Leucine and exercise improve skeletal muscle function in the *mdx* mouse

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Human Nutrition, Foods and Exercise

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January 21, 2010
Blacksburg, VA 24060

Keywords: Leucine, *mdx*, Muscular Dystrophy, Calpains, mTOR, Skeletal Muscle

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ABSTRACT

Duchene muscular dystrophy (DMD) is a lethal X-linked disease that afflicts approximately 1 in 3500 newborn males. Boys with DMD will become progressively weaker causing wheelchair dependence by their early teens and death by their mid to late twenties. Currently there is no cure for DMD, the exact mechanism of disease action remains elusive, and treatments to improve quality of life are limited. Two areas of DMD research that could begin to fill this void and provide simple, cost effective therapy aimed to improve quality of life are neutriceutical and exercise therapies.

We hypothesized that leucine, a branched chain amino acid (BCAA) with anabolic properties, given to sedentary and exercised x-linked dystrophic mice (*mdx*) over 4 weeks would improve skeletal muscle function and decrease markers of skeletal muscle degradation. In sedentary *mdx* mice, leucine improved tetanic extensor digitorum longus (EDL) stress ($p < 0.05$), gastrocnemius mammalian target or rapamycin (mTOR) phosphorylation ($p < 0.05$), while decreasing the rate of real-time calpain activity in flexor digitorum brevis (FDB) fibers ($p < 0.05$) compared to sedentary mice given no leucine. In exercised *mdx* mice, leucine improved total running distance over the 4 week testing period by 40% ($p < 0.02$) and increased EDL stress at every frequency recorded ($p < 0.05$).

Our data lead us to the conclusion that the BCAA leucine can increase EDL muscle stress in dystrophic animals, and that the effects of leucine treatment are enhanced when leucine supplementation is combined with exercise. Leucine supplementation should be explored further and in higher order species of muscular dystrophy to determine if its use could provide clinical improvements in DMD patients.

Acknowledgments

I would like to thank all of my committee members for their input and encouragement during my training. I would especially like to thank Dr. Robert Grange for seeing talent in me as an undergraduate student in his exercise physiology class, and encouraging me to enter graduate school. His leadership and guidance has brought me to the place I am today and I am extremely thankful for that. I would also like to thank Dr. Christopher Ward for sharing his knowledge and helping collect data on numerous occasions. Your willingness to extend your hand and your resources to me has had an unimaginable impact on me as a person and a scientist.

To Andy Wolff, Nick Evans, Jarrod Call, Mary Pat Meaney, Sarah Misyak, and the undergraduate research assistants who I have worked with in the past, thank you for your help and support during my time as a graduate student. We have had many great times together, and you have all played a part in creating memories that will last a lifetime.

To my brother, who unknowingly has inspired me on a daily basis to be a better person, seeing you grow as a person and an artist has been one of the most amazing things in my life. You have always been there to offer support and advice when I needed it, and that has been invaluable to me.

To my parents, your unending love and support made this possible. Thank you for encouraging me and nurturing my talents from a young age, you have been there and seen me through the best and worst times in my life and always stood behind me 100%. Everything I have become and I do is a reflection of you and your love and support. To my extended family, thank you for caring for me and being a part of my life from day one. Whether it was extra gas money for my trips home, or reminding me of "the rule", your input, love and support has impacted me as a person and I hope I have made you all proud.

To Lorna, you have come into my life during one of my most trying times and weathered the storms with me. Thank you for your love, encouragement, and making me smile on a daily basis. I hope I can provide the same love and support for you as you go through life and face its many trials.

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Abbreviations

DMD – Duchenne's muscular dystrophy

mdx – X-linked muscular dystrophy mouse

DGC –Dystrophin Glycoprotein Complex

CK – Creatine Kinase

mTOR – Mammalian Target of Rapamycin

EDL – Extensor Digitorum Longus

FDB – Flexor Digitorum Brevis

BL/10 – C57BL/10 mice given normal drinking water

BL/10-L – C57BL/10 mice given leucine supplemented drinking water

mdx – *mdx* mice given normal drinking water

mdx-L – *mdx* mice given leucine supplemented drinking water

mdx Run – Exercised *mdx* mice given normal drinking water

mdx Run-L – Exercised *mdx* mice given leucine supplemented drinking water

WGA – Wheat germ agglutinin

EBD – Evan's blue dye

PO – Procion orange

Chapter 1 - Introduction

1.1 - Introduction

DMD is a lethal X-linked disease that afflicts 1 in 3500 newborn males[66,76]. The disease is characterized by a genetic mutation in the dystrophin gene, resulting in the loss of the sub-membranous protein, dystrophin[15]. The dystrophin protein is an important mechanical link from the intracellular cytoskeletal actin to the associated membrane proteins known as the dystrophin glycoprotein complex (DGC)[80]. In skeletal muscle, the loss of both dystrophin and the DGC is hypothesized to lead to mechanical or contraction-induced membrane rupture, and altered cellular signaling. These changes are believed to cause the progressive skeletal muscle weakness and mass loss in DMD boys. Currently, the exact mechanisms of disease onset and progression are not known and no cure or long-term treatment is available[38,102].

Boys affected by DMD have no major physical signs of the disease at birth, but will have elevated blood levels of the muscle specific enzyme, creatine kinase (CK), and may have difficulty holding their heads upright[1]. DMD boys take longer to learn to walk (~24 months), will develop a waddling gait, and use the Gowers' maneuver to pick themselves up from the floor, typically between ages 5 -7 years. Afflicted boys become wheelchair bound in their early teens, and typically die in their early to mid-20's[1]. Since the discovery of the genetic basis for the disease in the early 1980's, little advancement has been made in disease treatments.

A major goal across numerous laboratories has been the identification of strategies to delay the pathogenic progression of the muscle dysfunction in DMD. Despite the significant efforts in this area, the use of therapeutic exercise as well as nutraceutical interventions have been relatively unexplored. Herein, we focused on a potential nutritional supplement, leucine, endurance exercise and their potential synergistic interactions in DMD. Leucine is an essential

branched chain amino acid (BCAA) that shows promise as a supplement to increase skeletal muscle mass and strength. Leucine stimulates protein synthesis through the mammalian target of rapamycin pathway[113,11,6] (mammalian target of rapamycin -mTOR, Figure 1.1) and has shown promise as an inhibitor of calpain mRNA expression[98,97]. mTOR is a regulator of skeletal muscle growth and hypertrophy, and can be stimulated following mechanical overload (i.e., weight training) or through nutrient stimulation (i.e., insulin, leucine).[49] Calpains are ubiquitously expressed calcium-activated proteases which alter or cleave proteins in skeletal muscle that are then further degraded by the lysosomal or ubiquitin pathways. Dystrophic skeletal muscle fibers are believed to be susceptible to excessive calcium entry, thereby activating calpains and calpain-related proteolysis.[55,131,9] Because of leucine's ability to increase protein synthesis while depressing proteolysis, it could potentially slow the progressive loss of muscle mass and strength associated with dystrophic skeletal muscle.

A second reason leucine was targeted as a potentially beneficial nutritional therapy is that when used in conjunction with exercise, leucine enhances strength gains and shortens recovery time from exercise activities[4,16,34]. In the dystrophic mouse model (*mdx*), these mice adapt to exercise by increasing running distance each consecutive week of voluntary wheel running, while increasing muscle mass and strength[29,30,42,132]. The positive effects of exercise in the *mdx* mouse are most robust when exercise is initiated at an early age (i.e., weaning age 21 – 28 days), and when using a voluntary running wheel as opposed to forced treadmill running[132]. Interestingly, studies to determine if leucine can benefit dystrophic skeletal muscle under both sedentary and exercise states have not been performed.

We hypothesized leucine supplementation in sedentary *mdx* mice would increase extensor digitorum longus (EDL) stress production, mTOR activation, and decrease calpain-

related proteolysis. We also hypothesized that when leucine supplementation was combined with voluntary wheel running exercise, *mdx* mice would adapt to exercise more effectively, and the positive effects of exercise on EDL stress would be increased compared to exercise alone.

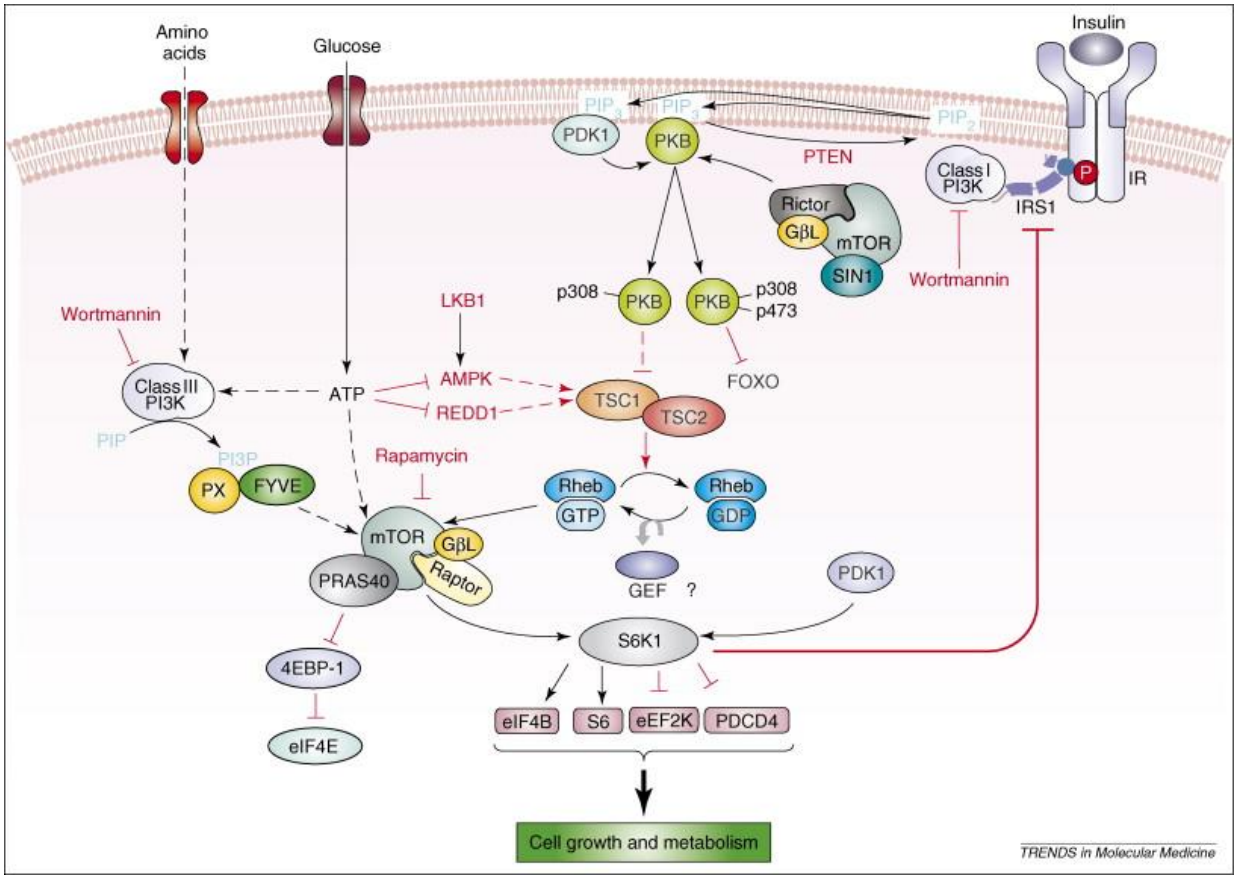

Figure 1.1 –An overview of the mTOR pathway in skeletal muscle. mTOR exists in two structurally distinct complexes. mTOR Complex1, which contains raptor, is regulated by amino acids (i.e., Leucine), ATP and insulin, all of which initiate diverse signaling cascades (broken arrows indicate the uncertainty of the mechanisms involved). Phosphorylation of S6K1 by mTOR Complex1 enables PDK1 to phosphorylate and activate S6K1. Abbreviations: eEF2K, eukaryotic elongation factor 2K; eIF4B, eukaryotic initiation factor 4B; FYVE, Fab1/YOTB/2K632.12/Vac1/EEA1 domain; GEF, guanosine nucleotide exchange factor; PDCD4, programmed cell death protein 4; PX, Phox homology domain[36].

1.2 - Research Goal

The overarching goal of this dissertation project was to determine if oral leucine supplementation in young *mdx* mice with or without voluntary run exercise, can increase skeletal muscle function. We tested this possibility in two different ways. First, in sedentary *mdx* mice, we will determine if 4 weeks of leucine supplementation increases EDL stress, mass, or cross-sectional area (CSA). We will also determine if leucine increases the phosphorylation of mTOR, and changes the content or activity of calpain proteins.

Second, evidence from the literature suggests exercise effects are enhanced when leucine supplementation is provided[16,78,34]; therefore we will provide mice access to a voluntary running wheel during 4 weeks of leucine supplementation. At the conclusion of the 4 weeks of combined leucine and voluntary run exercise, we will determine if there are changes in body mass, running distance, EDL stress production, morphology, and myosin heavy chain distribution.

1.3 - Study Design

The two studies performed for this dissertation are outlined below.

1.3.1 - Study 1 – Leucine increases *mdx* EDL stress production

Age 21 day, male *mdx* and C57BL/10 (BL/10) mice were separated into either leucine supplemented (*mdx*-L, BL/10-L) or non-leucine supplemented (*mdx*, BL/10) groups. The leucine supplemented groups had 114 mM leucine in their drinking water while the non leucine supplemented groups had normal drinking water for 4 weeks. These groups had their body mass recorded weekly, and at the conclusion of the 4 week study, animals were sacrificed and assays were performed. Because of limited tissue availability, these assays were conducted on three different muscles of the lower hind limb.

Table 1.1 – The assays performed for study 1

Whole Animal	Weekly Body Mass
Muscle	***Assays***
Extensor Digitorum Longus (EDL)	In Vitro Stress Production; Section Analysis; Myosin Heavy Chain Distribution
Gastrocnemius	Immunoblots (mTOR and Calpain:Calpastatin)
Flexor Digitorum Brevis (FDB)	Real-time calpain activity

1.3.2 - Specific Aims

To determine if 4 weeks of leucine supplementation will improve skeletal muscle morphology and contractile properties in *mdx* mice, and if these changes are related to changes in the leucine signaling pathway as measured by mTOR activation and protein degradation rates as measured by calpain-mediated proteolysis. We will determine in vitro EDL contractile properties, EDL morphological properties, and EDL myosin heavy chain distribution. We will also determine if leucine increases mTOR activation, and depresses calpain proteolysis or calpain content.

1.3.3 - Hypotheses

1: It is hypothesized leucine supplementation will increase body mass.

2: It is hypothesized leucine supplementation will increase EDL stress production.

3: It is hypothesized leucine supplementation will increase EDL mass and cross-sectional area.

4: It is hypothesized leucine supplementation will increase MHC type IIa and IIx content in EDL muscles.

5: It is hypothesized leucine supplementation will decrease that percentage of centrally nucleated fibers in EDL muscle.

6: It is hypothesized leucine supplementation will increase average fiber area in EDL muscle.

7: It is hypothesized that leucine supplementation will decrease the rate of calpain substrate cleavage in FDB muscle fibers.

8: It is hypothesized that leucine supplementation will decrease the ratio of calpain to calpastatin.

9: It is hypothesized that leucine supplementation will increase mTOR phosphorylation.

1.4.1 - Study 2 – Leucine combined with voluntary wheel running enhances the effects of exercise in the *mdx* mouse

Age 21 day, male *mdx* mice were separated into individual cages with access to voluntary running wheels. These animals were split into two groups; 1) exercised *mdx* mice with leucine supplemented drinking water (114 mM, *mdx* Run-L) and 2) exercised *mdx* mice with normal drinking water (*mdx* Run). Animals were housed for 4 weeks with daily running distance, and weekly body mass being recorded. After 4 weeks, tissues were collected and assays were performed.

Table 1.2 - The assays performed for study 2

Whole Animal	Weekly Body mass; Running Distance
Muscle	***Assays***
Extensor Digitorum Longus (EDL)	In Vitro Stress Production; Section Analysis; Myosin Heavy Chain Distribution

1.4.2 - Specific Aims

To determine if 4 weeks of leucine supplementation combined with voluntary wheel running exercise will enhance the effects of exercise in *mdx* mice. To determine if leucine has an impact on dystrophic mouse running capacity and dystrophic skeletal muscle adaptation, we will determine average running distance over 4 weeks, as well as in vitro EDL contractile and morphological properties, and myosin heavy chain distribution.

1.4.3 - Hypotheses

1: It is hypothesized leucine supplementation with exercise will increase body mass.

2: It is hypothesized leucine supplementation will increase the running distance of *mdx* mice.

3: It is hypothesized leucine supplementation with exercise will increase EDL stress production.

4: It is hypothesized leucine supplementation with exercise will increase EDL mass and cross-sectional area.

5: It is hypothesized leucine supplementation with exercise will increase MHC type IIa and IIx content in EDL muscles.

6: It is hypothesized leucine supplementation with exercise will decrease the percentage of centrally nucleated fibers in EDL muscles of *mdx* mice.

7: It is hypothesized leucine supplementation with exercise will increase the average fiber diameter in EDL muscle of *mdx* mice.

Chapter 2 - Review of Literature

2.1 - Introduction

This review summarizes the available literature on Duchenne's muscular dystrophy, the effects of dystrophy on skeletal muscle, the potential benefits of exercise for muscular dystrophy, the potential effects of leucine on dystrophic skeletal muscle, and the potential synergistic effects of leucine and exercise on dystrophic skeletal muscle function.

2.2 - Duchenne's muscular dystrophy

2.2.1 Brief disease history

DMD was first characterized by two different physicians in the mid-1800s. The first clinical descriptions of the disease appeared in the 1850's authored by the English physician Edward Meryon. Meryon described the clinical signs of DMD, and noted three specific characteristics: 1) the disease was "familial" and affected only males; 2) the nervous system was intact, therefore DMD specifically affected the musculoskeletal system; and, 3) muscle membranes were destroyed, and in many areas the muscle had been replaced by oil globules and granular matter. Even with this very early account, it appeared the disease was genetic, specific to skeletal muscle, and acted by breaking down muscle tissue[1].

In the 1860's, a French physician, Duchenne, described both the clinical signs of DMD and detailed muscle histology. Duchenne was the first physician to use a biopsy needle to obtain living tissue samples from DMD boys, which was very controversial at the time. Duchenne's characterization of DMD began when he treated a 9 year old boy who was losing his ability to walk because of a muscle wasting disease. This intrigued Duchenne and he sought out additional cases, and by 1868 Duchenne had observed and carefully recorded the signs and symptoms of 13 boys.. Duchenne combined his observations and published an extensive report on the disease[71].

Following the early disease descriptions by Meryon and Duchenne, it was not until the 1960's that DMD was found to be X-linked[90]. This was confirmed when a girl with Turner's syndrome, possessing only one x-chromosome, was afflicted with DMD[52]. Typically, females possess two X-chromosomes, so if one is mutated, the second x-chromosome will still possess an un-mutated dystrophin gene so that dystrophin protein can be expressed. With one X- and one Y-chromosome, males do not have the ability to compensate for the mutated dystrophin gene. However, it wasn't until 20 years later that the specific location of the dystrophin gene, locus Xp21, was discovered[91]. Subsequently, the location of the dystrophin protein was discovered to be sub-membranous, located on the cytosolic face of the muscle cell membrane[76,136]. The absence of the dystrophin protein from the cytosolic face of the muscle cell membrane is the primary cause of DMD, and because dystrophin attaches the skeletal muscle membrane and membrane associated proteins to the myofilaments inside the muscle cell, its absence results in progressive degeneration, regeneration, and death of muscle tissue that leads to the progressive loss of strength and ambulation in boys with DMD. However, the specific mechanism or mechanisms by which the progressive loss of muscle tissue occurs is still not yet defined.

2.2.2 - The Pathogenesis of Duchenne Muscular Dystrophy

DMD can be detected through genetic testing in fetuses as early as the second trimester[13]. DMD newborns are described as "floppy" because of difficulty holding their heads upright or still, and have elevated serum creatine kinase (CK) levels[102]. CK is a muscle specific enzyme that is believed to enter the blood stream following muscle tissue degradation or breakdown, but the exact mechanism of CK leakage is currently unknown. The combination of these signs and symptoms is considered to be the first clinical signs of DMD.

DMD is also associated with a delay in learning basic motor skills, and approximately 50 percent of DMD patients take 18 months to learn to walk, while 25 percent can take 2 years or more[44,45]. In contrast, 97 percent of healthy children are able to walk by age 18 months[99]. Between the ages of 3 and 5, DMD children typically demonstrate a waddling gait while walking, difficulty getting up and down stairs, and individuals with the disease never learn to run properly. This is soon followed by pseudohypertrophy (increased skeletal muscle size without improved function or strength) of the calf muscles, and proximal limb muscle weakness. Limb muscle weakness is bilateral and symmetrical, starting in the lower limbs and working from muscles proximal to the trunk inwards[46].

As the dystrophic process continues and muscles weaken, lumbar lordosis becomes more pronounced, and children require a wheelchair typically between the ages of 7 and 13[86]. In 90% of patients, severe spinal kyphosis results from wheelchair dependence. The age of wheelchair dependence is roughly correlated to the age of death, with the most severe and early cases, resulting in the youngest age of death[44]. Death from DMD can be caused by respiratory failure due to diaphragm and intercostal muscle weakness, or by cardiac complications. Some patients

use forced air respiration machines to assist breathing; the use of forced air respirators have helped prolong the life of a few DMD patients into their 30's[43].

The lifelong progressive muscle strength and mass loss with DMD is due to mutations of the dystrophin gene. The mutations to the dystrophin gene are at the root of disease progression and result in the lack of a stable dystrophin protein being produced; understanding the role of the dystrophin protein is vital to understanding the mechanism(s) of disease onset and progression.

2.2.3 - Dystrophin Gene and Protein

2.2.3.1 - Gene

The dystrophin gene is the largest gene in the human genome at 2.5 Mb, and consists of 79 exons, with introns making up the remainder of the gene[15,38,102]. The dystrophin gene is located on locus Xp21 in the X-chromosome[37], and approximately 60% of the mutations to the dystrophin gene are large insertion mutations that lead to frame shift errors. The remaining 40% of mutations to the dystrophin gene are random point mutations that can cause premature stop codons or small frame shift changes[66]. Either frame shift or point mutations lead to incomplete transcription of the dystrophin gene, and subsequently no functional dystrophin protein product.

The 14 kb mRNA sequence transcribed from the dystrophin gene is primarily found in skeletal and cardiac muscles, but also is present in the brain and in brain Purkinje cells. Expression in each of these tissue or cells is driven by 3 different full-length promoters that are followed by the same 78 exons. The three full-length promoters are for the brain (B), Purkinje cells (P), and skeletal and cardiac muscle (M) isoforms of dystrophin (Figure 2.2)[21,31]. The protein product from the brain promoter is primarily expressed in cortical neurons and in the hippocampus of the brain. The protein product from the Purkinje promoter is found in the

Purkinje cells in the cerebellum of the brain as well as in skeletal muscle. The muscle isoform, is expressed primarily in skeletal muscle and cardiomyocytes, with only low level expression in the glial cells of the brain[15]. Translation of any of the mRNAs that arise from these separate promoters results in the production of the specific isoforms of the dystrophin protein.

There are also four splice variant promoters that will produce a truncated COOH-terminal dystrophin protein (Figure 2.2). They are retinal (R), brain-3 (B3), Schwann cell (S), and general (G). These four proteins are much smaller in size than the full length protein, with masses of 260 kDa, 140 kDa, 116 kDa, and 71 kDa, respectively. The protein product that arises from the general promoter is expressed in most non-muscle tissues, while the retinal, brain-3, and Schwann isoforms are primarily expressed in central nervous tissues (Figure 2.2).

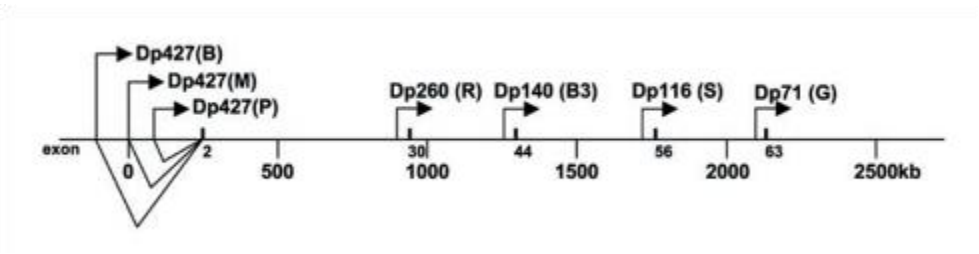

Figure 2.2-A schematic of the dystrophin gene with the three major promoter: brain (B), muscle (M), and Purkinje (P), as well as the four splice variant promoters; retinal (R), brain-3 (B3), Schwann (S), and general (G) regions indicated above the exonwhere transcription is initiated (used with permission)[15].

2.2.3.2 - Protein

The full length dystrophin protein is 427 kDa, that is composed of 3685 amino acids, and is present in all types of skeletal muscles. Dystrophin is a cytoskeletal membrane protein that is a member of the β-spectrin/α-actinin family. It is considered to be crucial to sarcolemmal integrity of muscle cells because of its association with the 13 other proteins known as the dystrophin glycoprotein complex (DGC, Table 2.3, Figure 2.3) and with cytoskeletal γ-actin[108,107,77]. Much like dystrophin, if any of the DGC proteins are absent from skeletal muscle,

other forms of muscular dystrophy can result, some more severe than others (Table 2.3). The most severe form of muscular dystrophy is Duchenne's because when dystrophin is absent, the proteins of the DGC are also greatly reduced. Therefore, a mechanical and signaling link from the interior to the exterior of the muscle cell is lost, highlighting the importance of dystrophin and its ability to anchor the proteins of the DGC to the membrane. As noted above, dystrophin is primarily expressed in skeletal muscle, but different isoforms can also be found in the brain and Purkinje fibers[21,31].

The dystrophin protein is comprised of four separate domains (Figure 2.3). These four domains include the actin binding domain at the NH_2 terminus, the central rod domain, the cysteine-rich domain, and the COOH-terminal domain. The NH_2 domain binds to the cytoskeletal protein, F-actin, providing a structural anchor for the dystrophin protein. The central rod domain is comprised of 24 spectrin-like repeating units that are believed to give the molecule its flexible structure. The cysteine-rich domain has several protein motifs associated with Ca^{2+} binding and the binding of other divalent cations such as Zn^{2+}, and also binds the protein calmodulin in a Ca^{2+} -dependent manner. The COOH-terminal domain is believed to form an α-helical coiled coil which binds dystrobrevin and may control binding to many other proteins of the DGC[102]. The primary research tool used to understand the dystrophin protein and its function has been the mouse model of muscular dystrophy, the *mdx* mouse.

Table 2.3 - This table lists dystrophin and the proteins of the DGC including their names, masses, locations and associated dystrophy types, when specific proteins are absent. (BMD – Beckers muscular dystrophy)

Protein	Molecular Mass	Location	Mutation Associated Diseases
Dystrophin	427 kD	Intracellular	DMD/BMD
á-Dystroglycan	156 kD	Extracellular	Lethal
Â-Dystroglycan	43 kD	Transmembrane	Lethal
á-Sarcoglycan	50 kD	Transmembrane	á-Sarcoglycanopathy
â-Sarcoglycan	43 kD	Transmembrane	Â-Sarcoglycanopathy
ã-Sarcoglycan	35 kD	Transmembrane	ã-Sarcoglycanopathy
ä-Sarcoglycan	35 kD	Transmembrane	ä-Sarcoglycanopathy
Sarcospan	25 kD	Membrane Integrated	Normal
á-Dystrobrevin	90 kD	Intracellular	Dystrophic Phenotype
á-Syntrophin	60 kD	Intracellular	Normal
$â_1$-Syntrophin	60 kD	Intracellular	unknown
$â_2$-Syntrophin	60 kD	Intracellular	unknown
n-NOS	161 kD	Intracellular	unknown
Caveolin-3	22-24 kD	Membrane Integrated	Limb-Girdle MD

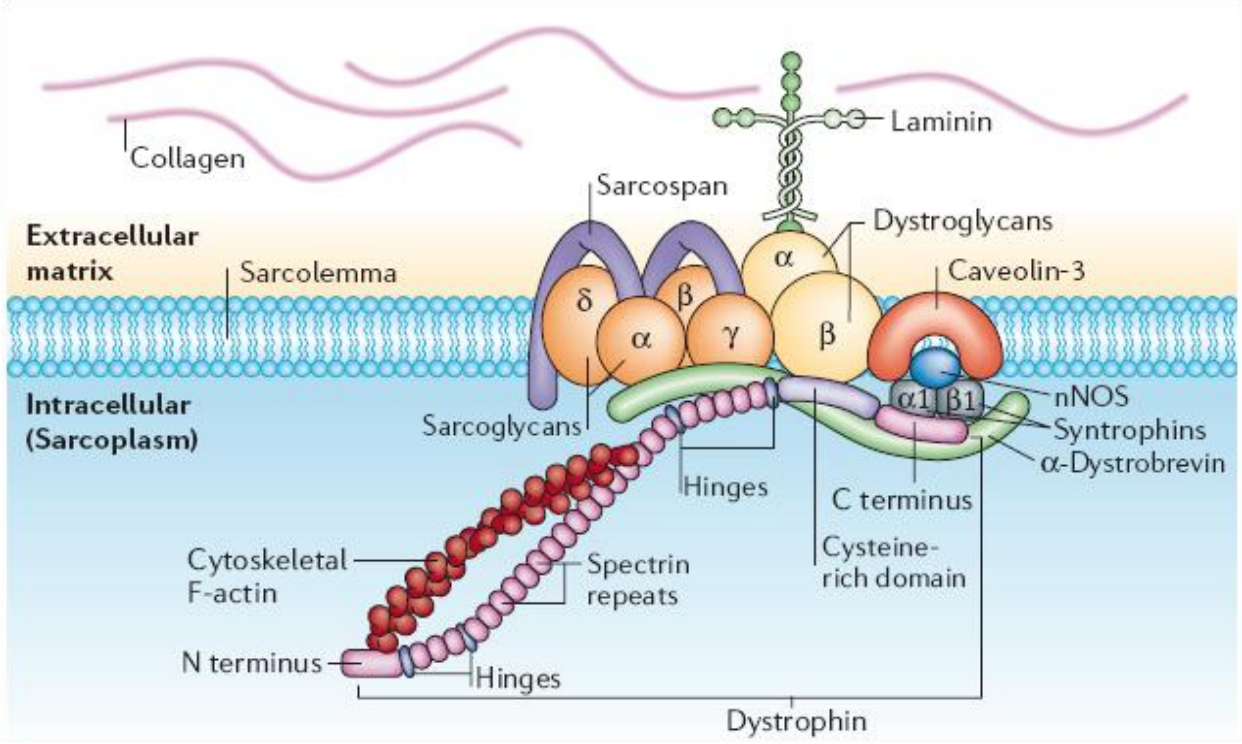

Figure 2.3 - The four regions of the dystrophin protein: (1) NH2 domain, (2) central rod domain, (3) cysteine-rich domain, and (4) the COOH-terminal domain. The NH2 domain attaches to cytoskeletal F-actin, then the cysteine rich domain associates with the proteins of the DGC, and the COOH-terminal domain also binds dystrobrevin. The binding of both cytoskeletal and membrane bound proteins to dystrophinsupports its role as a structural protein[38].

