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## Muscle Unloading Induced Sex Specific Neurophysiological and Myofiber Profile Adaptations

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**MUSCLE UNLOADING INDUCED SEX SPECIFIC NEUROPHYSIOLOGICAL AND  
MYOFIBER PROFILE ADAPATIONS**

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of  
Science with Honors in Kinesiology and Health Sciences from  
The College of William and Mary

By

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### ABSTRACT

Muscle unloading affects a muscle's ability to produce a contractile force and it affects the muscle's endurance. The objective of this project was to investigate the differences between males and females and their neurophysiological adaptations to hindlimb suspension, an effective model of muscle unloading. Thirty nine young adult Wistar rats were divided into the following four groups: 1) male control, 2) female control, 3) male unloading, 4) female unloading. The unloading groups were subjected to a hindlimb suspension model. Soleus muscles were surgically removed to quantify neuromuscular function, and fluorescent fiber type staining was performed to quantify the cross-sectional area and fiber type composition. By using different stimulation protocols, muscle contraction was induced either directly or indirectly (via motor nerve terminals) and muscular force was quantified by a force transducer. Fluorescent staining was used to image type I and type II fibers. The results showed that over a 5 minute stimulation protocol, muscle fatigue was greater during indirect stimulation than direct stimulation, indicating that the motor neuron fatigues at a faster rate than the muscle fibers it innervates. Hindlimb suspension affected the females more than the males whether the muscle was stimulated directly or by the nerve. Unloading significantly increased the neuromuscular block over the five minute fatigue train only in the females. There was significant atrophy in the unloaded groups, but no sex-specific significant differences and no fiber type transitions. In summary, the muscle fatigue is likely due to fatigue in the neuron's ability to stimulate the muscle, and females are more affected by the hindlimb suspension than males. There was also unloading induced atrophy but it was not sex specific.

## INTRODUCTION

The central nervous system controls skeletal muscle by sending action potentials through the central and peripheral nervous systems which then terminate in a neuromuscular junction. Excitation-contraction coupling is the following series of events that allow an action potential to result in contraction of the muscle. The action potential in the nerve triggers a release of acetylcholine, stored in vesicles at the pre-synaptic nerve terminal into the synaptic cleft. When the acetylcholine binds to the motor end plate on the muscle fiber the ion permeability changes on the fiber membrane and creates an endplate membrane potential. The acetylcholine is broken down by post-synaptic acetylcholinesterases, allowing the choline to be reabsorbed. The endplate potential, in turn, results in an action potential on the sarcolemma that is conducted across the entire myofiber (Wood & Slatter, 2001). The next important structure in this process of excitation-contraction coupling is the transverse tubule, or the T-tubule.

The T-tubule is a membranous structure where the surface membrane folds into the cell to run perpendicular to the surface. When the action potential reaches the T-tubule, the membrane potential changes are quickly transmitted to the interior of the cell. The permeability changes are then transmitted to an internal structure closely associated with the T-tubule, the sarcoplasmic reticulum. The sarcoplasmic reticulum is a modified form of the endoplasmic reticulum. The T-tubule has membrane proteins called dihydropyridine (DHP) receptors that act as a voltage sensor to sense the change in voltage along the membrane in the T-tubule. The DHP receptor is associated with ryanodine receptors on the sarcoplasmic reticulum. The DHP receptor will induce a

conformational change in the ryanodine receptor which results in the release of calcium ions from the sarcoplasmic reticulum (Block et al., 1988).

According to what is known as the sliding filament theory, the calcium that is released from the sarcoplasmic reticulum is what starts the muscle contraction of the contractile filaments, actin and myosin. The calcium binds to troponin and moves it so that binding sites on tropomyosin are exposed. Both troponin and tropomyosin, along with actin, are considered thin myofilaments. Myosin is considered the thick myofilament. Energized cross bridges on the myosin are then able to bind to the exposed binding site on actin. The movement of the cross bridge is called the power stroke and slides the actin over the myosin, which is why it is called the sliding filament theory. In skeletal muscle contraction, is stopped when the cytosolic calcium is removed by being reabsorbed by the sarcoplasmic reticulum (Huxley, 1995).

There is a slight lag in time between the arrival of the action potential and the muscle beginning its contraction, known as the latency period. Following the latency period the muscle begins to contract and produce force. The time between the end of the latency period (start of contraction) and the peak force created by the muscle is the contraction time. The relaxation time is the time from the peak force to when the muscle completely relaxes. Figure 1 illustrates this.

The muscle as a whole unit contracts to produce force and sometimes movement in the body. The force of the contraction is determined by several factors. More specifically, the number of motor units recruited is proportional to the amount of force produced. A motor unit is a single motor neuron that originates in the spinal cord and all

of the myofibers that it innervates (Scott et al., 2001). When the nerve fires an action potential all of the fibers in the motor unit are stimulated. A greater number of motor units that are recruited results in greater force production. Increasing the frequency of neuronal action potential firing also increases the force produced. The muscle length also plays a role in determining the amount of force produced. If the muscle is longer or shorter than its optimal length, it will not be able to produce an optimal amount of force because it will have too few or too many thick and thin filaments overlapping respectively. Contraction speed plays a variable role in force production depending on the type of contraction. For concentric contractions (as studied in this paper), an increased speed of contraction decreases the force produced in a hyperbolic pattern. Fiber type composition of the muscle also has an effect on the amount of force produced (Salo, 2015). Fiber types will be further explored later.

### Adaptability of Neuromuscular System

One situation that affects the ability of the muscles to contract to produce strength is when the muscle is not stimulated or used for a prolonged length of time. In this instance the muscle is said to be unloaded, because it is not experiencing any load on it. Humans may experience muscle unloading in the case of bed rest or immobilization following surgery or injury. Disuse as a result of inactivity may also be increasing with the current increase in chronic illnesses including cardiovascular disease, diabetes, obesity and osteoarthritis (Blair et al., 1995). Orthopedic surgeries are one such surgery that often requires prolonged immobilization. For example, total knee arthroplasty, or knee replacement, is an orthopedic surgery that requires an average of three months before patients are considered totally recovered. In the first two to four

weeks, patients are advised to use a walker or crutches and then transition to a cane (Total Knee Replacement). During the recovery time the patient is greatly limited in mobility and their muscles experience the same unloading effects as the rats in this study. In the United States there are more than 600,000 knee replacements each year and with an increasingly aging and obese population, by 2030 the demand for knee replacement surgery is expected to exceed 3 million (Beyond Surgery Day). Given the massive number of knee replacements alone, the results of this study and their future impact on clinical care will be essential in improving surgical outcomes.

