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Junctophilin Damage Contributes to Early Force Deficits and Excitation-Contraction Coupling Failure after Performing Eccentric Contractions

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ACCEPTANCE

This dissertation, JUNCTOPHILIN DAMAGE CONTRIBUTES TO EARLY FORCE DEFICITS AND EXCITATION-CONTRACTION COUPLING FAILURE AFTER PERFORMING ECCENTRIC CONTRACTIONS, by BENJAMIN T. CORONA, was prepared under the direction of the candidate's Dissertation Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Education, Georgia State University.

The Dissertation Advisory Committee and the student's Department Chair, as representatives of the faculty, certify that this dissertation has met all standards of excellence and scholarship as determined by the faculty. The Dean of the College of Education concurs.

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ABSTRACT

JUNCTOPHILIN DAMAGE CONTRIBUTES TO EARLY FORCE DEFICITS AND EXCITATION-CONTRACTION COUPLING FAILURE AFTER PERFORMING ECCENTRIC CONTRACTIONS
by
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Junctophilins (JP1 & JP2) are expressed in skeletal muscle and are the primary proteins involved in transverse (T)-tubule and sarcoplasmic reticulum (SR) membrane apposition. During the performance of eccentric contractions, the apposition of T-tubule and SR membranes may be disrupted, resulting in excitation-contraction (EC) coupling failure and thus reduced force-producing capacity. In this study, we made three primary observations: 1) Through the first three days after the performance of 50 eccentric contractions *in vivo* by the left hindlimb anterior crural muscles of female mice, both JP1 and JP2 were significantly reduced by ~50 and 35%, respectively, while no reductions were observed after the performance of non-fatiguing concentric contractions; 2) following the performance of a repeated bout 50 eccentric contractions *in vivo*, only JP1 was immediately reduced (~30%) but recovered by 3d post-injury in tandem with the recovery of strength and EC coupling; and 3) following the performance of 10 eccentric contractions at either 15 or 35˚C by isolated mouse EDL muscle, isometric force, EC coupling, and JP1 and JP2 were only reduced after the 35˚C eccentric contractions. Regression analysis of JP1 and JP2 content in TA and EDL muscles from each set of

experiments indicated that JP damage is significantly associated with early (0 – 3d) strength deficits after performing eccentric contractions ($R = 0.49$; $P < 0.001$). As a whole, the results of this study indicate that JP damage plays in role in early force deficits due to EC coupling failure following the performance of eccentric contractions.

JUNCTOPHILIN DAMAGE CONTRIBUTES TO EARLY FORCE DEFICITS AND EXCITATION-CONTRACTION COUPLING FAILURE AFTER PERFORMING ECCENTRIC CONTRACTIONS

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A Dissertation

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ABBREVIATIONS

ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CICR	Calicium induced calcium release
DHPR	Dihydropyridine receptor
EC coupling	Excitation contraction coupling
JP1	Junctophilin 1
JP2	Junctophilin 2
JP-45	Junctional protein-45
K^{+}	Potassium
MG29	Mitsugumin-29
Mg^{2+}	Magnesium
Na^{+}	Sodium
RyR1 & 2	Ryanodine Receptor 1 & 2
SERCA	Sarco/endoplasmic reticulum calcium ATPase pump
T-tubule	Transverse-tubule
VICR	Voltage induced calcium release

CHAPTER 1

THE ROLE OF JUNCTOPHILINS IN EARLY STRENGTH DEFICITS AFTER PERFORMING ECCENTRIC CONTRACTIONS

Introduction

The general topic of contraction-induced muscle injury has been studied extensively. Using the following search parameters, (skeletal muscle) AND (damage OR injury) AND (contraction OR exercise), the United States National Library of Medicine database (PubMed) yielded over 3,500 research articles. The abundance of interest and attention this topic has received by the research community is likely related to the fact that most everyone, if not all people, experience exercise-induced muscle injury at some point in their lives.

Contraction-induced muscle injury is usually not of a clinical magnitude in healthy populations, although there are specific groups of individuals that are prone to debilitating chronic muscle injury (e.g., Duchenne muscular dystrophy patients) or acute severe muscle damage (i.e., rhabdomyolysis). For all populations, contraction-induced muscle injury can result in a myriad of perturbations to skeletal muscle including muscle damage (Lieber *et al.*, 1996; Lowe et al., 1995), sarcoplasmic reticulum (SR) Ca^{++} dysregulation (Balnave & Allen, 1995; Ingalls et al., 1998b; Lynch *et al.*, 1997; Warren *et al.*, 2002a), resting cytosolic Ca^{++} perturbation (Balnave & Allen, 1995; Ingalls et al., 1998b; Lynch *et al.*, 1997; Warren *et al.*, 2002a), plasmalemma damage (Armstrong *et al.*, 1983; Warren *et al.*, 1993b; Warren et al., 2002a), and mitochondrial disruption (Duan *et al.*, 1990; Warren *et al.*, 1996). Ultimately, this

type of muscle injury results in immediate and prolonged force deficits (Balnave & Allen, 1995; Corona et al., 2008a; Ingalls et al., 1998a; Ingalls et al., 1998b; Ingalls et al., 2004b; Lowe et al., 1995; McCully & Faulkner, 1986). Loss of the ability to produce force can have a profound impact on sports performance, occupational tasks, and job performance. For groups prone to contraction-induced muscle injury, functional deficits can be life-threatening. For example, decrements in force producing capacity of the respiratory muscles (i.e., diaphragm) consequent of chronic muscle injury necessitates ventilation assistance at a young age in Duchenne muscular dystrophy patients (Petrof, 1998).

Immediate and prolonged force deficits that occur after contraction-induced muscle injury appear to be of dissimilar etiology (Armstrong *et al.*, 1991; Proske & Morgan, 2001; Warren et al., 2002b). Skeletal muscle force production relies on SR Ca^{++} release, which under normal circumstances is voltage-induced. The process in which plasmalemma and T tubule membrane depolarization directly leads to SR Ca^{++} release is called excitation-contraction (E-C) coupling. Force deficits immediately and three days after injury are due primarily to E-C coupling disruption and to a lesser extent to damage to force-bearing proteins (Balnave & Allen, 1995; Ingalls et al., 1998b; Warren et al., 1993c). In contrast, prolonged strength deficits are due to loss of contractile protein (Ingalls et al., 1998a; Lowe et al., 1995) mediated by an inflammatory response. It has been shown that E-C coupling disruption is due to a loss of communication between the T tubule (dihydropyridine receptor; DHPR) and the SR terminal cisternae (SR Ca^{++} release channel; ryanodine receptor; RYR1) (Balnave & Allen, 1995; Ingalls et al., 1998b; Ingalls et al., 2004a; Warren et al., 2000; Warren et al., 1995). However, it is not known how the communication between the DHPR and RYR1 is altered in injured muscle.

The following literature review provides general information about contraction-induced muscle injury, focusing primarily on the effects of muscle injury on force producing capacity. Specifically, the impact of injurious muscle contractions on early strength deficits will be highlighted with in-depth discussion of the underlying cause of early strength deficits. The effects of muscle temperature during injury induction, as well as the effect of a repeated bout of injurious contractions on the initial force deficits and E-C uncoupling will be discussed. The purpose of this literature review is to support the theory that E-C uncoupling observed immediately after injurious contractions are due to alterations of the triadic proteins junctophilin 1 and 2 (JP1 & JP2).

Excitation Contraction Coupling

Structure of the Triad

Adult skeletal muscle fibers are designed to rapidly disperse an electrical signal throughout the cell. The electrical stimulus, in turn, prompts the activation of a chemical 2nd messenger system (i.e., SR Ca^{++} release), which then removes inhibition of mechanical force production. The process in which an electrical signal, or muscle action potential, elicits SR Ca^{++} release is called excitation contraction (E-C) coupling.

Skeletal muscle possesses a transverse (T) tubule system, which is a system of continuous intracellular conduits that are extensions of the plasmalemma and whose lumen is continuous with the extracellular fluid, that allows an entire muscle fiber to respond rapidly to electrical stimuli. The T tubule membrane is excitable and functions to propagate action potentials from the plasmalemma into the depths of the muscle fiber (Stephenson, 2006). The majority of the T tubule system is arranged in perpendicular to the longitudinal axis of the muscle fiber (i.e., transverse tubules), which surround the myofibrils. Additionally, there are longitudinal tubules

that connect adjacent transverse tubules making the T tubule system a continuous network (Stephenson, 2006).

T tubules are intimately associated with specialized endoplasmic reticulum called sarcoplasmic reticulum (SR) in striated muscle. The SR has three distinct regions: the longitudinal reticulum, which is oriented in parallel with the myofibrils, the terminal cisternae, which comprise the ends of the SR and therefore "cap" the longitudinal reticulum, and the lumen, which is the space encapsulated by the SR membrane. Electron micrographs illustrate that a T tubule associates with two terminal cisternae of adjacent SR, forming structures called triads (Flucher *et al.*, 1993; Franzini-Armstrong, 1970). In mammalian skeletal muscle, triads are observed at the sarcomeric A-I band interface. Thus, one SR longitudinal reticulum centers on the M line and the adjacent SR longitudinal reticulum centers on the Z band of a given sarcomere. Helping to maintain this organization, even during lengthening and shortening of the fiber or sarcomere, the SR appears to attach to the M line and Z band by an intricate interaction between small ankyrin proteins that have an SR transmembrane domain and a protein called obscurin that can bind with titin (Armani *et al.*, 2006; Bagnato *et al.*, 2003). Importantly, this structural arrangement brings the excitable membrane of the T tubule, the SR terminal cisternae, and the myofilaments into close proximity to each other, which, as will be discussed, is crucial for muscle force production.

The SR is adept at storing high concentrations of Ca^{++}. Within the lumen of the SR, total $[Ca^{++}]$ is ~10 or 20 mM in slow and fast muscle fibers respectively, which is quite large compared to normal resting cytosolic $[Ca^{++}]$ (10 – 100 nM) under resting conditions (Allen *et al.*, 2008; Gissel, 2005; Rossi & Dirksen, 2006). This steep concentration gradient is achieved because the SR is outfitted with Ca^{++} ATPase pumps (SERCA) and calsequestrin, a low-affinity

high-capacity Ca^{++} binding protein. SERCA localizes to the longitudinal reticulum of the SR and actively pumps Ca^{++} from the cytosol into the SR lumen, which is the primary means by which resting cytosolic [Ca^{++}] is restored following activation. However, the effectiveness of SERCA to pump Ca^{++} into the SR lumen is greatly enhanced by Ca^{++} buffering by calsequestrin. Calsequestrin Ca^{++} binding effectively lowers the luminal free [Ca^{++}] to ~0.3 – 1 mM, which reduces the gradient that SERCA must pump Ca^{++} against by ~10 – 100 fold (Allen et al., 2008; Rossi & Dirksen, 2006). In tandem, these proteins allow the SR to store an extraordinary amount of Ca^{++} in a cell in which the cytosol is nearly devoid of this ion, thereby creating a large potential for Ca^{++} signaling within the cell.

The key proteins involved in skeletal muscle E-C coupling are the dihydropyridine receptor (DHPR, or Cav1) and the ryanodine receptor (RYR1). The DHPR is an L-type voltage-gated Ca^{++} channel located in the T tubule at the triad. The DHPR has five subunits (α_1, β, δ, γ, α_2) (Dulhunty, 2006). Of note, the α_{1s}–subunit comprises the transmembrane ion channel and contains the channel's voltage sensor (Bannister, 2007). Electrophysiological experiments that have characterized DHPR function in single muscle fibers highlight that the skeletal DHPR charge movement is initiated at ~-60 mV, while the channel Ca^{++} conductance is not initiated until ~-20 mV (Z. M. Wang *et al.*, 1999). More commonly known as the SR Ca^{++} release channel, RYR1 is a homotetramer (~560 Kda per subunit) that spans the SR terminal cistern membrane (Hamilton, 2005). RYR1 is noted for having a large cyoplasmic component known as the "foot" with a small transmembrane component forming the ion pore (Block et al., 1988; Franzini-Armstrong, 1970; Hamilton, 2005). At the triad, the DHPR and RYR1 are stereologically associated: Four DHPRs form a unit (i.e., tetrad) that aligns directly across from

a single RYR1, one DHPR per subunit of RYR. In skeletal muscle, a quaternary unit of DHPRs opposes every other RYR1 (Bers & Stiffel, 1993).

There is evidence to suggest that the skeletal muscle DHPR and RYR1 are physically coupled. Electron micrographs illustrate that the T tubule and SR membranes are only ~12 nm apart and the large cytoplasmic, or foot region of RYR1 spans this gap, presumably making physical contact with DHPR (Block et al., 1988; Franzini-Armstrong, 1970; Ikemoto *et al.*, 1997; Lu et al., 1994). Biochemical studies have indicated that there are indeed specific interactions between skeletal muscle DHPR subunits and RYR1 (Bannister, 2007; Dulhunty, 2006; Suda et al., 1997; Takekura et al., 1995; Tanabe et al., 1988). For example, the cytoplasmic loop between transmembrane domains II and III of the α_{1s} of DHPR has been found to increase the open probability of purified RYR1 in lipid bilayers and to increase [H^3] ryanodine binding in skeletal muscle SR microsomes (Suda et al., 1997; Takekura et al., 1995). While the α_{1s} subunit has been identified as crucial for DHPR-RYR1 physical coupling, other studies have also indicated that interactions amongst the multiple DHPR subunits and between these multiple DHPR subunits and RYR1 are likely involved in physically coupling DHPR and RYR1 in skeletal muscle (Bannister, 2007; Dulhunty, 2006).

While the DHPR and RYR1 appear to physically couple at the triad, these channels do not appear to be solely responsible for the development of this close spatial relationship. Two similar studies were conducted in which skeletal muscle isoforms of DHPR and RYR were expressed in Chinese hamster ovary cells (Suda et al., 1997; Takekura et al., 1995). The collective results of these studies revealed that the DHPR located to the plasmalemma and exhibited a normal L-Type Ca^{++} current and that the RYR1 located to the ER/SR membrane and could be activated with Ca^{++} or caffeine (*see below*). However, when both channels were co-

expressed in these cells no junctions between the channels were present. These findings suggest that expression of muscle specific accessory proteins are required for physical coupling between DHPR and RYR1 *in vivo* (Suda et al., 1997; Takekura et al., 1995).

DHPR – RYR1 physical coupling requires the formation of triads. The number of identified proteins that are associated with membrane junction complexes (i.e., diads and triads) is growing rapidly (Weisleder *et al.*, 2008). Of these proteins, junctophilins (JPs) appear to be the primary proteins that maintain the diad and triad structures in cardiac and skeletal myocytes respectively (Ito et al., 2001; Komazaki et al., 2002; Takeshima et al., 2000). Amino acid sequence analysis of the JPs reveal that the C-terminus contains a single-pass membrane domain that crosses the SR membrane and the N-terminus possesses MORN (membrane occupation and recognition nexus) motifs, which form non-covalent bonds with phospholipids in the plasmalemma or T tubule membrane (Takeshima et al., 2000). The middle of the protein is thought to form an alpha helix secondary structure (Weisleder et al., 2008). JP1 (72 kDa) and JP2 (90 kDa), which are different gene products, are both expressed in mature skeletal muscle, while only JP2 is expressed in cardiac muscle (Ito et al., 2001; Takeshima et al., 2000). Both of these proteins localize to the triad structure in skeletal muscle fibers (Ito et al., 2001). Based on the structure and cellular location of JP1 and JP2, it would appear that these proteins serve as a "molecular spring" that tethers the T tubule and terminal cistern membranes in skeletal muscle fibers.

Takeshima et al. (2000) first demonstrated the role of junctophilins in forming junctional complexes. They injected plasmids with JP cDNA into amphibian embryonic cells and observed the formation of junctions between the plasmalemma and ER membrane. No junctions were observed in cells injected with control plasmids, or in cells injected with truncated JP cDNA

plasmids that did not express the C terminus of JP1. Interestingly, the gap distance between the ER and plasmalemma membrane in embryonic cells expressing JP was ~7 nm (Takeshima et al., 2000). Ryanodine receptors were not present on the ER membrane in these cells. In dyspedic muscle cells (i.e., ryanodine receptors are not expressed) the gap distance between the T tubule and SR membrane is ~7 nm, however in muscle cells that express RYR1 the gap distance is 12 nm (Ikemoto et al., 1997). These findings indicate that JPs are involved in the formation of membrane junction complexes and suggest that JPs have an elastic property that may apply tension on the adjacent membranes.

Further evidence that JP1 and JP2 are specifically involved in triad and diad formation in skeletal muscle cells was provided by two studies in which JP1 gene expression was ablated in mice (Ito et al., 2001; Komazaki et al., 2002). JP1 deficient mice ($JP1^{-/-}$) died within one day after birth. Electron micrographs of neonatal hindlimb muscles indicated that the number triads that formed in $JP1^{-/-}$ muscle was reduced by ~90% compared to WT values, in spite of the fact that JP2 expression and diad formation were not reduced in these $JP1^{-/-}$ mice (Ito et al., 2001; Komazaki et al., 2002). These findings indicate that JP1 is required for triad formation, while JP2 mediates diad formation. Additionally, JP1 and JP2 do not appear to be functionally redundant, as JP2 expression cannot mediate triad formation or sustain post-natal life.

Targeting of DHPRs to the T tubule in the triad and alignment of DHPRs over RYR1 subunits is required for the direct physical coupling between DHPRs and every other RYR1 (Anderson et al., 2006; Delbono et al., 2007). While it is not entirely understood how these processes occur, junctional protein 45 (JP45; 45 kda) is thought to play a role in DHPR T tubule membrane targeting. JP45 is first expressed early during development (~15 days into embryonic development), associates in the cell with the DHPR and calsequestrin in the SR lumen, and

therefore has an SR transmembrane domain (Anderson et al., 2003). More specifically, JP45 binds to the I-II loop and C-terminus of α_{1s} subunit as well as the β_{1s} subunit of DHPR (Anderson et al., 2006). This association is of significance because the α_{1s} C-terminus and β_{1s} subunit have been implicated in DHPR triad targeting and membrane expression respectively (Chien et al., 1995; Flucher et al., 2000; Flucher et al., 2002). Both knock-down of JP45 in myotubes and chronic ablation of JP45 expression in $JP45^{-/-}$ mice has reduced DHPR protein expression in microsomal vesicles containing T tubule membranes, as well as DHPR peak molecular charge movement (- ~40%) in myotubes and mature muscle fibers (Anderson et al., 2006; Delbono et al., 2007). Of note, JP45 ablation is not lethal, does not appear to have an effect on protein expression of other key triadic proteins (e.g., RYR1, SERCA, triadin, calsequestrin), and does not appear to reduce SR Ca^{++} load in adult muscle fibers (Delbono et al., 2007). These results suggest that JP45 expression is involved in the proper positioning of DHPR in the triad, but may have some functional redundancy with another triadic protein.

Current Theory of E-C Coupling

The current theory of skeletal muscle E-C coupling indicates that T tubule membrane depolarization induces an intermolecular charge movement in DHPR, which corresponds to a conformation change in the DHPR, that then physically opens RYR1 (Bannister, 2007; Chandler et al., 1976; Dulhunty, 2006; Z. M. Wang et al., 1999). Although the skeletal muscle DHPR is an L-type Ca^{++} channel, skeletal muscle E-C coupling is not dependent on influx of Ca^{++} through this channel, and therefore is classified as "voltage" induced Ca^{++} release. As noted above, the DHPR charge movement occurs at a more negative membrane potential than the L-type Ca^{++} current, indicating that the DHPR charge movement occurs before the L-type Ca^{++} current. Careful examination of the membrane potential at which voltage-induced whole cell Ca^{++}

transients (i.e., RYR1 Ca^{++} release) begin reveals that SR Ca^{++} release is initiated before the L-type current in skeletal muscle, suggesting that RYR1 may reciprocally augment the L-type current (i.e., regrograde coupling) (Z. M. Wang et al., 1999). Retrograde coupling does not occur in dyspedic myotubes in which the L-type Ca^{++} current and voltage-induced Ca^{++} release is abolished, but may be restored by expression of RYR1, but not the cardiac RYR isoform (RYR2) (Nakai *et al.*, 1998). Thus, retrograde coupling is isolated to skeletal muscle, although the physiological significance is not yet clear.

It is important to contrast the mode of E-C coupling in skeletal muscle to cardiac muscle to highlight the distinct characteristics of each mode. While skeletal muscle employs an extracelluar Ca^{++} independent mode of E-C coupling (i.e., votage-induced SR Ca^{++} release), cardiac muscle relies on extracellular Ca^{++} dependent mode of E-C coupling (i.e., Ca^{++} induced Ca^{++} release; CICR). Briefly, CICR is the process wherein an action potential depolarizes the cardiac myocyte membrane. The drop in membrane potential opens the cardiac DHPR allowing extracellular Ca^{++} to enter the cytosol. The localized increase in Ca^{++} entry proceeds to open the cardiac RYR (RYR2), which results in a SR Ca^{++} efflux into the cytosol providing a robust stimulus for force production (Lamb, 2000). There are number of contrasting characteristics of both skeletal and cardiac DHPRs and RYRs that support their respective mode of E-C coupling. For example, the cardiac DHPR exhibits a greater Ca^{++} current that is initiated at a more negative membrane potential than the skeletal muscle DHPR (Tanabe *et al.*, 1990). Additionally, RYR2 has a greater sensitivity to Ca^{++} activation in the presence of physiological levels of Mg^{++} than RYR1 (Lamb, 2000). However, as demonstrated above, potentially the most important difference between these tissue specific channel isoforms is the ability for the skeletal muscle DHPR and RYR1 to physically couple (Suda et al., 1997; Tanabe et al., 1988).

Physical interaction between DHPR and RYR1 is crucial for the initiation of E-C coupling in skeletal muscle. Based on the discussion above, it is clear that the specific skeletal muscle DHPR and RYR isoforms promote physical coupling. The functional implication of this coupling is demonstrated by Tanabe et al. (1990) who reported that expression of the α_{1s} and not α_{1c} (cardiac version) subunit of DHPR in dysgenic cells was required for voltage-induced SR Ca^{++} release in the absence of extracellular Ca^{++}, as well as Nakai et al. (1998) who reported that expression of RYR2 in dyspedic myotubes cannot support voltage-induced Ca^{++} release.

