Oxygen Consumption, Muscle Fibrosis, and Oxidative Stress in the mdx mouse:
Influence of Treadmill Running

THESIS

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Abstract

Dystrophin protein absence in the *mdx* mouse leads to myofiber necrosis and oxidative stress, and exercise accelerates a cascade of events resulting in muscle damage. Examining the influence of exercise on oxygen consumption, oxidative stress and muscle fibrosis will help to enhance the understanding of these pathological mechanisms.

PURPOSE: To test the effects of a four-week treadmill exercise program on oxygen consumption and oxidative stress, and muscle fibrosis in the *mdx* mouse in attempt to improve endpoint measures of the disease that can be used to monitor treatment effectiveness. METHODS: Seven *mdx* (EX) mice aged four weeks were subjected to four weeks of twice-weekly treadmill exercise for 30 minutes at 12 m/min on a rodent treadmill (Columbus Instruments). Nine *mdx* (SED) mice and five wild type C57-BL/10 mice (WT) served as non-exercise sedentary controls. All mice underwent two graded V02 max tests; at three days prior to four weeks of age, and again at three days after eight weeks of age. O2 consumption and CO2 production were measured continuously during max testing. Total time to exhaustion (TTE) on the treadmill was also measured. Fibrotic skeletal muscle damage in isolated quadriceps was determined by the degree of hydroxyproline deposition. Oxidative stress was measured in quadriceps, cardiac, and abdominal muscle by measuring glutathione oxidation. Muscle cryosections of approximately 8 μm were stained for intracellular immunoglobulin G (IgG) and Collagen...
I to determine muscle damage and fibrotic scarring, respectively. RESULTS: At four weeks of age there was a significant difference in TTE between mdx and WT (16.5 ± 1.2 mins vs. 24.8 ± 0.9 mins, p < 0.01), however there was no difference in either baseline or peak oxygen consumption. At eight weeks, compared with SED mice, EX mice had significantly lower baseline O2 consumption (42.4 ± 6.0 ml•kg⁻¹•min⁻¹ vs. 79.2 ± 12.6 ml•kg⁻¹•min⁻¹, p < 0.05) and TTE (18.8 ± 2.6 mins vs. 25.3 ± 6.4 mins, p < 0.05). Quadriceps muscle from EX mice displayed an improved glutathione oxidative state (29.5 ± 5.0 vs. 18.1 ± 2.3, p < 0.05). Collagen I staining in EX mice heart tissue was significantly higher than SED mice (2.40 ± 0.8% vs. 0.57 ± 0.1%, p<0.05). Hydroxyproline content in EX quadriceps tissue was significantly higher than SED mice (2.97 ± 0.1 µg/mg vs. 2.54 ± 0.1 µg/mg, p<0.05) as was heart tissue (3.70 ± 0.4 µg/mg vs. 2.11 ± 0.2 µg/mg, p<0.01). CONCLUSION: Exercised mdx mice exhibit decreased exercise capacity, increased fibrosis, and improved oxidative profiles in select muscles. These mice adapt differently to exercise and adverse outcomes may be associated with exercise increases in those with muscular dystrophy.
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Publications


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Fields of Study

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Table of Contents

Abstract ........................................................................................................................................... ii

Acknowledgments .......................................................................................................................... iv

Vita .................................................................................................................................................. v

List of Tables .................................................................................................................................... ix

List of Figures ................................................................................................................................... x

Chapter 1: Introduction .................................................................................................................... 1

Chapter 2: Review of Literature ...................................................................................................... 4
  Overview of Duchenne Muscular Dysrophy ................................................................................. 4
  DMD Mouse Models ...................................................................................................................... 5
  Effects of Dystrophin Absence on Muscle Function and Oxidative Stress ............................ 7
  Exercise and Free-radical Homeostasis ......................................................................................... 9
  Exercise and the Mdx Mouse ......................................................................................................... 12

Chapter 3: Research Manuscript .................................................................................................. 16
  Introduction ................................................................................................................................. 16
  Materials and Methods .............................................................................................................. 18
Results ................................................................................................................................. 22

Discussion.......................................................................................................................... 31

Chapter 4: Reflection and Discussion ............................................................................... 35

Bibliography....................................................................................................................... 39
List of Tables

Table 3.1 Summary Data.................................................................26
List of Figures

Figure 2.1 Overview of Dystrophin.................................................................5

Figure 2.2 Overview of Events Leading From Dystrophin Deficiency to Inflammation,
Necrosis, and Fibrosis.........................................................................................9

Figure 2.3. Acute Versus Chronic Bouts of Exercise and the Effects on Oxidative Stress
............................................................................................................................11

Figure 2.4. Exercise as an Antioxidant..............................................................11

Figure 3.1. Exercise and Metabolic Testing at Four Weeks-of-age......................26

Figure 3.2. Exercise and Metabolic Testing at Eight Weeks-of-age....................27

Figure 3.3. EPR Spectral Intensity Ratios Used to Investigate Oxidative Stress ....28

Figure 3.4. Hydroxyproline Deposition in Several Muscles of Exercised and Sedentary
Mdx Mice.............................................................................................................29

Figure 3.5. Hematoxylin and Eosin and Collagen I Immunostaining of Heart Sections
from Exercised Mice Show Greater Amounts of Collagen Scarring at Eight weeks-of-age
.............................................................................................................................30

Figure 3.6. Quantification of Cardiac Tissue Damage and Fibrosis Histological Analysis
.............................................................................................................................31
Chapter 1: Introduction

Neuromuscular disorders encompass a variety of diseases that affect both voluntary and involuntary muscles, and their associated nerves. Prevalent conditions include amyotrophic lateral sclerosis, multiple sclerosis, myasthenia gravis, spinal muscular atrophy, and the muscular dystrophies. These diseases cause debilitating side effects and almost always result in early death or a significant drop in standard of living – primarily by loss of ambulation and muscle control. The muscular dystrophies are mainly composed of Duchenne, Becker, facioscapulohumeral, and limb girdle muscular dystrophies. These diseases have genetic causes and it has been estimated that 1 in 3500 of the population will develop a neuromuscular disorder either at childhood, or later in life [1].