2.2.4 - The *mdx* mouse model of Muscular Dystrophy

To study muscular dystrophy, several animal models are used. The two most common animal models are the *mdx* mouse and the golden retriever model of muscular dystrophy (GRMD dog). In this project, the *mdx* mouse was used exclusively, so this review will focus on the details of this dystrophic model.

2.2.4.1 - Discovery and Characterization of the *mdx* mouse

The *mdx* mouse was discovered in 1984 by Bulfield et al. while screening for enzyme mutations in the glycolytic metabolic pathway. While screening for glycolytic mutations, Bulfield discovered certain mice had a 3-fold increase in blood levels of pyruvate kinase (PK). It was determined that the elevated PK was a muscle specific isoform, and the animals that expressed this trait also had a recessive X-chromosome linked mutation. Bulfield was intrigued by these findings and found that elevated blood PK levels mirrored a human muscle disease, DMD[61]. Following the discovery that the mutant mice mirrored human DMD, Bulfield carried out a complete skeletal muscle histological analysis. The muscle histological analysis revealed additional similarities to human DMD. For example, mice aged 4 days, had no irregularities in the muscle, which is similar to DMD newborns where there is little early physical evidence of the disease. Mutated mice, aged 3 weeks (i.e., weaning age/early maturation), began to show changes in the limb muscles compared to control animals, including skeletal muscle atrophy and necrosis. The mutated mice also had a high percentage of limb muscle fibers with centralized nuclei, which are present following skeletal muscle fiber degeneration and regeneration (Figure 2.4)[22]. When the mutated mice reached the age of 9 weeks, centralized nuclei, atrophy, and weakness were evident in a number of skeletal muscles. Following these discoveries, the mutated animals were inbred to create the *mdx* or X-linked muscular dystrophy mouse model[24].

Mdx mice outwardly appear physically similar when compared to control animals, have no obvious movement weakness, and live a slightly shortened lifespan, which is different from the human form of the disease. Pastoret et al. followed 23 C57BL/10 and 24 *mdx* mice over their lifespan[104]. At age 78 weeks, all animals from both groups were still alive, but by 104 weeks 17 C57BL/10 remained alive compared to only 2 *MDX* mice.

As *mdx* mice age, they lose body and muscle mass, especially as they near the end of their lifespan; a similar pattern is seen in DMD boys. When Pastoret et al. were determining the lifespan of *mdx* mice, they also measured the body and tibialis anterior (TA) muscle mass. Body mass significantly decreased in *mdx* mice compared to C57BL/10 mice following age 50 weeks, and by week 100 *MDX* body mass was approximately 1/3rd of C57BL/10 mice. In *mdx* mice, the TA muscle saw a marked pseudohypertrophy peaking around age 20 weeks (~80 mg *mdx* vs. ~50 mg C57BL/10), by week 100 *mdx* TA mass was approximately 1/2 of C57BL/10 TA mass (~20 mg *mdx* vs. ~40 mg C57BL/10)[104].

In addition to loss of body and skeletal muscle mass as *mdx* mice age, *in vitro* analysis of muscle force production is decreased in *mdx* mice when it is normalized to the cross-sectional area of the muscle (i.e., stress production, g/mm^2). For example, Lowe et al. reported a significant 20% drop in 21 and 35 day old *mdx* EDL muscle stress production ($p < 0.001$) when compared to C57BL/10 EDL stress production[82]. Also, Lynch et al. reported a depression in *mdx* EDL force production at age 6, 17, and 24 months ($p < 0.05$) compared to C57BL/10, and soleus force production was also depressed at 6, 17, 24 and 28 months ($p < 0.05$) compared to WT soleus muscles[84].

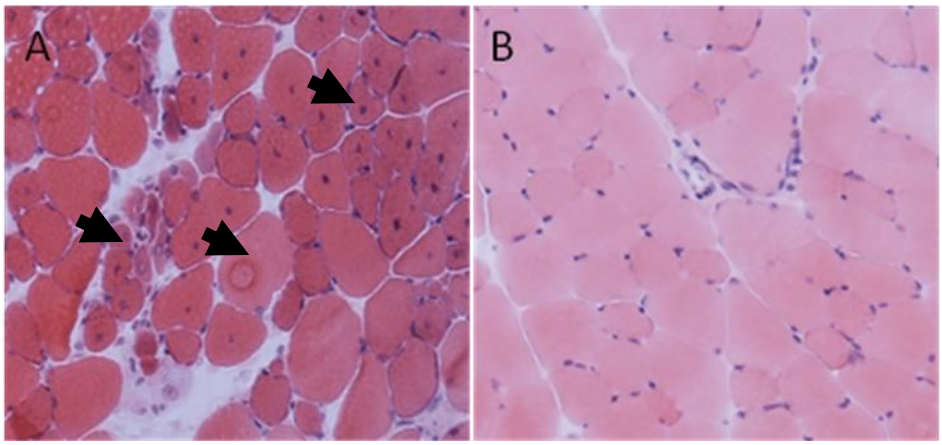

Figure 2.4 - Hematoxlyn and Eosin stained sections of a 7 week old *mdx* EDL muscle (A) and a 7 week old WT or control EDL muscle (B). In the *mdx* EDL, there are centrally nucleated fibers, areas of fiber breakdown and regeneration, as well as swollen fibers(areas indicated by large arrowheads). In the WT EDL muscle, the nuclei are located at the periphery of the muscle fibers, and fibers possess similar shape and size.

2.2.5 - Muscle specific effects of Duchenne Muscular Dystrophy

The *mdx* mouse has been extensively used to study many of the muscle specific effects of muscular dystrophy. In addition to the changes in muscle mass and function, dystrophic skeletal muscle demonstrates several key changes in morphology. Cross-sections of dystrophic skeletal muscle reveal individual muscle fibers which are inconsistent in shape and size compared to healthy skeletal muscle. The inconsistency in size and shape could be due to an increase in bifurcated and malformed muscle fibers[81]. In addition to the changes in size and shape, dystrophic skeletal muscle cross sections stained with hematoxylin and eosin reveal infiltration of immune cells and a large percentage of muscle fibers with centralized nuclei (Figure 2.5).

Dystrophic skeletal muscle has a high rate of protein degradation. Following skeletal muscle injury, the muscle must regenerate, but over time, the ability of dystrophic skeletal muscle to regenerate declines because of premature depletion of the muscle satellite cell pool[2]. Satellite cells are myogenic precursor cells, and when muscle injury occurs they migrate to the area of injury and begin to proliferate. Once satellite cells have reached the injured area and proliferated, they fuse and repair the damaged muscle fiber. The depletion of the satellite cell

pool may play a role in the replacement of skeletal muscle with fat and connective tissues that causes the loss of ambulation and strength in dystrophic muscles. As muscle loss becomes pronounced, contractures will occur across the joints of DMD boys. Contractures are caused by an imbalance of muscle strength across a joint, and typically force the limb into a flexed position. Contractures inhibit movement at the knees, hips, feet, elbows, wrists, and fingers[87].

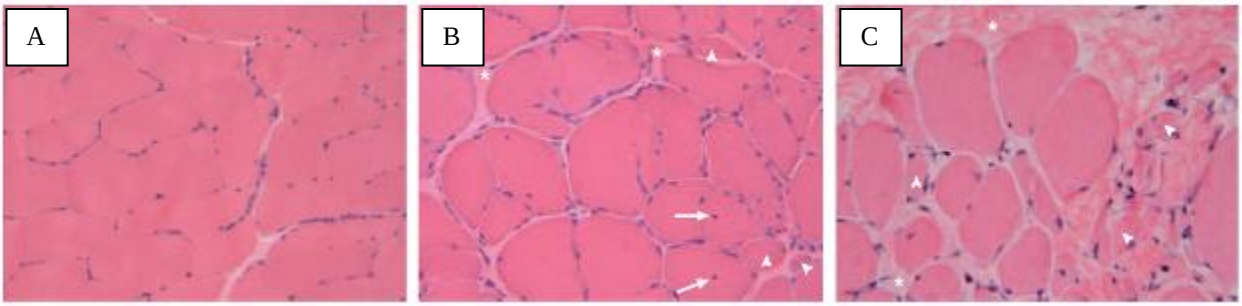

Figure 2.5 - Hematoxlyn and Eosin staining of skeletal muscle from (A) a healthy boy and (B-C) a boy with DMD. Arrows point to centralized nuclei; arrow heads demonstrate the variation in fiber size, and the asterisks indicate increased connective tissue[38].

2.2.6 - Hypotheses on dystrophic muscle degradation

There are two major hypotheses to explain why dystrophic skeletal muscle continually degenerates and regenerates: (1) mechanical and, (2) signaling. Under the mechanical hypothesis muscles waste and break down because dystrophin, the physical link from the actin cytoskeleton to the DGC located in the membrane of the muscle cell, and the proteins of the DGC are absent. The absence of these proteins causes membrane disruptions and ruptures that lead to muscle cell death[15]. To explore this hypothesis, measurements of serum CK, membrane impermeable dyes such as procion orange (PO) or Evans blue dye (EBD), muscle histology, and changes in myoplasmic ion concentrations have been used. Under the signaling hypothesis the components of the DGC may play an important signaling role in skeletal muscle. When the components of the DGC are lost, there is aberrant signaling which could lead to an increase in cytosolic calcium concentrations ($[Ca^{2+}]_i$) as well as an increase in reactive oxygen species (ROS) and their activity. Although there is evidence to support both the mechanical and

signaling hypotheses, there is no clear definition of the mechanism or series of events that leads to the continuous cycles of skeletal muscle degeneration and regeneration in DMD. The cycle of skeletal muscle degeneration and regeneration also takes place in *mdx* mice, but is isolated to several weeks during early life. Following the time period of degeneration and regeneration, skeletal muscle stabilizes, allowing *mdx* mice to live a fairly normal lifespan[50]. Therefore, studies in *mdx* mice should be focused during the time period when skeletal muscle degeneration and regeneration is most prevalent.

2.2.7 - Exercise and *mdx* mice

Mdx mice adapt well to voluntary wheel running[29,96,132,64,30,42,63], but the effects of enforced treadmill running seems to be detrimental to the function of *mdx* muscle[53,110,130,40]. When *mdx* mice begin voluntary wheel running at a young age (21 – 28 days old), typically they can sustain muscle strength later into life, and show functional and morphological improvement over their age-matched, sedentary counterparts.

2.2.7.1 - Voluntary Wheel Running

To demonstrate the effects of training on *mdx* mice at early (i.e., starting at 4 weeks) versus late ages (i.e., starting at 6 months), Carter et al.[30] used voluntary wheel running for 4 weeks. Both groups positively adapted to exercise by running more distance each consecutive week. The young *mdx* mice that ran voluntarily exhibited significant skeletal muscle hypertrophy in the soleus (11.6 ± 0.4 mg exercised vs. 8.4 ± 0.4 mg sedentary, $p < 0.05$) and produced more tetanic tension (17.4 ± 1.8 g exercised vs. 14.6 ± 1.2 g sedentary, $p < 0.05$) as well. The old *mdx* mice that ran voluntarily also had a hypertrophic response in the soleus (18.2 ± 1.0 mg exercised vs. 13.7 ± 1.2 mg sedentary, $p < 0.05$), but there was no improvement in soleus force production. There were no changes exhibited in the EDL muscle of young *mdx*

mice, but the older *mdx* EDL muscle exhibited hypertrophy (17.3 ± 0.5 mg exercised vs. 16.8 ± 1.0 sedentary, $p < 0.05$), although it was coupled with a loss in tetanic tension (29.6 ± 2.6 g exercised vs. 34.1 ± 2.1 g sedentary, $p < 0.05$). Carter's data demonstrated that starting training at an early age benefits dystrophic muscle, but Dupont-Versteegden et al.[42] studied *mdx* mice after long term voluntary wheel running. Specifically, they assessed changes in *mdx* diaphragm muscle function after voluntary wheel running after 40 weeks (mice started running at age 4 weeks). The diaphragm is considered the most clinically relevant muscle in the *mdx* mouse, and following the run training, there was an ~30% increase in active tension of the diaphragm muscles in the exercised mice compared to sedentary controls (~1.0 kg/cm^2 sedentary vs. ~1.3 kg/cm^2 exercised, $p < 0.05$). They also noted that there were no changes in force production in soleus muscles (~2.4 kg/cm^2 sedentary vs. ~2.5 kg/cm^2 exercised) following the 40 weeks of voluntary wheel running. The data of Dupont-Versteegden et al. shows long term exercise can benefit *mdx* diaphragm skeletal muscle strength, and this was true in the most clinically relevant muscle in the *mdx* mouse. Therefore, the above data suggest that *mdx* mice can tolerate voluntary exercise starting at a young age, and that running leads to no obvious detrimental effects on skeletal muscle mass or *in vitro* force production during long term training. But, what molecular adaptations take place in the *mdx* skeletal muscle, and do the adaptations delay the dystrophic pathology?

Hayes et al. [64]also used voluntary wheel running in young (age 4 weeks) *mdx* mice and examined the changes in muscle soleus and EDL fiber-types. Skeletal muscle contains a mixture of fiber types that are specific to the type of work the muscle performs. Major fiber type categories are type I or "slow twitch", and type II or "fast twitch." Type II fibers have primarily glycolytic metabolism and can be divided into type IIa (also oxidative metabolism), IIx, and IIb

fibers. Type I fibers are primarily oxidative and perform repetitive long term work well, while type II fibers are more explosive and are related to power or strength[72]. Hayes et al. noted an increase in type I fibers as a percent of the total fibers (63.1 ± 4.1% exercised vs. 52.4 ± 2.5% sedentary, $p < 0.05$) and a decrease in type IIa fibers (34.2 ± 2.0 exercised vs. 43.8 ± 2.3% sedentary, $p < 0.05$) in the soleus. In the EDL, a fast twitch muscle, a significant increase in type IIa fibers occurred following exercise (60.2 ± 2.5% exercised vs. 47.6 ± 2.9% sedentary, $p < 0.05$) with decreased type IIb fibers (38.1 ± 3.0% exercised vs. 51.0 ± 2.9% sedentary, $p < 0.05$). This fiber type shift is important because slow twitch fibers are considered more resilient to the dystrophic pathology[128].

Call et al. [29] looked at the effect of green tea extract (GTE) on voluntary wheel running distance and EDL force production in *MDX* mice. *Mdx* mice ran voluntarily running starting at age 3 weeks for a short time period of 3 weeks. Running alone increased serum antioxidant capacity (156 mm Trolox exercised vs 128 mM Trolox sedentary, $p < 0.001$), EDL tetanic stress (24.0 g/mm^2 exercised vs. 20.5 g/mm^2 sedentary, $p < 0.05$), contractile protein content, and the percentage of type I (~5% exercised vs. ~2% sedentary, $p < 0.05$) and IIa/x (~42% exercised vs. ~35% sedentary, $p < 0.05$) fibers. These studies demonstrate the benefit of voluntary, low-load running exercise on young (age 3-4 weeks) *mdx* skeletal muscle.

2.2.7.2 - Enforced treadmill running

In contrast, enforced treadmill running elicits muscle damage in *mdx* mice. Brussee et al.[23] ran *mdx* mice downhill on a 15% slope at 10 meters/min for 3 days to assess sarcolemma damage. Twenty-four hours before the final bout of exercise, the mice were injected intravenously with Evans Blue Dye (EBD). EBD is a non-toxic compound that is believed to enter muscle cells that have had sarcolemmal or membrane rupture. If the muscle cell membrane

remains intact, no dye will be detected in the muscle fibers. The downhill running requires eccentric contractions (i.e., lengthening contractions) which increases the likelihood of muscle membrane damage. Following the enforced treadmill running, EBD increased in cross section of diaphragm strips from 1.6% to 29.4% ($p < 0.001$), in gastrocnemius cross-sections from 13.9% to 23.8% ($p < 0.001$), and in soleus cross-sections from 2.8% to 12.8% ($p < 0.001$).

Quinlan et al. [110] also subjected *mdx* mice to downhill treadmill running. These authors tested the efficacy of the membrane sealant, poloxamer 188. Poloxamer 188 is intended to increase the stability of the sarcolemma of dystrophic muscle, thus preventing sarcolemmal damage. Mice ran on a treadmill at a 10% downward slope and initial speed of 8 m/minute for the first 10 minutes; then the speed was increased 0.5 m/minute every 5 minutes until either a total of 90 minutes of running had been performed or the animals were exhausted and could no longer continue running. Using EBD, Quinlan examined the quadriceps muscle, and on average there was between 14% - 20% EBD positive fibers following eccentric exercise with poloxamer 188, and 17% and 20% EBD positive fibers without the poloxamer. These results were significantly greater than non-exercised *mdx* mice which averaged around 4% - 9% dye positive fibers ($p < 0.05$). Thus, the poloxamer 188 failed to prevent entry of EBD. De Luca et al. [40] ran mice on a level treadmill at a rate of 12 m/minute, two times a week for 4 to 8 weeks. Following the exercise protocol, forelimb strength in the exercised *mdx* mice was approximately 60% less compared to unexercised *MDX* mice ($p < 0.05$). From these data, it would appear that voluntary, low load exercise, initiated at an early age rather than enforced treadmill running is the best exercise paradigm to counter the dystrophic condition in *mdx* mice.

2.2.8 - Conclusion

Skeletal muscle from *mdx* mice, much like that of DMD boys, is most functional at an early age. It has been established that the skeletal muscle of *mdx* mice will adapt positively to voluntary wheel running and the running is more effective if initiated at a young compared to an old age. If similar responses were evident in DMD boys, these findings would suggest that light physical activity training might be most effective if initiated at a young age.

2.3 - Leucine

Leucine is one of three essential branched chain amino acids (BCAA). Isoleucine and valine are the other two BCAA. Leucine has a molecular mass of 131.14 Da and occurs as 7-8% of total amino acids in common proteins. In addition to being an essential amino acid, leucine has signaling properties that affect both the hypertrophic and proteolytic pathways in skeletal muscle[26,54,75,113]. The anabolic properties were first described in 1975 by Buse et al., when they incubated rat hemi-diaphragm muscle in 0.5 mM leucine. Buse et al. observed a 25% increase of labeled [^{14}C]-Lysine incorporation into the muscle when incubated in the presence of leucine. When the rat hemidiaphragms were incubated in the presence of the other two BCAA's, isoleucine and valine, labeled [^{14}C]-Lysine incorporation into muscle was -12.5% and 2.9%, respectively[26]. These results were the first evidence that leucine might effect protein synthesis and degradation. Today, it is established that leucine acts through the mammalian target of rapamycin (mTOR) signaling pathway, but the mechanism of action is still not completely understood [33,113,95]. Studies have shown that signaling through mTOR is essential for skeletal muscle growth and protein synthesis[93,70,17,8] (Figure 2.6).

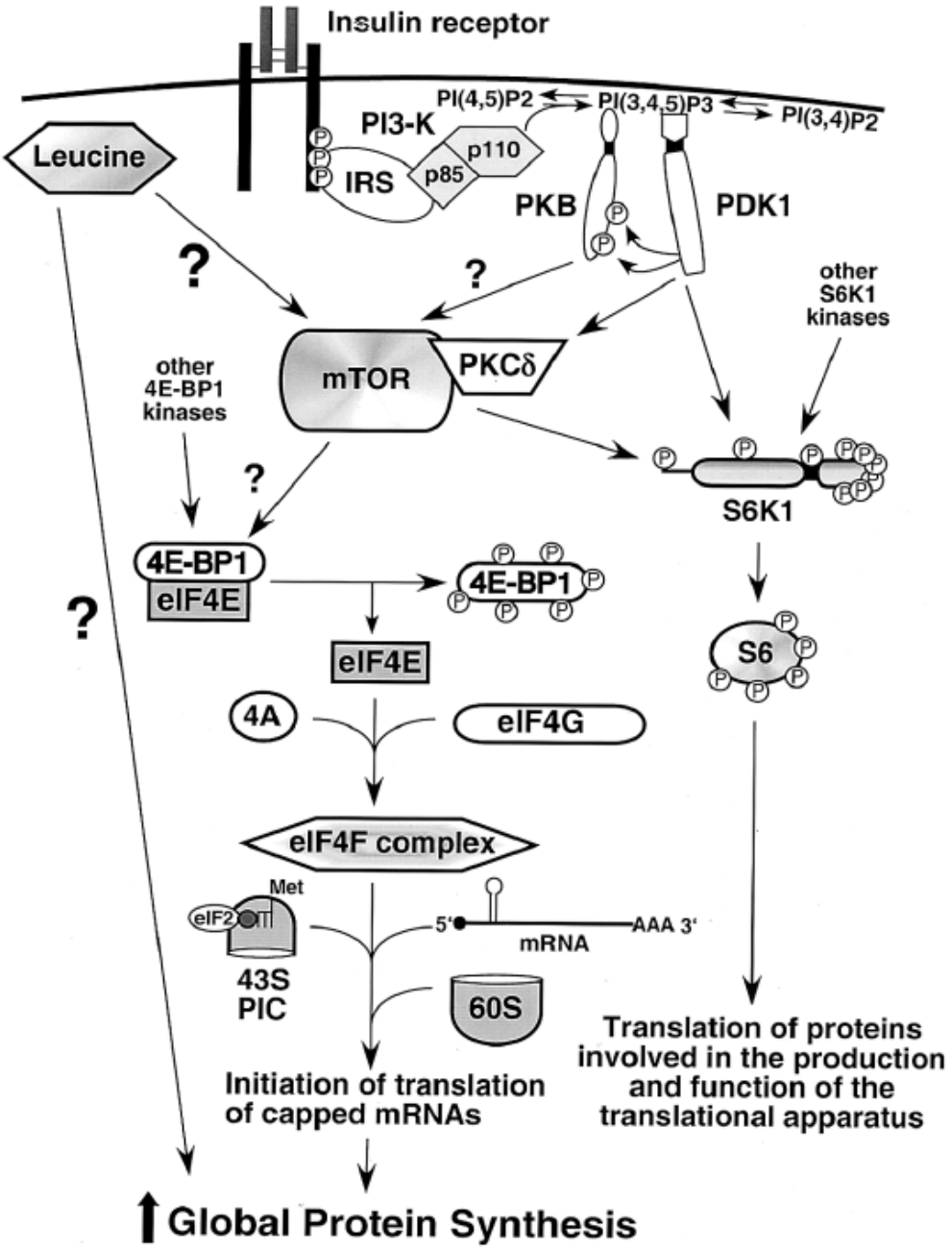

Figure 2.6 – This figure illustrateshow leucine may act on the mTOR pathway to increase protein synthesis either directly through mTOR or through other mechanisms. Abbreviations: IRS-Insulin receptor, PI3-K - Phosphoinositide 3-kinase, PKB – protein kinase B, PDK1 – pyruvate dehydrogenase kinase 1, PKCδ – protein kinase C delta, mTOR – mammalian target of rapamycin, S6K1 – ribosomal protein S6 kinase 1, S6 – ribosomal protein S6, 4E-BP1 – 4E binding protein 1, eIF4E – eukaryotic initiation factor 4e, 4A – eukaryotic initiation factor 4A, eIF4G – eukaryotic initiation factor 4G, eIF4F complex – eukaryotic initiation factor 4F complex, 43S PIC – 43S pre-initiation complex, 60S – 60S ribosomal subunit[6].

2.3.1 - Leucine and Skeletal Muscle Signaling

Leucine acts through the mTOR pathway, but is the mechanism or mechanisms are not presently known. The mTOR pathway is critical in regulating protein synthesis, and cell growth/hypertrophy in skeletal muscle[18,56,70,89]. Signaling through mTOR can be initiated by skeletal muscle mechanical overload (i.e., anaerobic exercise)[18,122,19] and nutrient sensitive mechanisms[124,60].

2.3.1.1 - mTOR

To activate mTOR, first phosphatidylinositol-3 kinase (PI3K), a serine/threonine kinase, must be activated by skeletal muscle mechanical overload or insulin, and will be active for a short period of time (< 15 minutes)[103]. PI_3K will phosphorylate another serine/thronine kinase known as protein kinase B (Akt). Akt will phosphorylate the tuberous sclerosis complex 2 (TSC2) which prevents the formation of the TSC1-TSC2 heterodimer, which is a negative regulator of mTOR. Studies that have ablated or inhibited either TSC1 or TSC2 in skeletal muscle demonstrate enhanced mTOR activation[120]. There are two different branches of the mTOR signaling cascade, mTORC1 and mTORC2. The mTORC1 pathway is leucine sensitive, binds raptor, and is inhibited by rapamycin. When activated, mTORC1 is responsible for ribosome biogenesis, translation initiation, and the inhibition of autophagy. mTORC2 is both leucine and rapamycin insensitive, and binds rictor, and is responsible for cytoskeletal organization[134]. Phosphorylated mTORC1 can inhibit either eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1), releasing the eukaryotic initiation factor 4E (eIF-4E), or activate the ribosomal protein S6 kinase 1 (S6K1).

By inhibiting 4EBP-1, eIF-4E becomes active, and binds with eIF-4A and eIF-4G, forming the eIF-4F complex. These three subunits are important because 1) eIF4A is a RNA

helicase that unwinds the secondary structure in the 5'-untranslated region of mRNA, 2) eIF-4E binds directly to the m^7GTP cap structure present at the 5' end of most eukaryotic mRNA's, and 3) eIF-4G is a scaffolding protein anchoring eIF-4A, eIF-4E, mRNA and the 43s subunit of the ribosome[123]. eIF-4G activity is dependent on phosphorylation at Ser-1108 in the COOH terminus; phosphorylation and activity are maximized in rat skeletal muscle following either leucine hindlimb perfusion or a leucine bolus[35,20]. The formation of the eIF-4F complex, driven by leucine supplementation, increases the capacity of the cell to translate mRNA to protein. S6K1 will activate both S6 and eukaryotic translation factor 4B (eIF-4B). S6's role in increasing mRNA translation is still not clearly elucidated, but it may act through the EIF-4F complex. eIF-4B will bind to the 48s ribosomal subunit, further enhancing mRNA translation[56].

Related to muscular dystrophy, Betzinger et al.[10] created both raptor and rictor null mice to explore the differences in the two branches of mTOR signaling in skeletal muscle. The removal of raptor (mTORC1) resulted in a dystrophic like phenotype, including muscle atrophy, spinal kyphosis, early death, and depressed muscle force, while the removal of rictor (mTORC2) caused no changes compared to control animals. To date, it is not known if a deficiency in raptor or mTORC1 is partly responsible for dystrophic pathogenesis. Nevertheless, the mTORC1 signaling cascade appears to be important for skeletal muscle function and growth.

Additionally, recent work on the upstream mTOR activator, protein kinase b (Akt), has elucidated effects of the PI3K/Akt/mTOR signaling pathway on skeletal muscle of *mdx* mice and the canine model of muscular dystrophy, the GRMD dog. Blaauw et al.[14] induced activation of Akt in *mdx* mice, which limited force depression following eccentric contractions, but also increased concentrations of cytoskeletal proteins dysferlin, desmin, and utrophin. Peter et al.[106] similarly upregulated activation of Akt in *mdx* mice and noted increased utrophin and related

glycoprotein complex protein concentrations; utrophin is a homolog of dystrophin and can replace dsytrophin in the DGC (ref). In addition, entry of EBD into muscle fibers was reduced, suggesting improved sarcolemmal stability. Feron et al.[51] determined Akt phosphorylation was depressed in the GRMD dog compared to healthy littermates. This decreased phosphorylation was related to depression in the downstream target of mTOR, ribosomal protein s6 kinase (p70S6K1), and these alterations in PI3K/Akt signaling were related to increased Akt inhibitor phosphatase and tensin homolog (PTEN). These studies on the Akt pathway in conjunction with alterations in skeletal muscle function and morphology following raptor ablation, reinforce the importance of mTOR signaling to maintain healthy and functional skeletal muscle, and suggest that regulation of mTOR and its related signaling pathway may be altered in dystrophic skeletal muscle.

2.3.1.2 - Leucine acts on the mTOR pathway

As indicated in Figure 2.6, leucine acts on the mTOR pathway, increasing the phosphorylation of 4EBP-1 and S6K1. This activation seems to be regulated by the intracellular concentration of leucine[11]. To demonstrate the potency of leucine signaling through mTOR, Atherton et al.[7] recently tested how all of the essential amino acids (EAA;amino acids not synthesized endogenously) affected the mTOR signaling cascade in C2C12 muscle cells. Atherton et al. demonstrated that leucine was the only EAA that increased phosphorylation of mTOR and 4EBP-1, 1.67 and 2.5 fold respectively. Leucine also increased the phosphorylation of S6K1 by 5.9 fold, the greatest amount of any of the EAA[7]. How leucine increases phosphorylation of these downstream targets is not entirely understood.

To determine how leucine acts on the mTOR pathway, rapamycin, an inhibitor protein that abolishes or severely inhibits the activity of mTOR, has been used. Anthony et al. [5] fasted

rats and split them into control or rapamycin treated groups, then administered a leucine or saline bolus and measured rates of protein synthesis. Rapamycin plus saline animals had protein synthesis rates that were 76% of those for the control plus saline animals. The rapamycin plus leucine group had statistically similar values of protein synthesis to those of the control plus saline group. The control plus leucine group had protein synthesis rates that were 142% of those for the control plus saline group. These data suggest that leucine dependent stimulation of muscle protein synthesis is rapamycin sensitive, and acts through the mTOR pathway. Rapamycin also reversed leucine induced phosphorylation of 4EBP-1, and the association of eIF-4G with eIF-4E to control levels, and abolished phosphorylation of S6K1. These results further support the idea that leucine acts either directly upon mTOR or on upstream regulators of mTOR[5].

