, the gene *Sprr2a2,* encoding for small proline rich protein 2A2, is heavily downregulated (Figure 6a). *Sprr2a2* is expressed in the gut and intestine (Informatics.jax.org, 2020). Small proline rich protein 2A is a stress-inducible protein regulated by the gp130 signaling pathway in the heart and is possibly involved in mitochondrial apoptosis (Pradervand et al., 2004; Luo et al., 2013). From this finding, it is possible that with exercise, mitochondrial biogenesis, fusion and fission increases, thus reducing the activation of mitochondrial apoptosis pathways by lowering ROS levels. This would elicit benefits affecting cell differentiation, the activation of the immune system and adaptation in metabolism (Ajith, 2018).

Another gene that was heavily upregulated in the liver is the gene *Gm7002*. The gene encodes for a protein that has only been predicted but its function still remains unknown (Alliancegenome.org, 2020; Informatics.jax.org, 2020). While this gene is expressed in the

alimentary system particularly in the gut, it still remains to be seen if the gene is also expressed in the intestine (Informatics.jax.org, 2020). It is widely known that *Gm7002* is upregulated in the liver, yet it is not well characterized as to what this gene encodes specifically for.

Another gene of interest, *GSTP3* (Gstp3 glutathione S-transferase pi 3), is significantly upregulated in the liver (Ncbi.nlm.nih.gov, 2020). *GSTP3* is part of the GST families which are enzymes that play a major protective role in responding to oxidative stress particularly in the liver and are induced by activation of the Nrf2/Keap1 pathway (Xu et al., 2018). Excessive ROS levels may induce oxidative stress resulting in mtDNA damage, mitochondrial lipids and proteins as well as causing a detrimental feedback loop in the OXPHOS pathway leading to greater ROS production (Ajith, 2018). From this finding, a suggested mechanism is that there is a crosstalk between the Nrf2 pathway that induces the gene *GSTP3* and the mitochondria. This mechanism would occur by activation of the Nrf2/Keap1 pathway via PGC-1α during exercise, which would induce the expression of *GSTP3* and thereby protect mitochondria against oxidative-stress induced degradation and promote mtDNA content. Such a mechanism is plausible as exercise can stimulate an increase of PGC-1α expression, resulting in increased co-activation of the transcription factors Nrf1/2 in the nucleus leading to downstream expression of mitochondrial genes involved in the OXPHOS pathway and TFAM on the nuclear genome. Consequently, expression of OXPHOS and TFAM related genes will result in increased mtDNA replication and transcription, mitochondrial biogenesis, and mtDNA content levels (Zamora and Villena, 2014; Wahwah et al., 2020; Granata et al., 2018). Considering that the Nrf2 pathway regulates expression of genes that provide protection against ROS induced oxidative stress and the Keap1-Nrf2 pathway has a close cross-talk with mitochondrial proteins responsible for

regulating apoptosis or maintenance, it is entirely possible that such a mechanism may occur (Itoh et al., 2015).

In the quadriceps skeletal muscle tissue, the gene *Sln* encoding for the protein sarcolipin is heavily upregulated in the skeletal musculature (Figure 6b) (Informatics.jax.org, 2020). Sarcolipin is also found to increase mitochondrial biogenesis and fatty acid oxidation through sarcoplasmic reticulum (SR)-Ca^{2+}cycling and cross-talk with mitochondria (Maurya et al., 2018). Furthermore, an over-expression of sarcolipin results in an increase in mitochondrial number and size as well as an increase in expression of PGC-1α and peroxisome proliferator-activated receptor δ (PPARδ) (Maurya et al., 2015). It is also evident that sarcolipin can be enhanced with exercise, creating a positive feedback loop of increased sarcolipin expression and greater exercise capacity (Maurya et al., 2018). In addition, a knock-out of sarcolipin can lead to fewer cristae and lower levels of enzymes that are involved in fatty acid transport and oxidation (Maurya et al., 2018). From this, exercise may enhance upregulation of the gene *Sln* to further enhance mitochondrial biogenesis and fatty acid oxidation ultimately resulting in increased levels of mtDNA content in quadriceps skeletal muscle tissue. This positive relationship between exercise and sarcolipin expression would enhance signaling pathways that activate PGC-1α and PPARδ. Once again, the upregulation of PGC-1α and PPARδ would lead to expression of the mitochondrial genes in the nuclear genome responsible for fatty acid oxidation and promote transport of fatty acids into the mitochondria (Gan et al., 2018; Maurya et al., 2018; Zamora and Villena, 2014; Vega et al., 2000).

There are some weaknesses in the current study that should be considered. One possible limitation is that the combination of strains used could lead to outliers due to poor power and skewing of the entire data set. Another weakness in this study was that all the mice were female

leading to a lack of sex differences among a genetic variation. Some genes found in this study were only known as predicted genes, and this makes it challenging to completely explain the mechanism by which exercise may directly influence these genes. Minor differences between body and tissue weights could be from water content as mice were fasted from food but not water thus signifying another possible flaw in the study. Nevertheless, further research is warranted to study the mtDNA levels between males and females and observe for any sex differences in the expression of the potential candidate genes that may promote mitochondrial biogenesis and dynamics found from this study. Furthermore, more in-depth studies into sarcolipin, small proline rich protein 2A2 and Gstp3 glutathione S-transferase pi 3 and the predicted genes using GWAS is necessary. Additionally, mitochondrial biochemical markers would be empirical in future research. Future studies could focus on mtDNA levels in an additional tissues such as adipose tissue and cardiomyocytes after voluntary exercise training and the potential mechanisms of which mitochondrial biogenesis and dynamics occurs in those respective tissues. Accordingly, the data collected would be compared to the data from this study. Nonetheless, this study highlights that mitochondrial biogenesis and dynamics are influenced by exercise in a tissue-specific manner.

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