Duchenne muscular dystrophy (DMD) remains one of the most widespread of all the muscular dystrophies with a prevalence of 1 in 3,500 live male births [2]. Males are only afflicted with DMD and patients with the disease lack the muscle protein dystrophin, which causes a cascade of events – mechanical fragility, chronic inflammation, and unbalanced oxidative stress all leading to muscle damage [3]. Over 80% of DMD sufferers are confined to a wheelchair by 14 years of age, and only half live past the age of 24 [4]. The leading causes of death associated with DMD are respiratory or cardiac failure brought on by declines in skeletal and cardiac muscle function [5, 6]. Secondary
conditions, such as pneumonia, can also become life threatening for those afflicted with the disease. Current treatment options are limited and typically focus on maintaining ambulation with glucocorticoid corticosteroid use without really attempting to repair the underlying mechanisms that cause disease. Steroid treatment with prednisone has shown to improve muscle function and strength [7] and improve walking and stair-climbing ability in patients with muscular dystrophy [8].

Dystrophin absence causes muscle degeneration by a host of mechanisms. Myofiber necrosis in dystrophic muscle appears to result mainly from contraction-induced injury. Improper blood flow resulting from muscle ischemia also adds to disease pathology via disruptions in nitric oxide function [9]. Dystrophin presence is shown to protect muscle, specifically the sarcolemma, from damaging lengthening contractions [10, 11] like those associated with exercise. Healthy muscle adapts to exercise by increasing muscle regeneration and subsequent function and force. Animal studies done examining how exercise might affect dystrophic muscle show contradictory findings with some studies displaying improvements in dystrophic pathology with exercise while other’s do not [12-16]. Exercise intensity, duration, and modality appear to be important variables modulating the effects exercise has on diseased muscle with further study warranted in examining how this stimulus interacts with dystrophic muscle. One thing remains certain, diseased muscle adapts to exercise differently than healthy muscle and may be more vulnerable to changes brought on by physical activity [11, 17, 18].

Exercise training has been shown to induce a host of other beneficial adaptations on several physiological systems not primary to muscle – oxidant homeostasis,
angiogenesis, and cardiovascular fitness [19-21]. Most, if not all, of these innate systems remain perturbed in the context of disease. A link seems to be establishing itself between exercise, muscle damage and repair, and oxidative stress and oxygen metabolism that may be even heavily modulated in the context of diseased muscle. Several mechanisms that link these events are poorly understood even in healthy tissue and more research needs to be done examining how both healthy and diseased tissue adapts to exercise differently.

The purpose of this study was to begin examining how exercise influences oxygen metabolism, muscle health, and oxidative stress within several dystrophic muscles in attempt to discern how exercise could reduce, or induce muscle pathology. Present research has gaps concerning how diseased muscle adapts differently to exercise. Prior studies utilizing this exercise protocol have shown deleterious impacts on certain outcomes of interest [12]. We are attempting to use the same protocol but investigate other aspects of disease pathology that encompass different areas related to applied physiology and muscle function. Examining how this interaction occurs in diseased muscle will bring about important conclusions that could have profound implications on disease treatment and management. Therapeutic strategies for DMD, and other muscle diseases, will continue to advance and improve muscle function, clinical outcomes, and patient ambulation. Understanding how an increase in physical activity will interplay with disease mechanisms remains crucial to maintain patient improvement, help understand the nature of the disease itself, and improve outcomes within the neuromuscular diseases.
Overview of Duchenne Muscular Dysrophy

Duchenne Muscular Dysrophy (DMD) is a degenerative muscle disease affecting 1 in 3,500 [2] live male births with an average life expectancy of 19 to 25 years [22]. DMD is caused by mutations in the dystrophin gene that result in protein deficiency and initiate a cascade of pathological events that lead to muscle damage and necrosis. Dystrophin associates with a complex of molecules forming the dystrophin-glycoprotein complex (DGC), which is responsible for attaching the muscle extracellular matrix to the cytoskeleton of the muscle fiber [23] (Figure 2.1). Petrof et al. assessed sarcolemmal damage in mdx mice and showed increased susceptibility to contraction-induced sarcolemmal damage [10, 11], very much like the damage associated with exercise. Absence of dystrophin ultimately results in muscle death, failed regeneration, and fibrotic scarring through events that are not fully understood. Cardiac muscle is equally affected as skeletal muscle, thus making treatment of DMD with drugs specific to cardiac disease important in maintaining the heart function. Respiratory failure appears to be the overwhelming cause of death in DMD patients, with estimates of 55-90% mortality from respiratory failure between ages 16 and 19 and irregularly after age 25 years [24-29].

Currently there is no cure for DMD even though several treatments are at varying stages of development. The current standard-of-care treatment remains glucocorticoid
corticosteroid administration to maintain patient ambulation for as long as possible.
Steroid treatment has had success in improving muscle strength and function. Manzur et al. produced a meta-analysis of three randomized controlled trials showing improvement in muscle strength and function over six months using glucocorticoid corticosteroids [7]. These drugs are not without side effects and better treatments and cures for DMD are emerging that focus on specific disease mechanisms.

**Figure 2.1.** Overview of Dystrophin.

*DMD Mouse Models*

The most widespread animal model of DMD remains the *mdx* (X-chromosome-linked muscular dystrophy) mouse. Research investigating specific pathogenic mechanisms of DMD as well as possible treatment strategies has utilized the *mdx* mouse as a model [30-33]. These animals mirror the protein loss seen in human patients with DMD and are fully deficient of dystrophin. Even though these animals suffer from the
same deficiency their phenotype does not resemble what is found in humans. *Mdx* mice display signs of muscular dystrophy early on in life [23]; however partially recover while having reduced longevity compared to wild-type mice. Chamberlain et al. aged both wild-type and *mdx* mice under normal conditions and found that, along with reduced life span, older *mdx* mice were also prone to develop muscle tumors resembling the human form of alveolar rhabdomyosarcoma [34]. Other *mdx* crosses have been made including knockouts for the muscle-specific transcription factor MyoD that exhibit worse regenerative capabilities and more severe dystrophy [35].

One theory for the mildness of the *mdx* phenotype involves the protein utrophin and its function in muscle. Utrophin may compensate for the lack of dystrophin and be responsible for the reduced phenotype seen in *mdx* mice. Deconinck et al. produced double-knockout (DKO) mice for both utrophin and dystrophin that exhibit a phenotype much more similar to DMD patients. These animals have a greatly reduced lifespan, increased cardiomyopathy, and severe muscle weakness [36]. Even though DKO mice replicate key features of DMD their reduced life span and difficulty breeding make them less than ideal mice for animal studies.

