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Sarcopenia and current research using Ames dwarf mice: a research study

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SARCOPENIA AND CURRENT RESEARCH USING AMES DWARF MICE:
A RESEARCH STUDY

by

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Bachelor of Science, Concordia University Wisconsin, 2011

A Scholarly Project Submitted to the Graduate Faculty of the

Department of Physical Therapy
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In partial fulfillment of the requirements for the degree of

Doctor of Physical Therapy

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This Scholarly Project, submitted by Amanda Sodemann in partial fulfillment of the requirements for the Degree of Doctor of Physical Therapy from the University of North Dakota, has been read by the Advisor and Chairperson of Physical Therapy under whom the work has been done and is hereby approved.

(Graduate School Advisor)

(Chairperson, Physical Therapy)
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Department Physical Therapy

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ABSTRACT
This research project focused on the differences observed in muscle fiber composition and surface area between wild type mice and Ames dwarf mice as they aged. A mouse model was utilized because the skeletal muscle framework of mice closely resembles that of human muscle; thus, the observations seen within the mice may also be seen within humans as they age. Comparisons between the wild type and dwarf mice include a smaller cross-sectional muscle area in a dwarf mouse and no drastic change in the percentages of fast and slow twitch muscle fibers as the dwarf mouse ages. Furthermore, differences in fiber length and cross-sectional muscle area between wild type and dwarf mice were revealed. However, exact conclusions on muscle fiber composition and surface area as mice age cannot be drawn secondary to the limited number of lower extremity musculature that was obtained. Current knowledge of sarcopenia and its effects were also explored throughout this paper.
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Sarcopenia has become increasingly prevalent in the population secondary to increased longevity of life seen in communities. It is expected that sarcopenia will continue to steadily escalate in the population as the anticipated life expectancy rises; furthermore, the population of adults aged 65 and over will double within the next 15 years.\(^1\) With these projected outcomes, age-related losses in skeletal muscle mass and function will become a crucial aspect in the provided health services. These functional declines can contribute to the increased hospitalizations secondary to falls and subsequent hip fractures; hence, there may be an increased prevalence of gait disorders and balance disturbances in the elderly. Currently, the term sarcopenia is being used interchangeably with loss of muscle mass, strength, and function. Loss of muscle mass and strength, cognitive decline, and a decrease in overall physical functioning are associated with the aging process. The loss of muscle mass and strength can occur as early as the third decade of life but is most commonly seen in the fourth decade and beyond.\(^2,3\) However, there is conflicting evidence surrounding the age at which cognitive decline begins. Some researchers argue cognitive decline starts in the sixth decade of life; whereas, others have suggested it can appear as early as the second or third decade.\(^4\)

Research has proposed decreased participation in physical activity leads to the gradual loss of muscle mass (atrophy); however, several other factors may also contribute
to loss of strength and muscular atrophy. Clinically relevant questions center on how to define sarcopenia. Once sarcopenia is defined, how does one medically treat it and are there therapeutic rehabilitative approaches? Are there ways to prevent sarcopenia? The following paragraphs will aim to answer these questions. Furthermore, the meaningful role that Ames dwarf mice play in sarcopenia research will be discussed.
CHAPTER II

RESEARCH DESCRIPTION

Research’s efforts have revealed that by the time a person reaches the age of 50, there is an average decline of 1% to 2% of muscle mass per year. By age 80, a person’s muscle mass decline has reached approximately 50%. Once muscle mass falls below two standard deviations of the mean of a standardized young control group and gait speed declines to 0.8 m/s², a clinical diagnosis of sarcopenia is concluded. It is estimated that 5% to 13% of persons aged 60 to 70 years are affected by this disorder; this percentage increases to 11% to 50% for persons aged 80 years and older. Another group of researchers conclude that females aged 75 years and older lose approximately 0.64% to 0.7% in muscle mass per year; whereas, males aged 75 years and older tend to lose 0.8% to 0.98% per year. Furthermore, the loss of muscular strength is considered to be 2 to 5 times greater than the loss of muscle mass. Advanced skeletal muscle loss may affect a person’s quality of life, the need for support services, and eventually long term care. This loss of muscular strength is a consistent risk factor for the development of disabilities associated with sarcopenia. Ultimately, the ability to climb stairs, ambulate, maintain proper balance and posture, and transfer from sit to stand will become increasingly strenuous and difficult.