Equally as important, although potentially less tissue specific, is the expression of triadic proteins that promote the union of the key skeletal muscle E-C coupling proteins. In the absence of associating triadic proteins DHPR-RYR1 junctions do not form (Suda et al., 1997; Takekura et al., 1995). Importantly, in these studies the DHPR exhibited a L-Type Ca^{++} current and RYR1 was capable of being activated with Ca^{++} or caffeine. However, electrical stimulation only elicited a Ca^{++} -induced Ca^{++} release from the ER, and when electrical stimulation was performed with 0 mM extracellular Ca^{++}, no ER Ca^{++} release was observed (Suda et al., 1997; Takekura et al., 1995). Therefore, it appears that in spite of expressing skeletal muscle DHPR and RYR1, if skeletal muscle accessory triadic proteins are not expressed then the cell does not support voltage-induced Ca^{++} release.

In support of this finding, ablation of JP45, which decreased the density of DHPRs presumably in the triadic T tubule membrane, also significantly decreased DHPR peak charge movement, RYR1 peak Ca^{++} transient, and whole muscle twitch and tetanic force production, suggesting that proper localization of DHPRs in the triad is critical to voltage-induced E-C coupling (Delbono et al., 2007). Additionally, JP1 and JP2 knock-down in adult muscle, which resulted in marked triad deformation, reduced voltage induced Ca^{++} release in single fibers by

~50% (1 Hz; 0 mM $[Ca^{++}]_o$) (Hirata et al., 2006). Thus, it is clear that accessory triadic proteins are required for *in vivo* skeletal muscle E-C coupling.

RYR1 is a Molecular "Hub"

The ryanodine receptor appears to be a molecular "hub" in the cell that is modulated by a number of factors including endogenous molecules (i.e., Ca^{++}, Mg^{++}, & ATP), pharmaceutical agents (e.g., caffeine & 4-chloro-m-cresol), cytosolic and luminal proteins (e.g., FKBP12, calmodulin, and calsequestrin), enzymes (glyocgen phosphorylase), and cellular redox status.

On the cytosolic portion of RYR1, each subunit is thought to have two ATP binding sites, indicating that a total of 8 ATP molecules may bind with RYR1 (Dias *et al.*, 2006). ATP may activate RYR1, in the absence and presence of cytosolic Ca^{++} (Godt & Maughan, 1988). Additionally, the cytosolic portion of each RYR1 subunit bears an activation site as well as an inactivation site that may bind Ca^{++} or Mg^{++} (Lamb, 2000). Ca^{++} binds with a greater affinity than Mg^{++} to the activation site (Laver *et al.*, 1997). While Mg^{++} binding does not open RYR1, Ca^{++} binding (1 μM Ca^{++}) to the activation site of isolated RYR1 increases the open probability of the channel. Conversely, the inactivation site binds Ca^{++} and Mg^{++} with equal affinity. Isolated RYR1 are inactivated by 1 mM cytosolic $[Ca^{++}]$ (Laver et al., 1997; Meissner, 1986, 1994). However, under resting conditions where cytosolic $[Ca^{++}]$ and $[Mg^{++}]$ are approximately 50 nM and 1 mM respectively, Mg^{++} would predominantly bind and inactivate the channel. Interestingly, in the presence of Mg^{++}, isolated RYR1 open channel probability is greatly reduced ($P_o \approx 0.10$) across a physiologically relevant range of $[Ca^{++}]$ ($\sim 10^{-7} - 10^{-2}$ M), which suggests that at physiological concentrations of Mg^{++} isolated RYR1 are "stuck" in a closed state (Lamb, 2000). Clearly, this is not the case *in vivo*, as RYR1 opens in response to the DHPR charge movement. Therefore, DHPR-RYR1 coupling and potentially associative proteins (e.g.,

FKBP12) must reduce the sensitivity of Mg^{++} inhibition on RYR1 (Lamb & Stephenson, 1994). Of course, this would allow for coupled receptors to become more responsive to cytosolic Ca^{++} (for both activation and inactivation) and more importantly to enter an open state upon activation, but does not speak to the inhibitory effect of Mg^{++} on the open probability of uncoupled RYR1. To date, it is unknown how uncoupled RYR1 are opened during E-C coupling.

Luminal calcium content may also regulate SR Ca^{++} release and force production. During repeated activation of a muscle fiber, not all of the released Ca^{++} is returned to the SR. Instead, other organelles (e.g., mitochondria) and Ca^{++} binding proteins (e.g., parvalbumin) may buffer some Ca^{++} or Ca^{++} may leave the cell via various ion channels or pumps, which can result in SR Ca^{++} store depletion (Gissel, 2005). Demonstrating that reductions in SR Ca^{++} content regulates RYR1 Ca^{++} release, Dutka et al. (2005) reported that a ~35% reduction in SR Ca^{++} content resulted in an ~45% reduction in peak tetanic force, despite the fact that there was still enough Ca^{++} stored in the SR to elicit maximal tetanic force.

A portion of the luminal Ca^{++} regulation is mediated via interaction between RYR1 and calsequestrin. At resting levels of free SR Ca^{++} (~1mM), RYR1, calsequestrin, junctin, and triadin form a quaternary complex that inhibits SR Ca^{++} release (Rossi & Dirksen, 2006). Both junctin and triadin bind to RYR1 and therefore localize calsequestrin (and a large amount of Ca^{++}) near the release channel. Triadin and junctin binding to calsequestrin appears to be [Ca^{++}] dependent, whereby higher and lower concentrations will result in the release of calsequestrin and thus the removal of RYR1 inhibition (Zhang *et al.*, 1997).

Decrements in SR Ca^{++} stores are clearly not advantageous for force production. To counteract the lose of Ca^{++} during repeated fiber activation, the SR Ca^{++} stores are “monitored”

and presumably upon store depletion a signal is sent from the SR to the T tubule membrane to allow the entry of extracellular Ca^{++} for SR store replenishment. This process is called store operated Ca^{++} entry (SOCE). The molecular mechanism underlying this process is not entirely understood. Currently, stromal interaction molecule 1 (STIM1) is thought to be the SR Ca^{++} sensor involved in SOCE (Stiber *et al.*, 2008). However, the T tubule membrane channel has not been conclusively determined. Candidates include Orai1 and transient receptor ion channels (TRPCs), and it may be that complexes of both proteins comprise the SOCE channel (Liao *et al.*, 2008). Malfunction of SOCE is thought to play a role in high intensity (frequency) fatigue (Germinario *et al.*, 2008). Interestingly, animal models that no longer express triadic proteins known to assist in the development and maintenance of triads [JP1, JP2, & mitsugumin 29 (MG29)] exhibit reductions in SOCE and greater fatigability (Hirata et al., 2006; Nagaraj *et al.*, 2000). Conversely, in one animal model in which ablation of a SR luminal protein (sarculumenin), whose function is unknown, resulted in an increased expression of MG29, SOCE was enhanced and fatigability was reduced (Zhao *et al.*, 2005). While purely speculative, these findings do suggest that high-intensity exercise may disrupt triads, hinder SOCE, and thus promote fatigue in active muscle fibers.

RYR1 channel function can be modulated by a number of pharmacological agents. For example, at nanomolar concentrations, the plant alkaloid, ryanodine, activates RYR1, while at higher concentrations (>100 microM), ryanodine closes RYR1 (Carroll *et al.*, 1991). Ruthenium red has been used to study RYR1 function because of its ability to close RYR1 (Stoyanovsky *et al.*, 1997). Additionally, dantrolene has been found to be an effective treatment for malignant hyperthermic episodes because of its ability to stabilize RYR1 in a closed state (Cherednichenko *et al.*, 2008).

Both 4-chloro-*m*-cresol and caffeine have been used to study E-C coupling because each of these compounds directly activate RYR1 and therefore by-pass the voltage-gated DHPR (Balnave & Allen, 1995; Ingalls et al., 1998b). This strategy allows for the assessment of SR Ca^{++} content, RYR1 functionality, and, under some assumptions (e.g., constant Ca^{++} sensitivity), contractile protein integrity. Both compounds appear to increase SR Ca^{++} release in response to voltage and Ca^{++} activation (Corona et al., 2008a; Lamb *et al.*, 2001), however, 4-cholor-*m*-cresol is a 10 – 25% more potent activator of RYR1 than caffeine (Fessenden & Ehleringer, 2003; Westerblad *et al.*, 1998).

Ryanodine receptor binding by various proteins in the cytosol such as FKBP12/12.6 and calmodulin, and in the SR lumen, such as calseqeustrin, junctin, and triadin, appear to be crucial for normal channel function. Skeletal muscle expresses a 12 and 12.6 kDa protein that binds to the immunosuppressant drug FK506 (FKBP12 and FKBP12.6). Each subunit of RYR1 has an FKBP12/12.6 binding site, which has a greater binding affinity for FKBP12.6 than FKBP 12, however, because there is a higher concentration of FKBP12 than FKBP12.6, FKBP12 predominantly binds RYR1 (Chelu *et al.*, 2004). FKBP12 appears to modulate isolated RYR1 channel function by stabilizing the channel in a closed state and by coordinating the actions of each RYR1 subunit (Ahern *et al.*, 1997; Brillantes *et al.*, 1994; Gaburjakova *et al.*, 2001). Additionally, FKBP12 may play a significant role in RYR1 coupled gating, or the gating of uncoupled RYR1s by RYR1s coupled to DHPRs (Marx *et al.*, 1998). Maintenance of a resting closed channel state via FKBP12-RYR1 binding appears to be important for E-C coupling gain, as myotubes and EDL muscle in which FKBP12-RYR1 interaction was eliminated show signs of diminished E-C coupling gain (Avila *et al.*, 2003; Tang *et al.*, 2004). However, these alterations were not observed in the diaphragm or soleus muscles from FKBP12 knock-out mice raising the

possibility that FKBP12 may augment E-C coupling gain in a muscle specific manner (Tang et al., 2004).

Calmodulin is a 17 Kda protein with four Ca^{++} binding domains (E-F Hands) that is prevalent in skeletal muscle (Tang *et al.*, 2002). Each subunit of RYR1 has a calmodulin binding domain on its cytoplasmic face (Wagenknecht *et al.*, 1994). Single channel and binding assay studies indicate that calmodulin activates and inhibits RYR1 at nanomolar and micromolar [Ca^{++}] respectively (Buratti *et al.*, 1995; Tripathy *et al.*, 1995). Additionally, calmodulin may also bind to the α_1-subunit of the DHPR, although the functional consequence of this binding has not been determined (Tang et al., 2002). Interestingly, the binding sites for calmodulin on the skeletal muscle DHPR and RYR1 may be in close proximity to where these channels interact, suggesting that the effects of calmodulin binding to either channel *in vivo* may be different than those identified in single channel studies (Tang et al., 2002). The exact role that calmodulin plays in skeletal muscle E-C coupling has not been determined.

Muscle glycogen depletion has been associated with reduced force producing capacity during fatiguing exercise, however, it is not entirely understood how glycogen depletion alters the production of force (Allen et al., 2008). Partially explaining the effects of the decrease in force, mouse flexor digitorum brevis muscle that performed repeated tetanic contractions exhibited a 25% decrease in glycogen content and a 30% decrease in tetanic force. Muscle fibers from these mice exhibited a ~25% decrease in voltage-induced Ca^{++} transients, suggesting that glycogen content may modulate RYR1 channel activity (Chin & Allen, 1997). Alternatively, reductions in glycogen associated with the SR could also result in T tubule membrane depolarization, since the Na^+ - K^+ pump is thought to require glycolysis derived ATP (Dutka & Lamb, 2007), which would also result in a reduction in voltage-induced SR Ca^{++} release.

The reductive and oxidative (redox) conditions surrounding RYR1 can alter channel function. Each RYR1 subunit possesses 100 cysteine residues, which have thiol groups that may be reactive to redox conditions (i.e., reactive thiol groups may be directly oxidized or covalently modified via S-glutathionylation & S-nitrosylation) (Stamler & Meissner, 2001). Oxidation or reduction of these thiol groups can effectively alter RYR1 conformation promoting a greater probability of channel opening or closure. Both reactive oxygen species (ROS) and nitric oxide (NO) are produced constitutively in skeletal muscle, with increased production of both ROS and NO occurring during activity (Reid, 1998, 2001; Reid & Durham, 2002; Smith & Reid, 2006; Stamler & Meissner, 2001). ROS are primarily produced in the mitochondria, but may be produced in the cytosol as well (e.g., via sarcolemmal NADPH oxidase) (Smith & Reid, 2006). NO is also produced in the mitochondria and cytosol by different nitric oxide synthase (NOS) isoforms (endothelial and neuronal NOS respectively), in a Ca^{++} and calmodulin dependent manner (Stamler & Meissner, 2001; J. G. Tidball *et al.*, 1998). Studies in which RYR1 is oxidized *in vitro* via application of redox reactive agents (e.g., hydrogen peroxide) have indicated that RYR1 oxidation increases RYR1 open probability, sensitivity to activators (e.g., caffeine), and Ca^{++}-induced Ca^{++} release (CICR), while decreasing the sensitivity of the channel to inhibition by Mg^{++} (Aracena *et al.*, 2003; Donoso *et al.*, 2000; Favero *et al.*, 1995; Oba *et al.*, 2002; Zaidi *et al.*, 1989). Similar studies describing the effect of NO on RYR1 function indicate that NO may either reduce or increase channel activity (Meszaros *et al.*, 1996; Stoyanovsky et al., 1997). The disparity of these results is likely tied to the different experimental conditions used in each study (Stamler & Meissner, 2001). In the case where NO reduces RYR1 channel activity, this effect may be the direct result of NO modification of the channel or the result of antioxidant effects of NO.

ROS and NO alter force production differently. ROS appear to increase maximal and submaximal force production (Reid *et al.*, 1993). However, ROS may exert this effect at the level of the myofilaments as opposed to E-C coupling proteins (Andrade *et al.*, 2001; Smith & Reid, 2006). In support, Posterino et al. (2003) reported that ROS (H_2O_2) do not increase RYR1 Ca^{++} release during voltage-induced twitch or tetanic contractions, although force production is increased. Thus, it would appear that, in non-fatigued muscle under conditions in which the T tubule membrane is fully depolarized, ROS exert their influence on troponin C Ca^{++} sensitivity and not E-C coupling gain. On the other hand, NO appears to depress submaximal force production in skeletal muscle (Kobzik *et al.*, 1994). NO may depress force production by increasing production of cyclic GMP, which reduces force production through an unknown mechanism, and a cyclic GMP independent mechanism, which may include reducing SR Ca^{++} release and/or opposing the effects of ROS on RYR1 and the myofilaments (Kobzik et al., 1994; Reid *et al.*, 1998; Stamler & Meissner, 2001).

While RYR1 oxidation does not appear to influence SR Ca^{++} release under normal conditions, RYR1 Ca^{++} release is likely augmented by oxidizing agents when E-C coupling is disrupted (Posterino et al., 2003). Posterino and colleagues (2003) reported that application of hydrogen peroxide to mechanically skinned muscle fibers enhanced CICR and, when E-C coupling was disturbed (i.e., the T system was only partially depolarized), augmented twitch force. Presumably, under conditions in which voltage induced Ca^{++} release would be diminished, augmentation of twitch force by oxidizing agents is the result of a greater reliance on RYR1 Ca^{++} induced Ca^{++} release.

Muscle Actions and Force Production

Skeletal muscle can perform isometric, shortening (concentric), or lengthening (eccentric) muscle actions (contractions) to produce force. An isometric contraction has no length change and thus a shortening or lengthening velocity of zero. During this type of contraction, muscle fiber force production is determined by muscle length, in addition to other factors (e.g., physiological cross-sectional area, PCSA). The isometric muscle length-tension relationship indicates that tension rises, plateaus, and then descends at shorter, optimal, and longer muscle lengths respectively (Gordon *et al.*, 1966; Josephson, 1999). The force produced corresponds to sarcomere lengths at which actin-myosin overlap progressively increases until it is optimal and then decreases (Gordon et al., 1966). During concentric contractions, the fiber shortens and therefore has a positive velocity. In this case, the amount of force produced during a concentric contraction is not only dependent on the initial length of the muscle, but also the magnitude of shortening velocity. A.V. Hill described the effects of shortening velocity on muscle force production in 1938. Notably, muscle force production falls precipitously as shortening velocity increases (e.g., ~10% of maximal velocity shortening results in a ~35% decrease in force from P_o), indicating from a practical perspective that an object with a relatively large mass must be moved at a relatively slow shortening velocity (Hill, 1938). Because concentric contractions by definition must have a positive velocity, maximal peak isometric force (P_o) is greater than concentric force, assuming that all other things are equal (i.e., muscle size, architecture, etc.)

In contrast to isometric and concentric contractions, during an eccentric contraction the force produced by the muscle is less in magnitude and opposite in direction than the force applied by an external load, which results in active lengthening of the muscle. Eccentric

contractions therefore have a negative velocity. However, interestingly maximal eccentric force is relatively velocity-independent and is significantly greater in magnitude (≥ 50%) than maximal isometric force (P_o) (Corona et al., 2008a, 2008b; Friden & Lieber, 1992).

The greater peak force and velocity independence of force production during eccentric contractions may be explained by differences between contraction types at the cross-bridge level. The cross-bridge theory proposed by Huxley (1957) does not appear to accurately describe the mechanics and metabolism of eccentric contractions. Part of the cross-bridge theory indicates that for myosin and actin to dissociate ATP must first bind to myosin (Huxley, 1957). However, detachment of the myosin head from actin is not necessarily propagated by ATP hydrolysis during an eccentric contraction. Instead actomyosin bonds may be pulled apart as the sarcomeres are actively lengthened (Flitney & Hirst, 1978). It has been shown in single muscle fiber experiments that detachment of myosin heads from actin without ATP hydrolysis results in an enhanced rate of reattachment in a strong binding state (Lombardi & Piazzesi, 1990). Thus, during eccentric contractions there may be more myosin heads strongly bound to actin that are producing force than during an isometric contraction of similar duration (Linari *et al.*, 2000).

Active force production in skeletal muscle requires that myosin and actin interact. From the abbreviated description above of the cross-bridge cycle, it is apparent that ATP plays a key role in this process. However, it is important to remember that Ca^{++} must bind with troponin C, which removes tropomyosin from the myosin binding site on actin, for myosin and actin to interact. Thus, at the most basic level, skeletal muscle active force production results from the repetitive interaction of a motor (myosin) with a highly organized molecular pathway (actin), which is dependent on energy (ATP) and an electrical signal (voltage-induced Ca^{++} release).

General Description of Contraction-Induced Muscle Injury

Skeletal muscle injury is defined as "damage or harm to a tissue that functions to produce motion, and is associated with a prolonged (i.e., days to weeks) impairment of muscle function" (Warren *et al.*, 1999b). Muscle may be injured by a variety of stressors (i.e., crush injury, cryo-injury, chemical injury); however, the most commonly observed muscle injury is induced by contractions. Immediately after contraction-induced muscle injury, force-bearing proteins are damaged (Lieber et al., 1996), T Tubule and SR membrane cellular arrangement is disorganized (Takekura et al., 2001; Warren et al., 1995), and sarcolemmal integrity may be compromised (Armstrong *et al.*, 1983; Warren et al., 2002a). A number of exercise-induced muscle injury symptoms are frequently documented in the literature to indicate the presence of muscle injury. For example, increased serum levels of creatine kinase and muscle soreness are commonly used as evidence of muscle injury in human studies (Warren et al., 1999b). However, the most meaningful manifestation of muscle injury is the loss of muscle force producing capacity. Following the performance of eccentric contractions, strength is immediately diminished and remains depressed for an extended amount of time (days to weeks). In a well-documented mouse anterior crural muscle eccentric contraction injury protocol, the greatest deficit in peak isometric force is observed early (0-3 days) after the muscle is injured and does not fully recover until ~28 days later (Ingalls et al., 1998a; Lowe et al., 1995). Over the course of this period of time, the primary cause of strength deficits changes from failure to activate myofilaments to the actual loss of contractile protein (Balnave & Allen, 1995; Ingalls et al., 1998a; Ingalls et al., 1998b; Lowe et al., 1995; Warren et al., 2002b; Warren et al., 1993c). The underlying cause of strength deficits through time will be discussed in greater detail below.

Basis of Contraction-Induced Muscle Injury: Metabolic vs. Mechanical

Metabolic Basis of Injury

The exact mechanism explaining how eccentrically biased exercise injures muscle has not been determined. However, Armstrong et al. (1991) has proposed that the initial stage of the injury mechanism is believed to be mechanical, as opposed to metabolic, in nature. In theory, a metabolic initiation of muscle injury is based on a deficiency to meet the increasing energy demands of the exercise. It is suggested that a growing disparity between ATP supply and ATP demand will result in an inability to energize various endergonic cellular mechanisms (i.e. Na^+-K^+ ATPase and Ca^{++} ATPase) thereby jeopardizing the homeostatic integrity of the cell (Armstrong et al., 1991). However, the metabolic cost to perform concentric contractions is greater than the cost for eccentric contractions (Constable *et al.*, 1976; Enoka, 1996; Newham *et al.*, 1983b; Ryschon *et al.*, 1997), and yet it is the performance of eccentric contractions and not isometric or concentric contractions that induces muscle injury (Armstrong et al., 1983; Warren et al., 1993c).

Patel and colleagues (1998) tested the hypothesis that muscle injury is metabolically induced in a clever study that took advantage of the metabolic plasticity of muscle. They observed that type II fibers were predominantly injured during eccentric exercise, and postulated that this preferential injury could have been due to a lower oxidative capacity in these fibers. To test this hypothesis they increased the oxidative capacity of rabbit anterior compartment muscles by pre-treating the muscles with low frequency (10 Hz) electric stimulation intermittently for four weeks. Increased oxidative capacity was indicated by significant increases in citrate synthase activity and capillary density. However, P_o deficits in animals with higher oxidative

capacity were not different than control animals following injury, indicating that an increased oxidative capacity does not protect fibers from injury (Patel et al., 1998).