A newer *mdx* model, one that is haploinsufficient for utrophin and still fully deficient of dystrophin, *utrn*/: *mdx* “het” mice, has also been developed. These mice present with a much more aggressive dystrophy than *mdx* mice, yet still retain most of the longevity of the *mdx* mouse [37, 38]. Het mice also have increased cardiomyopathy and myofiber fibrosis and have become valuable for testing potential therapies due to the exaggerated phenotype over *mdx* mice.
Effects of Dystrophin Absence on Muscle Function and Oxidative Stress

Dystrophin plays an important role in muscle biology by linking the internal cytoskeleton to the muscle extracellular matrix (Figure 1). Dystrophin acts like a molecular spring and contributes to membrane elasticity by redistributing force within the cell. The amino-terminus of dystrophin binds to F-actin and the carboxyl terminus to the DGC. The DGC becomes destabilized when dystrophin is gone and causes diminished levels of other muscle proteins as well as impaired signaling with other molecules, many of which center around intracellular calcium handling [39, 40].

Mokri et al. used high resolution phase microscopy to show that patients with DMD to have increased muscle fiber fragility and plasma membrane abnormalities [41]. Additionally, dystrophic muscle has a compromised ability to produce force [42, 43] and sustain eccentric contraction, suggesting that exercise-induced damage will have large implications on disease management [44].

Muscle fiber damage within DMD that leads to muscle degeneration and necrosis will ultimately result in the formation of fibrotic scars composed of adipose and connective tissue [45]. Satellite cell proliferation is unable to compensate for constant muscle fiber breakdown within DMD [46] providing the muscle with only a limited capacity for recovery. Fibrotic tissue is unable to contribute to muscle force development and leads to an impairment of muscle function caused by increased stiffness and decreased elasticity. Overstrain of dystrophic muscle causes events leading to elevated
calcium that acts as a secondary messenger to activate native inflammatory processes that contribute to fibrotic tissue remodeling (Figure 2.2).

Muscle weakness and damage in animals lacking dystrophin is proposed to be a consequence of inflammation and damage repair cycling [47-49]. The link between muscle damage and fatigue injury and inflammation is unresolved however oxidative stress has been proposed as a contributing factor [50-52]. Whitehead et al. showed that tibialis anterior muscle from mdx mice had increases in subunits of a specific enzyme related to oxidative stress, NADPH oxidase, even as early as 19 days-of-age. Oxidative stress is defined as an upset in the relative amounts of endogenous antioxidants and the free radical species that they buffer, primarily reactive-oxygen (ROS) and nitrogen (RNS) species.

Increases in markers for oxidative stress are a consistent finding in both patients with DMD [53] and mdx mice [54, 55]. Peroxidation of lipids and oxidation of proteins, as well as oxidized DNA are increased in mdx mice and DMD patients. Rodriguez et al. [56] have shown an increase in a specific marker of DNA oxidative damage, 8-hydroxy-2’-deoxyguanosine, in DMD patients compared to healthy controls. In mdx mice specifically, Ragusa et al. [57] showed that TBARS, byproducts of lipid peroxidation, were consistently higher in mdx skeletal muscle. Dystrophin deficiency causes a marked increase in the amount of reactive oxygen species (ROS) via mechanisms that are not entirely understood [58]. Additionally, muscle membrane damage activates intracellular pathways leading to inflammation and ROS generation by macrophages and neutrophils in order to enhance phagocytosis [50]. Treatment of DMD with anti-oxidant compounds
have had limited success during clinical trials possibly compounded by the poor understanding of oxidative stress and its relationship with disease pathology.

Administration of anti-oxidant compounds have shown some success in mouse studies of DMD, with clinical success yet to be substantiated [15, 59-63].

**Figure 2.2.** Overview of Events Leading from Dystrophin Deficiency to Inflammation, Necrosis, and Fibrosis.

*Exercise and Free-radical Homeostasis*

Exercise has a paradoxical effect on the redox environment of normal muscle. Bouts of acute exercise cause a build-up of ROS in the body suggesting initial damage, yet persistent bouts produce an up-regulation of ROS-buffering capabilities [64]. One reason for an acute increase in ROS from exercise is due to increased oxygen consumption and cellular metabolism from exercise. Exercise is also thought to boost free radicals via increases in catecholamines that metabolically activate and produce
oxidant molecules. Also, the production of lactic acid from muscle contraction can convert superoxide molecules to hydroxyl radicals, which are stronger at damaging cells [65]. Additionally, strenuous exercise can lead to the oxidation of glutathione and increases in cytosolic enzymes that are signs of cell damage [66] leading to the notion that exercise intensity could be a large mediator of oxidative stress generated from physical activity.

Oxidative stress also plays an integral role in the body’s adaptation to exercise training. Stress from exercise causes increases in muscle superoxide dismutase (SOD) which acts as an antioxidant to buffer further oxidant attack. Oxidative stress resulting from exercise also increases markers of nitric oxide activity (eNOS and iNOS) that are important in healthy adaptation to exercise [66]. The oxidative response to several different intensities and rest durations also appears to differ and affect the redox reaction to exercise [19] with larger intensities of exercise causing higher magnitudes of oxidative stress. The constant assault of oxidant molecules on cells induces a wide array of antioxidant and muscle adaptation responses that differ between acute and chronic bouts of exercise [19] (Figure 2.3). Cells are naturally equipped with enzymatic antioxidant systems that appear to increase activity with higher exposure to exercise. In this way exercise alone can act as an antioxidant through activation of cellular pathways pertaining to oxidant buffering increases (Figure 2.4).
Figure 2.3. Acute Versus Chronic Bouts of Exercise and the Effects on Oxidative Stress

Figure 2.4. Exercise As an Antioxidant
Exercise and the Mdx Mouse

The impact of physical activity on DMD has been studied for a few decades with most experiments focusing on the *mdx* mouse and its response to differing exercise modalities. Voluntary wheel-running (VWR) was among the first modalities tested in the *mdx* mouse. Carter et al. showed that young (4 weeks-of-age) *mdx* mice only run ~70% of the distance ran by wild-type controls, and old (6 months-of-age) *mdx* mice only run ~40% of the distance ran by wild-type controls [67] showing *mdx* mice to have blunted exercise capacity. The amount of rest time between run sessions and the intensity of running may also be key factors influencing muscle damage as a response to exercise in *mdx* mice [13].

