ADAPTATION TO REPEATED ECCENTRIC EXERCISE

IN MALES AND FEMALES

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ABSTRACT

Unaccustomed eccentric exercise results in damage to skeletal muscle ultrastructure. One bout of eccentric exercise can induce adaptations in skeletal muscle, such that if the bout is repeated indices of muscle damage are attenuated. However, little is known about the pathways mediating these "protective" adaptations. The purpose of this thesis was to further characterize adaptations to repeated eccentric exercise and to investigate the hypothesis that increased stress protein content is associated with attenuated indices of muscle damage following the second exercise bout. Untrained male (N = 8) and untrained female (N = 8) subjects performed two bouts of unilateral, eccentric leg press and unilateral, eccentric knee extension exercises separated by 5.5 wk. Biopsies were taken from the *vastus lateralis* of the exercised and control (rest) leg. Blood samples and strength data were also collected. Following the second exercise bout the rise in serum CK activity, the post exercise force deficit and the extent of ultrastructural disruption as characterized by Z-disk streaming were attenuated, however ubiquitin conjugated protein content was elevated. Serum CK activity was lower in females as compared to males and ubiquitin conjugated protein content was greater in males as compared females. No gender difference was detected in the extent of Z-disk streaming or the relative magnitude of post exercise force deficit. Muscle macrophage counts were elevated over resting values 24 h post exercise bout 1 and 2 in both males and females. Whereas, neutrophils counts were only elevated over resting values following the second exercise in females. HSP72 protein content did not change in response to eccentric exercise, but it tended to be greater in males as compared to females ($p=0.053$). HSP27 protein content

increased in response to exercise in males only, although females had greater basal content. Following the second exercise bout bcl-2 protein content was greater in females as compared to males. In conclusion, adaptations to repeated eccentric exercise are associated not only with attenuated serum CK activity and force deficit as previously described, but also with decreased ultrastructural damage, increased ubiquitin conjugated protein content and changes in stress protein content.

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1.1. Introduction

Unaccustomed eccentric contractions can cause transient muscle damage which is characterized by delayed onset muscle soreness (DOMS), decreased muscle performance, muscle creatine kinase (CK) release into plasma, myofibrillar disruption, increased protein degradation and an acute phase immune response (5). Protective adaptations can occur after a single bout of eccentric exercise, such that if the bout is repeated some of the aforementioned indices of exercise induced muscle damage are attenuated (18;19;21;26;78). However, little is known about the biochemical pathways underlying these adaptations.

Gender-dependent differences in exercise induced muscle damage have been observed in humans and animal models (20). Differences in serum CK activity (17), inflammatory response (23) and ultrastructural disruptions (12) have been reported. Some of these differences have been attributed to the female sex hormone 17-β-estradiol (29). 17-β-estradiol may reduce cellular membrane damage, because of its antioxidant and membrane stabilizing properties (29).

The purpose of this thesis was to further characterize adaptations to repeated eccentric exercise and to attempt to elucidate some of the mediating pathways. A secondary purpose of this work was to gain additional insight into gender differences in exercise induced muscle damage.

1.2 Exercise Induced Muscle Damage

1.2.1.a) Initiating Events: Mechanical Damage

Eccentric contraction induced injury is initiated by mechanical factors with chemical or metabolic factors contributing to secondary or delayed onset muscle injury (28;44). Injury to a muscle fiber following eccentric contractions is localized to small groups of sarcomeres (28;135), whereas injury initiated by chemical or metabolic factors, such as calcium, free radicals and elevated temperature, is different (77). In isolated single muscle fibers one active stretch (i.e. eccentric contraction) is sufficient to cause damage, characterized by a loss in maximum isometric tetanic force (P_0) and ultrastructural disruptions (77). Such a model excludes confounding metabolic or chemical factors and thus lends support to the mechanical hypothesis of eccentric contraction induced muscle injury. Mechanical factors implicated in the initiation of fiber injury include average or peak force developed by the fiber (135), initial fiber length (44), strain (62) and work done by the fiber (29).

For a stretch to damage a fiber, the fiber must be active (i.e. there must be cross-bridge cycling). In general, passive stretches are not damaging (29;77). Initial fiber length can mediate the magnitude of contraction induced injury sustained (87). Using an *in situ* EDL muscle preparation, K. D. Hunter et. al. (44) observed that if work input was held constant, force deficit would be greater in muscle that had been activated at longer lengths. Muscle damage may also be related to the amount of active strain experienced by the fiber. The active strain can be defined as the displacement (i.e. difference between

initial and final fiber length) experienced by the muscle fiber during contraction. If strain was held constant but the amount of force generated by the muscle was varied, damage was similar (44). However, if force was held constant and the amount of strain was varied, then the muscles which experienced greater strain were damaged more (62). The work done by a fiber is dependent on the amount of strain and average force generated by the fiber. Work done by a fiber has also been shown to correlate well with contraction induced injury (77). When fibers were stretched under relatively low amounts of strain, then force developed by that fiber was the critical determinant of the magnitude of the subsequent loss in maximum tetanic isometic force (P_0). But if the strain was great, very little force would damage the muscle fiber (77).

Numerous investigators have attempted to elucidate the initial site of mechanical failure during eccentric contractions; sarcomeres (87), the sarcolemma (25), and the cytoskeleton (62) have all been implicated. The sarcomere hypothesis is compelling, because sacromeric disruptions can occur following a single contraction (77). Sarcomere length within a muscle fiber is not uniform and longer sarcomeres are thought to be more vulnerable to eccentric contraction induced injury. The tension generated by a sarcomere decreases with increasing length, because as actin and myosin overlap decreases so does the ability to form cross-bridges (86). During eccentric contractions, longer sarcomeres will be preferentially lengthened. Some sarcomeres may be stretched beyond actin and myosin overlap such that tension is maintained passively rather than by cross-bridges. This may compromise the structural integrity of the sarcomere. While many of the over-stretched sarcomeres return to normal length upon relaxation, some are irreversibly

damaged (87). This process has been referred to as sarcomere popping and is thought to occur extremely quickly (73;74). It has been shown that regions within a fiber which contained the longest sarcomeres, will have the greatest number of damaged sarcomeres following eccentric contractions (76). Disrupted sarcomeres place increased stress on neighboring sarcomeres making them more vulnerable to damage, thus initiating a cascade of events that may eventually damage the cytoskeleton, sarcoplasmic reticulum, and sarcolemma (87).

Others have hypothesized that the cytoskeleton is the initial site of mechanical failure. Eccentric contractions may disrupt the cytoskeleton and this would cause subsequent myofibril derangement (62). Indirect evidence cited in support of this hypothesis is that the loss of titin, a cytoskeletal protein, by irradiation results in ultrastructural damage to resting skeletal muscle which is histologically similar to eccentric contraction induced injury (42). Changes in the cytoskeletal proteins occur very quickly, desmin ghost fibers have been detected in the tibialis anterior of rabbits following only 5 min of cyclic eccentric contractions (63). Desmin is an intermediate filament protein involved in maintaining Z-disk integrity (97). The presence of desmin "ghost" fibers in the absence of contractile protein disruptions was thought to support the cytoskeleton hypothesis. However, light microscopy was used to evaluate contractile protein disruptions (63) and this technique is not sensitive enough to detect disruptions to individual or small groups of sarcomeres (141). Thus the issue as to whether cytoskeletal disruptions precede sarcomere damage has not, to date, been adequately addressed.

Finally, disruption of the sacrolemma by mechanical factors could lead to an influx of extracellular calcium and stimulate the activation of calcium sensitive proteases and phospholipases (25). Post exercise increases in plasma CK activity and toponin I are thought to be indicative of transient sarcolemma disruptions (137).

1.2.1.b) Initiating Events: Disruptions in Calcium Homeostasis

Loss of calcium homeostasis results in morphological and biochemical alterations which may contribute to exercise induced muscle damage. Ultrastructural disruptions characteristic of increased intracellular calcium include swollen mitochondria, dilated sarcoplasmic reticulum (SR) and t-tubules and myofilament derangement including Z-disk streaming (13). Increased intracellular calcium may stimulate the activation of calcium dependent proteases and phospholipases (26). Phospholipase A_2 produces prostaglandins and leukotrienes which are pro-inflammatory and may promote cellular degradation (50). The source of elevated intracellular calcium may be from the extracellular space or from the SR. Extracellular calcium may enter the muscle fiber down its concentration gradient through stretch-sensitive channels (25) or through sarcolemmal disruptions (21). Following strenuous exercise muscle pH may decrease, temperature increase and metabolism may also be altered and these may also contribute to changes in SR structure and function including failure of the SR to sequester intracellular calcium (13).

In rats, elevations in mitochondrial calcium concentration were seen following an acute bout of downhill walking and these were associated with increased muscle damage (25).

The mitochondria can act as a calcium sink, and thus elevated mitochondrial calcium content is indicative of increased intracellular calcium content. If calcium chelators were administered prior to the exercise bout, indices of muscle damage were attenuated (25). Intracellular calcium concentrations were elevated and P_0 was depressed in mice 48 h following an acute bout of downhill running (72). Fourteen weeks of downhill running training attenuated the post exercise increase in incellular calcium and the loss in P_0 (72). Whether such adaptions can occur after a single bout of ecccentric exercise is unknown thus far.

1.2.1.c) Initiating Events: Reactive Oxygen Species

Reactive oxygen species (ROS) may contribute to secondary or delayed exercise induced muscle damage (141). ROS can attack the unsaturated bonds of membrane lipids, oxidize proteins and damage DNA (114). To counter the deleterious effects of ROS, cells have evolved a complex system of enzymes, including superoxide dismutase, catalase, glutathione peroxidase, and take advantage of biological compounds with potent antioxidant properties, including, vitamin E, glutathione, and perhaps estrogen (51). Potential sites of endogenous free radical generation include the electron transport chain of mitochondria, the respiratory burst of neutrophils and xanthine oxidase (10).

Under normal physiological conditions over 98% of the oxygen used by the mitochondrial electron transport chain is reduced to form ATP and water. The remaining oxygen is available for one or two electron reduction, which will lead to the formation of the superoxide free radical (O_2^*) or hydrogen peroxide. NADPH oxidase and ubiquinone

are the two components of the electron transport chain which are susceptible to free radical formation (1). Xanthine dehydrogenase is an enzyme involved in purine catabolism in primates. If the muscle is stressed, xanthine dehydrogenase will begin to function as xanthine oxidase, which is capable of generating ROS. Inhibition of xanthine oxidase has been shown to attenuate muscle damage (64).

Following eccentric exercise, infiltrating neutrophils would probably be the most significant source of ROS. Both the NADPH oxidase system and the neutrophil granules can generate ROS (104). The NADPH oxidase system is a membrane associated complex which can produce of O_2^*, H_2O_2, and OH^- (53). It cooperates with the granule enzyme myeloperoxidase, which reacts H_2O_2 and chlorine to form HOCl and water. HOCl is a biological oxidant and can react further to form another group of oxidants known as chloroamines (104).

Increased oxidative stress, measured directly using electron paramagnetic resonance spectroscopy or indirectly using markers such as plasma or tissue MDA content and cellular glutathione status, following strenuous endurance exercise has been well documented in human and animal models (26;37;51;102;133). Training status of the subjects, the intensity and duration of the exercise protocol can affect the severity of the oxidative stress experienced (114). However, the contribution of ROS to the secondary or delayed injury following eccentric contractions is inconclusive.

Two paradigms have been used to investigate the effect of oxidative stress on the etiology of eccentric exercise induced muscle damage. Markers of oxidative stress can be measured directly to assess whether they increase following eccentric contractions or subjects can be supplemented with antioxidants and improved antioxidant status should be associated with decreased muscle damage. Polyethylene glycol-superoxide dismutase (PEG-SOD) supplementation attenuated the post exercise loss in P_0 and morphological damage in young, adult, and old mice 3 d following *in situ* lengthening contractions (141). PEG-SOD was believed to decrease muscle damage because of its antioxidant properties. Vitamin E supplementation of male rats did not attenuate the magnitude of ultrastructural fiber damage or the loss in P_0 following eccentric contractions (134;137). However, vitamin E supplementation may decrease CK release from muscle in animals and healthy adults (15;134). A limitation of these studies is that they did not measure markers of oxidative stress directly. Plasma and muscle MDA concentrations were not increased and tissue antioxidant status was not compromised 3 d to 12 d following eccentric knee extension exercise in humans, suggesting that eccentric contractions do not increase oxidative stress in skeletal muscle (17). By waiting for 3 d post exercise to begin measurements the authors of this study (17) may have missed peak neutrophil infiltration and thus a major source of ROS. Others have also failed to see an increase in plasma MDA concentration 1 h to 12 d following an acute bout of downhill running (15). Thus, the contribution of ROS to the etiology of eccentric exercise induced muscle damage remains an enigma.

1.2.2.a) Biochemical Consequences: Acute Phase Inflammatory Response

Unaccustomed eccentric exercise will induce an acute phase immune response. Post exercise, the concentration of circulating lymphocyte sub-populations increases (88;89), cytokine synthesis is upregulated (31) and neutrophils and macrophages infiltrate into damaged skeletal muscle (31).

Cytokines are the regulatory proteins of the immune system. They are secreted by white blood cells, including neutrophils and by tissues at the site of injury. Cytokines that are responsive to exercise include interleukin-1β (Il-1β), Il-6, and tumor necrosis factor-α (TNF-α). Following strenuous eccentric exercise, TNF-α is secreted first, followed by Il-1β and Il-6. All three are pro-inflammatory, they stimulate the activation and migration of inflammatory cells and further cytokine production (116;117). TNF-α and IL-1 could mediate increased proteolysis of skeletal muscle by activating the ubiquitin proteosome pathway (85). Cannon et. al. detected increased plasma TNF-α content 24 h following downhill running (14). Il-1β content in muscle and plasma was also elevated following an acute bout of downhill running and significant increases were detected as late as 5 d post exercise (31). Increases in plasma Il-6 content were greater following eccentric cycling as compared to concentric cycling, perhaps because of greater muscle damage sustained during eccentric cycling (12).

Strenuous, unaccustomed exercise stimulates the accumulation of inflammatory cells in skeletal muscle (121), there they facilitate the breakdown and remodeling of damaged tissue (132). Neutrophils infiltrate skeletal muscle first followed by macrophages (115).

Muscle neutrophil content is elevated as early as 45 min post eccentric exercise (31) and is thought to peak by 24 h post exercise. The life span of an activated neutrophil is relatively short, however, lasting only 1 to 2 d. Macrophages can be observered in muscle longer and peak later as compared to neutrophils. Elevated muscle macrophage counts have been detected in mouse muscle up to 7 d following an eccentric contraction protocol (121).

Inflammatory cells have been hypothesized to contribute to the pathophysiology of eccentric exercise induced muscle damage, because of they may be programmed for overkill and they are capable of generating ROS (132). However, a causal relationship between increased oxidative stress and eccentric exercise induced muscle damage is rather tenuous. Furthermore, macrophage invasion does not appear to contribute to muscle membrane damage. Inhibition of macrophage infiltration failed to improve sarcolemma integrity, as quantified by Evan's blue staining, following hindlimb unloading and reloading (129). The inflammatory process appears to be restricted to areas of damage and does not appear to have a large impact on force production. The fact that 48 h post exercise P_0 returned to baseline values while inflammatory cell infiltration is peaking supports this concept (70). Furthermore, inhibition of inflammatory cell infiltration may compromise tissue regeneration (84).

1.2.2.a) Biochemical Consequences: Activation of Proteolytic Pathways

An acute bout of eccentric exercise can increase protein turnover. In rats protein degradation is elevated 48 h to 5 d post eccentric exercise (70). In human volunteers

mixed muscle fractional breakdown rate is elevated 3 h and 24 h post eccentric resistance exercise (99). There are three major proteolytic enzyme pathways in skeletal muscle; lysosomal proteases which include cathepsins and acid hydrolases, calpain, and the ATP-dependent ubiquitin proteosome pathway (9).

Exercise has been shown to result in increases in lysosomal protease activity. β-Glucoronidase, cathepsin B and D activities are elevated following strenuous exercise (9). However, lysosomal proteases are a minor contributors to skeletal muscle protein degradation when compared to the ubiquitin proteosome pathway (9).

Calpain activation may be one of the initiatal events in post exercise muscle protein degradation (8). Skeletal muscle expresses three isoform of calpain; μ-calpain, m-calpain, and p94-calpain, which is a muscle specific isoform (8). p94 Calpain binds to titin and this is thought to regulate its proteolytic activity (120). In addition, p94 calpain mRNA content is at least one order or magnitude greater than that for μ-calpain and m-calpain. However, p94-calpain protein is detected only in very small amounts in skeletal muscle as it undergoes rapid autolysis following translation and disappears (9). Its half-life is approximately 10 min (120). Unlike μ-calpain and m-calpain, p94-calpain is a monomer, it is not inhibited by calpastatin, and it has proteolytic activity at physiological calcium concentrations (9).

μ-Calpain and m-calpain are composed of a unique 80 kD subunit and a common 30 kD subunit. The 80 kD subunit is composed of 4 domains (I, II, III, and IV); domain II has

cysteine protease activity and domain IV binds calcium. The 30 kD subunit is composed of two domains (V and VI); domain VI binds calcium. The two subunits associate at their calcium binding domains (120). The difference between μ-calpain and m-calpain is the calcium concentration required for their activation, 10^{-6} M and 10^{-3} M respectively. Physiological intracellular calcium concentrations in skeletal muscle ranges from 10^{-8} M to 10^{-6} M. μ-Calpain and m-calpain activity in skeletal muscle is controlled by the intracellular calcium concentration, autolysis, calpastatin content and the availability of hydrolysable substrate. Autolysis of the N-terminal of the 80 kD subunit may decrease the calcium requirement for activation of proteolysis. Calpastatin is a specific inhibitor of μ-calpain and m-calpain. Calpain substrates include desmin, vimentin, nebulin, and α-actinin; all these protein are Z-disk components or closely associated with Z-disks.

Calpain may have a pivotal role in the disassembly and remodeling of the cytoskeletal matrix following exercise (9). Desmin negative fibers have been detected in skeletal muscle shortly following eccentric contractions (63). Desmin is a preferred calpain substrate. If skeletal muscle is incubated with calpain *in vitro*, Z-disks are removed completely and quickly without any other visible derangement of myofibril ultrastructure (38). Calpain does not degrade proteins to small peptides or individual amino acids rather it cleaves them at specific sites leaving large protein fragments. While calpain can release proteins from the myofibril network, the ATP-dependent ubiquitin proteosome pathway is thought to be necessary for complete protein degradation (139).

Strenuous exercise increases total calpain activity and may also lower the calcium requirement for activation (8). In rats which were run to exhaustion, calpain activity was elevated immediately post exercise but by 24 h it had begun to return to baseline levels. Also, post exercise muscle samples were more vulnerable to calpain degradation as compared to baseline or 24 h post exercise muscle samples (7). Increases in calpain activity post exercise correlates well with increases in MPO activity (r=0.83) and if calpain activity was inhibited, MPO activity decreased correspondingly (103). Others have also observed a positive correlation between MPO activity and protein degradation as quantified by tyrosine release (70). Moreover, the N-terminal peptides cleaved during autolysis of calpain have been shown *in vitro* to have chemotactic properties (58;59). Therefore, calpain-derived peptides may be a factor initiating neutrophil infiltration. Thus, calpain may not only initiate protein turnover following exercise, but it may also contribute to the inflammatory response.

The ATP dependent ubiquitin proteosome pathway can degrade abnormally folded or damaged proteins, short lived regulatory proteins, and long lived proteins including myofibril components (60;140). Ubiquitin binds covalently to proteins and targets them for degradation by the 26S proteosome (124). A ubiquitin carboxy-terminal glycine residue is conjugated to a lysine residue on the substrate protein. A single conjugated ubiquitin is not a very strong signal for proteolysis, at least five ubiquitin proteins need to be linked together in chain to mark the protein for degradation (60). The conjugation process requires ATP and three enzymes. E1 hydrolyses ATP and activates ubiquitin to form carboxy terminal ubiquitin adenylate. The activated ubiquitin is transferred to a

carrier protein, E2, which facilitates contact between ubiquitin and substrate protein. E3, a ligase, covalently binds ubiquitin and protein (60;85). The activation or suppression of this pathway is regulated by E2 and E3 (85). Ubiquitin conjugated proteins are then degraded by the 26S proteosome, which is composed of the 20S proteosome and two regulatory components PA700 and PA28 (118). PA700 and PA28 bind to one or both ends of the 20S proteosome. PA700 selectively binds to ubiquitin conjugated proteins and facilitates their unfolding (118). The function of PA28 is somewhat unclear, though it may be involved in MHC1 molecule production (118). The 20S proteosome is composed of two inner β rings which contain active sites for protein hydrolysis and two outer α rings which form a small opening through which the unfolded substrate protein must pass (60). The 20S proteosome cleaves proteins into 3 to 25 amino acid long peptides which are rapidly hydrolyzed in the cytosol by non-specific proteases into individual amino acids (60).

Numerous models including starvation (140), denervation (140), sepsis (128), unloading, (124), chronic electrical stimulation (94), and exercise (110;112;127) have been used to demonstrate that the ubiquitin proteosome pathway is involved in muscle protein turnover. Following 9 d of hindlimb unloading, protein breakdown increased by 66 % and the ubiquitin proteosome pathway accounted for approximately 82 % of this increase (124). *In vitro,* the ubiquitin proteosome pathway cannot degrade intact myofibrils or complexed contractile proteins (i.e. actin and myosin or troponin and tropomyosin), it can only degrade myofilament monomers. Therefore, the ubiquitin proteosome pathway is thought to function in concert with calpain (139). Thus far, no experimental evidence

exists to support this hypothesis, perhaps because the time course for these two proteolytic pathways is different.

The effect of acute exercise on the ubiquitin proteosome pathway has not been well studied. Only changes in ubiquitin conjugated protein content have been assessed. Both free ubiquitin and ubiquitin conjugated protein content were elevated 48 h following an acute bout of eccentric elbow flexor exercise in humans (127). Protein ubiquitination was also increased in sarcoplasmic and myofibrillar fractions 48 h following a 16 h treadmill run in healthy and dystrophic mice (112). However, how exercise affects the rate of ATP-dependent proteolysis and 26S proteosome, E1, E2, and E3 mRNA and protein content is still unknown.

1.3.1. Adaptation to Repeated Eccentric Exercise

Adaptations to eccentric exercise are rapid, such that if the bout is repeated indices of muscle damage will be attenuated. The pathways mediating these adaptations are hypothesized to involve cellular and neural changes (19). Cellular adaptations may include structural changes in the cytoskeleton and contractile apparatus, such that the tension or shear stresses experienced by the muscle fiber during lengthening contractions are attenuated. These may include altered expression of "stabilizing" cytoskeletal proteins, the addition of sarcomeres in series (87), and increased stress protein content. A direct consequence of enhanced muscle fiber stability would be an attenuation of other indices of muscle damage, including serum CK activity (19) and the inflammatory response (100). Neural adaptations may involve changes in motor unit recruitment

patterns which may make the muscle more resistant to injury (136). Adaptations to eccentric exercise probably involve a combination of cellular and neural factors.

1.3.2. The Neural Hypothesis

During isometric or concentric contractions, type I motor units are recruited first, followed by type IIa and finally IIb motor units as force production increases. However, during a novel bout of eccentric exercise the order of recruitment may be reversed and there may be a greater reliance on type II motor units as compared to type I. Following the second exercise bout type I motor unit recruitment may be enhanced. This hypothesis depends on two factors, that type II fibers are more susceptible to contraction induced injury as compared to type I fibers (136) and that during voluntary eccentric contractions not all motor units are recruited (19;136). Some groups have reported greater susceptibility of type II fibers to injury as compared to type I fibers (77). For a given amount of strain, a greater force deficit develops in type II fibers as compared to type I fibers (77). Eccentric contractions may be more damaging to the relatively larger type II fibers, because the surface:volume ratio is greater in these fibers as compared to the smaller type I fibers (96). Furthermore, type II fibers may contain a smaller less compliant isoform of titin (97) and therefore may be less compliant. However, when animal models were used to investigate adaptations to repeated eccentric exercise, indices of exercise induced muscle damage were attenuated despite the fact the all motor units were recruited during involuntary muscle stimulation (106). Therefore, cellular adaptations must be involved.

