Examination of sex differences in quadriceps fatigability and Hsp70 content in response to intense intermittent isometric exercise

by

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Authors Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Abstract

The purpose of this study was to determine if there are sex differences in induced heat shock protein 70 (Hsp70) expression in human skeletal muscle under basal conditions and in response to intense intermittent isometric exercise. Furthermore, this study examined potential sex differences in muscle fatigability and sarcoplasmic reticulum (SR) function for up to 9 days following the bout of exercise. In total, 6 male (20 ± 0.5 years of age, 70.88 ± 10.25 kg, mean ± SE) and 6 female participants (19 ± 0.25 years of age, 58.02 ± 5.82 kg, mean ± SE) were recruited for this study to do one legged intermittent isometric exercise with a 50% duty cycle (5 sec contraction: 5 sec relaxation) at 60% of their maximal voluntary contraction (MVC) for 30 minutes. Muscle biopsies, blood samples and muscle stimulation measurements were taken prior to starting exercise for assessment of baseline values. These same measures were taken immediately POST exercise and at 24(R1), 72(R3), 144(R6) and 216(R9) hours following the exercise. Muscle samples were analyzed for exercise and recovery response of Hsp70, sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA)1 and SERCA2 protein content, as well as measurements of maximal Ca2+ ATPase activity and Ca2+ uptake. Blood samples were also analyzed for serum estrogen and creatine kinase concentrations. The results from this study show that there are no differences in basal Hsp70 protein content between males and females, and that females have a blunted (no increase up to 9 days post exercise) Hsp70 response following a bout of intense exercise in comparison to males who had a robust response. Immediately following exercise females had smaller decrements in MVC and electrically stimulated force (10 and 100Hz). It was also found

that at low frequencies of stimulation (10Hz), females were able to recover at a quicker rate than males. There was no evidence that the decrements in force or the differences in recovery time between males and females were due to alterations in SERCA protein content or function. This thesis is the first study in humans to show that there is sexual dimorphism in the exercise induced Hsp70 response to exercise.

Acknowledgements

First and foremorst I must thank my family. They have always been behind me 100%. Without their help there is no way I would be here right now.

Secondly, I have to thank Dr. Russ Tupling and his team of researchers, who have taught me the skills and knowledge needed to make this thesis possible.

I would also like to thank my supervising committee (Dr. Ken Stark and Dr. Marnia Mourtzakis) who "raised the bar" for my academic writing and planning.

Dedication

I would like to dedicate this thesis to the entire Hopf family, whose love and support helped me through my academic and professional development.

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Chapter 1: Introduction

The adult human being is comprised of a series of complex physiological systems. As humans progress through life, it is well documented that males and females have numerous differences in structural, functional and psychological physiology (Denton et al, 2007, Wheeler et al., 1991, Tarnopolsky et al, 2007). The term sexual dimorphism has commonly been used to define a scenario where genetic or biological differences exist between sexes (Nickerson et al, 2006). In comparison, the term gender refers to the sociological status of male and females and their place in society and should not be used to describe biological differences between males and females. Examples of physiological sexual dimorphism that are well established include differences in muscle mass and strength (Perez-Gomez et al, 2008), fibre type distribution (Komi et al, 1978), and contractile properties (Clark et al, 2003). Overall it appears that adult females have 40% less overall strength and significantly less muscle cross sectional area, when compared to males (Komi et al., 1978). Females may also be more susceptible to decrements in muscle mass and strength with aging compared to males (Mazaretti et al., 2009). This process is referred to as sarcopenia and it may have functional consequences as older adults lose their ability to be independent (Mazaretti et al, 2009, Lee et al., 2007 and Doherty et al., 2003). Another example is the difference between males and females in skeletal muscle fatigability, as females may be more fatigue resistant than males although this finding is inconsistent (Clarke et al., 2005; Tiidus et al., 2007). The differences in development and physiology are not just limited to the skeletal muscle. The risk of developing pathologies such as osteoporosis and coronary artery disease also appear to be sex dependent (Reviewed by Polk et al., 2005 and Balasch et al, 2003).

Role of Estrogen in Skeletal Muscle Physiology

The distinct differences between sexes have led researchers to examine potential biological mechanisms underlying the sexual dimorphism commonly observed in disease and function. The ovarian sex hormone estrogen, (17β-estradiol), and its effects on various physiological pathways in the human body have been proposed as one of the primary mechanisms behind the differences between sexes in muscle metabolism (Tarnopolsky et al, 2008, Paroo et al, 2002 and Tiidus et al., 2009). Estrogen (or 17β-estradiol) is a four ringed steroidal hormone (Figure 1) produced by developing granulosa cells in the ovaries of females. Estrogen is a molecule that contains an extra hydroxyl that may serve as a natural reducing agent in the muscle cell (Knowlton et al 2005). As a female matures and begins to menstruate, estrogen is released in a cyclical fashion. The female menstrual cycle is described in detail in Silverthorn et al., (2007). Briefly, every 28-30 days the female goes through the menstrual cycle where estrogen rises slowly during the first 7 days and spikes from days 8-14 to promote development of the uterine lining (Figure 2). During this time, referred to as the follicular phase, follicle stimulating hormone (FSH) increases as well to promote early maturation of oocytes. Once serum estrogen concentrations have peaked, concentrations of luteinizing hormone (LH) surge. This surge in LH stimulates the final stage of ovum maturation and is an important step to initiate ovulation. Following ovulation, production of other ovarian sex hormones (progesterone and FSH) increases to promote further development of the uterine wall. If fertilization and implantation does not occur, menstruation takes place and all of the ovarian sex hormones return to baseline concentrations, and the cycle repeats itself. As females age and follicular maturation ceases, endogenous production of estrogen is

greatly reduced. This transition is called menopause and is associated with many changes to the female body (Copeland et al, 2004).

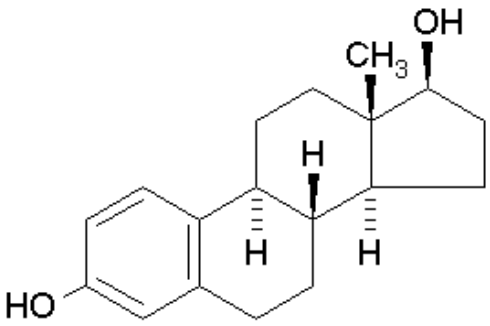

Figure 1: 17B-Estradiol (Estrogen) Biochemical Structure: http://healthpsych.psy.vanderbilt.edu/SoyBreastCancer_files/image002.gif

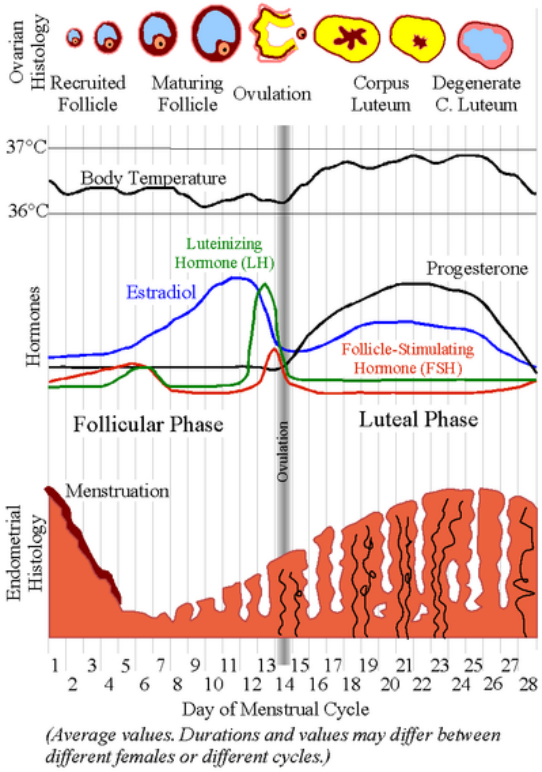

Figure 2: The female menstrual cycle- This figure illustrates the typical patterns of ovarian sex hormone release into the blood stream throughout the 28 day cycle. *http://en.wikipedia.org/wiki/Menstrual_cycle*

As a result, females may supplement estrogen via oral or transdermal methods to maintain their circulating estrogen levels to help offset some of the complications known to be caused by a lack of the hormone. Estrogen is also responsible for the development of secondary sex characteristics in females, as well as numerous other physiological processes such as bone and muscle metabolism (Ropero et al., 2007 and Tarnopolsky et al, 2007). Estrogen is also known to influence the function of organs throughout the body including bone, brain, heart and skeletal muscle (Leung et al, 2007, Polk et al., 2005 and Balasch et al, 2003). LH, progesterone, and FSH may also contributed to sexual dimorphism in neural tissue (Mitsushima et al, 2003 and Gonzalez-Hernadez et al, 2000), but very little research has been done on the role of these hormones in skeletal muscle physiology. The remainder of this thesis will focus on the role that estrogen plays in sexual dimorphism involving skeletal muscle physiology.

Recently it has been shown that skeletal muscle expresses estrogen receptors (ERs) (Stice et al., 2008). Skeletal muscle has two types of ERs; ERα and ERβ. These ERs are responsible for transducing an extracellular signal to modulate various intracellular signalling pathways. One of the primary effects of estrogen in skeletal muscle, which appears to be acting primarily through the NFκβ pathway, is to upregulate various stress proteins and possibly attenuate the inflammatory processes caused as a result of various physiological stressors such as exercise (Paroo et al, 2002, Voss et al, 2003 and Knowlton et al., 2001), ischemia reperfusion, acidosis and hyperthermia (Knowlton et al., 2001 and Stice et al., 2008). Furthermore, estrogen has been described as a membrane stabilizing molecule (Tiidus et al., 2003). Due to its lipophilic properties, estrogen has the ability to interact with the phospholipid bilayer of cells and possibly protect them from

damaging stressors. Cumulatively, these properties of estrogen suggest that females may be less susceptible to stress and better able to retain function or be more resistant to fatigue compared to males. One of the primary proteins associated in the stress response in Heat Shock Protein 70 (Hsp70). Therefore, it stands to reason that the Hsp70 response may display sexual dimorphism following periods of stress.

Heat Shock Proteins and the cellular stress response

Heat Shock Proteins (HSPs) are a family of highly conserved proteins that are upregulated in response to various physiological insults to a cell and are ubiquitous among all mammalian species (as reviewed by Noble et al., 2007). The first HSP was discovered in 1962 when *Drosophila Melanogaster* larvae were exposed to an intense heat stress. The resulting upregulation of these proteins in response to heat is what gave them the name "Heat Shock Proteins". Much research has been conducted in the over forty plus years following the first discovery and there is now evidence to suggest that HSPs function to protect cells against a variety of biological and environmental stresses. There are many different isoforms of the HSPs including Hsp27, Hsp32, Hsp70 and Hsp90 to name just a few, which are named based upon their molecular weights.

There are only a limited number of studies investigating the role of these proteins in human skeletal muscle physiology and the majority of studies have focused on Hsp70. Hsp70 is highly inducible and rapidly upregulated when cells are exposed to various forms of stress such as exercise (Noble et al., 2007 and Locke et al., 1991), hyperthermia (Morton et al., 2007), hypoxia (Iwaki et al, 1993) and reactive oxygen species (Madamanchi et al, 2001). Hsp70 has been shown to be responsible for folding un/misfolded proteins, chaperoning newly synthesized proteins to their target location

and preventing damaged proteins from aggregating which would render them unfunctional (Lui et al., 2006). There are two types of Hsp70 in skeletal muscle, a non-inducible isoform present under basal conditions, termed heat shock cognate 70 (Hsc70) and an inducible form (Hsp70) which is up regulated in response to myocellular stress (Reviewed in Noble et al., 2007). This isoform is also commonly referred to as Hsp72 in the literature based on its molecular weight and difficulty distinguishing it from Hsp70 during western blotting procedures (Noble et al, 2007). The remainder of this thesis will focus on the inducible form, Hsp70. Under basal, unstressed conditions, Hsp70 molecules are bound to a transcription factor named heat shock factor 1 (HSF1) in the cytosol of a cell. When a physiologic stress is imposed upon the muscle and oxidative damage occurs, causing protein denaturation, Hsp70 identifies hydrophobic residues of denatured proteins, dissociates from HSF1 and binds to denatured proteins (Noble et al., 2007 and Knowlton et al., 2001). Unbound HSF1 then trimerizes, becomes phosphorylated and translocates to the nucleus. A "transcriptional complex" then forms and gains the ability to bind with the transcriptional factor Heat Shock Element (HSE), causing the upregulation of *hsp70* messenger ribonucleic acid (mRNA) (Figure 3). This cycle will continue to occur until the stimulus to induce the Hsp70 response has ended (Noble et al., 2007).

Numerous studies have shown that exercise can directly increase the Hsp70 content in the muscle cell (Noble et al., 2006, Tupling et al., 2007, Paroo et al, 2002 and Punschart et al, 1996). The extent of the Hsp70 response may be accentuated with exercise of longer duration (Febbraio et al., 2000), higher intensity (Lui et al., 1999) and using a more eccentric type of exercise compared to concentric (Paulsen et al., 2007). It

has also been demonstrated that the exercise induced Hsp70 response is muscle fibre type specific, with a more robust response seen in type I fibres compared to type II fibres (Tupling et al, 2007 and Bombardier et al, 2009). Type I fibres are characterized by the slow myosin heavy chain I and have a higher oxidative capacity compared to the more anaerobic type II fibres which express a fast type II myosin heavy chain isoform (Reviewed by Fitts et al., 1994). It was speculated that the greater response observed in type I fibres was due to higher levels of oxidative stress compared to type IIA and IIX fibres (Tupling et al., 2007). Moreover, protein isoforms expressed in type I fibre types may be more susceptible to oxidative damage compared to protein isoforms expressed in type II muscle fibres. For example, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) protein is highly susceptible to oxidative stress (Holloway et al., 2005) and research has shown that high levels of oxidative stress and nitration (via reactive nitrogen species) can decrease its activity, therefore resulting in functional decrements of the muscle cell, such as decreased force production, rate of relaxation (-dF/dt), and rate of force production (+dF/dt) (reviewed by Tupling et al, 2004). These findings show that exercise of various intensities and types is capable of inducing an Hsp70 response.

Sex Differences in Heat Shock Protein 70: Basal and exercise induced responses

Recently, the number of studies comparing the male and female response to various forms of stress has increased (Reviewed by Noble et al, 2007). More specifically, researchers have been trying to determine if the Hsp70 stress response in various tissues, including heart and skeletal muscle is sex dependent. To date, much of the research has been done using a rodent model, either by using direct male/female comparisons or using an ovariectomized model (Paroo et al, 2002, Voss et al., 2003 and Knowlton et al, 2001).

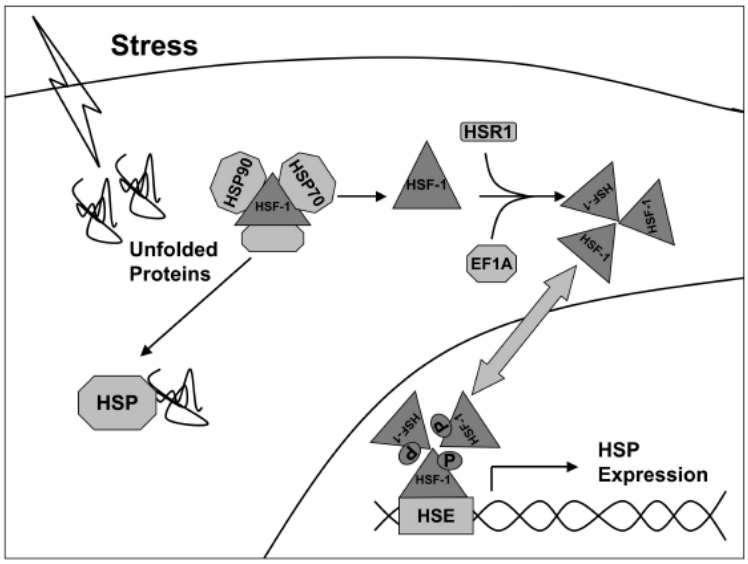

Figure 3: HSF1 dissociation/binding cascade and *hsp70 mRNA* production. *Stice et al. (2008) Mol. Med. 14(7-8)*. This diagram illustrates the events following stress that lead to Hsp70 binding to proteins and the resulting upregulation of *hsp70 mRNA.* HSF-1-Heat Shock Factor 1; HSE- Heat Shock Elemement.

Ovariectomized rats do not have an endogenous source of estrogen and researchers use this model to replicate the male response. The ovariectomized rat can also be supplemented with other ovarian sex hormones via time release pellet or injection, which will bring the hormone of interest back to physiologic levels. This allows for assessment of the independent effects of a single ovarian sex hormone (i.e. estrogen) (For full review see Paroo et al, 2002 or Bombardier et al, 2009). Another less commonly used method utilizes pre/post menopausal women to examine the extent of the changes which occur once a female loses her ability to produce endogenous sources of estrogen. Finally, males can be supplemented with an exogenous source of estrogen to test the effect of an acute estrogen exposure on various physiological pathways. Considering sex differences in cardiovascular disease is a very popular area of research, a number of

studies have examined sexual dimorphism in Hsp70 protein and *hsp70 mRNA* expression in cardiac muscle. In contrast, only a few studies have investigated if sexual dimorphism exists in the Hsp70 stress response in skeletal muscle and these have been limited to rodent models.