Some studies also show differing effects of VWR on different muscles in the *mdx* mouse. Long-term VWR may be deleterious to diaphragm function, have no effect on most other skeletal muscles, yet improve cardiac muscle function. Selsby et al. subjected *mdx* mice to VWR for one year and found a 60% decrease in diaphragm specific tension, yet a 15% increase in cardiac mass and 20% increase in left ventricular chamber size [14]. There is also a strong link between exercise capacity and oxidative stress in the *mdx* mouse. *Mdx* mice treated with green tea extract and exposed to VWR showed improvements in endurance capacity, increased serum antioxidant levels, muscle tetanic stress, and total contractile protein content [15]. Exercise treatment may be an appropriate therapy when supplemented with other strategies and when exercise intensity and rest duration are controlled.
Other exercise modalities have also been investigated in the *mdx* mouse. Mice subjected to swimming exercise have showed beneficial effects on muscle primarily by enhancing muscle regeneration, the proportion of oxidative fibers, and reducing muscle fragility [68]. Indeed, further research with *mdx* mice suggest that non-weight-bearing, low-intensity exercises like swimming may have no real deleterious effects on muscle function and may be useful therapeutic strategies for those with DMD [69].

A new alternative to both VWR and swimming is the use of controlled treadmill running (TR). Experiments utilizing TR can look deeper into the relationship between exercise time and intensity and outcomes on the dystrophic phenotype. Researchers can fine-tune the exact dose of exercise and what effects that may have on different outcome variables. A widely used *mdx* TR protocol utilizes 30 minutes of steady-state running at a speed of 12 meters per minute [18, 70]. Exercise at this intensity and duration caused increases in several negative markers associated with dystrophy – inflammatory cytokines, myofiber necrosis, creatine kinase, and muscle morphology [71]. Another marker of muscle function – forelimb strength was also decreased after TR exercise. This result leads to the notion that certain levels of TR may be a way to exacerbate the dystrophic phenotype and serve to make the *mdx* a better model of DMD. Indeed researchers have used this protocol as a way of inducing muscle weakness to test the efficacy of drug compounds meant to increase muscle force [12, 18, 72, 73].

Experiments using this exercise protocol have also shown increases in dystrophic muscle protein thiol oxidation, an indicator of oxidative stress [71]. TR using the given protocol appears to be a successful way to induce greater amounts of muscle weakness
and oxidative stress in the *mdx* mouse. There remain gaps in the research utilizing this particular treadmill protocol. Prior research has not examined oxidative or fibrotic effects on other skeletal muscles important to movement – the abdominal muscles. Prior research studies using this specific protocol have focused on plasma and histological analysis of ROS levels, not whole-muscle homogenate. Also, using highly sensitive magnetic resonance techniques to study the oxidative environment of tissues is a novel method that has not been utilized previously in exercised *mdx* muscles. Additionally, there have been no published studies examining how this exercise protocol impacts changes in resting and maximal oxygen consumption or further exercise ability on a graded maximal stress test. Filling these gaps would increase the ability to use these measures as outcomes of interest in DMD to monitor disease progress.

Exercise, like its effects on oxidative stress, appears to be a double-edged sword in the *mdx* mouse. Early research done with low-impact, low-intensity exercises like VWR and swimming show how exercise can invoke beneficial responses in diseased muscle by improving muscle regeneration and function. TR running, at least at specific speeds, has shown the opposite effects by inducing muscle damage and oxidative stress and decreasing muscle function. Many physiological variables exhibit dose-responses to exercise and it remains no surprise that this seems to be the case with the *mdx* mouse.

Too much exercise, coupled with insufficient recovery time, exacerbates dystrophic pathology and decreases functional outcomes in *mdx* mice. Newer advances in treatment strategies for DMD will ultimately improve muscle function and allow patients to undergo increases in physical activity. Understanding how this change in physical
activity might interplay with disease pathology and affect clinical measures remains crucial to maintain the best outcomes for DMD patients.
Chapter 3: Research Manuscript

Introduction

Duchenne muscular dystrophy (DMD), a progressively debilitating neuromuscular condition, remains one of the most common adolescent genetic muscle diseases with a prevalence of approximately 1 in 3,500 male births [1, 2]. Patients with DMD lack the muscle protein dystrophin, that associates with a complex of molecules forming the dystrophin-glycoprotein complex (DGC). The DGC is responsible for attaching the muscle extracellular matrix to the cytoskeleton of the muscle fiber and acts as a force-transducer to protect muscle fibers from repeated contraction-induced injury [10, 11]. Patients afflicted with DMD typically do not live past their late 20’s with an average life expectancy of 19 to 25 years [22]. The genotypic animal model for the disease – the mdx mouse has been studied extensively to advance possible treatments that might translate to human patients [30-33].

Exercise presents a platform to study how varying amounts of muscular contraction influence the progression of disease pathology in DMD differently. Important disease features of DMD include the development of non-functional fibrotic lesions within the muscle [46, 74], increases in oxidative stress [58], and perturbed oxygen utilization [75]. Additionally, dystrophic muscle fibers have shown to be more vulnerable to exercise [11, 17, 18], making it important to study how they adapt to changes in physical activity. Early studies with exercise utilizing wheel-based running and mdx mice
have showed beneficial effects on contractile function and fatigability [76, 77].
Experiments done using a specific treadmill exercise protocol in \textit{mdx} mice have shown
increases in muscle damage and protein oxidation as a result of treadmill running [18, 
71]. Prior research utilizing this treadmill exercise protocol does have gaps, and has not
examined oxidative or fibrotic consequences of exercise on another skeletal muscle
important to ambulation – the abdominal muscle. Also, using highly sensitive magnetic
resonance techniques to study the oxidative environment of tissues is a novel method that
has not been utilized previously in exercised \textit{mdx} studies. Prior research studies using this
specific protocol have focused on plasma and histological analysis of ROS levels. Here,
we used entire muscle homogenates to gain better insight on whole-muscle oxidative
stress. Additionally there have been no published studies examining how this exercise
protocol impacts changes in both resting and maximal oxygen consumption. Prior
research has attempted to identify alterations in further exercise ability from treadmill
exercise [73], however we developed a newer graded treadmill test to look closer at
exercise capacity. These applied physiologic parameters would be useful outcome
measures to monitor disease progression.