1.3.3.a) Cellular Hypothesis: Enhanced Stress Protein Content

Heat shock protein (HSP) synthesis is stimulated by numerous stresses including heat, hypoxia and disruptions in metabolism. Increased HSP content allows cells to better cope with subsequent exposure to the stress and to survive exposures which would have been lethal without preconditioning (83). Animals which have been "heat shocked" (i.e. preconditioned) prior to exposure to cardiac ischemia, will sustain significantly less cardiac muscle damage, mitochondrial disruption, and will have lower serum CK activity as compared to animals which were not heat shocked (83). HSP content in skeletal muscle can increase following training or an acute bout of exercise (39;66;79). Therefore, increased HSP content may be one of the pathways mediating adaptation to repeated eccentric contractions.

There are six HSP families based on the molecular size of the proteins. The HSP70 group is the most abundant and includes proteins that are 66 and 78kD in weight. Two prominent members of this family are HSP72 and HSP73. They are involved in the dissociation of aggregates, protein folding and traffic and protein translocation into the mitochondria and the endoplasmic reticulum. HSP72 is readily inducible whereas HSP73 is constitutively expressed (61). Metabolically active tissues such as skeletal muscle, heart and liver have quite high levels of HSP70 protein content (39). Regional distribution of heat shock proteins has been observed in skeletal muscle. Type I fibers have greater HSP72 and HSP73 protein content as compared to type II fibers (39), perhaps because of greater recruitment of type I fibers (69).

HSP70 protein and/or mRNA content has been shown to increase following an acute bout of exercise and in response to training. HSP72 mRNA content is increased in human skeletal muscle following an acute bout of aerobic exercise (30;101). The increases in HSP72 mRNA content reported are very large, up to 200 to 400 % (30;101). However, increased mRNA content does not necessarily result in increased protein content. Thus far in humans, corresponding increases in HSP72 protein content, subsequent to increased mRNA content, have not been detected (101). A more frequent or intense stimulus may be required to induce lasting increases in HSP72 protein content. HSP72 protein content was increased in the *vastus lateralis* of elite rowers following 4 wk of intense training (66) and increases were related to the intensity of the training stimulus (65;66). Increased HSP72 protein content may protect against exercise induced muscle damage, as training induced increases in HSP72 were accompanied by decreased serum CK activity. Unlike humans, increased HSP72 protein content has been observed in rodents following a single session of treadmill running (39;68;95).

HSP27 is a low molecular HSP which in unstressed cells forms oligomers 80 to100 kD in weight. If the cell is severely stressed HSP27 associates into aggregates that weigh in excess of 2 million kD (48). Heat shock, oxidative stress, and estrogen exposure stimulate HSP27 synthesis (35). As compared to HSP72, HSP27 induction kinetics are slower, but increases in protein content are maintained for much longer (67). HSP27 is abundant in numerous tissues including skeletal (24;49;54), cardiac (71) and smooth (48) muscle. HSP27 associates with several smooth muscle contractile proteins, including actin, myosin, caldesmon, and tropomyosin (48). In rodent and human cardiac muscle

and in *c. elegans* skeletal muscle HSP27 has been localized to the Z-disks (24;71). Thus, HSP27 may be involved in maintaining myofibrillar integrity.

HSP27 activity depends on cell type, intracellular concentration, and phosphorylation state (113). HSP27, like HSP72, has molecular chaperone properties. HSP27 binds and stabilizes denatured proteins and in conjunction with HSP70 can participate in the refolding of denatured proteins (27). HSP27 alone, however, cannot refold denatured proteins. *In vitro*, phosphorylated HSP27 has been shown to inhibit actin polymerization by acting as a capping protein and thus helping maintain cytoskeletal integrity (46). HSP27 may improve the antioxidant status of a cell, as increased HSP27 protein content correlates well with increased intracellular glutathione (GSH) concentrations (16). GSH is a potent antioxidant. HSP27 may also help maintain protein and mRNA synthesis during stress. In vitro, cells with high HSP27 protein content recover basal rates of RNA and protein synthesis much faster following heat shock than cells which do not (16). All of these properties and the fact that HSP27 is expressed by skeletal muscle, suggest a potential role for HSP27 in the rapid adaptation to repeated eccentric exercise.

Bcl-2 is an anti-apoptosis oncoprotein with antioxidant properties. It is localized to intracellular sites of oxygen free radical generation, including the mitochondria and endoplasmic reticulum. Bcl-2 has been shown to protect cells from H_2O_2 in cell culture. Bcl-2 does not inhibit the formation of H_2O_2, but it blocks its downstream effects and attenuates lipid peroxidation (40). Bcl-2 positive myofibers have been detected in

patients with peripheral neuropathy (125) and Duchenne Muscular Dystrophy (DMD) (111).

The effect of exercise on bcl-2 expression has not been well studied. One rodent study found no difference in bcl-2 expression between normal and dystrophin deficient *mdx* mice at rest. However, after a night of treadmill running a significant decrease in blc-2 protein content was detected in the *mdx* muscle only, and this corresponded to an increasein the number of apoptotic nuclei (112). Bcl-2 positive staining fibers were detected in males 48 h following an acute, eccentric exercise bout (123). The functional significance of these positively stained fibers is unknown. Since skeletal muscle can express the proteins of apoptosis and it may be capable of undergoing programmed cell death (112;125). Bcl-2 is also upregulated by inflammatory cells as they are removed from skeletal muscle by apoptosis, therefore increased bcl-2 content may be correlated with increased leukocyte turnover (130).

HSP72, HSP27 and bcl-2 are not the only stress proteins which may mediate adaptations to repeated eccentric exercise, however, they were the ones investigated in this thesis. Others include HSP60, HSP90, and αB crystallin (67). HSP60 and HSP90 protein content is increased in skeletal muscle following endurance exercise or training (67;79;109). αB Crystallin is a low molecular weight stress protein which has been localized to skeletal and cardiac muscle (49;54;71). αB Crystallin is a molecular chaperone which associates with contractile and cytoskeletal proteins including desmin, actin, and vimentin, and it may prevent the formation of protein aggregates (49;71).

Like HSP27, αB crystallin protein content is increased in the EDL following sciatic nerve transections in rats (49). However, a detailed discussion of these and others stress proteins is beyond the scope of this thesis.

1.3.3.b) Cellular Hypothesis: Cytoskeletal Proteins

Rapid adaptation to repeated eccentric exercise may be mediated by increased "stabilizing" cytoskeletal protein content or by the synthesis of more stress resistant isoforms of "stabilizing proteins". Talin and viniculin protein content in the soleus and plantaris muscle of rats was elevated 3 d and 7 d following eccentric contractions (32). Viniculin and talin are cytoskeletal proteins involved in the linkage of cytoskeleton to sarcolemma (32) and this linkage may be quite vulnerable to contraction induced injury (34). Viniculin and talin protein content is 2 fold greater in type I fibers as compared to type II fibers, this may contribute to the greater threshold to contraction induced injury in type I fibers (32). Increased talin and viniculin protein content may be part of the adaptive response to contraction induced injury (32).

Desmin contributes to the structural integrity of the myofibril lattice by forming inter-Z-disk connections (97). Desmin is also linked to the nucleus, mitochondria and sarcolemma (34). Although, desmin is sensitive to eccentric contractions (63), desmin staining intensity, thus protein content, is increased 3 d post exercise in humans (33). Like talin and viniculin, increased synthesis of desmin may increase the muscle fiber's tolerance of mechanical stress. Whether other cytoskeleton proteins respond similarily to an acute bout of eccentric exercise remains to be investigated.

Titin is a large cytoskeletal protein which links myosin to the Z-disk (41). Titin helps maintain passive tension within a myofiber (97). The elasticity of muscle is determined in part by the length of the PEVK (proline, glutamate, valine, lysine) region within the titin protein. In cardiac muscle the PEVK region is 163 amino acids long, whereas in the soleus it is 2000 amino acids long. Soleus muscle is approximately 100 times more compliant than cardiac muscle (97). Adaptations to repeated eccentric exercise may involve the synthesis of isoforms of titin with longer PEVK regions.

1.3.2.c) Cellular Hypothesis: Addition of Sarcomeres in Series

An increased number of sarcomeres in series would make the muscle more resistant to contraction induced injury, because for a given eccentric contraction the degree of stretch experienced by individual sarcomeres would be attenuated due to a shift to the left along the length-tension curve, thus decreasing the probability of sarcomere popping (73;74). Five days of decline training (i.e. downhill running) has shown to increase the number of sarcomeres in series in the vastus intermedius muscles of rats by ~10 % (73;74). Muscle which had been decline trained and therefore had an increased number of sarcomeres in series, sustained less muscle damage following high force eccentric contractions (74). Others failed to find a consistent increase in the number of sarcomeres in series in rabbit dorsiflexor muscles following 12 wk of twice weekly eccentric training (55). Furthermore, increased "stabilizing" cytoskeletal protein content following an acute bout of eccentric exercise may be indicative of the addition of new sarcomeres in series (32).

It is still not known whether a single bout of eccentric contractions can increase sarcomere number, or whether a more intense or prolonged stimulus is required.

1.3.3.d) Cellular Hypothesis: Accelerated Rate of CK Clearance

Repeated eccentric exercise attenuates the rise in post exercise serum CK activity (47;78) for up to 6 mo following the initial bout (19). Serum CK activity is a function of enzyme release and clearance. An enhanced clearance rate may contribute to the blunted CK response observed following repeated eccentric exercise (47). When human volunteers repeated a bout of eccentric forearm flexor exercise with their contralateral arm, serum CK activity post exercise was attenuated, but other indices of muscle damage, including force deficit and muscle soreness, were not different from the initial bout (91). Enhanced clearance rate of CK was hypothesized to mediate these findings. The muscle CK isoenzyme (CK-MM) exits in three isoforms, CK-MM1 is released from muscle and CK-MM2 and CK-MM3 are formed by lysine or arginine cleavage in the blood. Serum CK-MM1 content is indicative of enzyme release from muscle and CK-MM3 content is indicative of enzyme clearance (47). To elucidate the contribution of CK release and clearance to the blunted CK response following exercise the CK-MM1 and CK-MM3 isoforms would need to be analyzed separately. However, serum CK activity is an indirect marker of muscle damage and greater understanding of CK response yields limited insight into rapid adaptation of repeated eccentric exercise.

1.4. Gender-Mediated Differences in Exercise Induced Muscle Damage

Gender differences in exercise induced muscle damage may be mediated at least in part by estrogen. 17-β-Estradiol is the primary human estrogen and has the most potent estrogenic effects. Estrogen has antioxidant and membrane stabilizing properties. Estrogen may stabilize intracellular membranes by intercalating into the phospholipid bilayer, much like vitamin E or cholesterol (131). Estrogen has a hydroxyl group on its phenolic "A" ring which makes it structurally similar to vitamin E. Reduction and oxidation of this hydroxyl group may give estrogen its antioxidant properties. *In vitro* experiments using human plasma show that estradiol is as effective as α-tocopherol (the active form of vitamin E) in preventing fatty acid peroxidation and far more effective at preventing cholesterol peroxidation (4). Physiological levels of estrogen can also compensate for vitamin E deficiency, lipid peroxidation was shown not increase in response to training or an acute bout of exercise, in vitamin E deficient female rats when compared to control females.

CK activity at rest, following an acute bout of exercise and during training, is lower in females than in males (20). Following 2 h of treadmill running, serum CK activity was greater in male rats compared to female rats. When male rats were supplemented with estrogen their CK response was blunted (6). Serum CK activity was lower in females as compared to males after 8 wk of resistance training (122). Gender differences in basal serum CK activity have also been observed in male and female varsity rowers who were involved in a strenuous sport specific training protocol (23). Gender differences in serum

CK activity cannot be explained by gender differences in lean body mass or activity level, thus estrogen may have a mediating effect (20).

Gender mediated differences in response to eccentric exercise have also been observed in the post-exercise inflammatory response. Following unilateral, eccentric hindlimb contractions, fewer myofibers were invaded by acid phosphatase-positive leukocytes in female mice as compared to male mice. The time course of cellular infiltration was also different between genders with males exhibiting a peak inflammatory response earlier than females (121). Estrogen has been shown to attenuate skeletal muscle myeloperoxidase activity following exercise. Post exercise myeloperoxidase activity was lower in female rats as compared to male rats and a two weeks of estrogen administration decreased myeloperoxidase activity in male rats to female levels (133). Following an acute bout of downhill running, focal inflammation and fiber necrosis also was greater in male rats as compared to female rats and the female rats had lower muscle β-glucoronidase activity.

In animals, cytoskeletal disruptions are greater in males as compared to females following eccentric exercise. Discontinuous dystrophin staining, desmin desolution, and rearrangement of actin was greater in the male rats as compared to female rats following downhill running (56). However, studies using human subjects have not demonstrated greater ultrastructural disruptions in males as compared to females (123). Following an acute bout of eccentric, quadriceps exercise the extent of Z-disk streaming was similar

between genders (123). Clearly, further research in potential gender mediated differences in eccentric contraction induced muscle injury is required.

1.5. Hypotheses

Following the initial exercise bout, it was hypothesized that force output would be attenuated and that serum CK activity, Z-disk streaming and inflammatory cell infiltration into muscle would be increased. The magnitude of the relative force deficit and the extent of Z-disk streaming were expected to be similar between genders. However, it was proposed that serum CK activity and inflammatory cell infiltration would be greater in males as compared to females. Markers of protein degradation, ubiquitin conjugated protein content and calpain 30 kD regulatory subunit protein content, were hypothesized to be elevated 24 h post eccentric exercise. Following the second exercise bout, an attenuation in force deficit, serum CK activity, Z-disk streaming inflammatory cell infiltration and proteolytic enzyme protein content was predicted. It was also predicted that indices of muscle damage would be similarly attenuated between genders following the second exercise bout.

Increased stress protein content was hypothesized to mediate the repeated bout effect to eccentric exercise. It was hypothesized that HSP27 protein content would be greater at rest in females as compared to males and that HSP72 and bcl-2 protein content would be greater in males as compared to females following eccentric exercise.

Chapter 2: RAPID APAPTATION TO ECCENTRIC EXERCISE INDUCED MUSCLE DAMAGE

2.1 Abstract

One bout of eccentric exercise stimulates protective adaptations in skeletal muscle, whereby the magnitude of subsequent muscle damage is attenuated when the bout is repeated. Changes in ultrastructural damage, inflammatory cell infiltration, and proteolytic enzyme protein content were hypothesized to characterize these adaptations. Untrained male (N = 8) and untrained female (N = 8) subjects performed two bouts of 36 repetitions of unilateral, eccentric leg press and 100 repetitions of unilateral, eccentric leg extension exercise (at 120 % of the single repetition maximum) separated by 5.5 wk. Biopsies were taken from the *vastus lateralis* of each leg 24 h post exercise. Following repeated eccentric exercise the force deficit and the rise in serum creatine kinase (CK) activity were attenuated. Females had lower serum CK activity as compared to males ($p<0.05$), but there was no gender difference in the relative magnitude of the force deficit. The amount of Z-disk streaming was elevated only after the first exercise bout ($p<0.05$), with no gender difference. Muscle neutrophil counts were significantly greater in females 24 h after the second bout as compared to the first or rest ($p<0.05$), but they did not increase in males. Muscle macrophages were elevated in males and females following exercise bout 1 and 2 ($p<0.05$). Protein content of the regulatory calpain subunit did not increase in response to exercise; whereas ubiquitin conjugated protein content did, but only after the second bout ($p<0.05$). We conclude that adaptations to

eccentric exercise are associated with attenuated serum CK activity, decreased ultrastructural damage and increased activity of the ubiquitin proteosome pathway.

2.2. Introduction

Unaccustomed, eccentric exercise can cause muscle damage, which is characterized by delayed onset muscle soreness, decreased muscle force production, increased serum CK activity (126), ultrastructural disruption (36;43), inflammation (31;75) and increased proteolytic enzyme activity (7;14;108;112;127). If the exercise bout is repeated, indices of muscle damage are reduced (19;21;43). Some of these adaptations, such as attenuated CK response, are quite long lasting (19). Potential changes in ultrastructural disruption, acute phase inflammatory response and proteolytic enzyme activity or protein content in humans have, to our knowledge, not been systematically investigated or quantified.

Gender-based differences in muscle damage have been observed in humans and animals following an acute bout of aerobic or eccentric exercise (20). Differences in plasma CK activity (92), inflammatory response (121), and ultrastructural disruptions(56) have been reported. Some of these differences may be attributed to the sex steroid hormone 17β-estradiol (131). However, the influence that gender may have on rapid adaptation to eccentric exercise has not been investigated in humans.

Cellular and neural hypotheses have been put forward to explain the rapid adaptations to repeated eccentric exercise (19). The cellular hypothesis suggests that adaptations to eccentric exercise involve changes in the cytoskeleton or the contractile apparatus, such

that the tension or shear stresses experienced by the muscle fiber during lengthening contraction are reduced. These may include altered expression of "stabilizing" cytoskeletal proteins such as titin, talin, viniculin and desmin (34). Addition of sarcomeres in series may also attenuated eccentric contraction induced muscle damage (87). As a direct consequence of increased fiber stability, other indices of muscle damage, including serum CK activity (26;78) and inflammatory response (100), will also be attenuated.

The primary purpose of this study was to investigate the effect of repeated eccentric exercise on the eccentric exercise-induced force deficit, serum CK activity, Z-disk streaming, neutrophil and macrophage infiltration and proteolytic enzyme protein content. A secondary purpose of this study was to further characterize gender-based differences in exercise-induced muscle damage. We hypothesized that following the second exercise bout, all indices of muscle damage would be attenuated and that gender differences would exist. Force deficit, serum CK activity, inflammatory cell infiltration, and proteolytic enzyme expression would be greater in males as compared to females, however, the extent of Z-disk streaming would be similar between genders (123).

2.3. Materials and Methods

Subjects

Healthy, non-smoking male (N=8) and female (N=8) university students volunteered to participate in the study and gave informed, written consent. The study was approved by the McMaster University Research Ethics Board. None of the subjects had participated in a regular, structured exercise program for at least 6 mo prior to participating in the study. Five of female subjects were oral contraceptive users and all were tested as close as possible to the mid-follicular phase of their menstrual cycle (7 to 11 days following menses).

Testing Protocol

Two weeks prior to beginning the study all subjects were required to report to the testing lab for a familiarization session where they were acquainted with the isokinetic dynanometer *(Biodex Medical Systems Inc., Shirley, NY)* and other testing apparati. All subjects were required to perform, using their weakest leg, several concentric isokinetic leg extensions at a velocity of $30\ °\cdot sec^{-1}$ such that each subject would be eliciting peak torque by the fifth contraction. Each subject was allowed to perform only 5 contractions during the familiarization session. Subjects also had their single repetition maximum (1 RM; the maximum weight that can be lifted in one repetition) determined for each leg independently while performing a unilateral leg press and unilateral knee extensions. Briefly, subjects performed 10 warm-up repetitions for each leg on each exercise before their 1 RM was determined. Subject's 1 RM was first estimated from the values and procedures described by Mayhew et. al. (80). Once a predicted 1 RM was

determined, the actual 1 RM was determined based on the predicted value. Using this procedure a subject's 1 RM could be determined with only 1 to 2 efforts.

On the testing day, subjects were required to report to the lab where they had a baseline venous blood sample taken by venipuncture prior to engaging in 10 minutes of light (75 W) cycle ergometry. After completing the cycling, subjects were seated in the isokinetic dynamometer so that the peak isokinetic torque of the knee extensors could be determined. Briefly, subjects were seated upright in the isokinetic dynanometer *(Biodex Medical Systems Inc., Shirley, NY)* with their leg positioned at 90 ° from horizontal so that the axis of rotation corresponded to the centre of the subject's knee. Subjects had a lap belt to prevent movement of their pelvis, and shoulder straps to prevent movement of their upper body. Once they were comfortable each subject performed several warm-up concentric contractions (consisting of 3 to 4 submaximal contractions) at 30 °·sec^{-1} prior to determining peak torque. After the warm-up subjects were then asked to elicit their maximal voluntary torque at 30 °·sec^{-1}. Subjects were given three opportunities to achieve their peak torque, the amount of rest between each effort was not held constant but was at least 20 sec. The maximum torque of the three efforts, for both legs, was taken as the subject's maximum torque.

Following the preliminary (PRE) testing session, the subjects performed a series of resistance exercises designed to elicit muscle damage of the knee extensor muscle group. Subjects performed all exercises using their weakest leg while their stronger leg acted as non-exercised control. The first set of resistance exercises was using a standard leg

extension machine *(Nautilus)* and required that the subjects lower a mass equivalent to 120 % of their pre-determined unilateral concentric 1 RM. To perform an eccentric muscle action, subjects had the weight lifted for them by the investigators after which they lowered the weight through through an arc of approximately ~75 °. Hence, subjects were seated in the Nautilus machine such their legs were initially at ~15 ° angle from horizontal and the subjects then flexed their legs to ~90 ° at which time the experimenters lifted the weight for the subject while the subject's foot rested passively on the foot pedal. Each subject performed 36 (3 sets x 12 repetitions per set) eccentric muscle actions using the leg press machine with 3 min rest between each set. Subjects were required to lower the weight at a fixed cadence (1 sec to raise the weight and then 2 sec to lower the weight) which was verbally given by one of the investigators. Subjects then performed 100 eccentric muscle actions (10 sets x 10 repetitions per set) using their weakest leg on a standard knee extension machine *(Nautilus).* Again subjects were required to lower a weight, that was lifted for them by the investigators, equivalent to 120 % of their pre-determined unilateral 1 RM. Subjects sat on the apparatus and required flexion of their leg through an arc of ~75 °, from ~15 ° from horizontal to fully extended at ~90 °. The same lifting cadence was maintained and subjects were verbally encouraged to maintain their effort throughout the range of motion. If subjects were deviating from the required cadence of lowering the weight then a brief (30 sec) rest was allowed during the set, so that the subject could complete the set while still giving an appropriate effort. A maximum of two rests per set were allowed. Subjects also had 3 min of rest between each set. Each subject experienced some degree of fatigue during the lifting protocol, but all subjects were able to complete the protocol. We acknowledge that it is possible that

the subjects could have applied less force while lowering the weight when they were becoming fatigued, however, we tried to control for this by timing the motion. It appeared that all subjects maintained a high level of effort, in that they maintained a constant rate at which the load was lowered.

Following cessation of the resistance exercise, subjects rested for 30 min before they had their maximum isokinetic peak torque at a velocity of 30 $°\cdot sec^{-1}$ determined using the isokinetic dynamometer, as described above. In addition, subjects had a venous blood sample taken ~30 min post-exercise for determination of serum CK activity (see below).

Subjects reported back to the testing center 24 h following the performance of the resistance exercise to have their isokinetic peak torque determined, as described above. Subjects also had a venous blood sample drawn to determine serum CK activity. At this time muscle biopsies were taken from the *vastus lateralis* of both the non-exercised (rest) and exercised legs for the determination of muscle damage, Western blotting, and histochemical characteristics. Only the rest muscle samples from the first exercise bout were analyzed, as the two rest samples were assumed to be equivalent. Subjects had their skin anesthetized using 1 % lidocaine and a small (~4 to 5 mm) incision was made ~20 to 25 cm proximal to the subjects knee on the lateral aspect of the lateral thigh. A 5 mm Bergström biopsy needle was used for muscle sampling. The muscle biopsy was blotted with a gauze pad to remove any excess blood and dissected free of any visible adipose or connective tissue. A small (~10 mg) longitudinal section of each muscle biopsy was immediately placed into 2 % glutaraldehyde for determination of muscle damage and

stored at 4 °C until processing. Another portion (~50 mg) of the muscle biopsy was placed in liquid N_2 and stored at –80 °C for analysis of protein content by Western blotting. A further piece of muscle was oriented in cross section in OCT embedding medium and snap frozen in isopentane cooled by liquid N_2 and stored at –50 °C prior to histochemical analysis.