Some researchers have contended that the initiation of muscle injury does have a metabolic component, especially during prolonged endurance exercise. Theoretically, enhanced free radical production, via the electron transport chain, during prolonged exercise can damage membrane lipids and induce a cascade of events leading to muscle injury (Close *et al.*, 2005). There are reports of prolonged cycling and endurance running resulting in muscle soreness, increased blood levels of muscle-borne proteins (e.g., creatine kinase and lactate dehyodrogenase), prolonged strength deficits, and even satellite cell infiltration (Goodman *et al.*, 1997; Mackey *et al.*, 2007; Mastaloudis *et al.*, 2006; Romano-Ely *et al.*, 2006; Saunders *et al.*, 2007). However, antioxidants, which should alleviate ROS induced muscle damage, do not appear to reduce muscle injury after a 50 km run (Mastaloudis et al., 2006). Additionally, we have observed that inhibition of nitric oxide synthase activity during and after eccentric contractions actually enhances EDL muscle *in vitro* force deficits, although this may be due to the attenuation of antioxidant effects of NO (*unpublished findings*).

Nevertheless, it should be noted that eccentric contractions are performed during the stance phase of the gait cycle (Josephson, 1999) and that the number of eccentric contractions performed is directly related to the magnitude of muscle injury (Hesselink et al., 1996; Warren et al., 1993b). Therefore, during a marathon in which it is estimated that ~10,000 eccentric contractions are performed (Horita *et al.*, 1999) it is likely that muscle injury is due to the repetitive performance of eccentric contractions and not metabolic stress.

Mechanical Basis of Injury

Performance of maximal eccentric contractions places great mechanical stress and strain on force-bearing components in skeletal muscle, which may result in injury to these cellular constituents (McCully & Faulkner, 1986; Warren *et al.*, 1993a). Mechanical strain refers to the "stretchiness" of the muscle tendon complex and is defined as the change in length relative to initial length. Mechanical stress refers to the amount of the force generated by the muscle at a given muscle length and is defined as force divided by the cross-sectional area of the tissue. A stress-strain curve indicates that muscles may stretch while activated and return to the initial length until the stress exceeds the yield strength. However, when both strain and stress increase to the point where stress equals the tensile strength of the muscle, components of the active fiber may fail (Warren et al., 1993a). Thus, mechanically sensitive elements in muscle fibers may be damaged by exposure to both high stress and strain during eccentric contractions.

There are a number of studies to date that have determined that the unique mechanical properties of eccentric contractions cause muscle injury (Hesselink et al., 1996; Lieber & Friden, 1993; McCully & Faulkner, 1986; Talbot & Morgan, 1998; Warren et al., 1993a; Warren et al., 1993b). However, within the literature there appears to be some debate as to whether peak mechanical stress or peak mechanical strain during an eccentric contraction is the primary determinant of the magnitude of muscle injury. The argument between peak mechanical stress and strain is exemplified by the findings of McCully and Faulkner (1986). Using an *in situ* mouse extensor digitorum longus muscle preparation, muscles were electrically stimulated (150 Hz) and lengthened (20% of fiber length) at varying contraction velocities and durations. The magnitude of injury was assessed 3 days post-injury by measuring histological alterations and reductions in isometric strength. The results indicated that the peak force generated during the

eccentric protocol was primarily associated with the reductions in isometric strength (r = -0.70). Interestingly, when the amount of force produced between eccentric, concentric, and isometric muscle contractions were similar (85% P_o), only the eccentric contractions resulted in muscle injury (McCully & Faulkner, 1986). These results were interpreted as circumstantial evidence that the lengthening component of an eccentric contraction and not just the peak force generated promotes the incidence of muscle injury.

Suggestive that muscle strain is the predominant cause of injury, Lieber and Friden (Lieber & Friden, 1993) reported that the magnitude of mechanical strain was the primary predictor of reductions in isometric force following eccentric contractions performed by isolated rat tibialis anterior muscle. In this study, muscles were allotted to a 25% or 12.5% strain group, with the 25% strain group being comprised of a high force (~1500 g) and moderate force (~1300 g) sub-group and the 12.5 strain group being comprised of a moderate force (1300 g) and low force (1200 g) sub-group. Because the 25% strain group exhibited greater strength deficits than the 12.5% strain group (~60 vs. ~40% respectively) regardless of peak force production, the data suggest that peak strain was the primary determinant of muscle injury (Lieber & Friden, 1993). However, by not controlling for force production while manipulating strain, the interpretation of these results may be fundamentally flawed.

Interestingly, the same research group reported in a subsequent study that strain experienced during a lengthening contraction protocol did not associate with the incidence of injury (Peters *et al.*, 2003). In this study, fibers from EDL and TA muscles were stained using immunochemistry to detect the absence or presence of desmin and embryonic myosin heavy chain respectively. The researchers found that although the EDL muscle experienced twice the

amount of strain than the TA muscle, EDL muscle fibers did not show any alteration in staining pre- to post-injury while the TA muscle fibers showed evidence of injury (Peters et al., 2003).

Comparatively, Talbot and Morgan (1998) investigated the effects of varying strain during eccentric contractions using toad sartorius muscle and found that the initial length at which the muscle begins an eccentric contraction mostly determines the degree of isometric force deficit. Peak tension during the first stretch of the lengthening protocol was not significantly correlated with the magnitude of strength loss even though the correlation coefficient was still high ($r = -0.75$; $p = 0.98$). However, there are methodological concerns when applying these findings to mammalian skeletal muscle. First, to assess the contribution of initial muscle length to the magnitude of muscle injury, muscles were lengthened by as much as ~33% of L_o before being actively stretched by 10% L_o (Talbot & Morgan, 1998). Thus, during each eccentric contraction muscles were lengthened to ~140% of L_o, which is much greater than would likely be experienced by a mammalian muscle under a "physiological" setting. This "over-stretching" of muscle could result in overestimation of the emphasis of initial muscle length on immediate force deficits. Second, these experiments were conducted at 20 to 22 ºC, which is suitable for amphibian muscle but grossly underestimates the temperatures at which mammalian muscle normally functions (human & mouse; ~37˚C). Because temperature is directly related to peak eccentric force production and consequent isometric force deficits (Warren et al., 2002a), it is possible that these lower temperatures masked a significant relationship between peak eccentric force and isometric force deficits.

Warren and colleagues (1993a) conducted a highly-controlled study to determine which mechanical factor, strain or stress, predominantly induced muscle injury. Forty-two different eccentric protocols, each controlling for peak eccentric contraction force, length change,

lengthening velocity, and initial muscle length were employed to injure rat isolated soleus muscle. The results revealed that immediate peak isometric force deficits were primarily related to peak forces (stress) produced during the eccentric contractions. Importantly, these experiments were conducted at 37˚C and muscle lengths were kept within a physiological range. The results of this study indicate that 1) muscle injury is indeed mechanically induced and 2) peak force during eccentric contractions performed at 37˚C is the primary determinant of the magnitude of mammalian muscle injury when muscle length is kept within a physiological range (Warren et al., 1993a).

Studies comparing level versus downhill treadmill running highlight that exercise-induced muscle injury has a mechanical basis. At similar running speeds, the rate of oxygen consumption (dotVO_2) during uphill running, which has a pronounced quadriceps concentric contraction component, is higher than during downhill running, which has a pronounced quadriceps eccentric contraction component. Yet, it has been shown in humans and animals that downhill running often results in skeletal muscle injury (Armstrong et al., 1983; Green *et al.*, 2008; Minetti *et al.*, 2002). Based on these findings, contraction-induced muscle injury most likely stems from the high mechanical loads placed on muscle tissue during the performance of eccentric contractions.

Experimental Factors Affecting the Magnitude of Force Deficits

Mechanical Factors

There are a number of experimental factors that can be manipulated in an injury protocol that have an impact on the magnitude of force deficits. From the above discussion on stress and strain, clearly peak eccentric force, the initial muscle length, and the total change in muscle length can play a role in determining the severity of strength deficits (Lieber & Friden, 1993;

McCully & Faulkner, 1986; Talbot & Morgan, 1998; Warren et al., 1993a). For instance, rat soleus muscle strength deficits were ~50% greater when initial peak eccentric force was ~150% versus 125% of P_o during five eccentric contractions (Warren et al., 1993a). Additionally, Talbot and Morgan (1998) clearly demonstrated the effect of initial muscle length on strength deficits (albeit at non-physiological lengths), as they reported a significant correlation between initial muscle length and force deficits ($r = 0.960$).

Number of Eccentric Contractions

The number of eccentric contractions performed during an injury protocol may also determine the magnitude of immediate force deficits (Hesselink et al., 1996; Warren et al., 1993b). Whereas peak eccentric force may injure muscle fibers under the "normal stress theory mechanism of failure", which states that failure occurs when the tensile strength is greater than the yield strength of a cellular component, repetitive eccentric contractions may injure muscle under a "materials fatigue mechanism" (Warren et al., 1993a). Demonstrating materials fatigue, rat tibialis anterior muscle exhibited an increase in the % of damaged fibers (Hematoxylin & Eosin staining) from 1.2 to 14.8 and 36.2% twenty-four hours after performing 60, 180, and 300 *in vivo* eccentric contractions respectively (Hesselink et al., 1996). Similarly, rat soleus muscle exhibited a precipitous decrease in *in vitro* isometric tetanic force production after the performance eight eccentric contractions, but not after contractions 1 through 7 (Warren et al., 1993b). Based on these studies, it appears that the number of eccentric contractions performed can partly determine the magnitude of muscle injury.

Repeated Eccentric Bouts

Skeletal muscle may adapt to a single bout of eccentric contractions, whereby the muscle is injured significantly less following a subsequent injury bout. The "protection" from eccentric contractions afforded by an initial injury bout is termed the repeated bout effect (Nosaka & Clarkson, 1995). In humans, eccentric contraction-induced muscle injury is often characterized by increases of muscle proteins in the blood (e.g., creatine kinase), muscle stiffness, soreness, and strength deficits (Clarkson & Sayers, 1999; Green et al., 2008; Warren et al., 1999b). An initial bout of eccentric contractions can attenuate these perturbations following a second injury bout (Ingalls et al., 2004b; McHugh, 2003). For example, peak tetanic force produced by mouse anterior crural muscles following a single injury bout of 150 eccentric contractions is reduced by ~50%, while force is only reduced by ~40% following an identical injury bout (Ingalls et al., 2004b). However, some studies have reported that immediate strength deficits following subsequent injury bouts are not significantly reduced after an initial injury bout (Corona et al., 2008b; Newham *et al.*, 1987). For example, following each of three bouts of maximal voluntary eccentric contractions performed by humans with their elbow flexors, immediate strength deficits were similar (~50%) (Newham et al., 1987). Therefore, it appears that there is little to no change in immediate strength deficits and repeated injury bout (Corona et al., 2008b; Ingalls et al., 2004b). Nevertheless, the rate at which force is recovered after repeated bouts of eccentric contractions is enhanced. For example, three days after four weekly bouts of eccentric contractions mouse anterior crural muscle isometric tetanic torque was only reduced by ~20% as opposed to ~50% following an initial injury bout (Corona et al., 2008a).

Muscle Temperature during Eccentric Contractions

Experiments performed at sub-physiological temperatures (e.g., 15 – 30˚C) indicate that hypothermic conditions will, in general, reduce the deleterious effects of a variety of injury models on skeletal and cardiac muscle. For example, there are numerous reports of hypothermic conditions during skeletal muscle ischemia helping to maintain sarcolemmal integrity and muscle function upon reperfusion at physiological temperatures (Bolognesi *et al.*, 1996; Gurke *et al.*, 2000; Wilson *et al.*, 1997). Similarly, maintenance of isolated cardiac muscle at 22˚C attenuated sarcolemmal damage in response to the removal and subsequent replenishment of extracellular [Ca^{++}] from the bathing medium (i.e., subphysiological temperatures protect muscle from the Ca^{++} paradox) (Ganote & Sims, 1984).

Warren et al. (2002a) addressed the temperature dependency of contraction-induced muscle injury. *In vitro* studies were performed with mouse EDL muscle across a range of temperatures (15 – 37˚C). EDL muscles were either injured by performing five eccentric contractions or used as exercised controls by performing five concentric contractions. When EDL muscles were injured at 15˚C, isometric force deficits were not different from isometric control force values. However, as the experimental temperature increased the force deficits generally increased predominantly for the injured muscle. For instance, at 25 – 30˚C injured EDL muscles exhibited a ~15% decrease of P_o, while controls exhibited no deficit. At 37˚C, P_o was reduced by ~30% for the injured muscles and ~5% for the isometric control muscles (Warren et al., 2002a).

In an effort to explain the temperature sensitivity of contraction induced muscle injury, the authors determined that the temperature dependent force deficits were not associated with sarcolemmal damage, intracellular Ca^{++} accumulation, reductions in SR Ca^{++} re-uptake rates, or

increases in Ca^{++} sensitive neutral protease activity (Warren et al., 2002a). Additionally, it does not appear that an enzymatic process mediated the temperature sensitive loss of force, as decreasing the temperature to 15˚C between eccentric contractions performed at 37˚C did not grossly attenuate force deficits (lowering the temperature during the rest interval should theoretically reduce enzyme activity). Instead, it appears that the force deficits are primarily due to a "failing component within the muscle whose integrity is temperature dependent" (Warren et al., 2002a). The authors suggest that the T tubule membrane may undergo a phase transition at temperatures between 25 – 30˚C that presumably plays a role in mediating force deficits (Warren et al., 2002a).

Causes of Eccentric Contraction-Induced Force Deficits

Myofiber Damage

Strength deficits following the performance of eccentric contractions are the most pronounced immediately and during the first few days after injury, but may persist for prolonged periods of time depending on the muscle injured, the novelty of the injury, and the severity of the injury protocol. Historically, immediate force deficits associated with muscle injury were thought to be due solely to damage of force bearing proteins (Armstrong et al., 1991). This assertion was based on the principle that muscle force production is directly related to a given muscle's functional area. However, throughout the literature there appears to be dissociation between the degree of damage and the magnitude of early force deficits.

There are reports of the magnitude of force deficits being greater than the presence of muscle damage (Hesselink et al., 1996; Lowe et al., 1995; McCully & Faulkner, 1986; Warren et al., 1994). For example, following the performance of 150 eccentric contractions *in vivo* by mouse anterior crural muscles, Lowe et al. (1995) reported that force was reduced immediately

by ~55% while less than 1% of the muscle fibers in the TA showed signs of damage. Similarly, Hesselink et al. (1996) indicated that rat anterior crural muscles (TA muscle) injured *in vivo* with 60 or 120 eccentric contractions exhibited damage in < 1 to ~5% of muscle fibers 24 hours after injury respectively. However, isometric torque was reduced by ~35 to 45%, when correcting for fatigue, for each respective condition at this time (Hesselink et al., 1996). Additionally, Warren et al. (1994) also reported that EDL muscle that had performed 15 eccentric contractions *in vitro* exhibited greater specific force deficits (~60%) than could be explained by the number of fibers that showed signs of damage per cross section (≤ 3%) immediately after the injury.

It is also possible to have robust muscle damage without a marked reduction in force production (Hortobagyi *et al.*, 1998; Newham *et al.*, 1983a; Newham et al., 1983b; Street & Ramsey, 1965). Street and Ramsey reported in 1965 that muscle damage per se does not necessarily disrupt force production. In these experiments, frog muscle fiber bundles underwent a unique crush-injury that resulted in the retraction of myofibrils from the point of injury, which left an empty tube surrounded solely by the sarcolemma. Amazingly, the majority of the fibers or fiber bundles analyzed retained 70 to 80% of their force producing capacity – One fiber even maintained 100% of its pre-injury force. The ability of the muscle to generate near maximal force despite being damaged was attributed to lateral transmission of force propagated by the still-intact sarcolemma (Street & Ramsey, 1965).

The contribution that damage to force bearing structures makes to immediate force deficits may also be determined via a functional approach. Application of maximally activating Ca^{++} or caffeine to skinned or intact fibers directly activates the myofilaments. Thus, a decrease in peak Ca^{++} of caffeine induced- force indicates that force bearing proteins have either been damaged or lost from the cell. Warren et al. (1994) found that EDL muscle voltage-induced

maximal isometric force was reduced by ~70% after the muscle performed 15 eccentric contractions *in vitro.* Meanwhile, maximal Ca^{++} activated force produced by single fibers from these injured EDL muscles was only decreased by ~34% (Warren et al., 1994). These findings indicate that damage to force bearing proteins may account for ~50% of the immediate force deficits with this injury model.

In comparison, Balnave and Allen (1995) reported that caffeine-induced force produced by single mouse flexor digitorum brevis (FDB) muscle fibers was not altered after performing 10 eccentric contractions *in vitro* (length change of 25% L_o), although voltage-induced peak isometric force (100 Hz) was reduced by ~20%. These findings suggest that damage to force-bearing structures did not contribute to the immediate strength deficits. Together the studies of Warren et al. (1994) and Balnave & Allen (1995) indicate that 0 to 50% of immediate force deficits are explained by damage to force-bearing proteins.

The interpretation of the above single fiber measurements assumes that the Ca^{++} sensitivity of the myofilaments is not altered immediately after performing eccentric contractions. However, it is possible that myofilament Ca^{++} sensitivity could be reduced by elevations of factors known to decrease the sensitivity of the myofilaments, such as inorganic phosphate or oxidative stress, after injury (Andrade et al., 2001; Godt & Nosek, 1989; Perkins *et al.*, 1997). Importantly, Balnave and Allen (1995) reported that myofilament Ca^{++} sensitivity was not significantly reduced in single fibers following a physiological muscle injury protocol, a finding made by measuring intracellular Ca^{++} transients during a force-frequency test.

Morgan and colleagues have proposed a theory explaining how muscle damage contributes to initial strength deficits (Morgan, 1990). Based on the previous observation that sarcomere inhomogeneity may exist on the descending limb of the sarcomere length tension

relationship (Gordon et al., 1966), Morgan proposed that when muscle fibers are lengthened, weaker sarcomeres will lengthen more than shorter stronger serial sarcomeres (Morgan, 1990), a finding recently supported by Faulkner's laboratory (Panchangam *et al.*, 2008). According to Morgan, weak sarcomeres will become even weaker as they move further down the descending limb of length tension curve, eventually reaching a point where thick and thin filaments do not overlap. Beyond this point, the passive structures of the lengthened sarcomere bear the stress and strain of adjacent myofibrils and serial sarcomeres respectively. As a result, the lengthened sarcomere may fail (Morgan & Allen, 1999; Proske & Morgan, 2001).

Morgan and colleagues postulate that 1) weak sarcomeres are located randomly throughout a muscle fiber, 2) that sarcomeres will become disrupted progressively based on initial vulnerability, 3) and that the sarcomere damage will eventually spread adjacently, damaging parallel myofibrils, the sarcolemma, and structures involved in the E-C coupling process (Morgan & Allen, 1999; Proske & Morgan, 2001). This group of researchers contend that sarcomeric damage may be the initial event in contraction-induced muscle injury.

It is possible that force production by the myofibrils is not diminished following muscle injury, but the transmission of force from the myofibrils ultimately to the muscle tendon is impeded (Gao *et al.*, 2008). Desmin is an integral part of the cyoskeleton that connects adjacent Z disks together and connects the Z disks to the sarcolemma forming a structure called a costamere and is involved in lateral transmission of force (K. Wang & Ramirez-Mitchell, 1983). Lieber et al. (1994) indicated that eccentric contractions reduced desmin protein content (Western blot) by 23 and 35% at 1 and 3 days after injury induction and that the reduction in desmin content was highly correlated with force deficits. These findings indicate that eccentric contractions may indeed damage desmin, which may result in force deficits and lends support to

the radial propagation of damage from "popped" sarcomeres (Morgan & Allen, 1999; Proske & Morgan, 2001).

However, in a subsequent study conducted by Lieber's research group, it was reported that only ~250 of ~15,000 fibers in rat TA muscle demonstrated abnormal immunohistochemical staining for desmin 12 hours after eccentric contraction injury induction (Peters et al., 2003). It was concluded by the authors that desmin could not possibly explain the ~45% isometric strength deficit observed immediately after the injury protocol, as complete functional failure of the desmin negative fibers would only explain a 2% force deficit (Peters et al., 2003).

Loss of Contractile Protein

In response to the physical damage to force-bearing proteins and potentially the sarcolemma, skeletal muscle exhibits a degenerative and regenerative process that initially removes proteins within and surrounding focal sites of injury and then eventually rebuilds the degraded areas (Ingalls et al., 1998a; Lowe et al., 1995; Newham et al., 1983a; J.G. Tidball, 2005). To partially explain the degeneration of muscle proteins following injury, it has been thought that contraction-induced sarcolemmal damage may allow extracellular Ca^{++} to accumulate within injured muscle fibers (Armstrong et al., 1991). In support, the performance of eccentric contractions has been shown to increase intracellular [Ca^{++}] (Balnave & Allen, 1995; Ingalls et al., 1998b; Lynch et al., 1997), total muscle [Ca^{++}] (Lowe *et al.*, 1994), and mitochondrial [Ca^{++}] (Duan et al., 1990) immediately and for 2 – 3 days after injury. Increases in intracellular [Ca^{++}] may activate Ca^{++} - sensitive degradative pathways (e.g. calpain, phospholipase A2, ROS production, proteasome), which may further damage force-bearing proteins and the sarcolemma (Armstrong et al., 1991; Clarkson & Sayers, 1999; Costelli *et al.*, 2005; K. J. Davies, 2001).

Because elevated intracellular [Ca^{++}] is observed immediately after injury, it has been suggested that myofiber Ca^{++} accumulation is driving initial functional deficits (Armstrong et al., 1991). However, it does not appear that a rise in intracellular [Ca^{++}], at least when the source of Ca^{++} is extracellular, plays a role in immediate strength deficits. Lowe et al. (1994) reported that increasing extracellular [Ca^{++}] to 5.0 mM from 1.25 mM following the performance of eccentric contractions did not promote greater strength deficits. Moreover, Ca^{++} activated neutral proteases (i.e., Calpain) do not appear to play a role in immediate strength deficits as incubation of EDL muscle with calpain inhibitors (e.g., luepeptin, calpeptin) before and during the performance of 5 eccentric contractions *in vitro* at 37˚C did not attenuate immediate force deficits (~ -30% P_o) (Warren et al., 2002a). Nevertheless, while eccentric contraction induced intracellular [Ca^{++}] elevations may not be involved in initial force deficits, the activation of Ca^{++} sensitive degradative pathways may contribute to prolonged force deficits.