The present study aimed to determine how a simple treadmill exercise protocol
might affect several facets of DMD disease pathology differently in the \textit{mdx} mouse in
attempt to produce better outcome measures of the disease. Previous research done using
\textit{mdx} mice and this exercise protocol have shown a link between exercise workload and
adverse events [12] that warrants more research to examine how this might interplay with
oxygen consumption, exercise ability, and muscle fibrosis and oxidative stress in other
skeletal muscles. The understanding of how oxidative stress is linked to DMD is still unclear. The accumulation of oxidized protein is an important outcome of interest for several diseases relating to cardiovascular and skeletal muscle biology – stroke, atherosclerosis, diabetes, amyotrophic lateral sclerosis, and essential hypertension [78, 79]. Exercise has been shown to improve the degree of oxidative stress [80, 81] as well as exercise capacity but might further increase muscle damage due to contraction-induced injury. Investigating systemic, whole-animal basal and maximal oxygen consumption would also shed light on oxygen metabolism in this DMD model as it responds to exercise. Evaluating these outcome measures is important as improvements in treatment strategies for DMD allow for greater amounts of physical activity and may exacerbate negative aspects of the disease that need to be monitored.

Materials and Methods

Ethics Statement:

All experiments were approved by the Institutional Animal Care and Use Committee of The Ohio State University conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Animal procedures and groups

All experiments were performed on 4- to 8-week-old male and female dystrophic mdx mice and non-dystrophic control C57Bl/10 mice. Mice from each litter were given numbered ear tags at 3 weeks-of-age. Groups subjected to exercise were started at 4 weeks-of-age and continued until 8 weeks-of-age. Animals were housed at The Ohio State University under standard conditions with unlimited access to food and water, and a
12-hour light/dark cycle. All mice were sacrificed by cervical dislocation, as per approved protocol so as to not chemically contaminate tissues, at 8 weeks-of-age after metabolic measurements. Heart, quadriceps, diaphragm, extensor digitorum longus, tibialis anterior, and abdominal muscle were excised immediately. Half of each tissue was immediately flash frozen in liquid nitrogen for biochemical and oxidative stress measurement, with the other half embedded in optimal cutting temperature (OCT) cryoprotectant and frozen in liquid nitrogen-cooled isopentane for histological sections. The following groups were used: sedentary C57Bl/10 (n=5), sedentary mdx (n=10), and exercised mdx (n=7).

Exercise protocol

The TREAT-NMD recommended protocol “Use of treadmill and wheel exercise for impact on mdx mice phenotype M.2.1_001” http://www.treat-nmd.eu/research/pre-clinical/SOPs exercise regimen consisting of 30 min treadmill running at a speed of 12 m/min was used. The rodent treadmill was an Exer 3/6 from Columbus Instruments (USA). Running lanes were separated with clear dividers so that mice could see one another while exercising. The treadmill remained horizontal (0 percent incline) and mdx mice were ran in groups of 2 or 3. If during the 30 min exercise session a mouse fatigued and could no longer run, the procedure was as follows: turn the treadmill belt down to 6 m/min for 2 minutes, then increase the speed to 12 m/min for the remainder of the 30 minute bout. Exercise consisted of 4 weeks of twice-weekly boats at 12 m/min with no more than 48-72 hours between bouts, for a total of 8 bouts. The electrical shock grid was not used. Mice that could not perform all bouts of exercise were excluded.
Metabolic measurements

To determine basal and maximal oxygen (VO$_2$) and carbon dioxide (VCO$_2$) consumption animals were housed for a period of 5 minutes, then subsequently ran until exhaustion inside the clinical laboratory animal monitoring system (CLAMS). All mice completed a maximal exercise test at 4 weeks and 8 weeks. The maximal exercise test first consisted of a 5 minute stabilization period where the mouse remained motionless inside the chamber. After this phase the speed increased to 6 m/min while remaining at 0% grade. After 12 minutes the incline was increased to 20% grade and remained there for test duration. Additionally, at the 12 minute mark the speed was increased to 7 m/min and increased 1 m/min for every minute until exhaustion. Exhaustion was defined as the inability to remain on the treadmill belt while sitting on the electrical shock grid for at least 5 seconds, a plateau of VO$_2$, and/or a respiratory exchange ratio (RER) of >= 0.95. During the test measurements of VO$_2$, VCO$_2$ and heat were taken every 15 seconds.

Hydroxyproline analysis

Quantification of quadriceps, diaphragm, abdominal, and cardiac hydroxyproline was performed using the method of Reddy and Enwemeka [82]. Muscle samples were homogenized, weighed, and hydrolyzed for 24 hours in 2N sodium hydroxide at 100 degrees C. Hydrolyzate (20 µL) was mixed with 450 µL of 0.056M chloromine T reagent (Sigma-Aldrich) in citrate buffer and oxidized at room temperature for 25 minutes. 500 µL of 1M Ehrlich’s reagent [15 g of 4-(dimethylamino) benzaldehyde (Sigma-Aldrich), 33 mL n-propanol, 66 mL perchloric acid] was added to each sample, mixed, and incubated at 65 degrees C for 20 minutes, followed by spectrophotometric measurements.
taken at 550 nm. A standard curve (0–5,000 nM, trans-4-hydroxy-l-proline; Sigma-Aldrich) was included for each assay. Measurements of hydroxyproline were normalized to tissue weight and results are reported in µg hydroxyproline/mg tissue.

**Oxidative stress measurements**

To evaluate oxidative stress levels and redox state of heart, abdominal and quadriceps tissue we measured the ratio of probe compound (RSSR) to reduced glutathione protein (RSH) as a relative indicator of reduced (GSH) and oxidized (GSSG) glutathione levels. Relative amounts of glutathione oxidation have been shown to be a major indicator of cellular redox state with lower GSH, higher GSSG, and a lower GSH/GSSG ratio being found in many different disease pathologies [83, 84]. Analysis was done using electron paramagnetic resonance spectroscopy (EPR). EPR exploits the magnetic properties of electrons and their absorption of microwave radiation in the presence of a magnetic field. Entire muscle homogenates (5-10 mg) were incubated in Newcastle buffer (4 M urea, 75 mM Tris, pH 6.8, 3.8% SDS) for 35 minutes. A disulfide biradical of imidazoline (RSSR, Enzo Life Sciences, NY) was used as the probe that detects GSH levels. Isolated muscle cellular proteins were incubated with 10 µM RSSR and loaded into an EMX X-band EPR spectrometer (Bruker Biospin, Ettlingen, Germany) with multiple scans being made for each sample. The same volume amount of protein isolate was used for all samples. EPR scans were analyzed using WinEPR software specifically designed for EPR spectra analysis (Bruker Biospin, Ettlingen, Germany). Analysis of the relative spectral peak intensities of both the monoradical, and biradical products determined tissue oxidation.
Histology

Heart and skeletal muscle tissue were embedded in optimal-cutting-temperature medium and frozen on liquid-nitrogen cooled isopentane for histological prepared sections. 8 μm muscle cryosections were stained for intracellular immunoglobulin G (IgG) and Collagen I. Immunostaining was performed using a CY3-conjugated antimouse IgG antibody (1:100, Jackson research laboratories) as previously described [85] with costaining with anti-Collagen I antibody (1:150). The percentage of IgG, as well as Collagen I, stained pixels was quantified using Image J.