Experimental research using molecular genetics has revealed several pathophysiological disturbances that occur with sarcopenia. Molecular genetics seeks to
identify alterations in protein arrangements and the effects these alterations have on the organism. In the molecular genetics research of sarcopenia, there have been several discoveries which have aided in establishing further research projects and a more broad-base of knowledge in muscle tissue proteomics. These discoveries include the association of age-dependent muscular wasting with impaired muscle protein synthesis, impaired metabolic pathways, disrupted ion homeostasis, unbalanced levels of growth factors and hormones, excitation and muscular contraction uncoupling, reduced cellular stress response, heightened susceptibility to apoptosis, and diminished regenerative capacity. Through the use of molecular genetics and proteomics, sarcopenia is now more accurately defined.

What is sarcopenia and how it is classified?

Researchers have created several possible definitions for sarcopenia. One definition states that decreased muscle strength, (which was represented by knee extension isometric torque in this study), handgrip strength, lower extremity muscle power, and physical performance measured by gait speed are indicative of sarcopenia. Another definition states muscle strength or function has a direct correlation with muscle mass; therefore, if muscle mass decreases, it can be assumed that the strength of the muscle would also diminish. For example, one with low muscle mass would most likely have a decreased gait speed secondary to the reduced amount of muscular power produced by muscles. Muscular power is lost before muscular strength. Muscle power is defined as the ability to generate as much force as quickly as possible; whereas, strength is defined as the ability to generate as much force as possible under no time constraints. Mathematically, power is calculated by dividing work by time; strength is calculated by
multiplying the mass and distance it has traveled (or work it has done). While both muscle power and strength are important for functional daily activities, power is better at predicting the ease in which functional activities are performed; thus, a person who has decreased gait speed more than likely has a decreased ability to generate adequate muscle power to efficiently contract his lower extremity muscles to move in a forward progression.2,8

However, different researchers have argued the term sarcopenia describes alterations in muscle structure, function, and body composition which lead to weakness and dysfunction. At the time, the term was mainly used as a major determinant in the decline of physical function and seen as a risk factor for disability and disease. Other early definitions include any type of muscle loss due to disease, any person who required the use of an assistive device, and any person who has had a history of falls.9

In 1998, researchers began developing methods of classification and diagnostic criteria for sarcopenia; these are still utilized in determining the severity of the disease. Sarcopenia classification began with using mathematical formulas. Different mathematical formulas have been suggested to define the rate of sarcopenia. One formula demonstrates sarcopenia as appendicular skeletal mass (ASM), which is the sum of lean mass for both arms and legs, divided by the height squared (kg/m²).2,10 Values that were at least two standard deviations below the mean of a reference population were classified as having sarcopenia. Another formula that was introduced used muscle mass; this formula corrected muscle mass for a combination of height, fat mass, or total body mass.2 Formulas used to compute this percentage included ASM (kg) = 0.2487 (weight) +
0.0483(height) -0.1584(hip circumference) + 0.0732(grip strength) +2.5843(sex) + 5.8828 for determination of muscle mass and percentage of body fat = 0.2034(waist circumference) + 0.2288(hip circumference) + 3.6827(In triceps skinfold) - 10.9814(sex) - 14.3341.10 The latter formula was derived using the bioelectrical impedance analysis (BIA) equation which was cross-validated with magnetic resonance imaging (MRI) measures of whole body muscle mass and body mass index. This particular study found a correlation of 0.93 and a 9.0% standard error of the estimate between MRI and the BIA equation. From this study, absolute muscle mass in kilograms (kg), was normalized for height and termed skeletal muscle index (SMI).11 These formulas have been utilized to generate criteria to diagnose sarcopenia.

Diagnostic criteria correlate to the understanding of related consequences low muscle mass has on a person’s ability to execute daily and vocational functions. Evidence has shown muscle mass is associated with a person’s self-reported physical disability and functional impairments.2 It has been recently suggested the main criteria for sarcopenia diagnosis include low muscle mass greater than two standard deviations below the mean, low muscle strength, diminished physical performance, and gait speed less than 0.8m/s.5 Contributing factors to this diagnosis are chronic inflammation, reduced protein intake, motor neuron atrophy, and immobility.12