2.3.2 - Leucine stimulated protein synthesis in skeletal muscle

In 1975, Buse et al.[26] studied the effect of the BCAAs on protein turnover in isolated rat diaphragms using labeled [^{14}C]-lysine or [^{14}C]-acetate. Diaphragms were either pre-incubated with or without 0.5 mM leucine, then placed in the presence of 0.3 mM combined leucine, isoleucine or valine, or 0.5 mM leucine, isoleucine, or valine individually for 1 hour. The uptake of labeled precursors incorporated into the diaphragms was measured. The combined 0.3mM BCAAs or 0.5 mM leucine by itself resulted in a 20.4 ± 8.4% and 25.3 ± 7.1% increase of labeled protein, respectively compared to control muscles. (see Table 2.4). The addition of the other two BCAA, valine and isoleucine demonstrated either no significant increase or a decrease in labeled protein incorporation into the muscle cells of the rat diaphragms. These results indicated that leucine alone or the combination of all three BCAA were necessary to elicit an increase in both protein synthesis and the rate of muscle protein turnover.

Table 2.4 - Table adapted from Buse et al. 1975. Uptake of labeled tracer proteins in Rat hemidiaphragms under both fed and fasted conditions. Muscles were incubated in ringers solution that contained a mixture of all three BCAA's or each individual BCAA. Note the leucine treated animals had the highest percent of a [14C] lysine tracer uptake in both fed and fasted states..

	Medium	**^{14}C incorporated into proteins**	**% change**
Experiment 1 - Fasted	Control	41.4 ± 2.7	+20.4% ± 8.4
	Leucine + Isoleucine + Valine (0.3 mM each)	46.4 ± 2.9	
Experiment 1 - Fasted	Control	47.0 ± 3.9	+25.3% ± 7.1
	Leucine 0.5 mM	58.2 ± 4.7	
Experiment 1 - Fasted	Control	45.2 ± 3.3	-12.5% ± 3.3
	Isoleucine 0.5 mM	39.4 ± 3.0	
Experiment 1 - Fasted	Control	47.6 ± 5.7	+2.9% ± 4.8
	Valine 0.5 mM	46.4 ± 5.9	
Experiment 2 - Fed	Control	50.4 ± 2.3	+18.5% ± 5.6
	Leucine 0.5 mM	58.8 ± 1.4	

In 1984, Hong et al. [67] incubated soleus and EDL skeletal muscles from fed rats with and without 0.5 mM leucine in the incubation media. The leucine treated soleus and EDL muscles incorporated 48% and 22% of labeled [^{14}C]-tyrosine into muscles respectively. The leucine group also showed significant stimulation of protein synthesis after the rats were starved for 24 (59% soleus, 29% EDL, $p < 0.05$) and 72 hours (24% Soleus, $p < 0.05$). These results demonstrated that both EDL and soleus muscles are affected by leucine treatment, therefore both fast and slow twitch muscles should show benefits from leucine supplementation.

In addition to increasing protein synthesis, leucine also blunts muscle protein degradation. Nakashima conducted two separate studies in which leucine, and its metabolite α-ketoisocaproate (α-KIC) were provided to young chicks by gavage after a 24 hour starvation period. In both studies, the key finding was the expression of calpain mRNA in skeletal muscle was depressed by leucine and α-KIC [97,98]. In the first study, Nakashima et al.[97] incubated chick myotubes in 1.0 mM leucine and saw a significant depression in m-calpain mRNA. Control

chick myotube m-calpain mRNA was normalized at 100%, while the treated chick myotube m-calpain mRNA was expressed between 75% and 80% of the control mRNA level ($p < 0.05$). In his second study, Nakashima et al. [98] starved 14 day old chicks for 24 hours and then provided by gavage 225 mg/100g body mass of leucine, the enantiomer D-leucine, leucine's metabolite α-KIC, or a control saline mixture and measured the relative expression of calpain mRNA. All three treatments significantly depressed m-calpain mRNA compared to the control condition. The m-calpain mRNA was depressed by ~40% following leucine gavage, ~50% following D-leucine gavage, and ~50% following α-KIC gavage (all three values $p < 0.05$).

Busquets et al. [27] measured the rates of proteolysis in excised EDL and soleus muscle from rats that were incubated in control Krebs-Henseleit buffer, Krebs-Henseleit buffer plus 5 mM leucine, or Krebs-Henseleit buffer plus 10 mM leucine. In all treatment conditions, muscles were pinned at resting length by attaching clamps to both tendons. Total lysosomal proteolysis in EDL muscles was depressed 29%, calpain proteolysis was down 27% and ATP- dependent proteolysis was depressed 14% ($p < 0.05$). Busquets et al.[28] also studied EDL calpain mRNA following 48 hour starvation in rats. Control muscles were incubated in normal Krebs-Henseleit buffer and treated muscles were incubated in Krebs-Henseleit buffer plus 10 mM leucine. Treatment with leucine depressed calpain mRNA expression from 233 ± 18 AU to 137 ± 43 AU ($p < 0.05$), again indicating leucine may have an effect on the calpain proteolytic system. These data indicate leucine blunts degradation as much as it plays a hypertrophic role in skeletal muscle, although leucine's exact mechanism of action is still not presently known.

Can leucine and its effects on muscle protein synthesis as well as its specific depression of calpain mRNA benefit *MDX* skeletal muscle? If leucine is combined with exercise, do these effects still occur? These questions have not yet been answered in the literature, and therefore

assessement of the effects of leucine on the calpain pathway, muscle force output, and run exercise adaptions in *mdx* mice may provide valuable clues to develop leucine as a treatment for DMD boys.

2.3.3 - Exercise and Leucine

Leucine oxidation is increased during exercise,[68,69,65] and when supplemented post exercise, it promotes increased protein synthesis[4,101]. By supplementing leucine following exercise, researchers have increased muscle performance and function specific to the type of training performed. The exact mechanism still is elusive, but researchers know leucine acts through the mTOR pathway and increases phosphorylation of the translation factors 4E-BP1 and S6K1[100,73]. Studies on the effect of leucine supplementation and exercise have ranged from mice and rats to humans. The majority of these data show that combined leucine and exercise provides benefits over exercise alone.

Anthony et al. [4] tested the hypothesis that leucine is important to increase post exercise protein synthesis by exercising rats. Rats ran on a treadmill for 2 hours at 36 m/min on a 1.5% upwards grade, and were immediately gavaged following exercise. The rats were gavaged with a saline control (EF), a carbohydrate only meal (2.63 g carbohydrate, EC), a leucine only meal (0.27 g leucine, EL), or a combined leucine and carbohydrate (2.36 g carbohydrate, 0.27 g leucine, ECL) meal following exercise. The leucine fed rats increased the fractional rate of protein synthesis in skeletal muscle over the carbohydrate only group (~9.5 %/d EL fed vs ~7.1 %/d EC fed, $p < 0.05$). Also, the leucine and carbohydrate group had a significant increase in muscle glycogen following exercise (~3.5 mg/g tissue ECL vs. 2.0 mg/g tissue EF, $p < 0.05$). These authors concluded that leucine was important for muscle recovery following exercise.

Crowe et al. [34] reported that competitive outrigger canoeists supplemented with 45 mg/kg bw/day of leucine following exercise increased peak power output (6.7±0.7 vs. 6.0±0.7 W/kg bw, $p < 0.05$) compared to placebo groups. There was also a significant increase in time to exhaustion in the leucine supplemented group (~65 minutes placebo vs. ~ 90 minutes leucine, $p < 0.05$) and a decrease in perceived exertion (15.0 ± 1.4 RPE placebo vs. 12.9 ± 1.4 RPE leucine, $p < 0.05$). There were also no reported side effects of leucine supplementation.

Koopman et al. [78] used untrained males to study the ingestion of a carbohydrate only (50 g carbohydrate, CHO), carbohydrate and protein drink (50 g carbohydrate and 33.3 g protein, CHO+PRO), or carbohydrate, protein and leucine drink (50 g carbohydrate, 33.3 g protein, 16.6 g leucine, CHO+PRO+LEU) following a bout of resistance exercise. The resistance exercise program consisted of eight sets of eight reps of leg press and eight sets of eight reps using the leg extension machine. The exercise weight was set at 80% of a one repetition max. The CHO+PRO+LEU beverage significantly increased the net protein balance following exercise (ratio of protein gain to protein loss + oxidation) over both the CHO and CHO+PRO (values not reported, $p < 0.05$), as well as significantly increased the fractional synthesis rate of protein over the CHO group (0.136 ± 0.007%/h CHO+PRO+LEU vs. 0.097 ± 0.013%/h CHO only, $p < 0.05$). Koopman et al. [79] followed up their previous study to determine if the combination of CHO+PRO+LEU was greater than CHO alone when given to young and old untrained males following exercise designed to simulate performing a series of daily activities. The exercise regimen included 6 sets of 10 repetitions on the horizontal leg press, and 6 sets of 10 repetitions on the leg extension machine. The first 2 sets were performed at 40% of their 1 repetition max, sets 3-4 were performed at 55% of their 1 repetition max, and sets 5-6 were performed at 75% of their 1 repetition max. Again, Koopman showed that the CHO+PRO+LEU drink significantly

increased the net protein balance in both old and young men (CHO+PRO+LEU vs. CHO only for both young and old, $p < 0.001$), and fractional synthesis of mixed muscle proteins in both young (0.08 %/h CHO+PRO+LEU vs. 0.06 %/h CHO only, $p < 0.001$) and old individuals (0.07 %/h CHO+PRO+LEU vs. 0.04 %/h CHO only, $p < 0.001$).

Pellegrino et al. [105] studied the effect of an essential amino acid mixture (containing 30.5% leucine) on the skeletal muscle of control mice and exercised mice. The mice were allowed free access to a running wheel in their cages for 8 weeks. The combination of wheel running and supplementation significantly affected fiber-type expression compared to both running alone and supplementation alone in soleus, tibialis anterior, gastrocnemius, and diaphragm muscles. These data are shown in Figure 2.7 and Figure 2.8. Pellegrino also noted that soleus type 1 fibers had a significant decrease in CSA while type IIx fibers had no change in CSA. These results fit with the hypothesis that endurance training does not elicit a hypertrophic effect[92,12], and the addition of essential amino acids does not alter this.

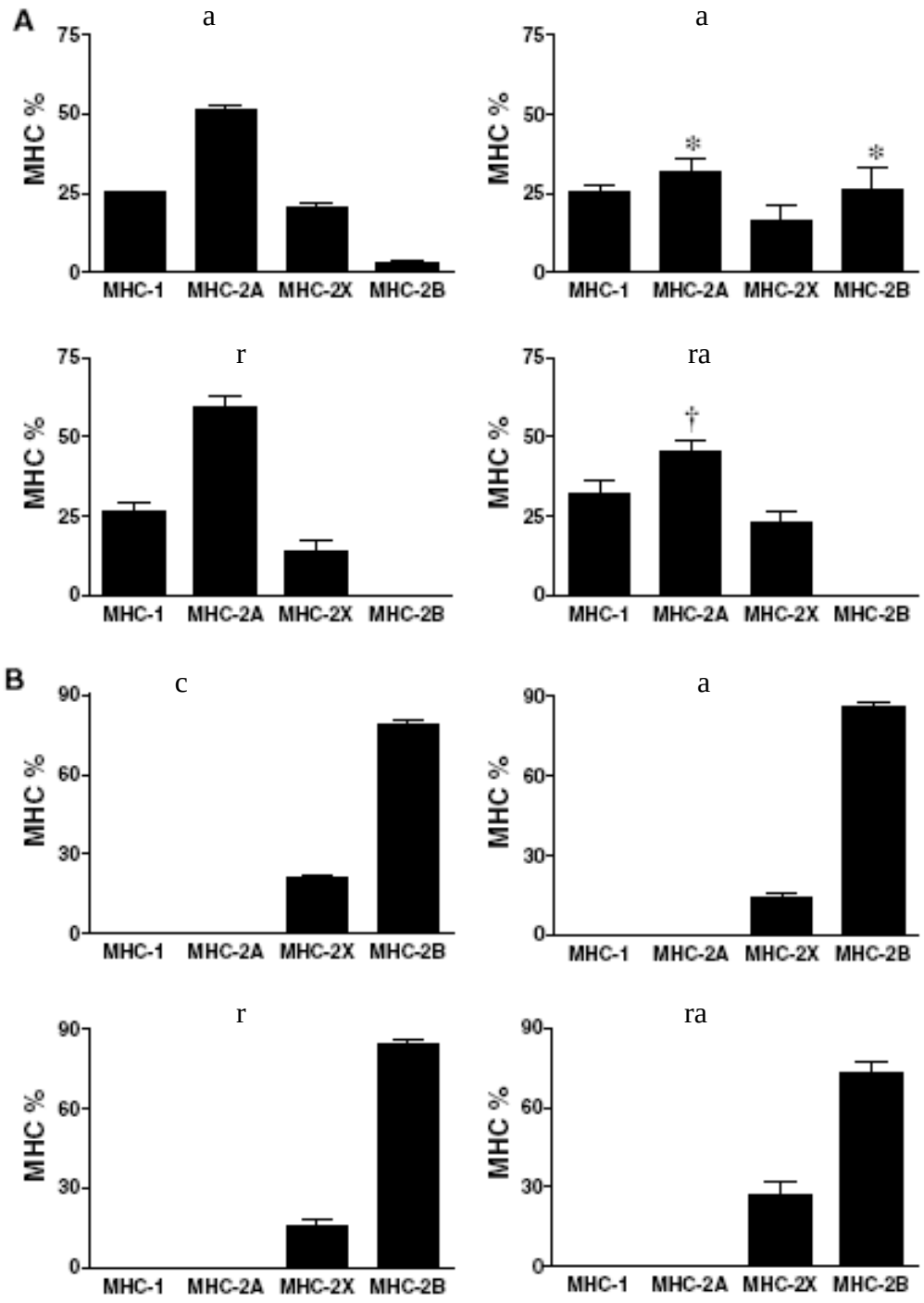

Figure 2.7 - Effects of EAA treatments on myosin heavy chain (MHC) distribution in the soleus (A) and tibialis anterior (B) muscles. *Significantly different from C group ($P<0.05$). †Significantly different from RA (running supplemented with amino acid mixture) group ($P<0.05$)[105]. **Abbreviations:** c – control animals, a – EAA supplemented animals, r – voluntary wheel run animals, ra – voluntary wheel run animals supplemented with EAA.

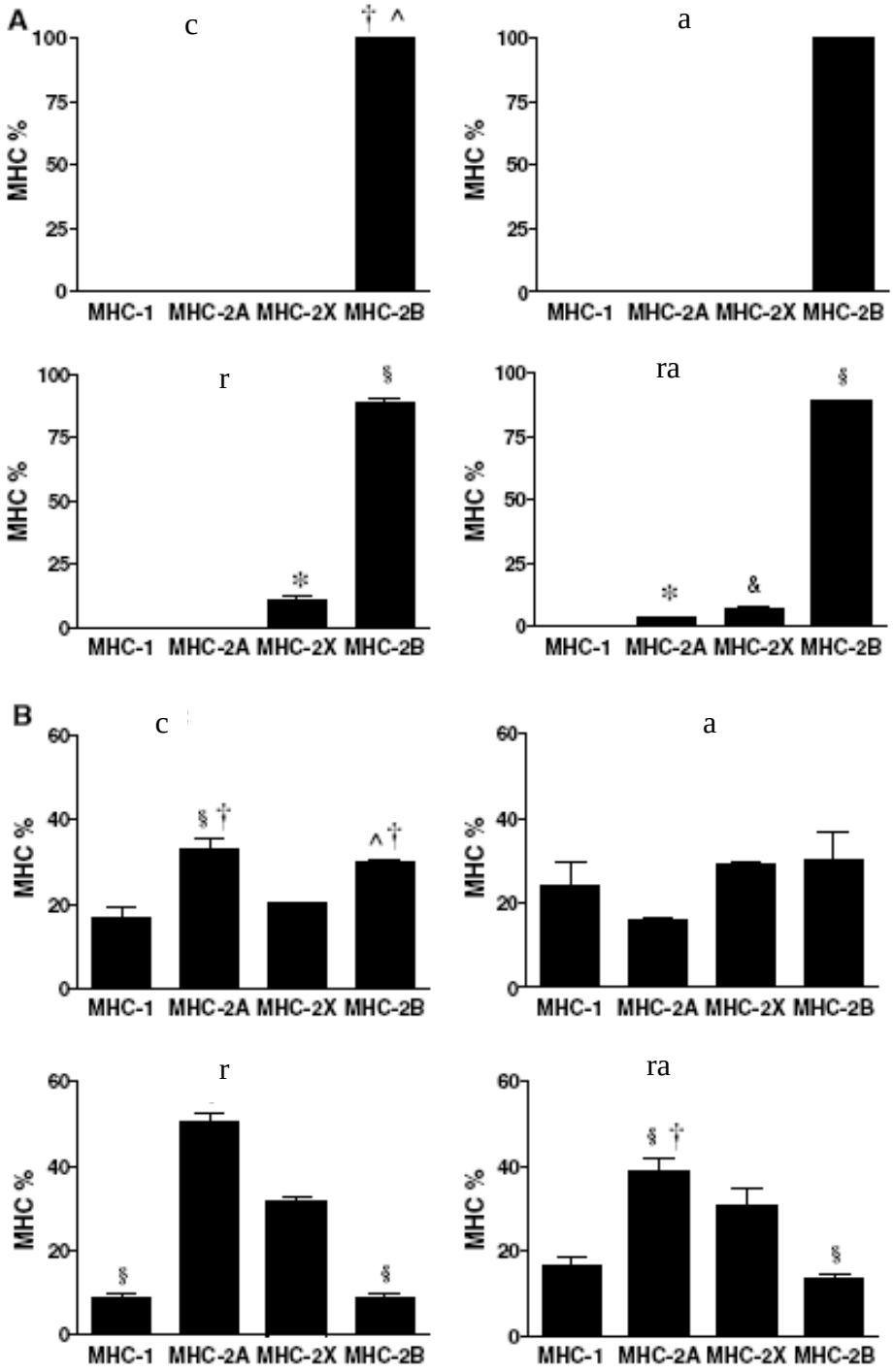

Figure 2.8 - Effects of EAA treatments on MHC distribution in the gastrocnemius (A) and diaphragm (B) muscles. *Significantly different from C group (P<0.05). §Significantly different from A (sedentary supplemented with amino acid mixture) group (P<0.05). ^Significantly different from R (wheel running) group (P<0.05). † Significantly different from RA group (P<0.05). [105] **Abbreviations:** c – control animals, a – EAA supplemented animals, r – voluntary wheel run animals, ra – voluntary wheel run animals supplemented with EAA.

Leucine has a definite positive impact on skeletal muscle function following resistance exercise.[101,113] But, its effects on endurance training are much less understood. As stated previously, Crowe et al. did note a lower rating or perceived exertion (RPE) following leucine supplementation in outrigger canoeists (endurance activity) yet these athletes still produced significantly more power. Collectively, the studies cited above suggest that research in the area of leucine and endurance exercise in humans is lacking. Furthermore, from the perspective of developing a potential therapeutic approach for DMD boys, no study has yet even directly addressed the effects of leucine in *mdx* mice following voluntary wheel running. Since leucine possesses the ability to increase protein synthesis following exercise, it may increase the ability of the *mdx* mouse to adapt to exercise. Data from cancer models treated with lecuine supports a similar use in a muscle wasting disease such as muscular dystrophy, although it is still not clear if beneficial effects of leucine would also prevail under exercise conditions.

2.3.4 - Leucine and Muscle Wasting Disease

Leucine has been used in muscle wasting disorders such as cancer cachexia and sepsis. In cancer cachexia studies, leucine given to cachexic rats have repeatedly shown benefits such as maintaining muscle mass and increasing the phosphorylation of eIF-4E.[116] Ventrucci et al. [126] in 2001 supplemented tumor-bearing rats diets with 3% leucine. There was a significant increase in lean body mass of leucine fed animals (37.7 ± 1.5g control tumor bearing, vs. 42.2 ± 1.5g leucine supplemented tumor bearing, $p < 0.05$), as well as the total nitrogen content (60.3 ± 3.6 mg/100 g mass control vs. 68.3 ± 3.5 mg/100 g mass leucine, $p < 0.05$). Gomes-Marcondes et al. [57] did a similar study in young tumor bearing rats and supplemented their diets with 3% leucine, and saw similar trends with significant increases in lean carcass mass (19.9 ± 0.6 g, tumor bearing vs. 23.1 ± 1.0 g tumor bearing leucine supplemented, $p < 0.05$), total protein of the carcass (42.72 ± 1.14 mg/g, tumor bearing vs. 43.47 ± 1.05 mg/g tumor bearing leucine supplemented, $p < 0.05$),

and gastrocnemius mass (0.152 ± 0.015 g, tumor bearing vs. 0.248 ± 0.033 g tumor bearing leucine supplemented, $p < 0.05$). In 2007, Ventrucci et al. [127] reported enhanced phosphorylation of EIF-4E and S6K1 in pregnant tumor bearing rats, showing leucine still plays a role in the hypertrophic response in skeletal muscle of tumor bearing animals. In an interesting parallel to muscular dystrophies, muscles in tumor bearing rats and mice show membrane abnormalities and have a reduced expression of dystrophin and the components of the DGC,[3] which is similar to the dystrophic pathology of the *mdx* mouse[3,80].

Sepsis also appears to benefit from leucine administration. Vary et al. [124] gavaged septic rats with 2.5 mL/100g body mass of a 400 mM leucine solution. By giving an acute dose of leucine, septic rats showed enhanced 4E-BP1 phosphorylation (63 ± 5 AU septic + leucine vs. 11 ± 3 AU septic, $p < 0.005$), allowing the formation of the eIF4E-4G complex (33 ± 8 AU septic + leucine vs. 2 ± 1 AU septic, $p < 0.005$). By increasing the phosphorylation of 4E-BP1, ribosome biogenesis should be upregulated, helping counteract the impaired mRNA translation that is present in septic skeletal muscle[39].

2.3.5 - Conclusion

Leucine is an effective supplement to increase post-exercise protein synthesis and the net gain of protein. The increase in protein synthesis is accomplished by leucine acting on the mTOR pathway, but how leucine turns on the mTOR pathway is currently not known. If the effect of leucine and exercise can help with muscle protein balance in the *MDX* mouse following exercise, then leucine treatment for dystrophic skeletal muscle should be explored more extensively as a potential treatment especially when combined with exercise therapy.

2.4 - Summary

The pathogenesis of muscular dystrophy is still unclear; the main hypotheses include sarcolemmal damage that results in muscle injury leading to calpain activation, and muscle fiber proteolysis. Over time, the capacity of the muscle to regenerate decreases and results in muscle weakness. The unique capability of leucine to enhance muscle protein synthesis, thus expediting muscle recovery, and to depress calpain mRNA transcription, possibly slowing down calpain related proteolysis, strongly suggests leucine has a potential role as a therapy for DMD. The dystrophic *mdx* mouse adapts to run exercise, and leucine provides significant gains when used as a post workout supplement on the basis of human and rat studies. In the two chapters that follow, the beneficial effects of leucine treatment with and without run exercise in *mdx* mice are described. The intent of these studies was to substantiate the use of leucine and run exercise in *mdx* mice first, with the idea that these data would provide support for subsequent leucine and light physical activity training in DMD boys

Chapter 3 – Leucine increases *mdx* EDL stress production

Leucine increases *mdx* EDL muscle stress production

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3. 1 - Abstract

Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disease for which there is no cure. We tested the hypothesis that the branched chain amino acid leucine could potentially improve muscle strength in dystrophic muscles because of its anabolic effects. Dystrophic (*mdx*) and control (BL/10) mice starting at age 21 days were provided either drinking water with leucine (114 mM, L), or no leucine for 4 weeks. After 4 weeks, the maximal tetanic stress produced by *mdx*-L compared to *mdx* EDL muscles was increased ~20% ($p < 0.05$). mTOR phosphorylation in *mdx*-L gastrocnemius muscles was increased ~20%, but surprisingly muscle masses of *mdx*-L gastrocnemius, TA and EDL muscles were unchanged. However, myosin and actin contents assessed in *mdx*-L TA muscles were both increased ~10%. In addition, calcium activated calpain (CAC) proteolysis rates ($p < 0.05$) in isolated FDB fibers were decreased ~40%. Our data suggest that leucine produces anabolic effects in dystrophic skeletal muscle, possibly by driving expression of contractile proteins, while slowing degradation by blunting rates of calpain cleavage. Together, these effects increased stress production of dystrophic muscles. Leucine supplementation may be a simple but effective nutriceutical approach to improve the performance of dystrophic muscles.

Keywords: Calpain, mTOR, skeletal muscle, function

3.2 - Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disease that afflicts 1 in 3500 new born males [37,76,117]. DMD causes the skeletal muscle of affected individuals to continually degenerate and regenerate, resulting in progressive skeletal muscle wasting and weakness [104,117]. In boys, DMD onset is between the ages of 2 and 5, followed by patients becoming wheelchair bound around the age of 12, and dying in their early to mid-20's[50]. There is no current cure for DMD, and interventions to improve the quality of life of patients are limited.

DMD is caused by mutations to the 427 kb dystrophin gene which results in the absence of a dystrophin protein[76]. The dystrophin protein is located on the inner surface of the skeletal muscle membrane and attaches cytoskeletal actin to the membrane bound dystrophin glycoprotein complex (DGC)[15,38]. In the absence of dystrophin, the mechanical link from the cytoskeleton of the muscle cell to the membrane and the components of the DGC is missing[47]. The continual degeneration and regeneration of skeletal muscle caused by the absence of dystrophin and the DGC is hypothesized to result from 1) a fragile or weakened skeletal muscle membrane,[108,107] or 2) altered cell signaling caused by the absence of the DGC. To date, the mechanism(s) of DMD related muscle wasting remains unknown [131].

We wanted to determine if nutritional supplementation of the branched chain amino acid (BCAA) leucine would benefit dystrophic mice (*mdx*) by increasing muscle strength and structural integrity. Leucine has been reported to depress calpain mRNA expression and increase protein synthesis that could counter or slow the loss of muscle mass and improve quality of life in DMD patients[28,67,97]. Leucine Leucine acts on the components of the hypertrophic mTOR signaling pathway, activating the ribosomal protein S6, and 4EBP-1, enhancing global protein synthesis[26,54,5]. [28,67,97]. Also, in animal models of other muscle wasting disorders, such as sepsis

and cachexia, supplementation with leucine has resulted in beneficial effects on maintaining muscle mass [126,57,116,127,39,124]. However, it is not presently known if long term leucine supplementation will benefit either skeletal muscle function or mass in dystrophic *mdx* mice.

Mendell et al. [88] in 1984 ran a controlled clinical trial on leucine in DMD patients to determine if leucine had therapeutic effects. There was a transient increase in muscle strength reported over the first month of the 12 month trail for the leucine treated group. Past the 1 month point, additional differences were attributed to an unusual rate of decline in the placebo treated group. Mendel also used boys from a wide range of ages instead of focusing on one age group in particular, which could have affected the results of his study based on each patient's individual state of DMD progression. Since Mendel's controlled clinical trial, there have been considerable advancements in knowledge on both leucine and DMD. We wanted to test if leucine given to dystrophic mice would cause a similar increase in muscle strength and determine what changes were present in the muscle.

With the progression of muscular dystrophy, skeletal muscle mass is replaced by fibrous and fatty tissues, therefore the timing of leucine supplementation is critical to have maximal impact on the maintaining or improving skeletal muscle mass, function, and quality of life. Disease onset in *mdx* mice begins at a young age (around weaning age, 3 to 4 weeks), therefore we chose to begin supplementation of leucine at weaning age of 3 weeks and continue till age 7 weeks. This is a known period of heightened skeletal muscle degeneration and regeneration in *mdx* mice and thus, a time when the most robust effects of leucine supplementation on protein synthesis and decreased muscle degradation could be observed[50].

The objectives of the current study were to determine if four weeks of leucine supplementation would (1) increasing extensor digitorum longus (EDL) stress (2) decrease markers of EDL muscle degradation (e.g. centralized nuclei, fiber size) and (3) decrease the rate of calpain dependant muscle degradation.

3.3 - Methods

3.3.1 - Mice and Housing

At weaning age (21 days) control (BL/10) and *mdx* mice were randomly assigned to either a leucine (-L) or no leucine () group. At this time the -L mice were provided drinking water with L-Leucine (sigma) dissolved into the drinking water at a final concentration of 114 mM, while the no leucine group was given unsupplemented drinking water. Mice were then kept in-house for 4 weeks and water consumption was monitored daily. The body mass of all mice was recorded every 7 days, and then on the 49^{th} day mice were anesthetized utilizing a ketamine/xylazine cocktail (2mg xylazine and 20mg ketamine per 100g body mass) injected intrapertoneally, and tissues were excised and experiments were performed. Due to tissue size, experiments were performed across three muscles of the lower leg all of which are considered "fast" or "glycolytic". All studies were approved by the Virginia Tech Animal Care and Use Committee.

3.3.2 - Function

Muscle Prep: Extensor digitorum longus (EDL) muscles were surgically excised from anesthetized mice and secured via 4-0 suture to a dual-mode servomotor (Aurora Scientific: Aurora, Ontario) as previously described [58,133].

Muscles were then equilibrated in an oxygenated (95% O_2-5% CO_2) physiological salt solution (PSS; previously described [58]) bath for 10 minutes prior to data collection. Following the equilibration period, muscles were subjected to three twitches and two tetani, each separated by 1 minute. After a 5 minute rest, one EDL from each mouse was subjected to a stretch protocol while the remaining muscles were subjected to force frequency protocols.

Force frequency protocol: EDL muscles were stimulated for 800 ms at 1, 30, 50, 80, 100 and 150 Hz, each separated by 1 minute. After a 5 minute rest, muscles were subjected to one twitch and one tetani (separated by 1 minute) and rested for 6 minutes.