Subsequently, subjects reported back to the testing center at 48 h, 96 h, and 7 d following the performance of the resistance exercise bout, during which time they had a venous blood sample taken for CK activity and had their peak isokinetic torque, at 30 °·sec^{-1}, determined.

To examine the effect of a previous bout of eccentric resistance exercise on a subsequent bout of eccentric exercise, subjects rested for 5 to 6 weeks after performing the initial eccentric resistance exercise bout, and then returned to the testing center to repeat the entire protocol including strength testing, blood samples and muscle biopsies. Between exercise bouts subjects refrained from doing any strenuous physical activity.

Blood

Blood samples were collected via a 21 Ga plastic catheter placed into the antecubital vein. Blood was collected into tubes, allowed to clot, and then centrifuged at 10,000 RPM (at 4 °C) for ~10 min. The serum was stored at –20 °C until analysis.

Serum Creatine Kinase (CK) Activity

Serum was assayed spectrophotometrically (λ=340 nm) for creatine kinase using a commercially available kit *(#DG147-UV, Sigma Diagnostics, Columbus, OH).* All samples were run in triplicate and the intra-assay coefficient of variation for six identical samples was 6 %.

Toluidine Blue Stained Microscopy

The glutaraldehyde fixed muscle was post fixed in 1 % osmium tetroxide, dehydrated in graded alcohol, and embedded in plastic resin (Spurr's). Longitudinal, semi-thin sections (~1 μm in thickness) were cut with a glass knife and stained with toluidine blue for light microscopic evaluation. The amount of extensive muscle damage was quantified by visual analysis using light microscopy at 600x power and it was defined as any area of Z-disk streaming encompassing more than two adjacent Z-disks. Areas reported as Z-disk streaming by toluidine blue staining, have been previously confirmed to represent Z-disk streaming by cutting ultra-thin sections from the same blocks and staining them with uranyl acetate and lead acetate and examining them using a transmission electron microscope (123).

Results are expressed as number of areas of extensive Z-disk streaming per mm^2 of muscle tissue. Entire muscle sections were captured with a digital camera *(Spot, v2.2, Diagnostic Instruments, Sterling Heights, MI)* and the area was quantified using a computerized image analysis system *(ImagePro Plus, v4.0, Media Cybernetics, Silver Springs, MD).* Mean tissue area was 1.93 ± 1.81 mm^2; (range 0.30 mm^2 to 2.82 mm^2).

The entire data set was counted and statistically analyzed by two blinded, independent investigators. The number of areas of Z-disk streaming counted by the two investigators were in concordance with each other as confirmed by an independent t-test ($p>0.05$). (Only the counts from a single reviewer, N. S., are presented here.)

Immuno-histochemistry

Frozen muscle was serially cross-sectioned to 5 μm thickness using a cryostat *(Microm HM 500 OM, Microm International, Walldorf, Germany)*. Negative control sections were included in all analyses. The slides were dried overnight and stored at –80 °C until analysis. Slides were fixed in cold acetone for 15 min. Endogenous peroxidase activity was blocked using a liquid DAB substrate kit *(#00-2014, Zymed Laboratories Inc., San Francisco, CA)*. The slides were blocked with 1 % goat serum *(#D3002S, Dako Diagnostics Canada, Mississagua, ON))* for 15 min. The primary antibody was diluted in goat serum and positive slides were incubated for 30 min. Then slides were incubated with secondary goat anti-mouse antibody *(#95-6543-B, Zymed Laboratories Inc., San Francisco, CA)* for 15 min and with peroxidase *(#95-6543-B, Zymed Laboratories Inc., San Francisco, CA)* for an additional 15 min. An AEC Kit *(#00-2007, Zymed, San Francisco, CA)* was used for color development. The primary antibodies used were monoclonal mouse anti-human myeloperoxidase *(#M0748, Dako Diagnostics Canada, Mississagua, ON)* at a 1:300 dilution for neutrophil detection and monoclonal mouse anti-human CD68 *(#M0814, Dako Diagnostics Canada, Mississagua, ON)* at a 1:100 dilution for macrophage detection.

The number of neutrophils and macrophages in the total cross-sectional area were counted and expressed as number of positive cells per mm^2 of muscle. Entire muscle cross-sections were captured with a digital camera *(Spot, v2.2, Diagnostic Instruments, Sterling Heights, MI)* and area was quantified using a computerized image analysis system *(ImagePro Plus, v4.0, Media Cybernetics, Silver Springs, MD)*. To determine to determine test re-test reliability, ten representative slides were randomly chosen and each slide was counted twice 2 wk apart, the coefficient of variation between the two counts was less than 10 % for each slide.

Polyacrylamide Gel Electrophoresis and Western blotting

Biopsies were homogenized in 600 µl 5 % SDS-phosphate buffered saline (PBS; 152 mM NaCl, 2.99 mM KCl, 85.0 mM $NaHPO_4$, 4.00 mM KH_2PO_4) using a glass homogenizer. It was then heated at 70 °C for 10 min and protein content was determined using the Bradford protein assay *(#500-0006, Bio-Rad, Hercules, CA)*. All samples were run in triplicate and the coefficient of variation was less than 10 % for each sample. Crude muscle homogenate was stored at –80 °C until analysis, care was taken to minimize the number of freeze-thaw cycles.

Proteins were separated on a 10 % SDS-polyacrylamide separating gel and a 4 % SDS-polyacrylamide stacking gel. 40 µg of protein was run in each lane. Rest, bout 1 and bout 2 samples for a male and female subject were always run on the same gel, along with a broad range molecular weight standard *(#161-0319, Bio-Rad, Hercules, CA)*. The gels were run at 100 V for 1 h at room temperature.

Gels stained for the 30 kD regulatory subunit of calpain were transferred to PVDF membranes *(polyvinylidene difluoride; #162-0177, Bio-Rad, Hercules, CA)* for 1 h, at room temperature, at 100 V. Gels stained for ubiquitin conjugated proteins were transferred overnight, on ice, at 25 V. The PVDF *(#162-0177, Bio-Rad, Hercules, CA)* membranes were blocked with 9 % gelatin *(#G-7765, Sigma)* dissolved in Tris-buffered saline with 0.1% Tween *(#170-6531, Bio-Rad, Hercules, CA)* (TTBS; 500mM NaCl, 20mM Tris HCl, pH 7.5) for 1.5 h.

The 30kD regulatory subunit of calpain antibody *(#sc-7530, Santa Cruz Biotechnologies, Santa Cruz, CA)* was diluted to 1:500 in TTBS and the ubiquitin antibody *(#NCL-UBIQm, Novacastra Laboratories Ltd., New Castle Upon Tyne, UK)* dilution was 1:50 in TTBS. Blots were incubated with the primary antibody overnight. Blots for calpain were incubated for 1.5 h with a secondary donkey, anti-goat with a conjugated biotinylated-streptavidin alkaline phosphatase enzyme *(#sc2020, Santa-Cruz Biotechnologies, Santa Cruz, CA)*, which was diluted 1:3000 in TTBS. Blots for ubiquitin were incubated for 1.5 h with a secondary donkey, anti-mouse antibody with a biotinylated-streptavidin alkaline phosphatase enzyme *(#sc2316, Santa-Cruz Biotechnologies, Santa Cruz, CA)*, which was diluted 1:3000 in TTBS. Blots were developed using an alkaline phosphatase conjugate substrate kit with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) *(#170-6432, Bio-Rad, Hercules, CA).*

Band Quantification

Blots were digitized using a scanner and a computerized image analysis system *(LabWorks, UVP Laboratory Products, Upland CA)* was used to determine the band density and molecular weight. Because of the high variability between blots, only within blot comparisons were made. To determine relative changes in protein expression within a subject following exercise, the density value of the rest band was set to unity (i.e. 1), and the values of bout 1 and bout 2 bands were expressed as bout 1/rest and bout 2/rest. Gender differences in protein expression were determined by dividing the absolute density of the male band for a given time point by the absolute density of the female band for same time, with all female rest and exercise band densities were set to unity.

Statistics

Computerized statistical software was used for all analysis *(Statistica v5.0, Statsoft).* The absolute force data was analyzed using four-way ANOVA with the between factor being gender (2 levels) and the within factors being exercise bout (2 levels), contraction type (eccentric or concentric; 2 levels) and time (6 levels). The relative force deficit was expressed as percent of initial (pre) force and analyzed using a four-way ANOVA similar to absolute force data. The CK activity data was analyzed using three-way ANOVA with the between factor being gender (2 levels) and the within factors being time (6 levels) and conditition (rest, bout 1, bout 2; 3 levels). The ultrastructural damage and cellular infiltration data was analyzed using two-way ANOVA with the between factor being gender (2 levels) and the within factor being condition (rest, bout 1, bout 2; 3 levels). Changes within a subject in ubiquitin conjugated protein or calpain regulatory subunit

expression were analyzed using a two-way ANOVA with the between factor being gender (2 levels) and the within factor being condition (rest, bout 1, or bout 2; 3 levels). Gender differences in ubiquitin conjugated proteins or calpain regulatory subunit expression were analyzed using a two-way ANOVA with the between factor being gender ratio (male/female or female/female; 2 levels) and the within factor being condition (rest, bout 1, or bout 2; 3 levels). A Newman-Keuls post hoc test was used to locate pair-wise significant differences, where appropriate. The level of significance was set to $p<0.05$. All data in texts, graphs and tables are presented as MEAN ± SD.

2.4. Results

Subject Characteristics

The descriptive subject characteristics are presented in *Table 1*. The males were significantly taller, heavier and had greater 1RM values for leg press and leg extension exercise ($p<0.05$).

	Females	Males
Age (yrs)	23.2 ± 2.0	22.2 ± 2.0
Height (cm)	168.4 ± 4.9*	178.3 ± 8.1
Weight (kg)	65.1 ± 7.9*	82.5 ± 10.3
Leg Press 1RM (kg)	69.7 ± 14.8*	112.5 ± 18.3
Knee Extension 1 RM (kg)	21.9 ± 4.6*	36.6 ± 5.3

Table 1. Subject descriptive characteristics. The subjects were matched for age, however the males were taller, heavier, and stronger ($p<0.05$).

Force data

Absolute force output was depressed 30 min, 24 h, 48 h, 96 h, and 7 d post exercise bout 1 ($p<0.05$). Following the second exercise bout, force was depressed 30 min, 24 h, 48 h, and 96 h post exercise ($p<0.05$). The force output was decreased at 48 h, 96 h, and 7 d post exercise following the second exercise bout as compared to the first ($p<0.05$; *see Fig. 1).* There was no difference in baseline force output prior to exercise bout 1 and bout 2. Males were stronger than females ($p<0.05$; main effect) and force output was greater for eccentric contractions as compared to concentric contractions ($p<0.05$; main effect). However, the magnitude of the relative force deficit was not affected by gender or contraction type (i.e. eccentric vs. concentric).

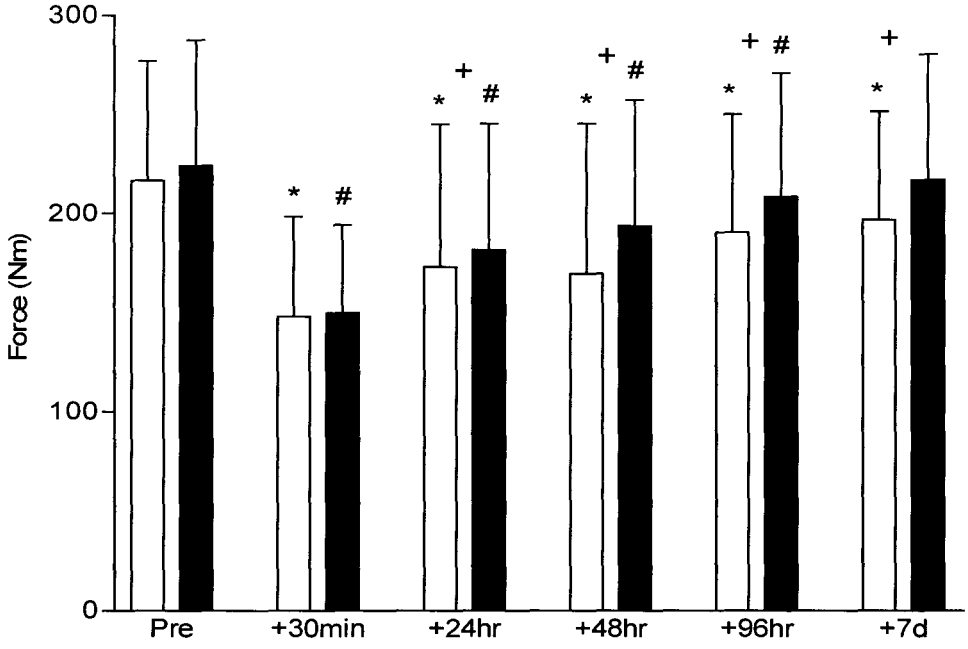

Fig. 1. Knee extensor following eccentric exercise collapsed across gender. Open bars: bout 1, closed bars: bout 2. * Significant decrease as compared to pre bout 1 ($p<0.05$). # Significant decrease as compared to pre bout 2 ($p<0.05$). + Significant difference between bout 1 and bout 2 at specified time point ($p<0.05$).

Serum CK Activity

Males had higher serum CK activity as compared to females ($p<0.05$; main effect). Following the first exercise bout, serum CK activity increased significantly over baseline values at 48 h and 96 h post exercise, yet by 7 d it was not significantly different from resting values. Following the second exercise bout, serum CK activity was different from resting values only at 96 h post exercise ($p<0.05$). Serum CK activity was lower 48 h and 96 h post exercise after the second exercise bout as compared to the first ($p<0.05$; *see Fig. 2.).*

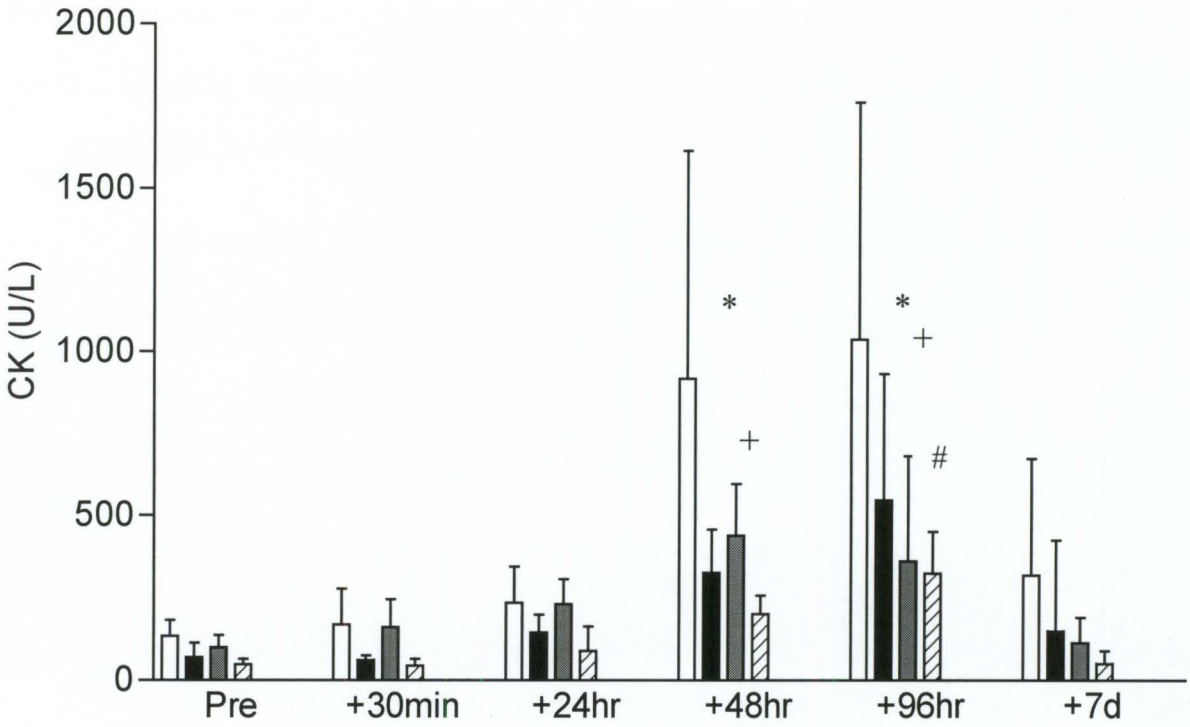

Fig. 2. Serum CK activity following eccentric exercise. Open bars = bout 1 male; closed bars = bout 1 female; grey bars = bout 2 male; hatched bars = bout 2 female. * Significant increase as compared to pre bout 1 when collapsed across gender ($p<0.05$). # Significant increase as compared to pre bout 2 when collapsed across gender ($p<0.05$). + Significant difference between bout 1 and bout 2 when collapsed across gender ($p<0.05$).

Toluidine Blue Sections

There was more Z-disk streaming in the exercised leg as compared to the control leg following the first exercise bout ($p<0.05$). Following the second exercise bout, the amount of Z-disk streaming in the exercised leg was attenuated and no longer different from the rest leg ($p=0.08$) *(see Fig. 3.)*. However, there was no significant difference in the amount of Z-disk streaming in the exercised legs between bout 1 and bout 2. There was no difference between genders in the extent of Z-disk streaming at any time point.

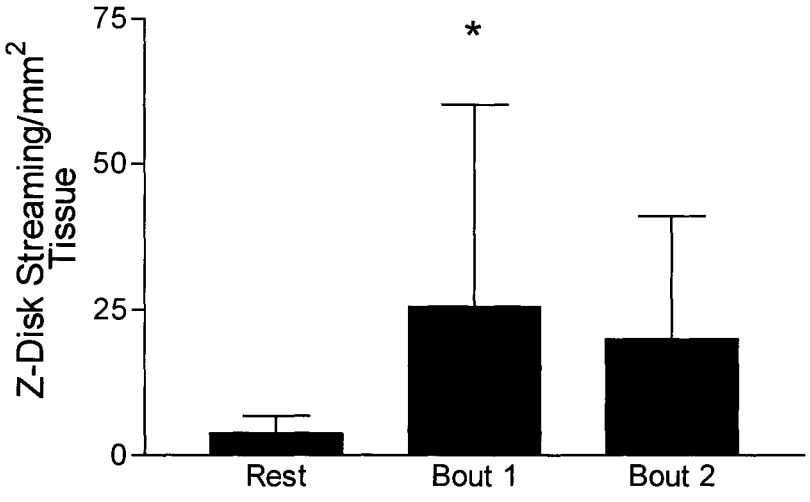

Fig. 3. Extensive Z-disk streaming following eccentric exercise collapsed across gender.
* Significant increase in damage as compared to rest values ($p<0.05$).

Immunohistochemistry (Neutrophil and Macrophage Infiltration)

The number of neutrophils per mm^2 of tissue did not increase significantly over baseline values in males, 24 h after the first or second exercise bout. In females, the number of neutrophils per mm^2 of tissue was significantly greater after the second exercise bout as compared to the first and versus rest ($p<0.05$). Furthermore, females had greater neutrophil counts than males after the second exercise bout ($p<0.05$). Males and females had similar neutrophil counts at rest and following the first exercise bout *(see Fig. 4.)*.

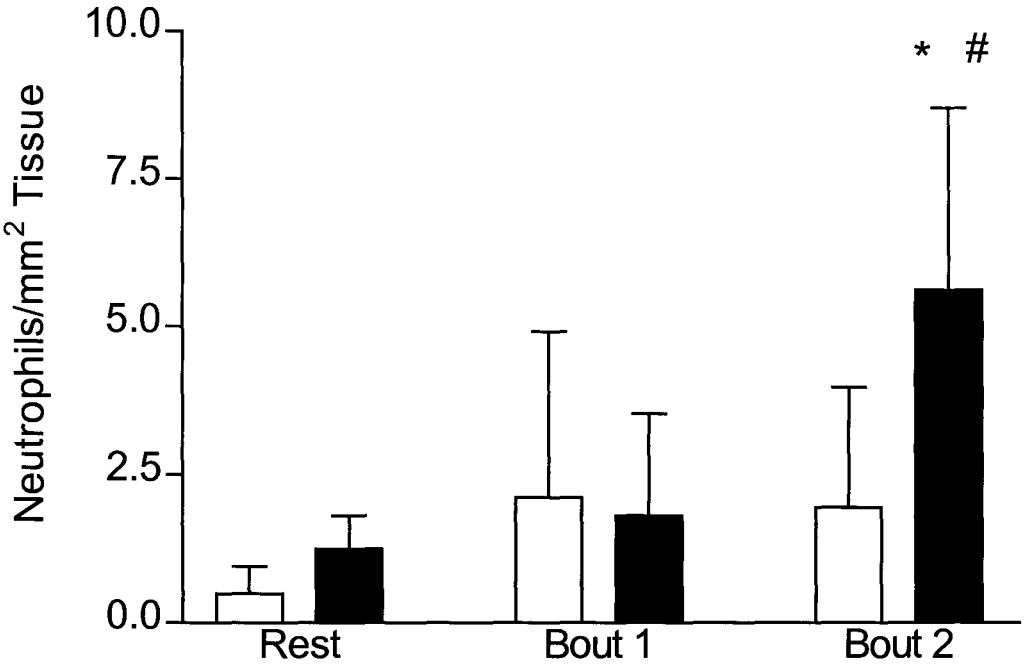

Fig. 4. MPO positive cells (neutrophils) in muscle following eccentric exercise. Open bars = males; closed bars = females. * Significant increase in the number of neutrophils/mm^2 of tissue as compared to female rest values ($p<0.05$). # Significant gender difference ($p<0.05$).

The number of macrophages per mm^2 of tissue was elevated over baseline values after the first and second exercise bout in both males and females ($p<0.05$; main effect). The number of macrophages per mm^2 of tissue was greater (almost double) in females after the second bout as compared to the first ($p=0.11$), whereas the macrophage counts were similarly elevated after both bouts of exercise in males *(see Fig. 5.)*.

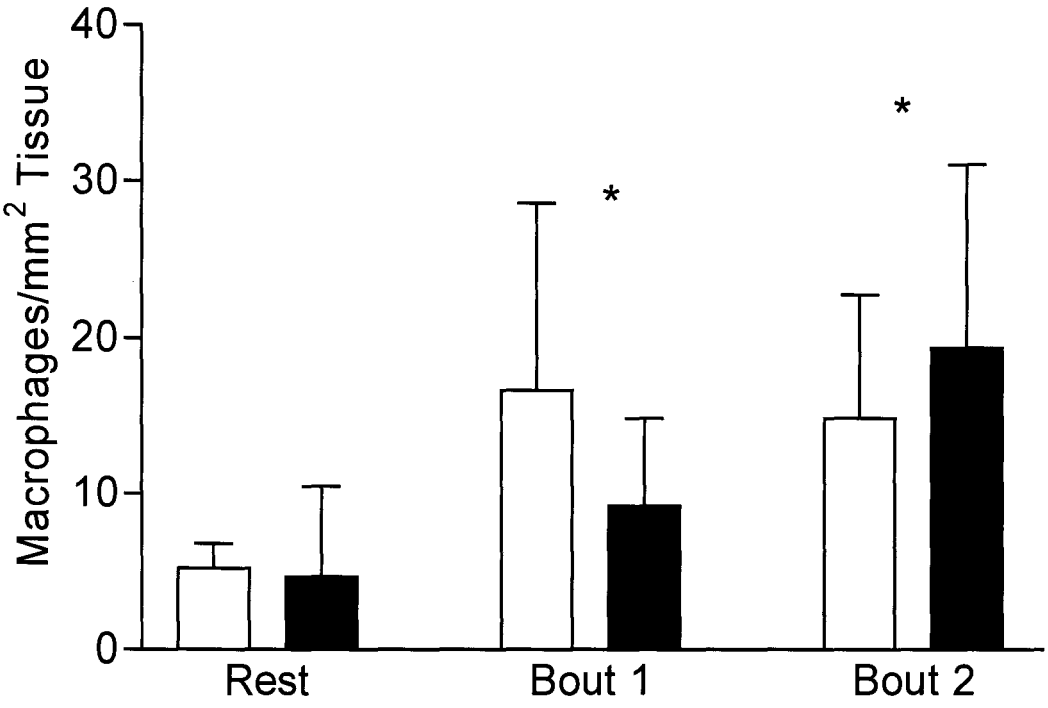

Fig. 5. CD68 positive cells (macrophages) in muscle following eccentric exercise. Open bars: males; closed bars: females. * Significant increase as compared to rest values when collapsed across gender ($p<0.05$).