There is evidence suggesting that Hsp70 can be upregulated in heart using various stresses such as ischemia reperfusion, heat (Mestril et al, 1994), pharmacologic treatments, or a pre conditioning exercise bout in cardiac muscle (Paroo et al., 2002). However, following a bout of treadmill exercise, Hsp70 protein content in cardiac muscle is increased twice as much in males than it is in females. It has also been shown in rodents that basal Hsp70 levels are higher in females hearts compared to male hearts (Voss et al., 2003 and Paroo et al., 2002).

Consistent with cardiac muscle, under resting conditions in skeletal muscle there is some Hsp70 available to cope with low levels of stress. The only study to date that directly compares male and female rodents has shown that there is no significant difference in basal Hsp70 protein content (Voss et al., 2003). In comparison, a separate study using an ovariectomized rodent model (no endogenous estrogen) that is supplemented with a time released estrogen pellet, showed that rodents with estrogen had a significantly higher level of basal Hsp70 compared to sham rodents (no estrogen) (Bombardier et al., 2009). These two studies are the only studies that have examined the differences in basal Hsp70 content in rodent skeletal muscle and to date no study has examined this in human skeletal muscle.

The exercise induced Hsp70 response in skeletal muscle is similar to that of cardiac muscle. To date, research that has been done in rodent models suggests that

females have a blunted Hsp70 response following a bout of intense exercise (Paroo et al., 2002 and Nickerson et al., 2008). Conversely, males display a significant elevation in Hsp70 following a similar bout of exercise. The blunted Hsp70 response in females also appears to be mediated by estrogen. Bombardier et al., (2009) showed that soleus muscle of ovariectomized female rats displayed an exercise induced Hsp70 response similar to that observed in males of other studies. When these ovariectomized females were supplemented with estrogen, the Hsp70 response was blunted. A separate study by Paroo et al., (2002) showed that administering an estrogen receptor blocker (Tamoxifan) to gonadally intact females, resulted in an Hsp70 response similar to males.

The data showing that females have a significantly higher level of basal Hsp70 and a blunted Hsp70 response to exercise have all been accumulated from rodent studies. To date, no controlled study using human participants has examined sex differences in the Hsp70 response to an intense bout of exercise. Since Hsp70 is a cytoprotective protein capable of binding to and protecting muscle proteins, it stands to reason that female skeletal muscle function may be better protected than males when exposed to exercise.

Sexual Dimorphism in Hsp70 and muscle fatigability: Is there a link?

When a contraction cycle is initiated, an action potential is transmitted from the post synaptic neuron, down the sarcolemma of the muscle. Through activation of the dihydropyridine receptor and the ryanodine receptor a resulting influx of calcium into the cytosol of the fibre ensues, causing activation of the contractile apparatus. Once the stimulus from the neuron has ceased, the SERCA pump acts to sequester Ca^{2+} ions back into the sarcoplasmic reticulum, allowing relaxation to occur. The result of numerous

sequential contractions is an increase of various metabolic by-products such as hydrogen ions (Bangsbo et al., 1993), reactive oxygen species (Andrade et al.,2001), lactate, and inorganic phosphate (Pi) (Murphy et al., 1999). These metabolic by-products and other structural/functional changes to either the contractile proteins or the proteins involved in excitation-contraction coupling have been proposed as some of the main contributors to the development of fatigue.

Fatigue is defined as the inability to produce a desired level of force (Allen et al., 2007). The mechanistic basis of fatigue is complex and is dependent upon numerous factors including exercise intensity, contraction type, rest intervals (Sale et al., 1987) and individual muscle fibre type profile (Essen et al., 1975). Decrements in force are commonly observed after periods of strenuous activity. In most cases, the ability to restore its maximal force producing capacity of skeletal muscle is restored within 30 minutes following exercise, depending on the exercise intensity (Baker et al., 1993). In some cases when the exercise intensity is extreme, although maximal force producing capacity may recover relatively quick, other indices of fatigue may be present for longer periods of time (Edwards et al., 1977).

Along with maximal voluntary contraction (MVC) and supramaximal twitch force, low frequency fatigue (LFF) and high frequency fatigue (HFF) are two measurements commonly used when assessing fatigue. HFF is a term used to describe force decrements which occur when a muscle is electrically stimulated at high frequencies (50 and 100 Hz). It has been suggested that HFF is likely a result of an impairment of the Na^+/K^+ ATPase, which is located within the sarcolemma of skeletal muscle (Fowles et al., 2002). Impairment of the Na^+/K^+ ATPase would result in an

inability to maintain the proper ion gradient needed to repolarize the cell and allow action potentials to be transmitted down the T-tubules. Low frequency fatigue is a term used to describe force decrements which occur when a muscle is electrically stimulated at low frequencies (10 and 20 Hz). It has been suggested that LFF is caused in part by impairments of calcium release from the SR and decrements in the ability of the SERCA pumps to sequester Ca^{2+} back into the lumen of the SR (Tupling et al., 2000, Enns et al., 1999, Tupling et al., 2004). The inability to sequester Ca^{2+} back into the SR can result in prolonged calcium transients within the cytosol of the cell and a loss of Ca^{2+} homeostasis, which may activate calpains/apoptotic pathways (Murphy et al., 2006) and decrease the ability to transmit signals from the t-tubules to the SR to initiate calcium release (as reviewed by Tupling et al, 2004).. The mechanistic basis of fatigue is complex but clearly SERCA pumps are extremely important in the performance of skeletal muscle and decrements in their ability to sequester Ca^{2+} may result in fatigue.

As previously mentioned, although controversial, there is evidence to suggest that sex differences exist in skeletal muscle fatigability following intense exercise. More specifically, females may be more resistant to fatigue when compared to males, in that they have longer endurance times and less force reduction following a bout of intense exercise. (Clark et al, 2005, Russ et al, 2003 and Hunter et al, 2001). Most of these studies utilized a low intensity sub-maximal contraction protocol and measured endurance time as an indicator of fatigue. In contrast, some evidence suggests that no sex difference exists in human skeletal muscle fatigability following exercise (Phillips et al., 2003), which was also replicated in rodent EDL muscles (Tiidus et al, 1999).

Hsp70 is known to be a cytoprotective protein that can bind to and protect functional proteins within the skeletal muscle. Recently it has been shown that Hsp70 has the ability to bind to SERCA1 (Tupling et al., 2004) and SERCA2a (Fu et al., 2007) isoforms and prevent thermal deactivation of both isoforms. More specifically, it was shown that human embryonic kidney cells (HEKs) which were transfected with their respective SERCA isoform cDNAs and then exposed to a heat stress, had a significant decrease in maximal SERCA activity; however when these same cells were cultured with Hsp70, they found that the heat induced decrements in SERCA activity were attenuated.

Therefore, the findings that LFF is, at least in part, caused by decrements in SERCA pump activity and the notion that Hsp70 is a cytoprotective protein capable of protecting SERCA pumps against damaging stress, suggests that the sexual dimorphism observed in skeletal muscle fatigue could be a result of the higher basal Hsp70 levels observed in females compared to males (**Figure 4**). This would theoretically allow females to be more protected and maintain skeletal muscle performance better than males.

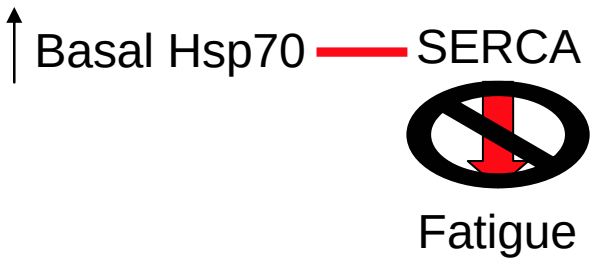

Figure 4: **SERCA:Hsp70 Interaction and Fatigue**- illustrates potential interaction between higher basal Hsp70 concentrations, SERCA and fatigue, and suggests Hsp70 may protect SERCA function in females which may help attenuate fatigue.

Previous work by Tupling et al (2000 and 2007) using a single legged isometric knee extension exercise model, where participants contract at 60% of their MVC, has

proven to be effective in inducing a robust Hsp70 response in males (Tupling et al, 2007), as well as post contractile depression (PCD) in both males and females. PCD is defined as prolonged fatigue at both low and high frequencies of stimulation following numerous tetanic contractions (Tupling et al, 2000). This model has shown that females display PCD for up to at least 1 hour following exercise (Tupling et al, 2000 and Fowles et al., 2002) and that males can display PCD for up to 6 days following the same exercise bout. Since these studies did not assess the time course of recovery beyond 1 hour in females (Tupling et al, 2000), it remains to be determined if sex differences exist in the time course of PCD and when full recovery of force occurs. Moreover, this study did not assess the Hsp70 response to this type of exercise in females. Therefore the current thesis will utilize the same exercise protocol to determine if sex differences exist in the time course of PCD and if differences exist, are they associated with basal and exercise induced Hsp70 content within human skeletal muscle.

Purposes:

1. To determine if there are sex differences in basal and exercise induced Hsp70 levels in human skeletal muscle.

2. To determine if there are sex differences in skeletal muscle fatigability in response to an acute bout of intense intermittent isometric exercise in human quadriceps.

3. To determine if there are sex differences in the effects of exercise on SERCA pump function in human skeletal muscle.

4. To determine if there are sex differences in the time course of recovery for all variables following intense intermittent isometric exercise.

Hypotheses:

1. Basal Hsp70 levels will be higher in females than males and females will have a blunted Hsp70 response in comparison to the more robust response displayed by males.

2. Females will demonstrate less fatigue and recovery muscle contractile function more quickly than males in response to the intense isometric exercise.

3. Females will have smaller decrements in maximal SERCA activity and Ca^{2+} uptake following the exercise bout compared with males due expected higher basal Hsp70 expression in females.

4. Females will recover markers of PCD and SERCA function earlier in the time course of the study compared to males.

5. Recovery of all parameters will be complete within 9 days following the exercise in both males and females.

Chapter 2: Methods

Participant Info

Twelve untrained but healthy participants (6 male and 6 female) were recruited for this study and all completed the study. The height, weight and peak VO_2 of the male participants were 180.3 ± 2.36 cm, 70.88 ± 1.71 kg, and 41.95 ± 1.54 ml/kg/min, respectively (Mean ± S.E.). For the females, height, weight and VO2 peak were 164 ± 0.97cm, 58.02 ± 0.97 kg and 37.9 ± 2.77 ml/kg/min (Mean ± S.E.), respectively. All participants were asked to refrain from exercise, caffeine and alcohol for one week prior to the exercise day and throughout the remainder of the entire study. Participants were informed of all risks associated with the study and were asked to sign an information consent form before partaking in the experiment. All females were asked to come in between days 1 and 3 of their menstrual cycles (See results for serum estrogen concentrations).

Experimental Design

On the Thursday, 5 days prior to the exercise test, participants took park in an introductory session in the laboratory. During this session participants performed single legged isometric knee extensions each both legs and maximal voluntary contraction (MVC) was assessed (see Muscle Stimulation and Force Analysis Section for procedure details). A stimulation voltage that would elicit a peak force during 100 Hz stimulation that was comparable to ~ 60% MVC was also determined. On the following Tuesday, participants came back to the laboratory to complete the exercise session. Legs were randomly assigned to either the exercise (E) condition or the non-exercising control (C) condition. Prior to the exercise, MVC and force frequency measurements were made on

both E and C legs (see Muscle Stimulation and Force analysis), muscle biopsies were taken from the C leg only and venous blood samples were taken to serve as baseline (PRE) levels. The participants then underwent a 30 minute intermittent exercise protocol, where they performed a single legged isometric knee extension exercise at 60% of their MVC. The study utilized a 50% duty cycle, in which participants contracted for 5 seconds and relaxed for 5 seconds. The control leg remained unstrapped and was relatively inactive for the exercise protocol. Using an oscilloscope to monitor force output, the participant was encouraged to maintain the preset force for as long as possible throughout the exercise protocol. On average, participants (males and females) could maintain the target force for ~ 20-25 minutes; however, all participants completed the full 30 minutes of exercise. Immediately following the exercise (Post), MVC and force frequency measurements were made on both legs, muscle biopsies were taken from the exercise leg only and venous blood samples were taken. The participants were then asked to come back to the laboratory on recovery days 1 (R1), 2 (R2), 3 (R3), 6 (R6) and 9 (R9) following the exercise protocol and MVC, force-frequency measurements, and muscle biopsies were taken from both E and C legs and venous blood samples were taken (except day 6, where only force measurements were taken) (See **Figure 5**).

Muscle Stimulation and Force Measurements

The protocol used for electrical stimulation and force measurements has been described previously in detail (Tupling et al., 2000; Fowles et al, 2002). Briefly, subjects were seated in a specially designed straight-backed chair such that their hips and legs were secured and their knee was at ~90^{o} to their thigh.

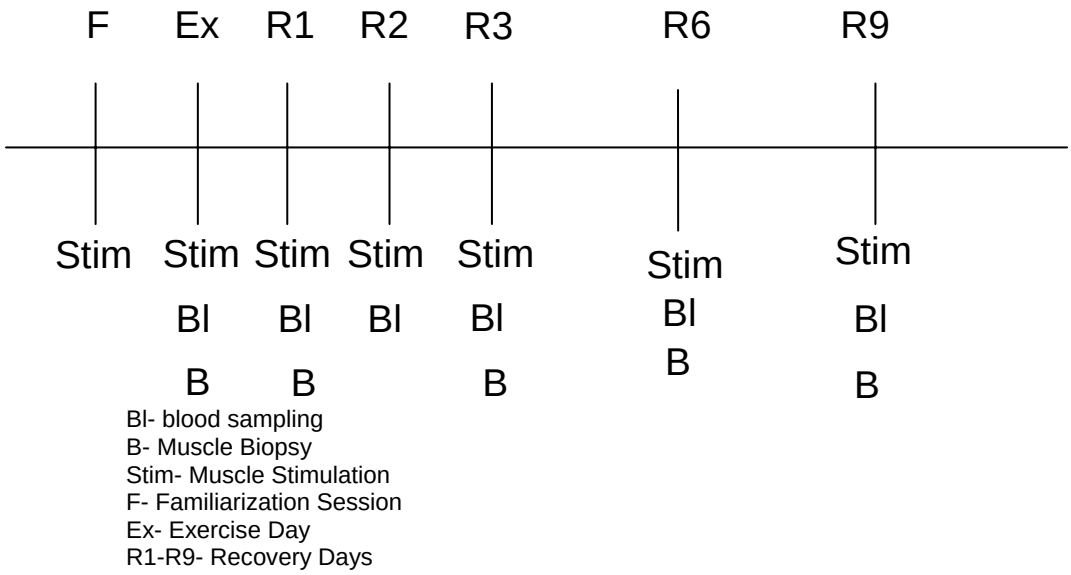

Figure 5: Experimental Design: Illustrates the time course for experimental protocols and measurements throughout the study.

A 5 cm plastic cuff was placed around the lower leg just proximal to the ankle. Voluntary and electrically evoked force production was measured using a linear variable differential transducer (LVDT). The LVDT signal was passed through a Daytronic carrier amplifier (11 Hz), converted to a digital signal and recorded on a laboratory computer for analyses. Electrical stimulation was applied over a range of frequencies (150 V supramaximal twitch, 10-100 Hz) using two aluminum chloride electrodes (8 x 13 cm) and a Grass model S48 stimulator. The ground electrode was positioned centrally on the anterior aspect of the thigh just proximal to the patella, whereas the active electrode was placed toward the hip on the belly of the vastus lateralis. The electrodes were coated in pre warmed electrode gel and secured in place using a high quality athletic tape. Locations of the electrodes were marked prior to exercise with permanent marker to ensure minimal

variability in placement on recovery days. At the beginning of each experimental day, all equipment was calibrated.

Measures of muscle activation were taken using electromyography (EMG). Two EMG electrodes (Ambu A/S, Denmark) were placed across the belly of the vastus medialis of both the control and exercise legs and a reference electrode was placed on the head of the fibula. All EMG electrode locations were shaved, abraded and cleaned with alcohol to maximize the signal. The locations of the biopsy sample sites and the muscle stimulation pads prevents measuring EMG from vastus lateralis, so the medialis muscle was chosen for measurement of muscle activation. To maintain signal reliability the location of the electrodes was marked with a black marker to minimize day-to-day variation.

Muscle Biopsies and Sample Preparation

Tissue samples (~50mg) from the vastus lateralis of the C leg were obtained via needle biopsy technique under suction immediately prior to the 30 minute exercise protocol (will serve as PRE exercise baseline values), and from the E leg immediately upon stopping exercise. On recovery days 1, 3, 6 and 9 muscle biopsies were obtained from both the E and C legs. Each biopsy was taken from a separate sampling site, under local anaesthetic (1% xylocaine). The muscle samples were diluted in a pre made sample buffer (PMSF) (250 mM Sucrose, 5mM HEPES, 0.2mM PMSF and 0.2% NaN_3; pH 7.5) and homogenized in a crucible immersed in an ice bath. The diluted muscle homogenate was aliquoted into microtubes and immediately frozen in liquid nitrogen, then stored at -80°C for later analysis of protein and Hsp70 content and calcium handling properties.