Stress output (g/mm^2) was calculated as the force output (g) for a given stimulation normalized to the estimated cross sectional area (CSA, mm^2) of the muscle. Cross sectional area of each muscle was determined by the following equation: muscle cross sectional area = muscle mass in g (1.056 g/mm^2 x muscle length in mm). When all functional data was completed, the muscles were frozen in liquid nitrogen cooled isopentane, and stored at -80^{o}C until they were ready to be used.

3.3.3 - Fiber Type Analysis

We determined the proportional content of MyHC isoforms as previously described [119]. Briefly, frozen EDL muscles obtained from BL/10, BL/10-L, *mdx*, *mdx*-L mice aged 49 days were thawed on ice, homogenized (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 6.81), assayed for total protein (BCA Assay, Peirce), and then diluted in sample buffer to a final concentration of 0.04 mg/ml. Ten microliters of each sample was subjected to SDS-PAGE using 4% stacking and 8% separating gels. Samples were run on a mini-gel electrophoresis unit (BioRad) for 24 h at 80 V (constant voltage). Gels were silver stained and scanned using a FluorChem image analysis system (Alpha Innotech). Band densities for each MyHC isoforms were expressed as a percentage of total MyHC band density, the myosin heavy chain bands were detected by running purified rabbit myosin as a standard..

3.3.4 - Sectioning

EDL muscles were mounted in OCT (Andwin Scientific) and at the point of maximal CSA (visually discerned) 8µm serial sections were taken using a Microm 505M cryostat set at -20^{o}C. Sections were collected on superfrost plus slides (Richard Allen Scientific) and allowed to air dry for 1 hour. Following air drying slides were stored at -80^{o}C.

3.3.5 - Membrane Staining

Each EDL was stained using rhodamine labeled wheat-germ agglutinin (WGA, Vector Labs). Sections were fixed for 5 minutes in a 4% formaldehyde/PBS solution, then blocked for 1 hour using a 5% BSA/PBS solution, then stained using 1:1000 WGA:PBS dillution. Sections were then washed 2 x 5 minutes in fresh PBS. Sections were briefly air dried before mounting coverslips (Vectamount) and imaging.

3.3.6 - H&E staining

EDL sections were stained using hematoxylin and eosin (H&E). First slides were dipped in hematoxylin (Fisher Scientific, Hematoxylin) for 30 seconds, washed in fresh tap water for 1 minute, followed by 30 seconds in eosin (Fisher Scientific, Eosin), and then washed for 1 minute in fresh tap water. Sections were allowed to air dry, then were coverslipped and imaged.

3.3.7 - Immunoblots

10% precast SDS-PAGE gels with 50µL wells (BioRad) were used. Protein concentration of samples was determined using a BCA assay (Pierce), then samples were mixed in a 1:1 ratio with 2X sample buffer (240mM β-mercaptoethanol, 1M Tris (pH 6.8), 20% glycerol, 0.1% bromophenol blue, and 10% SDS). Next samples were boiled for two minutes. Ten µL of broad range molecular markers (7kD-206kD, Bio-Rad) was loaded into well 1, and a total of 30 ug of sample protein was loaded per sample well. Gels were run on ice at a constant 150 V for 90 minutes in an electrophoresis apparatus.

Proteins were then transferred to a PVDF membrane. The transfer was run at a constant 100 V for approximately 90 minutes. Following transfer, the PVDF was washed in Tris buffered saline (TBS, pH 7.5) for 15 min at room temperature. Then the membrane was incubated in blocking buffer (3-5% non-fat dry milk in TBST (TBS + 0.05% Tween 20) for an hour with shaking, then washed 3 times for 15 min in TBST. The membrane was incubated in primary antibody (mTOR, Abcam; Ser2448 mTOR, Abcam; calpain, SCBT; calpastatin, SCBT; all primary antibodies were used at a 1:1000 dilution, and raised in rabbit) in blocking buffer overnight at 4ºC, and then was washed in TBST 3 times, each for 15 min. The membrane was incubated with secondary antibody (goat anti-rabbit HRP, 1:10000) in blocking buffer for 1.5 hours. After incubation, the membrane was washed in TBST 3 times, each for 15 min. The presence or absence of the protein of interest was visualized using enhanced chemiluminescence (ECL, peirce) and visualized on a ChemiDoc XRS Imaging System (BioRad, Hercules, CA).

3.3.8 - FDB dissociation

Flexor digitorum brevis (FDB) muscles were removed and incubated for 1-2 hours at 37°C in a modified Krebs solution (in mM, 135.5 NaCl, 1.2 $MgCl_2$, 5.9 KCl, 11.5 glucose, 11.5 HEPES, 1.5 $CaCl_2$, pH 7.3) containing 0.2% collagenase type IV. Muscles were washed twice in Krebs buffer, suspended in Dulbecco Minimum Essential Medium (DMEM) with Ham F12 complement (DMEM/HAM F12) supplemented with 2% fetal bovine serum and mechanically dissociated by repeated passages through fire-polished Pasteur pipettes with progressively decreasing diameter. Dissociated fibers were plated onto tissue culture dishes coated with extracellular matrix basement membrane (ECM, Harbour Bio-products, Norwood, M1, USA) and allowed to adhere to the bottom of the dish for 2 h. Culture dishes will be kept in an incubator, with 5% CO_2 at 30°C.

3.3.9 - In-situ Calpain activity

Measurement of in-situ calpain activity was done as described previously [55]. In detail, the synthetic substrate 7-amino-4-chloromethyl-coumarin-*t*-butoxycarbonyl-$_L$-leucyl-$_L$-methionine amide (Boc-Leu-Met-CMAC) measures real-time activity of both μ- and m-calpain activity. In its native form Boc-Leu-Met-CMAC is permeable to the cell where it is modified to Boc-Leu-Met-MAC-SG by the glutathione S-transferase, transforming the substrate to an impermeable form. The MAC-SG (7-amino-4-methylcoumarin glutathione conjugate, excitation and emission wavelengths, 380 nm and 480 nm respectively) will be cleaved from the Boc-Leu-Met moiety by calpain, and over time the fluorescence increase of MAC-SG inside the cell will represent the increase in calpain activity.

The substrate was added to the fiber chamber to a final concentration of 10 μM in Krebs solution. Fluorescence was recorded within 30 seconds of the substrates addition to the cell chamber. Detection was made with a photon counter that was restricted by a rectangular box that surrounded the fiber parallel to fiber's long axis. Transient changes in calpain activity were recorded at 100 Hz, for recordings over longer time periods, measurements were made for only 6 seconds in every 1 minute to prevent photobleaching of the sample.

3.3.10 - Statistics

Two-way and mixed-model analyses of variance (ANOVAs; SAS 9.1 software, SAS Institute, Inc., Cary, North Carolina) were used to compare genotype and treatment data. Differences in mechanical properties and maximum tetanic stress at a given age between the mouse genotypes were analyzed by a two-way ANOVA. The Tukey's post hoc test was applied to all significant main effects to determine differences between means. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SE.

3.4 - Results

Body Mass and EDL contractile and morphological features –To determine if leucine supplementation would increase muscle mass and function, mice were fed a normal chow diet with or without leucine in drinking water for 4 weeks. At the conclusion of the 4 week intervention, body mass and masses of the selected muscle were determined. No differences in body mass were observed between control and experimental groups (Figure 3.9).

To test if muscle stress production was altered by leucine supplementation, EDL muscles were hung in an *in vitro* bath system to measure muscle stress production (Figure 3.10). Independent of diet, there was no leucine effect on muscle stress production, but there was a genotype effect. BL/10 mice independent of leucine supplementation produced more muscle stress at all frequencies measured ($p < 0.01$) than in *mdx* mice. *Mdx*-L EDL muscles did produce more stress than the *mdx* EDL muscles at 80 (21.7 vs 18.7 g/mm^2, $p < 0.05$), 100 (24.6 vs. 21.1 g/mm^2, $p < 0.05$), and 150 Hz (29.0 vs. 24.9 g/mm^2, $p < 0.03$). In addition to determining stress production of EDL muscle we also measured the contractile and morphological properties of the EDL muscles tested.

Analysis of the morphological and contractile properties of EDL muscles (Table 3.5) showed a genotype effect independent of leucine supplementation on EDL muscle length ($p < 0.05$) and twitch stress ($p < 0.001$) but mesuredment showed there were no leucine or interaction effects. The *mdx*-L EDL muscles were longer than either of the BL/10 EDL muscles ($p < 0.05$). There were no differences in contractile properties such as half-relaxation time (HRT), time-to-peak tension (TPT), and active or passive stiffness. Because there were no gross changes in the contractile or morphological properties we explored possible changes in fiber type as well as section analysis or centralized nuclei and individual fiber size.

EDL Fiber Properties – To determine if stress changes in EDL muscles of *mdx*-L mice were due to a shift in fiber type we electrophoretically separated the myosin heavy chains (MHC) and determined the proportions of the individual MHC types (Figure 3.11). Independent of leucine supplementation there was a genotype difference in the distribution of MHC in EDL muscles of BL/10 mice. BL/10 mice had more MHC IIb and less MHC IIx compared to *mdx* mice ($p < 0.05$). There were no leucine or interaction effects on the distribution of MHC following 4 weeks of leucine supplementation in either BL/10 or *mdx* mice. A higher proportion on MHC IIb fibers would explain the higher stress production in the BL/10 EDL muscles, but not the increase in *mdx* stress. Therefore we determined if EDL cross-sections had changes in either their proportion of centrally nucleated fibers or if there were changes in individual fiber cross-sectional area (CSA).

The was a genotype effect independent of diet ($p < 0.0001$) and an interaction effect ($p < 0.05$) on the percentage of centralized nuclei present in EDL muscles of *mdx* mice compared to BL/10 mice (Figure 3.12). The *mdx* EDL had the highest proportion of centrally nucleated fibers compared to all other groups ($p < 0.05$), and the *mdx*-L animals had a lower percentage centrally nucleated EDL fibers compared to *mdx* animals, but still had a higher proportion than either BL/10 group ($p < 0.05$). A lower portion of centrally nucleated fibers could partially explain the increase in muscle stress production for the *mdx*-L animals tested. In addition to the centrally nucleated fiber proportion, we also wanted to determine if individual fiber area was changed. Individual fiber CSA is a more sensitive measure of CSA than the calculated whole muscle CSA that was used as a morphological measure of muscle size.

After calculating the average individual fiber area, independent of leucine supplementation there was a genotype effect, with BL/10 EDL fibers having a greater diameter than the *mdx* EDL

fibers (Figure 3.12), and interaction effects ($p < 0.05$). Individually the BL/10 EDL fibers had the greatest size compared to all other groups, the BL/10-L fibers had a greater CSA than both *mdx* groups. The *mdx* EDL fibers had greater CSA than the *mdx*-L group. Even though the *mdx*-L animals had the smallest CSA, there have been previous reports where *mdx* muscles with smaller CSA produce more stress[129]. After thoroughly analyzing the EDL morphological and contractile features, we also decided to measure markers of muscle degradation, the calpain pathway, and hypertrophy or growth, mTOR pathway. These measurements were made to determine if leucine supplementation affected the growth and degradation of *mdx* skeletal muscle, and if they could also be playing a role in the change of muscle stress production.

Measurement of muscle degradation and hypertrophy – It has been previously reported that the calpain proteolytic pathway is overactive in *mdx* muscle fibers[55] and that calpain mRNA is depressed in skeletal muscle following leucine gavage[98,97]. So, we tested the real-time rate of calpain cleavage in isolated flexor digitorum brevis (FDB) fibers of *mdx* mice. Following the 4 weeks of leucine supplementation *mdx*-L mice had a 42.4% drop in the rates of real-time calpain cleavage compared to *mdx* mice (Figure 3.13, $p < 0.01$). To see if this was due to a change in the content of calpain or its endogenous inhibitor calpastatin we determined their protein content through immunoblotting.

The ratio of calpain to calpastatin (Figure 3.14) had a main genotype effect, where the BL/10 animals had a lower ratio of calpain to calpastatin ($p < 0.01$), therefore there is more endogenous inhibitor present for the respective content of calpain. The *mdx*-L group had a higher ratio of calpain compared to calpastatin compared to the BL/10-L group ($p < 0.05$, Figure 3.14). Since leucine is a well established activator of the mTOR pathway we measured the ratio of mTOR phosphorylated on Ser 2448 compared to total mTOR content to determine if leucine is

increasing activation of the mTOR pathway and this might contribute to therapeutic effect on muscle function in *mdx* mice.

The ratio of phosphorylated mTOR to total mTOR had no genotype or interaction effects, but there was a significant depression of mTOR phosphorylation in the *mdx* group ($p < 0.05$, Figure 3.14). Leucine could be normalizing the activity of mTOR in *mdx* mice, enhancing muscle growth and strength in *mdx* mice. This, in addition to changes in calpain cleavage rates and the percentage of centrally nucleated fibers can explain the increase in EDL muscle stress in the *mdx*-L group.

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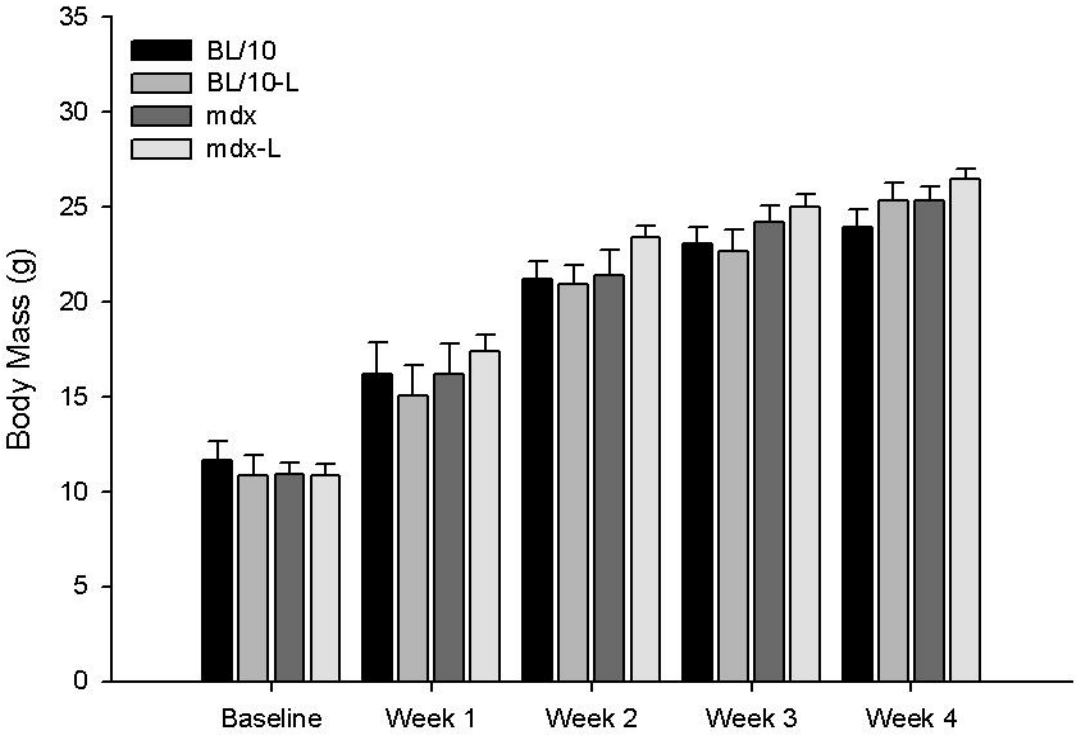

Figure 3.9 – Body mass of BL/10 and *mdx* mice during the 4 week study. There were no significant differences in body mass during the testing period. All values are means ± SEM, $p < 0.05$, BL/10 and BL/10-L n = 4; *mdx* n = 8; *mdx*-L n = 9.

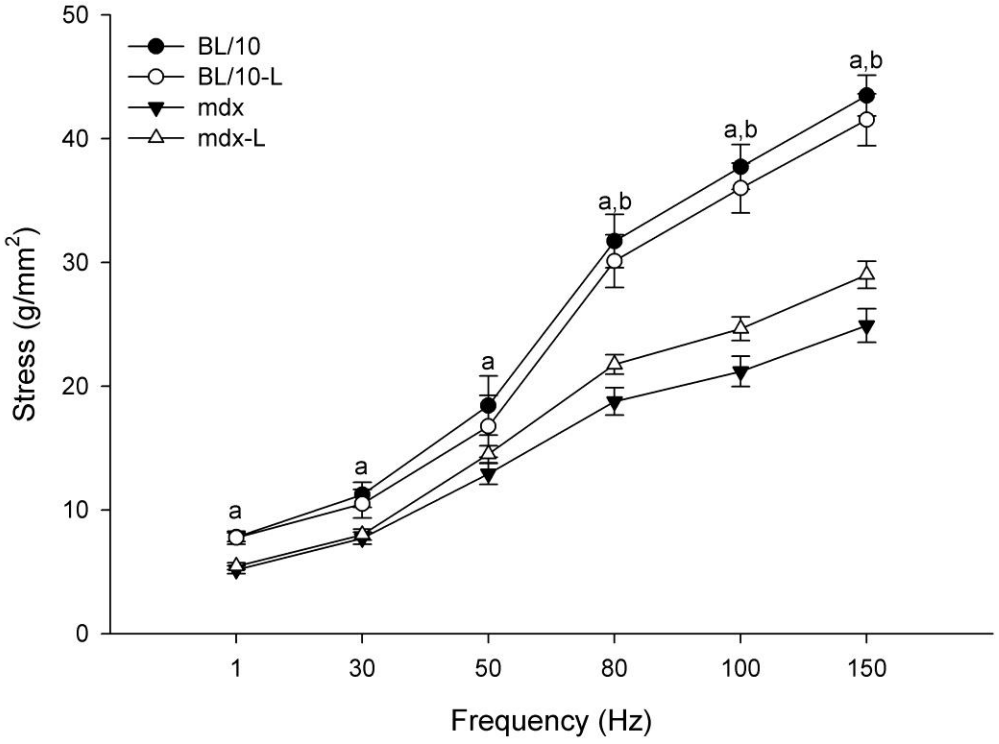

Figure 3.10 – Extensor digitorum longus stress frequency profiles for *mdx* and BL/10 mice given access to either normal or leucine supplemented drinking water. Four weeks of leucine supplementation increased *mdx*-L EDL stress production in vitro at 80, 100 and 150 Hz ($p < 0.05$). There were no effects of leucine supplementation on specific force production of in-vitro BL/10 EDL skeletal muscle at any frequency tested. aindicates the BL/10 group edl stress was greater than the *mdx* group edl stress, b indicates *mdx*-L edl stress was greater than *mdx* edl stress. All values are mean ± SEM; $p < 0.05$;BL/10 and BL/10-L, n = 4; *mdx*, n = 8; *mdx*-L, n = 9.

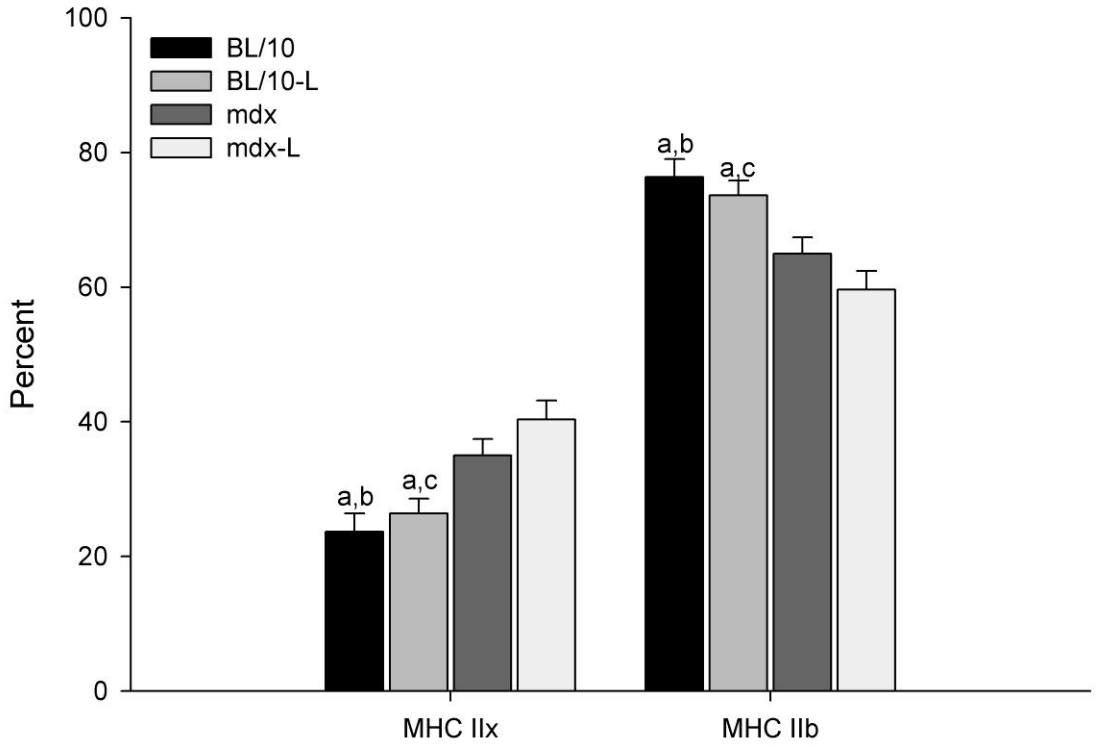

Figure 3.11 – EDL myosin heavy chain distribution as determined by electrophoretic separation in BL/10 and *mdx* animals following 4 weeks of normal or leucine supplemented drinking water. aThere was a significant difference between the genotypes independent of leucine supplementation. b Significantly different from either the *mdx* or *mdx*-L groups. c Significantly different from the *mdx*-L group only. All values are mean ± SEM; $p < 0.05$; all groups, $n = 4$.

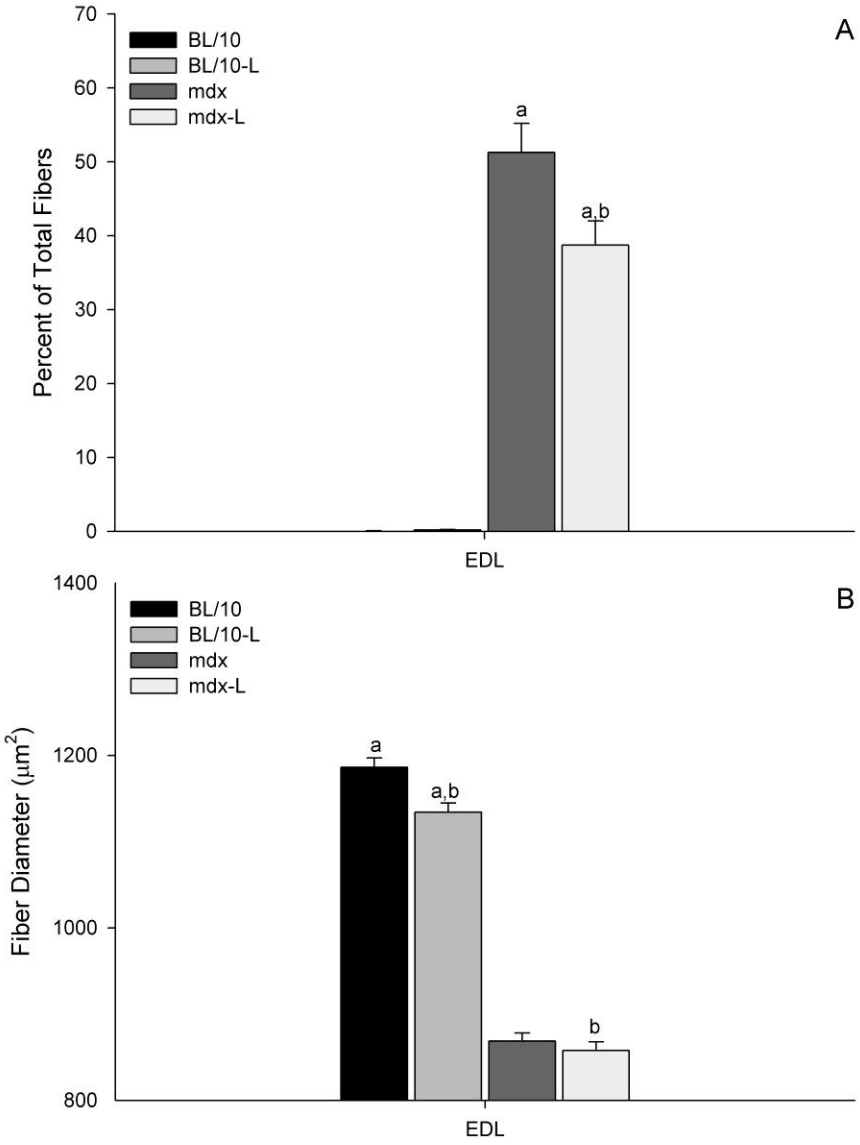

Figure 3.12 - (A) Percentage of centrally nucleated fibers in EDL cross sections stained with hematoxlyn and eosin from *mdx* and BL/10 mice after 4 weeks of normal or leucine supplemented drinking water. (B) The average diameter of EDL muscle fibers following 4 weeks of normal or leucine supplemented drinking water was determined by WGA membrane staining of EDL cross sections. a There was a significant difference between genotypes. b*mdx*-L EDL sections were depressed compared to *mdx* EDL sections. All values are mean ± SEM; $p < 0.05$; BL/10 and BL/10-L, n = 4, *mdx* and *mdx*-L, n = 5.

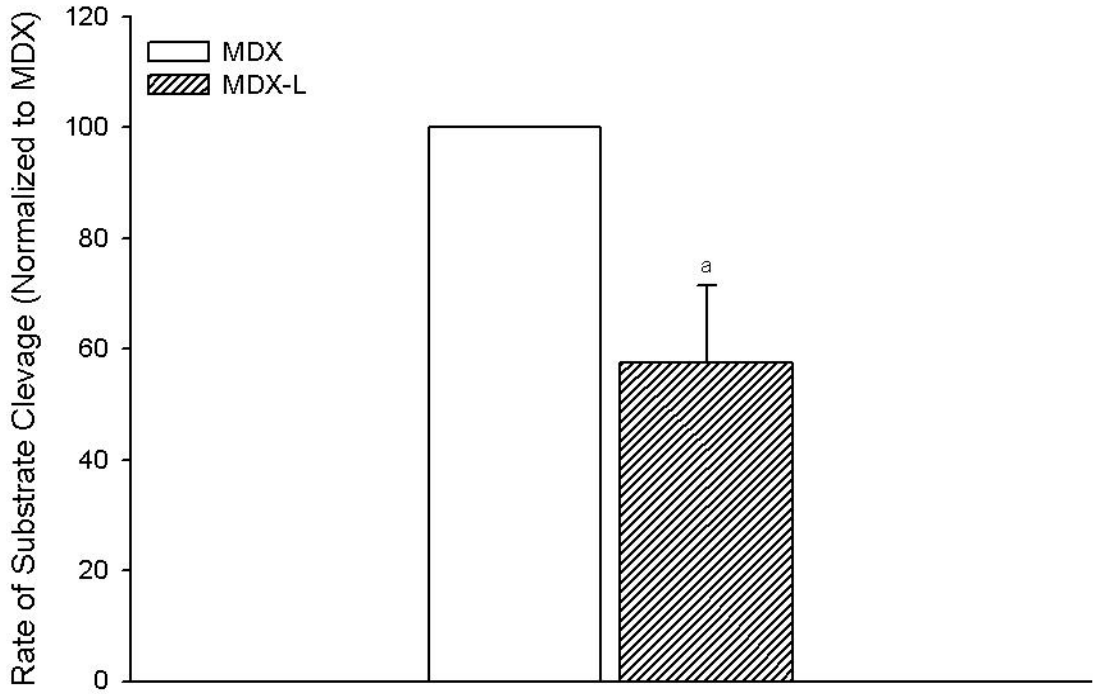

Figure 3.13 – (A) Percentage of centrally nucleated fibers in EDL cross sections stained with hematoxlyn and eosin from *mdx* and BL/10 mice after 4 weeks of normal or leucine supplemented drinking water. (B) The average diameter of EDL muscle fibers following 4 weeks of normal or leucine supplemented drinking water was determined by WGA membrane staining of EDL cross sections. a There was a significant difference between genotypes. b*mdx*-L EDL sections were depressed compared to *mdx* EDL sections. All values are mean ± SEM; $p < 0.05$; BL/10 and BL/10-L, n = 4, *mdx* and *mdx*-L, n = 5.

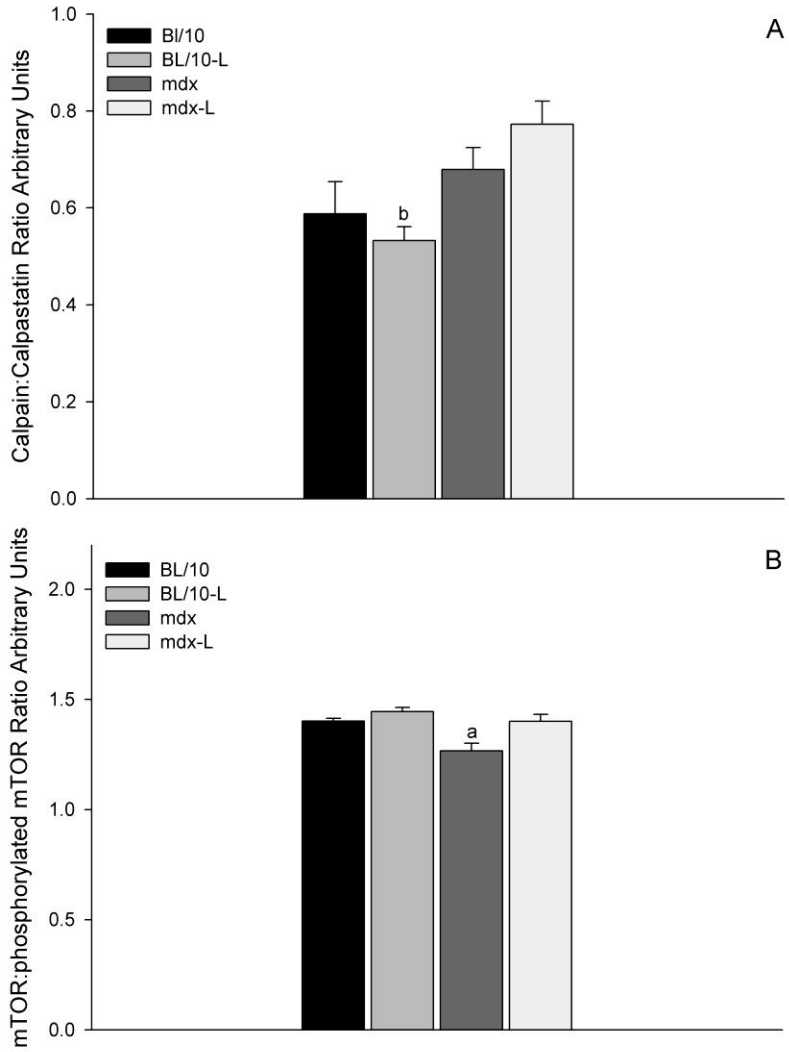

Figure 3.14- Effects of leucine treatment on the calpains and mTOR phosphorylation. Immunoblotting of Calpain, Calpastatin, mTOR and phosphorylated mTORSer2448 was conducted as described in Methods. Results are from immunoblots on the gastrocnemius muscle of treated and untreated BL/10 and *mdx* mice. (A) The ratio of calpain to the endogenous calpain inhibitor, calpastatin, was determined by dividing the intensity of the calpain band to the calpastatin band. b significantly different from the *mdx*-L group at $P < 0.05$. (B): The ratio of phosphorylated ser2448 mTOR to total mTOR in gastrocnemius muscle of BL/10 and *mdx* mice. a *mdx* mTORser2448 phosphorylation was significantly depressed compared to all other groups ($p < 0.05$). All groups, $n = 4$.