Ubiquitin Conjugated Proteins

Ubiquitin conjugated protein content was elevated over resting values following the second exercise bout ($p<0.05$; *see Fig. 6.a.)* Males had higher ubiquitin conjugated protein content at rest and following exercise as compared to females ($p<0.05$; main effect; *see Fig. 6.b.)*

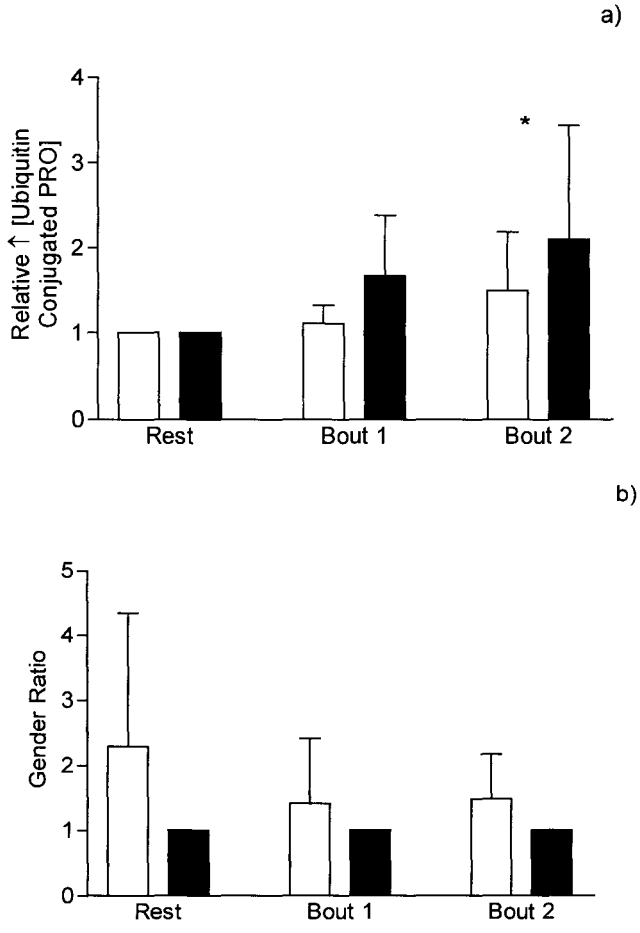

Fig. 6.a. Ubiquitin conjugated protein content following eccentric exercise; open bars = males, closed = bars females. * Significant increase in damage as compared to rest values (collapsed across gender; $p<0.05$).

6.b. Gender differences in ubiquitin conjugated protein content; open bars = male band density/female band density ratio for a given time point; closed bars = female band density/female band density ratio for a given time point.

30 kD Regulatory Calpain Subunit

The regulatory calpain subunit protein content did not change in response to exercise *(see Fig. 7.a.).* There was no gender-related difference in calpain expression at rest or following exercise *(see Fig. 7. b.).*

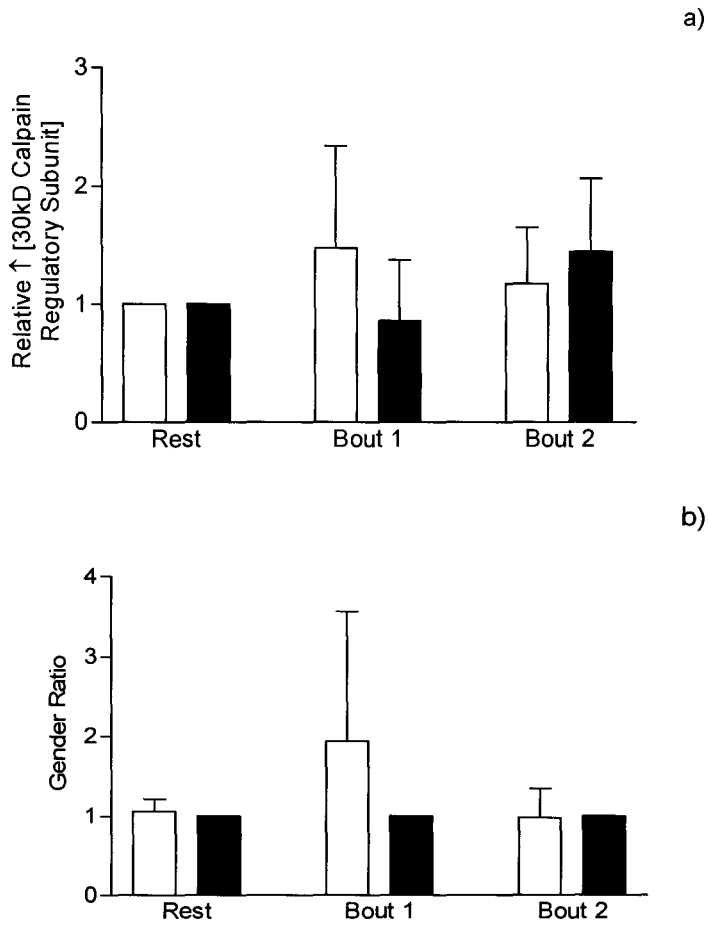

Fig. 7.a. Calpain regulatory subunit protein content following eccentric exercise; open bars = males; closed bars = females.

7.b. Gender differences in calpain regulatory subunit protein content; open bars = male band density/female band density ratio for a given time point; closed bars = female band density/female band density ratio for a given time point.

2.5. Discussion

The results of the current study indicate that a single bout of eccentric exercise induced adaptations in skeletal muscle that may have played a role in conferring protection resulting in less damage following a subsequent bout of eccentric exercise. Following the second exercise bout, force deficit, serum CK activity and Z-disk streaming were attenuated. Gender differences in serum CK activity, inflammatory cell infiltration and ubiquitin conjugated protein content were detected.

Repeated eccentric exercise attenuated the magnitude of the force deficit. Following the first exercise bout, force output was depressed at all time points, including 7 d post exercise. Following the second exercise bout, strength recovered to baseline levels by 7 d. Absolute force output was depressed 48 h, 96 h and 7 d post exercise bout 1 as compared to exercise bout 2. Attenuation of the exercise force deficit following repeated eccentric exercise has been well documented (19;21;26), however, the mechanism mediating this adaptation remains to be elucidated. Although males were stronger that females, the relative loss of strength following exercise bout 1 and 2 was similar between genders. Others have also observed no gender related difference in strength loss and recovery following an acute bout eccentric elbow flexor exercise (105).

Serum CK activity was elevated 48 h and 96 h after the initial exercise bout and returned to baseline values by 7 d post. After the second exercise bout, serum CK activity was elevated at 96 h post exercise only. Furthermore, the magnitude of the rise in serum CK activity was lower at 48 h and 96 h following the second exercise bout as compared to the

first. Decreased ultrastructural disruption and changes in inflammatory response may contribute to decreased serum CK activity following the second exercise bout. Others have suggested that the blunted CK response is due to, at least in part, an enhanced rate of clearance (47). However, most studies, including this one, only measure total CK activity and thus do not take into account changes in clearance and release rate. Nonetheless, serum CK activity is an indirect marker of damage and does not correlate with other indices of muscle damage (90), therefore it is essential to evaluate adaptations to repeated eccentric exercise using a variety of markers.

Females had lower serum CK activity as compared to males (main effect). CK activity at rest and during training has been previously reported to be lower in females as compared to males (2;6). In male rats, 17-β-estradiol administration can attenuate increases in plasma CK activity following a 2 h treadmill run (5;6). A blunted CK response in females may be due to the antioxidant properties of 17-β-estradiol (4). In support of this, one study did find lower plasma CK activity and plasma MDA concentration (a marker of lipid peroxidation and thus oxidative stress) in exercising females compared to males (23).

The amount of ultrastructural damage, as characterized by Z-disk streaming, was elevated after the first bout of eccentric exercise only. Following the second exercise bout, Z-disk streaming seemed to be attenuated as it was no longer different from rest values. However, the lack of a significant difference in the magitude of Z-disk streaming in the exercised leg following bout 1 and bout 2 weakened the strength of this finding. Great

intersubject variability in the magnitiude of Z-disk streaming following eccentric exercise probably contributed to the lack of difference in the exercised leg following repeated eccentric exercise. Some Z-disk streaming was observed at all time points measured, in accordance with previous research (36). This finding is in agreement with earlier research showing a trend of a three-fold attenuation in myofibrillar disruption following repeated, eccentric quadriceps exercise (43). These earlier findings, however, were only qualitative and not statistically significant due to a small sample size (N = 3) (43).

No gender differences in the amount of Z-disk streaming were observed at any time point. This finding is in agreement with our observation of a similar relative force decrement in both genders following exercise. Gender differences in serum CK activity are therefore unrelated to the amount of Z-disk streaming and the exercise-induced force-reduction, ostensibly the two hallmark measures of muscle damage. We have previously shown that the extent of Z-disk streaming was similar between genders 48 h post eccentric exercise (123). This may seem contradictory to reports of greater discontinuous dystrophin staining, desmin desolution, and rearrangement of actin and fibronectin in male rodents as compared to female rodents 6 h and 48 h after a bout of downhill running (57). This latter study measured cytoskeletal proteins by immunohistochemistry, which may not be directly correlated with Z-disk streaming *per se* (57). The downhill running protocol used in rodent studies (57) elicits a combined eccentric and oxidative stress, which together may be more damaging to the cytoskeleton. Using a unilateral eccentric knee extension and unilateral eccentric leg press model, we were unable to detect

discontinuous dystrophin staining nor significant desmin loss or a gender difference in either of these parameters *(N. Stupka and L. Beaton, unpublished observations, 2000).*

Exercise-induced muscle damage stimulates an acute phase inflammatory response, which includes infiltration into skeletal muscle by neutrophils and macrophages (31). In males, the number of neutrophils per mm^2 of tissue did not increase over baseline values 24 h post exercise in bout 1 or 2. In females, however, neutrophil counts were higher following the second exercise bout as compared to rest and or bout 1 and were also greater than male values at this time point. Neutrophil infiltration follows a relatively rapid time course peaking ~6 h to 12 h post exercise, therefore it is possible that in males neutrophils may have already returned to baseline levels by 24 h post exercise. The increase in infiltrating neutrophils seen in females after the second exercise bout may be due changes in the timing of the inflammatory response. In support of this notion, peak ER-BMDM1 leukocyte (a macrophage subset) infiltration is delayed in female mice as compared to male mice following an acute bout of exercise (121). Furthermore, a reduction in circulating neutrophils and a lower state of neutrophil and monocyte activation (i.e. CD18 and CD64 expression) have been reported after repeated eccentric arm exercise (100) which may also influence the time course of neutrophil infiltration. Our findings demonstrate that changes occur in the acute phase immune response following repeated eccentric exercise.

Muscle macrophage counts were elevated following exercise bout 1 and 2 in both males and females. This was expected, because the time course for macrophage infiltration is

slower than that for neutrophils. Macrophage counts peak ~18 h to 96 h post exercise (62). The number of macrophages per mm^2 of tissue in females tended to double after the second exercise bout as compared to the first, whereas in males macrophage counts were similarly elevated after both bouts. Although this finding was not significant, it does lend support to our hypothesis that adaptations to repeated eccentric exercise may be mediated in part by changes in the time course of inflammatory cell infiltration. To fully investigate this hypothesis, however, a time course study would be needed.

Ubiquitin conjugated protein content was elevated over resting values 24 h after the second exercise bout, but not the first. Ubiquitin binds covalently to damaged or abnormally folded proteins and targets them for degradation by the 20S proteosome via the ATP-dependent ubiquitin proteosome pathway (22). Increased free ubiquitin and ubiquitin conjugated protein expression have been detected in humans 48 h following an acute bout of eccentric elbow flexor exercise in humans (127) and in animals 48 h following overnight spontaneous treadmill running (112). Twenty-four hours following the initial exercise bout may not be enough time to detect increases in ubiquitin conjugated protein content. However, ubiquitin conjugated protein content may not be indicative of 26S proteosome activity, because ATP dependent proteolysis can be elevated prior to any significant accumulation of ubiquitin conjugated proteins (140).

Given the attenuation in the force deficit, serum CK and potentially Z-disk streaming following the second exercise bout, we had not expected to see an increase in ubiquitin conjugated protein content. Elevated ubiquitin conjugated protein content following the

second exercise bout, suggests that in response to repeated eccentric exercise the kinetics of ubiquitin proteosome pathway may be changed. An alternative and provocative hypothesis is that following repeated eccentric exercise a more "directed" form of proteolysis occurs, mediated via the ATP-dependent ubiquitin proteosome pathway as opposed to an inflammatory cell-mediated extracellular proteolysis. Increased ubiquitin conjugated protein content at 24 h may enhance removal of damaged contractile and cytoskeletal proteins and thus promote regeneration and remodeling. While protein degradation is attenuated with training, even in very well trained individuals an acute bout of resistance exercise will continue to stimulate increases in protein degradation (98). The relationship between muscle damage, protein degradation and the enzyme pathways responsible for this degradation requires further investigation.

Ubiquitin conjugated protein content was greater in males as compared to females. However, given the limitations of interpreting Western blots of ubiquitin conjugated protein, it is difficult to comment on the significance of this finding at this time. Further study of gender-dependent differences in proteolytic enzyme pathway response following eccentric exercise is required.

The 30 kD regulatory subunit of calpain protein content did not increase in response to exercise and there was no gender difference. Three isoforms of calpain exist in skeletal muscle: μM (low calcium requirement isoform), mM (high calcium requirement isoform), and p94 (a muscle specific isoform). The μM and mM isoforms are composed of an identical 30 kD regulatory subunit and a unique 80 kD catalytic subunit (103). The

30 kD subunit is the same for the two isoforms. Protein p94 content is very low (119). Thus the 30 kD subunit was chosen for Western blot analysis, because it would be the most general marker of increased calpain content. Twenty-four hours post exercise, however may be too late to detect increases in calpain protein content, because in rats calpain activity is increased shortly following the cessation of exercise (7) and returns to pre-exercise values within 24 h (7). Calpain activity post exercise has not, to our knowledge, been investigated in humans. It has been hypothesized that protein degradation, including removal of Z-disks (38), is initiated by calpain but subsequent proteolysis is dependent upon other proteolytic enzyme pathways, chiefly the ATP-dependent ubiquitin proteosome pathway (9).

The biochemical pathways mediating the rapid adaptation to eccentric exercise induced muscle damage have not been well characterized. This study extends previous work by examining the effects of a repeated bout of exercise on ultrastructural damage, inflammatory cell infiltration, and calpain and ubiquitin conjugated protein content. Serum CK activity, force deficit and Z-disk streaming were attenuated following repeated eccentric exercise. The increase in neutrophil infiltration in females following the second exercise bout suggests that adaptations to eccentric exercise induced muscle damage may include changes in the time course of inflammatory cell infiltration and protein degradation. The force deficit and the extent of ultrastructural disruption following exercise were similar between males and females. Thus, gender related differences in exercise induced muscle damage are found in the secondary responses to

eccentric contraction induced injury including serum CK activity, inflammatory cell infiltration and protein degradation.

Chapter 3: STRESS PROTEIN CONTENT IN SKELETAL MUSCLE FOLLOWING REPEATED ECCENTRIC EXERCISE

3.1. Abstract

Following repeated eccentric exercise indices of muscle damage are attenuated. The factors mediating attenuation are largely unknown, however, it is possible that increased expression of protective stress proteins, such as heat shock protein (HSP) 72, HSP27, and bcl-2, may be involved. We studied the effect of two eccentric exercise bouts separated by 5.5 wk in healthy, untrained male (N=8) and female (N=8) subjects using a unilateral, eccentric exercise design. Biopsies were taken from the *vastus lateralis* of each leg 24 h post exercise. Western blots were used to estimate muscle HSP27, HSP72, and bcl-2 content in the non-exercised (rest) and the exercised leg. HSP72 protein content did not change in response to acute exercise, but was higher in males as compared to females ($p=0.053$). Females had greater HSP27 content at rest ($p<0.05$); however, HSP27 content increased in response to exercise in males only, such that after the first and second bout there was no gender-based difference in HSP27 protein content. At rest and after the first exercise bout, bcl-2 content was similar between genders. After the second exercise bout, bcl-2 protein content was significantly greater in females as compared to males ($p<0.05$). Stress protein content is affected by eccentric exercise and gender, this needs to be evaluated further in future studies.

3.2. Introduction

Skeletal muscle adapts rapidly to numerous physiological stimuli and stressors, including exercise. One bout of eccentric exercise can induce "protective" adaptations in skeletal muscle, such that if the exercise bout is repeated indices of muscle damage, including elevations in serum CK and decreased force generating capacity are attenuated (19). Little is known about the pathways mediating these adaptations.

Gender differences in indices of muscle damage have been observed in humans and animals following an acute bout of aerobic or eccentric exercise. Differences in plasma CK activity (92), inflammatory response (121) and ultrastructural disruptions (56) have been reported. It has been hypothesized that at least some of these differences could be attributed to the female sex hormone, 17-β-estradiol. Estrogen may protect muscle cells by functioning as an antioxidant and/or a membrane stabilizer either directly or through receptor mediated pathway (131).

The effect of endurance exercise on HSP72 protein expression has been studied by a number of groups; however, the effect of repeated eccentric exercise on HSP72 protein content in humans has not been investigated. Puntshart et. al. reported that muscle HSP72 mRNA content was significantly elevated over baseline values in human males 4 min to 3 h following an acute bout of strenuous endurance exercise (101). HSP72 protein content, however, did not increase. Heat shock protein content did increase in elite male rowers in response to an intense 4 wk rowing training program (65;66). Increased HSP

synthesis has also been observed in rats following an acute bout of treadmill running (39) and after endurance training (52).

The effect of eccentric exercise on HSP27 expression has not been investigated. Similar to HSP72, HSP27 has molecular chaperone properties (27). Cells with high endogenous HSP27 content are far more tolerant of oxidative stress and maintain cytoskeletal integrity better than cells with low endogenous HSP27 content (45). HSP27 may attenuate exercise induced muscle damage, by stabilizing the cytoskeleton and contractile machinery during the lengthening contractions (46), by improving cellular antioxidant status and thus protecting the cell from ROS generated by infiltrating neutrophils and macrophages (45) and by promoting protein and RNA synthesis following exercise (16). HSP27 has an estrogen response element (35) and thus greater HSP27 protein content in females may help explain gender-related differences in eccentric exericse induced muscle damage.

Exercise may alter bcl-2 protein content in skeletal muscle. Following a night of treadmill running a significant decrease in blc-2 content was detected in *mdx* (dystrophic) muscle, and this corresponded to an increase in apoptotic nuclei (112). Bcl-2 positive staining fibers were detected in males 48 h following an acute, eccentric exercise bout (123). Since skeletal muscle can express the proteins of apoptosis and it may be capable of undergoing programmed cell death (112;125). Bcl-2 is also upregulated by inflammatory cells while they are removing themselves from skeletal muscle by apoptosis (130).

The primary purpose of this study was to investigate stress protein expression following two repeated bouts of eccentric, quadriceps exercise separated by 5.5 wk. The working hypothesis was that adaptations to repeated eccentric exercise are mediated by increased expression of HSP72, HSP27, and bcl-2, synthesis of these proteins is induced quite rapidly in response to stress and they help maintain cellular homeostasis. A secondary purpose of this study was to further investigate gender-mediated differences in exercise induced muscle damage. With regards to gender differences, it was hypothesized that males would have greater HSP72 (95) and bcl-2 (123) content post exercise as compared to females and that basal levels of HSP27 (35) would be higher in females.

3.3. Materials and Methods

Subjects

Healthy, non-smoking male (N=8) and female (N=8) university students volunteered to participate in the study and gave informed, written consent. The study was approved by the McMaster University Research Ethics Board. None of the subjects had participated in a regular, structured exercise program for at least 6 mo prior to participating in the study. Five of female subjects were oral contraceptive users and all were tested as close to the the mid-follicular phase of their menstrual cycle (7 to 11 days following menses).

Testing Protocol

Prior to starting the study, all subjects participated in a familiarization session, where the unilateral, concentric single repetition maximum (1RM) strength was determined for both legs and for both exercises (leg press and leg extension). To maximize the damage response, the subject's weaker leg was exercised and the stronger leg served as the control. On the testing day, following a brief warm-up consisting of 10 min of light (75 W) cycle ergometry, subjects performed 36 (3 sets x 12 repetitions per set) unilateral, eccentric leg extensions and 100 (10 sets x 10 repetitions per set) unilateral, eccentric leg presses using standard leg and knee extension machines *(Nautlis)*. For both exercises, the subjects lowered a mass equivalent to 120 % of their unilateral, concentric 1RM. The investigator performed the concentric phase of the exercise.

The subjects returned to the testing laboratory 24 h after the exercise bout. The *vastus lateralis* of the exercised and rest leg was biopsied using a suction modified Bergstrom needle. Approximately, 50 mg of the muscle biopsy was allotted for Western blotting, it was dissected free of visible fat and connective tissue, quenched in liquid N_2, and stored at –80 °C until the time of analysis. Only the rest sample from the first biospy was analyzed using Western blotting, as it was assumed that the two rest biospies would be similar.

The subjects rested for 5 to 6 wk after the initial bout and then returned to the testing center to repeat the entire protocol. Between exercise bouts subjects refrained from performing any vigorous physical activity.

Polyacrylamide Gel Electrophoresis and Western blotting

Biopsies were homogenized in 600 µl of 5 % SDS-phosphate buffered saline (PBS; 152 mM NaCl, 2.99 mM KCl, 85.0 mM $NaHPO_4$, 4.00 mM KH_2PO_4) using a glass homogenizer. The homogenate was heated at 70 °C for 10 min, centrifuged at 14,000 RPM for 10 min and the supernatant was saved. Supernatant protein content was determined using the Bradford protein assay *(#500-0006, Bio-Rad, Hercules, CA)*. All samples were run in triplicate and the coefficient for variation for the protein assay was less than 10 % for each sample.

Forty µg of supernatant protein was loaded onto a 7.5 % SDS-polyacrylamide separating gel and a 4 % SDS-polyacrylamide stacking gel. Rest, exercise bout 1 and exercise bout 2 samples for a male and female subject were always run on the same gel, along with a broad range molecular weight standard *(#161-0319, Bio-Rad, Hercules, CA)*. Gels were run in duplicate. The gels were run at 100 V for 1 h at room temperature.

Proteins were transferred to PVDF membranes *(polyvinylidene difluoride; #162-0177, Bio-Rad, Hercules, CA)* membranes overnight, on ice at 25 V. The PVDF membranes were blocked with 9 % gelatin *(#G-7765, Sigma)* dissolved in Tris-buffered saline with 0.1% Tween *(#170-6531, Bio-Rad, Hercules, CA)* (TTBS; 500 mM NaCl, 20 mM Tris-HCl, pH 7.5) for 1.5 h.