Statistical Analysis

All values are presented as means +/- SEM. Oxygen consumption and exercise capacity at four weeks-of-age, oxidative stress, immunohistology, and hydroxyproline content were analyzed with student t-tests. Oxygen consumption and exercise capacity at eight weeks-of-age was analyzed using ANOVA with Bonferroni post-hoc tests. A P-value <0.05 was used to determine significance level.

Results

Oxygen Consumption and Exercise Capacity

At four weeks-of-age there were no significant differences between sedentary mdx (Mdx) mice and sedentary wild-type C57BL/10 mice (WT4) in body weight, basal or maximal oxygen consumption (Table 3.1). Sedentary mdx mice did exhibit highly blunted exercise capacity when compared to WT4 mice, reaching similar maximal oxygen consumption values at much lower workloads (Figure 3.1A, p=.0045). Dystrophic mice
have shown deficits in muscle force [43] and seeing their diminished capacity on a graded maximal exercise stress test supports deficits in cardiorespiratory fitness.

At eight weeks-of-age and after the exercise intervention, exercised *mdx* (Ex) mice showed significantly lower exhaustion times (Figure 3.2A, \(p=.043\)) and basal oxygen consumption (Figure 3.2B) compared to sedentary *mdx* (Sed) mice. There were no significant maximal oxygen consumption differences between groups at eight weeks-of-age (Figure 3.2C) once again indicating that Ex mice utilize similar amounts of oxygen at much lower workloads when compared to Sed mice. Adaptation from exercise training typically increases an animal’s ability to further perform exercise and the results here indicate that this particular training protocol induced the opposite effect in the *mdx* mouse and supports how dystrophic animals adapt differently to exercise training.

*Oxidative Stress*

Chronic exercise has been shown to improve oxidant levels, specifically reactive oxygen species (ROS), and increase endogenous buffering of oxidant molecules systemically [80, 86]. We evaluated the redox state of several muscles in attempt to elucidate whether this advantageous exercise adaptation still persisted in dystrophic mice. Quadriceps muscle from Ex mice had significantly better oxidative profiles when compared to Sed *mdx* mice (Figure 3.3, \(P=.0361\)). Abdominal muscles from Ex *mdx* mice followed the same trend and were also significantly better than Sed mice with regards to oxidative stress (Figure 3.3, \(p=.0020\)). Ex mice also metabolize less oxygen during rest than Sed mice (Figure 3.2B) which could ultimately dampen the amount of ROS generated overall and improve the oxidant environment of muscles.
Due to its increased utilization during steady-state aerobic exercise it might be expected that cardiac muscle follow the same trends as other skeletal muscles. While cardiac muscles from Ex mice trended to be better than Sed mice, the difference was not statistically significant (Figure 3.3, P=.2170). This could be due to the small amount of exercise time and intensity the animals were subjected to and simply was not a large enough dose of exercise to elicit effects on the myocardial oxidative state.

*Hydroxyproline Content*

Contraction-induced muscle injuries, much like those associated with treadmill running, may be a large factor controlling the degree of fibrotic scarring seen in dystrophic muscles [46]. Hydroxyproline is a main constituent of fibrotic scars and the biochemical quantification of it remains an important way to quantify muscle fibrosis. Assays performed on skeletal and cardiac muscle showed that, when compared with Sed mice, Ex mice had significantly higher hydroxyproline deposition in both quadriceps (Figure 3.4, p=.0397) and heart tissue (Figure 3.4, p=.0047). This is not surprising given the contributions both of these muscles make in performing steady-state endurance exercise. Abdominal muscle hydroxyproline content was not significantly different between Ex and Sed mice (Figure 3.4), and could potentially be due to the differences in how this muscle may be utilized during exercise when compared to the heart and quadriceps.

*Immunohistology*

Myofiber necrosis and collagen scar formation remain hallmark features of dystrophic disease pathology. Dying muscle fibers become replaced with non-contractile
connective tissue and cause a blunting in muscle function. Interestingly, exercise has shown to have antifibrotic effects in normal muscle by enhancing the muscle microenvironment’s vascular supply, promoting the secretion of favorable growth factors, and limiting fibrotic scar formation resulting from muscle injury [87]. If exercise increases fibrotic scar damage in dystrophic mice than we would expect to see this reflected in both biochemical hydroxyproline assessment and histological investigation. Heart muscle histological sections from Ex mice showed significantly higher collagen-I scar formation (Figure 3.6, p=.0325) than Sed mice indicating that exercise increases the formation of these non-functional scars. The accumulation of these pathogenic scars in cardiac muscle might ultimately manifest themselves in diminished exercise capacity, as seen in exhaustion time deficits in Ex mice (Figure 3.2A). Levels of dying myocytes, as indicated by the amount of IgG staining, were higher in Ex hearts compared to Sed however the difference was not significant (Figure 3.6A, 0.27% versus 2.20%, p=.0958).

Analysis of both collagen-I and IgG staining in quadriceps was not different between groups (p=.7586 for Collagen-1; p=.6773 for IgG). There also remains a tight link between oxidative stress and fibrosis [46] and the much improved oxidative profile of Ex mice quadriceps (Figure 3.3) muscle may be aiding in dampening the genesis of fibrotic scarring and myofiber necrosis as these muscles did not display significant increases in either collagen-I or IgG staining.
Table 3.1: Summary data. Maximal (VO$_{2\text{max}}$) and basal (VO$_{2\text{basal}}$) oxygen consumption, body weight, and exhaustion time on a graded exercise test for sedentary mdx and C57BL/10 mice at 4 weeks-of-age, and exercised mdx, sedentary mdx and C57BL/10 at 8 weeks-of-age. * indicates P<0.05 versus corresponding control. Mdx: four week old sedentary mdx, n=7; WT4: four week old sedentary C57BL/10 mice, n=3; Ex: exercised eight week old mdx mice, n=6; Sed: sedentary eight week old mdx mice, n=7; WT8: sedentary eight week old C57BL/10 mice, n=3.