Additionally, in response to physical damage to force bearing proteins an immune response is activated that also results in further degeneration of proteins in the injured muscle fiber (Lowe et al., 1995; J.G. Tidball, 2005). The time-course of the immune response is typified by macrophages invading injured skeletal muscle fibers within 24 hours after injury, with the presence of these phagocytic cells peaking between 2 to 3 days after injury induction (Armstrong et al., 1983; Lowe et al., 1995; McCully & Faulkner, 1985). Endogenous proteases and enhanced phagocytic activity in injured muscle fibers results in a marked increase of the rate of protein degradation by 2 to 3 days after injury induction (Ingalls et al., 1998a; Lowe et al., 1995). Consequently, contractile protein content begins to decrease in injured muscle around this time (~3 – 5 days), and remains depressed over the next two weeks (Ingalls et al., 1998a). Because contractile protein content is not significantly reduced until ~5 days after injury induction,

degeneration of force-bearing proteins does not likely play a significant role in early (0 – 3 days) strength deficits, but is the predominant cause of prolonged strength deficits (Ingalls et al., 1998a; Warren et al., 2002b).

While the immune response plays a large role in mediating the degeneration of injured muscle, it also appears to play a role in tissue regeneration as well (Summan *et al.*, 2006; J.G. Tidball, 2005; Warren *et al.*, 2005; Warren *et al.*, 2004a). For example, selective depletion of peripheral monocytes with systemic injections of liposomal clodronate resulted in a prolonged clearance of necrotic muscle fibers during the weeks after freeze-injury (Summan et al., 2006). This finding is possibly reflective of a depressed immune response-mediated satellite cell activation, which is required for muscle regeneration after freeze- and contraction-induced muscle injury (Rathbone *et al.*, 2003).

Excitation-Contraction Coupling Failure

Human studies in which low-frequency force was decreased by a greater extent than high-frequency force following eccentric contractions initially suggested that E-C coupling was disrupted in injured muscle(C. T. Davies & White, 1981; Newham et al., 1983b). For example, Newham et al. (1983b) reported that the twitch (10 Hz) to tetanic (50 Hz) force ratio was depressed ~20% more in an eccentrically than concentrically exercised leg. In order to assert that greater force deficits at low- than high-frequencies are indicative of E-C coupling, 1) the high frequency force must be located on the plateau region of the force-frequency relationship, 2) the low-frequency force must be located on the steep slope of this relationship, and 3) there must be a uniform decrease in the SR Ca^{++} transient at all stimulation frequencies (e.g., the Ca^{++} transient is reduced by 10% at all frequencies). Under these conditions, a similar reduction in SR Ca^{++} transient will reduce force production at lower frequencies to a greater extent than at

maximal frequencies. It should be noted, however, that this interpretation has been challenged as the greater deficits at lower frequencies could also be due to an increased compliance of muscle fibers after injury (Morgan & Allen, 1999; Proske & Morgan, 2001).

Warren et al. (1993c) were the first to provide evidence of E-C coupling failure immediately after the performance of eccentric contractions. In this study, voltage-induced tetanic (150 Hz) and caffeine-induced (50 mM) contracture force produced by mouse soleus muscle was assessed after the performance of 20 eccentric contractions or 20 isometric contractions *in vitro* (37˚C). Peak isometric force was reduced by ~43% immediately after performing 20 eccentric contractions, while there was no significant decrease in P_o after 20 isometric contractions (~ -4%). Caffeine-elicited force, which was ~65% of P_o, was not reduced after the performance of either eccentric or concentric contractions (Warren et al., 1993c). Because caffeine-induced force bypasses upstream components of E-C coupling by directly inducing SR Ca^{++} release, these results suggest the immediate deficits in voltage-induced strength was due to E-C coupling failure.

Substantiating that E-C uncoupling plays a predominant role in immediate force deficits induced by eccentric contractions Balnave et al. (1995) reported that voltage-induced peak SR Ca^{++} transients are reduced in injured muscle fibers. For example, intracellular [Ca^{++}] measured during tetanic isometric contractions in isolated mouse FDB muscle fibers was reduced across a range of stimulation frequencies (30 – 100 Hz) after performing 10 eccentric contractions (22˚C; stretch of 25% L_o) (Balnave & Allen, 1995). Applying 10 mM caffeine during the 100 Hz stimulation restored the magnitude of the intracellular Ca^{++} transient back to pre-injury values. In the same fibers, it was also shown that peak isometric force (100 Hz) was reduced by ~20%, which could be restored back to pre-injury values by the addition of caffeine during 100 Hz

stimulation. Ca^{++} sensitivity of the contractile apparatus was not disturbed after injury (Balnave & Allen, 1995). Therefore, these findings indicate that immediate force deficits of injured fibers are solely due to a reduced voltage-induced SR Ca^{++} release, under these experimental conditions.

While *in vitro* experiments are useful because experimental conditions may be precisely manipulated and controlled, the conditions in which isolated muscles are injured may not be physiological (i.e., low temperature or too great of a length change). In an effort to injure muscle under a more physiological setting (at least in regards to temperature and length changes), Ingalls et al. (Ingalls et al., 1998b) investigated the effects of 150 eccentric contractions performed *in vivo* by mouse anterior crural muscles (TA and EDL muscles) on voltage induced or caffeine (or 4-chloro-m-cresol) elicited force and EDL SR Ca^{++} transients. Immediately after injury, *in vivo* anterior crural muscle torque was reduced by ~50%. Immediately and three days after injury, isolated EDL muscle peak isometric force was reduced by ~50%, while caffeine-induced force was reduced by ~15%. The authors therefore asserted that E-C coupling disruption accounted for ~75% of the strength deficits at this time (Ingalls et al., 1998b). In support, peak tetanic (100 Hz) Ca^{++} transients, measured in superficial fibers from the EDL muscle simultaneously, were reduced similarly in all fibers immediately (~25%) and three days after injury (~50%). Ca^{++} transients were restored to uninjured values during caffeine-like compound contractures immediately after injury, although three days after injury SR Ca^{++} release was not fully restored upon chemical exposure (Ingalls et al., 1998b). These findings indicate that an *in vivo* injury model results in force deficits that are primarily explained by disruption of E-C coupling and that this disruption persists for days after injury induction. Also, these findings and others (Balnave

& Allen, 1995) suggest that E-C coupling failure is experienced by all actively injured muscle fibers, and is therefore not focal in nature.

E-C coupling failure does not appear to significantly adapt to repeated bouts of injurious exercise. For example, following a single injury bout (150 maximal eccentric contractions *in vivo*), mouse EDL muscle specific tetanic force and caffeine contracture force may be reduced by ~50 and ~15% respectively, through the first five days after injury induction (Ingalls et al., 1998b; Ingalls et al., 2004b). Thus, ~75% of force deficits observed during the first five days of injury are explained by E-C coupling failure. However, following five repeated bouts of the same injury protocol, EDL muscle specific tetanic force was reduced by 33% immediately after five injury bouts and recovered rapidly over the next five days, although there was still significant force deficits three (17%) and five (7%) days post-injury. Meanwhile, caffeine-induced (50 mM) force was reduced by ~5%, at most, at these times after the fifth injury bout (Ingalls et al., 2004b). Therefore, E-C coupling failure explains ≥85% of strength deficits after the fifth injury bout, but appears to recover at an enhanced rate.

Additionally, E-C coupling failure induced by eccentric contractions appears to be temperature sensitive. In a study in which EDL muscles were injured by performing eccentric contractions *in vitro* at either 15 or 37˚C, voltage-induced isometric tetanic force deficits were only induced at 37˚C (Warren et al., 2002a). However, caffeine contracture force (50 mM at 37˚C) was similar between muscle injury temperature groups and was not different from an isometric control group (Warren et al., 2002a). Therefore, it appears that E-C uncoupling involves failure of a temperature sensitive component.

Sites of Excitation-Contraction Coupling Failure After Eccentric Contractions

It is clear that SR Ca^{++} transients are diminished in injured muscle during the first three days after injury and that the reduction in SR Ca^{++} release most likely contributes to early force deficits (Balnave & Allen, 1995; Ingalls et al., 1998b). However, the above experiments do not indicate where the breakdown in the E-C coupling process occurs. Potential sites of E-C coupling failure include disruption of the plasmalemma or T tubule network, a loss or reduced function of DHPRs or RYR1s, or disruption to the communication between DHPR and RYR1.

Plasmalemma and T Tubule

E-C coupling could fail because of a reduced ability of the plasmalemma to depolarize or propagate an action potential. Supporting this possibility, McBride et al. (2000) reported that rat tibialis anterior muscle membranes remained partially depolarized for up to 24 hours after perfoming eccentric contractions. In contrast, Warren et al. (1993c) reported that the resting membrane potential of injured soleus muscle was not different than the resting membrane potential of control muscle (~74 mV). These conflicting results may stem from the differences in the contractile protocols employed by each study [e.g., 100 Hz, 2.5 s train vs. 300 Hz, 120 ms train; (McBride et al., 2000; Warren et al., 1993c), respectively].

To further assess the excitability of skeletal muscle after eccentric contractions, mouse tibialis anterior muscle electrical activity (EMG) was measured during electrically stimulated isometric contractions before, immediately after, and during the days after performing eccentric contractions *in vivo* (Warren *et al.*, 1999a). Peak isometric torque was reduced by ~50%, while the root mean square of the electromyographic signal (EMG RMS), which is indicative of the amplitude of the muscle action potential, was reduced by ~20% immediately after eccentric contractions. However, EMG RMS was reduced to a similar degree immediately after the

performance of concentric contractions that did not induce muscle injury. One and three days after the eccentric contractions, EMG RMS was not different from pre-injury values, while anterior crural muscle torque remained reduced, indicating that muscle action potential generation and plasmalemma propagation were not affected by muscle injury per se (Warren et al., 1999a).

Similarly, the propagation of action potentials by and the structural integrity of the T tubule system do not appear to be adversely affected immediately after muscle injury. Evidence for this is provided by *in vitro* experiments in which EDL muscle K^+ contractures were performed immediately after eccentric contractions were performed (Ingalls et al., 1998b). Compared to contralateral control force values, peak isometric force produced by the EDL muscle was reduced by ~50% and K^+ contracture force was reduced to a similar magnitude. Because K^+ -induced force directly stimulates the T tubules to depolarize, a similar decrease in voltage-induced and K^+ induced force immediately after injury likely indicates that the site of failure is located at or below the DHPR (Ingalls et al., 1998b). However, it should be noted that K^+ -induced contracture could simply "skip-over" a non-excitable component of the T tubule membrane by diffusing past this disrupted component. Under this circumstance, only voltage-induced force production would be impeded.

A decreased K^+ contracture force (Ingalls et al., 1998b) accompanied with an unaltered EMG RMS and membrane potential (Warren et al., 2000; Warren et al., 1993c) suggests that action potentials may be delivered throughout the fiber and that E-C uncoupling is due to a disturbance at the site of the voltage sensor or below. However, it is also possible that the T tubule membrane may be damaged or may become sheered-off between myofibrils during eccentric contractions resulting in failure to deliver the action potential to a portion of the fiber

(Proske & Morgan, 2001). To address this possibility, lipophilic fluorescent markers were used to observe the structural integrity of the plasmalemma and T tubule network in soleus muscle following the performance of 20 eccentric or 20 isometric contractions *in vitro* (Warren et al., 1995). In unexercised muscle, the staining pattern was reflective of T tubules crossing transversely across the muscle fiber at regular intervals, which corresponded with the presumed distance between each A-I band interface of serial sarcomeres. However, immediately after performing either eccentric or isometric contractions, there does appear to be a loss of membrane integrity, as the staining pattern became longitudinally oriented in 20 – 80% of the fibers observed in each muscle, suggesting that the fluorescent label that was confined to the T tubule is now binding to SR membrane (Warren et al., 1995). Therefore, it does appear that T tubule disruption results from contractile activity, although this disruption may be transient and not specific to eccentric contractions.

Specific to performing eccentric contractions, the same study reported regions of fibers (10 – 20% of fibers in the muscle) that appeared swollen with an isolated increased intracellular [Ca^{++}] (Warren et al., 1995). Interestingly, there was an absence of fluorescent membrane markers next to the swollen regions of the fiber, indicating that the T tubule membrane system had circumvented the damaged area of the fiber (Warren et al., 1995). These findings are fascinating because they suggest that the membrane network may become re-organized to isolate the damaged area of a fiber.

A separate study has also documented distinct disruptions of the T tubules following the performance of eccentric contractions (Yeung *et al.*, 2002). In this study, the T tubular system in single muscle fibers was visualized following the performance of eccentric contractions with a length change of either a 40% or 25% of L_o. The most prominent physical change following the

40% L_o eccentric contractions was the appearance of vacuoles associated with the T tubule membrane. However, these vacuoles were not present following eccentric contractions with only a 25% change in L_o, even though peak tetanic isometric force was reduced by 65% (Yeung et al., 2002). Thus, it does not appear the vacuolization of T tubules per se is instigating force deficits. Additionally, experiments were performed with the dye already loaded in the T tubules of the fiber prior to performing eccentric contraction. If the dye remained in the fibers then this would provide evidence for a "sealing" off of T tubules following the performance of eccentric contractions. The results, however, indicated that all of the dye eventually diffused out of the visualized portion of the T tubule system (Yeung et al., 2002). Therefore it does not appear that the T tubules become "sealed off" after eccentric contractions (Warren et al., 1999a; Warren et al., 1995; Yeung et al., 2002).

DHPR and RYR1 Content and Function

E-C coupling failure could result from a reduced function or reduced presence of DHPRs or RYR1s following injury. Measurement of SR Ca^{++} release and re-uptake rates with a Ca^{++} sensitive minielectrode indicated that release (6%) and re-uptake (12%) are reduced immediately after injury induction (Ingalls et al., 1998b). Three days after injury induction the reductions in SR Ca^{++} release (20%) and re-uptake (17%) rates worsen. While the reduced rate of SR Ca^{++} release could contribute to immediate force deficits, the small reduction observed immediately after injury (at most 6%) cannot account for the ~50% reduction in peak isometric force observed in this study (Ingalls et al., 1998b). Additionally, a separate study indicated that the binding sensitivity and amount of DHPR and RYR1 immediately after injury induction does not appear to be adversely affected (Ingalls et al., 2004a). At this time, RYR1 content is not altered while DHPR content actually increases 20%. Three days after injury induction, RYR1 content

does decrease (16%) and DHPR content remains increased by 20% (Ingalls et al., 2004a). Therefore, it does not appear that a loss of DHPR or RYR1 or a reduced functional capacity of RYR1 leads to eccentric-contraction induced E-C coupling failure immediately after injury, but does not rule out a reduced functional expression of DHPRs, as the location of DHPRs in the muscle was not determined.

DHPR and RYR1 Communication

Physical coupling between DHPR and RYR1 is paramount for skeletal muscle E-C coupling (*see the beginning of this review*). However, the reductions in voltage-gated SR Ca^{++} transients accompanied by relatively un-changed caffeine-induced Ca^{++} transients observed in injured muscle suggests that DHPR-RYR1 coupling has failed (Balnave & Allen, 1995; Ingalls et al., 1998b). Following the performance of eccentric contraction-biased exercise, Takekura et al. (2001) reported that the organization of the T tubule membrane system was structurally disrupted in rat forelimb triceps brachii muscle. Electron micrographs of the injured tissue demonstrated the appearance of longitudinal T tubular segments unaccompanied by SR terminal cisternae, disoriented tetrad structure (i.e., T tubule no longer perpendicular to sarcomere at A-I band), and irregular pentad and heptad structures. These robust perturbations were observed throughout the injured muscle (i.e., the disruptions were not focal), but were not observed in muscle from mice that only performed level treadmill running (Takekura et al., 2001). These findings indicate that the high mechanical stress and strain placed on muscle fibers during eccentric contractions can alter the architecture of E-C coupling membranes and thus the spatial arrangement between DHPR and RYR1. Additionally, dissociation of T tubule and SR membranes suggests that eccentric contractions directly damage the proteins that maintain the triad structure.

Junctophilins Involvement in Force Production and E-C Coupling

Strength deficits due to E-C coupling failure are potentially the functional effect of damage to triadic proteins that appear to span the gap between the T tubule and SR membranes. As mentioned previously, JP1 and JP2 are involved in the development and maintenance of triads and diads respectively in skeletal muscle (Hirata et al., 2006; Ito et al., 2001; Komazaki et al., 2002). The lack of triad formation in JP1 deficient mice profoundly affected hind-limb neonatal skeletal muscle function and Ca^{++} homeostasis (Ito et al., 2001). Neonate vastus medialis muscle bundles from JP1 deficient mice were weaker at low frequency stimulation compared to muscle from WT mice (i.e., twitch force was reduced by 50% for $JP1^{-/-}$ muscle), although maximum force was not different between groups. JP1 deficient neonatal muscle was also more sensitive to extracellular [Ca^{++}] than WT muscle. For example, when $[Ca]_o$ was increased from 2.5 to 5.0 mM twitch tension increased to a greater extent (~30%) over the following 30 minutes for JP1 deficient muscle than WT muscle. Conversely, when extracellular Ca^{++} is removed, twitch tension measured over the following 20 minutes decreased at a faster rate and to a greater extent for JP1 deficient muscle than WT muscle (Ito et al., 2001). Collectively, these findings suggest that JP1 deficient muscle has a reduced E-C coupling efficiency, an increased dependence on Ca^{++} induced Ca^{++} release, and a reduced capacity to restore SR Ca^{++} content.

Evidence that junctophilins are involved in maintaining normal E-C coupling in adult muscle fibers was demonstrated in a study conducted by Hirata and colleagues (2006). Mature flexor digitorum brevis (FDB) and extensor digitorum longus (EDL) mouse skeletal muscles (5 - 7 weeks old) were injected with adenovirus containing shRNAs targeted at JP1 and JP2 mRNA. Three to five days after injection, JP1 and JP2 protein content was reduced by ~50 and ~60%,

respectively. As a result of this diminished expression of JP1 and JP2, a two-fold increase in triad deformation was observed, which was accompanied by an elevated resting cytoplasmic [Ca^{++}] and a ~50% reduction in voltage-induced Ca^{++} release (1 Hz; 0 mM $[Ca^{++}]_o$) in single fibers from these muscles. Additionally, store operated Ca^{++} entry was depressed in JP1 and JP2 knock-down muscles (Hirata et al., 2006). These findings indicate that knock-down of JP1 and JP2 expression disrupts triad structure (i.e., DHPR-RYR1 arrangement), cellular Ca^{++} regulation, and E-C coupling in adult skeletal muscle fibers.

The relationship between junctophilin expression and E-C coupling efficiency has also been demonstrated in a cardiac pressure overload-induced hypertrophy model (Xu *et al.*, 2007). This model is characterized by a progressive degeneration from compensated (CHT) to decompensated hypertrophy (DHT) states, in which left ventricular hypertrophy is accompanied by normal or depressed contractile indices, respectively. Using whole-cell patch clamping techniques, Xu et al. (2007) indicate that CHT E-C coupling gain is not altered while DHT e-c coupling gain is reduced by ~50%. However, by employing loose-patch clamp techniques with confocal imaging, which allows for the simultaneous measurement of L-type Ca^{++} channel transients (LCC) (sparklet) and subsequent whole-cell [Ca^{++}] changes via RYR2 Ca^{++} release (sparks) (i.e., Ca^{++} induced Ca^{++} release; CICR), it was found that the probability of LCC sparklets inducing an SR spark progressively decreased from ~67 % in control fibers to ~46% and ~36% in CHT and DHT fibers, respectively (Xu et al., 2007). These findings indicate that CICR is disrupted in both hypertrophy conditions.

In an effort to determine the underlying mechanism resulting in decreased CICR efficiency in the CHT condition, SR Ca^{++} content, T tubule structure, and JP2 expression were analyzed (Xu et al., 2007). SR Ca^{++} content, as determined by caffeine induced calcium release

was not different between control, CHT, and DHT groups. T tubule structure was not degraded in this early stage of cardiac hypertrophy, as has been observed in later stages of heart failure. However, JP2 expression normalized to GAPDH was decreased in CHT fibers by ~50% compared to control fibers. Based on these findings, the authors suggest that reduced JP2 expression prompts a subtle physical alteration of the E-C coupling membranes that reduces CICR efficiency (Xu et al., 2007).

JP1 may also augment RYR1 Ca^{++} release via redox sensitive association with RYR1 (Phimister et al., 2007). RYR1 has numerous reactive cysteine residues that render the channel sensitive to cellular redox regulation. Phimister et al. (2007) indicated that thiol sensitivity was reduced when junctional SR complexes were incubated in a solution promoting an RYR1 open state (100 μM Ca^{++}, 10mM caffeine, and 5mM ATP) compared to a solution promoting a closed state (10 mM Mg^{++}). However, when oxidants (e.g., nitric oxide & hydrogen peroxide) were added to the closed state incubation medium thiol reactivity was reduced, like when the open state medium was applied. JP1 was identified as one of the proteins with reactive thiols in the junctional SR complex along with RYR1. It was determined using SDS-PAGE, in-gel tryptic digestion, high pressure liquid chromatography, and peptide sequencing using mass spectrometry that JP1 possesses three highly reactive cysteine residues (101, 402, 627) and a CXXC motif, which are known to form inter- and intra-molecular disulfide bonds with other reactive cysteine residues (Phimister et al., 2007). RYR1 has five CXXC motifs suggesting that JP1 and RYR1 may bind to one another under oxidizing cellular conditions. To determine if RYR1 and JP1 are physically associated, RYR1 was immunoprecipitated in junctional SR complexes under open state, closed state, and oxidized conditions. Immunoblots revealed that JP1, but not JP2, co-precipitated with RYR1 under all conditions. However, more JP1 was bound to the same

amount of RYR1 under open-state and/or oxidized conditions, as opposed to closed state conditions (Phimister et al., 2007). These findings suggest, but do not conclusively indicate, that cellular redox conditions coordinate physical binding of JP1 to RYR1 and that this association augments RYR1 function.

Junctophilin binding to the plasmalemma may be sensitive to mechanical or chemical stress. JP1 and JP2 contain eight MORN motifs, each comprised of 14 amino acids, near the N-terminus of the protein. MORN motifs have been recognized in only a few proteins that are expressed across a broad range of organisms (Im *et al.*, 2007; Takeshima et al., 2000). For example, the plant *Arabidopsis* expresses phosphatidylinositol-4-phosphate (PIP) 5-kinase, which possesses seven MORN motifs, each comprised of 24 amino acids (Im et al., 2007). In both JP and PIP 5-kinase, MORN motifs result in binding of the protein to the plasmalemma (Im et al., 2007; Takeshima et al., 2000). Interestingly, when plant cells experience hyperosmotic stress, PIP 5-kinase exhibits a ~30% reduction in plasmalemmal binding and a similar increase in endomembrane binding (e.g., endoplasmic retiuculum) (Im et al., 2007). Although speculative, these findings suggest that JP binding to the plasmalemma is designed to be plastic, in which the stress-sensitive MORN motifs shift from binding the plasmallema to the SR when the cell membrane is stressed.