A small subset of the population also experiences unloading due to microgravity when outside the Earth's gravitational field in outer space. Unloading occurs because gravity is no longer providing resistance, so the muscles are no longer forced to produce the force to overcome gravity to support the body. Continued space exploration and experimentation have resulted in an increased interest of these effects of microgravity on the body during longer flights and stays on the International Space Station. Under NASA's Human Exploration and Operations Mission Directorate, NASA has had an increased interest in understanding of the detrimental effects of spaceflight on the neuromuscular system. In total, 547 people have been in space for an approximate total of 123 person-years (Astronaut/Cosmonaut Statistics). These people and the increasing number of people going into space provide further incentive to study the effects of unloading on skeletal muscle.

### Effects of Unloading

Atrophy It has been established that unloading causes atrophy or the loss of muscle tissue. After a hindlimb suspension of 12.5 days, soleus muscles of rats showed significantly lighter weights, smaller myofiber cross-sectional areas, angular myofibers, myofibril disruption and more myofibers expressing fast myosin (Krippendorf, 1993). Similar results were found when the rats were sent into space to experience microgravity, which is what unloading simulates. Staron et al. (1998) measured atrophy in rats orbiting Earth for 10 and 14 days and found significant muscle atrophy compared to control groups in the ground, but did not find any significant differences between the groups in space for 10 or 14 days. Similar results of unloading induced atrophy have been found in other muscles in the leg including the extensor digitorum longus (EDL) and gastrocnemius (Schuenker et al., 2009).

Function Another aspect of unloading is its effect on the amount of force that the muscle produces, or its ability to function. A study that measured the contractile properties of soleus muscle following 14 days in microgravity found a significant decrease in maximal isometric tension (-37%), and an increase in maximal shortening velocity (+20%) (Caiozzo et al., 1996). The increased shortening velocity has been attributed to greater expression of the fast isoform of myosin heavy chain. Another study that examined rats after a 6-day spaceflight mission measured the soleus muscles' in situ contractile measurements. The maximum force produced was decreased by 24% and maximal shortening velocity was increased by 14%. The muscles were subjected to a two minute endurance test which showed a significant



increase in fatigability in those exposed to microgravity. This experiment showed that the changes due to microgravity can happen in a little as one week (Caiozzo, 1994).

Some studies have isolated soleus muscle fibers in vitro and measured the contractile force of isolated, single muscle fibers. Holy and Mounier (1991) studied fibers (not specified by type) when under maximal calcium activation conditions. They found a decrease in maximum tension and proposed that this may have been caused by a decrease in fiber diameter. They also found an increase in time to maximum force, again, likely due to increased expression of fast myosin heavy chain.

Fiber Composition There are now four different classification systems that categorize muscle fibers based on their morphological, histochemical, biochemical or physiological characteristics. Often the same fiber can fall into multiple different categories depending on the classification system used. Differences in myofiber composition allow for the differences in muscle capability, and different muscles have different myofiber compositions to allow the muscle to perform differently (Delp & Duan, 1996). Frequently fibers are divided into three categories: slow-oxidative (SO, type I), fast-oxidative-glycolytic (FOG, type II), and fast-glycolytic (FG, type IIb). The type I, II and IIb schemes correspond to isoforms identified by myosin ATPase staining, and to myosin heavy chain isoforms. The SO, FG, and FOG scheme is based on biochemical metabolic pathways. Both humans and rats have type I and type II fibers. However, humans do not express type IIb and instead express the analogous type IIx fibers (Scott et al., 2001).

The nerves innervating these different fiber types also can be assigned to different categories featuring different structural and functional properties. Fast twitch fibers are innervated by nerves that have an above average pre-terminal axon diameter and fire at a rate that is six times faster than the smaller neurons which innervate the slow-oxidative fibers (Eccles et al., 1958). Additionally, differences in myoglobin content permit fibers to be classified as either red or white muscle tissue (Glancy & Balaban, 2011).

In reaction to changing demands, myofibers can change their size (atrophy, or hypertrophy, as previously discussed) or fiber type composition. Changing the fiber type composition involves changing fibers' phenotypic types from one type to another, called fiber type transitions. Fibers change in an orderly graded fashion along the fast-slow spectrum with respect to a functional transition, as they change from slow twitch to fast twitch or vice-versa. This can be seen in the changes of the myosin heavy chain expressed (Pette & Staron, 1997). Fibers may also change in a similar orderly fashion along a metabolic spectrum in the direction of glycolytic or aerobic-oxidative. The resulting transition depends on the fiber phenotype as well as its initial location on the fiber spectrum (Pette, 2002). Following hindlimb suspension, studies have found that there is a slow to fast transformation of muscle fibers (Nemirovskaia, 2005, Kasper, 1993). The increase in fast twitch fibers accounts for the increased contractile velocity observed in fibers that have been unloaded.

### Gender Differences

It is important that unloading research address the potential differences between sex specific adaptations. Comparing the differences between male and female neuromuscular systems and their response to spaceflight was not important when NASA was first founded, but since Sally Ride became the first American woman in space in 1983, women have been joining the ranks of NASA astronauts venturing into space (Maugh). To date NASA has sent 48 women into space (Barry). Figure 2 illustrates a rapid growth rate in the percentage of space flights occupied by women, with 1 of 5 current rides occupied by females. Accordingly, NASA is placing an increased emphasis on the female personnel that participate in spaceflight missions. NASA's Women@NASA website, created under the White House's Council on Women and Girls, claims to "aspire to inspire" young women and is the "federal response to issues that have a distinct impact on women and girls" (Women@NASA). However, it is important for NASA to directly address the biological needs of females traveling in space, acknowledge that the female body is different from that of males, and not simply try to recruit women without properly taking care of them. NASA has a responsibility to the 48 women they have already sent to space, and the many more that surely will go in the future. Before being asked to participate in spaceflight missions, women should have an explanation of how microgravity will impact their ability to perform the physical tasks assigned to them, and any long term effects they may experience.