Western Blotting and Protein Content Determination

Western blot analysis was performed to measure the muscle protein content of Hsp70, SERCA2a and SERCA1 in whole muscle homogenate. Band linearity and loading concentrations were determined prior to running homogenate samples. The diluted samples were run on a 7.5% polyacrylamide gel and the proteins were separated using standard SDS-page protocols (Laemmli et al., 1970) and then transferred to polyvinylidene difluoride membranes (Roche Diagnostics, Mannheim, Germany). After blocking with 10% skim milk suspension, the membranes containing the high molecular weight proteins (above 60 kDa) were incubated for either 16 hours with anti-Hsp70 monoclonal antibody SPA-810 (Stressgen Biotechnologies) or 1 h with either anti-SERCA1a monoclonal antibody A52 (Zubrzcka-Gaarn, E, 1984) or anti-SERCA2a antibody 2A7-A1 (Affinity Bioreagents) and the membranes containing lower molecular weight proteins (below 60 kDa) were incubated for 1 hour with anti-α-sarcomeric actin antibody 5C5 (Sigma) to control for protein loading. Membranes were then washed for 30 minutes using Tris-HCl, pH 7.5 and Tween (TBS-T). The membranes were treated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Hsp70-1:2000, SERCA1-1:10000, SERCA2a-1:2000), washed again with TBS-T and then the protein bands were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) using a bio-imaging system. Densitometric analysis was performed using a software program (GeneSnap) to measure the band density, allowing for quantification of the specific protein relative to total whole muscle homogenate protein. All specific protein measurements were expressed relative to the values obtained in the pre-exercise muscle biopsy and normalized to the concentration of α-actin and standard.

The standard used was a known amount of each protein of interest that was loaded on every gel. The standard was used so direct comparisons could be made between male and female protein contents.

Measurement of SERCA Activity

The protocol for measuring SERCA activity in homogenates prepared from human vastus lateralis muscle is summarized by Duhamel et al., 2007. Briefly, maximal SERCA activity was measured using a spectrophotometric assay technique developed by Simonides and van Hardeveld (1990). Homogenate (25 μL) was added to a calcium ATPase reaction buffer containing 100mM KCl, 20 mM HEPES, 15 mM $MgCl_2$, 1 mM EGTA, 10 mM NaN_3 and 10 mM of phosphenolpyruvate (PEP) (pH 7.0) at 37^{o}C. Immediately prior to initiating the reaction, 18 μL lactate dehydrogenase (LDH), 18 μL pyruvate kinase (PK), 10.5 μL calcium ionophore, (Sigma-A23187) 100 μL ATP and 25μL of whole muscle homogenate were added to 5 mL of the pre-made calcium ATPase reaction buffer, vortexed vigorously and kept on ice. A range (27-30.6μL) of calcium chloride ($CaCl_2$) concentrations was added to microtubes. This pre-determined range of $CaCl_2$ additions was intended to achieve a plateau and subsequent decline in Ca^{2+} ATPase activity were observed (Vmax). Also 2 μL of cyclopiazonic acid (CPA) a specific inhibitor of SERCA activity was added to the two highest $CaCl_2$ additions to represent a baseline ATPase value, based on Duhamel et al (2007). A total volume of 300 μL of the reaction cocktail (calcium ATPase buffer plus other additions), was added to the varying calcium concentrations. After vortexing vigorously, 100 μL of this cocktail was pipetted in duplicate into a 96 well plate, 1 μL of 0.3mM NADH (previously made that day and kept in a dark environment) was then pipetted into each well, and quickly

inserted into the plate reader and read at a wavelength of 340 nm to determine the fluorescence of NADH. Ca^{2+} ATPase activity was calculated as the difference between the total ATPase activity measured without CPA and the basal ATPase activity with CPA. All measurements of Ca^{2+} ATPase activity were expressed relative to whole muscle homogenate protein concentrations determined via the Lowry Protein Assay (Schacterle and Pollock, 1973).

SERCA Calcium Uptake Analysis

Calcium uptake measurements were made using a Photon Technology International (PTI) dual photon flourometer, as described in detail by Duhamel et al (2007). Briefly, fluorescence measurements were collected on a dual-emission wave-length spectrofluorometer. The excitation wavelength was set at 355 nm and 405 and 485 nm correspond to the emission wavelengths for bound (F) and free (G) indo-1, respectively. Photon counts were collected simultaneously for each wavelength. Before each trial session, the background fluorescence was determined in the absence of INDO-1 and subtracted prior to starting of each analysis. In brief, a reaction buffer consisting of 200mM KCl, 20mM HEPES, 10mM NaN_3, 7uM TPEN, 5mM Oxalate, 15mM $MgCl_2$; (pH 7.0 at 37°C), was made prior to the measurements and stored at -20°C. Whole muscle homogenate (30µL), ~2.5µL of 10mM $CaCl_2$ and 1µL of INDO-1, was added to a 2mL four sided cuvette containing 1.9mL of the reaction buffer. The cocktail was warmed to 37°C before starting the reaction by adding 40µL of 5mM ATP. As Ca^{2+} decreased because of active SR Ca^{2+} transport, F decreases and G increases. Using the PTI software, the ratio of F/G (R) is used to calculate the decrease in free calcium. Free calcium was determined by the software using the following equation:

$$(Ca^{2+})_f = K_d \times (G_{max}/G_{min})(R-R_{min})(R_{max}-R)$$

Where, R_{min} and R_{max} represent the min and max F/G ratios respectively, K_d represents the equilibrium constant for the interaction between Ca^{2+} and indo-1 (Set at 250) and G_{min} and G_{max} represent the min and max values for free indo 1

Measurements of calcium uptake were taken at 2000, 1500, 1000 and 500 nM free Ca^{2+} concentrations and rates were determined as reviewed by Tupling et al (2007). All uptake measurements were done in duplicate. All measurements of calcium uptake are expressed relative to whole muscle homogenate protein concentrations.

Serum Creatine Kinase Measurements

To detect the severity of sarcolemmal damage caused by the intermittent exercise protocol, measures of serum creatine kinase concentrations were taken. Venous blood samples were taken during each testing session, in order to track the changes in serum creatine kinase concentrations which were measured using a fluorometric assay according to the methods of Szasz et al. (1976).

Measures of serum estrogen

In order to assess the consistency of the menstrual cycle stage between females, circulating estrogen levels were determined. Venous blood samples were taken from the anti-cubital vein to measure serum estrogen concentrations. Using a commercially available radioimmunoassay kit (Coat-a-Count, Inter Medico, Markham, ON), estrogen levels were analyzed prior to exercise and on subsequent recovery days, to confirm there was no large variability within the study period.

Data Analysis

Three two-way repeated ANOVAs were performed for each of the Hsp70 measures (except basal Hsp70, where a t-test was used). More specifically, within the male and female groups, exercise and control legs were compared across the entire time course, to determine the effect of exercise and time. Males and females were then compared across the time course of the study, by just analyzing the exercise leg with each value being normalized to female PRE measurements. For all enzyme activities (CK and SERCA activity), estrogen concentrations and force measurements two way repeated measure ANOVAs were used. When comparing within group force response for males and females, force was expressed as absolute values. When comparing males and female force responses, all values were expressed relative to within group PRE values. Neumann-Kewls post-hoc comparisons were then done to compare specific means for each ANOVA. The level of significance was established at p values less than 0.05.

Chapter 3: Results

Heat Shock Protein 70 Sexual Dimorphism

When examining the differences in basal Hsp70 expression between male and female participants it was found that there were no statistical differences (p=0.123). (**Figure 6**) (Note: representative protein blots can be found in Appendix II).

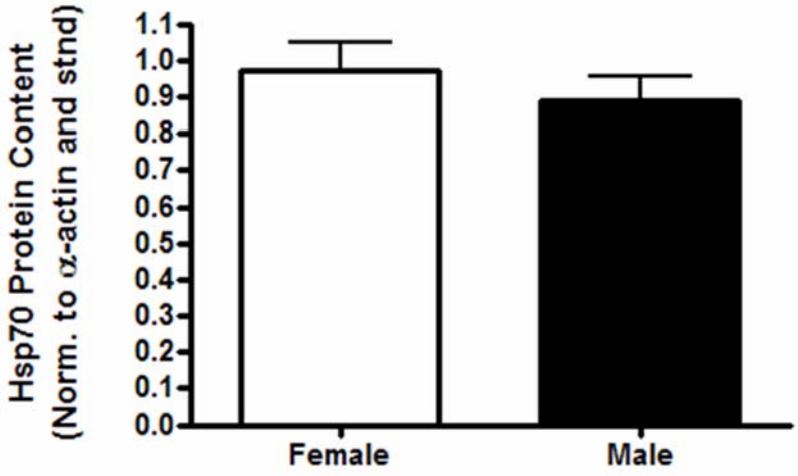

Figure 6: Male vs. Female Basal Hsp70 Content- Measurements were taken Pre exercise as baseline (basal) values. Values are expressed as Mean ± SE.

In males, intense isometric exercise caused a significant 35% increase ($p<0.05$) in Hsp70 content at R1 in the exercise leg compared to the pre exercise values, which remained elevated ($p<0.05$) until R3, decreased ($p<0.05$) to baseline at R6, then unexpectedly increased ($p<0.05$) again at R9 (**Figure 7A**). There were no differences ($p=0.873$) between PRE and POST levels. In females, there was a significant decrease ($p<0.05$) in Hsp70 content in the control leg at R3 but no significant changes ($p=0.812$) at any other time point in either the exercise or control leg (**Figure 7B**). Furthermore, it

was found that male Hsp70 content in the exercise leg was significantly higher ($p<0.05$) than females at R1 and R9 (**Figure 8**).

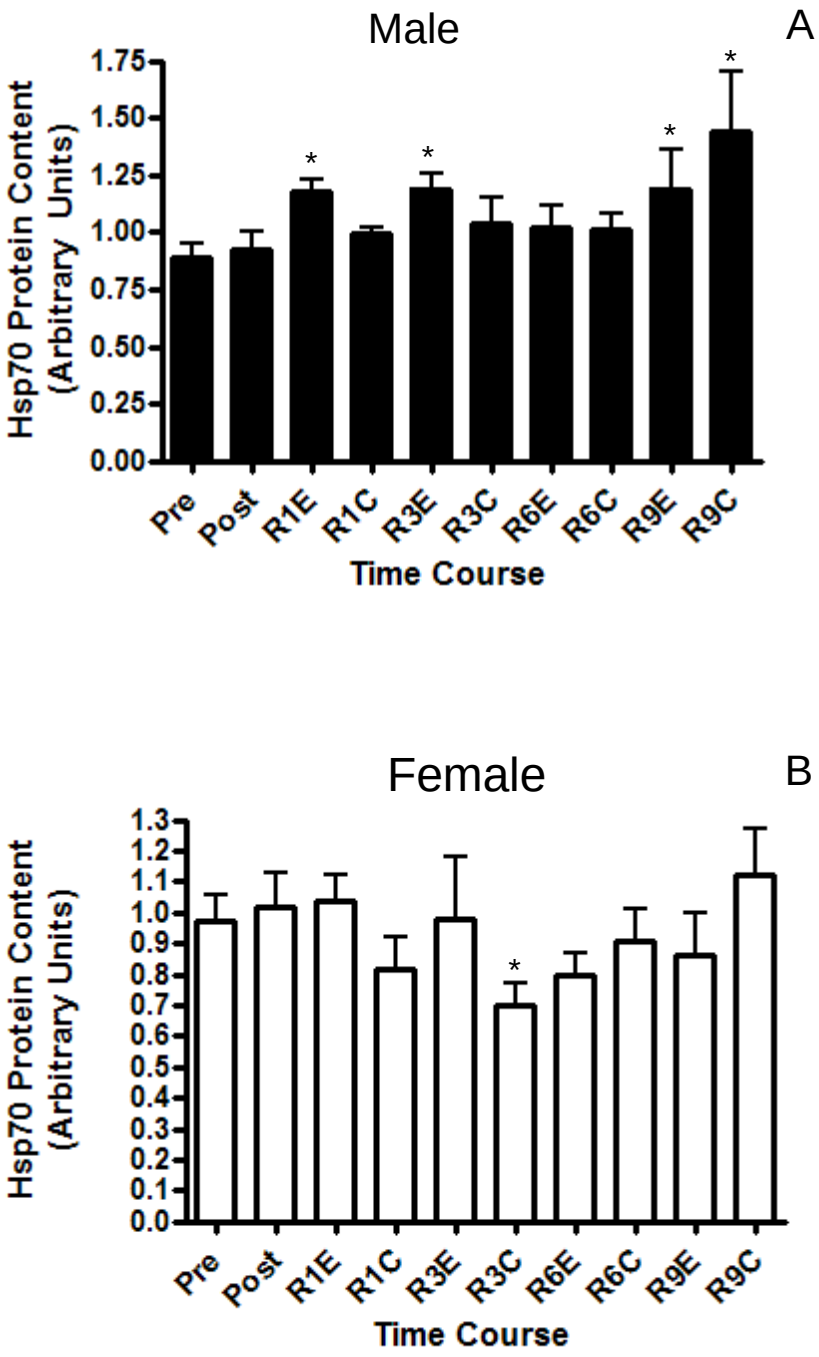

Figure 7 A/B: Male/Female Hsp70 Protein Content- Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216 (R9) h post exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE and are normalized to protein standard and α-actin levels. *Significantly different than Pre ($p<0.05$).

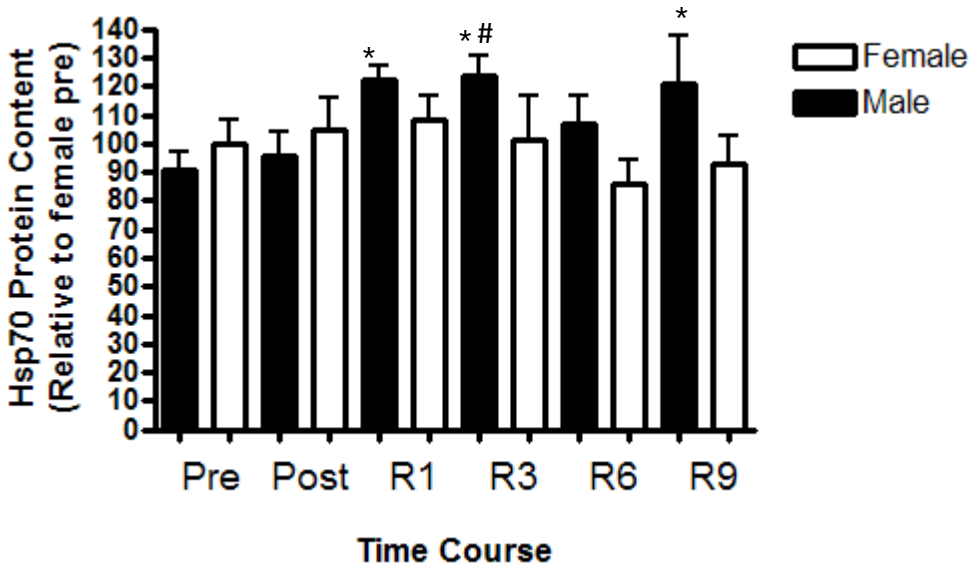

Figure 8: Male vs. Female Hsp70 Protein Content- Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216 (R9) h Post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are normalized to standard and alpha actin protein concentrations and expressed relative to female pre values. *Significantly greater than male Pre ($p<0.05$), # Significantly different than females ($p<0.05$).

Force Analysis

Maximal Voluntary Contraction (MVC)

Intense isometric exercise resulted in a 60% reduction ($p<0.05$) in MVC force of the exercise leg immediately POST exercise in males, which remained depressed ($p<0.05$) until R3 (**Figure 9A**). There was also a decrease ($p<0.05$) in MVC in the control leg immediately POST exercise in males, which recovered by R2. Females displayed an immediate ~25% reduction ($p<0.05$) in MVC in the exercise leg, which recovered completely by R1 (**Figure 9B**). There were no significant changes ($p>0.05$) in MVC force in the control leg of females at any time point. Furthermore, it appears that females are more fatigue resistant and able to recover force more quickly compared to males.

Figure 10 shows that females have less ($p<0.05$) reductions (~25%) in force in the exercised leg immediately following exercise compared to males (~60%), and completely recover force by R1 ($p<0.05$). Males did not fully recover maximal force until R3 of the exercise protocol.