The HSP72 antibody *(#sc-1060, Santa Cruz Biotechnologies, Santa Cruz, CA)* and HSP27 antibody *(#sc-1048, Santa Cruz Biotechnologies, Santa Cruz, CA)* dilution was

1:1000 in TTBS. Blots were incubated at room temperature overnight with the primary antibody and for 1.5 h with a secondary donkey, anti-goat antibody with a biotinylated-streptavidin alkaline phosphatase conjugated enzyme *(#sc2020, Santa-Cruz Biotechnologies, Santa Cruz, CA)* diluted to 1:3000 in TTBS.

Blots were incubated at room temperature with a bcl-2 polyclonal antibody *(gift from Dr. Brian Leber, Dept. Biochemistry, McMaster University)* diluted to 1:500 in TTBS overnight and for 1.5 h with a secondary donkey, anti-rabbit antibody with a biotinylated-steptavidin alakaline phosphatase conjugated enzyme *(#sc2020, Santa-Cruz Biotechnologies, Santa Cruz, CA)* diluted to 1:3000 in TTBS. Blots for all three proteins were developed using a colourmetric alkaline-phosphatase conjugate substrate kit with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) *(#170-6432, Bio-Rad, Hercules, CA).*

We confirmed that loading 40 μg of protein per lane would fall within the linear range of the optical density for HSP72, HSP27 and bcl-2 by loading increasing amounts of protein (20 μg to 75 μg) per lane, doing the respective Western blot and determining optical density. Increasing amounts of HSP72 protein standard *(# H8778, Sigma, St. Louis, MO)* ranging from 0.01 μg to 0.25 μg were also blotted and these too were found to fall within the linear range of optical density (r^2=0.971). Furthermore, five lanes containing the same sample and protein quantity (45 μg) were run on the same gel and Western blotted. The coeffcient of variation for band density was less than 10 %. Therefore, samples were prepared and loaded with good reproducibility.

Band Quantification and Statistical Analysis

Blots were digitized using a scanner and a computerized image analysis system *(LabWorks, UVP Laboratory Products, Upland, CA)* for band density and molecular weight. Because of the high variability between blots, only within blot comparisons were made. To determine relative changes in protein expression within a subject following the two exercise bouts, the density value of the rest band was set to unity (i.e. 1), and the values of bout 1 and bout 2 bands were expressed as bout 1/rest and bout 2/rest. Gender differences in protein expression were determined by dividing the absolute density of the male band, at a given time point, by the absolute density of the female band at the same given time with all female rest and exercise band densities set to unity. Since all gels were run in duplicate, mean values were used for statistical analysis. The coefficient of variation for the mean relative increase in band density over rest values for duplicate blots of HSP72, HSP27, and bcl-2 was 15 %, 19 %, and 19 % respectively.

Statistics

Computerized statistical software was used for all analysis *(Statistica v5.0, Statsoft, Tulsa, OK)*. Changes within a subject in HSP72, HSP27, or bcl-2 protein expression were analyzed using a two-way ANOVA with the between factor being gender (two levels) and the within factor being condition (rest, bout 1, or bout 2; three levels). Gender differences in HSP72, HSP27 or bcl-2 protein expression were analyzed using a two-way ANOVA with the between factor being gender ratio (male/female or female/female; two levels) and the within factor being exercise (rest, bout 1, or bout 2; three levels). A Newman-Keuls post hoc test was used to locate pair-wise differences, where appropriate.

Subject descriptive data were analyzed using one-way analysis of variance (ANOVA). The level of significance was $p<0.05$. All data in texts and figures are presented as MEAN±SD.

3.4. Results

Subject Characteristics

The descriptive subject characteristics are presented in *Table 1 (Chapter 2)*. The males were significantly taller, heavier, and had greater 1RM values for leg press and leg extension exercise ($p<0.05$).

HSP72

HSP72 expression did not increase in response to acute or repeated eccentric exercise. In both genders, HSP72 content was similar at rest and following exercise bout 1 and 2 *(see Fig. 8.a.)*. There was, however, a strong trend (NS; p=0.053) towards greater HSP72 protein content in males as compared to females *(see Fig. 8.b.)*.

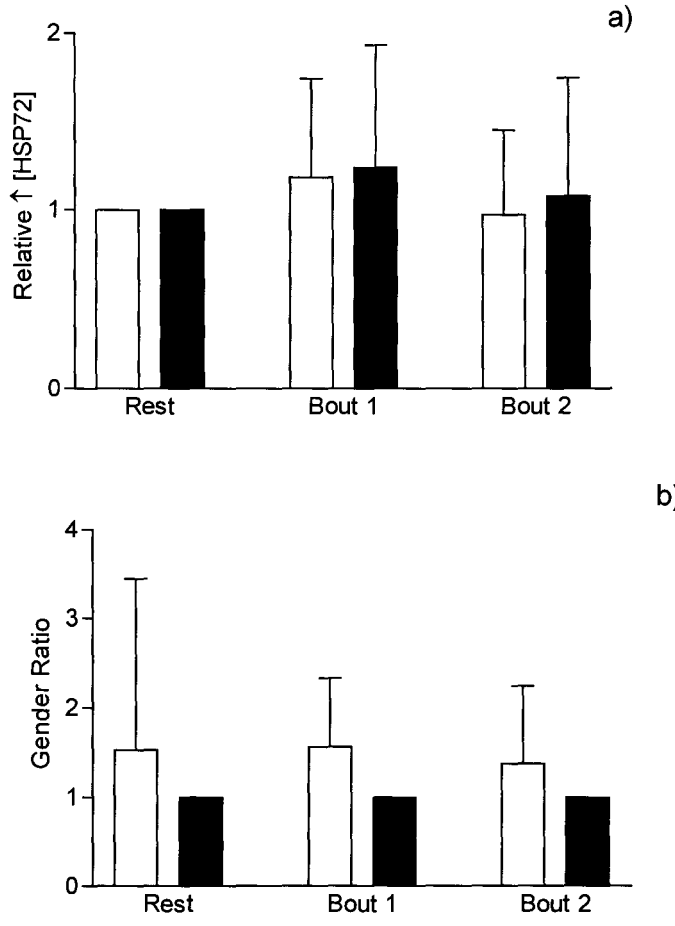

Fig. 8.a. HSP72 protein content following eccentric exercise; open bars = males, closed = bars females.

8.b. Gender differences in HSP72 protein content; open bars = male band density/female band density ratio for a given time point; closed bars = female band density/female band density ratio for a given time point.

HSP27

Basal HSP27 protein content was higher in females as compared to males ($p<0.05$). However, following exercise HSP27 content increased significantly in males only, such that after the first and second exercise bout there was no difference in HSP27 content between genders *(see Fig. 9.b.)*. Also, the relative increase in HSP27 expression following the second exercise bout was much greater in males than females ($p<0.05$; *see Fig. 9.a.)*.

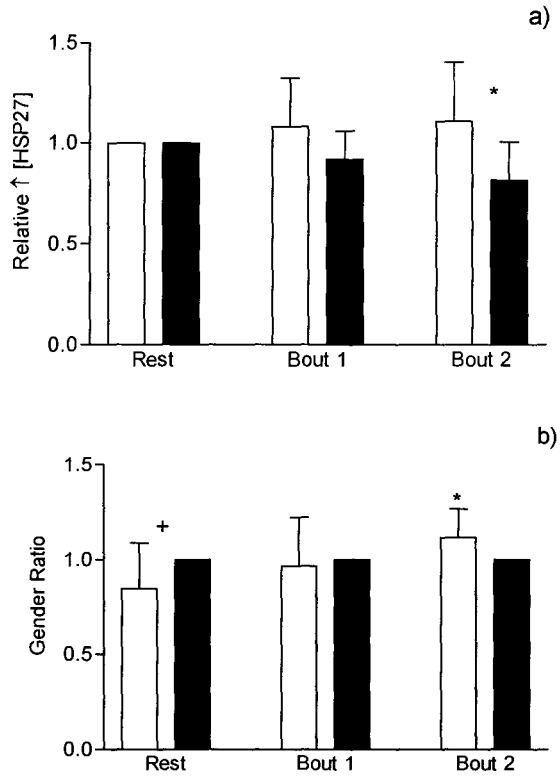

Fig. 9.a. HSP27 protein content following eccentric exercise; open bars = males; closed = bars females. * Significant difference in HSP27 content between genders after exercise bout 2.

9.b. Gender differences in HSP27 protein content; open bars = male band density/female band density ratio for a given time point; closed bars = female band density/female band density ratio for a given time point. * Significant difference in the male band density/female band density ratio as compared to rest and bout 1. + Significant gender difference ($p<0.05$).

Bcl-2

The relative increase in bcl-2 protein content following exercise tended to be greater in females as compared to males (NS; p=0.09; *see Fig. 3.a.).* The male:female ratio of bcl-2 band density was 1.09 at rest, but it decreased significantly to 0.80 following the second exercise bout (p<0.05; *see Fig. 3.b.).* Therefore, 24 h after the second exercise bout, bcl-2 protein content was higher in females as compared to males (p<0.05).

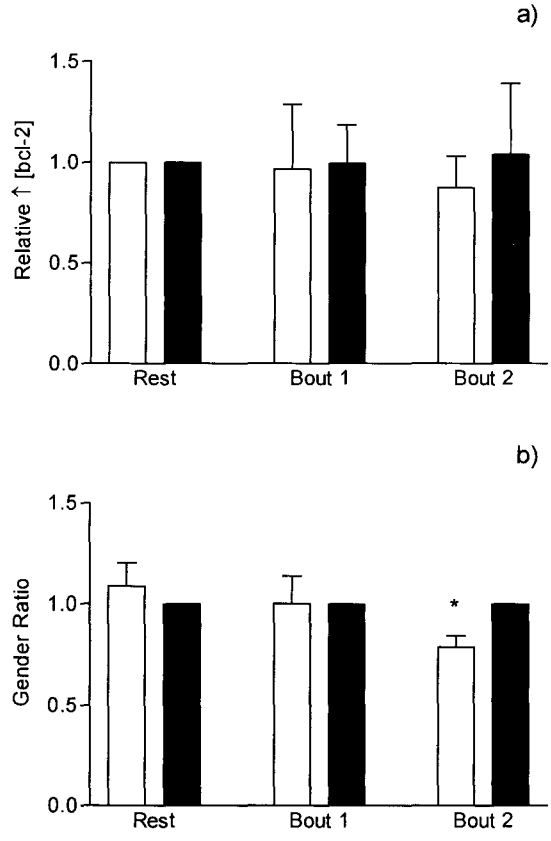

Fig. 10.a. Bcl-2 protein content following eccentric exercise; open bars = males, closed = bars females.

10.b. Gender differences in blc-2 protein content; open bars = male band density/female band density for a given time point; closed bars = female band density/female band density for a given time point. * Significant difference in the male band density/female band density ratio after the second exercise bout as compared to rest and exercise bout 1.

3.5. Discussion

Skeletal muscle adapts rapidly to repeated eccentric exercise. Previously, using this protocol, we observed an attenuation in various indices of exercise induced muscle damage *(see Chapter 2)*. Force deficit, the rise in serum CK activity and the amount of ultrastructural disruption were attenuated following the second exercise bout as compared to the first. It was hypothesized that this rapid adaptation to eccentric exercise may be mediated in part by increased in stress protein content.

Contrary to our hypothesis, HSP72 content was not elevated 24 h following eccentric exercise. Our study design is unique because a resistance exercise protocol was used. Most prior studies have used endurance exercise, where increases in HSP70 content may be related more to elevations in muscle temperature rather than muscle damage. Thus far in humans, increases in HSP72 protein content following an acute bout of exercise have not been detected (101). It is possible that 24 h post exercise may be too late to detect significant increases in HSP72 protein content, as by this time protein content may have peaked and returned to baseline values. In rats, peak increases in HSP72 protein content have been detected within a few hours following cessation of exercise. HSP72 protein content was increased by 150 % in the soleus and by 200 % in the EDL 4 h and 2 h respectively following an 60 min of treadmill running (39). Jackson et. al. observed that HSP72 protein content in the hindlimb muscles of rats peaked 4 h to 6 h post exercise (81). In addition, Locke et. al. reported increased HSP72 content in the soleus of male rats immediately following a 20 min run (68). However, others have detected significant increases in HSP72 content in the red and white *vastus* of male rodents as late as 24 h

following a 60 min treadmill run (95). Alternatively, a single bout of eccentric exercise may not be an adequate stimulus to induce long lasting increases HSP72 protein synthesis. In humans, more frequent and intense bouts of eccentric exercise may be required to stimulate lasting and measurable increases in HSP72 protein content (65;66).

HSP72 mRNA synthesis is induced very rapidly in response to endurance exercise (30;101;107). The increases in mRNA content in humans are quite large, up to 200 % to 400 % (30;101). In males, HSP72 mRNA content was elevated in the *vastus lateralis* 4 min to 3 h following a 30 min treadmill run at anaerobic threshold (101). Salo et. al. ran rats to exhaustion and observed that HSP70 mRNA content in the plantaris, soleus, and EDL muscles peaked 30 min to 60 min post exercise and it returned to baseline levels by 6 h post exercise (107). However, increases in mRNA content are often not accompanied by a proportional increase in protein content. A direct relationship between protein and mRNA content may not exist, as mRNA content can be elevated without corresponding increases in protein and vice versa (138).

If following a single bout of exercise, the increase in HSP72 content is rapid and transient, then perhaps the primary function of HSP72 in this context is as a molecular chaperone. Existing HSP72 will bind to damaged proteins and prevent the formation of protein aggregates. There may be a time lag in the processing of damaged proteins, resulting in depletion of the free HSP72 pool and this may stimulate increased HSP72 protein synthesis (67). Once the damaged proteins are processed, HSP72 content will return to baseline values.

Gender differences in HSP72 expression following exercise have been observed in animals. In male rats, estrogen attenuated post exercise increases in HSP72 protein content (95). We have observed gender differences in ubiquitin conjugated protein content following repeated eccentric exercise, with males having greater levels as compared to females *(see Chapter 2)*. Ubiquitin is responsive to heat shock because the ubquitin gene contains a heat shock promoter (11). Also, post exercise increases in HSP27 content were seen only in males. Therefore, heat shock proteins may be more readily induced in males as compared to females.

As hypothesized, females had greater basal HSP27 levels as compared to males. This gender-related difference may be due to the fact that HSP27 has an estrogen response element. When MCF-7 breast cancer cells were treated with estrogen, HSP27 mRNA and protein content increased two fold (35). Exercise stimulated increased HSP27 protein synthesis in males only. The induction kinetics for HSP27 are slower than those for HSP72, but protein content remains elevated longer (67). This may be why increases in HSP27 were detected, but not HSP72.

Post exercise increases in serum CK activity are lower in females as compared to males (3;134) and following the second exercise bout as compared to the first (19), enhanced HSP27 protein content may have a mediating role. HSP27 protects cells from ROS by increasing intracellular GSH content (82). The antioxidant properties of HSP27 may protect the sarcolemma from ROS, enhance membrane stability and thus decrease CK efflux.

HSP27 has been localized to the cytoplasm, the I-band, and the Z-disk (71). Thus, HSP27 may be involved in the assembly and function of the myofibril contractile apparatus (24). Changes in HSP27 protein content and phosporylation state may contribute to decreased ultrastructural disruption (i.e. Z-disk streaming) observed following repeated eccentric exercise *(see Chapter 2)*. HSP27 activity depends not only on its intracellular concentration but also on its phosphorylation state (113). HSP27 needs to be phosphorylized in order to stabilize actin filaments (45;46;113). Increased intracellular HSP27 protein content enhances cellular resistance against actin fragmentation (45). Despite, having higher basal HSP27 content, the extent of Z-disk streaming was not different in females as compared to males *(see Chapter 2)*. Prior to the first exercise bout, the unphosphorylated form of HSP27 may predominate and adaptations to repeated eccentric exercise may involve an increase in phosphorylation, perhaps due to increased protein kinase activity (45). Future studies will need to investigate potential changes in HSP27 phosphorylation state in response to repeated eccentric exercise.

Absolute bcl-2 content was greater in females as compared to males, 24 h following the second exercise bout and relative increases in bcl-2 content tended to be greater in females as compared to males ($p=0.09$). This may seem paradoxical in light of our previous findings, 48 h after an acute bout of eccentric exercise, using immunohistological techniques, bcl-2 positive staining fibers were detected in male subjects only (123). A limitation of Western blotting, when compared to

immunohistochemistry, is that the location of the protein cannot be identified. While bcl-2 can be expressed by muscle cells (112;125), it is also upregulated by inflammatory cells as they undergo apoptosis and remove themselves from muscle (130). The increase in bcl-2 content may have been related to the higher numbers of neutrophils seen in females after the second exercise bout *(see Chapter 2).* Neutrophils were increased approximately 5 fold over baseline and 3 fold over exercise bout 1 values. Thus, the greatest source of bcl-2 in muscle post eccentric exercise may be inflammatory cells undergoing apoptosis.

These results show that repeated eccentric exercise alters stress protein content and that gender biased differences in stress protein content exist. HSP27 is upregulated by males in response to repeated eccentric exercise, such that gender differences in basal HSP27 expression are abolished. HSP27 may interact with the contractile apparatus of myofibrils and thus attenuate Z-disk streaming following repeated eccentric exercise. The antioxidant properties of HSP27 may help maintain cell membrane integrity and may contribute to the decrease in serum CK activity following repeated eccentric exercise. HSP72 expression was greater in males as compared to females; however, HSP72 content was not elevated 24 h post exercise. Finally, following the second exercise bout bcl-2 expression was increased in females, although this response may be related to the removal of leukocytes from muscle. Future studies are needed to further characterize the gender-related differences in stress protein synthesis following repeated eccentrically biased exercise.

Chapter 4: CONCLUSIONS

In concordance with previously published reports we found that a single bout of eccentric exercise can induce protective adaptations in skeletal muscle, manifested by the attenuation of indices of muscle damage when the bout was repeated. Following the second exercise bout the rise in serum CK, the post-exercise force deficit and the amount of ultrastructural damage (i.e. Z-disk streaming) were attenuated. Post exercise serum CK activity was lower in females as compared to males, however, no gender-related difference was detected in the amount of Z-disk streaming and the relative magnitude of the force deficit. The first exercise bout may induce adaptations which minimize the tension or shear stresses experienced by the muscle fiber during lengthening contractions, including enhanced content of stress proteins. HSP27 protein content was greater in males and bcl-2 protein content tended to be greater in females following the second exercise bout as compared to rest. HSP72 protein content did not change in response to eccentric exercise. Two potentially paradoxical findings are, increased ubiquitin conjugated protein content in both genders and increased infiltrating neutrophils in females following the second exercise bout. Increased ubiquitin conjugated protein content 24 h post exercise may be indicative of changes in the kinetics of the ubiquitin-proteosome pathway or following repeated eccentric exercise a more "directed" form of proteolysis occurs, mediated via the ATP-dependent ubiquitin proteosome pathway as opposed to an inflammatory cell-mediated extracellular proteolysis. The increase in neutrophils seen in females after the second exercise bout, may be due changes in peak inflammatory response mediated by gender and repeated exercise. A delay in peak ER-

BMDM1 leukocyte (a macrophage subset) infiltration into muscle following an acute bout of exercise has been reported in female mice as compared to male mice (121). A reduction in circulating neutrophils and a lower state of neutrophil and monocyte activation (i.e. CD18 and CD64 expression) have been reported after repeated eccentric arm exercise (100).

The pathways mediating adaptations to eccentric exercise induced muscle damage are not well known. This study is novel in that it extends previous work by examining the effect of repeated bouts of exercise and gender on ultrastructural damage, inflammatory cell infiltration, stress protein and proteolytic enzyme content. Therein lies the strength and weakness of this thesis. Various aspects relating to muscle damage were examined, leading to the conclusion that adaptation to eccentric exercise is a dynamic process encompassing numerous and diverse physiological pathways. Since biopsies were collected at only one time point (24 h post exercise), this thesis raises more questions about the specifics adaptation to contraction induced injury than it answers.

A time course study would need to be undertaken to investigate the hypothesis that neutrophil and macrophage accumulation into skeletal muscle is delayed and possibly attenuated following the second exercise bout as compared to the first. Quantifying cytokine synthesis and adhesion molecule expression post execise would help elucidate a mechanism for a delay in inflammatory cell infiltration. A time course study would also be required to investigate the hypothesis that a more "directed" form of proteolysis occurs following the second exercise about. Enzyme assays as opposed to Western

blotting would be required to determine the contribution of the lysosomal, calpain and ATP-dependent ubiquitin proteosome pathways to post-exercise protein turnover following exercise bout 1 and 2. Other questions that this thesis leaves unresolved concern potential changes in HSP27 phosphorylation state in response to repeated eccentric exercise and the source of the increased bcl-2 protein content detected in females following the second exercise bout. Again the use of more specific biochemical techniques would help to resolve these questions.

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APPENDIX I

Methods: Western Blotting & Immunohistochemistry

WESTERN BLOTTING PROTOCOL

HOMOGENIZATION

Phosphate Buffered Saline (PBS):

16g NaCl (152mM)

0.4g KCl (2.99mM0

2.88g Na_2HPO_4 (85mM)

0.98g KH_2PO_4 (4.00mM)

- Dissolve in 1.8L of ddH_2O.
- Adjust pH to 7.2 with concentrated HCl.
- Make up to 2.0L with ddH_2O.

Homogenization Buffer:

10ml PBS

0.5ml 10% SDS

0.5ml ddH_2O

1. Weigh biopsy. (Record weight.)
2. Add 600µl of homogenization buffer to muscle and homogenize using a glass homogenizer (~100 passes).
3. Decant homogenate into a plastic Eppendorf tube and sonicate sample for 15sec.
4. Heat homogenate at 70°C for 10min.
5. Save 250µl of crude homogenate for ubiquitin and calpain analysis.
6. Centrifuge homogenate at 14,000RPM for 10min.
7. Decant supernatant into a new Eppendorf tube.
8. Run protein assay.
9. Prepare samples for Western blotting. (See below.)
10. Store samples at -70°C.

PROTEIN CONTENT DETERMINATION

The Bio-Rad protein assay (#500-0006, Bio-Rad, Hercules, CA) (aka Bradford Protein Assay) was used for protein content determination.

1. A standard curve using bovine serum albumin (BSA) *(#735-078, Boehringer-Mannheim)* was run with each new microtitre plate. Each point on the calibration curve was run in triplicate.
   a. Make up a 10mg/ml BSA protein standard (100mg BSA in 10 ml ddH_2O). Dilute further to yield desired protein concentrations.
   b. Calibration curve protein concentrations:

Desired Protein Concentration (mg/ml)	Volume of 10mg/ml BSA Standard (ml)	Volume of ddH_20 (ml)
10.0	1.00	0
7.5	0.75	0.25
5.0	0.50	0.50
2.5	0.25	0.75
1.0	0.10	0.90

   c. Dilute each point on the calibration curve 10x; 90µl ddH_2O and 10µl BSA. Make 3 separate dilutions for each point!
2. Dilute muscle homogenates (either supernatant or crude) 10x; 90µl ddH_2O and 10µl homogenate. Make 3 separate dilutions for each sample!
3. Dilute 25ml Bio-Rad stock reagent in 75ml ddH_20 (1:4) and filter. Store unused reagent at 4°C.
4. Pipette into the wells of the microtitre plates 10µl of diluted homogenate or standards. Leave 6 wells empty to determine blank values.
5. Using a multi-channel pipette add 200µl of diluted reagent into each well.
6. A microplate reader (Benchmark, Bio-Rad, Hercules, CA) was used for protein content analysis.
   a. Shake plate for 30 sec.
   b. Wait 5.0 min.
   c. Read absorbance at 595nm. (The computer will automatically calculate the linear regression of the standard curve and respective protein concentrations.)