It previously was established that when subjected to the same amount of unloading, women suffer greater losses of muscular strength than men (Deschenes et al., 2009, Deschenes et al., 2012). Using this model with men and women, it was

established not only that women suffer more severe losses in strength as a result of unloading, but also that declines in strength were significantly correlated with decrements in neural activation of the muscle as assessed by electromyography (EMG). This decreased EMG may be caused by a disturbance in nerve to muscle communication. This notion is supported by the work of Yasuda et al. (2005) who found that although women experienced significantly greater losses of muscle strength than men following unloading, muscle fiber atrophy did not differ between men and women. This additionally suggests that gender specific adaptations to unloading are likely to be based in the nervous system.

### Present Study

The currently available information shows that muscle unloading, both caused by hindlimb suspension and microgravity, changes the functional demands of skeletal muscles and consequently alters the muscles' capacity. This study uniquely investigates the effect of muscle unloading and sex on postural muscles on muscle function and myofiber profiles. When testing the muscle function, this study permits the determination of the difference of contraction when muscle is stimulated indirectly via the nerve or directly via the sarcolemma. This distinction allows the dissection of muscle and neuron fatigue. It allows us to determine if decreases in contractile force are due to fatigue of the contractile apparatus, or failure of nerve to muscle communication. The gender distinction represents an opportunity to determine if males and females respond to muscle unloading differently. This study in particular examines the soleus muscle of the rat because, as the primary postural muscle, it is the most affected by hindlimb suspension (Hennig, 1985).

This study is an *in vitro* rat study. Animal studies have been conducted in addition to human studies on muscle unloading for several reasons. Human studies can be hard to control for all possibly confounding variables. In order to measure unloading a healthy human must consent to immobilizing part of their body for a period of time. The situations that medically require immobilization, such as injury or surgery, may not be able to be predicted. Those who undergo a surgery may not be in the best of health, which may make it difficult to control for variables. Using animals allows for much more control, including their exact age, environment, and diet.

Jenny Haliski, a representative for the National Institutes of Health (NIH) Office of Laboratory Animal Welfare, defends rats as subjects in experiments to be applied to humans. She said, "Rats and mice are mammals that share many processes with humans and are appropriate for use to answer many questions" (Melina, 2010). Rats were also the animal chosen by Emily R. Morey when she created the hindlimb suspension model to emulate the effects of spaceflight. She was working for the National Aeronautics and Space Administration (NASA)-Ames Research Center and had been working with rats that had been orbiting Earth under microgravity, but wanted to develop an alternative method to study muscle and bone that did not require a very expensive and carefully planned spaceflight (1979). This model lifts the rats by the tail to produce a 30 degrees head-down tilt that shifts the cephalad fluid and prevents the hindquarters from bearing any weight. Morey and many others have investigated all of the detailed aspects of this model over the last several decades. As recently as 2002, Morey's lab supports this model for studying musculoskeletal unloading (Morey-Holton & Globus, 2002). Unloading, as imparted by unilateral lower suspension, simulates

microgravity in space by removing the effects of gravity that are usually experienced by the muscle when they support the body (Berg et al., 1991).

## METHODS

### Subjects

Four groups of young adult (6 month old) Wistar rats: 1) male control (N=10|group), 2) female control (N=10|group), 3) male unloading (N=10|group), 4) female unloading (N=9|group) served as subjects for this investigation. Unloading conditions were imparted by the hind limb suspension model of Morey that was created to simulate the effects of microgravity as previously discussed (1979). Control rats were subjected to normal housing conditions with two rats per tub. All rats consumed food and water ad libitum.

### Data Collection

Physiological Function Following a two week intervention period, rats were readied for testing of neurological function. Animals to be tested were anesthetized with a cocktail of ketamine and xylazine. Soleus muscles were quickly removed and weighed. They were then used for an ex vivo procedure (Aurora Scientific Instruments Model 807B *in situ* large rodent/small animal apparatus, Aurora, ON, Figure 3) to quantify neuromuscular function. By using unique stimulation protocols, muscle contraction was induced either directly or indirectly (by way of nerve terminal excitation) and muscular force was quantified by a force transducer and graphed using ASI Dynamic Muscle Control Data Analysis (Aurora Scientific Instruments, Aurora, ON, Figure 4). The muscle was stimulated indirectly by way of nerve terminal excitation, and directly by

stimulating the muscle. The nerve alone was stimulated by a shorter electrical pulse (0.2 ms) than the pulse that was used to stimulate the muscle (2 ms). The nerve has a lower threshold and therefore is able to produce an action potential by a weaker pulse. A longer electrical pulse stimulated both the nerve and the muscle by allowing the muscle to reach its threshold for an action potential without stimulation from the nerve.

In order to assess the muscle fatigue and whether fatigue is due to neural fatigue and not solely muscle fatigue, the stimulation protocol included a pre-test to establish a baseline function and a fatigue train to induce fatigue in the muscle and nerve, with the final two stimulations providing the post-test data. Each set of stimulations both stimulated the muscle indirectly via the nerve and then thirty seconds later stimulated the muscle directly. Each stimulation was 0.5 seconds long. The fatigue train started 30 seconds after the pre-test. The individual fatigue trains consisted of 9 indirect stimulations lasting 0.2 milliseconds every three seconds followed by a tenth and final single direct stimulation lasting 2 milliseconds. Therefore the fatigue train was 30 seconds long. The entire protocol consisted of 10 sets (trains) together lasting five minutes. The post-test was conducted one minute after the fatigue train. All of the muscle stimulations were set at 37 volts and 25 hertz.

The following parameters were used to assess the pre-test (before the five minute protocol), and post-test (last two stimulations of the endurance test): 1) maximum force, 2) specific tension, 3) neuromuscular block, 4) time to peak force. The maximum force is the maximum amount of force the muscle can produce and was measured in grams (g). The specific tension assesses the force produced per milligram of the weight of the muscle and was measured in grams per milligram (g/mg).

Neuromuscular block is the difference between the maximum force produced by indirect stimulation and direct stimulations over the maximum force produced by direct stimulation and is expressed as a percentage (%). This measure determines how much the nerve affected the amount of force the muscle could produce, in other words, the neuromuscular efficiency. The time to maximum force, measured in seconds (s) is a measure of muscle power and is the amount of time that it takes for the muscle to reach its maximum force production. To assess the muscle endurance, the percent difference was calculated between the first stimulations of the endurance test and the last stimulations.

After the stimulation protocol the muscle was quickly frozen at resting length in isopentane chilled with liquid nitrogen before being stored at  $-80^{\circ}\text{C}$ .