Working Hypothesis of E-C Coupling Failure After Eccentric Contractions

Eccentric contractions result in immediate force deficits that are predominantly due to E-C uncoupling. E-C uncoupling appears to be due to a disturbance of the communication between DHPR and RYR1. A direct physical coupling between the DHPR and RYR1 is required for voltage-induced Ca^{++} release, raising the possibility that E-C uncoupling is simply due to a loss of the physical contact between these channels. JP1 and JP2 play a role in maintaining voltage-

induced SR Ca^{++} release in skeletal muscle. The existing literature suggest a plausible explanation of early strength deficits after the performance of eccentric contractions is that eccentric contractions damage triadic proteins that are associated with both the T tubule and SR membranes. Damage to these proteins would presumably result in T tubule and SR membrane dissociation, disruption of DHPR-RYR1 communication, and thus, E-C coupling failure and strength deficits.

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CHAPTER 2

JUNCTOPHILIN DAMAGE CONTRIBUTES TO EARLY STRENGTH DEFICITS AND EC COUPLING FAILURE AFTER ECCENTRIC CONTRACTIONS

Abstract

Junctophilins (JP1 & JP2) are expressed in skeletal muscle and are the primary proteins involved in transverse (T)-tubule and sarcoplasmic reticulum (SR) membrane apposition. During the performance of eccentric contractions, the apposition of T-tubule and SR membranes may be disrupted, resulting in excitation-contraction (EC) coupling failure and thus reduced force-producing capacity. In this study, we made three primary observations: 1) Through the first three days after the performance of 50 eccentric contractions *in vivo* by the left hindlimb anterior crural muscles of female mice, both JP1 and JP2 were significantly reduced by ~50 and 35%, respectively, while no reductions were observed after the performance of non-fatiguing concentric contractions; 2) following the performance of a repeated bout 50 eccentric contractions *in vivo*, only JP1 was immediately reduced (~30%) but recovered by 3d post-injury in tandem with the recovery of strength and EC coupling; and 3) following the performance of 10 eccentric contractions at either 15 or 35˚C by isolated mouse EDL muscle, isometric force, EC coupling, and JP1 and JP2 were only reduced after the 35˚C eccentric contractions. Regression analysis of JP1 and JP2 content in TA and EDL muscles from each set of

experiments indicated that JP damage is significantly associated with early (0 – 3d) strength deficits after performing eccentric contractions ($R = 0.49$; $P < 0.001$). As a whole, the results of this study indicate that JP damage plays in role in early force deficits due to EC coupling failure following the performance of eccentric contractions.

Introduction

Force producing capacity of skeletal muscle is reduced both immediately and for a prolonged period of time after performing lengthening or eccentric contractions (9, 15, 25, 29). It is generally accepted that prolonged force deficits (5 days to 4 weeks) are primarily due to a loss of force-generating and force-transmitting proteins mediated by a well-characterized inflammatory response (15, 26, 32, 42). On the other hand, early force deficits (0 to 5 days) induced by a mouse anterior crural muscle *in vivo* injury model are primarily due to an inability to electrically stimulate sarcoplasmic reticulum (SR) Ca^{2+} release (i.e., excitation-contraction coupling failure), although damage to force-generating or force-transmitting proteins can also play a role in these early strength deficits (16, 25). To date, it is not completely understood how excitation-contraction (EC) coupling is impeded in injured skeletal muscle.

In skeletal muscle, excitation-contraction coupling involves the propagation of a muscle action potential along the plasmalemma and transverse (T)-tubule membranes to sites, called triads, where the T-tubule is met on each side by the terminal cisternae of two adjacent SR. At the triad, L-type Ca^{2+} channels (dihydropyridine receptors or Cav1.1) located in the T-tubule membrane physically couple with approximately every other SR Ca^{2+} release channel (ryanodine receptor, RyR1)(4, 6, 11). T-tubule membrane potential depolarization results in a conformational change in Cav1.1 that physically opens RyR1 and promotes release of Ca^{2+} from the SR (4, 11), which then incites force production.

Immediately after the performance of eccentric contractions, the primary components of the EC coupling process are still present and appear functional when tested in isolation. Specifically, immediately after performing short-duration (i.e., $\leq$ 200 ms) eccentric contractions the ability of the T-tubule to propagate an action potential does not appear to be impeded (16, 43,

44), Cav1.1 and RyR1 protein content are not diminished (17), and SR Ca^{2+} release and re-uptake rates are at most only modestly reduced (16). However, it does appear that the cellular organization of the T-tubule network is disrupted after the performance of eccentric contractions (35, 43). Thus, the EC coupling failure observed after eccentric contractions may result from a disruption in the communication between Cav1.1 and RyR1.

Although the physical coupling of Cav1.1 and RyR1 is essential for voltage-induced Ca^{2+} release (VICR), the expression of these ion channels in cell culture is not sufficient for channel co-localization or VICR, despite both channels exhibiting signs of independent functionality (e.g., Ca^{2+} induced Ca^{2+} release was observed) (34, 36). Therefore, expression of accessory triadic proteins in skeletal muscle is crucial for VICR. There are a number of triadic proteins that have been identified (45), however, in skeletal muscle two junctophilin isoforms, JP1 and JP2, are thought to be the primary proteins involved in embryonic development and post-natal apposition of T-tubule – SR membranes (19, 21, 37). Junctophilins possess a SR transmembrane domain at the C-terminus and a membrane occupation and recognition nexus (MORN) motif at the N-terminus that binds to the plasmalemma or T-tubule membrane (37). These characteristics allow JP1 and JP2 to tether the T-tubule membrane to the SR membrane, allowing direct interaction between Cav1.1 and RyR1.

Previous studies have indicated that reductions in junctophilin protein expression carry structural and functional consequences. For example, reduced expression of JP1 and JP2 in adult skeletal muscle results in loss of triad formation, reduced VICR, and diminished store-operated Ca^{2+} entry (13). Moreover, ablation of JP1, which is lethal, impedes SR – T-tubule membrane junction development and reduces force-producing capacity of neonatal skeletal muscle (19, 21). Similarly, in cardiomyocytes, reductions in junctophilin protein expression have been associated

with a failure to release Ca^{2+} from the SR in response to external stimuli and, therefore, to play a role in hypertrophic cardiomyopathy (28, 30, 46).

Based on the cellular location of junctophilins in skeletal muscle and the functional implications of junctophilin loss, we hypothesized that eccentric contractions would damage junctophilins, and that damage of junctophilins contributes to EC coupling failure and early strength deficits after injury. Therefore, our first objective was to characterize JP1 and JP2 content in mouse anterior crural muscles after the performance of a single bout of either eccentric or concentric contractions *in vivo*. Upon finding immediate reductions in JP1 and JP2 with only eccentric contractions, our next objective was to determine the content of JP1 and JP2 in mouse anterior crural muscle under conditions known to alter the magnitude and/or timing of E-C uncoupling and strength deficits.

Methods

Animals

Female CD1 mice 2 to 5 months old were used in this study. The mice were housed in groups of 10 animals per cage, supplied with food and water ad libitum, and maintained in a room at 20-22°C with a 12-h photoperiod. Mice were euthanized with an overdose of sodium pentobarbital (200 $mg \cdot kg^{-1}$) followed by cervical dislocation. All animal care and use procedures were approved by the institutional animal care and use committee and met the guidelines set by the American Physiological Society.

Experimental Design

Three studies were conducted to determine if junctophilins are damaged by eccentric exercise and to determine if junctophilin damage is associated with early strength deficits and EC

coupling failure. In *study one*, mice performed a single bout of 50 concentric or eccentric contractions with the left anterior crural muscles (TA & EDL muscles). Isometric torque produced by this muscle group as a function of stimulation frequency (20-400 Hz) was measured in anesthetized mice before and immediately after the concentric or eccentric contraction bout. The recovery of anterior crural muscle strength was also evaluated *in vivo* by measuring isometric torque as a function of stimulation frequency (20-400 Hz) in different groups of mice 1 day after the concentric contractions and 1 and 3 days after performing eccentric contractions.

For each respective group of mice, junctophilin protein content was assessed via Western blot and the extent of myocellular damage was assessed in uninjured and injured anterior crural muscles at 0, 1, and 3 days post injury. Additionally, isometric force production of isolated extensor digitorum longus (EDL) muscle as a function of stimulation frequency (10-300 Hz) and caffeine contracture force was assessed *in vitro* for uninjured muscles and muscles that had previously performed concentric (0 or 1d post) or eccentric (0, 1, or 3d post) exercise. Because caffeine elicits SR Ca^{2+} release by acting directly on RyR1 and therefore by-passes the upstream components of EC coupling (22), relative reductions in caffeine induced and electrically stimulated force were compared to evaluate EC coupling failure.

Upon finding that JP1 and JP2 exhibited reductions in protein expression during the first three days after eccentric exercise, we sought to perform experiments that would manipulate the magnitude and time-course of eccentric contraction-induced strength deficits and EC coupling failure. In *study two*, mice performed two bouts of 50 eccentric contractions *in vivo* separated by two weeks. It has been shown previously that the performance of repeated bouts of eccentric contractions results in at most a small attenuation in immediate force deficits but a robust enhancement in the rate of recovery of early force deficits (8, 9, 18). Therefore, in this study we

hypothesized that JP1 and JP2 protein content would be reduced immediately after performing a second bout of eccentric contractions, but would recover at a faster rate in conjunction with recovery of force and EC coupling. In this study, there were three groups of mice that performed a single bout of 50 eccentric contractions, with isometric torque-frequency testing occurring immediately before and after the contraction bout. Then, one group of mice performed an isometric torque-frequency test 14 days later, while the other two groups performed a second bout of 50 eccentric contractions with immediate pre- and post-injury isometric torque frequency testing. At this point, one of the repeated eccentric contraction groups ceased testing (i.e., 0d post eccentric bout 2) and the other group performed a follow-up isometric torque frequency test three days later. As in *study one*, on the final day of testing EDL muscles were isolated and underwent an isometric force-frequency and caffeine contracture test. JP1 and JP2 protein in the TA muscle was determined by Western blot.

In study three, the temperature that EDL muscles were injured *in vitro* was manipulated, as it has been shown previously that force deficits and EC coupling failure after performing eccentric contractions *in vitro* are temperature dependent (41). Specifically, force deficits and EC coupling have been observed when eccentric contractions are performed at ~35˚C, while significant force deficits and EC coupling failure is not observed when the same intensity eccentric contractions were performed at ~15˚C. Thus, we hypothesized that JP1 and JP2 protein content would only be reduced after the performance of eccentric contractions at 35˚C, further supporting that the loss of junctophilins after performing eccentric contractions is contributing to immediate force deficits and EC coupling failure. In this study, isolated EDL muscles performed 10 eccentric contractions at either 15 or 35˚C, or 10 isometric contractions at 35˚C. JP1 and JP2 protein content was then determined via Western blot.

Experimental Methodology

In vivo muscle strength analysis and injury induction

Contractile function (i.e., torque-frequency relationship) of the left anterior crural muscles was measured *in vivo* as previously described (8, 16). After mice were anesthetized (0.3 mg kg-1 fentanyl citrate, 16.7 mg kg-1 droperidol, 5.0 mg kg-1 diazepam), the left hindlimb was aseptically prepared. The mouse was then placed on a heated platform and halogen lamps were directed at the torso of the mouse in order to maintain core body temperature between 35 to 37˚C. The left knee was clamped and the left foot was secured to an aluminum "shoe" that is attached to the shaft of an Aurora Scientific 300B servomotor. Sterilized needles were inserted through the skin for stimulation of the left common peroneal nerve. Stimulation voltage and needle electrode placement were optimized with 5 to 15 isometric contractions (200 ms train of 0.1 ms pulses at 300 Hz). Contractile function of the anterior crural muscles was assessed by measuring peak isometric torque as a function of stimulation frequency (20-400 Hz). The anterior crural muscles performed either 50 concentric or eccentric contractions (38° angular movement at 2,000° s^{-1} starting from a 19° plantarflexed or dorsiflexed position, respectively). Core body temperature was monitored continuously during all functional testing with a mouse rectal thermocouple.

In vitro analysis of EDL muscle

EDL muscles were dissected free and studied using an *in vitro* preparation as previously described (8, 16). EDL muscles were mounted in a chamber containing a Krebs-Ringer bicarbonate buffer (pH 7.4) with (in mM) 144 Na^+, 126.5 Cl^-, 6 K^+, 1 Mg^{2+}, 1 SO_4^{2-}, 1 PO_4^{3-}, 25 HCO_3^-, 1.25 Ca^{2+}, 0.17 leucine, 0.10 isoleucine, 0.20 valine, 10 glucose, and 10 μg ml^{-1}

gentamicin sulphate and 0.10 U ml^{-1} insulin (the buffer was equilibrated with 95% O_2-5% CO_2 gas). The distal tendon was attached by silk suture and cyanoacrylate adhesive to a fixed support, and the proximal tendon was attached to the lever arm of a servomotor system (Aurora Scientific 300B). Optimal physiological muscle length (L_o) in the chamber was set with a series of twitch contractions (0.2 ms pulse at 150 V).

In studies one and two, peak isometric force as a function of stimulation frequency (10-300 Hz) was measured at 37˚C during isometric contractions (200 ms trains of 0.2 ms pulses), with 3 min between contractions. Caffeine sensitivity was assessed by measuring changes in baseline force during exposure to caffeine (50 mM) and twitch contractions at a rate of 0.2 Hz. Force (N) produced by the EDL muscle was normalized to physiological cross-sectional area (PCSA) as described previously (27).

In study three, once L_o had been set to peak twitch force, EDL muscles performed a twitch contraction followed 30 seconds later by a second twitch contraction, and then 30 seconds later by a tetanic contraction (300 Hz), all at 35˚C. Three minutes later a second tetanic contraction was performed. The muscle then remained at rest for 5 minutes while the organ bath chamber temperature was either changed to 15˚C or maintained at 35˚C. The muscles then performed either 10 isometric or 10 eccentric contractions. During the eccentric contractions, the muscle was shortened to 87.5% and then lengthened to 112.5% of L_o over 133 ms at 1.9 muscle lengths per second. Because peak mechanical stress during eccentric contractions has been shown to be a primary determinant of loss of force producing capacity (40), the stimulation frequency during the 35˚C eccentric contractions was reduced to 160 Hz in order to match peak eccentric force at 15˚C (62 Hz). The isometric control group performed 10 submaximal tetanic

contractions at 160 Hz at 35˚C. Caffeine contracture force was assessed for all treatment groups as described above for studies one and two.

Histology

Muscles intended for histological analysis were embedded in a cryomold with O.C.T. compound, frozen in liquid nitrogen chilled by 2-methylbutane, and stored at -80˚C until further use. Cryosections (10 μm) were obtained from the middle of the TA muscle and stained with hematoxylin and eosin (H&E) to estimate the extent of muscle damage caused by exercise. Myofibers were counted in the muscle cross-sections using BioQuant Nova Prime (V6.90.10) software. Discolored myofibers containing ≥ 3 internal nuclei were classified as active degenerating, or injured myofibers.

Western Blotting

Following dissection, muscles intended for Western blot analysis were weighed, frozen in liquid nitrogen, and stored at -80˚C until further use. Muscles were homogenized in a buffer of the following constituents (in mM): 0.1 sucrose, 46 KCl, 10 NaEDTA, 10 TrisCl (pH 7.4). Total protein content was determined spectrophotometrically using the Bradford assay. A portion of the whole muscle homogenate was diluted in SDS reducing buffer. 25 μg of protein from the whole muscle homogenate was loaded into a 7% polyacrylamide gel (Acrylamide to Bis-; 37.5:1) and separated according to molecular weight (100 V for 30 min; 150 V for 45 min). The protein was then transferred to a nitrocellulose membrane (100 V for 60 min). Following transfer, the membrane was blocked overnight at ~4˚C in 5% non-fat dried milk (w/v) in tris-buffered saline with 0.1% Tween-20 (TBS-T). The following morning, the membranes were then probed with rabbit anti-mouse primary antibodies for 2 hours at room temperature on an

orbital shaker. Junctophilin 1 and 2 (Invitrogen, Catalog #s 40-5100 & 40-5300, respectively) primary antibodies were diluted to 1:20,000, while tubulin antibody (Sigma, T 3526) was diluted 1:2,500 in TBS-T. Following incubation in primary antibody, membranes were washed with TBS-T (3 x 15 minutes) and then probed with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, G21234) diluted in TBS-T (1:25,000) for 1 hour at room temperature with shaking. The membranes were then washed as described above and treated with enhanced chemilumescent solution (Thermo Scientific) prior to detection with autoradiograpy film. Optical density of the blot was determined using SigmaGel (Jandel Scientific). JP1 and JP2 optical density was normalized to the tubulin content of each muscle.

Data Modeling and Statistics

Torque- and force-frequency relationships were modeled with the following equation:

$$f(x) = \min + (\max - \min) / [1 + (x / EC50)^{n}]. \qquad \text{Eqn. 1}$$

Where x is the stimulation frequency, min and max are the smallest (i.e., twitch) and largest (i.e., peak tetanic) respective forces estimated. EC50 is the stimulation frequency at which half the amplitude of force (max – min) is reached and n is the coefficient describing the slope of the steep portion of the curve.

Torque- and force-frequency parameters, anthropometric measurements, histological degeneration, and protein content values were analyzed separately using a one-way ANOVA. Eccentric and concentric contractions during each type of exercise bout in all three studies, as well as pre- and post-exercise protocol force values in study three were analyzed using a group by contraction or time split-plot ANOVA. Post-hoc means comparisons testing was performed when a significant ANOVA was observed. Statistical significance was achieved with $p < 0.05$. All statistical testing was performed with SPSS 12.0.

Results

Study One: A Single Bout of Concentric or Eccentric Contractions

In vivo Torque and Injury Induction

There were no significant differences in pre-exercise anterior crural muscle isometric torque values between concentric and eccentric groups (Figure 1 & Table 1). Peak eccentric torque was significantly greater than peak concentric torque and, over the 50 contractions, exhibited a significant decline (~40%), while concentric torque was not significantly reduced (Figure 2). Following the performance of concentric contractions, there were no significant alterations in isometric torque-frequency parameters compared to pre-exercise values (Figure 1 & Table 1). In contrast, throughout the three days following the performance of eccentric contractions both peak twitch and tetanic isometric torque values were significantly reduced and the stimulation frequency required to elicit half maximal force (EC_{50}) was shifted to higher frequencies, compared to pre-injury values (Figure 1 & Table 1). By three days after eccentric exercise all altered isometric torque parameters exhibited a significant but incomplete recovery.

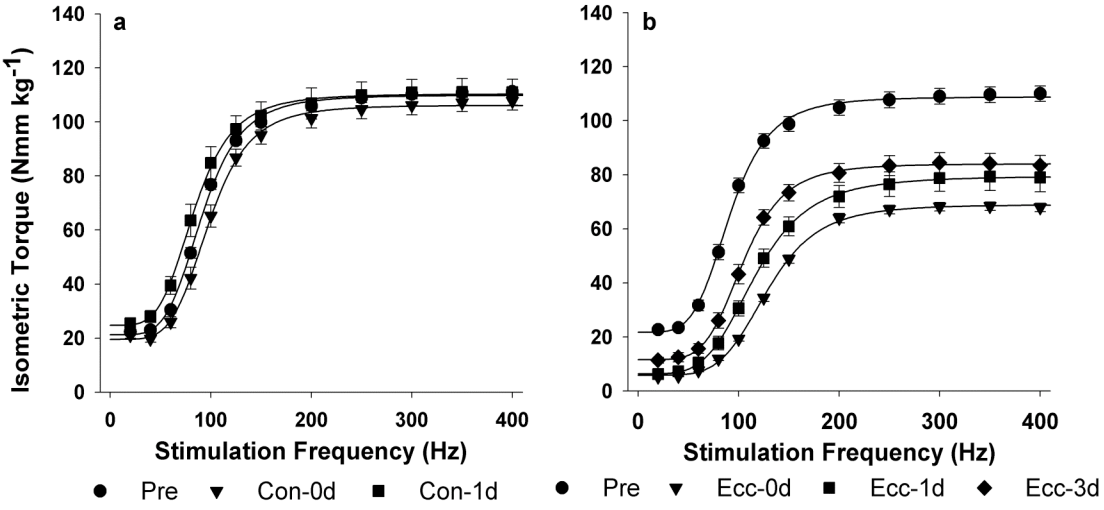

Figure 1. *In vivo isometric torque as a function of stimulation frequency. Measurements were made before (Pre), immediately (0d), and one day (1d) after a single bout of concentric (a) or eccentric contractions (b), as well as three days (3d) after a single eccentric bout. Each data set was modeled with Equation 1 listed in the methods. Values are listed as means ± SE.*

Table 1. *Study one in vivo torque-frequency parameters*

	Concentric			Eccentric			
	Pre	0d	1d	Pre	0d	1d	3d
Sample size n =	10	10	4	28	28	9	10
Body Weight (g)	30.8 ± 1.6	-	30.5 ± 0.9	28.6 ± 0.9	-	27.0 ± 1.5	29.3 ± 1.4
Min $_{measured}$ (N·mm·kg^{-1})	21.8 ± 1.0	19.4 ± 0.9	25.5 ± 1.5	22.1 ± 0.7	5.1 ± 0.2*	6.1 ± 0.5*	11.8 ± 1.2*,†,§
Max $_{measured}$ (N·mm·kg^{-1})	111.2 ± 3.1	107.7 ± 2.6	111.5 ± 4.8	111.9 ± 2.5	70.7 ± 1.6*	77.4 ± 3.1*,†	89.2 ± 3.9*,†
Min $_{estimated}$ (N·mm·kg^{-1})	20.9 ± 1.1	19.3 ± 0.9	24.6 ± 1.6	21.2 ± 0.7	5.8 ± 0.2*	5.9 ± 0.6*	11.8 ± 1.2*,†,§
Max $_{estimated}$ (N·mm·kg^{-1})	109.8 ± 3.0	106.0 ± 2.7	110.3 ± 5.0	110.2 ± 2.5	70.8 ± 1.6*	77.0 ± 3.2*	88.2 ± 3.9*,†
EC_{50} (Hz)	91.2 ± 1.9	97.9 ± 2.3	84.2 ± 3.4	91.0 ± 1.2	130.1 ± 1.4*	116.0 ± 3.0*,†	100.7 ± 3.3*,†,§
n coefficient	-4.8 ± 0.2	-5.1 ± 0.2	-4.5 ± 0.2	-4.9 ± 0.2	-5.2 ± 0.1	-4.6 ± 0.2	-5.1 ± 0.1

Values are listed as mean ± SE. The minimum (Min) and maximum (Max) torques measured and estimated represent twitch and peak tetanic torques, respectively. EC50 is the stimulation frequency at which half of the rise in amplitude of torque occurred. The Hill coefficient describes the slope of the steep portion of the torque-frequency curves depicted in Figure 1. * different than Pre Ecc; † different than Ecc 0d; and § different than Ecc 1d ($p < 0.05$).