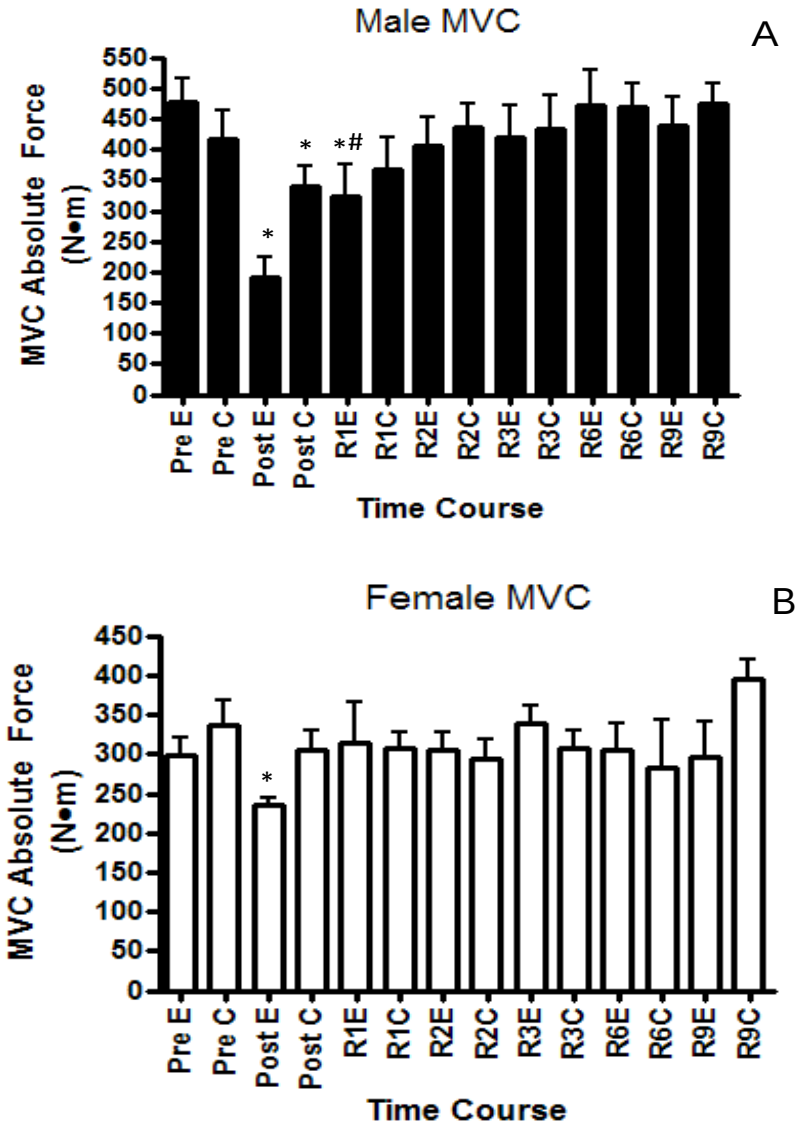

Figure 9A/B: Male/Female MVC- Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216(R9) h post exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE. *Significantly different than Pre ($p<0.05$). # Significantly different than Post E ($p<0.05$).

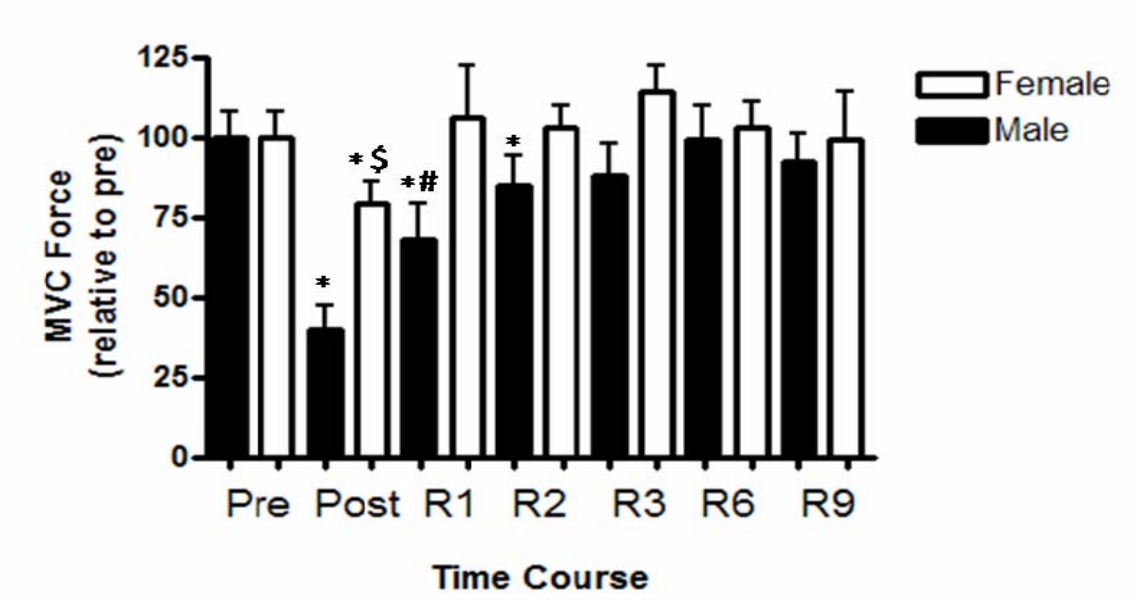

Figure 10: Male vs. Female MVC- Measurements were taken Pre, post, 24 (R1), 48 (R2) 72 (R3), 144 (R6) and 216 (R9) h post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are expressed relative to pre exercise values. *Significantly different than Pre ($p<0.05$). # Significantly different than Post ($p<0.05$). $ Significantly different than male Post ($p<0.05$).

Twitch

Intense isometric exercise resulted in a decrease ($p<0.05$) in peak twitch tension in the exercise leg immediately POST by 38% in males (**Figure 11A**) and 26% in females (**Figure 11B**). Peak twitch tension returned to baseline by R1 in the exercise leg of both male and female participants. The peak twitch tension of the control leg in male and female participants remained unchanged ($p>0.05$) following exercise and throughout the recovery period (**Figure 11A/B)**. Furthermore there was no change in +dF/dt or –dF/dt for males until R6, where there was an unexpected increase in both exercise and control leg for both measures (**Table 1**). Female +dF/dt decreased ($p<0.05$) at R1 in the exercise leg and R1 and R2 in the control leg ($p<0.05$). There were no further changes in either exercise or control leg for females in either +dF/dt or –dF/dt.

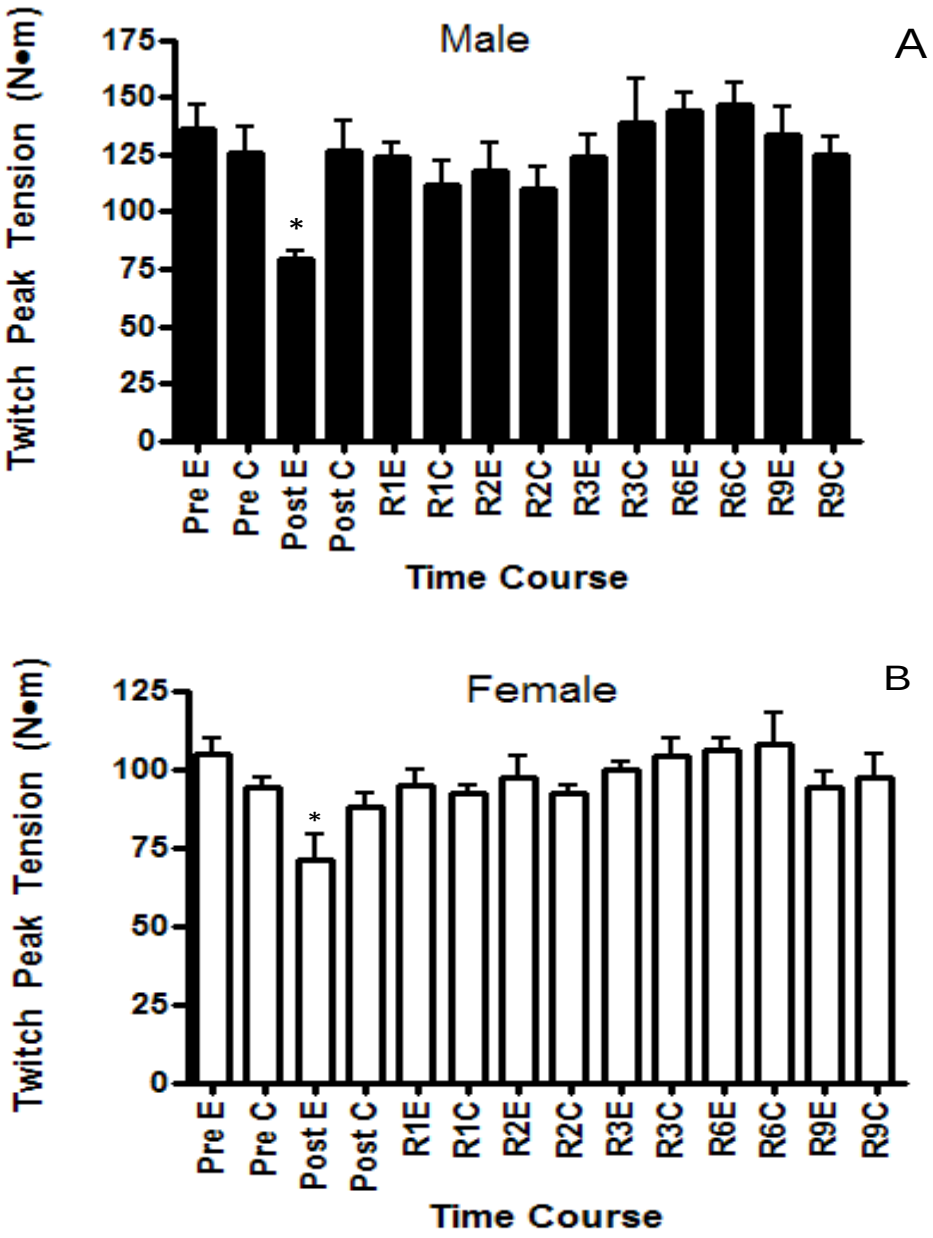

Figure 11A/B: Male/Female peak twitch tension (Po) - Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 h post exercise in both the exercise (E) and control (C) legs.. Values are expressed as Mean ± SE. * Significantly different than Pre ($p<0.05$).

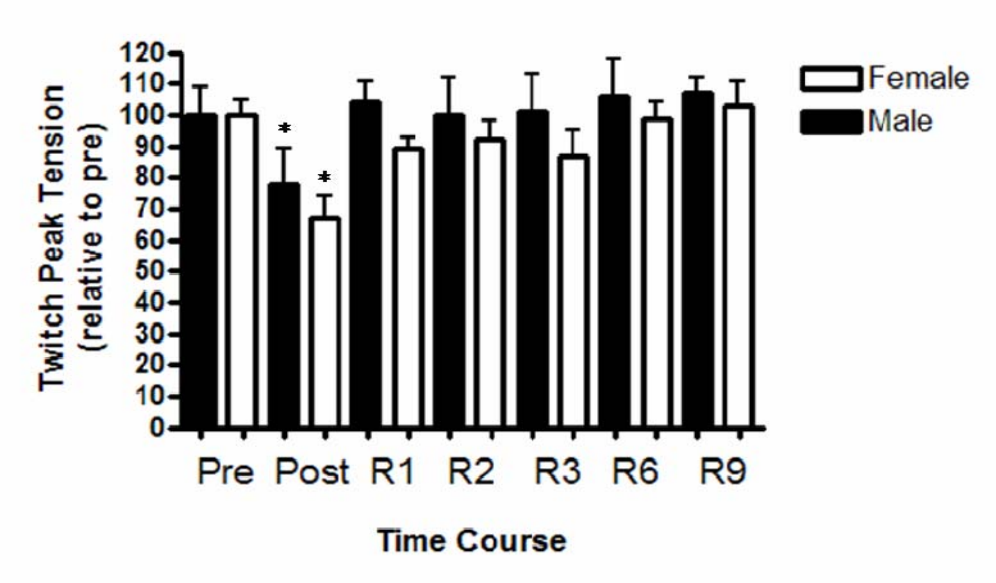

Figure 12: Male vs. female peak twitch tension - Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144 (R6) and 216 (R9) h post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are expressed relative to pre exercise values. *Significantly different than Pre ($p<0.05$)

Table 1: Twitch Kinetics (+dF/dt)- Measurements were taken PRE, POST, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 h POST exercise. Values are expressed as Mean ±SE. * Significantly different than PRE. $ Significantly different than R3.

	Male				Female			
	E	SE	Cont	SE	Ex	SE	Cont	SE
PRE	1734	115	1645	111	1688	106	1579	106
POST	1962	122	1752	163	1567	108	1599	116
R1	1890	124	1716	144	1494*	73	1424*	50
R2	1876	220	1782	154	1655	75	1401*	33
R3	1966	186	1875	48	1581	161	1548	100
R6	2446*	229	2456*	164	1485	102	1642	156

Table 2: Twitch Kinetics (-dF/dt)- Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 h POST exercise. Values are expressed as Mean ±SE. * Significantly different than PRE. $ Significantly different than R3.

	Male				Female			
	Ex	SE	Cont	SE	Ex	SE	Cont	SE
PRE	1017	197	1121	121	815	61	799	111
POST	1108	144	1097	97	725	58	775	68
R1	1035	92	962	75	833	93	681	68
R2	1016	108	973	122	768.2	81	647	47
R3	1102	111	850	58	792	51	887	87
R6	1291$^{\$}$	101	1127$^{\$}$	71	757	54	821	64

Low Frequency Force

At the low frequencies of stimulation (10 Hz) peak tension in the exercise leg was depressed ($p<0.05$) immediately post exercise in males and did not recover until R3 (**Figure 13A**). There was an unexpected decrease ($p<0.05$) in 10 Hz force at R3 in the control leg, but all other time points remained unchanged ($p>0.05$). Low frequency (10 Hz) force for females was significantly depressed ($p<0.05$) in the exercise leg immediately post exercise, which remained depressed until R2 (**Figure 13B**).

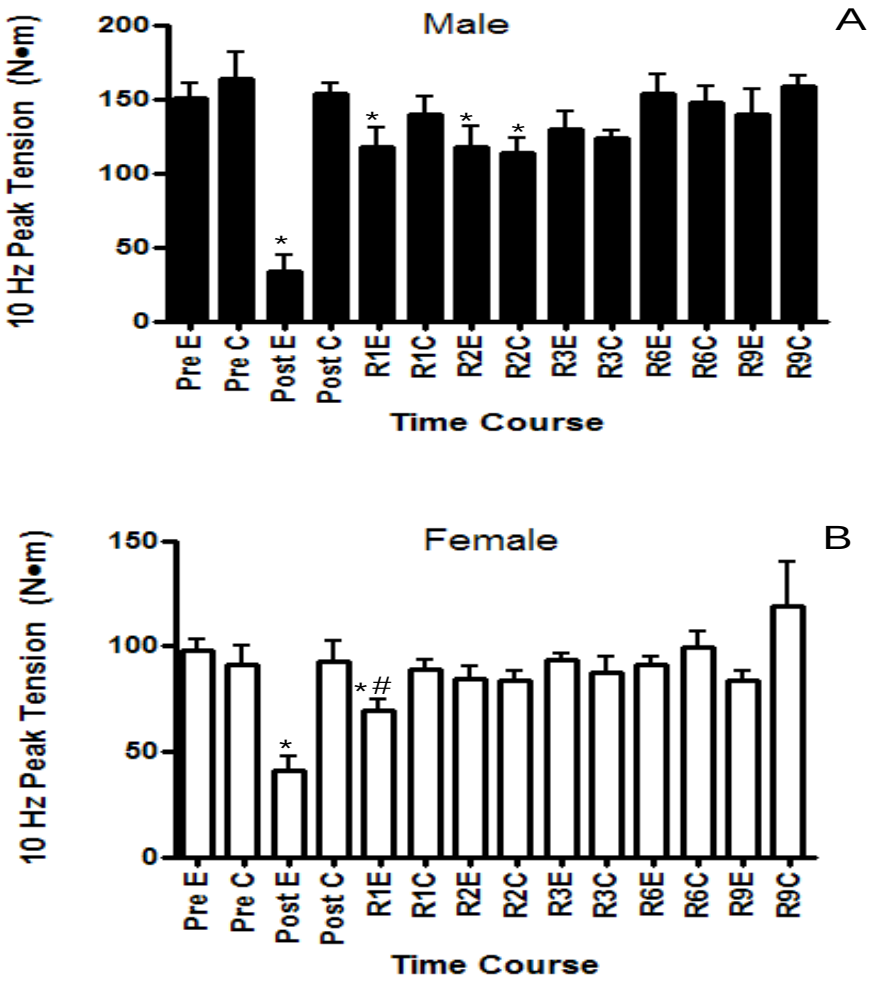

Figure 13A/B: Male/Female 10 Hz peak tension - Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 (R9) h post exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE. *Significantly different than Pre ($p<0.05$).

There were no significant changes ($p>0.05$) in 10 Hz force in the control leg at any time point during the protocol for females (**Figure 14**). Males also displayed a greater relative force reduction ($p<0.05$) immediately POST in the exercise leg compared to females (**Figure 14**).