Myofiber Profiles Following this physiological analysis of unloading-induced remodeling of soleus muscles in male and female rats, a morphometric analysis was performed in those same muscles. A Cryostat (Cryocut 1800; Reichert-Jung, NuBloch, Germany) was used to section the muscles cross-sectionally into 10  $\mu\text{m}$  thick slices onto a microscope slide pretreated with a 3% ethylenediaminetetraacetic acid (EDTA) to prevent muscle contraction as previously described (Pearson and Sabarra 1974). As a chelating agent, the EDTA binds to calcium metal ions in the muscle and prevents them from causing the fibers to contract (Haemek 2012). A Pap pen was used to create hydrophobic wells around the sections of muscles tissue. A 50  $\mu\text{L}$  sample of Superblock (ScyTek Laboratories, West Logan, UT) was applied to each section and the sections were incubated at room temperature for 60 minutes. The muscles were then washed 2 x 2 minutes in with phosphate buffered saline (PBS) with 1% bovine



serum albumin (BSA). The second incubation was for another 60 minutes in a humidified chamber at 37°C with a supernatant of the following primary antibodies and PBS with 1% BSA: BA-D5 (Developmental Studies Hybridoma Bank, University of Iowa) at a concentration of 1:10 to stain type I fibers, and SC-71 (Developmental Studies Hybridoma Bank, University of Iowa) at a concentration of 1:1 to stain type II fibers. The second washing was conducted with 50 µL of PBS with 1% BSA 3 x 5 minutes. The third incubation was for 30 minutes in a humidified chamber at 37°C with a supernatant of the following secondary antibodies and PBS with 1% BSA and 5% goat serum: Alexa 555 IgG2b (Molecular Probes, Eugene, OR) at a concentration of 1:500 to bind to the BA-D5 on the type I fibers, and Alexa 350 IgG1 (Molecular Probes, Eugene, OR) at a concentration of 1:500 to bind to the SC-71 on the type II fibers. The secondary antibodies were necessary to allow the fibers to fluoresce under a microscope. The third washing used 50 µL of PBS with 1% BSA 3 x 5 minutes. The microscope slides with the muscles were then washed with de-ionized water for 30 seconds and allowed to dry. The muscles were subsequently lightly coated with ProLong (Molecular Probes, Eugene, OR) and covered with a cover slip. After allowing the ProLong to dry the slides were examined under a microscope or stored at 4°C.

Microscopy An Olympus BX41 microscope (Olympus America, Melville, NY) was used to assess the myofiber profiles with a 100X objective. Images of the type I and type II were captured individually with Infinity Analyze software (Lumenera Corporation, Ottawa, ON). These images were overlaid using Adobe Photoshop Elements (Adobe, San Jose, CA) on one another to create a single image with all of the fiber types fluorescing (Figure 5). The myofiber cross-sectional areas of the type I and type II

fibers were quantified with the Infinity Analyze software, as shown in Figure 6. A random sample of 100-150 myofibers from each muscle was selected to be analyzed to determine the average myofiber size (cross-sectional area) and fiber type composition.

### Statistical Analysis

The physiological function and myofiber profile data collected was compared and analyzed using two-way ANOVA tests with main effects of unloading and gender. In the event of significant main or interactive effects, a Tukey post-hoc test was used to identify significant pair-wise differences. In all measures, significance was set at  $p \leq 0.05$ .

## RESULTS

### Function Performance

The results showed that hindlimb suspension resulted in a number of neurophysiological adaptations in both males and females. For the maximum force with neural stimulation, there were main effects for sex with the males exhibiting greater than the females, and for treatment with the control groups being greater than the hindlimb suspended groups. However, there was no interaction. Post-hoc analysis revealed that the male maximum force is greater than the female maximum force at the pre- but not the post-test. Post-hoc analysis also showed that the control groups produced more force than the hindlimb suspended groups at the pre- but not the post-test. For the maximum force produced with muscle stimulation, there was a main effect for treatment but not for sex. The post-hoc shows that the control groups produced more force in both the pre- and post-tests.

There were no significant differences between treatments or sex for specific tension when stimulated indirectly or directly. However, there were significant decreases between the pre- and post-test specific tensions of all four treatment groups when stimulated directly and indirectly.

The time to peak force showed a main effect between sexes when indirectly stimulated but not between the treatment groups, with no interaction. Post-hoc analysis shows the male hindlimb suspended group reached peak force faster than the female hindlimb suspended and control groups in the pre-test, but not the post-test. When stimulated directly, there was a pre- to post-test increase in time to peak force in all four treatment groups. When stimulated directly, there was a main effect for sex with females having a longer time to peak force, but no main effect for treatment and no interaction. Post-hoc analysis shows no treatment effect at the pre-test but a significant difference between treatments at the post-test, with the male hindlimb suspended being faster than the other three treatment groups. All of the groups got slower between the pre- and post-tests when stimulated directly.

There were no main effects for neuromuscular block for either sex or treatment, and no interaction. There was a significant increase in neuromuscular block between the pre- and post-tests for all four treatment groups. The muscle is able to produce a stronger contraction when stimulated directly than when the muscle is stimulated by the nerve.

The five minute endurance test showed that, when combined, neural fatigue is greater than muscle fatigue, as indicated by the larger percent decline when stimulated

by the neuron. The hindlimb suspended females only showed a significant difference from the rest of the treatment groups in the change in force produced over the endurance test. They did not display the same decrease in force production that the other treatment groups did. As a result, the neuromuscular block was significantly smaller than that of the female control group. Table 1 shows the data collected for the pre- and post-tests and the percent decline over the five minute endurance test.

### Myofiber Profiles

Cross-Sectional Area Data collapsed across myofiber type showed that significant unloading-induced fiber atrophy was seen in both genders. Significant fiber atrophy was also seen in both unloaded groups for both type I and type II fibers, but males and females showed the comparable amounts of atrophy (21% and 25%, respectively). There was no statistically significant difference between the male and female controls, or between the male and female unloaded. However there was a significant difference between the male control and female unloaded groups.

When considering the type I, or slow twitch, fibers alone, there were significant differences for unloading and gender, with the females having smaller fibers, and for unloading. Male and female fibers atrophied similarly again (21% and 18%, respectively). The male control group had larger fibers than the three other groups. There was no difference between the male and female unloaded groups.