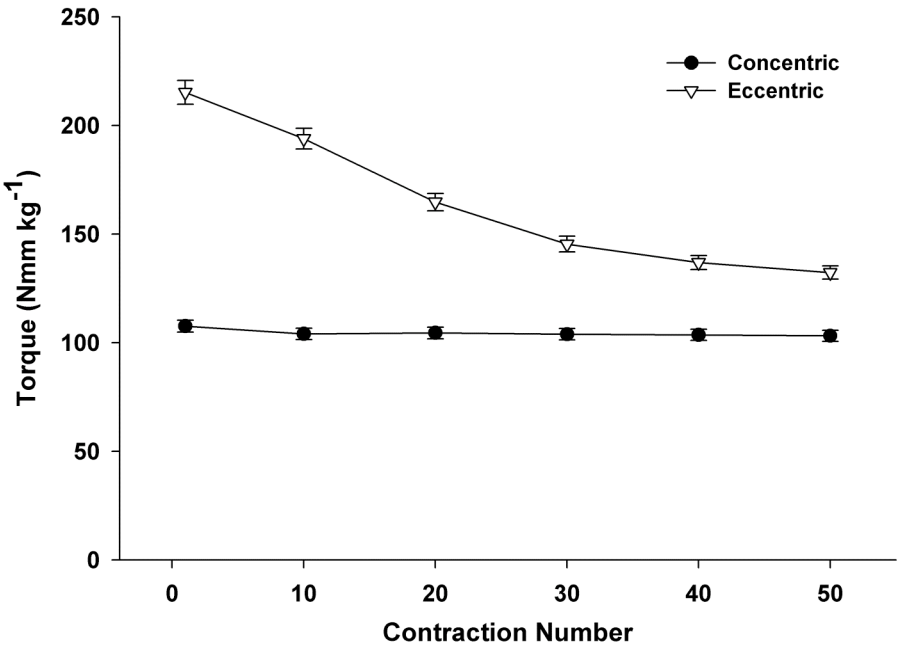

Figure 2. *Peak torque produced during either 50 concentric or eccentric contractions performed in vivo. Values are listed as means ± SE.*

In vitro EDL muscle force

EDL muscle isometric specific force was measured as a function of stimulation frequency for mice that had either previously performed *in vivo* concentric or eccentric exercise or had performed no prior exercise (control). Compared to control values the prior performance of concentric exercise did not significantly alter isometric specific force parameters (Figure 3; Table 2). However, within an hour after performing eccentric exercise peak twitch and tetanic isometric forces were significantly reduced and EC_{50} was significantly greater compared to control values. By three days after performing eccentric exercise, isometric peak twitch and tetanic specific force exhibited a significant but incomplete recovery, while EDL muscle EC_{50} recovered to control values one day after performing eccentric contractions (Figure 3; Table 2).

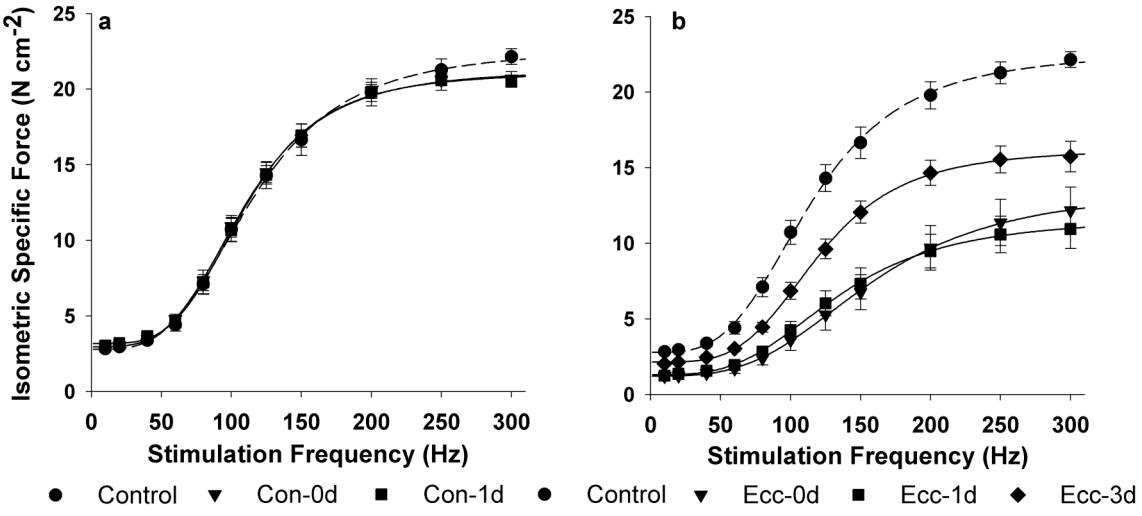

Figure 3. *In vitro EDL muscle isometric force as a function of stimulation frequency. Muscles were dissected and tested immediately (0d), and one day (1d) after a single bout of concentric (a) or eccentric contractions (b), as well as three days (3d) after a single eccentric bout. Additionally, measurements were made with EDL muscles that had performed no prior exercise (Control). Each data set was modeled with Equation 1 listed in the methods. Values are listed as means ± SE.*

Table 2. *TA and EDL muscle characteristics and in vitro EDL muscle force parameters*

	Control	Concentric		Eccentric Bout 1				Eccentric Bout 2	
		0d	1d	0d	1d	3d	14d	0d	3d
Sample size n =	4	4	4	4	4	4	5	5	5
TA muscle wt (mg)	55.0 ± 1.7	52.8 ± 3.3	59.0 ± 2.6	49.1 ± 3.0	58.2 ± 3.0	57.3 ± 2.7	60.4 ± 3.4	66.1 ± 3.5	65.4 ± 1.9
TA muscle protein (mg	24.1 ± 1.3	21.0 ± 1.3	21.2 ± 0.7	23.0 ± 1.7	22.8 ± 1.2	21.8 ± 0.6	22.2 ± 1.5	21.4 ± 2.4	22.8 ± 3.8
EDL muscle wt (mg)	9.0 ± 0.6	10.9 ± 0.6	11.4 ± 0.5	11.8 ± 1.6	10.9 ± 1.1	12.3 ± 0.2	10.3 ± 0.2	11.2 ± 0.4	11.2 ± 0.2
L_o (cm)	1.47 ± 0.02	1.52 ± 0.04	1.56 ± 0.02	1.47 ± 0.07	1.44 ± 0.07	1.56 ± 0.01	1.55 ± 0.34	1.55 ± 0.34	1.58 ± 0.02
$Min_{meas.}$ ($N \cdot cm^{-2}$)	2.8 ± 0.2	2.9 ± 0.1	3.1 ± 0.2	1.2 ± 0.2*	1.3 ± 0.1*	2.0 ± 0.1*,†,§	2.4 ± 0.1	1.7 ± 0.1*,†,‡	3.1 ± 0.4$^{\infty,\ddagger,\Diamond}$
$Max_{meas.}$ ($N \cdot cm^{-2}$)	22.2 ± 0.5	20.7 ± 0.4	20.8 ± 0.7	12.2 ± 1.5*	11.0 ± 1.3*	15.8 ± 1.0*,†	20.5 ± 0.6	14.0 ± 0.6*,‡	22.1 ± 0.9$^{\infty,\Diamond}$
$Min_{estim.}$ ($N \cdot cm^{-2}$)	2.8 ± 0.2	3.0 ± 0.2	3.2 ± 0.2	1.2 ± 0.2*	1.3 ± 0.1*	2.2 ± 0.1†,§	2.6 ± 0.1	2.0 ± 0.1*,†,‡	3.3 ± 0.4$^{\infty,\ddagger,\Diamond}$
$Max_{estim.}$ ($N \cdot cm^{-2}$)	22.8 ± 0.3	21.3 ± 0.5	21.2 ± 0.6	13.6 ± 1.6*	11.7 ± 1.4*	16.3 ± 1.1*	20.8 ± 0.7	14.9 ± 0.7*,‡	22.2 ± 0.8$^{\infty,\Diamond}$
EC_{50} (Hz)	116.5 ± 5.5	108.8 ± 5.1	109.9 ± 4.1	160.2 ± 6.0*	135.4 ± 5.1†	120.9 ± 5.3†	116.3 ± 2.7	140.1 ± 6.3*,‡	120.0 ± 5.5$^{\Diamond}$
n coefficient	-3.4 ± 0.2	-3.8 ± 0.2	-4.0 ± 0.2	-3.3 ± 0.1	-3.4 ± 0.2	-4.0 ± 0.2*	-4.3 ± 0.2*	-4.3 ± 0.1*,†	-4.5 ± 0.4*
Caffeine ($N \cdot cm^{-2}$)	7.7 ± 0.1	7.1 ± 0.4	7.5 ± 0.2	5.6 ± 0.2*	6.1 ± 0.3*	6.5 ± 0.3	7.3 ± 0.2	6.5 ± 0.2*,†,‡	7.9 ± 0.4$^{\infty,\Diamond}$

Values are listed as mean ± SE. TA muscle protein content was determined in whole muscle homogenate. L_o is the muscle length that coincided with peak twitch force. The minimum (Min) and maximum (Max) forces measured and estimated represent twitch and peak tetanic forces, respectively. EC_{50} is the stimulation frequency at which half of the rise in amplitude of force occurred. The n coefficient describes the slope of the steep portion of the force-frequency curves depicted in Figures 3 & 8. * different than Control; † different than Bout 1 Ecc 0d; § different than Bout 1 Ecc 1d; ∞ different than Bout 1 Ecc 3d; ,‡ different than Bout 1 Ecc 14d; and ◊ different than Bout 2 Ecc 0d ($p < 0.05$).

EDL muscle caffeine (50mM) contracture force was measured after the force-frequency test (Table 2). Compared to control values, there were no significant reductions of caffeine-contracture force ~1 to 24 hours after performing concentric contractions. In contrast, ~1 to 24 hours after performing eccentric contractions caffeine contracture force was significantly reduced but was not statistically different from control values three days after performing eccentric contractions. When comparing the caffeine contracture and peak tetanic force deficits to estimate EC coupling failure, ~40, 58, and 45% of the force deficit observed 0, 1, and 3 days after the injury bout is explained by EC coupling.

Histological Indications of TA muscle degeneration

Despite the reduction in caffeine contracture force immediately and one day after the performance of 50 eccentric contractions, the degree of muscle degeneration assessed via H & E

staining through the first three days after injury induction was not significantly different among groups. The percentage of total fibers counted exhibiting signs of degeneration for unexercised control, and 0d, 1d, and 3d post-eccentric groups was 0.2 ± 0.1, 0.5 ± 0.1, 1.0 ± 0.4, and 1.3 ± 0.3%, respectively. Approximately 2000 fibers were counted in each TA muscle cross-section. The relatively small amount of muscle fiber degeneration observed at three days after performing 50 eccentric contractions is in contrast to the degree of degeneration (~10% degenerating fibers) we have observed previously in wild type TA muscle three days after the performance of 150 eccentric contractions *in vivo* (8).

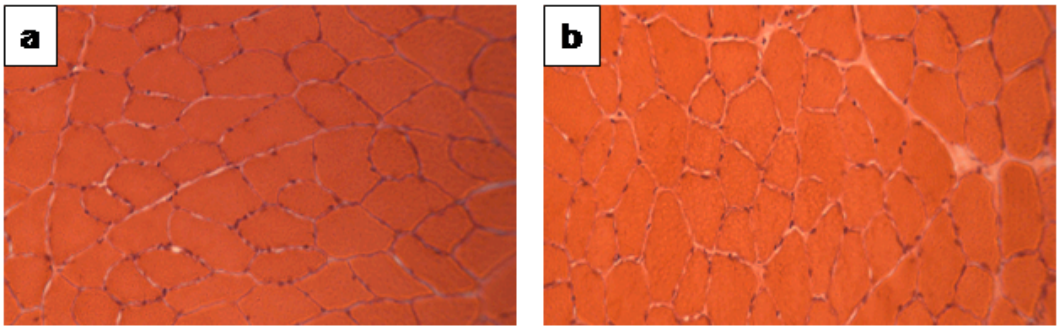

Figure 4. *Histological signs of muscle degeneration in TA muscle that had performed no exercise (A) or had previously performed 50 eccentric contractions in vivo three days before collection (B).*

Junctophilin Protein Content Alterations Following Exercise

Junctophilin 1 and 2 protein content normalized to tubulin content was not different from unexercised control values immediately or one day after performing 50 concentric contractions *in vivo* (Figure 5). In contrast, immediately, one day, and three days after performing 50 eccentric contractions JP1 and JP2 protein content was reduced by ~50 and 35% respectively. There were no significant differences among groups for tubulin protein content (ANOVA $p = 0.833$).

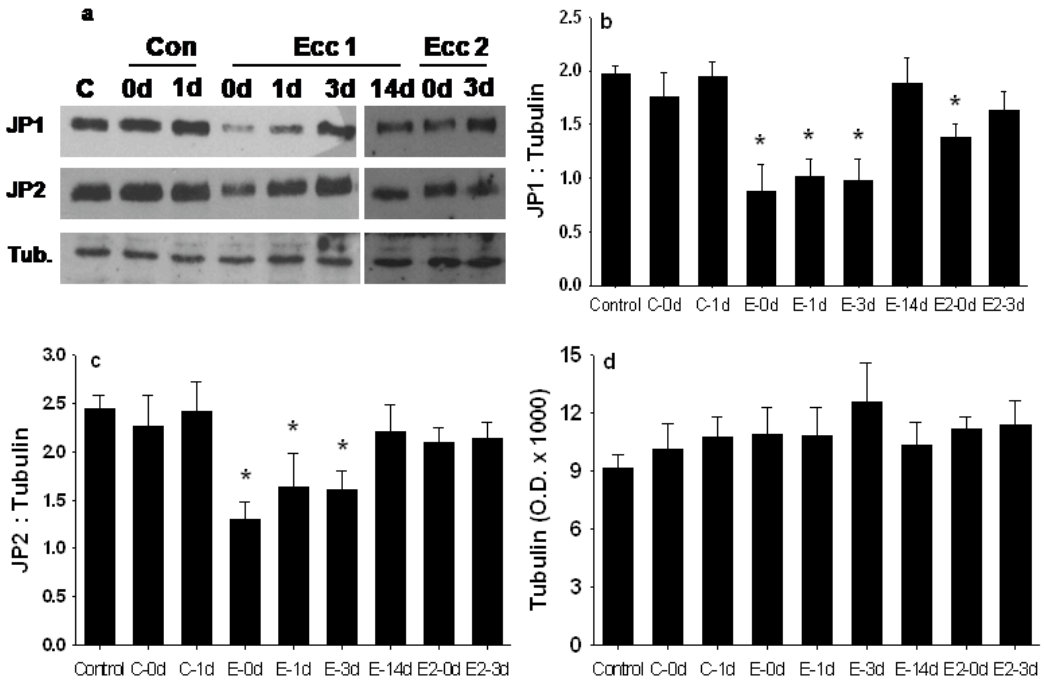

Figure 5. *Junctophilin protein content in TA muscle following in vivo concentric and eccentric exercise. Normalized JP1 (b) and JP2 (c) content, and tubulin (d) content were determined via Western blot (a) in unexercised muscle (control), in muscles collected immediately and one day after the performance of concentric contractions (C-0d & C-1d), in muscles collected immediately, one day, three days, and fourteen days after performing a single bout of eccentric contractions (E-0d, E-1d, E-3d, & E-14d), and immediately and three days (E2-0d & E2-3d) after performing a repeated bout of eccentric contractions. * Values are significantly different from control values ($p < 0.05$). Values are listed as mean ± SE.*

Study Two: Repeated Bouts of Eccentric Contractions

In vivo adaptation of force deficits

In this study, mice performed two bouts of 50 eccentric contractions *in vivo* separated by 14 days (Figure 6). Peak torque during the first eccentric contraction of both bouts were similar, although through the course of the 50 eccentric contractions eccentric torque was reduced to a greater extent during the first than the second eccentric bout (~ -36 vs. -25%).

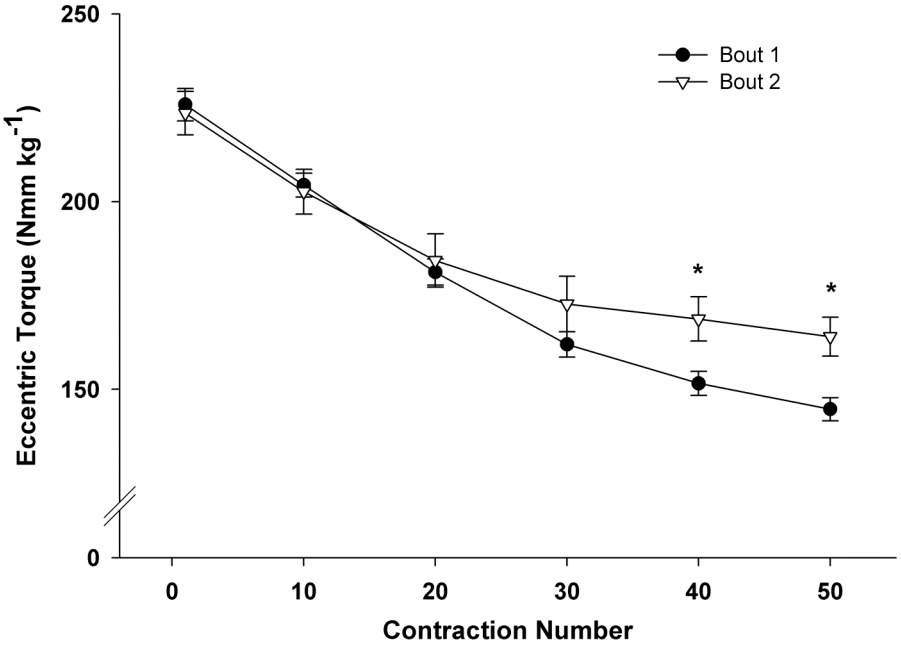

Figure 6. *In vivo eccentric torque produced during two bouts of eccentric contractions separated by 14 days. * Significantly different between bouts ($p < 0.05$). Values are listed as means ± SE.*

Isometric torque was measured as a function of stimulation frequency immediately before and after each eccentric bout, as well as three days after the second eccentric bout (Figure 7; Table 3). In response to the first eccentric bout, isometric torque parameters were immediately altered in a similar fashion as reported above for study one (Table 3). Fourteen days after the initial eccentric bout, peak tetanic torque was still reduced by ~10%, while twitch torque and all other isometric torque parameters had returned to pre-injury levels. In comparison to these fourteen-day post-injury values, immediately after the second eccentric injury bout all isometric torque parameters were altered similarly to that observed after the initial eccentric contraction bout (Table 3). Twitch and peak tetanic isometric torque values measured immediately after the first and second eccentric contraction bouts were similar, indicating that immediate strength deficits exhibited little adaptation. Three days after the second injury bout, twitch and peak

tetanic isometric torque was recovered to values observed before the initial eccentric contraction bout. This is in stark contrast to twitch and peak tetanic isomeric torque values that were still reduced by ~47 and 20%, respectively, three days after a single eccentric contraction bout in study one.

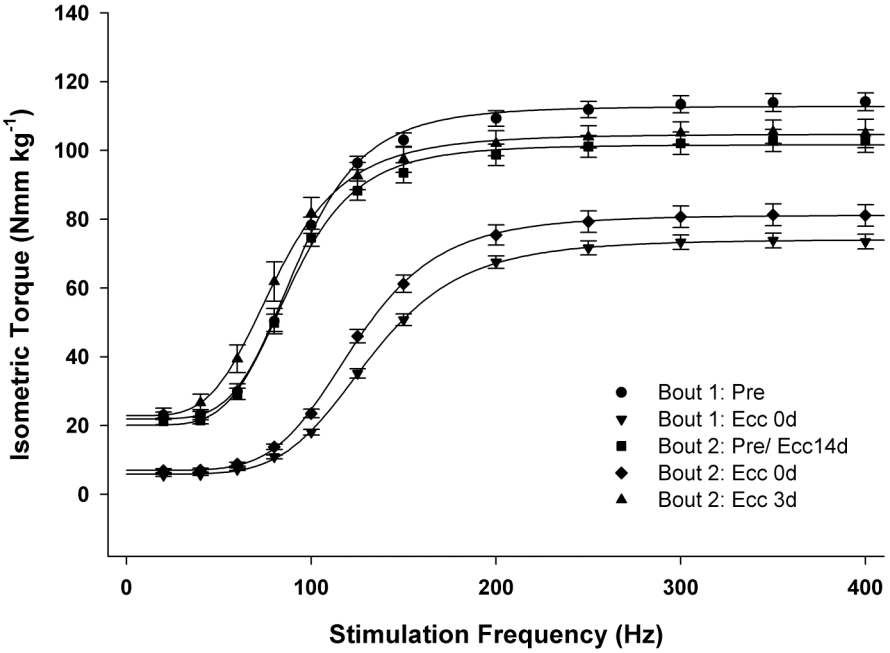

Figure 7. *In vivo isometric torque, as a function of stimulation frequency, produced before and after the performance of two eccentric contraction injury bouts separated by 14 days. Measurements were made before (Pre) and immediately (0d) after an initial and a repeated in vivo eccentric contraction bout, as well as three days (3d) after the repeated eccentric bout. Each data set was modeled with Equation 1 listed in the methods. Values are listed as means ± SE.*

Table 3. *Study two in vivo torque-frequency parameters*

	Eccentric Bout 1		Eccentric Bout 2		
	Pre	0d	Pre/14d	0d	3d
Sample size n =	15	15	15	10	4
Body Weight (g)	32.0 ± 0.5	-	32.8 ± 0.6	-	31.5 ± 1.2
Min $_{measured}$ ($N \cdot mm \cdot kg^{-1}$)	22.1 ± 1.1	5.5 ± 0.3*	20.6 ± 0.9	6.7 ± 0.6§	23.4 ± 1.7$^{\infty}$
Max $_{measured}$ ($N \cdot mm \cdot kg^{-1}$)	114.4 ± 2.5	74.0 ± 2.1*	103.3 ± 3.2*	81.4 ± 3.1§	105.7 ± 3.5$^{\infty}$
Min $_{estimated}$ ($N \cdot mm \cdot kg^{-1}$)	21.5 ± 1.0	5.9 ± 0.3*	20.0 ± 0.9	7.0 ± 0.5§	22.9 ± 1.8$^{\infty}$
Max $_{estimated}$ ($N \cdot mm \cdot kg^{-1}$)	112.6 ± 2.5	74.1 ± 2.1*	101.6 ± 3.2*	81.1 ± 3.1§	104.6 ± 3.5$^{\infty}$
EC_{50} (Hz)	91.7 ± 1.8	132.2 ± 1.6*	88.7 ± 1.4	123.8 ± 1.6†,§	81.7 ± 3.5$^{*,\infty}$
n coefficient	-5.3 ± 0.2	-5.4 ± 0.1	-5.1 ± 0.2	-5.4 ± 0.1	-4.4 ± 0.1$^{\dagger,\infty}$

Values are listed as mean ± SE. The minimum (Min) and maximum (Max) torques measured and estimated represent twitch and peak tetanic torques, respectively. EC50 is the stimulation frequency at which half of the rise in amplitude of torque occurred. The n coefficient describes the slope of the steep portion of the torque-frequency curves depicted in Figure 7. * different than Eccentric Bout 1 Pre; † different than Eccentric Bout 1 0d; § different than Eccentric Bout 2 Pre/14d; and ∞ different than Eccentric Bout 2 0d ($p < 0.05$).