<table>
<thead>
<tr>
<th></th>
<th>Mdx (n=7)</th>
<th>WT4 (n=3)</th>
<th>Ex (n=6)</th>
<th>Sed (n=7)</th>
<th>WT8 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exhaustion time (min)</td>
<td>16.5 ± 1.2 *</td>
<td>24.8 ± 0.9</td>
<td>18.8 ± 2.6*</td>
<td>25.3 ± 6.4</td>
<td>24.4 ± 1.3</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>16.3 ± 2.6</td>
<td>14.1 ± 2.1</td>
<td>22.9 ± 4.0</td>
<td>24.3 ± 2.6</td>
<td>21.9 ± 4.3</td>
</tr>
<tr>
<td>VO$_{2\text{basal}}$ (mL/kg/min)</td>
<td>64.9 ± 29.8</td>
<td>39.2 ± 3.7</td>
<td>42.4 ± 6.0*</td>
<td>79.25 ± 12.6</td>
<td>55.32 ± 5.3</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$ (mL/kg/min)</td>
<td>126.6 ± 12.9</td>
<td>133.5 ± 8.9</td>
<td>115.3 ± 4.4</td>
<td>123.0 ± 15.2</td>
<td>120.5 ± 6.2</td>
</tr>
</tbody>
</table>

Figure 3.1: Exercise and Metabolic Testing at Four Weeks-of-age. A: Exhaustion time on a graded maximal exercise test was significantly shorter in mdx mice, and at a much lower workload when compared to wild-type controls. * indicates P<.01. B: Mdx
and wild-type mice reach similar levels of oxygen consumption at maximal exercise output. WT4: 4 week old C57BL/10 mice, n=3; Mdx: 4 week old mdx mice, n=7.

**Figure 3.2: Exercise and Metabolic Testing at Eight weeks-of-age.**

A: Ex mice had significantly shorter exhaustion times compared to Sed mice on a graded maximal exercise test.* indicates P<.05 (ANOVA) B: Exercise negatively impacted basal oxygen consumption in mdx mice, causing a decrease in resting oxygen consumption compared to non-exercised mdx mice. * indicates P<.05 (ANOVA). C: Even after exercise intervention WT8, Ex, and Sed mice all reach similar levels of maximal oxygen consumption. WT8: 8 week old C57BL/10 mice, n=3; Sed: 8 week old sedentary mdx mice, n=7; Ex: 8 week old exercised mdx mice, n=6.
Figure 3.3: EPR Spectral Intensity Ratios Used to Investigate Oxidative Stress. A higher spectral intensity ratio of the probe compound (RSSR) compared to its bound glutathione product (RSH) indicates higher relative amounts of GSH and less oxidative stress from ROS. Quadriceps muscle from Ex mice was significantly less oxidized than Sed mice. * indicates P<0.05. Abdominal muscle from Ex mice followed the same trend to a higher degree. $ indicates P<0.01. Sed: 8 week old sedentary mdx mice, n=10; Ex: 8 week old exercised mdx mice, n=4-7.
Figure 3.4: Hydroxyproline Deposition in Several Muscles of Exercised and Sedentary Mdx Mice. Four weeks of treadmill running caused a significant increase in muscle hydroxyproline content within quadriceps and cardiac muscle. Abdominal muscle appeared largely unaffected. Hydroxyproline is a main constituent of collagen scarring that is seen in DMD as a result of muscle fibrosis. * indicates P<.05 versus corresponding control. Sed: 8 week old sedentary mdx mice, n=10; Ex: 8 week old exercised mdx mice, n=7.
Figure 3.5: Hematoxylin and Eosin and Collagen I immunostaining of Heart Sections from Exercised Mice Show Greater Amounts of Collagen Scarring at Eight weeks-of-age. Treadmill running increased the formation of collagen scarring in Ex mice as detected by immunofluorescence. Sed mice do not show significant amounts of collagen scarring by eight weeks-of-age and appear only slightly worse than WT8 mice. WT8: Sedentary 8 week old C57BL/10 mice; Ex: Exercised mdx; Sed: Sedentary mdx. Bar = 100 µM.
Figure 3.6 Quantification of Cardiac Tissue Damage and Fibrosis Histological Analysis. A: Cardiac myofiber damage in Ex mice, while higher, was not quite significant (P=.0958). B: Quantification of collagen I staining indicates that Ex mice had significantly higher cardiac muscle fibrosis compared to Sed mice. * indicates P<0.05. Sed: 8 week old sedentary $mdx$ mice, n=7; Ex: 8 week old exercised $mdx$ mice, n=6.

Discussion

A main finding of the present study showed that forced treadmill exercise increased the severity of dystrophic pathology in the $mdx$ mouse in most parameters, showing agreement with aspects of other studies utilizing this exercise protocol [12, 71]. Several studies using voluntary wheel-based running have shown exercise to be beneficial [76, 77, 88] even given the large distances these animals ran, in some cases up to 4km a day [77]. In this study the animals ran a total of 2.7km over the course of four weeks resulting
in large increases in fibrotic scarring and deficits in exercise capacity and oxygen metabolism. This difference in running distance ultimately points to the intensity and modality of exercise as playing a key role in how exercise affects dystrophic muscle. The given protocol appears too harsh and serves as a point of reference for any studies in the future looking to use exercise as a treatment strategy for DMD. Given that the \( mdx \) mouse typically has mild pathology compared to the human phenotype this protocol might serve well to induce a greater pathology and make exercised-\( mdx \) mice a better model of DMD for testing compounds meant to increase muscle strength.

To our knowledge, this is also the first study to examine how treadmill exercise effects whole-animal basal and maximal oxygen consumption in \( mdx \) mice. Maximal oxygen consumption remains one of the best predictors of all-cause mortality [89] and serves as an important marker of skeletal muscle and cardiovascular function. This exercise protocol had no effect on maximal oxygen consumption, rather a large effect on baseline oxygen consumption which was surprising given how exercise training typically enhances maximal oxygen consumption with little effect on basal oxygen consumption. A dampening of basal oxygen consumption is indicative of exercise producing disruptive changes in oxygen utilization and is bolstered by our findings on how treadmill exercise influences exercise capacity deleteriously, confirming results from previous research [73]. After several weeks of exercise, \( mdx \) mice were utilizing similar levels of oxygen as sedentary and wild-type mice, just at much lower workloads. The relationship between workload and oxygen consumption is well-established [90, 91] and seeing trained
animals consume more oxygen at a smaller workload shows the negative effect this exercise intervention had.