When considering the type II, or fast twitch fibers alone, there was a significant difference for unloading in both sexes, but there was no difference for gender. The control fibers were larger than the unloaded fibers. In males there was a 35% decrease

in cross-sectional area as a result of unloading, while hindlimb suspension elicited a 46% decrease in females. The cross-sectional area data is shown in Table 2.

Fiber Composition Fiber composition showed no significant changes for all of the groups. In all treatment groups, type I fibers were predominantly expressed in soleus muscles. These data are displayed in Table 3.

## DISCUSSION

As previously established, muscles experiencing different functional demands from usual usage, such as in microgravity or rest from injury or disuse, respond with alterations to their physiological functions and myofiber profiles. The disuse generally results in decreases in maximum contractile strength, greater fatigue and increases in time to peak force. These muscles also display atrophy seen as decreases in cross-sectional area, and sometimes alterations in muscle endurance (Caiozzo, 1994, Caiozzo et al., 1996, Krippendorf, 1993, Staron et al., 1998).

Unloading resulted in sex adaptations to the muscles' performance. The data on the maximum force reveal that the males were stronger than the females when stimulated indirectly and directly at the pre-test. This difference disappeared when looking at specific tension, so this difference is likely attributable to larger muscle mass in males. With regards to treatment and maximum force, the control groups produced more force than the hindlimb suspended groups when stimulated indirectly and directly at the pre- and post-tests. The control groups were stronger than the hindlimb suspended groups after the endurance test, but this difference was not seen when the muscles were

stimulated via the neuron. Therefore, the hindlimb suspension resulted in greater fatigue of the nerve.

The differences in maximum force production between the indirect and direct stimulations in the post-test indicate that fatigue is greater when stimulated via the nerve. Therefore in *in vivo* conditions the fatigue observed in continuously contracting skeletal muscle is more closely related to nervous system failure than failure of the muscle's contractile fibers. A step along the neuromuscular junction stimulation process is more responsible for the fatigue seen than the process of muscle filament contraction. It may be due to acetylcholine not being recycled fast enough to maintain the same amount of contraction. Further research will need to be done on the calcium and acetylcholine's role in this process.

Hindlimb suspension affected the females more than the males whether the muscle was stimulated directly or by the nerve at the end of the five minute stimulation protocol. Unloading significantly increased muscle endurance in the females only. This is likely related to weaker muscles that cannot produce as much force to begin with, maintaining force better. All of the muscles lost strength, but it is reasonable that the female unloaded muscles who started with less strength, resulted in less decline of strength over the prolonged series of muscle contractions.

Since unloading only increased the endurance in the females, there is likely a physiological difference between males and females, in the neuromuscular junction or possibly the contractile apparatus that makes the females more susceptible to the effects of unloading. Hunter (2014) reviewed and summarized some possible

mechanisms for this difference, and some of them many relate to changes that occur under unloading conditions. Men are usually stronger than women due to larger fiber size, and their ability to produce a larger force during the initial contractions leads to greater fatigue, but this is not always the case. The greater muscular strength of males was shown in this study in the pre-test in which the females had significantly smaller maximal contraction. Male muscles experienced larger declines in strength over the 5 minute protocol. However, muscle size and strength should not explain the differences in sensitivity to unloading because both male and female soleus muscles experienced similar unloading-induced atrophy. Hunter (2014) considered differences in blood flow and muscle perfusion as a possible mechanism. Decreased blood supply to a muscle increases its fatigability. Unloading may result in a change in the soleus' blood supply, possibly decreasing its nutrient supply and waste removal to a greater extent in females than in males. There may also be sex specific differences between the cardiovascular systems. Differing fiber types, metabolisms, and endocrine difference can also have an impact on fatigability, and could be differentially affected by unloading.

The absence of significant differences for specific tension, when stimulated both directly and indirectly, means that the force produced relative to the muscle mass was the same for males and females, and for the control and atrophied unloaded groups. However, significant differences did exist between the pre- and post-test groups. Therefore, regardless of gender or treatment, after an endurance test, less force is able to be produced relative to the muscle mass. This finding demonstrates the impact of muscle fatigue on performance, whereby "muscle quality" is unsurprisingly diminished in fatigued muscle.

The male hindlimb suspended group was significantly faster than the male control group and both female groups in the pre-test when stimulated by the neuron. Unloading caused the males to have a faster neural stimulation than the other groups. However, this difference disappears in the post-test. The endurance test may fatigue the neuromuscular junction and eliminate this speed in the post-test. All of the groups slowed from their pre-test to their post-test, supporting the hypothesis that the endurance test fatigues the muscle and the nerve, and therefore achieving maximum force takes more time and muscle power is reduced.

The absence of difference in neuromuscular block between male and female muscles in the pre- and post-tests, whether in rested or exhausted states, shows that sex did not have an impact on neuromuscular transmission. Each of the treatment groups showed significant increases in the percent of neuromuscular block. As a result, it is fair to say that while unloading alters neuromuscular transmission, it does so in a non-specific manner. The fact that there was no difference between treatment groups means that sex and unloading negatively influenced neuromuscular transmission equally.

Results from the strength decline in the five minute endurance test further supports that nervous system fatigue is more pronounced than muscle fatigue, indicating disturbances at motor nerve terminals. For each group, there was a greater percent decline when the muscle was stimulated indirectly than when stimulated directly. As a result, the neuromuscular block percentages showed large negative differences, indicating that the neuron fatigue is larger.



Regarding myofiber type profile, this study demonstrated that unloading caused significant atrophy in both types of fibers, as was to be expected based on previous literature (Krippendorf, 1993, Staron et al., 1998, Schuenker et al., 2009). Since males and females showed comparable amounts of atrophy, the mechanisms for atrophy appear not to be sex-specific with respect to unloading. Overall the males had larger muscle mass, which is likely the mechanism for the greater strength produced by males. Conversely the unloading-induced atrophy that was observed may be the cause of the unloading-induced loss of strength (Kasper, 1993).

The female groups had smaller type I fibers than the males. The fibers also showed unloading specific atrophy, but there was no difference in atrophy between the sexes. With respect to the type II fibers, there was a significant difference for unloading in both sexes but no difference for sex. The difference between the fibers shows that there were more unloading induced adaptations to the type II fibers than to the type I fibers.