Table 3.5 - EDL morphological properties. Leucine had no effect on any of the properties measured. a *mdx* versus BL/10 ($p < 0.05$). b *mdx*-L greater than either BL/10 group ($p < 0.05$). All values are means ± SEM; $p < 0.05$; BL/10 and BL/10-L, n = 4; *mdx*, n = 8; *mdx*-L, n = 9.

	Muscle Length (mm)	Muscle Mass (mg)	CSA (mm^2)	Twitch Stress (g/mm^2)	Time to Peak Tension (ms)	Half-Relaxation Time (ms)	Passive EPE (MPa)	Passive ESE (MPa)	Active Stretch (MPa)
Genotype									
BL/10	12.78±0.48	9.59±0.64	0.71±0.05	8.33±1.35	12.93±1.80	14.37±5.39	1.19±0.37	2.36±0.55	3.28±0.53
MDX	13.14±0.58[a]	9.84±1.06	0.71±0.08	5.52±1.19[a]	13.21±1.76	16.60±5.92	2.94±1.33	3.21±0.99	2.91±0.87
Treatment									
Control	12.97±0.60	9.99±0.88	0.73±0.06	6.42±1.94	13.18±1.99	16.08±6.02	1.87±0.87	2.56±0.54	2.77±0.51
Leucine	13.06±0.55	9.52±0.95	0.69±0.08	6.57±1.76	13.04±1.55	15.57±5.65	2.67±1.63	3.19±1.12	3.27±0.90
All Groups									
BL/10	12.69±0.59	9.78±0.51	0.73±0.04	8.42±1.19[b]	13.41±1.63	16.04±5.79	1.03±0.19	2.22±0.64	3.17±0.33
BL/10-L	12.88±0.35	9.40±0.73	0.69±0.05	8.24±1.58[b]	12.36±2.14	12.63±4.67	1.36±0.49	2.51±0.49	3.40±0.72
MDX	13.11±0.56	10.10±1.03	0.73±0.07	3.92±1.06	12.84±2.19	16.15±6.38	2.44±0.62	2.79±0.36	2.51±0.44
MDX-L	13.16±0.62[b]	9.58±1.07	0.69±0.09	3.90±0.75	13.32±1.37	17.17±5.67	3.33±1.62	3.52±1.21	3.20±1.01

3.5 - Discussion

Muscular dystrophy is the result of a missing dystrophin protein, and the utilization of leucine as a treatment was not expected to completely ameliorate all symptoms of muscular dystrophy. Instead the goal was to determine if the use of the BCAA leucine and its anabolic effects would have a positive impact on skeletal muscle function in *MDX* mice. The most significant finding in this study is that four weeks of leucine supplementation increases EDL stress production, mTOR phosphorylation and depresses CAC activity in *mdx*-L mice, but not in healthy BL/10-L mice. Therefore, the changes in stress output may be related to increased protein synthesis, decreased degradation, or a combination of both increased synthesis and decreased degradation.

Increased protein synthesis: It is well established that leucine acts on the mTOR pathway and its downstream targets resulting in increased ribosome biogenesis and rates of protein synthesis [89,26,54,67,94,6,11]. Increases in rates of protein synthesis are linked to an increase in protein mass and maturity in skeletal muscle[89]. Although our data show no measured muscle hypertrophy or changes to the distribution of EDL fiber types following 4 weeks of leucine supplementation in *mdx* or BL/10 mice, we did observe a significant increase in activation of mTOR measured by phosphorylation on ser2448 in the *mdx*-L group compared to the *mdx* group. The change in *mdx*-L mTOR phosphorylation provides evidence that leucine is effective at increasing the activation of the mTOR pathway in animals with muscular dystrophy. The increased rate of protein synthesis can affect dystrophic skeletal muscle in several ways.

First, increases in protein synthesis increase the rate at which muscle cells are regenerating. Following injury, skeletal muscle cells become centrally nucleated, then over time the central nuclei relocate to the periphery of the cell. We demonstrated that leucine supplementation actually decreases the percentage of centrally nucleated fibers in *mdx*-L mice. To our knowledge,

this is the first report of leucine affecting the number of centrally nucleated fibers in *mdx* mice. Since leucine is increasing mTOR activation, damaged and regenerated muscle cells could be maturing faster, which manifest as a decrease in the percentage of centrally nucleated EDL muscle fibers and increased skeletal muscle specific force production.

Second, there could be more contractile proteins. Leucine supplemented *mdx* mice had significantly more actin and myosin content than non-supplemented *mdx* mice in tibialis anterior (TA) muscle (data not shown). More contractile proteins are also consistent with a significant increase in EDL muscle stress production[29].

Finally, leucine could be compensating for an inherent defect in the mTOR pathway in dystrophic skeletal muscle. When mTOR is activated, it can form two complexes mTORC1 or mTORC2, and depending on the protein mTOR binds, the downstream effects are different. mTORC2 triggers cytoskeletal actin organization, but mTORC1 leads to ribosome biogenesis, increased protein synthesis, and transcription initiation[115]. Recently, mTORC1 pathway was ablated in mature mice and this resulted in a dystrophic phenotype with weakened specific force, centralized nuclei, and a shortened lifespan [10]. There has been little research on the mTOR pathway in muscular dystrophy, and it is currently not well understood if dystrophic skeletal muscle mTOR activation is changed. Our data demonstrate a decrease in phosphorylation of mTOR in *mdx* gastrocnemius muscle when compared to control BL/10 muscle. The depression in *mdx* mTOR phosphorylation is restored to statistically similar levels to control mice with leucine supplementation in the *mdx*-L group. These findings are significant and suggest that the mTOR pathway is important in the pathogenesis of muscular dystrophy and should be explored further.

Decreased protein degradation: In our study, leucine supplementation changed the rate of CAC cleavage but not the content of the endogenous CAC inhibitor calpastatin or total calpain. Because there are no changes in the amount of total protein, we hypothesize that leucine is having a direct inhibitory effect on calpain cleavage, but how leucine inhibits calpains remains unclear. Specifically, we measured the depression of CAC proteolysis in *mdx* FDB fibers, and the decrease in *mdx*-L CAC proteolysis could be responsible for the increase in *mdx*-L EDL stress production. By depressing calpain activity, specific force production in dystrophic and septic muscle increases [118,109,25,135]. Because CAC preferentially cleave the proteins of the sarcomere, leucine supplementation could be blocking their activity, therefore protecting the sarcomere and its proteins in the dystrophic state. By protecting the proteins of the sarcomere, there is a corresponding increase in stress output, and depression in centrally nucleated fibers. If leucine levels are increased in circulating blood, we could be preventing the secondary action of muscle injury in muscular dystrophy, thus preserving muscle strength.

Our results suggest more extensive research on leucine supplementation in muscular dystrophy is needed to determine if leucine supplementation is beneficial to DMD patients in extending ambulation or increasing strength when administered at a young age before or during the onset of the disease.

Chapter 4 - The combination of leucine and exercise increases *mdx* EDL stress production in *mdx* mice

The combination of leucine and exercise increases *MDX* EDL stress production in *mdx* mice.

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4.1 - Abstract

In this study, we examined the ability of the popular post-exercise supplement, leucine, on skeletal muscle strength and size in dystrophic *mdx* mice that were allowed voluntary low load exercise beginning at the young age of 3 weeks. Daily running distance, EDL stress, myosin heavy chain distribution, fiber size and centrally nucleated fibers were measured to determine if exercise benefited *mdx* skeletal muscle and whether it had any detrimental effects. *MDX* Run mice supplemented with leucine in the drinking water ran ~40% greater distance ($p<0.01$) over the 4 weeks tested, and produced significantly greater EDL stress at all frequencies recorded ($p < 0.05$). There were also no muscle specific markers of increased degradation in the *MDX* Run-L group compared to the *MDX* Run group. We conclude that exercise combined with leucine increases the effects of exercise in the dystrophic state, and that leucine supplementation may prove efficacious in DMD.

4.2 - Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disease that afflicts 1 in 3500 new born males.[37,76,117] DMD causes skeletal muscle to continually degenerate and regenerate, resulting in progressive skeletal muscle wasting and weakness. DMD onset is between the ages of 2 and 5 with patients becoming wheelchair bound around the age of 12 and dying in their mid-20's.[50] There is no current cure for DMD, and research aimed to improve quality of life of patients is limited.

DMD is caused by mutations to the 427 kb dystrophin gene which result in the absence of a dystrophin protein. The dystrophin protein is located on the inner surface of the skeletal muscle membrane and attaches cytoskeletal actin to the membrane bound dystrophin glycoprotein complex (DGC).[15] In the absence of dystrophin, the mechanical link from the cytoskeleton of the muscle cell to the membrane and the components of the DGC is missing.[47] The continual degeneration and regeneration of skeletal muscle caused by the absence of dystrophin in DMD is hypothesized to be a result of 1) a fragile or weakened skeletal muscle membrane, or 2) altered cellular signaling caused by the absence of the DGC, but the exact mechanism of DMD related muscle wasting remains unknown.[131]

One area of muscular dystrophy research has focused on using exercise as a way to increase muscle mass and strength. In the dystrophic mouse model (*mdx*), there have been several studies that conclude voluntary, low-load exercise (i.e. voluntary wheel running) assists in slowing the progression of muscular dystrophy. Voluntary wheel run mice typically have increased muscle stress production, and decreased levels of circulating creatine kinase (CK). Also, the greatest benefits are seen when exercise is begun at a young age (i.e. age 3-4 weeks).

Leucine and exercise increase *mdx* muscle stress

Following exercise, it is also well established that proper post-workout nutrition is a vital part of proper recovery and increasing muscle hypertrophy and strength in healthy subjects. But, it is not known if proper nutrition can further increase the effects of exercise in muscular dystrophy. The use of leucine, an essential branched chain amino acid (BCAA), post-workout has shown to increase release of insulin, shorten the time for glycogen replenishment, lead to increased muscle protein synthesis, hypertrophy and strength[4,32].

The objectives of this study were to determine if oral leucine supplementation in conjunction with exercise 1) increases *mdx* running distance/adaptation to exercise, 2) increases EDL muscle stress production, and 3) changes the morphological features of the *mdx* EDL muscles.

4.3 - Methods

4.3.1 - Mice

At weaning age (21 days), *mdx* mice were individually housed in cages with running wheels and randomly assigned to either a leucine (*mdx* run-L) or no leucine (*mdx* run) group. Both groups were provided drinking water ad libitum, but the *mdx* run-L mice were provided drinking water with a final concentration of 114mM L-Leucine (Sigma). Running distance was monitored daily over the 4 weeks, and body masses were recorded every 7 days. On day 49, mice were anesthetized with ketamine/xylazine (2mg xylazine and 20mg ketamine per 100g body mass) injected i.p., and tissues excised to conduct experiments. All studies were approved by the Virginia Tech animal care committee.

4.3.2 - Running Wheels

At age 21 day, mice were placed in individual 11 in. by 9 in. plastic cages with a running wheel (0.16 m diameter; Silent Spinner) mounted to the side of the cage. Mice were allowed free access to the wheel 24 h/day. A metal tab attached to the rear of the wheel was used to interrupt a light signal to a photoelectric gate. Each signal interrupt was recorded on a laptop computer at a sampling rate of 1 Hz using a digital data-acquisition card (National Instruments USB-6501) and a custom Labview program. The running distance and average running speed were saved to a data file every 120 s. At age 42 days, after 3 wk of voluntary run training or normal cage activity, mice were killed via CO_2 inhalation. Voluntary running is an endurance exercise that involves multiple limb skeletal muscles and increased activity of the cardiovascular system. We therefore assessed adaptations in several skeletal muscles involved in running, as well as the heart and serum (Table 1). For practical reasons (i.e., insufficient muscle mass), not all tissues could be assayed for all variables.

4.3.3 - Function Data

Muscle Prep: Extensor digitorum longus (EDL) and soleus (SOL) muscles were surgically excised from anesthetized mice and secured via 4-0 suture to a dual-mode servomotor (Aurora Scientific: Aurora, Ontario) as described previously.[58,133] Muscles were then equilibrated in an oxygenated (95% O_2-5% CO_2) physiological salt solution (PSS; previously described [58]) bath for 10 minutes prior to data collection. Following the equilibration period, muscles were subjected to three twitches and two tetani, each separated by 1 minute. After a 5 minute rest, one EDL and one Soleus from each mouse was subjected to a stretch protocol while the remaining muscles were subjected to force frequency and fatigue and recovery protocols.

Force frequency protocol: Muscles were stimulated for 800 ms at 1, 30, 50, 80, 100 and 150 Hz, each separated by 1 minute. After a 5 minute rest, muscles were subjected to one twitch and one tetani (separated by 1 minute) and rested for 6 minutes.

Stress output (g/mm^2) was calculated as the force output (g) for a given stimulation normalized to the estimated cross sectional area (mm^2) of the muscle. Cross sectional area of each muscle was determined by the following equation: muscle cross sectional area = muscle mass in g (1.056 g/mm^3 x muscle length in mm). When all functional data was completed, the muscles were frozen in liquid nitrogen cooled isopentane, and stored at -80^oC until they were ready to be used.

4.3.4 - Fiber Type Analysis

We determined the proportional content of MyHC isoforms as previously described [119]. Briefly, frozen EDL muscles obtained from BL/10, BL/10-L, *mdx*, *mdx*-L mice aged 49 days were thawed on ice, homogenized, assayed for total protein (BCA Assay, Peirce), and then diluted in sample buffer to a final concentration of 0.04 mg/ml. Ten microliters of each sample was subjected to SDS-PAGE using 4% stacking and 8% separating gels. Samples were run on a mini-

gel electrophoresis unit (BioRad) for 24 h at 80 V (constant voltage). Gels were silver stained and scanned using a FluorChem image analysis system (Alpha Innotech). Band densities for each MyHC isoforms were expressed as a percentage of total MyHC band density.

4.3.4 - Sectioning

EDL muscles were mounted in OCT (Andwin Scientific) and at the point of maximal CSA (visually discerned) 8µm serial sections were taken using a Microm 505M cryostat set at -20^{o}C. Sections were collected on superfrost plus slides (Richard Allen Scientific) and allowed to air dry for 1 hour. Following air drying slides were stored at -80^{o}C.

4.3.5 - Membrane Staining

Sections were fixed for 5 minutes in a 4% formaldehyde/PBS solution, then blocked for 1 hour using a 5% BSA/PBS solution, then stained using 1:1000 WGA:PBS dillution. Sections were then washed 2 x 5 minutes in fresh PBS. Sections were briefly air dried before mounting coverslips (Vectamount) and imaging.

4.3.6 - H&E staining

EDL sections were stained using hematoxylin and eosin (H&E). First slides were dipped in hematoxylin (Fisher Scientific, Hematoxylin) for 30 seconds, washed in fresh tap water for 1 minute, followed by 30 seconds in eosin (Fisher Scientific, Eosin), and then washed for 1 minute in fresh tap water. Sections were allowed to air dry, then were coverslipped and imaged.

4.3.7 – Statistics

To determine differences between groups a 1-way anova was performed, with significance set at $p < 0.05$. All tests were performed using the the statistical software package JMP 7, and values are reported as means ± SEM.

4.4 - Results

Running Distance and EDL muscle performance – Since leucine acts as an ergogenic aid, we first wanted to test to determine if leucine actually increased the distance run by *MDX* mice. *MDX* Run-L animals ran more average daily distance than *MDX* Run animals during weeks 2 ($p < 0.001$), 3 ($p < 0.002$), and 4 ($p < 0.0001$). The *MDX* Run-L animals also ran more total distance during the 4 week testing period ($p < 0.01$). The additional running had no negative effect on body mass of the exercised *mdx* mice (Figure 4.16). Since the leucine supplemented *MDX* mice did run a greater distance we determined if this also related to an increase in extensor digitorum longus (EDL) muscle stress production.

Following the 4 weeks of exercise and leucine supplementation The *MDX* Run-L group produced more EDL stress at every frequency recorded ($p < 0.05$, Figure 4.17). Therefore increases in exercise capacity were related to increases in muscle performance in *MDX* mice. We also looked at the morphological and contractile properties of the EDL muscles tested. There was an increase in *MDX* Run-L EDL twitch stress compared to *MDX* Run EDL twitch stress ($p < 0.02$), but all morphological measures and contractile properties of the EDL muscles were unchanged (Table 4.6). Because of the positive changes in muscle stress and running distance, we wanted to see if this related to changes in myosin heavy chain (MHC) distribution and histological marker of muscular dystrophy.

EDL Myosin Heavy Chain Distribution and Histological Features – Myosin heavy chain distribution was tested by electrophoretic separation on SDS PAGE gels. Typically increases in endurance capacity and running distance are related to changes in MHC distribution in *MDX* mice[64]. The addition of leucine to exercise had no effect on the percentage of MHC type IIx or

IIb expression in these animals. Therefore increases in running distance may be attributed to changes in motivation or effects on the central nervous or cardiovascular systems (Figure 4.18).

In addition to the MHC distribution, hematoxylin and eosin, and wheat germ agglutinin stains were performed to measure the amount of centrally nucleated fibers and average fiber count and fiber cross-sectional area (CSA). There were no effects of leucine on the percentage of centralized nuclei present in exercised *mdx* EDL muscle fibers (Figure 4.19). There were no effects of leucine on the absolute number of fibers in exercised *mdx* EDL muscles (Figure 4.19). The *MDX* Run-L group had a smaller average fiber area when compared to the *MDX* Run group ($p < 0.05$, Figure 4.19).

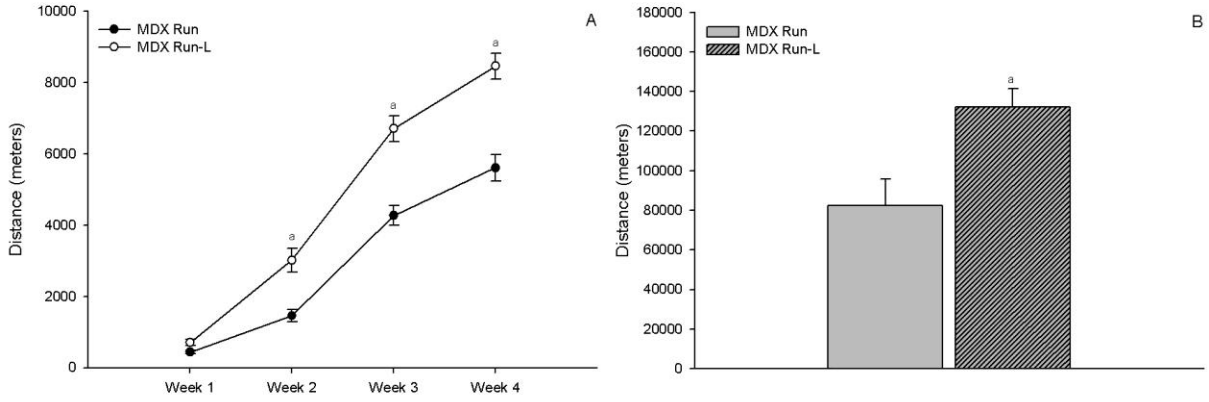

Figure 4.15 - Effects of leucine treatment on the calpains and mTOR phosphorylation. Immunoblotting of Calpain, Calpastatin, mTOR and phosphorylated mTORSer2448 was conducted as described in Methods. Results are from immunoblots on the gastrocnemius muscle of treated and untreated BL/10 and *mdx* mice. (A) The ratio of calpain to the endogenous calpain inhibitor, calpastatin, was determined by dividing the intensity of the calpain band to the calpastatin band. b significantly different from the *mdx*-L group at $P < 0.05$. (B): The ratio of phosphorylated ser2448 mTOR to total mTOR in gastrocnemius muscle of BL/10 and *mdx* mice. a *mdx* mTORser2448 phosphorylation was significantly depressed compared to all other groups ($p < 0.05$). All groups, $n = 4$

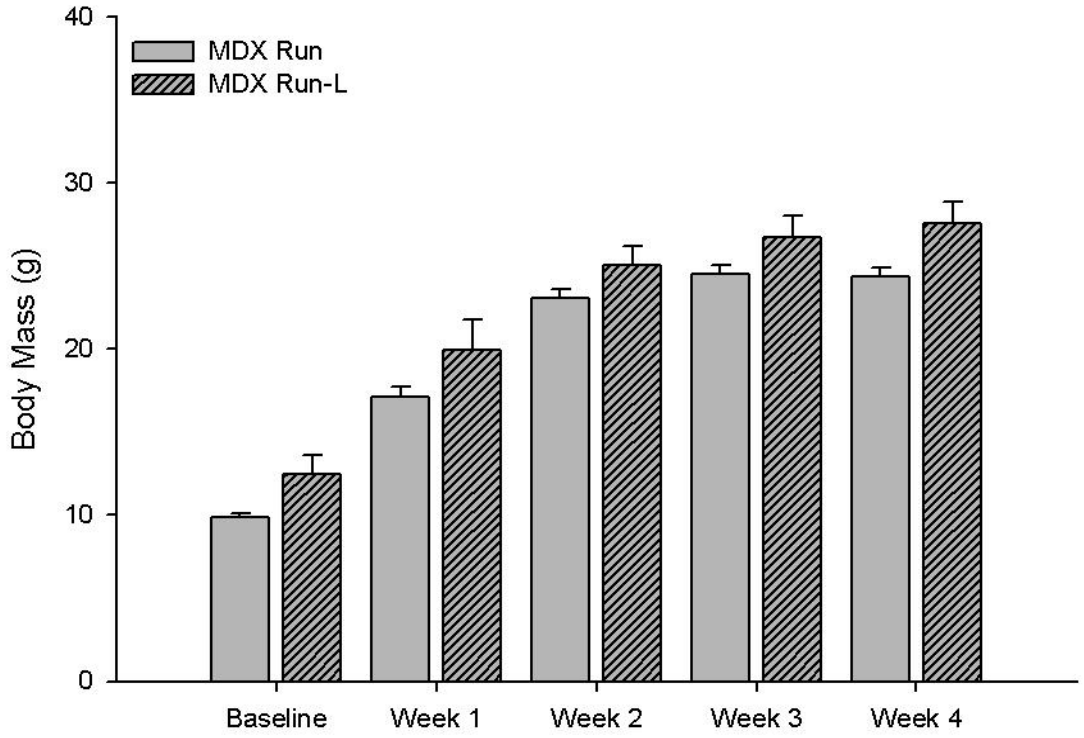

Figure 4.16 – Weekly body mass of exercised *mdx* mice during the 4 week study. There were no recorded mass differences between the *MDX* Run and *MDX* Run-L groups. All values are means ± SEM, $p < 0.05$, $n = 5$.

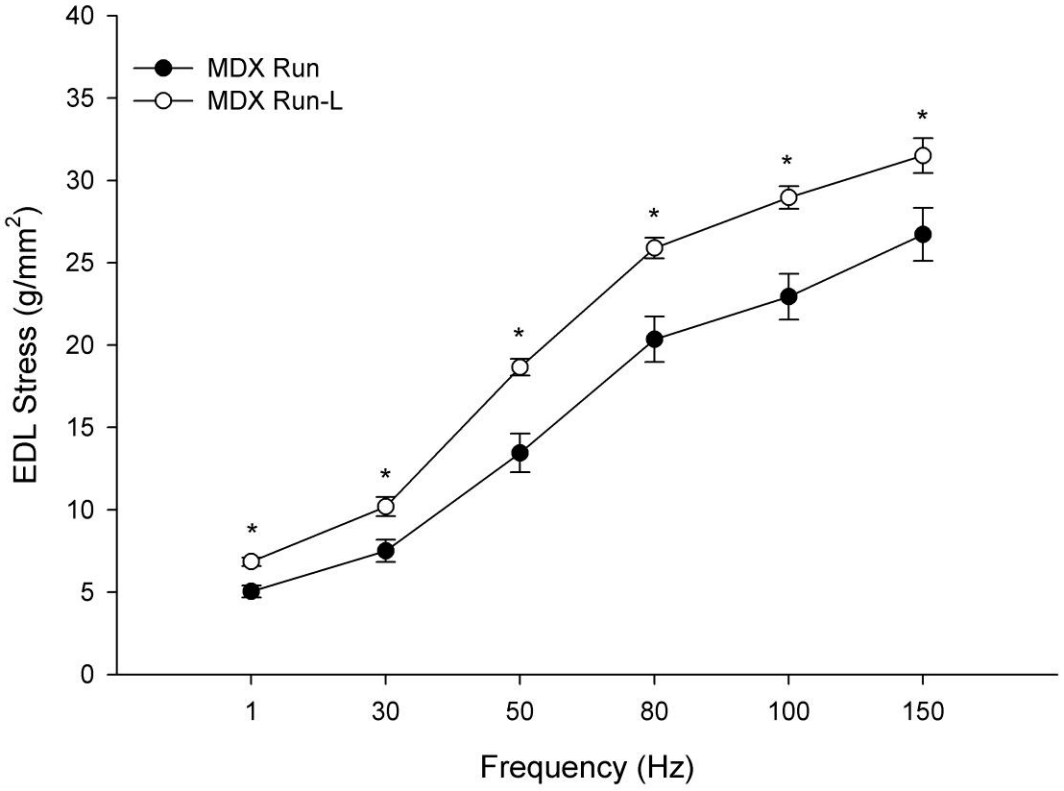

Figure 4.17 – Stress frequency profile of *MDX* Run and *MDX* Run-L EDL muscles. All tests were performed in vitro, in an oxygenated bath. **MDX* Run-L group was significantly greater than the *MDX* Run group. Values are reported as mean ± SEM; $p < 0.05$; $n = 5$.

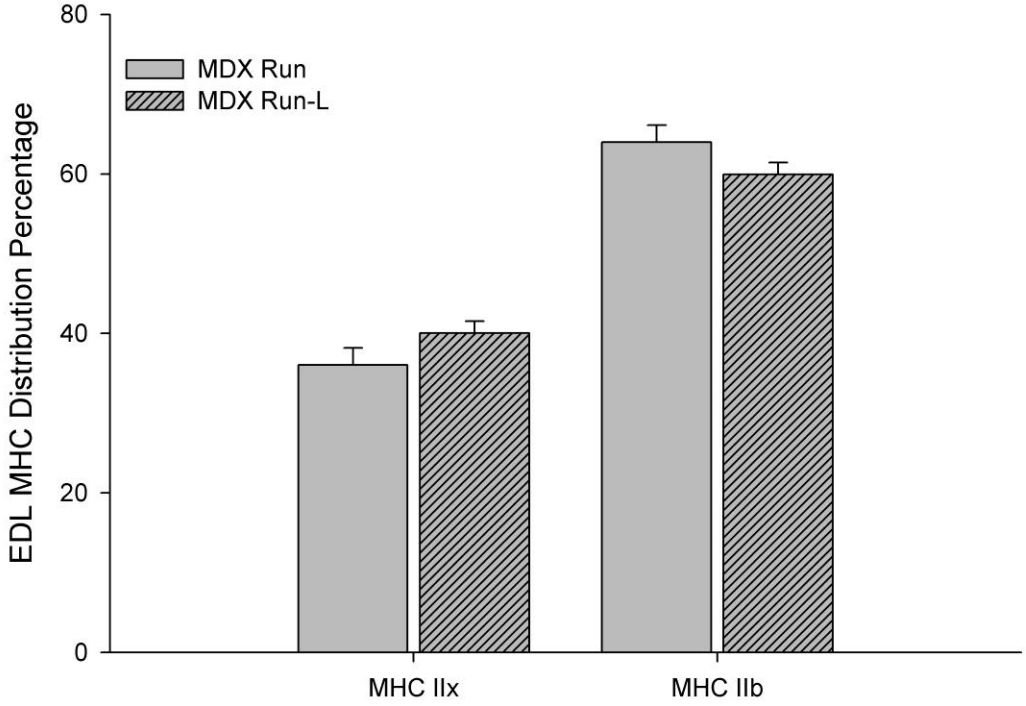

Figure 4.18 – Myosin heavy chain distribution of *MDX* Run and *MDX* Run-L EDL muscles determined by electrophoretic separation. There were no changes in the distribution of myosin heavy chain in EDL muscles following 4 weeks of voluntary wheel running and leucine supplementation. All values are mean ± SEM; and $p < 0.05$; $n = 4$.