Physicians are now classifying sarcopenia by severity using SMI. In order to determine “normal” skeletal muscle mass, SMI values were obtained from young persons aged 18 to 39 years old. Classification criteria for sarcopenia were derived using the SMI values from the young population.5 Class I sarcopenia is defined as SMI that is between
one and two standard deviations below the young adult values; it is most commonly associated with increased difficulty in stooping, crouching, kneeling, and ability to perform tandem stance. Class II sarcopenia is SMI that is greater than two standard deviations below the young adult values; it is most commonly associated with increased difficulty in climbing 10 stairs, lifting/carrying 10 pounds, rising to a stand from a chair, and decreased ability to perform household chores. A normal SMI is considered to be within one standard deviation of the gender-specific mean for young adults. The researchers utilized this approach because it was considered comparable to the use of bone mineral density of a young reference group for classifying normal bone density, osteopenia, and osteoporosis. It was discovered the prevalence of class I and class II sarcopenia increased from the third to sixth decades, but remained relatively constant in the seventh decade and beyond.6,9 Sarcopenia classification can be further aided by a physician’s prescription for diagnostic imaging.

Imaging is also routinely performed in the diagnosis of sarcopenia; the most common imaging techniques include MRI, dual energy X-ray absorptiometry (DEXA), and bioelectrical impedance analysis (BIA). MRIs allow the radiologist to determine segmental and total muscle mass, and assess overall muscle quality through calculating fat infiltration in muscle. DEXA has the advantage of analyzing body composition and is currently used as the procedure of choice for bone mineral density. BIA is currently recognized as the most specific exam to assess lean muscle mass and body fat. It is a routine exam that assesses body composition by focusing in on the molecular level, as a representation of the amount of muscle mass.12 It measures tissue resistance through an electrical signal to determine the proportion of body fat to lean mass. This calculation is
done as the signal’s current easily flows through parts of the body that are composed mainly of water such as blood, urine, and muscle. This technique is a reliable analysis in the study for lean mass versus muscle mass. BIA has several advantages including no radiation exposure, relatively inexpensive, requires no specialized staff, and is a portable machine; hence, the test can be completed at the patient’s bedside. In a study done by Janssen and colleagues, they revealed that the BIA has greater precision in classifying class II sarcopenia and estimating the percentage of skeletal muscle mass than the other aforementioned methods. Sarcopenia classification is accomplished through utilizing SMI young adult values attained through previous research. Type I sarcopenia is defined as SMI that is between one and two standard deviations below the young adult values and type II is classified as being greater than two standard deviations compared to young adult values. In another study, researchers concluded BIA using vector analysis was sensitive in determining body mass differences; however, it was not as sensitive in the assessing specific features of a person’s body composition. Furthermore, they discovered BIA could also be used in determining nutritional status in the elderly secondary to the ability to recognize individuals with different quantities of absolute mass. They concluded this newer technique of utilizing vector analysis may be applicable in finding several age-related conditions including sarcopenia and sarcopenic obesity.

Now that there is an understanding of how sarcopenia is classified and diagnosed, it is important to recognize how the muscle fiber and motor unit is structured, how much energy/power is required for muscle contraction, and how functional impairments occur as the muscle motor unit ages. Power for a muscular contraction occurs within the actin and myosin components of the sarcomere. Myosin plays an important role as it aids in the
differentiation of motor unit subtypes. Three motor unit types exist: slow oxidative, fast-fatigable, and fast fatigue-resistant motor units. In general, slow oxidative motor units have the smallest number of muscle fibers and are principally composed of type I myosin. These motor units will contract with slow velocity and are usually recruited for fine motor activities and gravity opposition. Unique characteristics of fast-fatigable motor units are high numbers of muscular fibers and the large cross-sectional areas. Additionally, they have the highest contractile velocity and are composed mainly of type IIb fibers, (now known as IIx in humans), and myosin, which transfer energy at a much higher rate than slow motor units. Any activity that requires a maximal power generation, such as weight-lifting or sprinting, will recruit fast-fatigable motor units. Fast fatigue-resistant motor units are composed of type I, type IIa, and type IIb muscle fibers. Transfer of energy occurs at a slower rate than fast-fatigable motor units, but faster than slow oxidative motor units.¹

Several aging processes can result in alterations in mass, composition, contractile properties, and mechanical properties of skeletal muscle tissue. One such process is the loss of slow and fast motor units and atrophy of muscle fibers as described above. Denervation of motor units result in this loss; in order to compensate, the remaining motor units will convert the denervated fiber type to that of the motor unit. Consequently, type II fast-fatigable fibers are converted into slow type I fibers.¹ This transition of fast-to-slow muscle fibers is apparent with physical exercise, low-frequency stimulation, hyper-excitability, and aging; these effects are most commonly seen in sarcopenic muscle. Researchers have suggested these muscle fiber transitions are most likely caused by a secondary occurrence. They are a result of faulty reinnervation mechanisms post
denervation, apoptosis-triggered loss of spinal motor neurons, and selective atrophy of type II fibers.