There were no unloading-induced changes in fiber type composition. This finding is in opposition to other studies that have found slow to fast fiber transitions (Baldwin et al., 1994, Kasper et al., 1993, Nemirovskaia et al., 2005). However, the significantly faster time to peak force in the male hindlimb suspended groups may be a result of slow to fast transitions that were not statistically significant enough to be found by the two way ANOVA and shorter period of unloading used in our study. Kasper et al. (1993) imparted unloading conditions on rats for 7, 14, and 28 days consecutively, while Nemirovskaia et al. (2005) examined rats after 19 and 33 days in microgravity and saw fiber transitions. When the muscle is unloaded for longer periods of time it has more time for the myosin heavy chains to make the transition from type I to type II fibers.

In summary, this study shows that unloading causes neuromuscular transition failure through greater nervous system fatigue than muscle fatigue. This effect is also more pronounced in females, suggesting that they are more susceptible to the effects of unloading. Unloading also causes atrophy, but there was no sex specific aspect to the atrophy. There was also no evidence of fiber transitions. The impact of these data may point to the need for women to specifically prepare for space flight or scheduled surgery. Such preparations may include prehabilitation which has been shown to reduce the effects of muscle disuse (Ditmyer et al., 2002, Santa Mina et al., 2014). Prehabilitation can be used to increase muscle mass and strength prior to expected periods of disuse. Still open for question though is whether prehabilitative training will prevent or mitigate disturbances in the function of motor nerve terminal ending during a protocol train of neural stimuli. Further research may allow the development of sex specific prehabilitation programs that will effectively target women.

The data also pinpoints one source of fatigue as somewhere along the neuromuscular transmission process. Identifying this can allow clinicians to develop more targeted programs to help prevent this. One such intervention can include the electrical stimulations that are used following spinal cord injury. Functional electrical stimulation helps improve function and decrease atrophy (Carty et al., 2013, Liu, 2013, Lee et al., 2015). This study suggests that muscles undergoing disuse from surgery, injury or microgravity can benefit from such stimulation by directly targeting the neuromuscular junction as a source of fatigue.

Other researchers have been investigating a wide variety of other effective interventions to support recovery following disuse. Insulin-like growth factor-I (IGF-I) is

being studied as a therapy to employ during disuse to attenuate atrophy and improve rehabilitation afterwards. It works by acting on satellite cell proliferation, differentiation, and survival as well as improving muscle fibers' protein synthesis and decreasing protein degradation. Parks et al. (2014) has focused on which form of IGF-I can have the greatest impact and has found that mature IGF-I is the best at preserving soleus muscle force production as well as expediting recovery upon reloading. They found that this was due to the fact that mature IGF-I downregulated atrophy promoting genes. Another study investigated the effects of tea catechins, an antioxidant found in green tea, on the negative effects of unloading. The tea catechins were found to minimize contractile dysfunction and atrophy. It was hypothesized that the antioxidant activity prevents oxidative modification of proteins in the muscle fibers (Ota et al., 2011). These and other studies show that therapeutic steps can be taken to prevent the negative effects of muscle disuse. This study emphasizes the need to recognize the differences between males and females and potentially developing sex specific protocols for such therapies. Moreover, interventions must be effective not only on muscle tissue, but also the motor neurons that excite muscle tissue.

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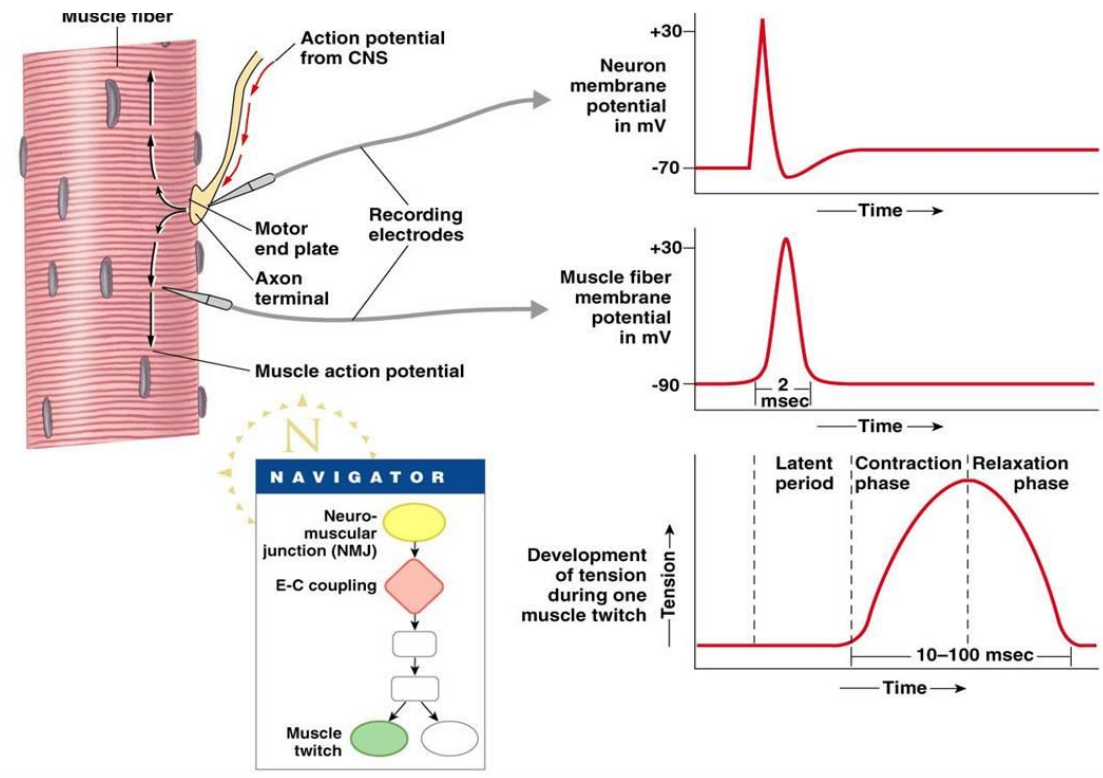
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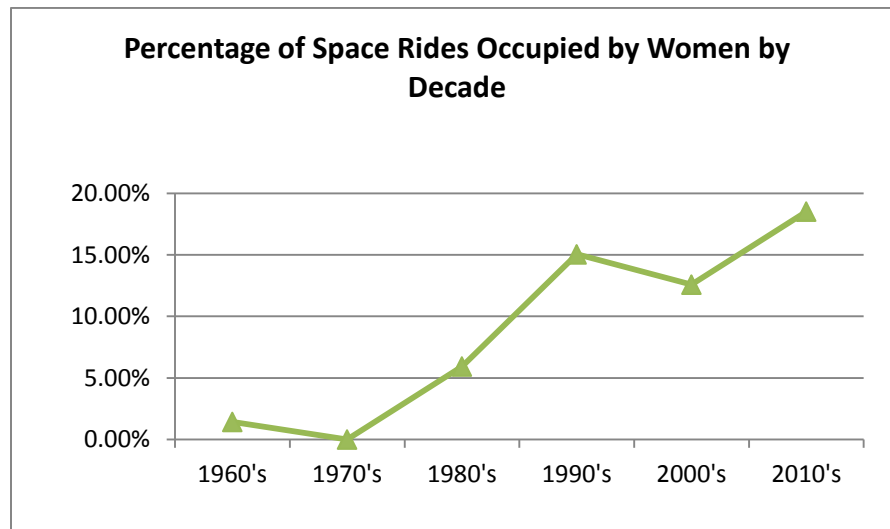
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**FIGURES AND TABLES**