SAMPLE PREPARATION FOR WESTERN BLOTTING

To ensure consistency between western blots, it is essential to minimize the number of freeze-thaw cycles. Repeated freeze-thaw cycles will affect the quality of the homogenate, making it very difficult to keep amount of protein loaded consistent. Therefore, the homogenate was prepared for Western blotting the same day that the Bio-Rad protein assay was run.

Sample Buffer (SDS Reducing Buffer):

3.55ml ddH_2O

1.25ml 0.5M Tris-HCl, pH 6.8 (see below)

2.5ml glycerol

2.0ml SDS (w/v) 10% (see below)

0.5ml Mercaptoethanol

- Mix well; store at 4°C.

Dilution Buffer:

2.796g (150mM) KCl

0.6055g (20mM) Tris Base

- Dilute in 250ml of ddH_2O; adjust pH to 7.0.

1. Want: 40µg of muscle protein/lane.
2. Volume of homogenate (µl) = 40µg / homogenate concentration (µg/µl)
3. Maximum volume per lane on a 10 lane, 1mm thick gel = 45µl.
4. Ratio of *homogenate + dilution buffer : SDS buffer* is *2:1* or *30µl : 15µl.*
5. Volume of dilution buffer = 30 – volume of muscle homogenate.
6. If volume of homogenate required is greater than 30µl then sample has to be evaporated in a rotor-evaporator, reconstituted with ~200µl (or less depending on original protein concentration) of homogenization buffer, sonicated for 15sec, and the new protein concentration has to be determined.

7. Gels are always run in duplicate, therefore the required volumes of homogenate, SDS buffer, and dilution buffer are multiplied by a factor of 2.3. Combine all three in a single Eppendorf tube and store at -70°C.
8. Always prepare a few extra samples.

SDS-POLYACRYLAMIDE GEL ELECTOPHERISIS

7.5 % separating gel and 4.5% stacking gel were used for: HSP72, HSP27 and bcl-2
10.0% separating gels and 4.5% stacking gel were used for: calpain (30kD regulatory subunit), and ubiquitin conjugated proteins.

To make up four 1mm thick mini-gels:

	7.5% Separating Gel	10% Separating Gel	4% Stacking Gel
ddH_2O	9.7ml	8.02ml	6.1ml
Separating buffer	5.0ml	5.0ml	2.5ml
10% SDS	200µl	200µl	100µl
30% Acrylamide/ 0.8%Bis	5.0µl	6.66ml	1.33 ml
10% AP Solution	100µl	100µl	50µl
Temed	10µl	10µl	10µl

30% Acrylamide/8% Bis:

87.6g Acrylamide

2.4g N'N'bis-methylene acrylamide

up to 300ml with ddH_2O

- Store in dark at 4°C.

Separating Gel Buffer:

27.33g Tris-base

80ml ddH_2O

- Adjust pH to 8.8 with concentrated HCl and make up to 150ml with ddH_2O.
- Store at 4°C.

Stacking Gel Buffer:

6g Tris-base

60ml ddH_2O

- Adjust pH to 6.8 with concentrated HCl and make up to 100ml with ddH_2O.
- Store at 4°C.

10% SDS:

10g SDS

- Dissolve in 90ml ddH_2O, then bring up to 100ml with ddH_2O.
- Store at room temperature.

10% APS (Ammonium Persulfate) Solution:

100mg APS

- Dissolve in 1.0ml ddH_2O.

5x Tris-Glycine Running Buffer:

45g Tris-base

216g Glycine

15g SDS

- Dissolve in 2500ml ddH_2O, then bring up to 3000ml with ddH_2O.
- Store at 4°C.

WORKING SOLUTION: Need approximately 1000ml per buffer chamber. Dilute 200ml 5x Tris-Glycine Running Buffer with 800ml of ddH_2O.

All reagents except AP and TEMED are available from hospital stores. Order Temed *(#T-9281)* and APS *(#A-3678)* from Sigma.

1. Assemble glass cassettes and casting stand. Use glass plates with a 1mm spacer.

2. Pour your separating gel, immediately overlay with water. Allow the gel to polymerize for 45min.
3. Dry separating gel with Kim wipe. Pour stacking gel and immediately insert a 1mm, 10 well comb. Allow gel to polymerize for 30 min.
4. Slide the gel cassettes into electrode assembly and buffer chamber, then add running buffer (working solution).
5. Heat your samples and broad range molecular weight standard *(#161-0319, Bio-Rad)* at 90°C for 7min.
6. Vortex samples well prior to loading into wells.
   a. Loading protocol:

Lane	Sample	Volume (µl)
1		
2	Female rest A	45
3	Female ex A	45
4	Female ex B	45
5		
6	Male rest A	45
7	Male ex A	45
8	Male ex B	45
9		
10	Molecular weight standard	15

7. Top up middle well with buffer. Connect assembly to power supply and run gels at 200V for ~40min.

WESTERN BLOTTING

Western Buffer A:

30.2g Tris

144g Glycine

- Adjust volume to 1000ml with ddH_2O.

Transfer Buffer (1000ml/buffer chamber):

100ml Western Buffer A

10ml 10% SDS

240ml Methanol

- Bring up to 1000ml with ddH_2O and chill.

TTBS:

Make fresh every time you use!

2.42g Tris

29.22g NaCl

- Dissolve in 900ml ddH_2O, adjust pH to 7.5 with concentrated HCl, then bring up to 1000ml with ddH_2O.
- Add 1.0ml TWEEN *(#170-6531, Bio-Rad)*.

9% Blocking Solution (5ml per blot):

- Dissolve 1ml Gelatin *(#G-7765, Sigma)* in 9ml TTBS.

1° and 2° Antibody Solution (5ml per blot):

- Dilute antibody in TTBS just prior to use. See below for appropriate dilutions.

1. Prior to use, the PVFD membrane *(#162-0184, Bio-Rad)* has to be immersed in methanol until it becomes translucent and then allowed to equilibrate in transfer buffer for ~5min.
2. Set up gel holders as follows: anode (black) – sponge – filter paper – gel – PVFD membrane – filter- sponge – cathode (white). Keep everything submerged in transfer buffer.
3. Assemble transfer apparatus.

a. *For HSP72, HSP27, Bcl-2, and ubiquitin conjugated proteins:* Place transfer apparatus in an ice bath. Transfer gels overnight at 25V. Transfer quality may be improved if the buffer is stirred continuously.

b. *For the 30kD regulatory subunit of calpain:* Keep transfer apparatus at room temperature and transfer at 100V for 1hr.

4. Block blot with 9.0% gelatin for 1.5 hr with agitation. (Use square petri dishes.)
5. Rinse 3x for 5-10min with (fresh) TTBS with agitation.
6. Apply diluted 1° antibody and incubate overnight with agitation.
7. Rinse 3x for 5-10min with (fresh) TTBS with agitation.
8. 2° antibody application and conjugate binding for molecular weight standard detection:
   a. Make a 1:3000 avidin-phosphatase dilution and a 1:3000 2° antibody dilution in the SAME volume of TTBS. The avidin with bind to the biotin conjugated to the molecular weigh standards. The 2° antibody amplifies the 1° Antibody signal.
   b. Incubate for 1.5 hr with agiation.
9. Rinse 3x for 5-10min with (fresh) TTBS with agitation.
10. Colour development using AP conjugate substrate kit *(#170-6432, Bio-Rad).* 5ml of colour development solution is required per blot.
    a. Dilute 0.40 ml of colour development buffer in 9.60ml of ddH_2O.
    b. Just prior to use add 0.10ml of colour reagent A and 0.10ml of colour reagent B.
    c. Carefully monitor colour development. Time varies depending on 1° antibody used.
    d. Stop colour development by rinsing blot in ddH_2O for 5 min.
    e. Allow blot to dry overnight.

BAND DENSITY DETERMINATION

Blots were digitized using a scanner and a computerized image analysis system *(LabWorks, UVP Laboratory products, Upland, CA)* was used to determine band density and molecular weight.

Because of the high variability between blots, only within blot comparisons can be made. One cannot compare between blots.

1. To determine relative changes in protein expression within a subject following exercise, the value of the rest band was set to one, and the values of bout 1 and bout 2 bands were expressed as bout 1/rest and bout2/rest.
2. Gender differences in protein expression were determined by dividing the density of the male band for a given time point by the density of the female band for a given time point. All female rest and exercise band densities were set to 1.
3. Computerized statistical software was used for all analysis *(Statistica v5.0, Statsoft).* A Newman-Keuls test was used to post hoc any significant results.

SUMMARY TABLE

Primary Antibody	Dilution	Secondary Antibody	Dilution	Homogenate Fraction	Sep. Gel	Transfer	Mol. Weight
Calpain reg (C-20) (Santa Cruz, #sc-7530)	1:500	Donkey anti-goat IgG (Santa Cruz, #sc-2020)	1:3000	Crude	10%	1hr @ 100V	30 kD
Ubiquitin (Novacastra, #NCL-UBIQm)	1:50	Donkey anti-mouse IgG (Santa Cruz, #sc-2316)	1:1000	Crude	10%	Overnight @ 25V	Variable
HSP 72 (Santa Cruz, #sc-1060)	1:1000	Donkey anti-goat IgG (Santa Cruz, #sc-2020	1:3000	Supernatant	7.5%	Overnight @ 25V	72kD
HSP 27 (Santa Cruz, #sc-1048)	1:1000	Donkey anti-goat IgG (Santa Cruz, #sc-2020	1:3000	Supernatant	7.5%	Overnight @ 25V	27kD
Bcl-2 (gift, Dr. B. Leber)	1:500	Donkey anti-rabbit IgG (Santa Cruz, #sc-2315)	1:3000	Supernatant	7.5%	Overnight @ 25V	26kD

IMMUNOHISTOCHEMISTRY PROTOCOL

CUTTING MUSCLE

1. Store OCT mounted muscle at -70°C.
2. Prior to cutting, place muscle in cryostat for at least 15 min and let it to warm up to ~20°C. Set knife temperature to -20°C. Cut sections to 5μm thick.
3. H+E stain the first few sections cut, to check for correct fiber orientation and for presence of freezing artifact. If freezing artifact is present, cut deeper into the muscle and redo the H+E stain. If less than 50% of the fibers are affected by freezing artifact after the second H+E, the muscle is suitable for further cutting.
4. Cut two sections per slide.
5. Allow slides to dry overnight. Wrap slides in aluminum foil and slides at -70°C.

HEMATOXYLIN AND EOSIN (H+E) STAINING

1. Fix freshly cut sections in absolute ethanol for at least 3 min.
2. Wash slides in running tap water (RTW) for 30 sec.
3. Rinse in ddH_2O.
4. Stain with Mayer's Hematoxylin solution *(#MHS-16, Sigma)* for 1 min.
5. Wash in RTW for 30 sec.
6. Place in 0.05M TBS solution (pH 7.6) for 1 min.
7. Wash in RTW for 30 sec.
8. Rinse in ddH2O.
9. Stain with Eosin 20 sec to 1 min. Eosin *(#HT110-2-16, Sigma)* needs to be acidified with 0.5ml of glacial acetic acid per 100ml.
10. Wash in RTW for 30 sec.
11. Rinse slides in 70% ethanol; 10 dips.
12. Rinse slides in 95% ethanol; 10 dips. (Repeat with fresh 95% ethanol.)
13. Rinse slides in 100% ethanol; 10 dips. (Repeat with fresh 100% ethanol.)
14. Rinse slides in xylene; 10 dips. (Repeat 2 more times with fresh xylene.)
15. Cover slip slides.
16. Evaluate slides under a microscope.

Note: Eosin should stain collagen pale pink, muscle pink, and red blood cells red. If samples are too pink, take them through the alcohol solutions again. If they are too pale, re-stain with Eosin and spend less time in the alcohol solutions. Hematoxylin stains nuclei blue, the intensity of the stain should be such that nucleous should still be visible.

ANTIBODY STAINING for Neutrophil Elastase, Myeloperoxidase, CD68, and LCA

1. Take slides out of the freezer and allow them to equilibrate to room temperature for ~15 min. Unwrap slides and let them rest for ~5 min.
2. Place slides in cold acetone for 15 min. Keep slides in cryostat. When acetone not in use, store at 4°C.
3. Wash in 0.05M TBS solution for 5 min.
4. Block endogenous peroxidase activity using liquid DAB substrate kit (Zymed; 00-2014):
    a. Add 1 drop of 20x buffer concentrate (Reagent A) to 1ml of distilled water and mix well.
    b. To this mixture add 1 drop of 20x DAB solution (Reagent B) and 1 drop of 20x hydrogen (reagent C) peroxide and mix well.
    c. Filter solution if necessary. The solution is light sensitive and should be used within 30 min of being made up.
    d. Arrange slides on a staining tray. Shake slide to remove 0.05M TBS solution. Use a Kim wipe to wipe around the slide, taking care not to remove the sample. Work quickly, do not allow the sample to dry out.
    e. Place 2 or 3 drops of the DAB solution (enough to cover the specimen) onto each slide. Colour should be adequately developed within 5 min.
5. Place slides in slide holder. Rinse 3 times with 0.05M TBS solution for 5 min at a time. Use fresh 0.05M TBS solution each time.
6. Blocking with goat serum; (the goat serum helps prevent non-specific binding):

a. Place slides on staining tray. Use separate trays for the positive and negative slides to prevent cross-contamination.
b. Shake slide to remove excess 0.05M TBS solution and use a Kim wipe to wipe around the slide, taking care not to remove the sample. Work quickly, do not allow the sample to dry out.
c. Place 2 or 3 drops of 1% goat serum on each slide and incubate for 15 min. Cover staining tray.
d. To make up 1% goat serum, mix 49.5ml of 0.05M tris buffer with 0.5ml of normal goat serum *(#D3002S, Dako)* in a 60ml dropper bottle. Keep refrigerated.

7. Primary antibody application:
   a. Shake positive slide to remove excess 1% goat serum. Use a Kim wipe to wipe around the slide, taking care not to remove the sample.
   b. Make up primary antibody dilutions using 1% goat serum as your diluting media.
   c. Place 2-3 drops of your diluted primary antibody onto the positive-labeled slides.
   d. The negative controls remain in the 1% goat serum protein block.
   e. Incubate.
8. Place slides in slide holder. Rinse 3 times with 0.05M TBS solution for 5 min at a time. Use fresh 0.05M TBS solution each time. Perform a pre-rinse using a squeeze bottle to prevent cross contamination. Take care not spray directly on the sample.
9. Pour the secondary antibody in a clean container. Incubate slides for 15 min. Secondary antibody is from the Histostain-streptavidin peroxidase bulk for mouse primary antibody kit *(#95-6543-B, Zymed)*. The secondary antibody solution can be re-used many times. As it ages, the incubation time needs to be increased.
10. Rinse 3 times in 0.05M TBS solution as before.
11. Pour the streptavidin peroxidase solution in a clean container. Incubate slides for 15 min. Streptavidin is from the Histostain-streptavidin peroxidase bulk for mouse primary antibody kit *(#95-6543-B, Zymed)*. The streptavidin-peroxidase

solution can be re-used many times. As it ages, the incubation time needs to be increased.

12. Rinse three times in 0.05M TBS solution as before.
13. AEC substrate solution *(#00-1111, Zymed)* is used for color development
    a. Aliquot AEC solution into a 60ml dropper bottle. The AEC solution is light sensitive; the dropper bottle needs to be covered in aluminum foil.
    b. Again arrange slides on staining tray. Shake slide to remove excess 0.05M TBS solution. Use a Kim wipe to wipe around the slide, taking care not to remove the sample. Work quickly, do not allow the sample to dry out.
    c. Place 2-3 drops of the AEC solution on each slide.
    d. Incubate for 5-10min.
14. Return slides to holder and rinse in running tap water for 5 min.
15. Rinse in distilled water, 10 dips.
16. Place in 50% Mayers Hemotoxylin for 3 min. Dilute Hemotoxylin *(#MHS-16, Sigma)* with equal parts of distilled water.
17. Rinse in running tap water.
18. Rinse in 0.05M tris buffer for at least 1min.
19. Rinse in distilled water.
20. Mount slides using Clearmount mounting solution *(#00-8010, Zymed)*.
    a. Blot-off excess water around tissue sections.
    b. Place slides in a horizontal position and apply Clearmount evenly over the tissue.
    c. Place slides in a drying oven set at 60°C for 30 min or until slides are thoroughly dried.

Inflammatory Cells:

Check your tonsil both positive and negative control. The negative control should have no red staining. The positive control should have many LCA positive inflammatory cells. Inflammatory cells will stain red. If the tonsil control does not look right, muscle samples cannot be evaluated. The muscle sections labeled positive will be quantified for

positive cells. Negative controls should be evaluated for the absence of red staining. Express results as *number of positive inflammatory cells/mm^2*.

ANTIBODY STAINING for Desmin and Dystrophin

Follow above procedure but omit steps 2 through 4 inclusive. Do not fix slides in acetone and it is not necessary to block endogenous peroxidase activity.

Dystrophin:

It is critical to incubate the dystrophin primary antibody for a full 24hr, else the staining will not be intense enough. To prevent the slides from drying out, tape down the lid of the staining tray. Dystrophin stained cells will have a continuous red rim around them. Discontinuous dystrophin staining (i.e. less than 50% of cell rim stained) is indicative of sarcolemmal damage. Do not evaluate cells in the edge of the section for discontinuous dystrophin staining. There should be an absence of red staining in the negative controls. Express results as *number of discontinuously stained cells/mm^2*.

Desmin:

Desmin will stain the entire muscle fiber red. Desmin ghost fibers (i.e. absence of red staining) are indicative of ultrastructural damage. There should be an absence of red staining in the negative controls. Express results as *number of ghost fibers/mm^2*.

0.05M TRIS BUFFER SALINE pH 7.6

Stock Solutions:

1. **0.5M Tris Base STOCK**
    a. Dissolve 121.1g of Tris Base in distilled water, make up to 2L
    b. Adjust pH to 7.6 with approximately 60-80ml concentrated HCl.

2. **8.5% NaCl**
    b. Dissolve 170g of NaCl in distilled water, make up to 2L.

Working Solution:

3. **0.05M Tris Buffered Saline pH 7.6**
    a. Take 250ml of 0.5M Tris stock
    b. Add 250 ml of 8.5% NaCl stock
    c. Add 2L of distilled water
    d. Check pH and adjust if required.

STAINING DILUTIONS:

1° Antibody	Dilution	Incubation Time	Control
Leukocyte Common Antigen (#M0701, Dako)	1:500	1 hr	Tonsil
Macrophage CD68 (#M0814, Dako)	1:100	30min	Tonsil
Neutrophil Elastase (#M0752, Dako)	1:200	30min	Tonsil
Neutrohil Myeloperoxidase (#M0784, Dako)	1:300	30min	Tonsil
Dystrophin (#NCLDys1, Novacastra Laboratories)	1:20	24hr	Skeletal muscle
Desmin (#NCL-DES-DERII, Novacastra Laboratories)	1:75	1hr	Skeletal muscle

COMPUTER ANALYSIS:

Section Area:

1. Focus image at 4x magnification.
2. Capture image with Spot camera *(Spot v2.2, Diagnostic Instruments Incorporated, Sterling Heights, MI)* if section is particularly large two pictures maybe required.
3. Use ImagePro Plus *(v4.0, Media Cybernetics, Silver Spring, MD)* to determine section area in μm^2.

APPENDIX II

Raw Data & ANOVA Tables

Subject Characteristics

Males

Subject	*Age*	*Ht (cm)*	*Wt (kg)*	*BMI (kg/m^2)*	*Leg Press 1RM (kg)*	*1RM/kg BW*	*Knee Ex 1RM (kg)*	*1RM/kg BW*
s1	23	170	85	29.41	130	1.53	42.5	0.50
s2	22	175	88.5	28.90	110	1.24	35	0.40
s3	23	182	98.3	29.68	125	1.27	35	0.36
s4	22	180	77.6	23.95	90	1.16	30	0.39
s5	21	188	85.2	24.11	80	0.94	37.5	0.44
s6	27	190	87.6	24.27	127.5	1.46	45	0.51
s13	21	173	72.4	24.19	117.5	1.62	37.5	0.52
s16	22	168	65.6	23.24	120	1.83	30	0.46
MEAN	**23**	**178.3**	**82.5**	**26.0**	**112.5**	**1.38**	**36.6**	**0.45**
SD	**2**	**8.1**	**10.3**	**2.8**	**18.3**	**0.28**	**5.3**	**0.06**

Females

Subject	*Age*	*Ht (cm)*	*Wt (kg)*	*BMI (kg/m^2)*	*Leg Press 1RM (kg)*	*1RM/kg BW*	*Knee Ex 1RM (kg)*	*1RM/kg BW*	*Oral Contraceptive*
s7	22	175	66.7	21.78	90	1.35	30	0.45	N
s8	19	167	59.5	21.33	80	1.34	20	0.34	Y
s9	22	165	63.4	23.29	55	0.87	17.5	0.28	Y
s10	21	172	56.2	19.00	60	1.07	20	0.36	Y
s11	23	164	60.9	22.64	55	0.90	20	0.33	N
s12	24	170	61.5	21.28	62.5	1.02	17.5	0.28	Y
s14	24	161	81	31.25	65	0.80	22.5	0.28	N
s15	27	173	71.4	23.86	90	1.26	27.5	0.39	Y
Mean	**23**	**168.4**	**65.1**	**23.1**	**69.7**	**1.08**	**21.9**	**0.34**	
SD	**2**	**4.9**	**7.9**	**3.6**	**14.8**	**0.22**	**4.6**	**0.06**	

Subject Characteristics T-tests

Height

	Variable 1	*Variable 2*
Mean	178.25	168.375
Variance	65.92857	23.98214
Observations	8	8
Pooled Variance	44.95536	
Hypothesized Mean Difference	0	
df	14	
t Stat	2.945618	
P(T<=t) one-tail	0.005319	
t Critical one-tail	1.761309	
P(T<=t) two-tail	0.010638	
t Critical two-tail	2.144789	

BMI

	Variable 1	*Variable 2*
Mean	25.96772	23.05333
Variance	7.888171	13.17286
Observations	8	8
Pooled Variance	10.53052	
Hypothesized Mean Difference	0	
df	14	
t Stat	1.796193	
P(T<=t) one-tail	0.047034	
t Critical one-tail	1.761309	
P(T<=t) two-tail	0.094068	
t Critical two-tail	2.144789	

Weight

	Variable 1	*Variable 2*
Mean	82.525	65.075
Variance	105.2593	62.73071
Observations	8	8
Pooled Variance	83.995	
Hypothesized Mean Difference	0	
df	14	
t Stat	3.808016	
P(T<=t) one-tail	0.00096	
t Critical one-tail	1.761309	
P(T<=t) two-tail	0.00192	
t Critical two-tail	2.144789	

Leg Press 1RM

	Variable 1	*Variable 2*
Mean	112.5	69.6875
Variance	333.9286	218.6384
Observations	8	8
Pooled Variance	276.2835	
Hypothesized Mean Difference	0	
df	14	
t Stat	5.151375	
P(T<=t) one-tail	7.36E-05	
t Critical one-tail	1.761309	
P(T<=t) two-tail	0.000147	
t Critical two-tail	2.144789	

Leg Press 1RM/kg

	Variable 1	*Variable 2*
Mean	1.3813	1.076418
Variance	0.080342	0.047525
Observations	8	8
Pooled Variance	0.063934	
Hypothesized Mean Difference	0	
df	14	
t Stat	2.411561	
P(T<=t) one-tail	0.015096	
t Critical one-tail	1.761309	
P(T<=t) two-tail	0.030193	
t Critical two-tail	2.144789	

Knee Extensor 1RM

	Variable 1	*Variable 2*
Mean	36.5625	21.875
Variance	28.45982	20.98214
Observations	8	8
Pooled Variance	24.72098	
Hypothesized Mean Difference	0	
df	14	
t Stat	5.908062	
P(T<=t) one-tail	1.91E-05	
t Critical one-tail	1.761309	
P(T<=t) two-tail	3.81E-05	
t Critical two-tail	2.144789	