In contrast, slow-to-fast muscle fiber transitions (type I to type II) are commonly associated with disuse atrophy, extensive bed rest, and microgravity. Muscular atrophy is characterized by a reduction in muscle fiber cross-sectional area, sarcomere dissolution, decreased mitochondria, and greater amounts of connective tissue within the muscle. Type I muscle fibers are the first to atrophy; type II fibers will start to atrophy later. Researchers suggest this finding is secondary to the extensive use of type I fibers; the majority of muscular contractions recruit type I fibers before type II fibers. Hence, it would be logical that with muscular atrophy, type I fibers lose their primary characteristics and begin to imitate the characteristics of type II fibers. The loss of both type I and type II fibers accelerates age-related alterations in muscle function. This progression of muscle function degenerative modification develops as muscle fibers undergo a shift from anaerobic-oxidative metabolism to aerobic-oxidative metabolism.

Aerobic-oxidative metabolism shifts have been researched through proteomics, the study of protein structures and functions. Emerging scientific research aims to observe ongoing intercellular transformations as the proteome is subjected to variable environments. Thus far, proteomic profiling has established extensive adjustments made in the expression patterns of specific proteins in aging skeletal muscle. These adjustments include enzymatic metabolic pathways, elements of the cellular stress response, and ion homeostasis regulatory components. One finding discovered that pyruvate kinase, a rate-limiting glycolytic enzyme, is significantly reduced in aged skeletal muscle; however,
essential mitochondrial proteins are amplified in sarcopenia. This discovery proposed a shift from anaerobic-oxidative to aerobic-oxidative metabolism, hence, altering the way in which muscle fibers respond to physical stressors.

**The role of Ames dwarf mice**

Numerous studies have been conducted on the protein arrangement of muscle fibers as they age. Comparisons between human skeletal muscle and mouse skeletal muscle are most common because the skeletal muscle in mice closely resembles that of human skeletal muscle. Since mice are frequently used for experimental research, these experiments are easily performed and provide a wealth of knowledge surrounding this particular diagnosis.16

Several mouse models, Ames dwarf mice included, carry specific genetic mutations which are associated with increased life span. Additionally, they have diminished levels of mitochondria and endogenous reactive oxygen species (ROS), and an increased resistance to oxidative stress. These mutations are beneficial in mediating longevity, thus extending their lifespan. Researchers have hypothesized that ROS-mediated oxidative damage is an essential component in determining longevity. They suggest that these physiological characteristics of mitochondrial function are developed early in life and continue throughout the life cycle. Moreover, Ames dwarf mice also have unique hormonal characteristics; they lack growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin. Ames Dwarf mice persistently live approximately 40%-60% longer than their normal littermates.17
Previous studies have also compared genetic versus environmental influences in the progression of sarcopenia. A group of researchers led by Edstrom detected the importance of metabolism and extent of muscle usage as the mouse aged. To determine the role of metabolism within the aging process, a litter of mice were put on restricted diets. The results of dietary restriction revealed a reduction in the appearance of several characteristic features in aging skeletal muscle through the restoration of transcriptional alterations that naturally occur with the aging process. Activity and exercise demonstrated similar results. Increasing levels of activity help maintain the current strength of muscle mass, thus, reversing the detrimental effects of aging on skeletal muscle.

For this research project, the lower extremity musculature of Ames dwarf mice was analyzed. The muscle tissues that were removed from the mice were placed on liver tissue to maintain resting length on a stable surface. The goal was to determine the number of fast-glycolytic and slow-oxidative muscle fiber cells located within the soleus (SOL) and extensor digitorum longus (EDL) muscles. Ames dwarf mice have a point mutation on chromosome 11 and characteristically have primary pituitary hormonal deficiency caused by reduced anterior pituitary cells. These cells produce and secrete prolactin, thyroid stimulating hormone (TSH), and growth hormone (GH). It has been suggested that these hormonal deficiencies play an important role in the longevity of Ames dwarf mice; in the cases of TSH and GH deficits, the lifespan may be influenced by lower metabolic rates and reduced premature aging. Furthermore, their small body size may also impact survival. Previous research has demonstrated significant differences in cardiac, liver, and kidney tissues between dwarf mice and normal mice;
these differences have been linked to life longevity, enzyme metabolism, and the absence of anterior pituitary hormones. Because Ames dwarf mice exhibit substantial tissue variances, current research is now asking, how does muscle tissue vary between wild type and dwarf mice and does it reveal aerobic-oxidative metabolic shifts or conversion of type II to type I muscle fibers? Does the delayed aging seen in Ames dwarf mice avoid the sarcopenic effects in muscle? If it does, how would this discovery contribute to the research in muscle health in humans? This was the area of focus in this research study. It aimed to detect differences in muscle fibers as they age. Observations of cross-sectional muscle area and fiber type composition of age-matched mice (3 month, 6 month and 12 month old) were analyzed and comparisons across ages were obtained.
CHAPTER III