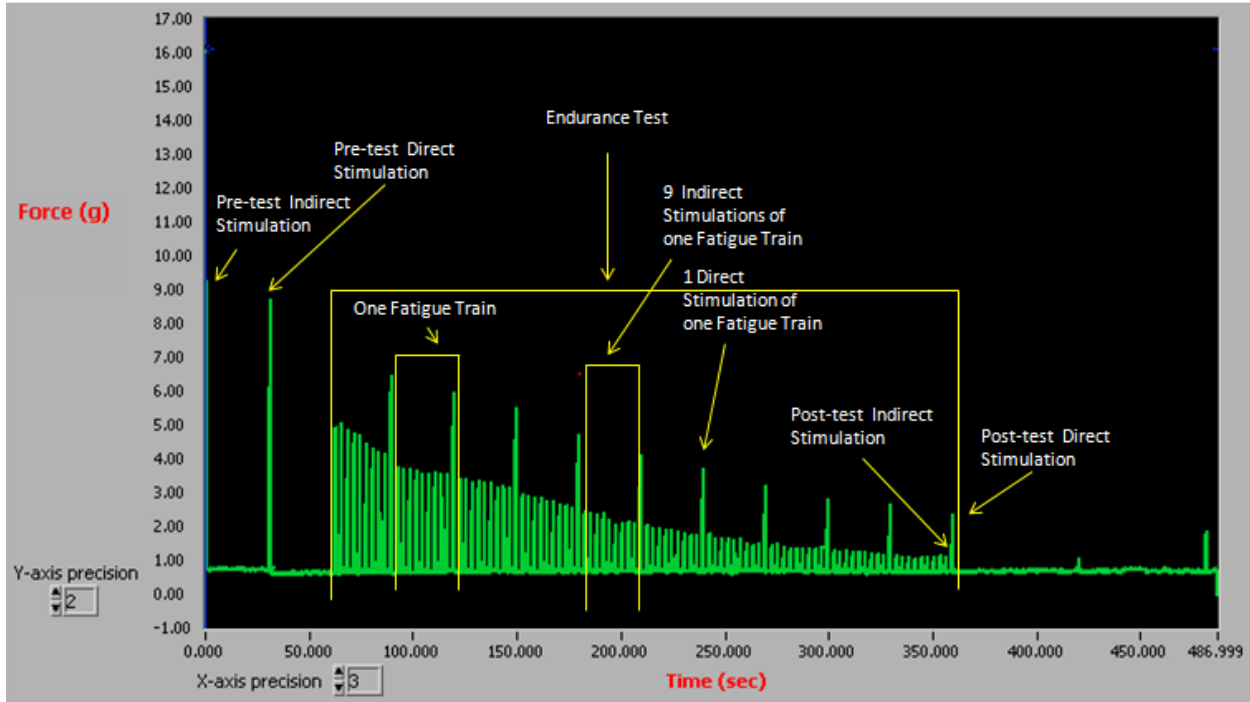
**Figure 1.** Graph illustrating latency period, Muscular System



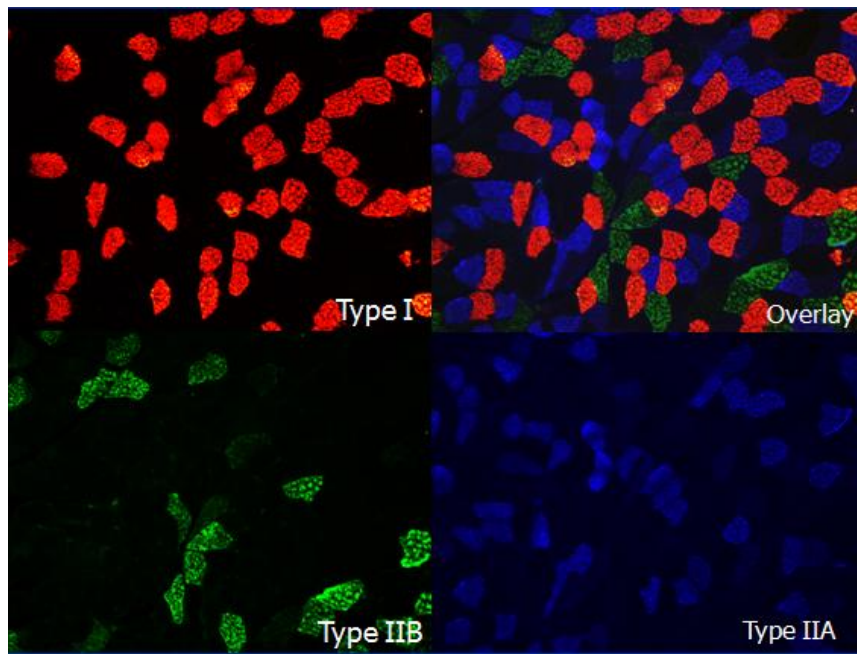
**Figure 2.** Percentage of Rides to Space Occupied by Women. (Based on individual ride data obtained from List of Female Astronauts; Braeunig.)