Force Absolute Data ($30°\cdot sec^{-1}$)

Males

	Subject	Bout 1 Rest	30 min	24 h	48 h	96 h	7 d	Bout 2 Rest	30 min	24 h	48 h	96 h	7 d
Conc.	s1	249.2	202.7	191.7	214.1	201.2	233.2	256.7	213.4	222.2	238.4	242.1	255.1
	s2	218.6	153.1	206.8	150.2	193.4	183.6	222.7	156	186.8	201.7	213.4	210.9
	s3	298.4	209.9	301.7	311.7	298.2	258.2	300.9	206.8	306.8	313.7	314.2	299.7
	s4	240.6	105.8	75.9	57.6	146	176.7	246.8	128.6	119.7	143.2	154.9	189.4
	s5	215.2	112.4	107.3	195.4	198.3	205.2	228.4	119.6	127.3	207.6	223.7	233.7
	s6	268.4	204.1	241.2	214.5	219.7	253.7	288.7	200.1	239	236.7	243.7	267.9
	s13	197.4	173.6	181.8	188.5	197.2	204.5	193.7	162.4	178.4	191.4	199.2	196.4
	s16	197.8	141.3	173.6	200.3	195.8	203.9	203.7	131.7	183.6	199.1	208.8	206.8
Ecc.	s1	267.5	217.6	248.1	260.6	212.8	226.3	277.2	196.4	250.8	273.4	264.3	273.3
	s2	262.3	183.7	248.2	180.2	232.1	220.3	264.8	209.1	251.8	233.7	244.0	251.6
	s3	401.8	282.6	406.3	419.7	401.5	347.7	419.5	270.2	368.7	401.2	413.5	423.5
	s4	283.9	116.9	89.6	68.0	172.3	208.5	289.7	122.3	106.8	124.7	155.4	168.7
	s5	233.9	122.2	148.0	212.4	215.6	223.1	229.3	130.1	159.6	198.7	225.7	236.8
	s6	330.1	251.0	296.7	224.5	270.2	312.1	360.4	228.3	307.2	303.7	334.6	359.7
	s13	236.9	208.3	218.2	226.2	236.6	245.4	258.4	190.4	233.4	248.5	267.4	266.1
	s16	260.1	163.8	205.8	245	240.6	266.2	251.3	143.7	219.4	186.7	222	264.9

(Conc. = concentric strength; Ecc. = eccentric strength)

Females

	Subject	Bout 1 Rest	30 min	24 h	48 h	96 h	7 d	Bout 2 Rest	30 min	24 h	48 h	96 h	7 d
Conc.	s7	174.5	102.4	133.3	117.6	138.4	143.6	234.1	129.4	199.9	213.4	245.4	241
	s8	177.1	160.3	144.7	140.2	139.8	138	205.4	165.9	187.3	162.4	173.6	170.4
	s9	152.2	106.2	134	126.2	151.3	144	168.9	109.9	136.2	130.2	168	159.8
	s10	142.2	77	85.7	58.6	118	117.3	164.9	88.2	95.2	72.8	133.7	144.6
	s11	150.7	108.2	130.2	118.4	134.7	144.4	177.3	137.4	153.2	139.3	158.6	169.9
	s12	172.5	93	108.9	106.9	113.8	130.2	191.2	103.4	128.6	118.4	136.4	144.2
	s14	112.4	95.1	101.3	102.6	110.6	115.9	172.9	133.1	148.9	150	159.6	178.3
	s15	174.5	139.1	162.4	169.6	175.2	179.8	198.9	132.4	145.2	126.7	199.4	206.4
Ecc.	s7	170.2	112.4	142	156	167.9	162.7	254	136.7	206.7	223.4	254.1	243.7
	s8	187.2	154.2	164.7	178.2	188.6	191	213.5	183.4	190	182.4	174.3	206.7
	s9	154.8	92.1	146.2	158.6	159.4	163.2	163.4	125.7	148.7	152.4	158.9	167.4
	s10	150.8	91.4	113.5	129.7	131.7	146.5	176.4	111.4	105.4	139.5	167.4	185.7
	s11	163.4	119.3	139.4	147.9	130.7	164	181.2	139.4	153.2	124.5	167.4	179
	s12	177.2	125.1	130.8	141	168.9	152.4	188.2	111.9	133.6	153.7	168.7	193.4
	s14	123.4	88.6	106.8	99.6	128.5	124.6	191.4	142.1	151.4	163.4	185.4	167
	s15	184.5	122.4	149.8	173	186.7	188.2	208.2	124.2	159.6	168.8	204.4	213.4

Absolute Force Data ANOVA Table: Gender x Bout x Contraction Type x Time

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	528092.8	14	40572.78	13.01594	0.002854
2 – Bout	1	17418.44	14	516.3132	33.73619	4.54E-05
3 – Contraction	1	105992.8	14	3931.378	26.96072	0.000136
4 – Time	5	41481.58	70	1521.381	27.26574	3.34E-15
12	1	143.7559	14	516.3132	0.278428	0.605996
13	1	4497.381	14	3931.378	1.143971	0.30291
23	1	173.8166	14	279.2396	0.622464	0.44329
14	5	1756.116	70	1521.381	1.15429	0.34041
24	5	1214.479	70	248.5411	4.88643	0.000683
34	5	953.6075	70	158.435	6.018921	0.000109
123	1	1.660106	14	279.2396	0.005945	0.939632
124	5	70.03804	70	248.5411	0.281797	0.921605
134	5	136.7174	70	158.435	0.862924	0.510482
234	5	53.0598	70	92.11684	0.576005	0.71815
1234	5	89.36929	70	92.11684	0.970173	0.442133

Newman-Keuls Post Hoc: Interaction - 24

		Bout 1						*Bout 2*					
		pre	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*	*pre*	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*
Bout 1	*pre*		0.0002	0.0001	0.0001	0.0001	0.0001	0.1280	0.0001	0.0001	0.0002	0.0415	0.8651
	30 min	0.0002		0.0002	0.0001	0.0001	0.0001	0.0001	0.6397	0.0001	0.0001	0.0001	0.0002
	24 h	0.0001	0.0002		0.3714	0.0002	0.0001	0.0001	0.0001	0.0375	0.0002	0.0001	0.0001
	48 h	0.0001	0.0001	0.3714		0.0002	0.0001	0.0002	0.0001	0.0098	0.0001	0.0001	0.0001
	96 h	0.0001	0.0001	0.0002	0.0002		0.2313	0.0001	0.0001	0.0272	0.4016	0.0002	0.0001
	7 d	0.0001	0.0001	0.0001	0.0001	0.2313		0.0001	0.0001	0.0013	0.4224	0.0043	0.0002
Bout 2	*pre*	**0.1280**	0.0001	0.0001	0.0002	0.0001	0.0001		0.0002	0.0001	0.0001	0.0009	0.0768
	30 min	0.0001	0.6397	0.0001	0.0001	0.0001	0.0001	0.0002		0.0002	0.0001	0.0001	0.0002
	24 h	0.0001	0.0001	0.0375	0.0098	0.0272	0.0013	0.0001	0.0002		0.0078	0.0001	0.0001
	48 h	0.0002	0.0001	0.0002	0.0001	0.4016	0.4224	0.0001	0.0001	0.0078		0.0011	0.0001
	96 h	0.0415	0.0001	0.0001	0.0001	0.0002	0.0043	0.0009	0.0001	0.0001	0.0011		0.0703
	7 d	0.8651	0.0002	0.0001	0.0001	0.0001	0.0002	0.0768	0.0002	0.0001	0.0001	0.0703	

Relative Force Deficit Data ($30°\cdot sec^{-1}$)

Males

	Subject	Bout 1 Rest	30 min	24 h	48 h	96 h	7 d	Bout 2 Rest	30 min	24 h	48 h	96 h	7 d
Conc.	s1	100	81.34	76.92	85.91	80.73	93.57	100	83.13	86.56	92.87	94.31	99.37
	s2	100	70.03	94.60	68.70	88.47	83.98	100	70.04	83.87	90.57	95.82	94.70
	s3	100	70.34	101.1	104.4	99.93	86.52	100	68.72	101.9	104.2	104.4	99.60
	s4	100	43.97	31.54	23.94	60.68	73.44	100	52.10	48.50	58.02	62.76	76.74
	s5	100	52.23	49.86	90.79	92.14	95.35	100	52.36	55.73	90.89	97.94	102.3
	s6	100	76.04	89.86	79.91	81.85	94.52	100	69.31	82.78	81.98	84.41	92.79
	s13	100	87.94	92.09	95.49	99.89	103.5	100	83.84	92.10	98.81	102.8	101.3
	s16	100	71.43	87.76	101.2	98.98	103.0	100	64.65	90.13	97.74	102.5	101.5
Ecc.	s1	100	81.34	92.74	97.42	79.55	84.59	100	70.85	90.47	98.62	95.34	98.59
	s2	100	70.03	94.60	68.70	88.47	83.98	100	78.96	95.09	88.25	92.14	95.01
	s3	100	70.34	101.1	104.4	99.93	86.52	100	64.41	87.89	95.63	98.56	100.9
	s4	100	41.16	31.54	23.94	60.68	73.44	100	42.21	36.86	43.04	53.64	58.23
	s5	100	52.23	63.26	90.79	92.14	95.35	100	56.73	69.60	86.65	98.43	103.2
	s6	100	76.04	89.86	68.00	81.85	94.52	100	63.34	85.23	84.26	92.84	99.80
	s13	100	87.94	92.09	95.49	99.89	103.5	100	73.68	90.32	96.16	103.4	102.9
	s16	100	62.97	79.12	94.19	92.50	102.3	100	57.18	87.30	74.29	88.34	105.4

(Conc. = concentric strength; Ecc. = eccentric strength)

Females

	Subject	Bout 1 Rest	30 min	24 h	48 h	96 h	7 d	Bout 2 Rest	30 min	24 h	48 h	96 h	7 d
Conc.	s7	100	58.68	76.38	67.39	79.31	82.29	100	66.03	83.43	91.65	98.64	95.59
	s8	100	90.51	81.70	79.16	78.93	77.92	100	82.37	87.98	95.19	100.7	102.0
	s9	100	69.77	88.04	82.91	99.40	94.61	100	59.49	94.44	102.4	102.9	105.4
	s10	100	54.19	60.26	41.20	82.98	82.48	100	60.61	75.26	86.00	87.33	97.14
	s11	100	71.79	86.36	78.56	89.38	95.81	100	73.01	85.31	90.51	79.98	100.3
	s12	100	53.91	63.13	61.97	65.97	75.47	100	70.59	73.81	79.57	95.31	86.00
	s14	100	84.60	90.12	91.28	98.39	103.1	100	71.79	86.54	80.71	104.1	100.9
	s15	100	79.71	93.06	97.19	100.4	103.0	100	66.34	81.19	93.76	101.1	102.0
Ecc.	s7	100	55.27	85.39	91.15	104.8	102.9	100	53.81	81.37	87.95	100.0	95.94
	s8	100	80.76	91.18	79.06	84.51	82.96	100	85.90	88.99	85.43	81.63	96.81
	s9	100	65.06	80.63	77.08	99.46	94.61	100	76.92	91.00	93.26	97.24	102.4
	s10	100	53.48	57.73	44.14	81.07	87.68	100	63.15	59.75	79.08	94.89	105.2
	s11	100	77.49	86.40	78.56	89.45	95.82	100	76.93	84.54	68.70	92.38	98.78
	s12	100	54.07	67.25	61.92	71.33	75.41	100	59.45	70.98	81.66	89.63	102.7
	s14	100	76.98	86.11	86.75	92.30	103.1	100	74.24	79.10	85.37	96.86	87.25
	s15	100	66.56	73.03	63.70	100.2	103.7	100	59.65	76.65	81.07	98.17	102.4

Relative Force Deficit Data ANOVA Table: Gender x Bout x Contraction Type x Time

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.199701	14	2346.868	8.51E-05	**0.992770135**
2 – Bout	1	896.1064	14	132.1165	6.782698	0.020800004
3 – Contraction	1	269.5053	14	164.3382	1.639943	**0.221143141**
4 – Time	5	8709.774	70	248.7263	35.01749	9.02832E-18
12	1	80.53023	14	132.1165	0.609539	0.447961986
13	1	12.78794	14	164.3382	0.077815	0.784359157
23	1	213.6868	14	116.6037	1.83259	0.197274014
14	5	45.78889	70	248.7263	0.184093	0.967720389
24	5	273.4608	70	46.70077	5.855594	0.000141543
34	5	52.32654	70	28.67433	1.824857	0.119152315
123	1	24.51202	14	116.6037	0.210216	0.653634429
124	5	29.79781	70	46.70077	0.638058	0.671368062
134	5	12.86144	70	28.67433	0.448535	0.812956035
234	5	16.60914	70	22.60176	0.73486	0.599790633
1234	5	20.04778	70	22.60176	0.887001	0.494585186

Serum CK Activity

Males

Subject	*Bout 1*						*Bout 2*					
	Rest	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*	*Rest*	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*
s1	189	148	120	596	302	213	109	287	213	635	137	85
s2	74	74	216	1968	2110	847	41	129	266	589	156	63
s3	107	381	197	2075	1937	909	115	290	266	320	246	234
s4	139	262	361	805	1087	159	45	145	303	243	474	33
s5	98	128	359	333	385	86	120	134	127	542	101	96
s6	150	49	66	585	401	41	134	90	322	564	276	77
s13	216	194	238	590	1456	243	104	159	246	303	1078	238
s16	109	118	331	381	612	66	133	57	115	301	426	83
MEAN	**135**	**169**	**236**	**917**	**1036**	**320**	**100**	**161**	**232**	**437**	**362**	**113**
SD	**48**	**109**	**110**	**697**	**726**	**352**	**37**	**85**	**76**	**159**	**319**	**78**

Females

Subject	*Bout 1*						*Bout 2*					
	Rest	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*	*Rest*	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*
s7	42	77	139	134	1367	768	36	49	169	178	545	25
s8	52	66	88	338	229	41	66	44	16	290	388	62
s9	164	82	123	566	623	74	44	66	49	139	202	14
s10	86	57	243	350	440	60	74	77	213	212	298	86
s11	52	45	123	260	344	41	41	25	44	128	172	119
s12	38	70	112	306	325	38	25	33	36	245	301	25
s15	52	41	193	338	503	27	57	22	98	214	367	30
MEAN	**100**	**161**	**232**	**437**	**362**	**113**	**49**	**45**	**89**	**201**	**325**	**51**
SD	**37**	**85**	**76**	**159**	**319**	**78**	**17**	**21**	**75**	**57**	**125**	**39**

Serum CK Activity ANOVA Table: Gender x Bout x Time

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 - Gender	1	1447704	13	166494.4	8.69521	0.011299
2 - Bout	1	1183321	13	147333.8	8.031565	0.014082
3 - Time	5	1244177	65	59128.76	21.04183	1.91E-12
12	1	232872.3	13	147333.8	1.580576	0.230796
13	5	132276.7	65	59128.76	2.237096	0.060979
23	5	238432	65	44193.5	5.395183	0.000328
123	5	81691.56	65	44193.5	1.848498	0.115738

Newman-Keuls Post Hoc: Interaction - 23

		Bout 1						*Bout 2*					
		pre	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*	*pre*	*30 min*	*24 h*	*48 h*	*96 h*	*7 d*
Bout 1	*pre*		0.9832	0.7775	0.0001	0.0002	0.5174	0.9297	0.9927	0.8717	0.0866	0.0492	0.7953
	30 min	0.9832		0.5935	0.0001	0.0001	0.4117	0.9829	0.8685	0.5605	0.0739	0.0468	0.9720
	24 h	0.7775	0.5935		0.0001	0.0001	0.5662	0.7335	0.6639	0.6961	0.2252	0.2043	0.7185
	48 h	0.0001	0.0001	0.0001		0.0306	0.0002	0.0002	0.0001	0.0001	0.0007	0.0007	0.0002
	96 h	0.0002	0.0001	0.0001	0.0306		0.0001	0.0001	0.0001	0.0001	0.0002	0.0001	0.0002
	7 d	0.5174	0.4117	0.5662	0.0002	0.0001		0.4296	0.4287	0.5989	0.2791	0.3427	0.4304
Bout 2	*pre*	0.9297	0.9829	0.7335	0.0002	0.0001	0.4296		0.9822	0.8693	0.0531	0.0269	0.9181
	30 min	0.9927	0.8685	0.6639	0.0001	0.0001	0.4287	0.9822		0.7338	0.0682	0.0399	0.9607
	24 h	0.8717	0.5605	0.6961	0.0001	0.0001	0.5989	0.8693	0.7338		0.1770	0.1347	0.8446
	48 h	0.0866	0.0739	0.2252	0.0007	0.0002	0.2791	0.0531	0.0682	0.1770		0.7526	0.0569
	96 h	0.0492	0.0468	0.2043	0.0007	0.0001	0.3427	0.0269	0.0399	0.1347	0.7526		0.0299
	7 d	0.7953	0.9720	0.7185	0.0002	0.0002	0.4304	0.9181	0.9607	0.8446	0.0569	0.0299	

Areas of Z-disk Streaming

Males

Subject	Area			No. areas of Z-disk streaming			No. areas of Z-disk streaming/mm^2		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s1	3.00	3.00		2.38	2.12	2.45	1.26	1.42	
s2	3.00	34.00	34.00	2.42	2.39	1.67	1.24	14.25	20.39
s3	2.00	0.00	6.00	1.23	0.44	1.03	1.63	0.00	5.82
s4	10.00	7.00	14.00	2.82	1.59	1.39	3.55	4.40	10.04
s5	4.00	168.00	1.00	1.59	1.32	1.34	2.52	127.32	0.74
s6	7.00	13.00	76.00	1.68	1.24	1.80	4.16	10.49	42.14
s13	10.00	15.00	0.00	1.01	1.58	1.33	9.87	9.50	0.00
s16	2.00	191.00	85.00	0.30	2.12	1.55	6.64	90.23	54.94
						MEAN	**3.86**	**32.20**	**19.15**
						SD	**3.04**	**48.52**	**21.52**

Females

Subject	Area			No. areas of Z-disk streaming			No. areas of Z-disk streaming/mm^2		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s7	4.00	14.00	116.00	0.83	0.87	1.65	4.83	16.06	70.51
s8	3.00	6.00	2.00	1.28	2.35	1.62	2.34	2.55	1.24
s9	14.00	42.00	65.00	2.46	2.06	2.29	5.70	20.39	28.34
s10		38.00	14.00	2.41	1.75	1.56		21.74	8.95
s11	3.00	71.00	27.00	2.21	1.99	1.40	1.36	35.71	19.27
s12	1.00	4.00	18.00	1.48	0.44	2.28	0.68	9.05	7.90
s14	12.00	82.00	5.00	1.28	2.48	0.90	9.38	33.02	5.58
s15	3.00	30.00	59.00	1.05	2.34	2.59	2.85	12.81	22.82
						MEAN	**3.87**	**18.92**	**20.58**
						SD	**3.01**	**11.35**	**22.22**

Z-Disk Streamining ANOVA Table: Gender x Exercise Bout

	Df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	367.4662	12	789.288	0.465567	0.507991
2 – Exercise Bout	2	2497.768	24	701.528	3.560467	0.044249
12	2	160.2623	24	701.528	0.228447	0.797479

Newman-Keuls Post Hoc: Main Effect – Exercise Bout

	Rest	*Bout 1*	*Bout 2*
Rest		0.044146	0.082871
Bout 1	0.044146		0.462769
Bout 2	0.082871	0.462769	

MPO Positive Cells (Neutrophils)

Males

Subject	*Area*			*No. ⊕ cells*			*Cells/mm²*		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s1	1.98	3.48	4.14	1.00	0.00	5.00	0.51	0.00	1.21
s2	2.56	1.17	3.50	3.00	9.00	8.00	1.17	7.68	2.29
s3	2.65	1.06	2.40	0.00	2.00	0.00	0.00	1.88	0.00
s4	2.06	3.44	3.50	1.00	6.00	4.00	0.48	1.74	1.14
s5	1.43	2.03	2.04	0.00	10.00	3.00	0.00	4.93	1.47
s6	3.32	4.29	3.05	1.00	3.00	20.00	0.30	0.70	6.57
s13		3.84	3.80		0.00	9.00		0.00	2.37
s16	4.70	1.46	5.05	5.00	0.00	3.00	1.06	0.00	0.59
						MEAN	**0.50**	**2.12**	**1.95**
						SD	**0.47**	**2.79**	**2.02**

Females

Subject	*Area*			*No. ⊕ cells*			*Cells/mm²*		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s7	5.85	4.29	5.37	7.00	8.00	37.00	1.20	1.87	6.89
s8	1.04	2.58	4.19	2.00	2.00	3.00	1.93	0.77	0.72
s9	2.31	1.27	1.86	3.00	7.00	11.00	1.30	5.52	5.92
s10	1.02	2.02	3.11	2.00	3.00	13.00	1.96	1.49	4.18
s11	3.68	6.85	3.27	2.00	12.00	12.00	0.54	1.75	3.67
s12	3.56	8.27		6.00	3.00		1.68	0.36	
s14	2.42	2.63	1.39	2.00	1.00	14.00	0.83	0.12	10.10
s15	1.51	3.72	2.41	1.00	10.00	19.00	0.66	2.69	7.88
						MEAN	**1.26**	**1.82**	**5.62**
						SD	**0.56**	**1.72**	**3.08**

Neutrophil ANOVA Table: Gender x Exercise Bout

	Df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	19.01259	12	4.0675601	4.6742010	0.0515276
2 – Exercise Bout	2	29.60073	24	4.4750165	6.6146631	0.0051512
12	2	15.95233	24	4.4750165	3.5647525	0.0441030

Newman-Keuls Post Hoc: Interaction – 12

		Males			*Females*		
		Rest	*Bout 1*	*Bout 1*	*Rest*	*Bout 1*	*Bout 2*
Males	*Rest*		0.455828	0.447369	0.54257059	0.54223573	0.00180995
	Bout 1	0.45582813		0.888632	0.70674079	0.73264027	0.00932622
	Bout 2	0.44736915	0.888632		0.54604882	0.90685964	0.01505041
Females	*Rest*	0.54257059	0.706741	0.546049		0.74784756	0.00552088
	Bout 1	0.54223573	0.73264	0.90686	0.74784755		0.0109483
	Bout 2	0.00180995	0.009326	0.01505	0.00552088	0.0109483	

CD68 Positive Cells (Macrophages)

Males

	Area			*No.⊕cells*			*Cells/mm²*		
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s1	1.98	3.48	4.14	8.00	84.00	68.00	4.05	24.14	16.44
s2	2.56	1.17	3.50	10.00	43.00	101.00	3.90	36.70	28.85
s3	2.65	1.06	2.40	12.00	6.00	55.00	4.53	5.65	22.88
s4	2.06	3.44	3.50	13.00	17.00	43.00	6.30	4.94	12.30
s5	1.43	2.03	2.04	12.00	56.00	19.00	8.36	27.61	9.30
s6	3.32	4.29	3.05	15.00	79.00	22.00	4.52	18.43	7.22
s13		3.84	3.80		26.00	59.00		6.77	15.51
s16	4.70	1.46	5.05	22.00	13.00	32.00	4.68	8.93	6.34
						MEAN	**5.19**	**16.65**	**14.86**
						SD	**1.60**	**11.93**	**7.85**

Females

	Area			*No.⊕cells*			*Cells/mm²*		
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s7	5.85	4.29	5.37	11.00	35.00	115.00	1.88	8.16	21.43
s8	1.04	2.58	4.19	1.00	18.00	17.00	0.97	6.97	4.05
s9	2.31	1.27	1.86	40.00	19.00	55.00	17.31	14.97	29.62
s10	1.02	2.02	3.11	1.00	35.00	78.00	0.98	17.34	25.09
s11	3.68	6.85	3.27	33.00	99.00	99.00	8.97	14.45	30.30
s12	3.56	8.27		15.00	25.00		4.21	3.02	
s14	2.42	2.63	1.39	5.00	23.00	8.00	2.07	2.78	5.77
s15	1.51	3.72	2.41	1.00	21.00		0.66	5.65	
						MEAN	**4.63**	**9.17**	**19.38**
						SD	**5.81**	**5.67**	**11.67**