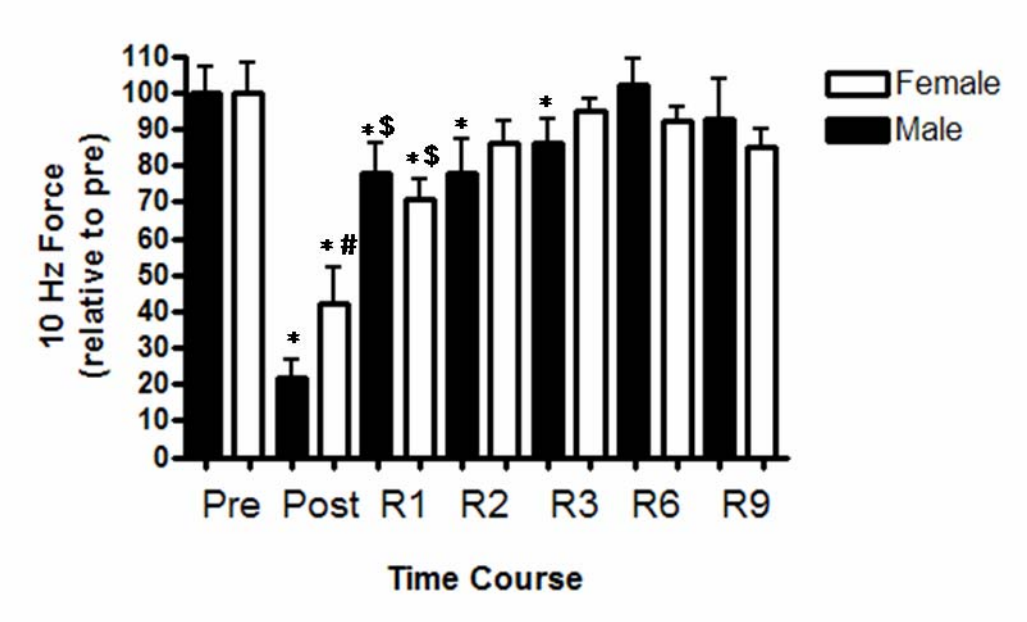

Figure 14: Male vs. Female 10 Hz peak tension - Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 (R9) h post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are expressed relative to pre exercise values. *Significantly different than Pre ($p<0.05$). #Significantly different than males ($p<0.05$).$ Significantly different than Post ($p<0.05$)

High Frequency Force

At the high frequencies (100 Hz) of stimulation, males had a significant 33% reduction ($p<0.05$) in force immediately POST exercise in the exercise leg, which

remained depressed ($p<0.05$) until R3 (**Figure 15A**). There were no significant decreases ($p>0.05$) in 100 Hz tension in the control leg of males, until R2

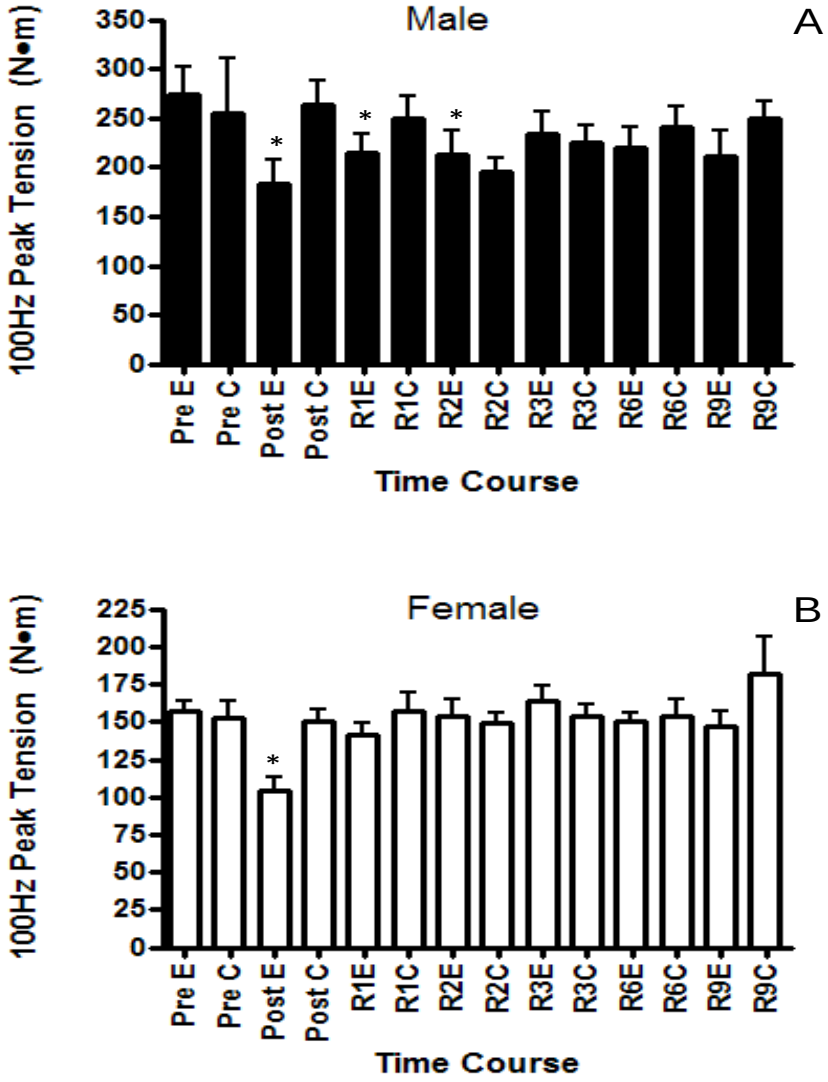

Figure 15A/B: Male/Female 100 Hz peak tension - Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 (R9) h post exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE. *Significantly different than Pre ($p<0.05$).

where there was an unexpected decrease ($p<0.05$). In females, there was an immediate 25% drop ($p<0.05$) in 100 Hz peak tension in the exercise leg, which recovered completely by R1 (**Figure 15B**). No changes ($p>0.05$) were observed in the control leg of

females throughout the recovery period. There were no interaction effects ($p>0.05$) when comparing the high frequency force between males and females (**Figure 16**).

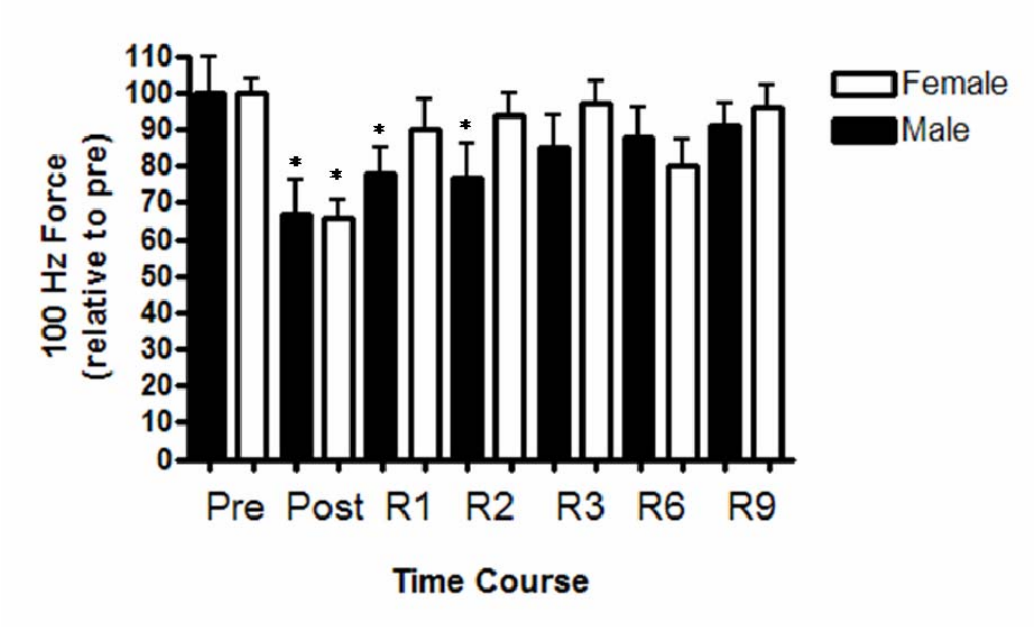

Figure 16: Male vs. Female 100 Hz peak tension (Po) - Measurements were taken Pre, Post, 24 (R1), 48 (R2) 72 (R3), 144(R6) and 216 h post exercise. Values are from the exercise leg only and are expressed as Mean ±SE. All values are expressed relative to pre exercise values. *Significantly different than Pre ($p<0.05$)

Figure 17 A/B shows that low frequency fatigue is more prevalent in males than females. More specifically, Figure 21A shows that low frequency fatigue is present in males at R1, but not in females, and Figure 21B shows that low frequency fatigue recovers in both males and females by R6.

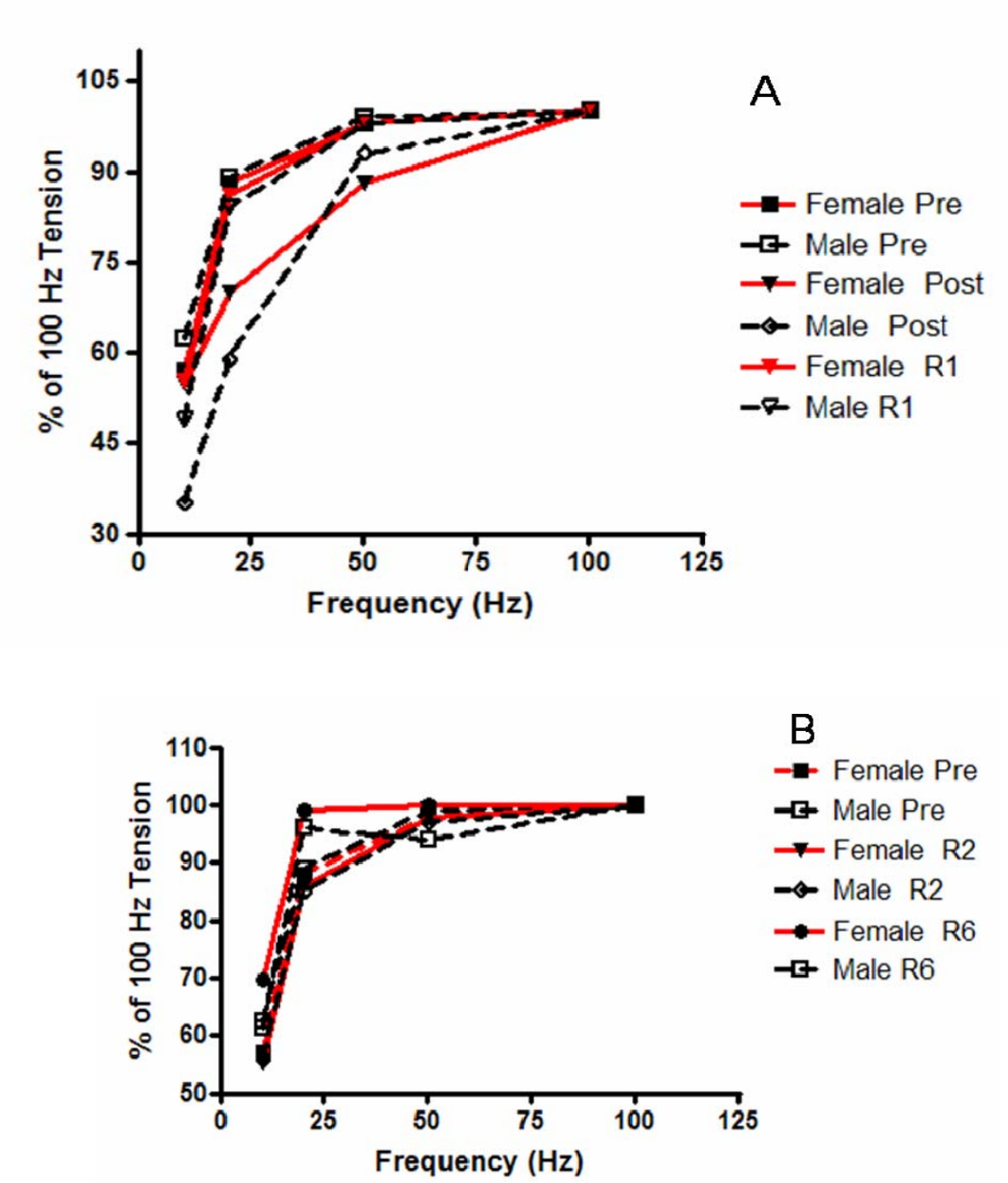

Figure 17: **Male and Female Force Frequency Response**: A, compares Pre to Post and R1 for males and females. B, compares Pre to R2 and R6. All force measurements (10, 20, 50 and 100 Hz) are expressed relative to pre 100 Hz levels. All male values are dashed lines and all female values are solid red lines.

Maximal SERCA Activity

Following exercise there were no changes ($p>0.05$) in maximal SERCA activity (V_{max}) at any time point throughout the experimental protocol in the exercise or control leg in either males or females (**Figure 18A**-male and **B**-female). Furthermore, there were

no significant differences ($p>0.05$) in the absolute V_{max} between male and female participants (**Figure 19**).

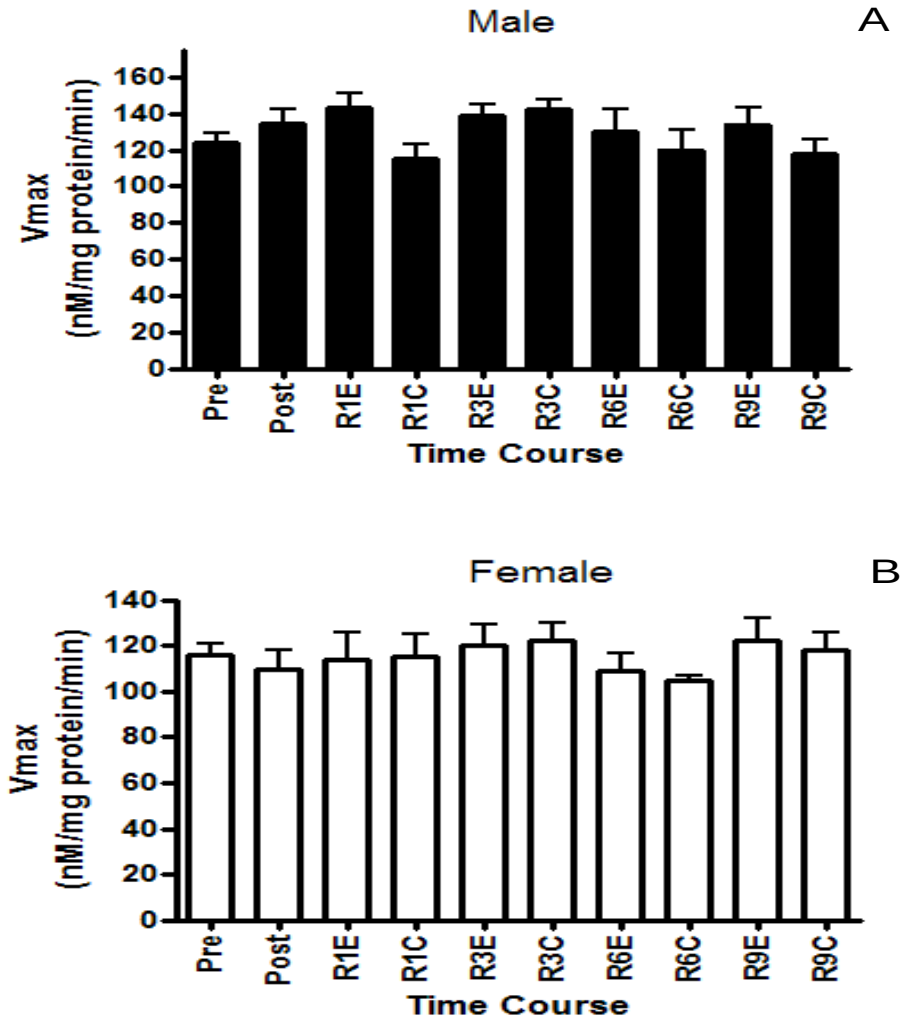

Figure 18A/B: Male/Female Maximal Ca^{2+} ATPase Activity - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216 (R9) h Post exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE.

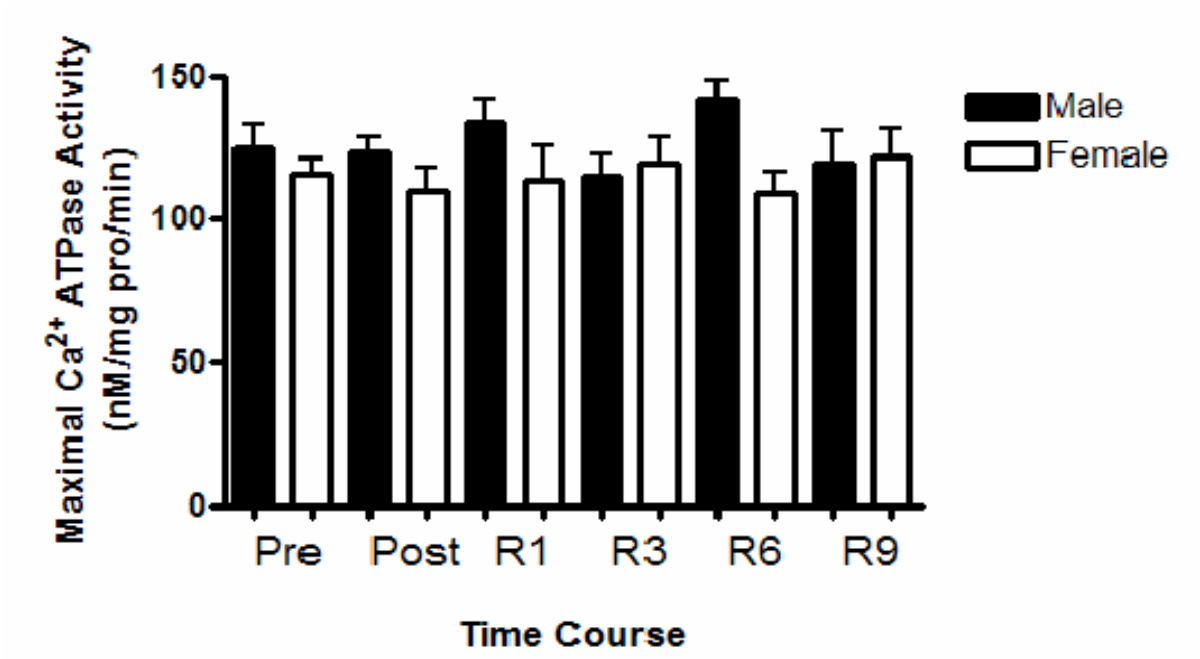

Figure 19: Male vs. Female Maximal Ca^{2+} ATPase Activity - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216h (R9) post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are expressed relative to pre exercise values.