METHODS

Muscle tissues were obtained from both wild type and Ames dwarf mice. Four muscles were extracted from bilateral lower extremities including SOL, gastrocnemius-plantaris (GP), EDL, and tibialis anterior (TA). After the muscles were extracted, they were placed on a slice of liver tissue that was surgically removed from a wild type mouse.

Skeletal Muscle Tissue Acquisition

Tribromoethanol, (2.5% in 0.9% saline per 100 µl/10g body weight) – [~300 µl – 400 µl for wild type mouse], was injected intraperitoneally to anesthetize the animals. Once the injection was given, the mouse’s pain and corneal reflexes were checked every couple minutes; when the mouse no longer responded to the stimuli, surgery proceeded.

Low angle cuts were made around the legs and ankles in order to free hind limb skin. The skin was detached from the muscles by inserting a scissors underneath the skin and spreading the scissors open to pull the skin away from the surrounding tissues. Cuts were made up the skin proximally towards the hip and the skin was peeled away. The distal attachment of the GP and SOL was severed by slicing the tendon at the heel. SOL was separated from the GP musculature at the knee joint; it was then cut at the knee and retracted towards the distal end. Another cut was made to remove the SOL. The tissue was then placed on a piece of liver, covered with Optimal Cutting Temperature (OCT) compound, and then frozen in isopentane in liquid nitrogen. Next, GP was removed from
its attachment at the knee. Following the above procedure, the tissue was then frozen in isopentane in liquid nitrogen. The third muscle extracted was the TA. Fascia surrounding TA was removed by toothed forceps; TA was freed by sliding the instrument along the tibia. The TA tendon was severed at both the ankle and knee. Again, the same freezing procedure as the GP was utilized. Finally, the EDL was located, removed from its attachments at the ankle and knee, and frozen in the same manner as the SOL.

**Slicing Muscle Tissue**

Muscle tissue was sliced using a Cryostat which was cooled to -25°C (optimal cutting temperature) for 8 to 10 hours. The cutting blade was rinsed with alcohol, inserted in to the Cryostat, and allowed to cool for 30 minutes. Tissue samples were refrigerated at -80°C; hence, they needed to warm up to -25°C prior to slicing. Once the appropriate temperature had been reached, the muscle tissue was set vertically on a metal plate with OCT to secure the sample. Slicing was done in 50-µm sections (trim) until arriving at the muscle tissue. The blade was then switched to 10-µm sections (fine). Fifty 10-µm sections were sliced to attain the main portion of the muscle. After reaching the main muscular portion, 10-µm serial sections were collected and placed on slides. The slides used for collection were Fisher Superfrost Plus Microscope slides (Fisher Scientific Pittsburgh, PA. Cat. No. 12-550-15 for 25 x 75 x 1.0 mm). Three sections were placed on each slide. When thirty sections had been compiled, another fifty 10-µm sections were skipped. Next, an additional 30 sections of 10-µm was recovered and placed on slides. This procedure was repeated until the end of the muscle was reached. The slides containing muscle tissue were then stored in a refrigerator at -20°C.
Hematoxylin And Eosin Staining

The slides were submerged in Harris hematoxylin for two minutes and then rinsed in distilled water twice. Next, the stained slides were transferred to acid alcohol (11 ml glacial acetic acid, 95 ml ethanol, and 5 ml distilled water) and dipped three times. Again, they were rinsed in distilled water. The slides were then dipped in ammonium water (0.588 ml of 28% NH₄OH in 250 ml dH₂O) six times and rinsed with distilled water. A working solution of eosin was mixed; it consisted of 100 ml stock eosin, 300 ml 80% ethanol, and 2.5 ml glacial acetic acid. The slides remained in the working solution for thirty seconds. Afterwards, the slides were rinsed twice in distilled water. Finally, the slides were dehydrated, for 15 seconds each, in 75%, 95%, and 100% ethanol and clear xylene. Once dehydrated, the slides were mounted with Permount and stored at room temperature.