Macrophage ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	6.706644	11	120.7913	0.055523	0.818048
2 – Exercise Bout	2	494.5486	22	48.20269	10.25977	0.000711
12	2	116.6417	22	48.20269	2.419817	0.11223

Newman-Keuls Post Hoc: Main Effect – Exercise Bout

	Rest	*Bout 1*	*Bout 2*
Rest		0.003	0.00087
Bout 1	0.003		0.341125
Bout 2	0.00087	0.341125	

Ubiquitin Conjugated Protein Content

Relative Increase in Ubiquitin Conjugated Protein Content Following Exercise Bouts 1 and 2 as Compared to Rest

Males

	Density						*Relative Increase in protein content*						*Mean Relative*		
	Blot A			*Blot B*			*Blot A*			*Blot B*			*Increase in protein content (Blot A & B)*		
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s13	37888	34377	22532	38287	25834	42252	1.00	0.91	0.59	1.00	0.67	1.10	1.00	0.79	0.85
s1	84835	74284		23435	41625		1.00	0.88		1.00	1.78		1.00	1.33	
s16	111000	98971	150000	158	200	141	1.00	0.89	1.35	1.00	1.26	0.89	1.00	1.08	1.12
s6	152000	151000	163000	127	210	246	1.00	0.99	1.07	1.00	1.66	1.94	1.00	1.33	1.51
s5	162000	291000	177000	376000	229000	232000	1.00	1.80	1.09	1.00	0.61	0.62	1.00	1.20	0.85
s3	38750	71753	147000	63783	50402	85900	1.00	1.85	3.79	1.00	0.79	1.35	1.00	1.32	2.57
s4	107000	85840	144000	93765	96396	112000	1.00	0.80	1.35	1.00	1.03	1.19	1.00	0.92	1.27
s2	80945	73211	188000				1.00	0.90	2.32	1.00			1.00	0.90	2.32
											MEAN		**1.00**	**1.11**	**1.50**

Females

	Density						*Relative Increase in protein content*						*Mean Relative*		
	Blot A			*Blot B*			*Blot A*			*Blot B*			*Increase in protein content (Blot A & B)*		
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s12	6189		10782	56576		37290	1.00		1.74	1.00		0.66	1.00		1.20
s10	41422	40957		24308	45756		1.00	0.99		1.00	1.88		1.00	1.44	
s14	45093	112000	189000	73	132	292	1.00	2.48	4.19	1.00	1.82	4.02	1.00	2.15	4.11
s9	227000	242000	161000	373000	359000	351000	1.00	1.07	0.71	1.00	0.96	0.94	1.00	1.01	0.83
s11	126000	236000	166000	154000	138000	202000	1.00	1.87	1.32	1.00	0.90	1.31	1.00	1.38	1.31
s7	37918	45335	49174	55004	40472	51202	1.00	1.20	1.30	1.00	0.74	0.93	1.00	0.97	1.11
s8	57938	99860	141000				1.00	1.72	2.43	1.00			1.00	1.72	2.43
s15	50904	153000	189000				1.00	3.01	3.71	1.00			1.00	3.01	3.71
											MEAN		**1.00**	**1.67**	**2.10**

Ubiquitin ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	2.059011	11	0.7943	2.592235	0.135687
2 – Exercise Bout	2	2.483346	22	0.328671	7.555722	0.003177
12	2	0.526609	22	0.328671	1.602238	0.224076

Newman-Keuls Post Hoc: Main Effect – Exercise Bout

	Rest	*Bout 1*	*Bout 2*
Rest		0.095263	0.00114
Bout 1	0.095263		0.043118
Bout 2	0.00114	0.043118	

Gender Differences in Ubiquitin Conjugated Protein Content at Rest and Following Exercise Bout 1 and 2

Gender Comparison (within blot)	*Blot A (Male Density/Female Density)*			*Blot B (Male Density/Female Density)*			*Mean (Blot A & B)*		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s1 to s10	0.96	0.91		2.05	1.81		1.51	1.36	
s1 to 13	0.41	1.12		13.71	6.89		7.06	4.00	
s13 to s10	1.58	0.56		0.91	0.84		1.24	0.70	
s13 to s12	6.12		2.09	6.12		2.09	6.12		2.09
s16 to s14	0.84	1.59	1.75	2.05	0.88	0.79	1.45	1.24	1.27
s6 to s14	0.48	1.51	2.18	2.81	1.35	0.86	1.65	1.43	1.52
s5 to s11	2.44	1.66	1.15	1.29	1.23	1.07	1.86	1.45	1.11
s5 to s9	1.01	0.64	0.65	0.71	1.20	0.73	0.86	0.92	0.69
s3 to s7	1.16	1.25	1.68	1.02	1.58	2.99	1.09	1.41	2.33
s4 to s7	1.70	2.38	2.19	2.82	1.89	2.93	2.26	2.14	2.56
s2 to s8	1.14	0.52	1.03				1.14	0.52	1.03
s2 to s15	1.30	0.34	0.72				1.30	0.34	0.72
						MEAN	2.29	1.41	1.48

Ubiquitin ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	1.717938	18	0.309675	5.547548	0.030052
2 – Exercise Bout	2	0.099673	36	0.045388	2.195996	0.12593
12	2	0.099673	36	0.045388	2.195996	0.12593

Newman-Keuls Post Hoc: Main Effect – Gender

Ubiquitin conjugated protein content is greater in males as compared to females.

Calpain 30 kD Regulatory Subunit Protein Content

Relative Increase in Calpain 30 kD Regulatory Subunit Protein Content Following Exercise Bouts 1 and 2 as Compared to Rest

Males

Subject	*Density*			*Relative Increase in Protein Content*		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s2	5.12	5.32	6.64	1.00	1.04	1.30
s3	6.44	7.74	7.36	1.00	1.20	1.14
s4	4.12	5.67	5.39	1.00	1.38	1.31
s5	1.59	1.27	3.30	1.00	0.80	2.07
s6	2.80	9.49	1.64	1.00	3.39	0.59
s13	1.27	1.56	1.04	1.00	1.23	0.82
s16	22.67	29.83	22.88	1.00	1.32	1.01
			MEAN	**1.00**	**1.48**	**1.18**

Females

Subject	*Density*			*Relative Increase in Protein Content*		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s7	7.36	1.70	9.57	1.00	0.23	1.30
s8	4.76	6.54	6.66	1.00	1.37	1.40
s9	1.28	1.81	3.10	1.00	1.41	2.43
s11	1.82	0.80	2.25	1.00	0.44	1.24
s12	2.36		1.79	1.00		0.76
s14	3.24	3.77	6.89	1.00	1.16	2.13
s15	19.19	10.06	17.16		0.52	0.89
			MEAN	**1.00**	**0.86**	**1.47**

Calpain ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.056655	10	0.211414	0.267981	0.615947
2 – Exercise Bout	2	0.537982	20	0.358398	1.501074	0.246954
12	2	0.279919	20	0.358398	0.78103	0.471409

Gender Differences in Calpain 30 kD Regulatory Subunit Protein Content at Rest and Following Exercise Bout 1 and 2

Gender Comparison (within blot)	*Mean (Blot A & B)*		
	Rest	*Bout 1*	*Bout 2*
s16 to s14	1.27	1.50	0.78
s6 to s14	0.99	0.86	0.33
s5 to s11	0.87	1.58	1.47
s5 to s9	0.89	5.20	0.96
s3 to s7	1.02	0.89	1.04
s4 to s7	1.18	1.57	1.33
s2 to s8	1.19		0.92
MEAN	1.06	1.93	0.98

Calpain ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*Df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.987981	11	0.418102	2.363012	0.152494
2 – Exercise Bout	2	0.918817	22	0.439139	2.092315	0.147278
12	2	0.918817	22	0.439139	2.092315	0.147278

HSP72 Protein Content

Relative Increase in HSP72 Protein Content Following Exercise Bouts 1 and 2 as Compared to Rest

Males

	Density						*Relative Increase in protein content*						*Mean Relative Increase in protein content (Blot A & B)*		
	Blot A			*Blot B*			*Blot A*			*Blot B*					
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s3	7.61	11.51	5.20	10.12	10.65	8.49	1.00	1.51	0.68	1.00	1.05	0.84	1.00	1.28	0.76
s4	14.62	11.98	5.22	12.11	14.23	6.68	1.00	0.82	0.36	1.00	0.82	0.36	1.00	1.00	0.45
s5	6.88	16.08	5.19	11.78	23.44	7.76	1.00	2.34	0.75	1.00	1.99	0.66	1.00	2.16	0.71
s2	9.93	4.21	6.34	6.19	3.34	4.78	1.00	0.44	0.67	1.00	0.54	0.77	1.00	0.49	0.72
s1	36.63	34.36		16.80	19.21		1.00	0.94		1.00	1.14		1.00	1.04	
s6	26.67	13.72	22.27	24.21	11.86	21.01	1.00	0.51	0.84	1.00	0.49	0.87	1.00	0.50	0.85
s13	5.45	8.16	9.53	9.73	13.67	15.82	1.00	1.50	1.75	1.00	1.41	1.63	1.00	1.45	1.69
s16	7.40	12.40	14.69	4.82	6.80	6.07	1.00	1.67	1.98	1.41	1.26		1.00	1.54	1.62
											MEAN		**1.00**	**1.18**	**0.97**

Females

	Density						*Relative Increase in protein content*						*Mean Relative Increase in protein content (Blot A & B)*		
	Blot A			*Blot B*			*Blot A*			*Blot B*					
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s9	3.46	4.10	4.15	5.66	7.59	9.35	1.00	1.18	1.19	1.00	1.34	1.65	1.00	1.26	1.42
s14	1.47	4.95	2.26	2.82	5.53	3.83	1.00	3.36	1.54	1.00	1.96	1.36	1.00	2.66	1.45
s15	4.77	6.42	9.14	8.36	10.30	19.69	1.00	1.34	1.92	1.00	1.23	2.36	1.00	1.29	2.14
s7	26.93	19.30	12.95	10.73	7.91	2.14	1.00	0.72	0.48	1.00	0.74	0.20	1.00	0.73	0.34
s10	39.53	33.57		16.92	15.56		1.00	0.85		1.00	0.92		1.00	0.89	
s8	16.67	22.03	22.03	21.90	27.68	25.14	1.00	1.32	1.32	1.00	1.26	1.15	1.00	1.29	1.23
s11	18.64	9.89	5.36	15.03	8.81	6.97	1.00	0.53	0.29	1.00	0.59	0.464	1.00	0.56	0.38
s12	15.17		8.23	9.41		4.73	1.00		0.62	1.00		0.57	1.00		0.60
											MEAN		**1**	**1.24**	**1.08**

HSP72 ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*Df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.08515	11	0.379527	0.224359	0.645
2 – Exercise Bout	2	0.21944	22	0.204851	1.071216	0.359798
12	2	0.028432	22	0.204851	0.138791	0.871166

Gender Differences in HSP72 Protein Content at Rest and Following Exercise Bout 1 and 2

Gender Comparison (within blot)	*Blot A (Male Density/Female Density)*			*Blot B (Male Density/Female Density)*			*Mean (Blot A & B)*		
	Rest	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s3 to s9	2.19	2.81	1.25	1.79	1.40	0.91	1.99	2.10	1.08
s4 to s14	9.95	2.42	2.31	4.30	2.57	1.74	7.12	2.50	2.03
s5 to s15	1.44	2.50	0.57	1.41	2.28	0.39	1.43	2.39	0.48
s5 to s7	0.64	2.03	2.45				0.64	2.03	2.45
s2 to s15	1.14	0.41	0.32				1.14	0.41	0.32
s1 to s10	0.93	1.02		0.99	1.23		0.96	1.13	
s6 to s8	1.60	0.62	1.01	1.11	0.43	0.84	1.35	0.53	0.92
s13 to s11	0.29	0.82	1.78	0.65	1.69	2.27	0.59	1.26	2.03
s16 to s12				0.59		1.28	0.59		1.28
s13 to s12	0.36		0.54				0.36		0.54
s16 to s11	0.60	1.71	2.86				0.60	1.71	2.86
						MEAN	**1.52**	**1.56**	**1.40**

HSP72 ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	5.541302	16	1.277688	4.336977	0.053699
2 – Exercise Bout	2	0.150457	32	0.737725	0.203947	0.816558
12	2	0.160353	32	0.737725	0.217362	0.805817

HSP27 Protein Content

Relative Increase in HSP27 Protein Content Following Exercise Bouts 1 and 2 as Compared to Rest

Males

	Density						*Relative Increase in protein content*						*Mean Relative Increase in protein content (Blot A & B)*		
	Blot A			*Blot B*			*Blot A*			*Blot B*					
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s3	8.55	11.97	13.81	17.79	20.11	23.42	1.00	1.40	1.62	1.00	1.13	1.32	1.00	1.27	1.47
s4	15.73	12.51	17.97	17.79	20.11	22.94	1.00	0.80	1.14	1.00	1.13	1.29	1.00	0.96	1.22
s5	29.15	31.47	33.31	17.75	24.72	35.73	1.00	1.08	1.14	1.00	1.39	1.45	1.00	1.24	1.30
s2	25.04	17.01	11.47	30.83	20.22	19.51	1.00	0.68	0.46	1.00	0.66	0.63	1.00	0.67	0.55
s1	7.92	12.61		14.26	19.34		1.00	1.59		1.00	1.36		1.00	1.47	
s6	10.33	12.90	12.56	14.73	11.52	12.35	1.00	1.25	1.21	1.00	0.78	0.84	1.00	1.02	1.03
s13	30.71	34.07	17.57	21.24	20.88	30.46	1.00	1.11	0.57	1.00	0.98	1.43	1.00	1.05	1.00
s16	13.77	14.4	23.3	26.49	24.68	19.33	1.00	1.05	1.70	1.00	0.93	0.72	1.00	0.99	1.21
											MEAN		**1.00**	**1.08**	**1.11**

Females

	Density						*Relative Increase in protein content*						*Mean Relative Increase in protein content (Blot A & B)*		
	Blot A			*Blot B*			*Blot A*			*Blot B*					
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s9	18.09	15.73	15.61	25.19	26.08	23.78	1.00	0.87	0.86	1.00	1.04	0.94	1.00	0.95	0.90
s14	21.09	22.08	16.87	25.33	26.11	24.75	1.00	1.05	0.80	1.00	1.03	0.98	1.00	1.04	0.89
s15	24.05	27.77	26.82	20.23	22.35	20.95	1.00	1.15	1.12	1.00	1.10	1.03	1.00	1.13	1.08
s7	19.87	10.45	9.44	24.71	22.14	26.67	1.00	0.53	0.48	1.00	0.90	1.08	1.00	0.71	0.78
s10	13.13	11.01		20.79	21.08		1.00	0.84		1.00	1.01		1.00	0.93	
s8	23.99	21.34	11.77	19.07	15.25	8.18	1.00	0.90	0.49	1.00	0.80	0.43	1.00	0.85	0.46
s11	30.77	33.08	25.81	24.21	14.62	16.52	1.00	1.08	0.84	1.00	0.60	0.68	1.00	0.84	0.76
s12	25.50		21.16	18.61		16.33	1.00		0.83	1.00		0.88	1.00		0.85
											MEAN		**1.00**	**0.92**	**0.82**

HSP27 ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*Df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.176559	11	0.058169	3.035271	0.109324
2 – Exercise Bout	2	0.005326	22	0.020039	0.265776	0.769037
12	2	0.073988	22	0.020039	3.692274	0.041432

Newman-Keuls Post Hoc: Interaction – 12

		Males *Rest*	*Bout 1*	*Bout 1*	*Females* *Rest*	*Bout 1*	*Bout 2*
Males	*Rest*		0.941938	0.517796	1	0.318937	0.063025
	Bout 1	0.941938		0.300277	0.744545	0.542392	0.081411
	Bout 2	0.517796	0.300277		0.362627	0.149637	0.01135
Females	*Rest*	1	0.744545	0.362627		0.57245	0.106652
	Bout 1	0.318937	0.542392	0.149637	0.57245		0.181649
	Bout 2	0.063025	0.081411	0.01135	0.106652	0.181649	

Gender Differences in HSP27 Protein Content at Rest and Following Exercise Bout 1 and 2

Gender Comparison (within blot)	*Blot A (Male Density/Female Density)*			*Blot B (Male Density/Female Density)*			*Mean (Blot A & B)*		
	Rest	Bout 1	*Bout 2*	*Rest*	Bout 1	*Bout 2*	*Rest*	Bout 1	*Bout 2*
s3 to s9	0.47	0.76	0.88	0.71	0.77	0.98	0.59	0.77	0.93
s4 to s14	0.75	0.57	1.07	0.70	0.77	0.93	0.72	0.67	1.00
s5 to s15	1.21	1.13	1.24	0.88	1.11	1.23	1.04	1.12	1.24
s1 to s10	0.60	1.14		0.69	0.92		0.64	1.03	
s6 to s8	0.43	0.60	1.07	0.77	0.76	1.51	0.60	0.68	1.29
s13 to s11	1.00	1.03	0.68	0.88	1.43	1.84	0.94	1.23	1.26
s16 to s12	0.54		1.10	1.42		1.18	0.98		1.14
s2 to s7	1.26	1.63	1.22	1.25	0.91	0.73	1.25	1.27	0.97
						MEAN	**0.85**	**0.97**	**1.12**

HSP27 ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.005758	12	0.045118	0.12763	0.727106
2 – Exercise Bout	2	0.057447	24	0.014171	4.053851	0.030425
12	2	0.057447	24	0.014171	4.053851	0.030425

Newman-Keuls Post Hoc: Interaction – 12

		Males			*Females*		
		Rest	*Bout 1*	*Bout 1*	*Rest*	*Bout 1*	*Bout 2*
Males	*Rest*		0.145786	0.001299	0.047079	0.053649	0.05856
	Bout 1	0.145786		0.033712	0.494242	0.519475	0.533214
	Bout 2	0.001299	0.033712		0.113429	0.102682	0.086443
Females	*Rest*	0.047079	0.494242	0.113429		1	1
	Bout 1	0.053649	0.519475	0.102682	1		1
	Bout 2	0.05856	0.533214	0.086443	1	1	

Bcl-2 Protein Content

Relative Increase in Bcl-2 Protein Content Following Exercise Bouts 1 and 2 as Compared to Rest

Males

	Density						*Relative Increase in protein content*						*Mean Relative Increase in protein content (Blot A & B)*		
	Blot A			*Blot B*			*Blot A*			*Blot B*					
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s3	14.64	18.60	15.15	18.38	17.07	13.61	1.00	1.27	1.04	1.00	0.93	0.74	1.00	1.10	0.89
s4	25.24	24.39	18.11	26.58	31.92	21.48	1.00	0.97	0.72	1.00	1.20	0.81	1.00	1.08	0.76
s5	10.52	6.35	7.03	16.74	8.70	9.36	1.00	0.60	0.67	1.00	0.52	0.56	1.00	0.56	0.61
s2	13.79	10.76	9.35	16.61	17.35	20.65	1.00	0.78	0.68	1.00	1.04	1.24	1.00	0.91	0.96
s1	7.74	14.37		10.58	14.27		1.00	1.86		1.00	1.35		1.00	1.60	
s6	9.57	5.37	8.88	12.40	14.94	14.90	1.00	0.56	0.93	1.00	1.20	1.20	1.00	0.88	1.06
s13	27.84	16.26	21.61	26.61	19.05	22.90	1.00	0.58	0.78	1.00	0.72	0.86	1.00	0.65	0.82
s16	11.16	10.68	10.21	12.03	10.89	13.35	1.00	0.95	0.91	1.00	0.91	1.11	1.00	0.93	1.01
											MEAN		**1.00**	**0.97**	**0.87**

Females

	Density						*Relative Increase in protein content*						*Mean Relative Increase in protein content (Blot A & B)*		
	Blot A			*Blot B*			*Blot A*			*Blot B*					
Subject	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*	*Rest*	*Bout 1*	*Bout 2*
s9	13.50	12.82	13.12	11.09	10.95	14.40	1.00	0.95	0.97	1.00	0.99	1.30	1.00	0.97	1.14
s14	19.78	18.42	27.34	25.65	27.73	31.11	1.00	0.93	1.38	1.00	1.08	1.21	1.00	1.01	1.27
s15	8.12	8.60	11.31	11.17	11.20	16.00	1.00	1.06	1.39	1.00	1.00	1.43	1.00	1.03	1.41
s7	24.88	24.93	15.22	43.72	33.69	24.57	1.00	1.00	0.61	1.00	0.77	0.56	1.00	0.89	0.59
s10	7.82	10.94		16.93	14.88		1.00	1.40		1.00	0.88		1.00	1.14	
s8	6.38	10.06	10.71	23.24	22.24	16.95	1.00	1.58	1.68	1.00	0.96	0.73	1.00	1.27	1.20
s11	22.23	12.85	20.91	23.35	17.65	32.22	1.00	0.58	0.94	1.00	0.76	1.40	1.00	0.67	1.16
s12	14.62		5.96	16.77		10.20	1.00		0.41	1.00		0.61	1.00		0.51
											MEAN		**1.00**	**0.99**	**1.04**

Bcl-2 ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*Df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.131788	11	0.039937	3.29986	0.096607
2 – Exercise Bout	2	0.026104	22	0.024804	1.052395	0.366027
12	2	0.05284	22	0.024804	2.130296	0.142659

Gender Differences in Bcl-2 Protein Content at Rest and Following Exercise Bout 1 and 2

Gender Comparison (within blot)	*Blot A (Male Density/Female Density)*			*Blot B (Male Density/Female Density)*			*Mean (Blot A & B)*		
	Rest	Bout 1	*Bout 2*	*Rest*	Bout 1	*Bout 2*	*Rest*	Bout 1	*Bout 2*
s3 to s9	1.08	1.45	1.15	1.66	1.56	0.95	1.37	1.50	1.05
s4 to s14	1.28	1.32	0.66	1.04	1.15	0.69	1.16	1.24	0.68
s5 to s15	1.30	0.74	0.62	1.50	0.78	0.59	1.40	0.76	0.60
s1 to s10	0.99	1.31		0.62	0.96		0.81	1.14	
s6 to s8	1.08	0.84	0.83	0.83	0.64	0.88	0.96	0.74	0.85
s13 to s11	1.25	1.27	1.03	1.14	1.08	0.71	1.20	1.17	0.87
s16 to s12	1.31		0.58	1.39		0.90	1.35		0.74
s2 to s7	0.55	0.43	0.61	0.38	0.51	0.84	0.47	0.47	0.73
						MEAN	**1.09**	**1.00**	**0.79**

Bcl-2 ANOVA Table: Gender x Exercise Bout

	df Effect	*MS Effect*	*df Error*	*MS Error*	*F*	*p-level*
1 – Gender	1	0.019592	12	0.079216	0.247318	0.627957
2 – Exercise Bout	2	0.075312	24	0.021479	3.506324	0.046139
12	2	0.075312	24	0.021479	3.506324	0.046139

Newman-Keuls Post Hoc: Interaction – 12

		Males			*Females*		
		Rest	*Bout 1*	*Bout 1*	*Rest*	*Bout 1*	*Bout 2*
Males	*Rest*		0.645769	0.012598	0.666954	0.49749	0.264192
	Bout 1	0.645769		0.028977	0.815271	0.969795	0.995291
	Bout 2	0.012598	0.028977		0.043743	0.075545	0.110227
Females	*Rest*	0.666954	0.815271	0.043743		1	1
	Bout 1	0.49749	0.969795	0.075545	1		1
	Bout 2	0.264192	0.995291	0.110227	1	1	