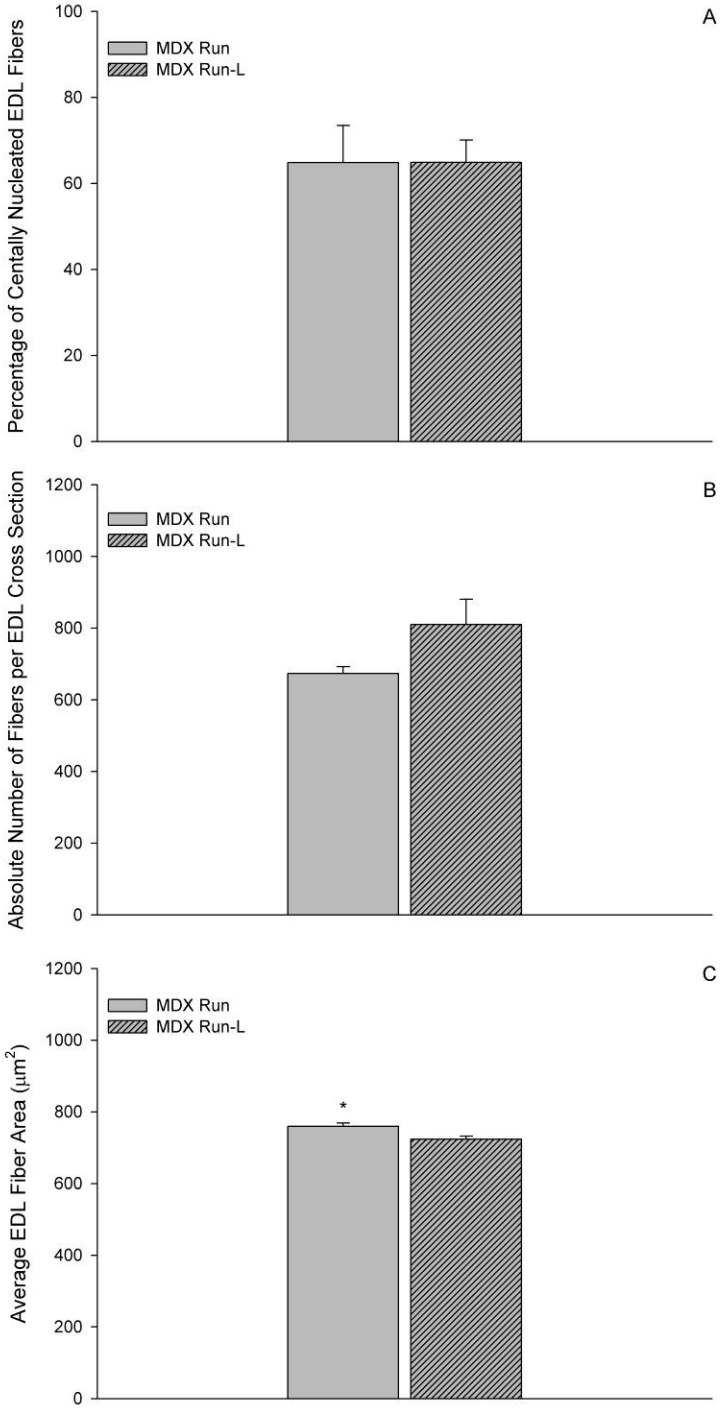

Figure 4.19 – EDL section analysis: (A) Percentage of centrally nucleated EDL fibers, (B) Total number of fibers per EDL section, and (C) Average EDL fiber area. * *MDX* Run values were significantly greater than the *MDX* Run-L group values; mean ± SEM; $p < 0.05$; $n = 5$..

Table 4.6 – *MDX* Run and *MDX* Run-L animals demonstrated no differences in muscle length, mass, or CSA. * The *MDX* Run-L group had greater EDL twitch stress compared to the *MDX* Run group ($p < 0.02$). All values are mean ± SEM; n = 5 in both groups.

	Muscle Length (mm)	Muscle Mass (mg)	CSA (mm^2)	Twitch Stress (g/mm^2)	Time to Peak Tension (ms)	Half-Relaxation Time (ms)	Passive EPE (MPa)	Passive ESE (MPa)	Active Stretch (MPa)
MDX Run	12.67±0.41	9.40±0.77	0.70±0.07	5.12±1.09	13.50±1.04	18.60±5.98	2.74±1.04	3.02±0.90	2.59±0.67
MDX Run-L	13.20±0.45	10.34±1.39	0.74±0.08	7.04±0.54*	13.40±1.34	18.40±5.51	2.42±0.75	2.92±0.70	2.43±0.27

4.5 - Discussion

The use of ergogenic aides in humans to increase muscle size, strength, and exercise performance is well established[114]. Surprisingly, the use of ergogenic aides in addition to exercise as a therapy for musculoskeletal diseases, such as muscular dystrophy, is very sparse. We applied the use of a popular ergogenic aid, leucine, to *mdx* mice given access to a voluntary running wheel for 4 weeks to determine if leucine supplementation could benefit exercised dystrophic skeletal muscle. The major findings in this study were that voluntarily exercised *MDX* mice that were given free access to leucine supplemented drinking water ran ~40% more distance over the 4 week testing period, produced greater EDL stress, and with the increase in exercise and stress production, there were no negative impact to the EDL measured by centralized nuclei.

Exercise in muscular dystrophy has been controversial, researchers are attempting to find a balance between exercise which will increase dystrophic muscle strength and stamina but not damage the already weakened muscle fibers[59]. We see that voluntary low load exercise (wheel running), when begun at a young age (i.e. age 3 – 4 weeks) benefits *mdx* mice, which has been previously demonstrated[29,30,42]. The new finding from this study is the demonstration that the addition of leucine to an exercise regimen benefits *mdx* muscle more than exercise alone. Notably, this is one of the few therapies in dystrophic mice that have resulted in a concurrent increase in EDL stress and running distance. Leucine has hypertrophic properties that benefit human and rodent skeletal muscle mass following exercise by increasing insulin release, glycogen replenishment, and power output [125,113,74,101,34,16,97,35]. We now show that exercised dystrophic skeletal muscle also benefits from leucine supplementation in a similar fashion to normal skeletal muscle.

Interestingly, with the increase in running distance we saw no significant shift in EDL MHC distribution. This could partially be due to the fact that the load induced on the EDL muscle during exercise is minimal; therefore MHC isoform shift was also minimal. Other studies that have exercised *mdx* mice for a similar time period have seen either small[64] or no[29] changes in EDL MHC distributions. We also saw a decrease in average fiber diameter of the leucine supplemented *mdx* mice. But, there are previous reports that decreased fiber diameter can be beneficial to *mdx* mice[129]. It has also been established in humans subjected to endurance training, that exercise will decrease fiber size while increasing muscle stress (Force/CSA)[62]. Consequently, these changes would be interpreted as beneficial for dystrophic skeletal muscle.

Very importantly, there were no changes in the percentage of centralized nuclei present between the *MDX* Run and *MDX* Run-L groups. Because centralized nuclei are indicative of muscle damage and regeneration, our animals were able to exercise more without a negative impact on the dystrophic skeletal muscle. These data are consistent with several other exercise studies in dystrophic mice which report benefits with treatment, yet no changes to the percentage of centrally nucleated fibers in muscles following exercise[25,64,30]. Seeing as the numbers of centralized nuclei are unchanged, we hypothesize that the additional distance ran and production of EDL stress has no negative or additional impact on the skeletal muscle of these *MDX* mice.

The results in this study support the hypothesis that exercise in muscular dystrophy can be utilized as a therapeutic approach to gain muscle strength, and improve quality of life, and the use of ergogenic aids such as the BCAA leucine, could benefit dystrophic patients. To determine proper load, type, frequency and nutritional support for DMD patients, exercise must first be explored in higher order species. Then these findings can be applied to DMD patients in treatment to increase muscle strength and function utilizing a proven training paradigm. The

data presented in this paper also emphasizes the importance of proper nutritional support for DMD patients, an area in which extensive research is lacking.

Chapter 5 - Conclusions

5.1 - Conclusions

Currently the most efficacious treatment for DMD is the use of the glucocorticoids prednisolone and deflazacort[86,83]. Although these drugs do extend ambulation, they are associated with several undesirable side effects such as weight gain, bone density loss, spinal fractures and cataracts[83,85]. It is imperative that the research community find new approaches that effectively counter the progression of DMD with minimal side effects and so increase the quality of life and lifespan of patients. Rennie et al. [112] studied protein synthesis rates in DMD boys and demonstrated that they were significantly suppressed. Therefore, we identified leucine as a potential treatment strategy because it has been proven to increase protein synthesis rates by acting on the mTOR pathway[7,41,113]. The goal of this dissertation was to demonstrate that leucine may be an effective therapeutic supplement for dystrophic skeletal muscle. We set out to prove this by measuring changes in muscle function, exercise capacity, histological and biochemical markers of muscular dystrophy in both sedentary and exercised states.

In the first study, (Chapter 3, specific aim 1) the overarching goal was to determine if 4 weeks of leucine supplementation would benefit skeletal muscle function of *mdx* mice. We chose to supplement *mdx* mice from age 3 weeks to age 7 weeks because this spans the critical period of muscle degeneration and regeneration in *mdx* mice[50]. Leucine was dissolved in drinking water at a concentration of 114 mM, and following the 4 week supplementation period animals were sacrificed and data was collected. Leucine supplementation had no effect on body mass of *mdx*-L mice, but increased in vitro EDL stress by 16% at 80 Hz, 100 Hz and 150 Hz compared to *mdx* mice. There was also a 25% decrease in the concentration of centralized nuclei of EDL muscles from *mdx*-L animals compared to *mdx* animals. Furthermore, the *mdx*-L animals had a 41.4% drop in the calpain cleavage rates in FDB fibers compared to *mdx* mice, and

mTOR phosphorylation in *mdx*-L gastrocnemius muscle was restored to control BL/10 levels. Combined, these data lead us to believe that leucine is effectively activating the mTOR pathway in *mdx* mice, restoring mTOR phosphorylation to levels of control animals, while controlling calpain-induced degradation, resulting in increased muscle stress production.

In the second study, (Chapter 4, specific aim 2) the overarching goal was to determine if leucine supplementation combined with exercise in *mdx* mice would result in ergogenic benefits. We applied the same supplementation method and timeline as in study 1, but added access to voluntary running wheels. The most significant finding was that *mdx* mice supplemented with leucine ran 40% more total distance during the 4 week testing period, and this resulted in increased EDL stress production at all frequencies tested. There were no changes in the percentage of centralized nuclei in the EDL, but there was a significant depression in EDL average fiber size. This demonstrates that even in a dystrophic state, leucine can provide ergogenic benefits that are not detrimental to skeletal muscle of *mdx* mice.

Leucine has previously been reported to increase protein synthesis in normal skeletal muscle[7,101,48,26], but it has yet to be thoroughly explored in a dystrophic population. Mendell et al. [88] conducted a clinical trial of leucine supplementation in dystrophic patients in 1984, and saw transient increases in muscle strength, but did not identify whether there were beneficial biochemical or morphological changes to skeletal muscles of treated DMD boys. That study also spanned several age groups; therefore boys were potentially at different stages of disease progression, possibly altering study outcomes. We decided to look at a specific time (early onset), and determine if changes of muscle stress were also related to changes in muscle morphology and biochemistry that would benefit a dystrophic patient. The studies presented in this dissertation demonstrate that leucine effectively increases dystrophic skeletal muscle stress,

and this result is compounded when animals are given leucine in addition to exercise training. Additionally, we see that leucine supplementation increases mTOR activation in *mdx* mice while depressing rates of calpain cleavage. These changes are novel, and can counteract the depressed rates of protein synthesis[111] and increased rates of calpain cleavage[55] found in dystrophic skeletal muscle. By altering the synthesis and degradation rates in dystrophic skeletal muscle, one could presumably extend ambulation and increase skeletal muscle function, thereby increasing the quality of life of dystrophic patients.

Chapter 6 - Future Directions

6.1 - Future Directions

The central objective in this study was to determine if leucine supplementation is beneficial to dystrophic skeletal muscle. We have shown that leucine supplementation, when started at weaning age, benefits *mdx* exercise capacity, muscle stress production, normalizes levels of mTOR phosphorylation, all while slowing calpain proteolysis in dystrophic muscle.

6.1.1 - Dystrophic skeletal muscle, exercise, and leucine

We are the first authors to show that utilizing leucine in conjunction with exercise in muscular dystrophy enhances the effects of exercise. Exercise for DMD patients is a controversial topic, although there is significant data in *mdx* mice that show low load, voluntary exercise benefits muscle strength, and the benefits are greatest when exercise is begun at an early timepoint in the animals lifespan (i.e. 3 – 4 weeks of age). This led us to hypothesize, that when exercise therapy or physical therapy is used with dystrophic patients, this must be combined with proper nutritional support.

We approached this study by providing leucine in the drinking water of *mdx* mice and saw benefits. In the future, determining a leucine dose curve and relating it to lean body mass would be an important step to see how much leucine is necessary to increase exercise capacity, and possibly shorten exercise recovery time in dystrophic skeletal muscle. By utilizing cages that measure oxygen exchange, we can determine if *mdx* mice that are provided with leucine utilize energy differently while exercising, and if this has a protein, or lean body mass sparing effect.

Determining the functional reason why exercise capacity increases is also imperative. The use of markers of muscle breakdown, such as creatine kinase and Evans blue dye, would

allow us to see whether leucine is protecting dystrophic skeletal muscle from exercise induced damage because of blunted rates of calpain cleavage. There is evidence that post workout supplementation of leucine also increases both insulin release from the pancreas and rates of glycogen restoration in skeletal muscle. Determining whether these effects also occur in dystrophic skeletal muscle would provide interesting and vital information to the research community.

6.1.3 - Leucine and calpain degradation

In this study we demonstrate that leucine supplementation depresses the rate of calpain proteolysis, but we saw no change in the actual calpain protein content. While depression of the amount of calpain mRNA present in skeletal muscle by leucine has been demonstrated previously, this is the first report that leucine actually depresses calpain proteolysis rates. This leads us to hypothesize that leucine is directly or indirectly inhibiting calpain proteolytic activity. Determining how and why leucine changes calpain related proteolysis rates would benefit both the dystrophic and leucine related literature.

Future studies utilizing calcium indicator dyes and testing resting calcium levels in leucine supplemented dystrophic FDB fibers would provide information if leucine is regulating myoplasmic calcium concentrations while the muscle is at rest. To determine if leucine signaling can protect the muscle from stretch induced calcium entry, we can osmotically stress the muscle and measure transient changes in calpain activation.

6.1.4 - Dystrophic skeletal muscle and mTOR

It is well documented that dystrophic skeletal muscle has significantly slower rates of protein synthesis, which results in progressive loss of muscle strength and mass, however why and how protein synthesis is depressed in dystrophic muscle is currently unknown. We have

shown that increased mTOR activation in dystrophic skeletal muscle leads to increased exercise capacity and muscle strength in dystrophic mice. Also, recent publications demonstrate that ablation of the rictor branch of the mTOR pathway causes muscular dystrophy in mice. This leads us to hypothesize that depression of the mTOR pathway could be responsible for the depression in protein synthesis in dystrophic skeletal muscle. Currently, the amount of research on the mTOR pathway in DMD is very limited.

Specific studies should include: 1) Determining whether specific upstream or downstream components of the mTOR pathway are affected in dystrophic skeletal muscle. 2) Determining if the components of the mTOR pathway are activated differently following mTOR stimulation under different conditions such as exercise, nutrient stimulation, or drug administration in dystrophic skeletal muscle. Also, it is recommended that these tests be carried out in a higher order species or actual patients with DMD. If there is an inherent defect in the mTOR pathway of dystrophic skeletal muscle, discovering exactly why would be important as would any potential therapy that could be easily applied to increase patient quality of life and lifespan.

6.1.5 - Differences between muscle types

As demonstrated in Appendix A, the soleus muscle of treated mice did not have the same response to leucine supplementation as EDL muscles in *mdx* mice. In the soleus, there were significant gains in muscle mass, but that caused the normalized values of muscle stress output to have no change, or be depressed when compared to control values. This in itself is interesting and the differences in leucine signaling between a fast twitch muscle such at the EDL and a slow twitch muscle like the soleus should be explored in both dystrophic and control skeletal muscle.

Appendix

Appendix A – Soleus Data

In addition to the EDL data presented in the two manuscripts in this dissertation, soleus data was also collected. The soleus of leucine supplemented mice did have interesting changes, but it was felt the data did not fit in with the EDL data in the manuscripts. The data is still significant and it is included below in this appendix. The data will be presented with the same approach as the manuscripts, one graph will present BL/10 vs *mdx* data, and the second graph will present the *MDX* Run data only.

Soleus Stress Frequency Curve

BL/10 vs *MDX*

There were no differences between genotypes or between treatments for any of the frequencies tested when *mdx* and BL/10 stress frequency curves are compared (Figure A.20).

Exercised *MDX*

When the *MDX* Run and *MDX* Run-L groups were compared the *MDX* Run animals produced more stress at 1 ($p < 0.05$), 50 ($p < 0.02$), 80 ($p < 0.03$), 100 ($p < 0.01$), and 150 Hz ($p < 0.01$, Figure A.20).

Soleus Morphological and Contractile Properties

BL/10 vs *MDX*

Soleus muscles from the BL/10 vs *mdx* group had several significant differences (Table A.7). Muscle mass ($p < 0.001$) and CSA ($p < 0.001$) were higher in the *mdx* group than then BL/10 group independent of diet. The *mdx*-L animals also had significantly greater muscle length ($p < 0.001$) and CSA ($p < 0.001$) when compared to both BL/10 groups individually. Half relaxation time (HRT) was slower in *mdx* mice compared to BL/10 mice independent of diet ($p < 0.05$), and

time-to-peak tension (TPT) was significantly faster in *mdx* mice compared to BL/10 mice independent of diet ($p < 0.01$).

Exercised *MDX*

The *MDX* Run-L animals had significantly greater muscle length, mass and CSA compared to *MDX* Run animals ($p < 0.001$, Table A.8), but there were no changes in HRT or TPT.

Myosin Heavy Chain Analysis

BL/10 vs *MDX*

In the soleus there was an increase in the proportion of MHC I ($p < 0.005$) and decrease in the proportion of MHC IIx ($P < 0.001$) in the BL/10 mice compared to the *mdx* mice independent of diet. Individually, the BL/10-L animals had more MHC I than the *mdx*-L animals ($p < 0.01$) and less MHC IIx than either the *mdx* or *mdx*-L groups ($p < 0.01$, Figure A.21)

Exercised *MDX*

Leucine supplementation had no influence of the MHC proportion of exercised *mdx* mice (Figure A.21).

Centralized Nuclei

BL/10 vs *MDX*

There was a genotype effect independent of treatment with *mdx* mice having a larger portion of centrally nucleated fibers compared to the BL/10 group ($p < 0.001$, Figure A.22). There were no other interaction or treatment effects.

Exercised *MDX*

There were no changes in the percentage of centrally nucleated fibers in exercised *mdx* mice with or without leucine supplementation (Figure A.22).

Average Fiber Area

BL/10 vs *MDX*

Independent of treatment, BL/10 animals had significantly greater average fiber diameter than *mdx* animals ($p < 0.002$), leucine supplemented animals independent of genotype had significantly larger fibers than no leucine supplemented animals ($p < 0.001$) and there was a significant interaction effect between the genotype and treatment groups ($p < 0.0001$). Individually, the *mdx* group had the smallest average fiber diameter compared to all other groups tested ($p < 0.001$), and there were no differences between the BL/10, BL/10-L, and *mdx*-L groups (Figure A.23).

Exercised *MDX*

The *MDX* Run-L group had greater average fiber area compared to the *MDX* Run group ($p < 0.0001$, Figure A.23)

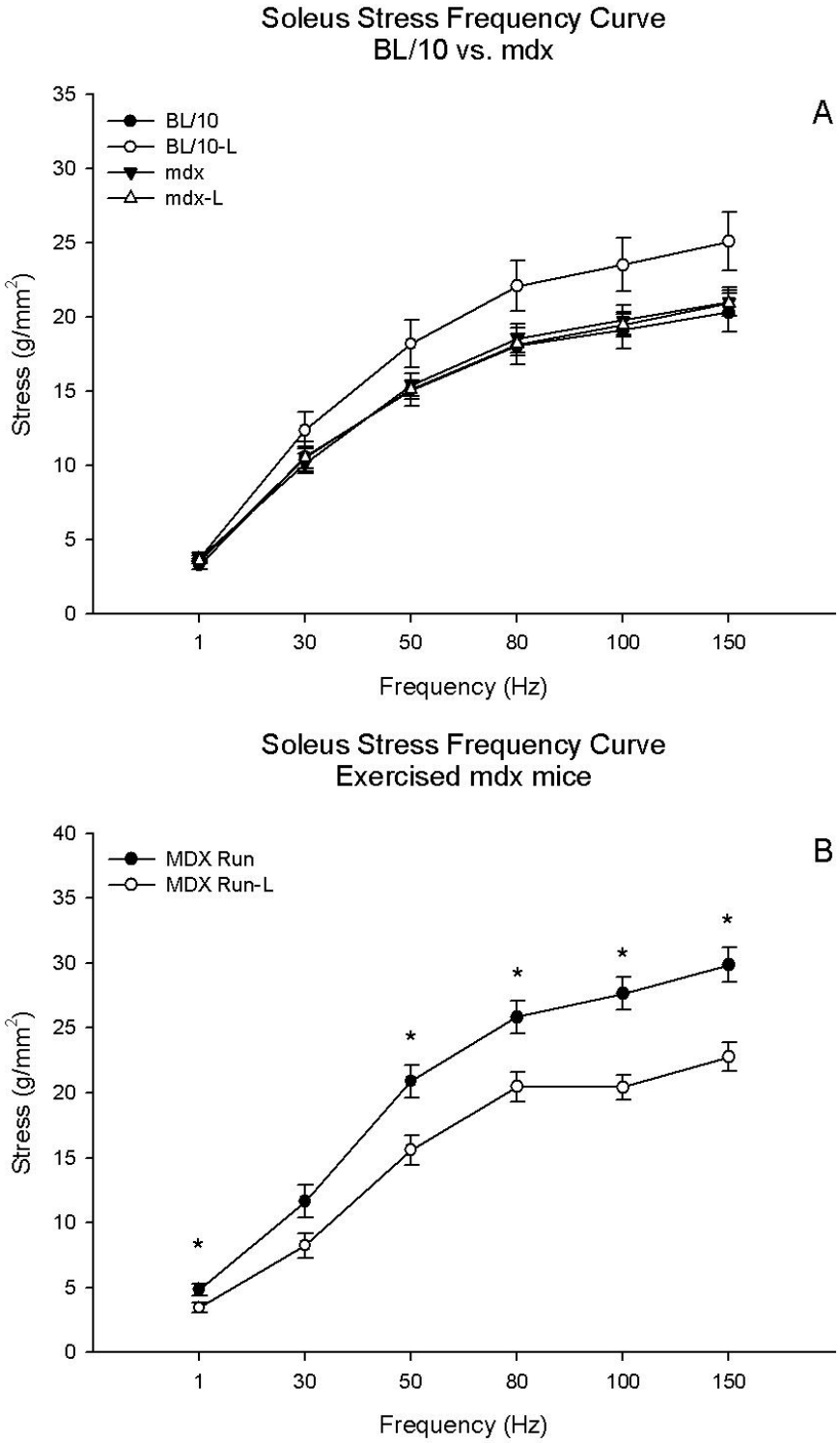

Figure A.20 – Stress frequency profiles from (A) BL/10 and *mdx* and (B) *MDX* mice given free access to a voluntary running wheel. * *MDX* Run animals produced more stress than *MDX* Run-L animals;all values are means ± SEM; $p < 0.05$.

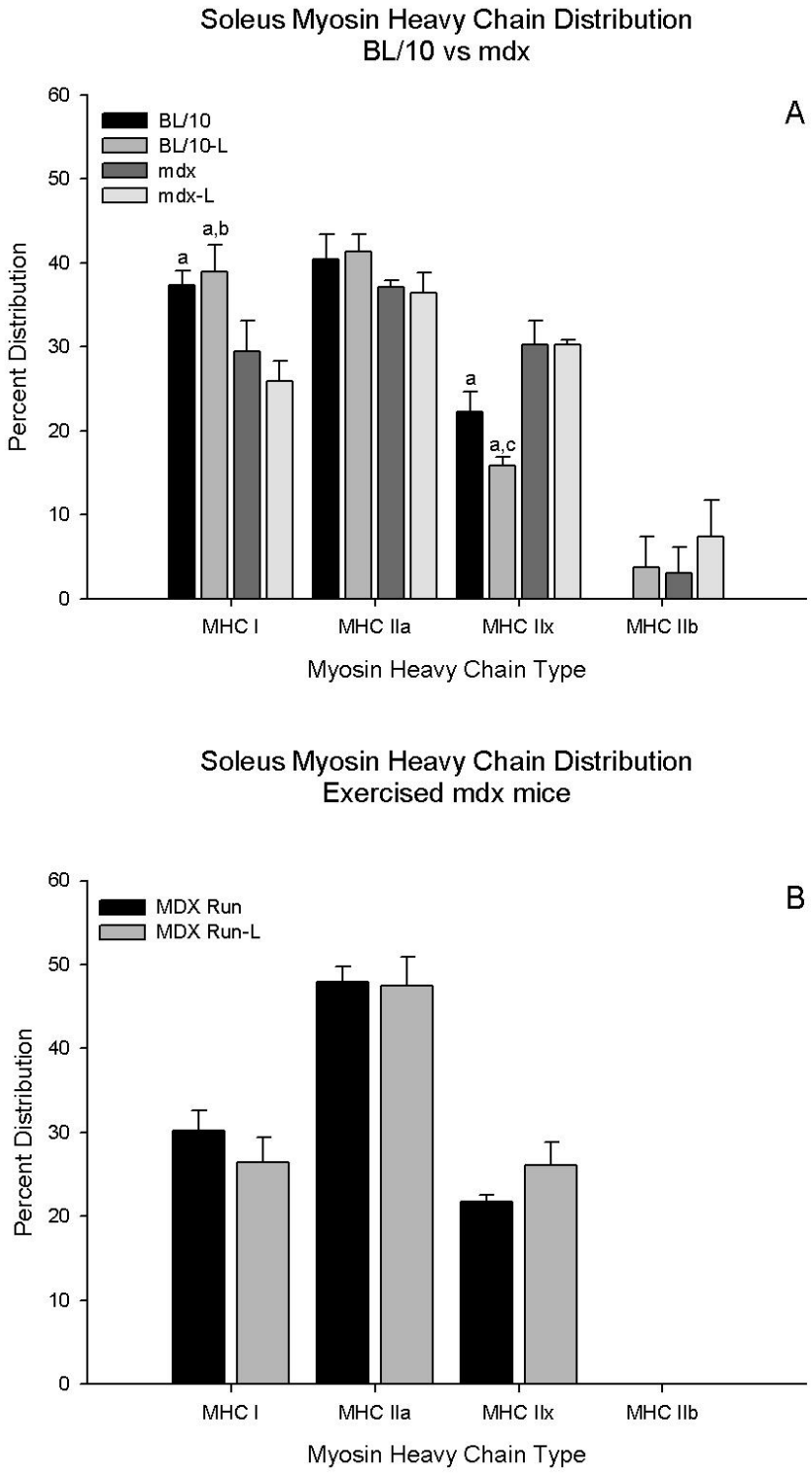

Figure A.21 – Myosin heavy chain distribution for BL/10 vs *mdx* mice (A) and exercised *mdx* mice (B) following 4 weeks of either normal or leucine supplemented drinking water. [a] There is a difference between genotypes, [b] BL/10-L is greater than *mdx*-L, [c] BL/10-L is less than either *mdx* group. All values are means ± SEM, $p < 0.05$.

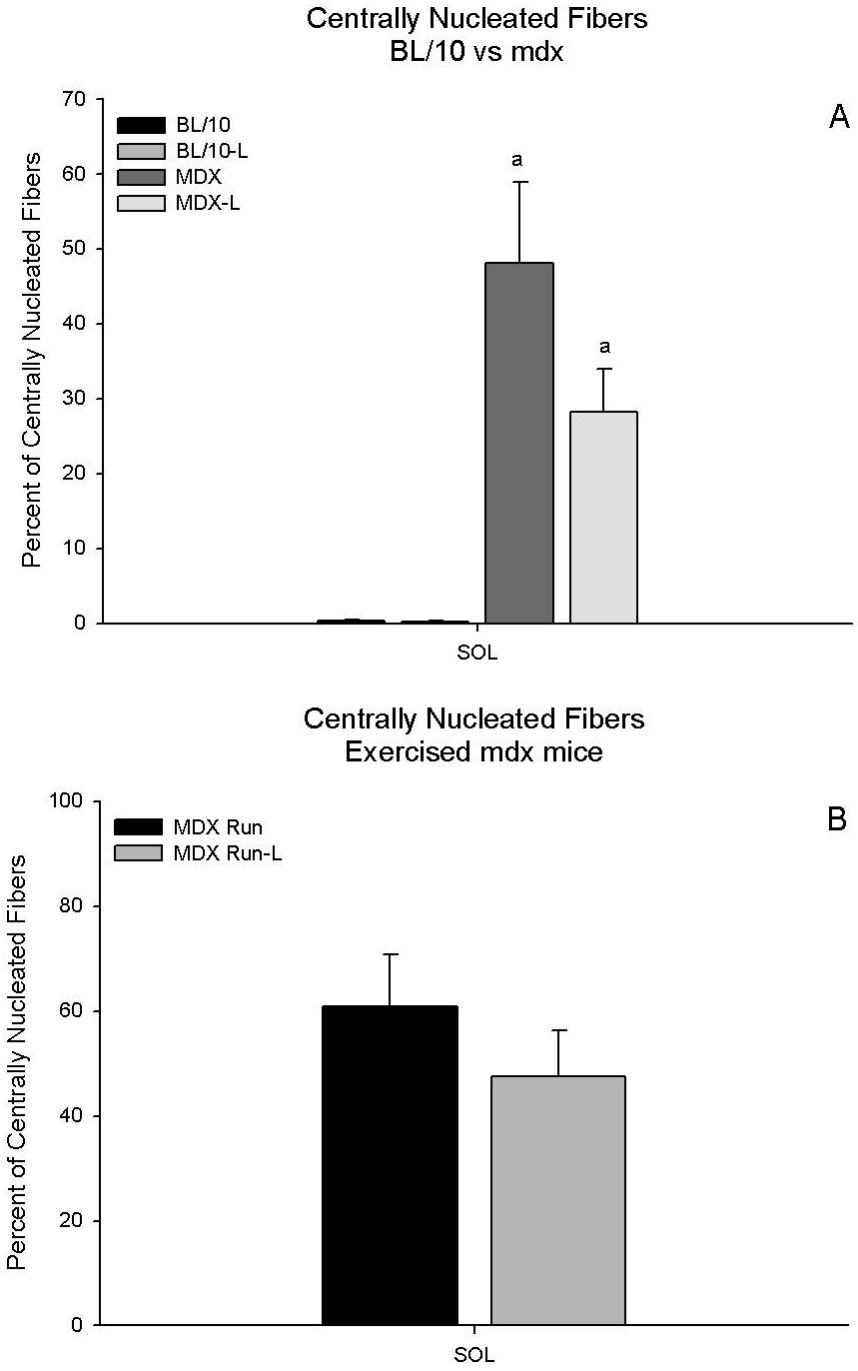

Figure A.22 The percentage of centrally nucleated fibers in the soleus muscle of BL/10 and *mdx* mice (A) and exercised *mdx* mice (B). [a]There was a genotype effect and the *mdx* animals were greater than BL/10 animals. All values are means ± SEM; $p < 0.05$.