Calcium Uptake

It was found that there were no differences ($p>0.05$) in the rates of calcium uptake (measured at 1500nM Ca^{2+}) immediately POST and throughout the recovery period in either males (**Figure 20A)** or females (**Figure 20B**). The only point of significance was at the R3 time point in females, where uptake was decreased ($p<0.05$) in the exercise leg. There were no significant differences ($p>0.05$) between the exercise and control legs in males or females. Furthermore, no statistical differences ($p>0.05$) were found when comparing the rates of Ca^{2+} uptake between males and females prior to exercise or at any time point throughout the recovery period (**Figure 21**). Results of measurements at other Ca^{2+} concentrations (2000, 1000 and 500nM) are not shown because they showed the same trends as the values measured at 1500nM.

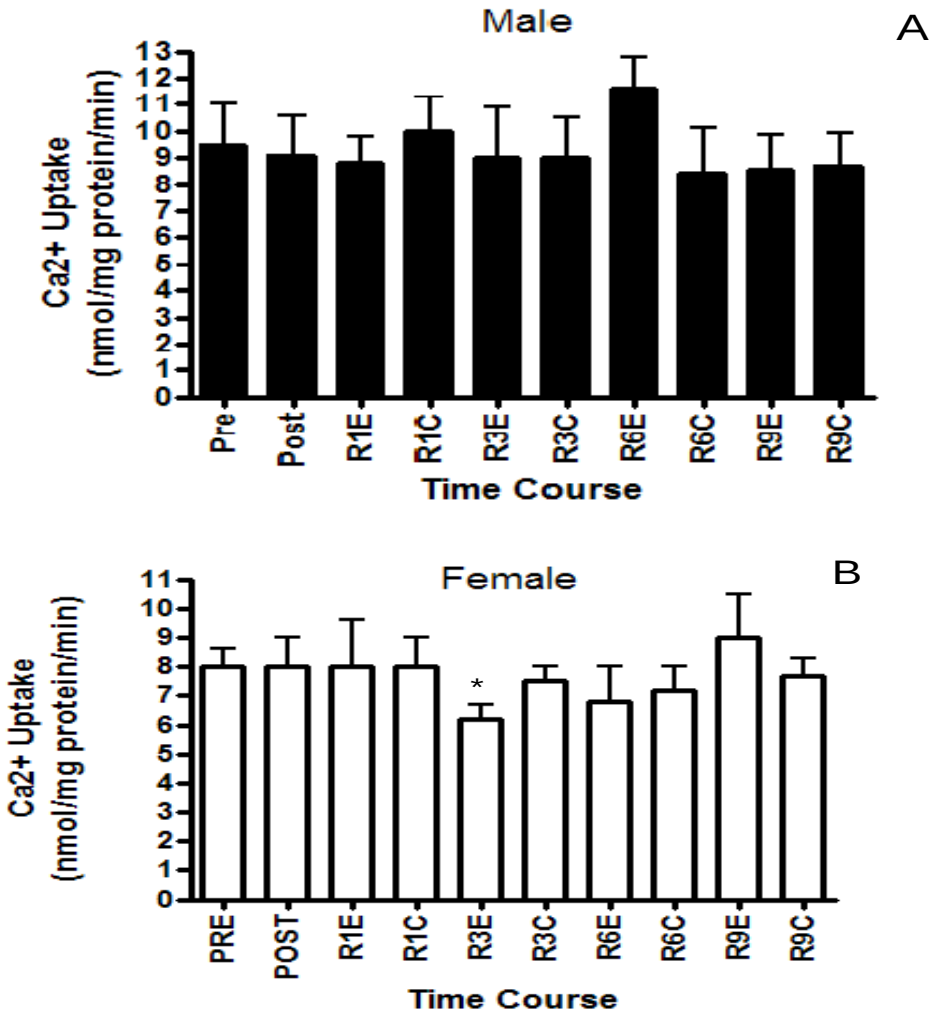

Figure 20A/B: Male/Female Ca^{2+} Uptake at 1500nM - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216 (R9) h POST exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE.

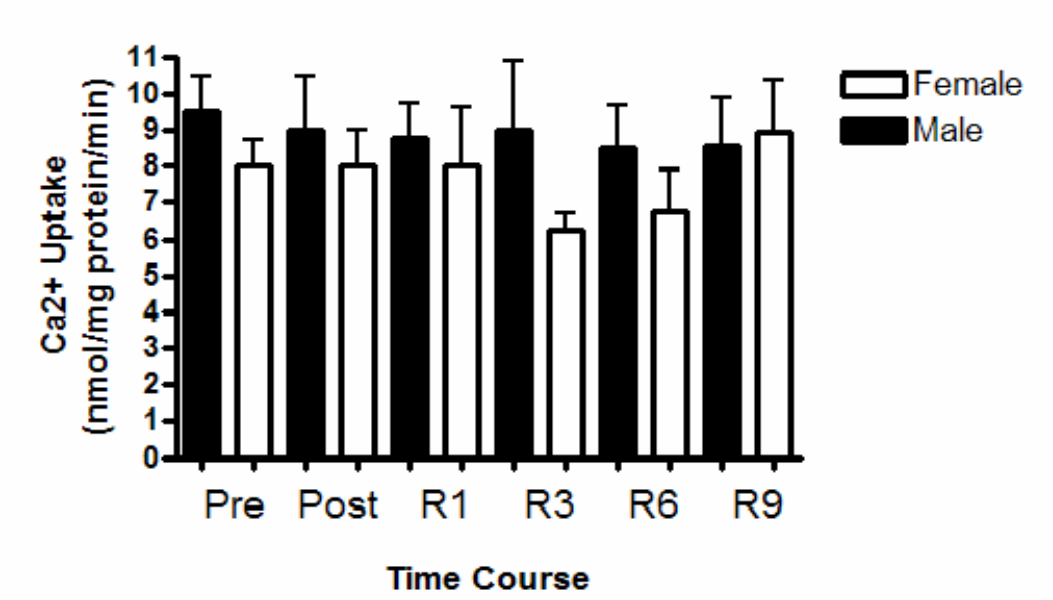

Figure 21: Male vs. Female Ca^{2+} Uptake at 1500nM - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216 (R9) h post exercise. Values are from the exercise leg only and are expressed as Mean ± SE.

SERCA Protein Content

Western blotting was performed on whole muscle homogenate to quantify SERCA1a and SERCA2a protein content in male and female vastus lateralis. SERCA1a protein content was unchanged ($p>0.05$) by exercise or recovery in either the exercise and control legs in both males **(Figure 22A)** and females (**Figure 22B**). Furthermore, there were no differences ($p>0.05$) in absolute SERCA1a protein content between males and females at any time point (**Figure 23). (Note: representative blots can be found in appendix II)**.

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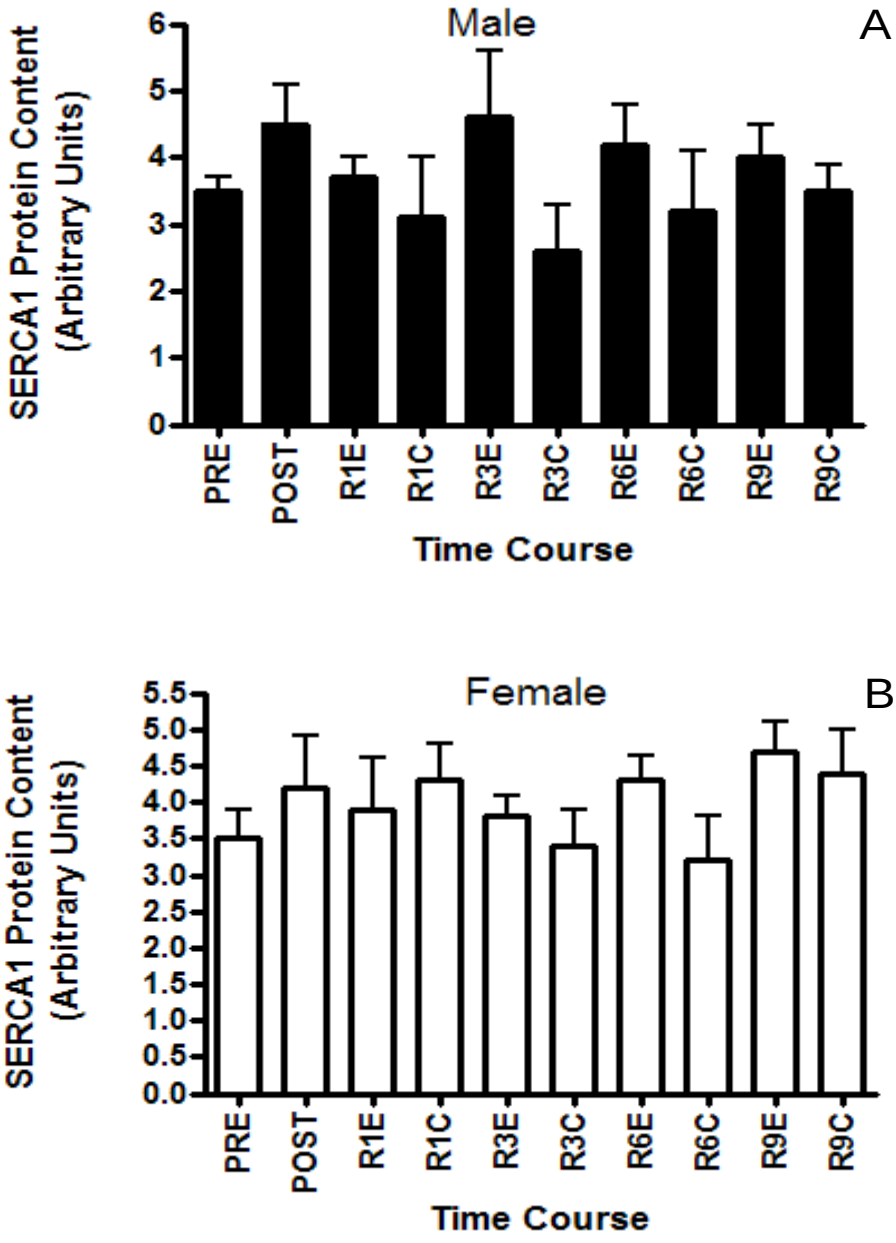

Figure 22A/B: Male/Female SERCA1a Protein Content - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144(R6) and 216 (R9) h POST exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE and are normalized to protein standard and α-actin levels.

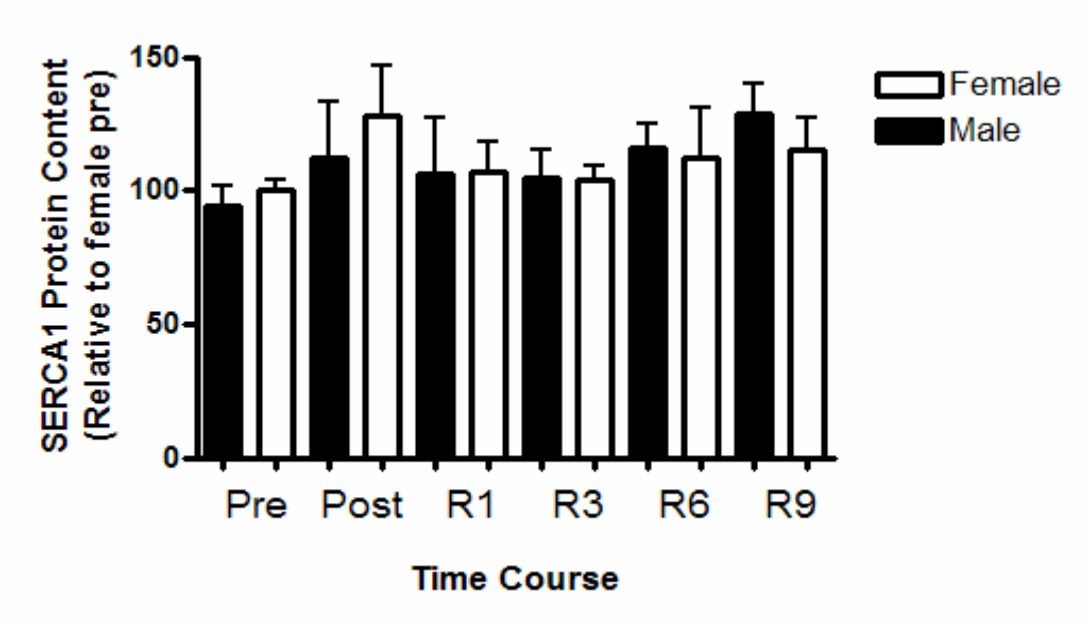

Figure 23: Male vs. Female SERCA1a Protein Content - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144(R6) and 216 (R9) h post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are normalized to standard and α-actin protein concentrations and expressed relative to female pre values.

In males, there was a significant increase ($p<0.05$) in SERCA2a protein content in the exercise leg at R1 and in the control leg at R3 and R6 (**Figure 24A**). In females, SERCA2a content was not different ($p>0.05$) in either the exercise or control leg at any time point throughout the study (**Figure 24B**). When comparing male and female SERCA2a protein content, it was found that females have significantly more ($p<0.05$) SERCA2a Pre and immediately Post (**Figure 25**).

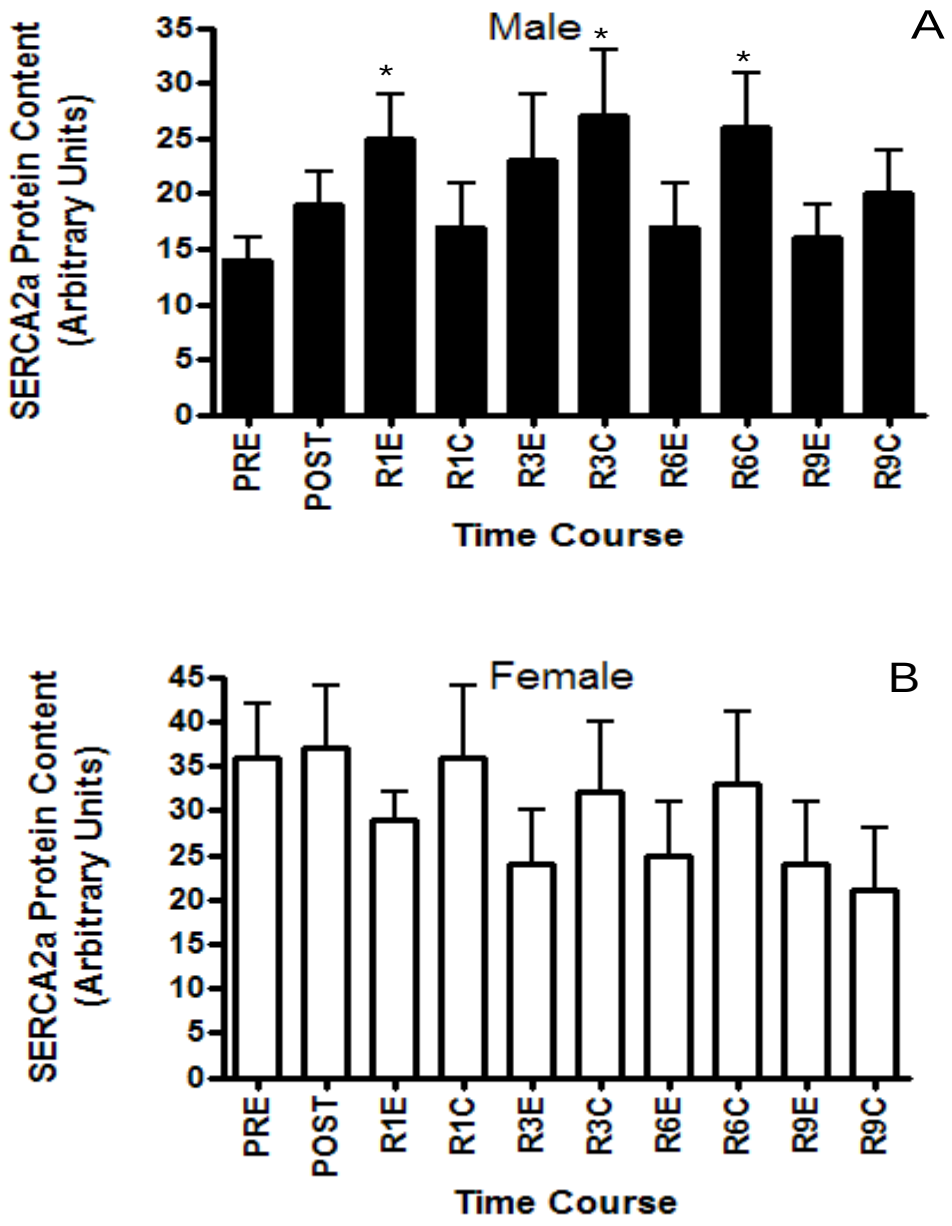

Figure 24A/B: Male/Female SERCA2a Protein Content - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144 (R6) and 216 (R9) h post exercise in both the exercise (E) and control (C) legs. Values are expressed as Mean ± SE and are normalized to protein standard and α-actin levels. *Significantly different than Pre ($p<0.05$).