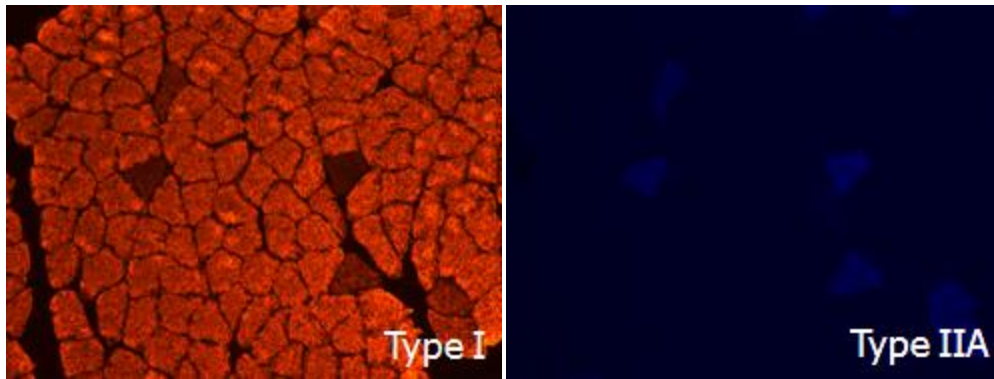
**Figure 3.** Aurora Scientific Instruments Model 807B *in situ* large rodent/small animal apparatus that was used to stimulate and measure the contractile force. The inset shows a muscle on the apparatus.



**Figure 4.** Annotated demonstration of the graph produced in ASI Dynamic Muscle Control Data Analysis.



**Figure 5.** Picture illustrating how individual images of fibers are overlaid to produce one image. This example is not from a soleus muscle due to the large presence of type IIB fibers. Magnification at 100x.



**Figure 6.** Image taken from a muscle in this study. Magnification at 100x.



**Table 1.** Data for the Pre- and Post-tests and the Percent Decline over the Five Minute Endurance Test.

Pre-Test	Male Control	Male HS	Female Control	Female HS
<b>Max Force (g)</b>				
<i>Neural Stimulation</i>	45.110±6.3	29.500±4.9 <sup>†</sup>	31.767±3.8 <sup>‡</sup>	14.840±2.2 <sup>†‡</sup>
<i>Muscle Stimulation</i>	48.560±6.2	30.060±5.0 <sup>†</sup>	38.167±4.3	20.630±3.0 <sup>†</sup>
<b>Specific Tension (g/mg)</b>				
<i>Neural Stimulation</i>	0.242±0.03	0.240±0.03	0.276±0.04	0.170±0.03
<i>Muscle Stimulation</i>	0.263±0.03	0.244±0.03	0.331±0.05	0.224±0.03
<b>Neuromuscular Block (%)</b>	5.470±11.7	-0.067±6.8	16.060±3.9	26.324±8.64
<b>Time to Peak Force (s)</b>				
<i>Neural Stimulation</i>	0.754±0.03	0.634±0.05 <sup>&amp;</sup>	0.802±0.01	0.780±0.02
<i>Muscle Stimulation</i>	0.751±0.03	0.754±0.01	0.816±0.05	0.804±0.004

Post-Test Data	Male Control	Male HS	Female Control	Female HS
<b>Max Force (g)</b>				
<i>Neural Stimulation</i>	12.070±2.6	9.048±2.5	10.508±2.2	6.830±0.8
<i>Muscle Stimulation</i>	21.900±3.0	14.950±3.1 <sup>†</sup>	19.344±2.2	14.750±2.2 <sup>†</sup>
<b>Specific Tension (g/mg)</b>				
<i>Neural Stimulation</i>	0.064±0.01*	0.073±0.02*	0.101±0.02*	0.075±0.01*
<i>Muscle Stimulation</i>	0.120±0.02*	0.124±0.03*	0.171±0.02*	0.153±0.02*
<b>Neuromuscular Block (%)</b>	48.580±6.0*	44.040±8.0*	40.044±5.7*	48.670±7.5*
<b>Time to Peak Force (s)</b>				
<i>Neural Stimulation</i>	0.802±0.01*	0.782±0.01*	0.829±0.01*	0.804±0.01*
<i>Muscle Stimulation</i>	0.817±0.01*	0.792±0.01* <sup>‡</sup>	0.838±0.01*	0.818±0.01* <sup>‡</sup>

Percent Decline over 5 Minute Endurance Test	Male Control	Male HS	Female Control	Female HS
<b>Max Force Decline (%)</b>				
<i>Neural Stimulation</i>	68.61±4.75	70.110±5.26	67.011±4.07	48.300±6.45 <sup>!</sup>
<i>Muscle Stimulation</i>	46.381±3.79	45.590±5.09	47.967±5.65	26.330±4.65 <sup>!</sup>
<b>Neuromuscular Block (%)</b>	-51.190±7.85	-62.400±12.71	-49.067±11.69	-154.069±58.73 <sup>#</sup>

Values are means ± SE, N=9-10/group. HS=Hindlimb suspended.

<sup>†</sup>indicates significant (p≤0.05) difference from control group of same sex

<sup>‡</sup>indicates significant (p≤0.05) difference from male control and male hindlimb suspended

<sup>&</sup>indicates significant (p≤0.05) difference from control of similar treatment

\*indicates significant (p≤0.05) difference from pre-test

<sup>#</sup>indicates significant (p≤0.05) difference from female control

<sup>@</sup>indicates significant (p≤0.05) difference from neural stimulation

<sup>!</sup>indicates significant (p≤0.05) difference from all other treatment groups

**Table 2.** Data for the Myofiber Cross-sectional Areas ( $\mu\text{m}^2$ )

	Male Control	Male HS	Female Control	Female HS
<b>Fiber Types Combined</b>	3300±478*	2615±602	2891±427	2179±599
<b>Type I</b>	3320±478 <sup>‡</sup>	2631±623	2673±949	2204±592
<b>Type II</b>	2368±652 <sup>†</sup>	1531±841	2218±459 <sup>†</sup>	1189±528

Values are means  $\pm$  SE, N=101 group. HS=Hindlimb suspended.

\* indicates significant ( $p < 0.05$ ) difference from female hindlimb suspended.

<sup>‡</sup> indicates significant ( $p < 0.05$ ) difference from all other groups.

<sup>†</sup> indicates significant ( $p < 0.05$ ) difference from unloaded.

**Table 3.** Data for the Myofiber Composition (%)

	Male Control	Male HS	Female Control	Female HS
<b>Type I</b>	97.3±3.6	98.7±2.1	97.8±3.2	95.9±7.7
<b>Type II</b>	3.6±3.2	2.6±2.3	4.6±1.7	7.3±9.1

Values are means  $\pm$  SE, N=101 group. HS=Hindlimb suspended.