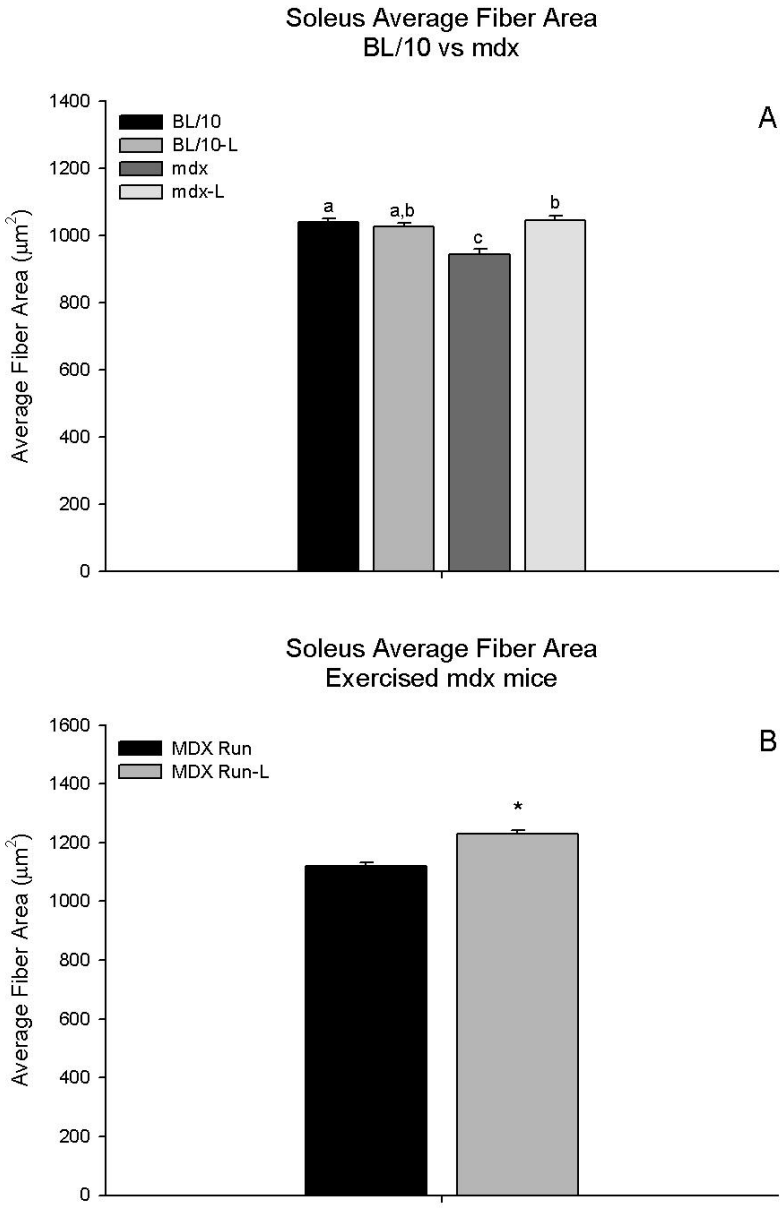

Figure A.23 – Average muscle fiber diameter of BL/10, *mdx* (A) and exercised *mdx* (B) mice following 4 weeks of either normal drinking water or leucine supplemented drinking water. [a] BL/10 is greater than *mdx* independent of treatment, [b] Leucine supplementation group is greater than the normal drinking water group independent of genotype. [c] *mdx* group had the smallest fiber area compared to all other groups. *The *MDX* Run-L group is greater than the *MDX* Run group. All values are means ± SEM, $p < 0.05$.

Table A.7 – Morphological and contractile properties of soleus muscles from BL/10 and *mdx* mice. Genotype and Treatment data are independent of each other. a Difference between BL/10 and *mdx*. b *mdx*-L is greater than either BL/10 group. All values are mean ± SEM; $p < 0.05$.

	Muscle Length (mm)	**Muscle Mass (mg)**	**CSA (mm^2)**	**Twitch Stress (g/mm^2)**	**Time to Peak Tension (ms)**	**Half-Relaxation Change (ms)**	**Passive EPE (MPa)**	**Passive ESE (MPa)**	**Active Stretch (MPa)**
Genotype									
BL/10	10.06±0.79	7.74±1.25	0.74±0.16	3.65±0.89	22.00±4.82	21.75±6.34	0.90±0.36	1.47±0.51	2.02±1.05
MDX	10.18±0.72	10.14±1.92a	0.95±0.20a	3.75±0.78	18.56±3.10[a]	24.74±3.86[a]	1.12±0.45	1.28±0.47	1.43±0.45
Treatment									
Control	10.01±0.72	8.84±1.79	0.84±0.17	3.66±0.76	19.50±4.26	24.46±4.39	1.08±0.42	1.31±0.42	1.48±0.45
Leucine	10.26±0.73	9.86±2.21	0.92±0.23	3.77±0.86	19.81±3.87	23.15±5.39	1.01±0.45	1.39±0.55	1.74±0.90
All Groups									
BL/10	9.88±0.88	7.65±1.46	0.75±0.18	3.57±1.41	22.29±4.80[e]	23.31±6.39	0.97±0.24	1.33±0.18	1.48±0.36
BL/10-L	10.25±0.71	7.83±1.36	0.73±0.17	3.92±1.00	21.70±5.20[f]	19.96±6.34	0.83±0.49	1.62±0.71	2.74±1.32
mdx	10.08±0.65	9.44±1.75	0.89±0.17	3.80±0.75	18.15±3.40	25.06±3.15	1.14±0.50	1.30±0.53	1.48±0.52
***mdx*-L**	10.27±0.77	10.77±1.91[b]	1.00±0.21[b]	3.71±0.82	18.87±3.08	24.39±4.41	1.11±0.43	1.27±0.45	1.41±0.43

Table A.8 - Morphological and contractile properties of soleus muscles from exercised *mdx* mice. a Difference between MDX Run and MDX Run-L. All values are mean ± SEM, $p < 0.05$.

	Muscle Length (mm)	**Muscle Mass (mg)**	**CSA (mm^2)**	**Twitch Stress (g/mm^2)**	**Time to Peak Tension (ms)**	**Half-Relaxation Time (ms)**	**Passive EPE (MPa)**	**Passive ESE (MPa)**	**Active Stretch (MPa)**
***MDX*-C Run**	9.93±0.49	10.55±2.15[c]	1.01±0.20[c]	4.86±1.31	18.25±3.45	21.88±1.96	0.86±0.11	1.14±0.12	1.37±0.12
***MDX*-L Run**	10.57±0.33	17.5±1.50[a]	1.57±0.15[a]	3.47±0.83	19.16±2.99	19.50±4.04	1.14±0.26	1.39±0.26	1.16±0.13

Appendix B – Methods

EDL Function tests

At age 7 weeks, after the 4 week testing period, mice were deeply anesthetized (2 mg xylazine-20 mg ketamine per 100 g of body mass, i.p.), and the fast-twitch extensor digitorum longus (EDL) muscles were excised. Additional muscles were also excised and assayed as noted below. EDL muscles were incubated at 30ºC in an oxygenated (95% O2-5% CO2) physiological salt solution (PSS) as previously described[58,133]. Non-absorbable braided silk suture (4-0) was tied to the distal and proximal tendons at the myotendinous junctions. EDL muscles were then fixed between a clamp and arm of a dual-mode servomotor system (300B, Aurora Scientific) at a resting tension (L_0) of 1.0 g. EDL muscles were maintained at L_0 by a stepper motor[133]. The servomotor arm and stepper motor were controlled by Dynamic Muscle Control software (DMC Version 4.1.6, Aurora Scientific) to obtain the position and force output data.

The stimulated muscle protocol consisted of 5 steps: (1) a pre-twitch and tetanus; (2) a single passive stress relaxation; (3) a single active stretch; (4) a stress frequency protocol; and (5) a post tetanus. The first and fifth steps were performed to determine the effects of steps 2 - 4 on contractile capability. In step 1, the stimulated muscle was subjected to three isometric twitches and tetani (150 Hz) spaced 1 minute apart. In step 2, the muscle was stretched instantaneously to 1.05 L0, held for 7.0 s, and then returned to 1.00 L0 to determine passive parallel elastic stiffness[133]. After 5 minutes at L_0, the muscle was stimulated at 80 Hz for 700 ms (step 3). During the final 200 ms, the muscle was stretched at 0.5 L_0/s to a total strain of 0.1 L_0 (i.e., an eccentric contraction) to determine active stiffness. In step 4, the muscle was subjected to stimulation at a frequency of 1, 30, 50, 80, 100, and 150 Hz, with each stimulation lasting 1 second and separated by 1 minute. After the stress frequency protocol, the muscle was quiescent

for 5 minutes at L_0, and was then subjected to a final tetanus (150 Hz, step 5). At the conclusion of the protocol, each muscle was measured to the nearest 0.1 mm and weighed to the nearest 0.1 mg using an A-200D electronic analytical balance (Denver Instruments, Denver, Colorado) and snap frozen in liquid nitrogen.

EDL contractile data analysis and morphological properties

Force and position output profiles were analyzed using Dynamic Muscle Analysis software (DMA Version 3.2, Aurora Scientific). For each twitch, the peak force, the time to peak stress (TPS), and half-relaxation time (HRT) were determined. Muscle cross-sectional area (CSA) was determined as previously described[58]. Twitch and tetanic forces were normalized to muscle CSA to obtain twitch and tetanic stress. Passive parallel elastic stiffness (g/mm2) for step 2, and active stiffness for step 3 were determined as previously described[58,133].

EDL myosin heavy chain distribution analysis

Myosin heavy chain was determined as previously described by Talmadge et al.[119]. In detail, EDL muscle from *mdx* and BL/10 mice were first minced in 9 volumes of ice-cold homogenization buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 6.8). Following mincing, muscles were further homogenized by glass tissue grinders, then the myofibrils were washed as described in Thomason et al.[121]. The washed myofibrils were then boiled for 2 minutes in sample buffer and a Bradford assay was used to determine total protein concentration. The final concentration of all samples was diluted to 0.125 mg/ml. 1 µg of samples was run in each lane on a 4% stacking, 8% separating SDS-PAGE gels were run at 70 V, for 24 hours at 4^{o}C. Gels were stained with Coomasie Blue stain and then scanned and densitometry was performed.

EDL muscle sectioning

EDL muscles were snap frozen in liquid nitrogen and stored at -80^{o}C until sectioning was performed. When sectioning was performed, EDL muscles were mounted with the long axis of the muscle perpendicular to the surface of the tissue sectioning chuck. Muscles were secured in the position by placing a small amount of optimum cutting temperature compound (OCT, Andwin Scientific) on the surface of the tissue sectioning chuck, then approximately 1/3rd of the EDL muscle was inserted into the OCT and immediately frozen by spraying with Cytocool freezing spray (Richard Allen Scientific). EDL muscles were then sectioned until the area of maximal cross-sectional area was reached; from this point forward 8 µm sections were cut and placed on super-frost plus slides (Richard Allen Scientific). Sections were allowed to air dry for 1 hour, then were either immediately stained or stored at -80^{o}C until used.

EDL Hematoxlyn and Eosin staining.

Previously cut EDL sections were briefly brought to room temperature and then were fixed by placing slides in a 4% parafomaldehyde/phosphate buffered saline (PBS) solution for 5 minutes. Following fixing, sections were washed 2 times, for 5 minutes each in fresh PBS. Slides were then placed in hematoxlyn for 5 minutes, briefly rinsed in tap water, then placed in eosin for 5 minutes and dehydrated in 95% ethanol for approximately 2 minutes. Following dehydration, stained sections were mounted using VECTASHEILD HardSet mounting medium (Vector Labs) and coverslipped.

EDL Membrane Staining

Each EDL was stained using rhodamine labeled wheat-germ agglutinin (WGA, Vector Labs). Sections were fixed for 5 minutes in a 4% formaldehyde/PBS solution, then blocked for 1 hour using a 5% BSA/PBS solution, then stained using 1:1000 WGA:PBS dillution. Sections were then washed 2 x 5 minutes in fresh PBS. Sections were briefly air dried before mounting coverslips (Vectamount) and imaging. Images were taken using a Nikon Eclipse TS100 inverted

microscope with an attached mercury burner bulb. Fluorescence was captured using a Texas Red filter cube with excitation wavelength of 540-580 nm and emission wavelengths of 600-660 nm. All images were digitally captured using the bundled NIS Elements BR 3.0 software (Nikon, USA). All image processing to determine individual fiber areas was done using NIH Image J software with the FIJI image analysis pack installed.

Immunoblots

Gastrocnemius muscles were first homogenized in 7 times their volume with a homogenization buffer (25 mM HEPES, 0.4% CHAPS, 1 mM DTT, 1X inhibitor cocktail, 50 mM thiamine pyrophosphate, 50 mM β-glycerophosphate, 94 nM microcystin, 0.71 mM sodium ortho-vanadate, 100 mM potassium fluoride, 1mM 2-chloroisocaproate) using a glass on glass homogenizer. Protein concentrations were then determined using a BCA assay kit (Pierce).

Samples were then mixed in a 1:1 ratio with Laemli sample buffer containing 5% β-mercaptoethanol, and 30 µg of sample was loaded into wells on a 10% precast SDS-PAGE gel (Bio-Rad). Gels were run using a Criterion Cell apparatus (Bio-Rad) filled with running buffer (3g/L Tris, 14.4g/L glycine, and 1.0g/L SDS) placed on ice and run at a constant 100 V for 1 hour.

Before transfer in a Bio-Rad Trans-blot cell, a piece of nitrocellulose membrane, two sheets of filter paper (cut to the size of the gel), and the gel were soaked in transfer buffer (3g/L Tris, 14g/LmM glycine, and 300ml/L methanol) for 15 min before transfer. The transfer sandwich was assembled as follows. Starting from the anode side: sponge, filter paper, nitrocellulose membrane, gel, filter paper, sponge, and ending on the cathode side. The transfer sandwich was positioned in the transfer chamber so that the negatively charged proteins would migrate from the gel toward the anode onto the nitrocellulose membrane. An ice pack was inserted into the transfer cell and the transfer/blotting buffer was added. The transfer was run at

a constant 100V for approximately 90 minutes. Following transfer, the nitrocellulose was washed in Tris buffered saline (TBS, pH 7.5) for 15 min at room temperature. The membrane was incubated in blocking buffer (5% non-fat dry milk in TBS + 0.05%Tween 20, TBST) for an hour with shaking. The membrane was washed 3 times, each for 15 min in TBST. The membrane was incubated with primary antibody in blocking buffer overnight at 4^{o}C, and then was washed in TBST 3 times, each for 15 min. The membrane was incubated with secondary antibody in blocking buffer for 1.5 hours. After incubation, the membrane was washed in TBST 3 times, each for 15 min.

Anitbodies

Primary Antibody	Dillution	Supplier	Product Number	Host Species
Calpain Antibody	1:1000	SCBT	Calpain (H-240), sc-30064	Rabbit
Calpastatin Antibody	1:1000	SCBT	Calpastatin (H-300), sc-20779	Rabbit
mTOR Antibody	1:1000	Abcam	Monoclonal [Y391] to mTOR, ab77741	Rabbit
mTOR Phosphorlated (Ser 2448) Antibody	1:1000	Abcam	Monoclonal to mTOR (phospho S2448), ab51044	Rabbit
Secondary Antibody				
Goat anti-Rabbit HRP	1:2500	Jackson Immunoresearch	111-05-003	Goat

FDB dissociation

Flexor digitorum brevis (FDB) muscles will be removed and incubated for 1-2 hours at 37°C in a Krebs solution (in mM, 135.5 NaCl, 1.2 MgCl2, 5.9 KCl, 11.5 glucose, 11.5 HEPES, 1.5 CaCl2, pH 7.3) containing 0.2% collagenase type IV. Muscles are then washed twice in Krebs buffer, suspended in Dulbecco Minimum Essential Medium (DMEM) with Ham F12 complement (DMEM/HAM F12) supplemented with 2% fetal bovine serum and mechanically dissociated by repeated passages through fire-polished Pasteur pipettes of progressively decreasing diameter. Dissociated fibers will be plated onto tissue culture dishes coated with extracellular matrix basement membrane (ECM, Harbour Bio-products, Norwood, MA, USA) and allowed to adhere to the bottom of the dish for 2 h. For Calpain and Ca2+ measurements,

cells were transferred to circular glass coverslips. Culture dishes will be kept in an incubator, with 5% CO_2 at 30°C.

In-situ Calpain activity

Measurement of in-situ calpain activity will be done as previously outlined in Gailly et al.[55]. In detail, the synthetic substrate 7-amino-4-chloromethyl-coumarin-t-butoxycarbonyl-L-leucyl-L-methionine amide (Boc-Leu-Met-CMAC) will measure real-time activity of both μ- and m-calpain activity. In its native form Boc-Leu-Met-CMAC is permeable to the cell where it is transferred to Boc-Leu-Met-MAC-SG by the glutathione S-transferase, transforming the sustrate to an impermeable form. The MAC-SG (7-amino-4-methylcoumarin glutathione conjugate, excitation and emission wavelengths, 380 nm and 480 nm respectively) will be cleaved from the Boc-Leu-Met moiety, and over time the fluorescence increase of MAC-SG inside the cell will represent the increase in calpain activity.

The substrate will be added to the fiber chamber to a final concentration of 10 μm in Krebs solution. Fluorescence will be recorded within 30 seconds of the substrates addition to the cell chamber. Detection will be made with a photon counter that will be restricted by a rectangular box that will surround the fiber parallel to fiber's long axis. To measure transient changes in calpain activity recording will be made at 100 Hz, for long recordings measurements will be made 6 s for every 1 minute to prevent photobleaching of the sample.

Appendix C – Raw Data

Weekly Body Mass – All Animals

Condition	Cage #	Mouse #	Start Weight	Week 1	Week 2	Week 3	Week 4
mdx	#1106	Mouse 1	12.8	21.7	28.1	28.4	28.8
mdx	#1124	Mouse 1	10.2	12.5	17.1	20.1	22.3
mdx	#1124	Mouse 2	11.5	13	19.3	24.5	26.3
mdx	#1124	Mouse 3	11.2	15.2	19.2	23.3	24.6
mdx	#1293	Mouse 1	13.7				
mdx	#1293	Mouse 2	13.6	18.8	23.7	27.1	X
mdx	#1502	Mouse 1	10.3	22.1	22.8	23.8	23.800
mdx	#1506	Mouse 1	12.2	22.2	25	26.5	28.4
mdx	#1515	Mouse 1	8.4	15.6	22.8	25	25.8
mdx	#1515	Mouse 2	7.8	6.3	13.2	20.3	23.2
mdx	#1515	Mouse 3	8.2	14	22.4	23.1	24.7
mdx-L	#1109	Mouse 2	13.5	20.8	26	27.2	27.3
mdx-L	#1109	Mouse 1	13.1	21	26.4	28.7	29
mdx-L	#1125	Mouse 1	10	13.3	20.1	23.2	25.8
mdx-L	#1125	Mouse 2	10.3	17.1	21.9	23.6	25.7
mdx-L	#1291	Mouse 1	13	16.5	24.2	26.7	27.7
mdx-L	#1291	Mouse 2	10.2	17.7	24.3	25.7	26.1
mdx-L	#1505	Mouse 1	10.9	19.4	23.3	23.9	26.2
mdx-L	#1505	Mouse 2	12.6	21.2	24.1	25.8	27
mdx-L	#1516	Mouse 1	8.4	15.2	22.8	24	26
mdx-L	#1516	Mouse 2	8.2	13.3	20.1	20.8	22.8
mdx-L	#1516	Mouse 3	9	15.5	23.7	25.6	27.7
MDX Run	#9	#9 - L	9.8	17.8	23.2	24.9	24.7
MDX Run	#13	#13 - L	10.3	15.7	22.3	24.6	25.1
MDX Run	#5	#5 - L	10.7	15.3	20.9	22.6	22.5
MDX Run	#9	#9 - L2	9.4	17.4	24.2	25.2	25.7
MDX Run	#13	#13 - L2	9.4	16.8	23	23.5	23
MDX Run	#16	#16 - L	9.8	19.6	24.8	26.4	25.3
MDX Run-L	#3	#3 + L	14	22.8	26.2	28	28.2
MDX Run-L	#12	#12 + L	13	17.3	24.4	26	27.5
MDX Run-L	#15	#15 + L	17	27.5	30.3	32.5	33.5
MDX Run-L	#16	#16 + L	9.2	16.3	22.8	25	26.2
MDX Run-L	#2	#2 + L	11.1	16.7	22.9	24.5	25.1
MDX Run-L	#5	#5 + L	10.5	19	23.5	24.5	24.7
BL/10	#1696	Mouse 1	10	13.5	19.3	21.4	22.4
BL/10	#1696	Mouse 2	10.8	15.4	22.6	24.2	23.6
BL/10	#1696	Mouse 3	11.2	14.9	20.1	21.7	23.1
BL/10	#1697	Mouse 1	14.5	21	22.8	24.8	26.6
BL/10-L	#1695	Mouse 1	10.7	14.5	21.7	23.8	25.5
BL/10-L	#1695	Mouse 2	9.2	13.3	20.1	21.5	25.3
BL/10-L	#1695	Mouse 3	9.4	12.6	18.7	20.1	22.9
BL/10-L	#1698	Mouse 1	14	19.7	23.3	25.2	27.5

This chart details the weekly body masses of animals used in both paper 1 and paper 2 of this dissertation.

Running Distance

DAY	MDX Run - L	MDX Run - L	MDX Run - L	MDX Run - L	MDX Run - L	MDX Run	MDX Run	MDX Run	MDX Run	MDX Run
	#3	#12	15	2	5	9	16	13	9	13
1	974.145	528.793	382.018	575.037	277.465	493.607	318.181	347.334	115.611	696.68
2	608.212	888.694	348.842	90.478	14.577	226.195	52.276	175.93	110.081	461.437
3	423.236	929.911	389.558	195.533	37.197	72.382	173.416	304.106	227.2	153.812
4	193.019	668.531	611.731	517.734	975.15	355.88	171.405	456.41	337.282	844.963
5	307.625	925.388	628.318	1018.379	1001.289	199.554	280.984	680.595	496.623	610.726
6	182.966	1407.433	447.866	476.014	929.408	545.38	383.526	1309.919	465.458	491.596
7	1254.627	2642.456	1086.237	1296.347	1276.744	486.57	778.612	1339.072	695.674	291.037
8	1024.41	6173.104	1486.35	3341.649	1772.36	330.747	34.181	1618.549	971.13	410.669
9	2522.825	7631.306	2122.712	52.276	1393.36	565.989	417.706	1971.412	592.63	613.742
10	4091.61	2352.424	2820.898	143.759	2247.369	1113.381	233.232	2409.727	729.854	1214.916
11	4394.711	1619.554	3870.945	409.162	2540.418	819.83	260.375	2645.472	1361.692	1764.319
12	5737.302	940.97	5059.724	677.578	2350.414	1073.671	269.926	2863.122	1442.117	2086.017
13	4956.68	3314.003	4885.805	1708.524	3528.134	1684.396	1143.037	2990.796	1880.432	2990.796
14	6734.569	4129.812	5954.951	1515.504	1958.846	2213.189	1291.32	3093.841	2140.304	3800.574
15	8765.797	4314.789	5761.933	2155.384	3934.279	2836.481	1692.439	3965.946	2040.778	3122.994
16	7969.593	5702.117	4968.742	3200.906	5654.867	3016.935	2129.246	5391.978	2246.365	4329.366
17	7482.519	5852.913	5960.481	2789.232	6613.429	4147.405	2637.429	5742.832	2394.647	4575.667
18	8383.278	8331.503	8060.07	3842.293	8331.504	4651.065	3740.758	5420.629	2488.142	5235.15
19	8939.213	9785.182	7995.228	4216.771	8005.784	5560.367	3708.084	5729.763	3005.373	5881.564
20	8439.072	9543.907	8674.314	4074.018	7881.125	5659.391	4274.074	5862.463	2996.828	7731.334
21	8177.691	8111.843	6231.412	6876.82	9483.588	6994.945	4082.563	6289.72	2577.614	7099.999
22	6484.247	6758.195	5705.635	7689.614	12453.273	7260.849	4058.435	6825.55	3024.474	8071.129
23	7742.895	7269.896	6096.198	8069.118	11369.047	5935.348	3922.215	6020.799	2933.494	8831.645
24	8160.099	7415.164	6850.18	9508.219	12556.318	6938.647	3176.779	5819.738	2985.769	7577.522
25	7417.677	7617.734	5758.916	8971.383	11942.576	6583.773	3523.61	5870.505	2675.632	9180.99
26	7041.189	8851.752	6059.001	9731.9	11826.965	6134.399	3841.288	8378.251	2306.18	6918.038
27	6369.14	9326.257	6019.292	9687.164	12598.038	6505.359	4123.278	7169.366	2487.639	8518.994
28	7370.93	7998.746	7038.675	8797.464	11362.512	6609.408	3846.817	8349.599	1914.11	7833.876
Total	142149.277	141032.377	121276.032	101628.26	154316.036	89015.143	54565.192	109043.424	47643.133	111339.552

All distances are recorded in meters. This is the running distance data from paper 2.

EDL Stress

Genotype	Muscle Length (mm)	Muscle Mass (mg)	FF Twitch (g/mm)	30 Hz (g/mm)	50 Hz (g/mm)	80 Hz (g/mm)	100 Hz (g/mm)	150 Hz (g/mm)
MDX Control	13.000	11.100	4.697	6.811	11.075	16.902	19.580	22.899
MDX Control	13.000	12.500	5.787	7.139	12.147	19.154	22.064	25.145
MDX Control	13.500	10.200	6.018	8.782	15.117	23.173	26.723	31.998
MDX Control	13.500	11.000	5.684	7.528	13.105	23.039	27.209	31.771
MDX Control	12.500	9.000	4.601	7.011	12.391	17.741	20.611	25.159
MDX Control	12.500	9.000	3.557	4.996	8.245	12.194	13.938	15.528
MDX Control	13.500	11.100	5.127	6.631	12.019	18.592	21.261	25.004
MDX Control	13.500	9.000	7.088	12.259	19.562	26.450	28.286	31.921
MDX Control	13.100	9.467	1.899	6.582	6.279	9.468	10.784	15.306
MDX Control	14.000	9.600	6.064	9.402	15.727	20.200	21.469	25.929
MDX Control	14.000	9.800	4.711	6.354	13.010	19.679	22.429	26.730
MDX Control	12.800	9.700	6.320	9.669	15.059	18.859	19.721	22.217
MDX Control	13.000	10.200	5.527	6.993	13.322	18.066	20.724	24.355
MDX Control	12.800	10.800	5.948	9.652	16.534	22.671	25.301	29.077
MDX Control	12.000	9.000	4.437	5.790	10.081	15.288	17.768	20.422
	13.113	10.098	5.164	7.706	12.912	18.765	21.191	24.897
	0.560	1.029	1.268	1.886	3.314	4.325	4.739	5.212
MDX Leucine	12.500	9.900	7.085	11.224	18.873	26.367	29.322	33.580
MDX Leucine	12.500	10.200	4.150	6.251	12.656	19.337	21.466	24.485
MDX Leucine	13.000	11.100	5.202	8.051	14.437	22.164	26.130	30.428
MDX Leucine	12.500	11.200	5.810	7.590	13.972	23.311	28.244	32.163
MDX Leucine	12.500	9.800	5.605	8.057	14.821	23.122	26.623	31.980
MDX Leucine	12.500	9.300	4.427	5.988	12.660	21.081	24.619	30.230
MDX Leucine	13.000	8.800	5.370	7.815	13.100	17.890	19.525	22.544
MDX Leucine	13.100	9.000	6.210	8.110	15.917	24.735	27.104	30.876
MDX Leucine	13.800	8.300	5.296	8.005	15.014	20.798	23.168	29.069
MDX Leucine	14.000	9.700	6.043	7.541	15.021	23.138	26.309	31.546
MDX Leucine	12.900	10.300	5.294	8.668	14.669	19.793	21.616	25.099
MDX Leucine	13.400	8.800	4.848	6.837	12.484	18.989	21.363	24.570
MDX Leucine	13.500	7.100	7.890	12.334	21.321	28.218	31.855	37.563
MDX Leucine	14.300	10.000	4.260	7.120	11.478	19.206	21.009	25.311
MDX Leucine	13.900	10.200	4.293	6.311	11.122	18.155	21.214	25.521
	13.160	9.580	5.452	7.993	14.503	21.754	24.638	28.998
	0.623	1.069	1.058	1.735	2.699	3.064	3.637	4.220
C57BL/10 Control	12.000	10.000	7.132	10.894	17.307	29.574	34.900	41.080
C57BL/10 Control	13.000	9.800	8.386	10.174	15.326	32.656	39.355	46.022
C57BL/10 Control	13.500	10.100	9.423	17.760	31.352	42.369	46.200	49.785
C57BL/10 Control	13.500	10.300	8.676	12.250	26.253	39.159	42.888	45.763
C57BL/10 Control	12.500	9.400	7.382	10.511	15.685	30.462	37.199	44.912
C57BL/10 Control	12.500	8.700	8.038	10.402	15.469	27.825	37.179	45.777
C57BL/10 Control	12.500	9.900	6.526	8.918	14.611	25.259	30.018	35.244
C57BL/10 Control	12.000	10.000	6.868	8.817	11.476	26.444	33.984	39.260
	12.688	9.775	7.484	10.754	17.719	29.981	35.469	43.480
	0.594	0.506	1.337	3.013	6.664	7.729	8.265	9.143
C57BL/10 Leucine	12.500	9.400	7.378	9.502	14.815	26.260	31.314	37.301
C57BL/10 Leucine	13.000	10.100	7.376	8.904	13.397	32.742	41.229	48.894
C57BL/10 Leucine	13.000	8.300	10.558	17.919	29.461	38.581	42.326	46.761
C57BL/10 Leucine	13.000	9.000	8.704	11.834	25.762	38.133	42.179	44.924
C57BL/10 Leucine	12.500	9.000	5.421	7.932	11.926	22.918	27.396	30.894
C57BL/10 Leucine	12.500	9.000	6.995	7.814	9.500	23.399	31.382	38.560
C57BL/10 Leucine	13.000	10.600	7.880	10.488	16.100	29.505	34.828	40.013
C57BL/10 Leucine	13.500	9.800	7.741	9.549	13.104	29.306	37.457	44.770
	12.875	9.400	7.756	10.493	16.758	30.105	36.014	41.515
	0.354	0.735	1.468	3.275	7.044	6.043	5.686	5.925

These are the raw and normalized (g/mm^2) values for EDL stress in paper 1.