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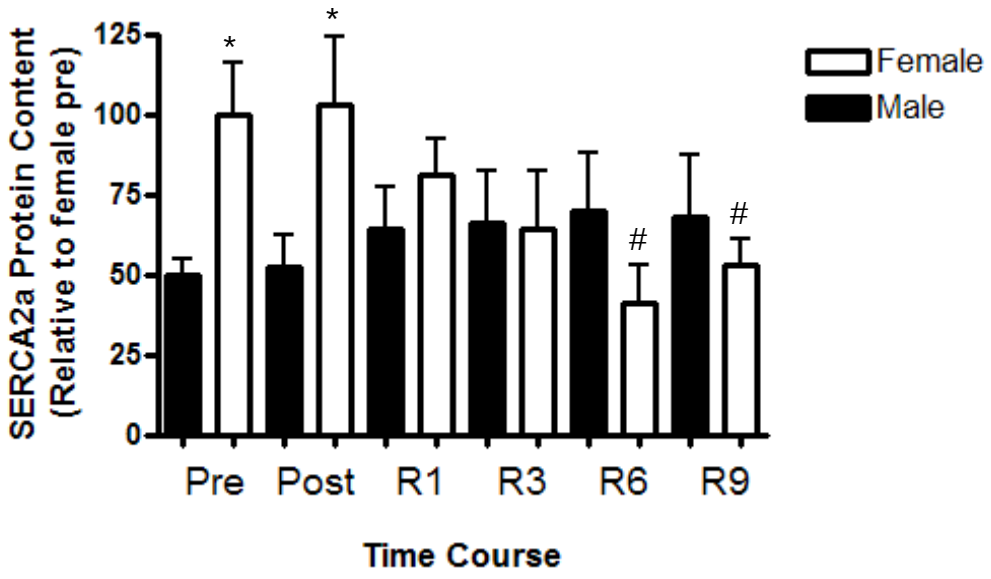


Figure 25: Male vs Female SERCA2a Protein Content - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144(R6) and 216 (R9) h post exercise. Values are from the exercise leg only and are expressed as Mean ± SE. All values are normalized to standard and alpha actin protein concentrations and expressed relative to female pre values. *Significantly different than male ($p<0.05$). # Significantly different than female Pre ($p<0.05$).

Serum Creatine Kinase

In males, serum creatine kinase activity was elevated ($p<0.05$) immediately Post exercise and returned to baseline values by R1, whereas in females, serum creatine kinase activity did not change ($p<0.05$) at any time point throughout the study (**Figure 26**).

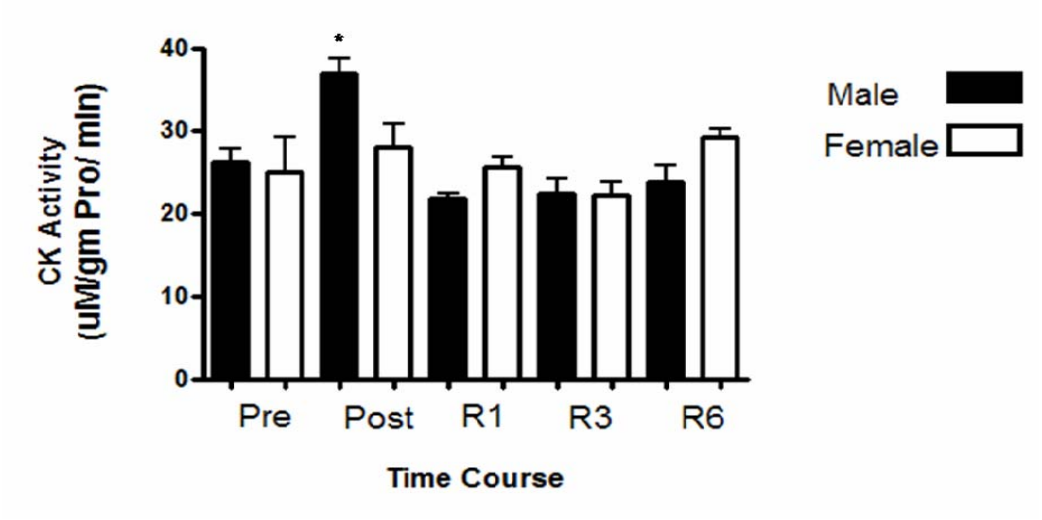


Figure 26: Serum Creatine Kinase (CK) Concentrations in Males and Females - Measurements were taken Pre, Post, 24 (R1), 72 (R3) and 144(R6) h post exercise. Values are expressed Mean ± SE. *Significantly different than Pre ($p<0.05$).

Serum Estrogen

Serum estrogen concentration was significantly higher ($p<0.05$) in females compared to males at all time points during the experimental protocol (**Figure 27**). Serum estrogen levels were stable over time in both males and females.

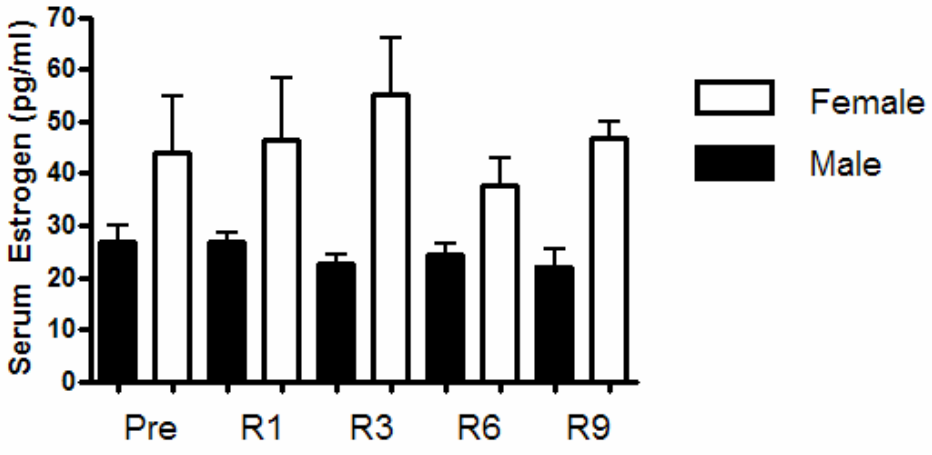


Figure 27: Serum Estrogen Concentrations in Males and Females - Measurements were taken Pre, Post, 24 (R1), 72 (R3), 144(R6) and 216 (R9) h post exercise. Values are expressed Mean ± SE. There was a significant main effect ($p<0.05$) for sex (Females>males).

Averaged electromyography (aEMG) during exercise

The results for aEMG show that neural activation of the vastus lateralis muscle increased in both males and females during the first 15 minutes of exercise, then reached a plateau during the last 15 minutes (**Figure 28**). There were no significant differences between aEMG activation between males and females at any time point during the exercise bout (**Figure 29**). Furthermore, it is apparent that the control leg was slightly active during the exercise protocol in both males and females.

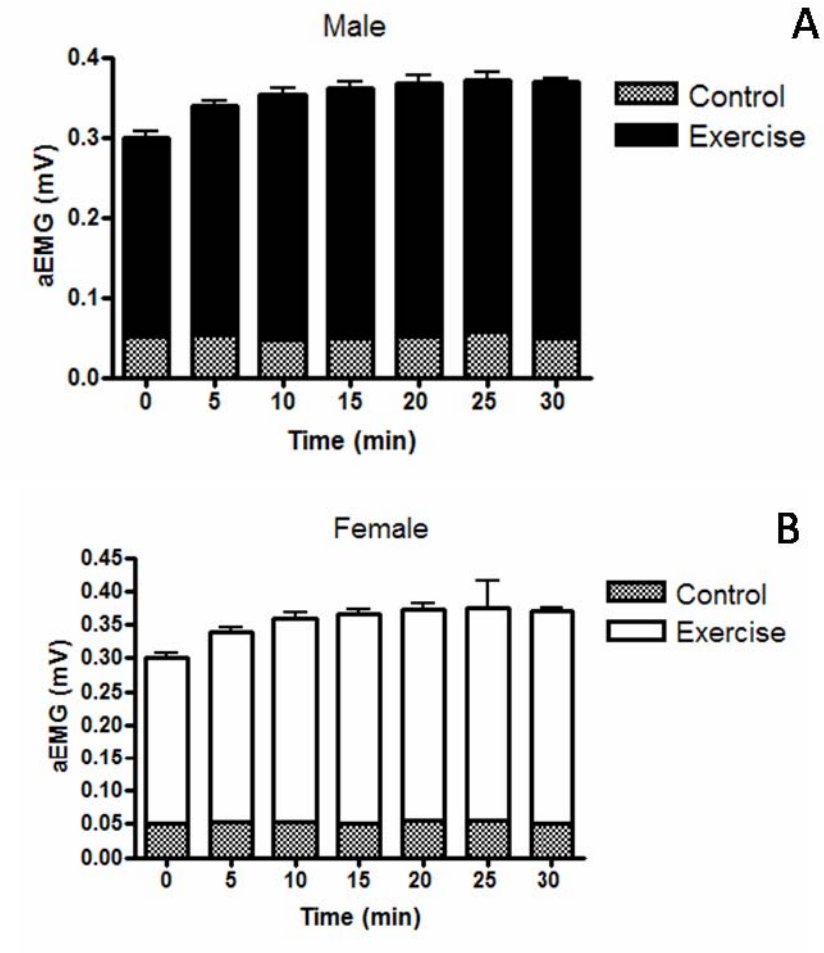


Figure 28A/B: Mal/Female aEMG during exercise: measurements were taken 0, 5, 10, 15, 20, 25, and 30 min throughout the exercise protocol. Values are expressed as Mean ± SE.

Chapter 4: Discussion

The major purpose of this study was to determine if sexual dimorphism exists in the basal and exercise induced Hsp70 protein content in human skeletal muscle. Furthermore, a major goal was to determine if any differences in Hsp70 content between males and females might be associated with skeletal muscle fatigability and mechanical function and changes in SERCA pump function in response to intense, intermittent, isometric exercise. The final purpose was to determine if the time course of recovery in all measurements differed between males and females.

Sexual dimorphism in basal Hsp70 protein content

It was found that there were no significant differences in basal Hsp70 content between males and females. In contrast with the initial hypothesis, pre-exercise muscle biopsy samples of the vastus lateralis showed that with no previous exposure to stress, there are no differences between males and females in Hsp70 expression. This finding is in agreement with one rodent study (Voss et al., 2003) which also showed no significant differences in the male/female basal Hsp70 content of skeletal muscle in rats. However, these results are in contrast to the findings by Bombardier et al (2009) who showed that ovariectomized rats that were supplemented with estrogen had a significantly greater amount of basal Hsp70 in soleus, compared to ovariectomized sham-supplemented females. The findings from this thesis also conflict with data from cardiac muscle, which showed that female rat heart had twice as much basal Hsp70 compared to male heart (Voss et al, 2003 and Paroo et al, 2002). Our hypothesis was also based on the finding that estrogen can activate the HSF1 pathway, which has been shown to increase basal levels of Hsp70 (Knowlton et al., 2003)

It was hypothesized that females would have higher basal Hsp70 levels due to the influence of estrogen on skeletal muscle physiology. As expected, compared with male participants, serum estrogen levels were two times higher in female participants; however, it's possible that basal Hsp70 expression in female skeletal muscle fluctuates over the course of the estrus cycle, and since female participants were tested between days 1-3 of their menstrual cycle, Hsp70 expression may not have been at its peak. It is unlikely that other sex hormones (progesterone and testosterone) have an influence on basal Hsp70 content in skeletal muscle. Milne et al. (2005) showed that castration of male rats (removing endogenous testosterone) had no effect on basal Hsp70 content in skeletal muscle and Bombardier et al. (2009) showed that administering exogenous progesterone to ovariectomized females did not increase basal Hsp70 content in soleus muscle. Bombardier et al. (2009) only measured Hsp70 content in rat soleus muscle, which is primarily composed of Type I muscle fibres. Since human vastus lateralis is composed of a mixture of fibre types (Green et al, 1981), it is possible that having more glycolytic fibre types dilutes the higher levels of Hsp70 in oxidative cells. Finally, it is possible that the observed lack of sexual dimorphism in basal Hsp70 expression in human skeletal muscle is due to the lack of statistical power and small sample size. In order to determine a significant difference of 25% in basal Hsp70, with a power of 0.90, it was calculated that a sample size of 15 male and 15 female participants would be needed. Therefore, this serves as a limitation of this study. Nevertheless, based on the results from this thesis, it is concluded that sexual dimorphism does not exist in humans with respect to basal Hsp70 content in skeletal muscle.

Sexual dimorphism in the exercise induced Hsp70 response

Previous studies have shown that exercise has the ability to upregulate Hsp70 protein content in order to protect the muscle against further damaging/lethal stresses in male human and rodent models (Paroo et al., 2002, Locke et al, 1991, Morton et al, 2006 and Tupling et al., 2007). It was hypothesized that following 30 minutes of intense intermittent single legged isometric knee extension exercise, females would have a blunted Hsp70 response compared with males. This study found that the exercise protocol employed has the ability to stimulate a robust Hsp70 response in male skeletal muscle, similar to findings from previous work which used the exact same exercise protocol (Tupling et al, 2007). Furthermore, as was hypothesized, this was the first study to show in humans that females have a blunted Hsp70 response following exercise.

Several mechanisms may be responsible for the blunted Hsp70 response in females. First, increased serum creatine kinase (CK) following intense exercise, which is indicative of sarcolemmal damage (Tiidus et al., 1996), was significantly increased one day after exercise in males, but not in females. This finding is consistent with previous work which shows that females have less overall damage compared to males following exercise (Tiidus et al., 1996, Bombardier et al, 2009 and Enns et al, 1999). This would suggest that since females have less muscle damage and potentially less protein denaturation, the stimulus to upregulate Hsp70 expression is lower in females compared with males. Secondly, estrogen has been described as an antioxidant molecule similar to vitamin E, capable of reducing the effects of increased levels of oxidative stress in skeletal muscle (Persky et al, 1999). Estrogen has these antioxidant properties because it has an extra hydroxyl group, capable of accepting free electrons (free radicals) which

accumulate in the cell during high periods of stress. If estrogen has the ability to minimize the accumulation of ROS/RNS which normally increase with exercise and can cause protein denaturation (Stice et al, 2008), this would suggest that less Hsp70 would be needed to counter the increase in ROS/RNS. Although ROS/RNS accumulation was not measured in this thesis, this may provide another explanation for the attenuated Hsp70 response in females.

The exercise induced increase in Hsp70 of male skeletal muscle probably occurred primarily in Type I fibre types. Using immunohistochemistry, Tupling et al (2007) showed that Type I muscle fibres had significantly more Hsp70 following exercise compared to type II fibres. This thesis did not confirm if fibre type differences occurred in either males or females so further work should be done to determine if the fibre type specific Hsp70 response displays sexual dimorphism. This study also found that there was a trend showing male Hsp70 would have completely recovered by R9 but instead there was an unexpected increase in Hsp70 at R9 in both E and C legs. This finding suggests that most likely participants did not follow pre-experimental instructions to not exercise during the experiment. Previous work by Tupling et al (2007) showed that Hsp70 protein content in male skeletal muscle was still higher than basal levels at R6, but was beginning to show signs of recovery.

Muscle Fatigability and Mechanical Function

Alterations to mechanical function in skeletal muscle are common following a bout of intense activity and can be associated with decreased force and slowing of muscle contraction (i.e. muscle fatigue) (Allen et al., 2007 and Tupling et al, 2004). It was hypothesized that female skeletal muscle function would be more protected compared to

males following intense exercise, which would be associated with less fatigue immediately post exercise, less decrements in low frequency fatigue (LFF) and a more accelerated force recovery compared to males. It was found that following the bout of exercise maximal voluntary contraction (MVC) was decreased by ~25% in females compared to the much larger ~60% decrease observed in males. It was also discovered that MVC of males took longer to return to baseline levels (recovered by R2), than females (recovered by R1). These findings support the hypotheses that females are more resistant to fatigue and recover mechanical function following fatiguing exercise more quickly than males.