Immunohistochemistry For Mouse Myosin Heavy Chains

Tissue slides were removed from the freezer and allowed to warm-up to room temperature. While the slides were warming, a working 1x phosphate buffered saline (PBS) solution was prepared. The solution was diluted with distilled water and potassium chloride to achieve a pH of 7.4. This solution was used throughout the procedure for rinsing the slides in between cellular staining. A total of 18 slides were stained; 8 slides were stained with fast-twitch, 8 with slow-twitch, and 2 were the control group.

Once the slides achieved room temperature, the tissue samples were encircled using a PAP pen and then rinsed in 1X PBS for 2 minutes. PAP pens are used for applying immunohistochemistry to slides; the hydrophobic properties of the pen allow
the user to draw barriers on a slide to confine the applied reagents to a defined area. The encircled area has a visible blue/green marking. For immunohistochemistry, the PAP pen should be applied following deparaffinization. A series of protein and blocking solutions were applied to the tissue samples, slides were incubated, and then rinsed in 1X PBS.

First, a mixed solution of 30% hydrogen peroxide and horse serum (30 µl H₂O₂ + 9 µl horse serum in 3 ml PBS) was added to the tissues and then incubated for 5 minutes. Slides were then rinsed in PBS solution twice for 2 minutes. Avidin blocking solution was allotted for 15 minutes; a 2 minute rinsing in PBS solution followed. Next, biotin blocking solution was placed on the samples for 15 minutes and then the samples were rinsed for 2 minutes in PBS. The slides were then incubated and covered for 60 minutes with a mixture consisting of 36 µl mouse IgG blocking reagent and 1 ml PBS. Subsequently, the tissues were rinsed in PBS twice for 2 minutes.

Thereafter, mouse on mouse concentrate (M.O.M.), which is a combination of 160 µl of protein concentrate and 2 ml PBS, was placed on the slides and allowed to sit for 5 minutes. Slides were then stained with either a fast-twitch or slow-twitch protein; 456 µl of M.O.M. diluent + 24 µl of slow antibody protein was added to 8 tissues slides and 425 µl of M.O.M. diluent + 47 µl of fast antibody protein was placed on another 8 tissue slides. These slides were incubated in 100 µl of primary antibody dilution and covered for 30 minutes. During the time of incubation, a solution of 1.25 ml of PBS, 1 drop of reagent A and 1 drop of reagent B was prepared and allowed to stand at room temperature for 30 minutes.
After the slides were rinsed in PBS solution twice for 2 minutes, they were incubated in 4 µl of M.O.M. biotinylated anti-mouse IgG reagent and 1 ml M.O.M. working diluent for 10 minutes. Again, slides were rinsed in PBS for 2 minutes. Next, the mixture of reagent A & B was applied to the slides for 5 minutes; afterwards, DAB solution was placed on the slides for 10 minutes. Following the application of DAB, the slides were rinsed in distilled water for 1 minute and then dehydrated in 70%, 95%, and 100% alcohols and xylene. Slides were mounted with Permount and stored at room temperature.

**Muscle Fiber Counting**

Once the slides had been stained, they were observed under a microscope. Tissue samples were enlarged for proper viewing. Stained muscle fibers were counted, stained and unstained. The stained fibers were considered to be either fast or slow twitch depending on the stain used on the tissue sample slides; unstained fibers were considered to be any muscle fibers that did not take to the specific stain. These fiber counts were compared between normal and dwarf mice. Please see the results section for further information.

**Determining Cross-Sectional Muscle Area**

Cross-sectional areas were measured using the software program, Scion. Ruler measurements were set at a known length of 1000 micrometers with .981 pixels/micrometers being the measured distance. Stained muscle fiber images were analyzed using a freehand line drawing tool. Five cells were representative of the entire tissue sample and were measured twice for increased accuracy in the actual cross-
sectional area of the muscle cell. The average of the 10 areas was used in the final t-test analysis.
CHAPTER IV

RESULTS

The following tables demonstrate the differences in muscle fiber counts: fast twitch versus slow twitch and average muscle fiber composition between wild type and dwarf mice. Comparisons can be seen across the lifespan in wild type and dwarf mice. Please see tables 1 through 6 for detailed information and the results section for further information regarding the laboratory observations.