In vitro force

Fourteen days after the initial eccentric contraction bout, EDL muscle twitch and peak tetanic isometric force and caffeine contracture force was similar to unexercised control values (Figure 8; Table 2). Immediately after the second eccentric contraction bout, twitch and peak tetanic isometric force and caffeine contracture force was reduced in comparison to control and 14 day post-injury values. The immediate reductions in peak tetanic isometric force was similar after the initial and second bout of eccentric contractions (Bout 1 vs. 2; ~ -45 vs. -37%), although there was less of a reduction in twitch (Bout 1 vs. 2; ~ -60 vs. -40%) and caffeine contracture force after the second than the initial eccentric bout (Table 2). Approximately 56% of the strength deficit is explained by EC uncoupling, as estimated by the comparison of caffeine contracture and peak tetanic force deificits. Three days after the second eccentric contraction bout peak twitch and tetanic isometric forces and caffeine contracture force was recovered to unexercised control values.

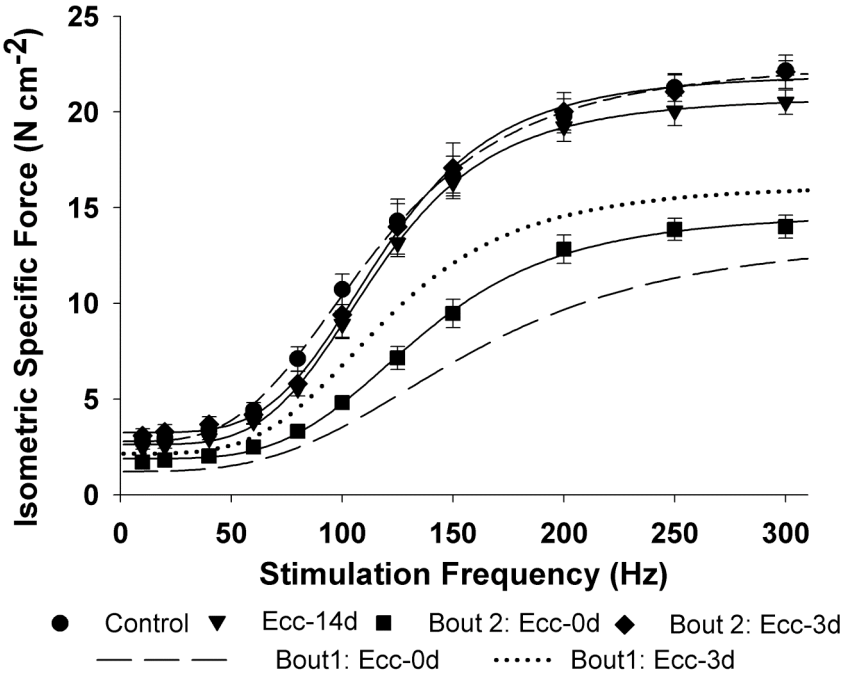

Figure 8. *In vitro EDL muscle isometric force as a function of stimulation frequency in response to a repeated bout of eccentric contractions performed in vivo. Muscles were dissected and tested 14 days (14d) after the initial injury bout and immediately (0d), and one day (3d) after the repeated bout of in vivo eccentric contractions. Additionally, measurements were made with EDL muscles that had performed no prior exercise (Control). Each data set was modeled with Equation 1 listed in the methods. Modeled curves for data immediately (0d) and three days (3d) after a single eccentric bout are illustrated for reference. Values are listed as means ± SE.*

Western blots

Fourteen days after the initial injury bout, JP1 and JP2 protein contents were recovered to control levels (Figure 5). Immediately after the second bout of 50 eccentric contractions, JP1, but not JP2, protein content was reduced compared to control values. Three days after the second injury bout, JP1 protein content was recovered to control levels.

Study Three: Temperature Dependence of Muscle Injury

In vitro Force

There were no significant differences among isometric or eccentric (15˚C and 35˚C) groups for average EDL muscle wet weight (10.6 ± 0.2, 10.5 ± 0.2, & 11.1 ± 0.3 mg, respectively), EDL muscle protein content (3.6 ± 0.2, 3.6 ± 0.1, & 3.6 ± 0.1 mg, respectively) and L_o (1.53 ± 0.02, 1.52 ± 0.01, & 1.55 ± 0.02 cm, respectively). As such, initial twitch and peak tetanic specific forces were similar among groups (Figure 9). Peak eccentric force produced during the 1st eccentric contraction was similar between 15 and 35˚C groups (Figure 10). From the 1st to 10th eccentric contraction there was a similar significant decrease in eccentric force at 15 and 35˚C. In contrast, submaximal isometric force was not significantly reduced from the beginning to the end of the isometric contraction protocol (Figure 10). Following the 10 isometric or eccentric contractions, twitch and peak tetanic forces produced at 35˚C were similar between the 35˚C isometric and 15˚C eccentric group and, in comparison, were significantly reduced for the 35˚C eccentric group (Figure 9). Despite the 35˚C eccentric contraction group exhibiting significant reductions in electrically stimulated force values, caffeine contracture force was similar among 35˚C isometric, 15˚C eccentric, and 35˚C eccentric groups (6.3 ± 0.1, 6.1 ± 0.1, and 6.1 ± 0.1 $N \cdot cm^{-2}$, respectively).

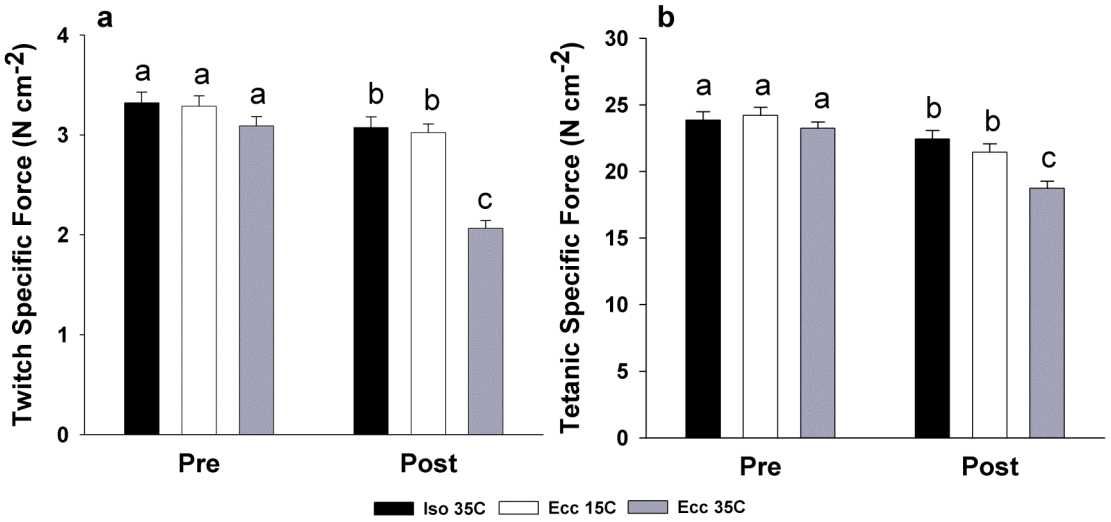

Figure 9. *In vitro isometric specific twitch and maximal tetanic force before and after 10 isometric or eccentric contractions performed at 35 or 15˚C. Values (mean ± SE) with different letters are significantly different ($p < 0.05$).*

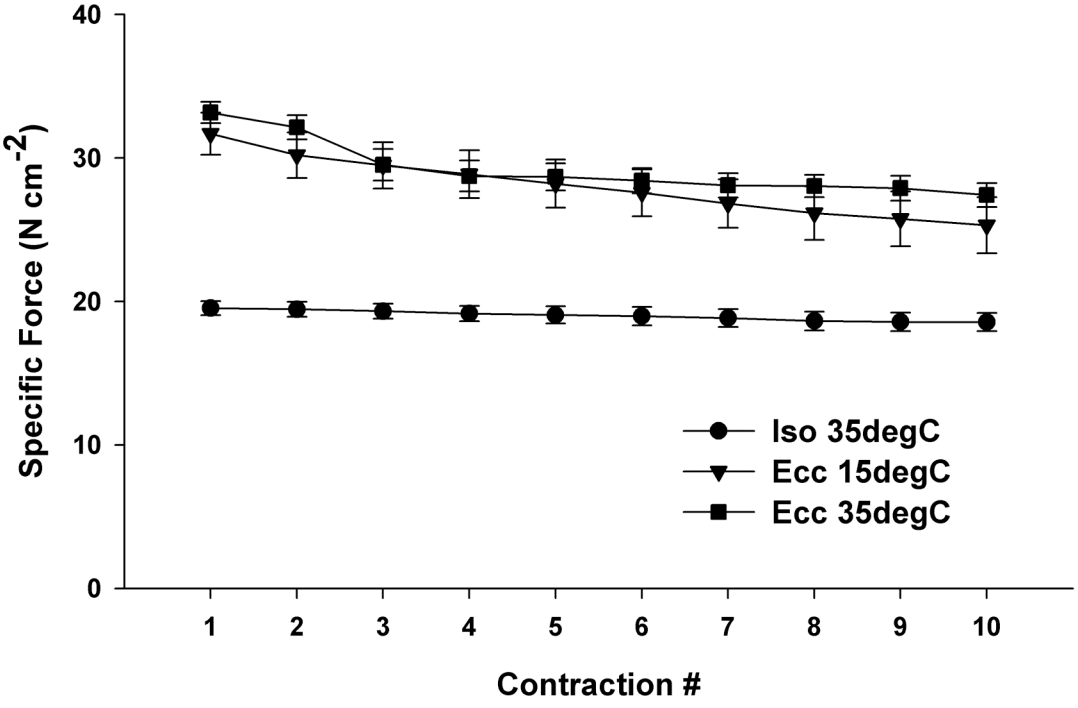

Figure 10. *In vitro specific isometric or eccentric force produced during a 10-contraction injury (eccentric; Ecc) or control (isometric; Iso) protocol at 35 or 15˚C. Values are listed as mean ± SE.*

Junctophilin Protein Content

The performance of 10 isometric contractions at 35˚C or eccentric contractions at 15˚C did not result in a significant reduction in JP1 and JP2 protein content in comparison to unexercised control values (Figure 11). However, within 15 minutes after performing eccentric contractions at 35˚C, both JP1 and JP2 protein content was reduced by ~25% in comparison to unexercised control, 35˚C isometric, and 15˚C eccentric group values.

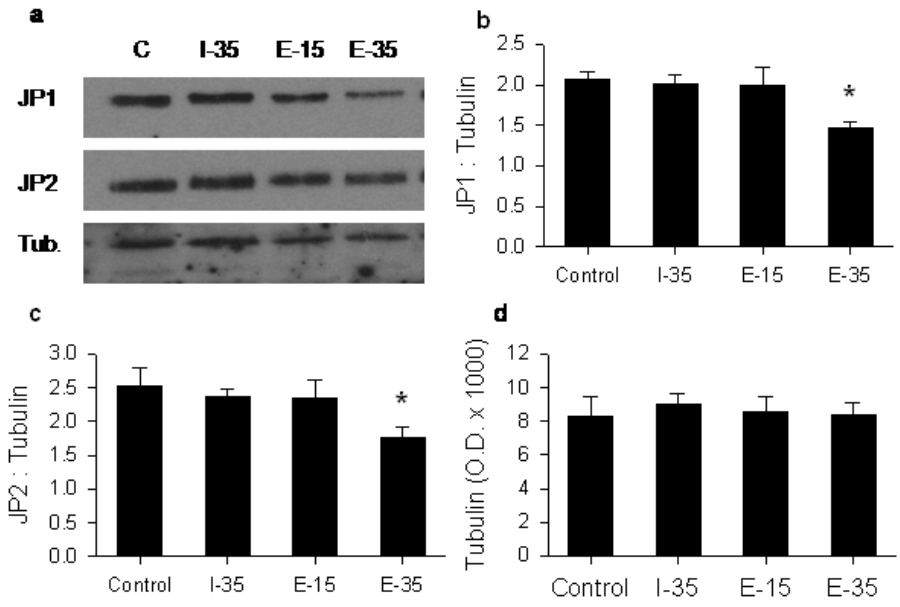

Figure 11. *Temperature dependence of EDL muscle JP1 and JP2 protein loss after performing eccentric contractions in vitro. Normalized JP1 (b) and JP2 (c) content, and tubulin (d) content were determined via Western blot (a) in unexercised EDL muscle (C or control), in muscles that performed 10 isometric contractions at 35˚C (I-35) or 10 eccentric contractions at 15 or 35˚C (E-15 or E-35, respectively). * Values (mean ± SE) are significantly different from control values ($p < 0.05$).*

Regression Analysis of JP Content and Force Deficits

JP content of all TA muscles from studies one and two and EDL muscles from study three that had a corresponding pre- and post-exercise functional measurement were included in a single regression analysis (Figure 12). Based on this analysis JP damage and force-producing capacity are significantly associated (Figure 12).

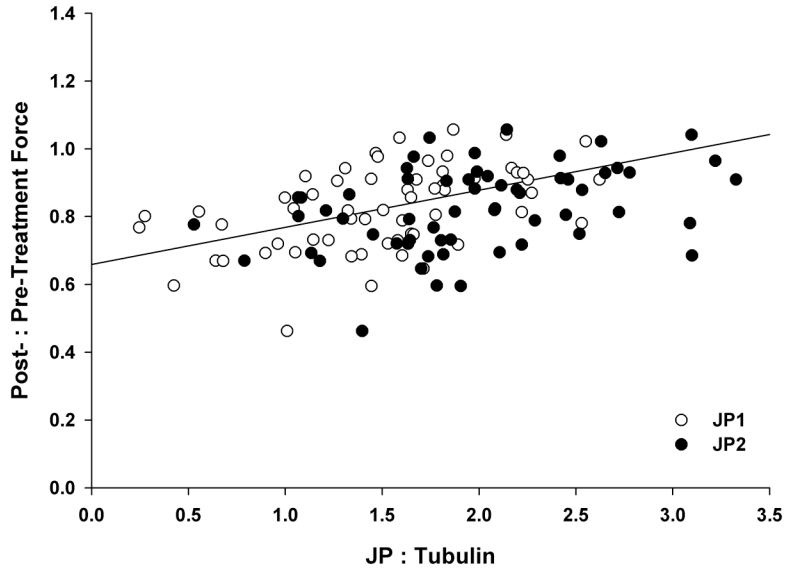

Figure 12. *Regression analysis of skeletal muscle JP1 and JP2 protein content to force producing capacity. All muscles in study one, two, and three that performed pre- and post-treatment force measurements were included in this analysis ($f(x) = 0.11x + 0.66$; $R = 0.486$; $SE_E = 0.11$; $p < 0.001$).*

Discussion

Skeletal muscle can be injured when the contractile stress and/or strain exceeds what the muscle normally accommodates. As seen in this study and numerous others, the functional consequence of this injury is immediate and prolonged impairment in force producing capacity (Figs. 1-3). Force producing and transmitting structures are known to fail with eccentric contractions and certainly contribute to the strength deficits (5, 24, 48). However, with our mouse injury model, we have documented that the majority of strength deficits stem from failure with EC coupling, specifically with the interaction between Cav1.1 and RyR1 (16). The primary findings of this study are that JP1 and JP2, proteins thought to localize triadic membranes, are damaged after a small number of eccentric contractions, and that the loss of JP1 and JP2 is associated with EC uncoupling and strength deficits.

The magnitude and time course of JP1 and JP2 damage following injury were comparable with force deficits and EC coupling failure. In this study, through the first three days after a single bout of eccentric contractions, peak tetanic force was reduced by ~30 to 45% and JP1 and JP2 content was reduced by a similar 33 to 55%. Additionally, in two separate studies that also observed an ~45% isometric tetanic force loss after eccentric contractions, electrically stimulated SR Ca^{2+} release was shown to be reduced by 25 to 40% immediately and 3 days after the injury (3, 16). Thus, during the first three days after injury induction, a 40 to 50% loss of JPs appears to result in a slightly lower relative disruption to EC coupling, which is in agreement with the ~50% reduction in electrically stimulated Ca^{2+} release reported after JP1 and JP2 have been knocked-down via shRNA in adult skeletal muscle to ~60% of control values (13). Furthermore JP1 and JP2 damage during the *in vitro* injury protocol used in this study induced a much smaller force deficit than observed after *in vivo* injury (i.e., only a ~15% force deficit), but

in concert with this smaller deficit only ~25% of JP1 and JP2 were damaged. Although, JPs exhibit a close circumstantial association with eccentric contraction-induced force deficits and EC coupling failure, regression analysis of JP content and force producing capacity indicated a significant but modest relationship (Figure 12).

The obvious means by which JP1 and JP2 damage can reduce EC coupling is through dissociation of Cav1.1 and RyR1. However, JP1 and JP2 damage after eccentric contractions may contribute to disruptions in electrically stimulated Ca^{2+} release and force deficits by disrupting store-operated Ca^{2+} entry (SOCE). It has been shown previously that knockdown of JP1 and JP2 to 60% of control values disrupts SOCE and therefore drastically reduced caffeine-induced Ca^{2+}release by ~60% (13). Comparably, caffeine-induced Ca^{2+} release is reduced immediately after injury in single muscle fibers by ~50%, which manifested a ~40% decrease in caffeine contracture force (3). In the current study, at nearly all time points that JPs were damaged following *in vivo* injury there was a concomitant reduction in caffeine contracture force. Because there were no significant signs of muscle degeneration after *in vivo* injury induction (Figure 4), caffeine contracture force deficits may result primarily from reductions in SR Ca^{2+} stores, which may have been due to disturbances in SOCE with JP damage.

It is remarkable that JP1 does not exhibit protection from damage immediately after a repeated bout of eccentric contractions, while JP2 appears to be fully protected from damage after a second bout of eccentric contractions. We are not aware of another protein that has been implicated in contributing to early force deficits that does not exhibit significant protection from damage immediately after repeated bouts of eccentric contractions. For example, it is has been shown that the cytoskeletal and structural proteins desmin, dystrophin, and titin all exhibit an immediate protective repeated bout effect (24). Immediate force deficits can be only minimally

attenuated following repeated bouts of eccentric contractions in humans (7, 31) and in animals (8, 9, 18) because EC coupling failure, in line with serving as a protective mechanism, is not significantly attenuated after injury (18). That JP1 content is reduced after a second injury bout, but then recovers at an enhanced rate, is telling in its role in EC coupling failure and strength deficits after injury.

Although we did not investigate potential causes of the loss of JP1 and JP2 in the current study, it is reasonable to assume that the proteins are either enzymatically cleaved or that chemical bonds that normally anchor JPs to the T-tubule are mechanically disrupted. Following both *in vivo* and *in vitro* eccentric contraction-induced muscle injury, intracellular Ca^{2+} concentration increases (3, 16, 27, 33, 41, 47), presumably as a result of activation of stretch-activated channels (33, 47). It has been demonstrated in mechanically skinned and in intact muscle fibers, that elevated intracellular Ca^{2+} can disrupt depolarization-induced Ca^{2+} release and may result in triad deformation (23, 47). Recently, pharmacological calpain inhibition has been shown to attenuate EC coupling failure in mechanically skinned muscle fibers with elevated cytosolic [Ca^{2+}] (38, 39) and to attenuate force deficits after injury in isolated EDL muscle (48). Given these results, it is possible that JP1 and/or JP2 are cleaved by calpains during the injury response.

Triad deformation has also been observed following the performance of eccentric contractions, raising the possibility that JP damage is a mechanically induced event (35). JPs possess MORN motifs on their N-terminus that non-covalently bind to the T-tubule membrane (12), and it has been shown in plant cells that MORN motif binding to the plasmalemma is disturbed by hyperosmotic stress (14). In the current study, JP1 and JP2 were damaged only when eccentric contractions were performed at physiological temperatures. We have shown that

the temperature dependency of force loss is not entirely explained by enzymatic processes, but may be related to a more readily compromised plasmalemma during eccentric contractions at physiological temperatures (41). Based on these observations, and because membranes are known to undergo a phase transition as temperature increases from 20 to 40˚C (20), it is interesting to speculate that the binding of the MORN motif to the plasmalemma is mechanically disrupted during eccentric contractions.