Similar results were observed for both low and high frequency force. At low frequencies of stimulation (10 Hz), there was a significant 75% decrease in peak tension for males with females showing only a 55% decrease immediately post exercise. Females also had full recovery of low frequency force by R2, while males did not fully recover until R3. This finding is similar to previous work from our lab which has shown peak tension elicited at 10 Hz to be attenuated for up to 4-6 days following the bout of exercise (Fowles et al, 2001). Intense isometric exercise also resulted in a significant decrease of peak tension of the vastus lateralis muscle stimulated at high frequencies (100 Hz) in both males and females. More specifically, males had a significant ~30% decrease in 100 Hz peak tension immediately post exercise, which remained depressed until R3 of the experimental protocol. Females displayed a similar ~30% decrease in 100 Hz peak tension immediately post exercise; however, force was fully recovered by R1. These findings are consistent with the results from Fowles et al (2001), which showed HFF to be present for up to 4 days following the bout of exercise.

Force depressions at both high and low frequencies that persist past one day of recovery have been termed post contractile force depressions (PCDs) (Fowles et al, 2002 and Tupling et al, 2000). Decrements in force stimulated at high frequencies have been associated with alterations to the Na^+/K^+ ATPase. The Na^+/K^+ ATPase is responsible for maintaining a proper ion gradient across the sarcolemma to maintain excitability of the t-tubules. Fowles et al (2002) suggested that decrements in Na^+/K^+ ATPase are at least in part due to accumulation of ROS. This may provide one mechanism by which females experience less fatigue and recover quicker than males.

Low frequency fatigue (LFF) is a prolonged reduction in force when stimulated at low frequencies (Tupling et al, 2004 and Allen et al, 2007). LFF is a common observation following a bout of intense exercise. At low frequencies of stimulation, force development relies upon the release of Ca^{2+} from the SR. In situations where LFF is present, Ca^{2+} release from the SR is impaired and the rise in intracellular Ca^{2+} needed to cause contraction is less. The result in an increase of intracellular calcium concentrations, which has been associated with the activation of various proteolytic calpains capable of further catabolic reactions (Murphy et al, 2006) The results of this thesis suggest that LFF is present in both males and females, but females recovery more quickly. Figure 16A/B uses values at various frequencies (10, 20, 50 Hz) expressed relative to force elicited at 100 Hz. Figure 16 A shows that male Post and R1 are not only lower than male Pre, but also lower than female Pre, Post and R1. Furthermore, in Figure 16B it is apparent that LFF does not exist in either males or females any further than R2 since force is recovered by R3. It is likely that since females did not have as much damage as males, as illustrated by less CK activity post exercise, the total amount of damage to membranous and

intracellular structures in females was less. This would potentially mean that there is less impairment of calcium release in females and less activation of calpains, which may help explain why females are more fatigue resistant than males.

Previous research has examined whether sexual dimorphism exists in skeletal muscle fatigability. The evidence that currently exists is equivocal, in that some studies show females to be more fatigue resistant than males (Clark et al, 2005, Russ et al, 2003 and Hunter et al, 2001), while others show no differences (Buckley-Bleiler et al., 1989, Savage et al, 2002 and Thompson et al, 1997). Most previous work supporting fatigue resistance in females used a much lower intensity of contraction (~25% MVC) and measured endurance time (time to failure) rather than directly measuring skeletal muscle contractile properties. These studies found that females were able to maintain the intensity of exercise longer than males, which they attributed to differences in muscular blood flow (Clark et al., 2005 and Russ et al, 2003). Conversely, studies which contended the notion that females are more fatigue resistant than males utilized higher exercise intensities and showed there were no differences in fatigue resistance between males and females during exercise. Furthermore, these same studies suggest that there is no difference in recovery of force in the days following exercise. In this thesis, a high intensity exercise protocol was employed but unlike other studies that employed higher intensity exercise protocols, the findings support the notion that females are more fatigue resistant immediately following exercise and that females recover force more quickly than males. Differences between this study and others that contend fatigue resistance in females is associated with the protective effects of estrogen may be due to a couple of reasons. First, previous work showing females were not fatigue resistant which used

isometric exercise used a longer work:rest ratio. Buckly-Bleiler et al (1989) used a 10:20 sec (work:rest ratio) compared to our 5:5 sec ratio. This longer work to rest ratio may have resulted in more recovery time in between contractions, which may be the reason no differences were detected. This same study contending fatigue resistance in females only used a total of 40 contractions, whereas this thesis had the participants doing 180 contractions. These two differences may result in less damage to skeletal muscle and more recovery during the exercise bout, which may help explain the discrepancy in results compared to this thesis. Finally, previous work suggesting there is no differences in fatigue between males and females following exercise, did not actually use a direct male to female comparison. These studies utilized pre-menopausal comparisons (Buckley-Bleiler et al., 1989, Savage et al, 2002 and Thompson et al, 1997) in comparison to the male-female comparison used in this thesis.

Another debate that currently exists in the literature is whether the examination of fatigue between males and females should be studied with respect to absolute or relative force production. This debate stems from the finding that under low intensity contractions, which is expressed relative to the individual's MVC, females are more fatigue resistant compared to males (Hunter et al., 2001, Clark et al., 2005, Russ et al., 2003). When the intensity is increased there appear to be no differences in fatigability between males and females (Maughan et al., 1986, Phillips et al., 1993). This argument is based on the notion that at low intensities, although both male and female subjects contract at a certain percentage of their MVC, males have higher absolute force production, which results in more occlusion of peripheral vasculature and therefore puts them under more ischemic conditions (Clark et al., 2005, Wust et al., 2008 and Maughan

et al., 1986). When intensity is increased, both males and females are shown to have the same amount of occlusion, which explains the lack of sexual dimorphism observed in fatigability at higher intensities (Maughan et al., 1986 and Russ et al., 2003). The results of this thesis cannot rule out the fact that males may have been under more ischemic conditions compared to females since male participants would have been producing higher absolute forces than females due to the relative force production of the exercise protocol. Although using a force expressed relative to an individual's MVC is generally accepted as a valid method of analysing fatigue between people, future studies may aim to recruit male and female participants that have similar MVCs. This type of design would rule out the fact that blood flow restrictions and absolute force production in males is what is causing the sexual dimorphism observed in skeletal muscle fatigability since both groups would be contracting with the same absolute force.

The observed sexual dimorphism in muscle fatigability and rate of fatigue recovery is not associated with Hsp70 as hypothesized, given that no differences in basal Hsp70 expression were observed between males and females. It is possible that the increased fatigue resistance observed in females can be explained by estrogen having a stabilizing effect on the cell membranes or acting as an antioxidant and preventing oxidative damage to key cellular proteins involved in excitation-contraction coupling.

Sex Differences and the Effects of Exercise on Calcium Handling Properties in Muscle

The decrements observed in force generated by low frequency stimulations have been associated with impairment to SERCAs ability to sequester Ca^{2+} back into the lumen of the SR (Allen et al, 2007, Duhamel et al., 2007, Tupling et al, 2000). More

specifically, it could be a result of impaired calcium release, which associated with a disabled excitation-contraction coupling mechanism. Since Hsp70 can bind to and protect SERCA function, and females may have higher basal Hsp70 compared males, it was hypothesized that SERCA function in females would be better protected during exercise when compared to males (Tupling et al., 2004 and Fu et al., 2007).

Contrary to the hypothesis that exercise would result in a decrease in maximal SERCA activity and Ca^{2+} uptake, there were actually no changes in either measure in both males and females at any time point throughout the study protocol. These findings do not agree with those found from other studies which showed that exercise causes impairment of SR pump function (decrease in activity and uptake) in human skeletal muscle (Tupling et al, 2000 and Duhamel et al, 2007). It is unlikely that the discrepancies observed in this study are due to analytical error since both maximal SERCA activity and calcium uptake showed similar patterns throughout the recovery period for both males and females. Another suggestion may be that Pre values of maximal SERCA activity and calcium uptake are lower compared to previous studies. Duhamel et al (2007) found that maximal SERCA activity (V_{max}) in males was ~150-170 μM/g protein/min prior to exercise and decreased to ~120 μM/g protein/min immediately Post exercise, a value which is closer to the Pre values of this thesis. Discrepancies between this thesis and previous work may be a result of participants not following pre experimental procedures or analytical error, in that Vmax was not actually reached in the Pre samples. It was assumed that the $CaCl_2$ additions which were used to determine calcium dependent SERCA activity would be the same for each participant but it is possible that some samples had different $CaCl_2$ requirements than others.

There were no differences between males and females in SERCA1a expression but SERCA2a content was higher in females than males at Pre. There were no changes with exercise or recovery in protein content of either SERCA isoform in males whereas in females SERCA2a content was decreased 6 days following the exercise and remained lower at R9. The significance of these results is unknown but further work investigating sexual dimorphism in the regulation of SERCA isoform expression is warranted.

Given the uncertainty in the SERCA activity results in this thesis, it is difficult to make any conclusions regarding the role of SERCA in sexual dimorphism in muscle fatigability. The mechanisms underlying sexual dimorphism in muscle fatigability may related to the ability of estrogen as an antioxidant to protect Ca^{2+} release from the SR or the Ca^{2+} sensitivity of the contractile apparatus. Unfortunately, due to limitations of this study, these proposed mechanisms cannot be confirmed.

Limitations

Due to time and cost restraints, this thesis had a relatively small sample size which may have precluded detection of potentially significant differences between males and females, especially in basal Hsp70 expression which tended to be higher in females. This thesis was also unable to determine fibre type differences in Hsp70 protein content through immunohistochemistry which is a more sensitive technique than Western blotting Therefore, it is possible that sexual dimorphism in basal Hsp70 expression does exist in different fibre types. The fact that Hsp70 increased at R9 suggests that participants did not fully follow pre experimental instructions. This unfortunate occurrence does not allow conclusions to be drawn regarding the time course of complete Hsp70 recovery following exercise in male skeletal muscle.

The finding that SERCA function (maximal SERCA activity and Ca^{2+} uptake) was unaltered by exercise disagrees with previous studies that employed the exact same exercise protocol (Tupling et al, 2000 and Duhamel et al., 2007). The results from this study on the effects of exercise on SERCA function are therefore questionable due to the reasons stated above. This thesis did not examine the role of calcium release as a mechanism for low frequency fatigue. Since other studies have found that LFF is associated with impaired calcium release, it would have been useful to determine if fatigue in this study was related to impairments in SR Ca^{2+} release.

This thesis was also unable to elucidate the effects of individual sex hormones (estrogen, progesterone, testosterone) on human skeletal muscle physiology. Future studies should aim to use different populations (pre/post menopausal women for example)

to determine effects of endogenous estrogen/progesterone depletion on the Hsp70 stress response and resulting effects on human skeletal muscle physiology.

Future Directions

Considering the most important finding from this study was that there is sexual dimorphism in the exercise induced Hsp70 protein content, future studies should focus on populations where muscle mass and function are important for independence and function. We were unable to detect any significant differences in basal Hsp70, therefore future studies should aim to increase sample size to determine if basal differences do exist between males and females. Furthermore, our laboratory has found that Hsp70 expression following exercise is fibre type specific in males. Therefore, future work may try to determine if the response in females is similar. Future studies may also try to look at different phases of the menstrual cycle to determine if varying serum estrogen concentrations in the blood effects the basal and exercise induced Hsp70 response in females.

Future studies should aim to work with the elderly individuals and comparing pre and post menopausal females to determine the significance of Hsp70 at that point in the life cycle. Future studies should also aim to elucidate the mechanism by which sexual dimorphism in muscle fatigability exists and the interaction of Hsp70 and SERCA between males and females.

Conclusion

The results of this thesis suggest that basal Hsp70 expression in skeletal muscle does not display sexual dimorphism in humans. However, there is sexual dimorphism in the exercise-induced Hsp70 response in skeletal muscle in humans as it was found that males have a more robust Hsp70 response in the recovery days following exercise compared with females. The findings of this thesis are most likely attributable to the differences in circulating estrogen between males and females.

The results of this thesis were also able to confirm sexual dimorphism in muscle fatigability and function. It was found that females were more fatigue resistant than males and were able to recover force at an accelerated rate. The causes of fatigue, which have commonly been associated with alterations in SERCA function and activity could not be confirmed in this thesis as exercise had no effects on maximal SERCA activity, Ca^{2+} uptake or SERCA protein content.

Appendix 1:

Homogenate Ca2+ ATPase Buffer

Reagent	Molecular Weight	Mass(g)	Concentration (mM)
KCl	74.56	1.6	100
HEPES	238.3	1.02	20
NaN3	65.01	.13	10
EGTA	380.4	.082	1
MgCl2	95.21	.2046	10
PEP	465.3	1	10

- All reagents were combined in a beaker and mixed with a magnetic stir bar, then 190mL of H20 was added
- Substance was heated to 37 ^{o}C and pH was brought to 7.0
- Mixture was then brought to a volume of 215 mL and aliquoted into tubes to be stored at -20^{o}C

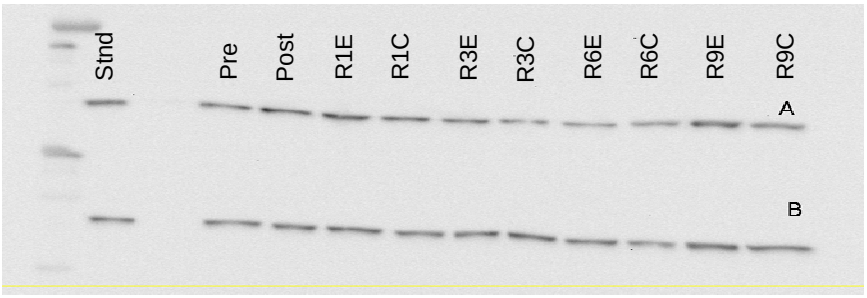
Homogenizing Buffer Recipe

Reagent	Molecular weight	Mass (g)	Concentration
Sucrose	343.3	85.6	250
HEPES	238.3	1.19	5
PMSF	174.19	.034	.2
NaN3	65.01	2	.2%

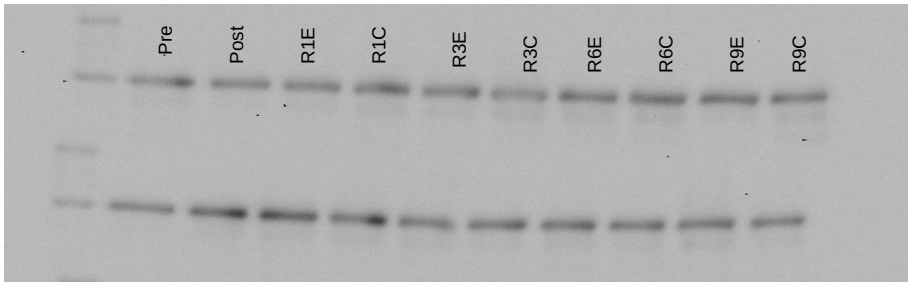
- PMSF was added to 800mL of Hs0 in a beaker and mixed with a magnetic stir bar. It was then covered with parafilm and placed on a hot plate set at a temperature of 35-40 ^{o}C.
- The PMSF was then brought to a pH of 7.5 with KOH and brought to a volume of 1L and pH was rechecked.

Appendix II- Representative Blots

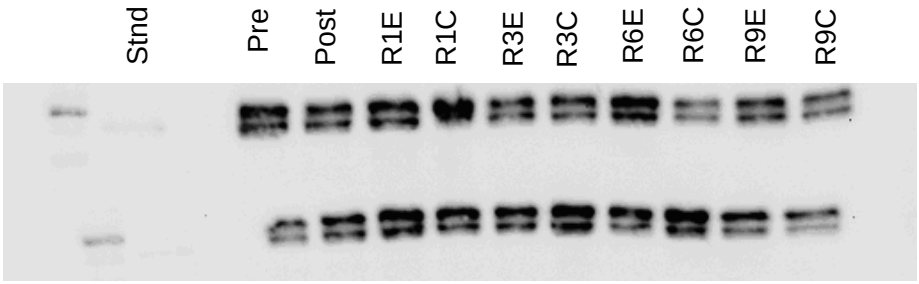
Hsp70



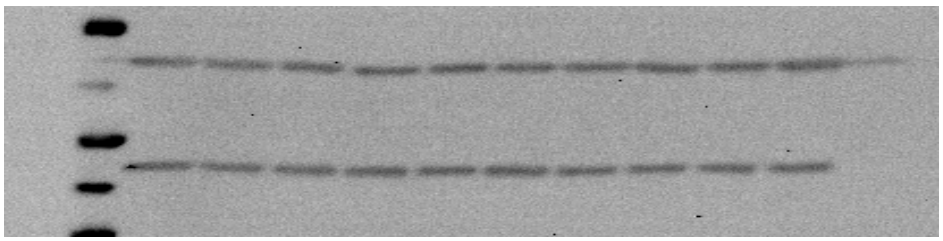
SERCA1



SERCA2a



Alpha-Actin



Appendix II: Western blotting for Hsp70, SERCA1, SERCA2a and Alpha Actin. All blots were done pairing one female and one male participant in each casting unit

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