Wild type Mouse Muscle Fiber Counts

Table 1: Muscle fiber counts for SOL in wild type mice. Fast vs. slow twitch.

<table>
<thead>
<tr>
<th>Animal #, Slide Section</th>
<th>Age</th>
<th>Fiber type</th>
<th>Right vs. Left</th>
<th># of stained fibers</th>
<th># of fast unstained fibers</th>
<th>Total # of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>N4 175</td>
<td>12 mo.</td>
<td>Slow</td>
<td>Right</td>
<td>143 (55.85%)</td>
<td>113 (44.14%)</td>
<td>256 (100%)</td>
</tr>
<tr>
<td>N4 176</td>
<td>12 mo.</td>
<td>Fast</td>
<td>Right</td>
<td>92 (42.2%)</td>
<td>126 (57.8%)</td>
<td>218 (100%)</td>
</tr>
<tr>
<td>N2 214</td>
<td>3 mo.</td>
<td>Slow</td>
<td>Left</td>
<td>204 (58.45%)</td>
<td>145 (41.54%)</td>
<td>349 (100%)</td>
</tr>
<tr>
<td>N2 213</td>
<td>3 mo.</td>
<td>Fast</td>
<td>Left</td>
<td>158 (45.4%)</td>
<td>190 (54.6%)</td>
<td>348 (100%)</td>
</tr>
</tbody>
</table>
**Dwarf Mouse Muscle Fiber Counts**

<table>
<thead>
<tr>
<th>Animal #, Slide Section</th>
<th>Age</th>
<th>Fiber type stained</th>
<th>Right vs. left</th>
<th># of stained fibers</th>
<th># of unstained fibers</th>
<th>Total # of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 10x</td>
<td>6 mo.</td>
<td>Fast</td>
<td>Right</td>
<td>104 (30.3%)</td>
<td>239 (69.7%)</td>
<td>343 (100%)</td>
</tr>
<tr>
<td>D1 10x</td>
<td>6 mo.</td>
<td>Slow</td>
<td>Right</td>
<td>235 (63.5%)</td>
<td>135 (36.5%)</td>
<td>370 (100%)</td>
</tr>
<tr>
<td>D2 10x</td>
<td>6 mo.</td>
<td>Fast</td>
<td>Right</td>
<td>194 (35.5%)</td>
<td>352 (64.5%)</td>
<td>546 (100%)</td>
</tr>
<tr>
<td>D2 10x</td>
<td>6 mo.</td>
<td>Slow</td>
<td>Right</td>
<td>387 (68.5%)</td>
<td>178 (31.5%)</td>
<td>565 (100%)</td>
</tr>
</tbody>
</table>

Comparisons between table 1 and 2 reveal composition differences in wild type and dwarf mice. As expected, the dwarf musculature has a higher percentage of slow twitch fibers. Although it is not a huge difference, the percentages do correlate with the normal percentage expected in both wild type and dwarf mice. The percentage of slow twitch fibers in SOL was 60-70% in dwarf compared to wild type of 50-60%.
Cross-sectional areas of soleus muscles in wild-type mice and Ames dwarf mice.

**Table 3:** Cross-sectional areas for 12 month old, wild type mouse N4. Five different cells, representative of the entire tissue sample were measured twice for increased accuracy in actual area for muscle cells. Measurements are in micrometers.

<table>
<thead>
<tr>
<th>Sample 12 H&amp;E R SOL N4</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell #</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2246</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2181</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3484</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3415</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1695</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1621</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1357</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1347</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3386</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3307</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average Cross-sectional Muscle Area</strong></td>
<td><strong>2403.9</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4:** Cross sectional area for 3 month old, wild-type mouse N2. Measurements are in micrometers. The bolded numbers are essential in the analysis for determining the cross-sectional area of muscle fiber cells.

<table>
<thead>
<tr>
<th>Sample 3 H&amp;E L SOL N2</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell #</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2471.01</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>2665.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2087.57</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>2238.25</td>
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<tr>
<td>5</td>
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<td></td>
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</tr>
<tr>
<td>6</td>
<td>3598.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2339.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2344.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2519.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2744.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average Cross-Sectional Muscle Area</strong></td>
<td><strong>2639.55</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Table 5: Cross sectional area for 6 month old, dwarf mouse, D1. Measurements are in micrometers. The bolded numbers are essential in the analysis of the cross-sectional area of the muscle fiber cells.