Another key finding was how exercise affects the oxidative environment of several dystrophic muscles. The various actions of ROS play an important role in several cellular processes in healthy tissue that respond favorably to oxidative stress resulting from exercise by increasing endogenous antioxidant system activity to maintain redox homeostasis after chronic bouts of exercise [92]. Multiple muscles from exercised mdx mice showed significantly better oxidative profiles, hinting that diseased muscle still adapts favorably to exercise with regards to oxidant buffering and redox homeostasis. Additionally, the lower levels of basal oxygen consumption seen in exercised mdx mice might be aiding this due to less oxygen being consumed and therefore less ROS being produced as a byproduct of mitochondrial respiration. This improvement in oxidative homeostasis from exercise seems contrary to previous studies [12, 71], potentially due to the differing technique used to examine oxidative stress and the different tissues investigated. We utilized entire-muscle preparations, rather than plasma or histological indicators of oxidative stress. Oxidative stress is an important outcome measure to evaluate the progression of DMD as it responds to exercise and could also be harnessed to examine outcomes related to differing treatment strategies.

The goal of this study was to comprehensively examine how a particular treadmill intervention affected the mdx mouse with regards to myofiber damage and fibrosis, oxygen consumption, exercise capacity, as well as oxidative stress in attempt to discover better outcome variables for disease management. Studies have shown that exercise can
be beneficial for DMD, yet our results indicate a tipping point where certain amounts of
exercise induce harmful adaptations. Due to the small amount of time and distance these
animals ran, the intensity of exercise most likely had the largest effect. In order to
optimize the potential of exercise as a therapeutic strategy for muscle disease future
studies need to investigate differing levels of exercise intensity and modality, and in turn
how they affects different parameters of muscle disease. Additionally, future advances in
treatment strategies for DMD will ultimately improve muscle function and would allow
patients to undergo increases in physical activity. Understanding how changes in physical
activity affect disease pathology, as well identifying novel outcome measures to monitor
disease status, remains crucial to maintain the best outcomes for DMD patients.
I have had the fortunate opportunity to be involved in biomedical research for the past six years. I transitioned to Ohio State after several successful years in the private sector to shift my research focus to neuromuscular disease and muscle physiology. I started off as a research associate in the department of Molecular and Cellular Biochemistry where my central focus was Duchenne’s muscular dystrophy (DMD). I played an integral role in the technical design, and implementation of experiments to test different therapeutic approaches for muscular dystrophy-associated heart failure. I consider myself a well-rounded researcher with experience in molecular biology and genetics, biochemistry, and applied muscle physiology. In all these previous pursuits I was never the sole lead researcher, as was the case with this project. Doing so provided a wealth of experience in how to successfully handle the burdens of a research project, interpretation of the results, and the forthcoming conclusions.

I have been incredibly fortunate at Ohio State to forge collaborations with researchers across multiple departments including: Human Sciences, Molecular and Cellular Biochemistry, Physiology and Cell Biology, and Pharmacology. These connections would prove to be pivotal in making this research project a success. True science requires multiple minds and the combined expertise of multiple labs. I was able to perform histology and biochemistry experiments in one lab, and oxidative stress and
metabolism experiments in other labs. This was crucial in determining the right data to collect and the proper conclusions to draw. I was able to learn several new skills and laboratory techniques that grew me as a person and scientist.

In addition to advances in my physical skillset I exposed myself to a brand new area of biomedical research. I was already quite familiar with both muscular dystrophy and exercise physiology, however, I was ignorant about the overlap between these two fields and how physical activity interplays with muscle disease. There is a massive, growing body of research on how exercise and physical activity modulate factors associated with muscle disease. I learned how different modalities and intensities of exercise affect muscle disease differently. Some exercise has proven to be beneficial, while others deleterious. I have grown my inner body of knowledge to encompass all aspects of exercise physiology and neuromuscular disease, and in turn have grown and matured as a scientist.

Scientific insight into how physical activity can influence disease states is a growing avenue of research. Specifically, neuromuscular disease presents an ideal platform to study how exercise and movement affect disease onset. Initially it might be assumed that exercise may be harmful to those with frail and diseased muscle. Surprisingly, most studies show that aerobic and strength gains can occur in those suffering from progressive disorders [93]. In order to preserve the best patient outcomes the exact time, intensity, and modality of exercise need to be further explored. Exercise can also be a key variable in assessing and managing neuromuscular disorders as they
progress and in different patient populations [94] as well as evaluating how well rehabilitation management is progressing in those afflicted [95].

Exercise investigation has a definite niche within the muscular dystrophy research community. For the past several decades researchers have started to begin examining how physical activity influences changes in diseased muscle. There remains definite evidence that exercise can even be used as a therapy for muscular dystrophy. Dying muscle remains a hallmark feature of DMD and exercise has been shown to do quite the opposite. Initial animal studies with differing modalities of exercise even show promise in reversing deficits in muscle function and increasing growth of muscle of specific muscles [14-16]. We chose to study treadmill running as it yields the ability to finely tune intensity and time of the exercise dose.

A main finding of the present study showed that forced treadmill exercise increased the severity of dystrophic pathology in the mdx mouse in most of our parameters, showing agreement with certain aspects of other studies utilizing this exercise protocol [71]. Also, to our knowledge, this is also the first study to examine how treadmill exercise effects whole-animal basal and maximal oxygen consumption in mdx mice. Another key finding was how exercise affects the oxidative environment of several dystrophic muscles. Multiple muscles from exercised mdx mice showed significantly better oxidative profiles, hinting that diseased muscle still adapts favorably to exercise with regards to oxidant buffering and oxidative homeostasis. Most importantly this study identifies oxidative stress as a novel outcome of interest that can be used to monitor new treatment strategies.
This project – “Oxygen Consumption, Muscle Fibrosis, and Oxidative Stress in the mdx mouse: Influence of Treadmill Running” was a tremendous learning experience. I improved existing collaborations with researchers, advanced new ones, and networked with other graduate students researching in my subject area. I find it incredible that I was able to take my previous skill set and add a new, fresh research focus to it. In doing so I advanced my skills, knowledge, and efficacy as a biochemist and applied physiologist.
BIBLIOGRAPHY