Genotype	Muscle Length (mm)	Muscle Mass (mg)	FF Twitch (g/mm)	30 Hz (g/mm)	50 Hz (g/mm)	80 Hz (g/mm)	100 Hz (g/mm)	150 Hz (g/mm)
MDX Runner	13.500	8.700	5.997	10.054	17.817	24.393	26.446	30.424
MDX Runner	12.500	8.300	5.662	9.479	16.318	22.700	25.130	29.356
MDX Runner	12.500	10.000	5.736	7.403	13.546	22.787	25.855	29.992
MDX Runner	12.500	9.700	5.477	7.791	14.608	22.719	25.702	30.284
MDX Runner	12.500	9.700	3.396	5.005	9.371	14.800	17.422	20.539
MDX Runner	12.500	9.400	4.227	6.721	12.364	18.063	20.650	24.432
MDX Runner	12.500	10.300	4.789	6.076	10.116	16.985	19.328	22.010
	12.750	9.350	5.163	7.650	13.964	21.214	24.129	26.720
	0.463	0.709	0.944	1.715	3.218	4.186	4.812	4.276
MDX Leucine Runner	13.000	8.500	6.915	11.538	19.507		28.529	33.903
MDX Leucine Runner	12.500	9.300	7.626	9.835	18.465	27.673	31.215	28.586
MDX Leucine Runner	13.500	10.700	7.141	11.438	20.127	25.852	29.754	34.015
MDX Leucine Runner	13.000	9.500						
MDX Leucine Runner	13.500	11.700	6.313	9.673	17.344	24.713	27.399	30.482
MDX Leucine Runner	13.500	11.500	6.243	8.474	17.840	25.307	27.869	30.555
	13.167	10.200	6.848	10.192	18.657	25.886	28.953	31.508
	0.408	1.295	0.580	1.295	1.153	1.279	1.543	2.373

These are the raw and normalized (g/mm^2) values for EDL stress in paper 2.

Soleus Stress

Genotype	Muscle Length (mm)	Muscle Mass (mg)	FF Twitch (g/mm)	30 Hz (g/mm)	50 Hz (g/mm)	80 Hz (g/mm)	100 Hz (g/mm)	150 Hz (g/mm)
MDX Control	9.500	12.900	3.970	8.819	13.438	15.932	16.909	17.995
MDX Control	10.500	11.300	4.302	7.888	15.719	20.031	21.454	23.361
MDX Control	10.000	11.300	2.977	7.395	12.205	14.812	15.933	16.989
MDX Control	9.500	10.200	3.317	7.961	13.806	16.891	18.111	19.629
MDX Control	9.000	6.500	3.589	11.112	15.231	17.405	18.426	19.716
MDX Control	9.500	7.200	4.383	13.099	19.063	22.495	24.158	25.552
MDX Control	9.000	8.300	3.442	8.961	14.851	18.377	19.573	21.321
MDX Control	10.000	8.200	3.790	10.216	17.770	23.312	24.847	26.291
MDX Control	9.800	9.600	3.196	9.407	13.045	15.252	16.299	17.357
MDX Control	10.800	9.200	3.675	7.801	11.978	14.641	15.638	16.946
MDX Control	10.500	9.800	3.761	12.037	17.382	20.606	21.240	22.850
MDX Control	10.800	10.600	3.659	8.375	15.410	18.768	20.169	22.049
MDX Control	10.300	6.700	5.595	17.971	23.723	27.800	29.465	29.422
MDX Control	11.000	9.200	3.943	12.315	17.776	21.169	22.539	22.731
MDX Control	10.900	9.800	3.088	9.361	12.543	14.422	15.469	16.510
MDX Control	10.100	10.300	3.136	9.352	12.828	15.215	16.423	17.806
	10.075	9.444	3.724	10.217	15.555	18.746	19.983	21.033
	0.653	1.750	0.664	2.782	3.193	3.863	4.047	3.843
MDX Leucine	8.500	12.800	2.475	5.344	9.170	11.585	12.607	13.684
MDX Leucine	9.500	12.300	2.400	6.771	11.586	15.446	16.422	18.528
MDX Leucine	9.500	13.700	3.108	8.388	12.507	14.482	15.292	16.416
MDX Leucine	9.500	13.100	2.773	7.081	13.144	16.601	17.900	16.705
MDX Leucine	10.500	9.000	5.227	17.729	17.837	20.904	22.308	24.205
MDX Leucine	10.000	8.800	3.816	16.468	16.554	20.095	21.907	23.414
MDX Leucine	10.000	9.000	3.548	10.336	13.996	16.159	17.458	18.629
MDX Leucine	10.000	9.000	4.128	12.542	19.600	23.637	25.255	27.008
MDX Leucine	11.800	11.400	4.429	12.866	18.975	22.513	23.779	25.918
MDX Leucine	10.400	10.200	3.668	11.312	17.525	21.081	22.489	24.193
MDX Leucine	11.300	12.100	3.327	9.165	13.576	16.054	16.857	18.071
MDX Leucine	10.800	13.900	2.890	7.293	12.477	15.284	16.431	17.805
MDX Leucine	10.500	10.400	3.673	10.878	15.296	18.137	19.446	21.163
MDX Leucine	10.400	10.400	3.469	10.123	15.477	18.512	19.829	21.499
MDX Leucine	10.000	7.200	4.885	13.375	18.192	21.380	23.005	24.778
MDX Leucine	10.300	9.600	3.703	10.245	15.650	18.737	20.088	21.837
MDX Leucine	11.000	11.300	3.421	10.378	14.641	17.119	18.458	20.280
MDX Leucine	10.900	9.600	3.084	9.335	15.837	19.270	20.822	22.708
	10.431	10.544	3.736	11.275	15.918	19.032	20.402	21.539
	0.623	1.911	0.689	2.965	2.167	2.530	2.680	3.311
C57BL/10 Control	8.500	7.100	1.868	6.241	9.470068117	11.478	12.351	13.365
C57BL/10 Control	9.500	10.000	2.467	7.127	11.77850198	15.158	15.874	17.192
C57BL/10 Control	11.500	6.100	3.890	13.740	17.11233142	19.418	20.348	21.085
C57BL/10 Control	10.500	7.650	3.753	14.396	18.9263343	22.108	23.082	24.124
C57BL/10 Control	10.000	7.650	3.496	11.059	15.77710824	18.718	19.979	21.477
C57BL/10 Control	10.000	6.300	3.786	11.639	17.61279269	20.828	22.147	23.764
C57BL/10 Control	9.500	8.200	3.202	10.030	14.22424434	17.053	18.256	19.479
C57BL/10 Control	9.500	8.200	3.816	11.221	16.70596172	20.659	21.894	23.035
	9.875	7.650	3.285	10.682	15.201	18.177	19.241	20.440
	0.876	1.234	0.741	2.861	3.195	3.500	3.621	3.672
C57BL/10 Leucine	9.000	10.000	3.817	11.392	18.804	23.215	25.386	27.358
C57BL/10 Leucine	10.000	6.100	2.990	9.595	15.535	20.944	22.408	23.547
C57BL/10 Leucine	11.000	7.000	4.152	15.386	19.245	22.582	23.845	25.006
C57BL/10 Leucine	10.000	8.100	2.136	8.328	11.768	14.458	15.141	15.743
C57BL/10 Leucine	11.000	6.700	5.110	16.847	23.548	27.169	28.862	30.678
C57BL/10 Leucine	11.000	7.000	4.316	13.940	22.208	27.189	28.712	31.314
C57BL/10 Leucine	10.000	8.400	4.643	15.741	22.058	25.481	26.913	28.584
C57BL/10 Leucine	10.000	9.300	2.943	7.818	12.500	15.907	17.017	18.701
	10.250	7.825	3.763	12.381	18.208	22.118	23.536	25.116
	0.707	1.358	0.998	3.559	4.503	4.819	5.131	5.579

These are the soleus stress (g/mm^2) values for the data in Appendix A (*mdx* vs. BL/10).

Genotype	Muscle Length (mm)	Muscle Mass (mg)	FF Twitch (g/mm)	30 Hz (g/mm)	50 Hz (g/mm)	80 Hz (g/mm)	100 Hz (g/mm)	150 Hz (g/mm)
MDX Runner	10.500	10.900	4.707	12.109	20.929	25.545	27.285	29.589
MDX Runner	10.500	13.900	3.786	6.613	17.173	23.131	24.892	27.038
MDX Runner	10.500	8.500	6.384	15.221	24.431	28.935	30.925	33.575
MDX Runner	10.000	10.400	4.475	10.026	20.424	26.960	29.348	31.683
MDX Runner	9.500	9.100	3.889	9.774	18.224	22.431	24.205	26.357
MDX Runner	9.500	10.200	4.338	10.629	20.580	25.876	27.813	30.023
MDX Runner	9.500	8.000	7.189	18.340	27.695	32.251	33.714	35.861
MDX Runner	9.500	13.400	3.911	10.511	17.832	21.629	23.125	24.634
	9.938	10.550	4.835	11.653	20.911	25.845	27.663	29.845
	0.496	2.152	1.264	3.617	3.574	3.560	3.595	3.794
MDX Leucine Runner	10.500	16.400	4.817	11.649	19.899	23.651	0.000	26.610
MDX Leucine Runner	11.000	18.800	3.144	6.871	14.615	19.019	20.522	21.937
MDX Leucine Runner	10.700	15.000	3.999	10.101	18.205	22.773	24.172	25.887
MDX Leucine Runner	10.700	17.800	3.098	7.455	13.947	18.034	19.416	20.999
MDX Leucine Runner	10.000	18.600	3.454	8.267	14.545	0.000	18.999	20.505
MDX Leucine Runner	10.500	18.400	2.257	5.186	12.333	18.943	19.091	20.706
	10.567	17.500	3.461	8.255	15.591	17.070	17.033	22.774
	0.333	1.501	0.873	2.319	2.855	8.665	8.568	2.745

These are the soleus stress (g/mm^2) values for the data in Appendix A (*MDX* Run vs *MDX* Run-L).

Myosin Heavy Chain Distribution

MDX Study Leucine Running				Percentage MyHC isoforms			
EDL	Number	Muscle	Group	I	2a	2x	2b
1	1695 M1	EDL	wt L	0.0	0.0	32.7	67.3
2	1698 M1	EDL	wt L	0.0	0.0	24.7	75.3
3	1695 M2	EDL	wt L	0.0	0.0	25.6	74.4
4	1695 M3	EDL	wt L	0.0	0.0	22.5	77.5
			MEAN	**0.0**	**0.0**	**26.4**	**73.6**

SOL	Number	Muscle	Group	I	2a	2x	2b
5.0	1695 M1	SOL	wt L	44.7	42.3	13.0	0.0
6.0	1695 M3	SOL	wt L	43.1	40.2	16.7	0.0
7.0	1695 M2	SOL	wt L	37.2	46.3	16.6	0.0
8.0	1698 M1	SOL	wt L	31.0	36.7	17.3	14.8
			MEAN	**39.0**	**41.4**	**15.9**	**3.7**

EDL	Number	Muscle	Group	I	2a	2x	2b
9	1696 M1	EDL	wt C	0.0	0.0	30.4	69.6
10	1697 M1	EDL	wt C	0.0	0.0	24.0	76.0
11	1696 M3	EDL	wt C	0.0	0.0	17.3	82.7
12	1696 M2	EDL	wt C	0.0	0.0	23.0	77.0
			MEAN	**0.0**	**0.0**	**23.7**	**76.3**

SOL	Number	Muscle	Group	I	2a	2x	2b
13.0	1696 M2	SOL	wt C	32.6	44.5	22.9	0.0
14.0	1696 M3	SOL	wt C	38.0	46.5	15.6	0.0
15.0	1696 M1	SOL	wt C	38.0	34.4	27.6	0.0
16.0	1697 M1	SOL	wt C	40.8	36.5	22.8	0.0
			MEAN	**37.4**	**40.5**	**22.2**	**0.0**

EDL	Number	Muscle	Group	I	2a	2x	2b
17	15 L	EDL	mdx run L	0.0	0.0	40.4	59.6
18	12 L	EDL	mdx run L	0.0	0.0	41.5	58.5
19	3 L	EDL	mdx run L	0.0	0.0	42.5	57.5
20	5 L	EDL	mdx run L	0.0	0.0	35.8	64.2
			MEAN	**0.0**	**0.0**	**40.1**	**60.0**

SOL	Number	Muscle	Group	I	2a	2x	2b
21.0	5 L	SOL	mdx run L	33.1	45.9	20.9	0.0
22.0	3 L	SOL	mdx run L	18.7	56.6	24.7	0.0
23.0	12 L	SOL	mdx run L	27.3	47.7	25.0	0.0
24.0	15 L	SOL	mdx run L	26.6	39.5	33.9	0.0
			MEAN	**26.4**	**47.4**	**26.1**	**0.0**

EDL	Number	Muscle	Group	I	2a	2x	2b
25	13 C	EDL	mdx run C	0.0	0.0	33.0	67.0
26	16 C	EDL	mdx run C	0.0	0.0	38.4	61.6
27	9 C	EDL	mdx run C	0.0	0.0	31.9	68.1
28	13 C (2)	EDL	mdx run C	0.0	0.0	40.8	59.2
			MEAN	**0.0**	**0.0**	**36.0**	**64.0**

SOL	Number	Muscle	Group	I	2a	2x	2b
29.0	9 C	SOL	mdx run C	33.3	46.1	20.6	0.0
30.0	5 C	SOL	mdx run C	34.1	44.0	21.8	0.0
31.0	13 C	SOL	mdx run C	23.7	52.5	23.8	0.0
32.0	16 C	SOL	mdx run C	29.9	49.1	21.0	0.0
			MEAN	**30.3**	**47.9**	**21.8**	**0.0**

EDL	Number	Muscle	Group	I	2a	2x	2b
33	1560 M2	EDL	mdx L	0.0	0.0	45.8	54.2
34	1563 M2	EDL	mdx L	0.0	0.0	38.4	61.6
35	1291 M1	EDL	mdx L	0.0	0.0	36.9	63.1
			MEAN	**0.0**	**0.0**	**40.4**	**59.6**

SOL	Number	Muscle	Group	I	2a	2x	2b
36.0	1516 M2	SOL	mdx L	24.6	31.8	30.5	13.1
37.0	1505 M3	SOL	mdx L	20.0	33.4	30.1	16.6
38.0	1516 M1	SOL	mdx L	26.9	41.6	31.6	0.0
39.0	1505 M1	SOL	mdx L	32.0	39.1	28.9	0.0
			MEAN	**25.9**	**36.5**	**30.3**	**7.4**

EDL	Number	Muscle	Group	I	2a	2x	2b
40	1124 M2	EDL	mdx C	0.0	0.0	5.6	64.4
41	1106 M1	EDL	mdx C	0.0	0.0	33.1	66.9
42	1562 M2	EDL	mdx C	0.0	0.0	30.0	70.0
43	1562 M3	EDL	mdx C	0.0	0.0	41.4	58.6
			MEAN	**0.0**	**0.0**	**27.5**	**65.0**

SOL	Number	Muscle	Group	I	2a	2x	2b
44.0	1502 M1	SOL	mdx C	38.1	8.6	23.2	0.0
45.0	1506 M1	SOL	mdx C	31.9	38.4	29.6	0.0
46.0	1515 M2	SOL	mdx C	27.3	36.1	36.6	0.0
47.0	1515 M3	SOL	mdx C	20.4	35.4	31.8	12.4
			MEAN	**29.4**	**29.6**	**30.3**	**3.1**

Real-time Calpain Clevage Rates

Nov. 2007		July. 2008				
MDX-L	MDX	MDX-L	MDX	MDX-L	MDX	
10.51	18.375	0.1956	0.13133	0.07555	0.07612	
13.219	20.96	0.18022	0.17665	0.01855	0.09067	
20.88	33.5	0.11205	0.17972	0.0223	0.06075	
19.711	37.95	0.04268	0.15732	0.11593	0.1573	
27.32	31.447	0.08234	0.15257	0.0269	0.17047	
24.391	57.42	0.05251	0.16634	0.06745	0.7785	
37.39	52.83	0.15907	0.18527	0.05852	0.75224	
39.16	24.19	0.1283	0.19143	0.06337	0.56909	
48.86	28.16	0.13428	0.23935	0.06236	0.12741	
40.9	19.1	0.10294	0.14472	0.08578	0.12678	
21.2	19.23	0.11364	0.14969	0.13452	0.09569	
18.78	47.77	0.0993		0.08394	0.09074	
17.496	25.07	0.09904		0.09037	0.11605	
33.05	45.15	0.03814		0.07313	0.20127	
14.45	38.26	0.051		0.08682	0.19658	
35.34		0.05257		0.08838	0.08322	
28.46		0.06429		0.07628		
21.759		0.0995		0.08621		
		0.14349		0.09335		
				0.13146		
				0.02268		
26.27089	33.29413	0.102682	0.170399	0.074469	0.230805	average rate

Cell Counts and Central Nuclei

Condition	Muscle	Central Nuclei	Normal Cells	Total Cells	Muscle	Central Nuclei	Normal Cells	Total Cells	EDL %	SOL %
MDX C										
MDX C	EDL	514	403	922	SOL	185	474	659	55.74837	28.07284
MDX C					SOL	328	316	644		50.93168
MDX C	EDL	416	277	693					60.02886	
MDX C	EDL	347	440	787	SOL	440	236	676	44.09149	65.08876
MDX C	EDL	271	329	600					45.16667	
MDX L	EDL	253	526	779	SOL	258	596	854	32.47754	30.21077
MDX L	EDL	285	375	660					43.18182	
MDX L					SOL	152	397	549		27.6867
MDX L	EDL	217	521	738	SOL	61	517	578	29.40379	10.55363
MDX L	EDL	326	386	712	SOL	146	414	560	45.78652	26.07143
MDX L	EDL	257	343	600	SOL	375	429	804	42.83333	46.64179
MDX C + Run	EDL	440	242	682	SOL	337	397	734	64.51613	45.91281
MDX C + Run	EDL	594	124	718					82.72981	
MDX C + Run					SOL	607	156	763		79.55439
MDX C + Run	EDL	260	365	625					41.6	
MDX C + Run	EDL	472	197	669	SOL	345	258	603	70.55306	57.21393
MDX L + Run	EDL	671	330	1001	SOL	508	267	775	67.03297	65.54839
MDX L + Run	EDL	536	145	681	SOL	523	385	908	78.70778	57.59912
MDX L + Run	EDL	414	323	737	SOL	303	437	740	56.17368	40.94595
MDX L + Run	EDL	473	349	822	SOL	188	537	725	57.54258	25.93103
WT C	EDL	0	934	934	SOL	2	860	862	0	0.232019
WT C	EDL	0	1114	1114	SOL	0	662	662	0	0
WT C	EDL	2	998	1000					0.2	
WT C	EDL	0	951	951	SOL	2	799	801	0	0.249688
WT L	EDL	0	627	627	SOL	0	799	799	0	0
WT L	EDL	3	796	799	SOL	1	820	821	0.375469	0.121803
WT L					SOL	4	791	795		0.503145
WT L	EDL	5	917	922	SOL	4	814	818	0.542299	

Appendix D – Statistics

Manuscript 1

Body Mass

Baseline

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Condition	1	1	0.7469394	0.1780	0.6765
Treatment	1	1	1.0939394	0.2607	0.6139
Condition*Treatment	1	1	0.7952727	0.1896	0.6669

Week 1

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Condition	1	1	7.5150027	0.4873	0.4916
Treatment	1	1	0.0034237	0.0002	0.9882
Condition*Treatment	1	1	8.3273711	0.5399	0.4693

Week 2

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Condition	1	1	9.5191878	1.0511	0.3151
Treatment	1	1	4.4049773	0.4864	0.4920
Condition*Treatment	1	1	7.2918194	0.8052	0.3781

Week 3

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Condition	1	1	18.273173	3.2912	0.0817
Treatment	1	1	0.271594	0.0489	0.8268
Condition*Treatment	1	1	2.026199	0.3649	0.5512

Week 4

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Condition	1	1	9.4747265	2.6484	0.1167
Treatment	1	1	9.1509855	2.5579	0.1228
Condition*Treatment	1	1	0.0660934	0.0185	0.8930

EDL Stress Frequency Curve

1 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	63.763843	44.5066	<.0001*
Treatment	1	1	0.151034	0.1054	0.7470
Genotype*Treatment	1	1	0.292965	0.2045	0.6535

30 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	94.177229	17.6339	0.0001*
Treatment	1	1	0.496206	0.0929	0.7620
Genotype*Treatment	1	1	2.660428	0.4981	0.4842

50 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	157.83794	7.1949	0.0104*
Treatment	1	1	0.01897	0.0009	0.9767
Genotype*Treatment	1	1	27.86410	1.2702	0.2661

80 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	1184.1319	54.6872	<.0001*
Treatment	1	1	4.9359	0.2280	0.6355
Genotype*Treatment	1	1	55.2368	2.5510	0.1177

100 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	2030.6712	93.8277	<.0001*
Treatment	1	1	7.9445	0.3671	0.5479
Genotype*Treatment	1	1	69.1381	3.1945	0.0811

150 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	2523.1457	103.3303	<.0001*
Treatment	1	1	11.8856	0.4868	0.4892
Genotype*Treatment	1	1	95.9910	3.9311	0.0540

EDL MHC Distribution

Type IIx

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	0.431026	0.0242	0.8793
Treatment	1	1	1.600256	0.0897	0.7701
Genotype*Treatment	1	1	80.985641	4.5397	0.0565

Type IIb

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	0.431026	0.0242	0.8793
Treatment	1	1	80.985641	4.5397	0.0565
Genotype*Treatment	1	1	1.600256	0.0897	0.7701

EDL Centrally Nucleated Fibers

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	7776.0427	234.3001	<.0001*
Treatment	1	1	145.6091	4.3873	0.0581
Genotype*Treatment	1	1	158.0145	4.7611	0.0497*

EDL Fiber Diameter

Calpain Cleavage Rate

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	1	1	14826.505	472181.7	<.0001*

Calpain:calpastatin Ratio

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	0.09545702	8.8295	0.0117*
Treatment	1	1	0.00225223	0.2083	0.6562

Phosphorylated mTOR to total mTOR ratio

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	1	1	0.01491050	3.3080	0.0920
Genotype	1	1	0.03014141	6.6870	0.0226*
Treatment*Genotype	1	1	0.02090597	4.6381	0.0506

Manuscript 2

Running Distance

Week 1

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	250390.4	250390	2.6677
Error	12	1126331.3	93861	**Prob > F**
C. Total	13	1376721.7		0.1284

Week 2

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	8461821	8461821	20.1914
Error	12	5028965	419080	**Prob > F**
C. Total	13	13490785		0.0007*

Week 3

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	20765080	20765080	16.5986
Error	12	15012135	1251011.2	**Prob > F**
C. Total	13	35777214		0.0015*

Week 4

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	28435536	28435536	304.4939
Error	12	1120635	93386.214	**Prob > F**
C. Total	13	29556170		<.0001*

Total Distance

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	6189921973	6.1899e+9	9.3540
Error	8	5293926075	661740759	**Prob > F**
C. Total	9	1.1484e+10		0.0156*

Body Mass

Baseline

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	19.763333	19.7633	4.8305	0.0526
Error	10	40.913333	4.0913		
C. Total	11	60.676667			

Week 1

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	24.08333	24.0833	2.2084	0.1681
Error	10	109.05333	10.9053		
C. Total	11	133.13667			

Week 2

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	11.407500	11.4075	2.2376	0.1656
Error	10	50.981667	5.0982		
C. Total	11	62.389167			

Week 3

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	14.740833	14.7408	2.5749	0.1397
Error	10	57.248333	5.7248		
C. Total	11	71.989167			

Week 4

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	29.767500	29.7675	4.9266	0.0507
Error	10	60.421667	6.0422		
C. Total	11	90.189167			

EDL Stress Frequency Curve

1 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	8.733226	8.73323	12.6563
Error	11	7.590350	0.69003	**Prob > F**
C. Total	12	16.323576		0.0045*

30 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	19.881960	19.8820	8.0138
Error	11	27.290777	2.4810	**Prob > F**
C. Total	12	47.172737		0.0163*

50 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	67.74093	67.7409	9.5781
Error	11	77.79740	7.0725	**Prob > F**
C. Total	12	145.53833		0.0102*

80 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	78.03042	78.0304	8.1964
Error	9	85.68127	9.5201	**Prob > F**
C. Total	10	163.71169		0.0187*

100 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	105.69816	105.698	11.5542
Error	10	91.48065	9.148	**Prob > F**
C. Total	11	197.17881		0.0068*

150 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Treatment	1	171.42528	171.425	14.8568
Error	12	138.46238	11.539	**Prob > F**
C. Total	13	309.88766		0.0023*

EDL MHC Distribution

Type IIx

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	32.40125	32.4012	2.4031	0.1721
Error	6	80.89750	13.4829		
C. Total	7	113.29875			

Type IIb

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	32.40125	32.4012	2.4031	0.1721
Error	6	80.89750	13.4829		
C. Total	7	113.29875			

EDL Centrally Nucleated Fibers

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	0.00042061	0.000421	0.0000	0.9989
Error	6	1218.3660	203.061		
C. Total	7	1218.3664			

EDL Fiber Count

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	37401.13	37401.1	3.5609	0.1081
Error	6	63019.75	10503.3		
C. Total	7	100420.88			

EDL Fiber Size

Appendix A – Soleus

Soleus Stress Frequency – BL/10 vs *mdx*

1 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	0.13417191	0.2447	0.6232
Treatment	1	1	0.88964361	1.6226	0.2091
Genotype*Treatment	1	1	0.08587002	0.1566	0.6941

30 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	0.1667030	0.2853	0.5958
Treatment	1	1	0.2389790	0.4090	0.5256
Genotype*Treatment	1	1	1.1859341	2.0298	0.1610

50 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	22.423729	2.1034	0.1538
Treatment	1	1	19.772556	1.8547	0.1799
Genotype*Treatment	1	1	29.893534	2.8041	0.1008

80 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	34.408412	2.5130	0.1198
Treatment	1	1	33.982162	2.4819	0.1220
Genotype*Treatment	1	1	51.286413	3.7457	0.0591

100 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	33.702882	2.2094	0.1440
Treatment	1	1	42.765308	2.8035	0.1008
Genotype*Treatment	1	1	58.014475	3.8031	0.0573

150 Hz

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	34.979209	2.1103	0.1531
Treatment	1	1	56.968808	3.4369	0.0702
Genotype*Treatment	1	1	61.908692	3.7349	0.0595

Soleus Stress Frequency – Exercised *MDX*

1 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	6.466830	6.46683	5.1759	0.0421*
Error	12	14.992904	1.24941		
C. Total	13	21.459735			

30 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	39.58864	39.5886	4.0094	0.0684
Error	12	118.48795	9.8740		
C. Total	13	158.07659			

50 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	97.04896	97.0490	8.9454	0.0113*
Error	12	130.18882	10.8491		
C. Total	13	227.23778			

80 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	263.98710	263.987	6.8248	0.0227*
Error	12	464.16967	38.681		
C. Total	13	728.15677			

100 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	387.42098	387.421	10.1612	0.0078*
Error	12	457.53024	38.128		
C. Total	13	844.95122			

150 Hz

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	171.42528	171.425	14.8568	0.0023*
Error	12	138.46238	11.539		
C. Total	13	309.88766			

MHC Distribution - BL/10 vs *MDX*

Type I

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	443.10250	13.4988	0.0032*
Treatment	1	1	3.61000	0.1100	0.7459
Genotype*Treatment	1	1	27.04000	0.8238	0.3820

Type IIa

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	68.062500	3.6163	0.0815
Treatment	1	1	0.062500	0.0033	0.9550
Genotype*Treatment	1	1	2.402500	0.1277	0.7271

Type IIx

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	504.00250	33.2977	<.0001*
Treatment	1	1	40.32250	2.6640	0.1286
Genotype*Treatment	1	1	39.69000	2.6222	0.1313

Type IIb

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	46.580625	1.1041	0.3141
Treatment	1	1	64.400625	1.5265	0.2403
Genotype*Treatment	1	1	0.390625	0.0093	0.9249

MHC Distribution – Exercised *MDX*

Type I

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	29.26125	29.2613	1.0197	0.3516
Error	6	172.17750	28.6963		
C. Total	7	201.43875			

Type IIa

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	0.50000	0.5000	0.0158	0.9042
Error	6	190.43500	31.7392		
C. Total	7	190.93500			

Type IIx

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	37.41125	37.4112	2.3111	0.1793
Error	6	97.12750	16.1879		
C. Total	7	134.53875			

Centrally Nucleated Fibers – BL/10 vs *MDX*

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	4800.0516	35.3410	0.0001*
Treatment	1	1	325.0678	2.3934	0.1529
Genotype*Treatment	1	1	328.2188	2.4166	0.1511

Centrally Nucleated Fibers – Exercised *MDX*

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	1	307.2471	307.247	1.0093	0.3612
Error	5	1522.1015	304.420		
C. Total	6	1829.3486			

Average Fiber Area – BL/10 vs *MDX*

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Genotype	1	1	3584661.0	10.0068	0.0016*
Treatment	1	1	4733242.8	13.2131	0.0003*
Genotype*Treatment	1	1	8424353.2	23.5171	<.0001*

Average Fiber Area – Exercised *MDX*

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment 2	1	21151656.4	21151656	48.0778	<.0001*
Error	7145	3143420254	439946.85		
C. Total	7146	3164571911			

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