<table>
<thead>
<tr>
<th>Cell #</th>
<th>Area</th>
<th>Average Cross-Sectional Muscle Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>808.43</td>
<td>715.84</td>
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<tr>
<td>2</td>
<td>778.29</td>
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<tr>
<td>3</td>
<td>484.23</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>457.21</td>
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<td>5</td>
<td>659.84</td>
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<tr>
<td>6</td>
<td>653.6</td>
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<td>7</td>
<td>871.81</td>
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<td>841.68</td>
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</tr>
<tr>
<td>9</td>
<td>814.66</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>788.69</td>
<td></td>
</tr>
</tbody>
</table>

Comparing tables 3 and 4 show the mean cross-sectional area differences between the 3 month old and 12 month old wild type mice were not significant ($P=.478$). The average muscle area for the wild type 3 month old was 2639.55; the average muscle area for the 12 month old was 2403.9. However, differences were significant between dwarf and wild type cross-sectional areas ($P<.001$) with the dwarf mean area being about 25% the size of the wild type. The dwarf average muscle area was 715.84. This result was expected considering the smaller nature of the dwarf mouse.

Comparisons were drawn between the 3 month and 12 month normal mice and the 3 month normal mouse and the 3 month dwarf mouse. Using a two-tailed t-test analysis, the statistics for comparing the normal mice had a $p$ value = 0.478, standard deviation of 905.05 for the 12 month old, standard deviation of 491.7 for the 3 month old, standard error of 286.2 for the 12 month old, and standard error of 155.5 for the 3 month
old. The preceding analysis may demonstrate the differences seen as a wild type mouse ages. The percentage of muscle fibers, specifically fast twitch fibers decreases; additionally, cross-sectional muscle areas in the SOL decrease as well. These results would be expected in a typical aging process of a wild type mouse.

Comparisons between the 3 month old wild type mouse and 3 month old dwarf mouse were calculated using a two-tailed t-test analysis. Statistics demonstrated a $p$ value of less than 0.0001. Standard deviation of 147.3 and standard error of 46.58 for the dwarf mouse and standard deviation of 491.7 and standard error of 155.5 for the wild type mouse was calculated.

Conclusions that can be drawn from the comparison between the wild type and dwarf mice include a smaller cross-sectional muscle area in a dwarf mouse and no drastic change in the percentages of fast and slow twitch muscle fibers as the dwarf mouse ages. This is considered normal for a dwarf mouse; the percentage of fast twitch and slow twitch muscle fibers remain relatively constant as the mouse ages. Cross sectional muscle area was expected to be smaller in the dwarf mice considering their smaller size in comparison to the normal mice. Furthermore, it was also expected for there to be differences in fiber length and cross-sectional muscle area between wild type and dwarf mice.
CHAPTER V
DISCUSSION

This study analyzed the counts of fast-twitch and slow-twitch muscle fibers and cross-sectional muscle fiber areas of tissue samples. It allowed the student researchers to perform microscopic surgery to remove the lower extremity musculature, obtain muscle tissue samples utilizing a Cryostat, prepare and stain tissue samples, and learn how to operate software programs to analyze results and draw comparisons. This study also demonstrated some main differences in muscle size and fiber composition between wild type and Ames dwarf mice. It revealed typically observed percentages of fast and slow twitch muscle fibers and muscle areas for the two mice samples. The wild type mice were expected to have a change in muscle fiber composition as they aged; additionally, an overall larger surface area in the lower extremity musculature was expected due to the bigger size of this mouse. In contrast, the dwarf mice did not demonstrate a significant change in muscle fiber composition as they aged and their muscle surface area was much smaller compared to the wild type mice.

Some limitations of the study include small sample size and younger age of the mice. All mice were between 3 months-12 months of age. Much of the specific aging processes could not be well observed in this study secondary to the age of the mice involved. Thus, conclusions on the aging process and sarcopenic effects in mouse musculature cannot be formulated. Furthermore, only one dwarf mouse soleus muscle’s
fiber composition was able to be analyzed due to the limited access of the software program and time constraints.

Future studies should incorporate bigger sample sizes and newer software programs for analysis to determine actual muscle fiber composition changes and what those changes might indicate. Studies should also be performed on human subjects willing to undergo dietary restrictions and exercise protocol to determine if muscle fiber composition changes, or lack thereof, can be observed.
REFERENCES