Given the complexity of Ca^{2+} handling in skeletal muscle fibers, it is likely that other triadic proteins may also be damaged during eccentric contractions and may therefore contribute to EC coupling failure and strength deficits in injured muscle (45). For example, JP-45 locates to the triad, binds to Cav1.1 in the T-tubule membrane and calsequestrin in the SR, appears to play a role in targeting Cav1.1 to the triad, and is required for normal EC coupling (1, 2, 10). Interestingly, Cav1.1 muscle content is increased by ~20% immediately after injury (17), which may be in response to improper cellular location due to JP-45 damage. Future study should be directed at assessing the contribution of other triadic proteins, especially those that cross the T-tubule membrane gap like JP-45, to EC coupling failure in injured skeletal muscle.

In the current series of studies, we have demonstrated that junctophilins are damaged by the performance of maximal and submaximal eccentric contractions, but not non-fatiguing concentric or isometric contractions, at physiological temperatures. Additionally, we have provided circumstantial evidence indicating that JP1 and potentially JP2 damage is involved in early strength deficits and EC coupling failure. This was shown by first demonstrating that JP1 is damaged to a similar degree following an initial and repeated bout of eccentric contractions, and then by demonstrating that JP damage, force reductions, and EC coupling failure are similarly temperature dependent. Lastly, a combined regression analysis for all three studies of

JP1 and JP2 content to muscle force producing capacity indicated that reductions in force producing capacity after injury are significantly associated with JP content (Figure 12). While we have not determined the role of other triadic proteins such as JP-45 (10, 45), the results of the current study indicate that JP damage plays a role in early force deficits due to EC coupling failure following the performance of eccentric contractions.

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APPENDIX A

ANIMAL USE PROTOCOL

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Mail: P.O. Box 3999
Atlanta, Georgia 30302-3999

In Person: Alumni Hall
30 Courtland St. Suite 232

Phone: 404.413.3508
E-mail: iacuc@gsu.edu
Web: http://www.gsu.edu/research/iacuc.html

February 03 2009

MEMORANDUM

TO: Dr. Christopher P. Ingalls
Kinesiology

FROM: Dr. Margo A. Brinton, Chair
Institutional Animal Care and Use Committee

RE: Approval of Protocol Number: A09001
Protocol Title: The Role of Triadic Proteins in Strength Deficits in Injured Skeletal Muscle
Animal Type and Quantity: 354 mice
Proposal Period: February 03 2009 - February 03 2012
Initial Review Period: February 03 2009 - February 03 2010

Your protocol referenced above was approved by Georgia State University's Animal Care and Use Committee on February 03 2009. This approval will remain in effect for the three year proposal period. However, you are required to submit a request for continuation of your protocol on an annual basis. To assure timely review by the IACUC, please submit the request for continuation at least 30 days before the protocol's annual renewal date.

Any changes to the current protocol must be approved before implementation. Submit changes on the amendment form.

Any unexpected adverse effects of the experiments described in this protocol must be reported to the Chair of the IACUC immediately.

Protocol approval does not guarantee animal housing space. Please contact the Assistant Director of the Department of Animal Resources, Dean Blake, to request animal housing space. Note: When ordering animals please refer to your full protocol number A09001

Georgia State University has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is **A3914-01**.

MAB/jtd

cc: Dean Blake
Michael Hart

Institutional Animal Care and Use Committee
Georgia State University

RESEARCH PROTOCOL FOR ANIMAL CARE AND USE

For Office Use Only
Date Received:
Biosafety Approval Needed: **Yes ☐ No ☐** **Approval Date_____ Approval Number_____**
Radiation Safety Approval Needed: **Yes ☐ No ☐** **Approval Date_____ Approval Number_____**

DATE: 12-22-2008
PRINICPAL INVESTIGATOR: Christopher P. Ingalls, PhD
DEPARTMENT: Kinesiology and Health
E-MAIL: cingalls@gsu.edu
PROTOCOL TITLE: The role of triadic proteins in strength deficits in injured skeletal muscle

Over a period of three (3) years I would like to use a total of 354 (number) animals.
common name Mouse
scientific name Mus Musculus

All mice used under this protocol will be: Female outbred CD1 (CF1, Swiss Webster Mice, CFW) mice aged two to six months or ~20 to 35 g body weight.

(PLEASE COMPLETE A SEPARATE PROTOCOL FOR EACH DIFFERENT TYPE OF ANIMAL.)
I would like to begin using these animals on February 1, 2009 (date).
I will obtain animals from Charles River (name of supplier).
I will breed these animals: ☐ yes X☐ no. (If yes, justify in question 7 below). If yes, from where will breeding stock be obtained (name of supplier).
List grants, if any, under which the procedures listed in this protocol will be used.

--

Protocol Number: Approval Date _____

Signature of Attending Veterinarian _____

Signature of IACUC Chair _____

Veterinary Care and Consultation: Principal Investigators must obtain consultation from the Attending Veterinarian **before** submission of the application to the IACUC.

X☐ I consulted with the Attending Veterinarian about this protocol.
Highest Pain Category within this protocol: D
Date of consultation: 1-2-2009

Certification of Compliance: I will comply with the procedures described in the NIH Guide for the Care and Use of Laboratory Animals, with PHS policy, the Animal Welfare Act, and applicable GSU IACUC policies. I acknowledge responsibility for the procedures described with and assure that the faculty, staff and students who perform these procedures are qualified (or will be adequately trained) to conduct them in a humane manner. Failure to comply may result in sanctions by the IACUC including, but not limited to suspension of research activities.

Signature of the Principal Investigator _____ Date 1-7-2009

1. Briefly state the objective and rationale for each of your major projects (e.g. grants) in **lay language** (*i.e.,* minimal scientific terms, define all acronyms)

   Begin with a broad statement concerning the overall problem (*e.g.*, "Pancreatic cancer kills ~50,000 Americans each year. We are addressing this problem by …."). Include a statement about the long term benefit(s) of this research.
   ➔

   Disruptions in the process that lead to calcium release inside skeletal muscle cells are known to be associated with human disease (e.g., malignant hyperthermia, central core disease), fatigue, and injury. Normally, force production in skeletal muscle is initiated when **electrical** signals from the nervous system are delivered to muscle cells. This **electrical** signal is then conducted into the interior of the muscle cell by a specialized membrane system called the transverse-tubular (T-tubule) system. The excitation signal from the T-tubule is then transferred to nearby structures **that store calcium inside the muscle cell** called the sarcoplasmic reticulum (SR). In skeletal muscle cells, two SR oppose the T-tubule creating an anatomical structure called the triad. The release of this stored calcium inside the muscle cell will then trigger certain proteins to interact resulting in force generation. In general, the amount of force produced by skeletal muscle cells depends on the amount of calcium released by the SR.

   Skeletal muscle injury **is a common occurrence when an individual performs exercise that is unaccustomed in intensity and/or duration. Injury to skeletal muscle during unaccustomed exercise (i.e., exercise-induced muscle injury) is known to stem from muscle "contractions" that are used to slow the body's limbs (called an eccentric contraction).** We have documented that the majority of strength loss early (i.e., 0-5 days) after exercise-induced injury of skeletal muscles stems from a failure in the signal from the T-tubule to the SR **(i.e., excitation-contraction coupling failure)**. However, the exact cause of **the communication failure between the T-tubule and SR** is unknown. A number of recently identified triadic proteins (e.g., JP1, JP2, SRP-27, MG29, JP45) are thought to help maintain the normal close association of the T-tubule and sarcoplasmic reticulum membranes in muscle cells. Of these proteins, junctophilin 1 and 2 (JP1 and JP2) appear to be crucial for normal triad development and maintenance. The goal of this research project is to determine if JP1 and JP2 are damaged or altered during the course of performing eccentric contractions in a manner that corresponds to

excitation-contraction coupling failure and **strength loss**. To achieve this goal, three studies will be conducted. Study 1 will characterize the general alterations to JP1 and JP2 that occur during the days after a single injury bout, while studies 2 and 3 will assess the relationships amongst JP alterations, functional deficits, and excitation-contraction coupling failure by injuring muscles under conditions known to manipulate the degree of functional deficits and excitation-contraction coupling (i.e., repeated injury bouts or temperature).

Justify the use of animals in these studies in general.

➔

The investigator is unaware of any alternative to the animal model proposed for this specific research question. Studying the effects of eccentric contractions on triadic protein functional expression should be examined *in vivo* in adult tissue, as injury recovery is a multi-system process and triad structure may not be fully developed in cell culture.

Justify the use of the proposed species in these studies.

➔

There are many reasons that make the mouse an appropriate model for the proposed study. First, the mouse model that will be used in this research is an accepted preparation for the study of skeletal muscle physiology. Second, the investigator has previously established the exercise model in the mouse (*J. Appl.Physiol*. 79:1260, 1995; *J. Appl. Physiol*. 80: 332, 1996; *J. Appl. Physiol*.80:1660, 1996; *J. Mus. Res. Cell Motil*. 19:215, 1998; *J. Appl. Physiol*.85:58, 1998; *J. Physiol*.515:609, 1999; *J. Appl. Physiol*.87:189, 1999; *J. Appl. Physiol*. 96: 1619-1625, 2004; *FASEB J.* 18:1597-9, 2004; *J. Appl. Physiol*.. 98: 1674-1681, 2005). Third, the functional response of mouse skeletal muscle to exercise-induced injury closely simulates the response of human limb muscle to the same injury stimulus.

☐ **I certify that the information provided herein is consistent with the information provided on the corresponding grant for sponsored research.**

_____ _____ _____

_____ _____ _____

OR

X☐ This protocol application is not associated with sponsored research.

Signature of the Principal Investigator _____ Date

2. **JUSTIFICATION FOR THE USE OF ANIMALS:**

Explain what alternatives exist in place of using animals.

➔ The investigator is unaware of any viable alternative to the animal model proposed for this specific research question. Studying the effects of eccentric contractions on triadic protein functional expression should be examined *in vivo* in adult tissue, as injury recovery is a multi-system process and triad structure may not be fully developed in cell culture (i.e., myotubes vs. myofibers).

Do any of the procedures cause more than momentary pain or distress? Please describe them.

➔ The exercise protocols themselves should not be associated with pain as the mice will be under fentanyl,
droperidol, and diazepam anesthesia. In humans, mild-to-moderate muscle soreness has been reported 1-3 days after novel eccentrically biased exercise. Muscle soreness is known to be absent or greatly attenuated in subsequent eccentrically biased exercise bouts.

Please provide a brief summary of the results of a recent database searches that you have used to verify the investigation of alternatives to painful or distressful procedures? (Alternatives incorporate some aspect of replacement, reduction or refinement of animal use in pursuit of the minimization of animal pain and distress. Alternatives or alternative methods include use of non-animal systems, less sentient animal species or techniques that lessen or eliminate pain or distress such as chemical instead of physical castration or reducing the size of implants and incisions).

Names of two databases used➔ PubMed and NLM Gateway
(Use PubMed **plus** one of the following: PsyInfo, AltWeb, NLM Gateway, AVAR, Galileo, AGRICOLA or AWIC). Date(s) of searches ➔12-10-2008

Search keyword(s): include hits for combinations of terms in this listing. **You must use 'alternative' and 'animal welfare'** (*i.e.*, Alternative AND Obesity; Alternative OR Animal Welfare AND Obesity etc.)

Summarize the hits below. (Note: this is a general summary, you do not need to go into the details of each hit but rather summarize meaning of hits by relevant groups)

➔ **Keywords:** (Alternative OR "Animal Welfare") AND (skeletal muscle) AND (injury OR damage) AND (eccentric OR lengthening)
PubMed: 2 hits
NLM Gateway: 14 hits

The hits from these searches describe findings relating to the topic of muscle injury (i.e., the inflammatory process) and do not provide alternatives for replacement, reduction, or refinement.

3. Please provide a brief summary of the results of recent searches of two databases that verify the proposed studies **do not** unnecessarily duplicate previous work.
Name of the database(s) ➔ PubMed and NLM Gateway
Date(s) of search ➔12/10/2008

Search keyword(s): include hits for combinations of terms in this listing (*i.e.*, Hamster AND Obesity; Hamsters AND Obesity AND Insulin)
➔ **Keywords:** (skeletal muscle) AND (injury OR damage) AND junctophilins
PubMed: 0 hits
NLM gateway: 1 hits

Keywords: Muscle AND junctophilin
PubMed: 15
NLM gateway: 19 hits

Summarize the hits below. (Note: this is a general summary, you do not need to go into the details of each hit but rather summarize hits by relevant groups)
➔ The published research studies on junctophilins in skeletal muscle have identified the protein as being crucial for triad formation during development and triad maintenance in adult muscle. Additionally, studies have illustrated that junctophilin 1 and 2 ablation during development is lethal

and reducing juntophilin 1 and 2 in adult muscle alters intracellular calcium homeostasis. No study has addressed the role of junctophilns in functional deficits of injured skeletal muscle.

Does the proposed work duplicate any previous work?
☐ Yes (please justify below)
X☐ No

4. Please describe in detail all of the work or procedures you will be doing with animals. Include for each procedure:
Procedure name: ➔ *In vivo* anterior crural muscle strength measurement
Infectious? ☐ yes X no (If yes, also see Question 6)
Radioactive? ☐ yes X no (If yes, also see Question 6)
Antibody production? ☐ yes X no
(If you are using intradermal or multiple injections of complete Freund's adjuvant (CFA), please state and justify)

Detailed description of EACH PROCEDURE including:
Will these animals be restrained during this procedure for any reason?
X Yes (If yes, explain below*)
☐ No
➔ Please see description of protocol below

Will animals be subjected to multiple survival surgeries (i.e. more than one penetration of a body cavity including the brain, or more than one procedure that impairs physical or physiologic function at different times)
☐ Yes (If yes, scientifically justify below*)
X No
➔

The maximum number of times the treatment/procedure will be done to any animal is:
➔Mice will be anesthetized a maximum of 3 times for this procedure. However, the maximum number of times that strength will be measured is 5 (twice during the first anesthetic induction for the first exercise bout, twice during the second anesthetic induction for the second exercise bout (2 weeks after first exercise bout), and one time point (either 3, 6, 24, OR 72 h) following the second exercise bout (Study 2).

If anesthesia is used, please describe:
➔ **Anesthesia:** Mice will be anesthetized with fentanyl (0.3 mg/kg ip), droperidol (16.7 mg/kg ip), and diazepam (5 mg/kg ip). Supplemental doses of fentanyl-droperidol cocktail will be administered to maintain the proper level of anesthesia (i.e., absence of pedal reflexes from each limb). We have found that this anesthetic regimen is superior to others (methoxyflurane, ketamine-xylazine, pentobarbital) in measuring peak muscle force in anesthetized mice (*J. Appl.Physiol*. 80:332-340, 1996). In addition, multiple anesthetic inductions are well tolerated by the mice, with mice showing no significant adverse health effects (e.g., no loss in body weight, decrease in grooming habits, or in muscle functional capacity of control mice) (*J. Physiol.* 515: 609, 1999; *J. Appl. Physiol*. 97: 1067-1076, 2004; *J. Appl. Physiol*. 98: 1674-1681, 2005; *J. Appl. Physiol*. 105:1542-1553, 2008; *J. Appl. Physiol*. 105: 527-537, 2008).

General description of procedure

➔ **Experimental design:** Two of the three studies proposed in this protocol will employ *in vivo* muscle testing. The first study (N=198) will characterize the effect of performing eccentrically or concentrically biased exercise *in vivo* on JP1 and JP2 protein content and cellular location in the anterior crural muscles [i.e., Tibialis anterior (TA) and Extensor digitorum longus (EDL) muscles] during the first 3 days after exercise. The second study (N=83) will characterize the effect of performing two bouts of eccentrically or concentrically biased exercise *in vivo* on JP1 and JP2 protein content and cellular location in the anterior crural muscles during the first 3 days after the second injury bout. (The third study (N=73) is described in the *"In vitro muscle testing"* procedure section). In all studies, the alterations to JP1 and JP2 will be compared to muscle function and excitation-contraction coupling failure.

Animal transport: On the day of the experiment, one cage of mice (n=1-4) will be transported to the Muscle Biology Laboratory (Room G19) in the Sports Arena building. The cage will be placed in a ventilated box and walked from the housing facility to the Muscle Biology Laboratory. It takes less than 5 minutes to reach the Sports Arena building from the animal housing facility. If the mice are not euthanized after the experiment, animals will be returned to the animal housing facility at the end of the same day of testing following recovery from anesthesia. The transport box will be decontaminated immediately prior to placing the mouse cage inside it by spraying the inside and outside surfaces with Clidox-S (diluted 1:18:1 and provided by DAR). The outside of the transport box will also be sprayed with Clidox-S immediately upon re-entry into the animal facility upon returning the animals.

***In vivo* muscle strength testing:** In study 1, contractile function (i.e., torque-stimulation frequency relationship) of the left anterior crural muscles will be measured *in vivo* immediately before and 0, 3, 6, 24, and 72 hrs after a single bout of eccentrically biased exercise, and before and 0 and 24 hours after a single bout of concentrically biased exercise (Table 1). In study 2, contractile function of the left anterior crural muscles will be measured *in vivo* immediately before and after two bouts of eccentrically biased exercise that are separated by two weeks. Contractile function of the left anterior crural muscles will also be examined at 3 or 6, 24 and 72 hours after the second injury bout. Additionally, in study 2, one group of mice will only perform *in vivo* strength testing immediately before, immediately after, and 14 days after the first injury, but will not perform the second injury bout or any further *in vivo* testing thereafter (Table 2). Therefore, in study 1, anterior crural muscle strength will be measured on either 1 or 2 different days. In study 2, anterior crural muscle strength will be measured on either 2 or 3 different days. On non-terminal experimental days, mice will be allowed to recover under a heat lamp before being transported back to the animal housing facility.

In study 1, 30 animals at both 3 and 6 hours after injury are required to investigate the ubiquination of JP1 and JP2 in injured and uninjured TA muscle. At these early time points after injury, Western blotting for JP1 and JP2 may not be sensitive enough to detect damage to these proteins. Proteins that have been damaged are tagged with ubiquitin for subsequent removal by proteasomes. Therefore, in addition to assessing muscle damage with Western blotting of JP1 and JP2, we will also assess the ubiquination of these specific proteins at 3 and 6 hours after injury induction. Because of technical limitations, 5 TA muscles will be pooled to form one sample, allowing 6 samples per time point. For this analysis, the left, injured TA muscles will be compared to the right, uninjured TA muscles.

The strength-testing regimen is well tolerated by the mice. Using a multiple use experimental protocol (i.e., *J. Physiol.* 515: 609, 1999; *J. Appl. Physiol.* 97: 1067-1076, 2004; *J. Appl. Physiol.* 98: 1674-1681, 2005; *J. Appl. Physiol.* 105:1542-1553, 2008; *J. Appl. Physiol.* 105: 527-537,

2008), we have observed no adverse effect on the health of the mouse (i.e., no loss in body weight, decrease in grooming habits, or in muscle functional capacity of control mice). Immediately before muscle strength testing, mice will be anesthetized, and then placed on a heating pad to maintain body temperature. The hair on the left hindlimb will be removed using clippers and a depilatory, and then the skin covering the left biceps femoris and lateral gastrocnemius muscles will then be aseptically prepared. After aseptic preparation of the left leg, the mouse is transferred to a temperature-controlled platform. The tail, forelimbs, and right leg will be secured to the platform a mouse rectal thermometer will be inserted 2-3 mm beyond the anus. (At the conclusion of the experiment, the mouse rectal probe will be removed and cleaned with alcohol). The ankle and knee will be held in place using a clamp device described in *J. Appl. Physiol.* 72:1205, 1992 and used previously by the investigator (i.e., *J. Appl. Physiol*. 79:1260, 1995; *J. Appl. Physiol*. 80:332, 1996; *J. Mus. Res. Cell Motil.* 19:215, 1998; *J. Appl.Physiol*. 84:2171, 1998; *J. Appl. Physiol*. 85:58, 1998; *J. Physiol*. 515:609, 1999; *Med. Sci. Sport Exerc*.,32: 820, 2000; *J. Appl. Physiol*. 97: 1067-1076, 2004; *J. Appl. Physiol*. 97: 1619-1625, 2004; *J. Appl. Physiol*. 98: 1674-1681, 2005). Two sterilized platinum subdermal needle electrodes will be inserted into the aseptically prepared skin proximal to the common peroneal nerve on the left hindlimb. Anterior crural muscle strength will be measured by stimulating the left common peroneal nerve via percutaneous needle electrodes. Anterior crural muscle torque will then be measured at the following stimulation frequencies: 20, 40, 60, 80, 100, 125, 150, 200, 250, 300, 350, and 400 Hz. For mice that will undergo *in vivo* strength tests on more than one day, on the initial day of testing, while the mouse is still anesthetized following that day's final *in vivo* strength test, the animal's ear/s will be systematically marked (holes and notches) for mouse identification.

In the tables that follow, the PI outlines the design of study one and two in this proposal. These tables identify the various experimental groups plus the measurements that are made on the mice in those groups. The number of mice needed for each group is also indicated; these numbers do not account for experimental failures.

Table 1. Study 1: Effects of a single injury bout on JP1 and JP2 protein expression and cellular location (N=180)

Treatment Group	Time after exercise protocol (hours)				
	0	3	6	24	72
Unexercised Control (n=15)	*In vitro* strength; protein analyses (n =15)				
Concentric (n=30)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)			*in vivo* strength; *in vitro* strength; protein analyses (n =15)	
Eccentric (n=135)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)	*in vivo* strength; *in vitro* strength; protein analyses (n =45)	*in vivo* strength; *in vitro* strength; protein analyses (n =45)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)

For all cells: 5 EDL muscles will not perform *in vitro* caffeine contracture testing and will be used for immunohistochemistry instead. Additinonally, 10 TA muscles in every cell will used for both JP1 and JP2 immunohistochemistry and Western blotting.
For Eccentric 3 and 6 hours: 30 mice from each cell will be used to study JP1 and JP2 ubiquination. The right, uninjured TA muscle will be used as a control for the left, injured TA muscle.

Table 2a. Study 2: Repeated bout effect on JP1 and JP2 protein expression and cellular location (N=75)

Treatment Group	Immediately before	Time after second injury bout (hours)			
		0	3 or 6	24	72
Eccentric (n=75)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)	*in vivo* strength; *in vitro* strength; protein analyses (n =15)

For all cells: 5 EDL muscles will not perform *in vitro* caffeine contracture testing and will be used for immunohistochemistry. Additionally, 10 TA muscles will be used for both JP1 and JP2 immunohistochemistry and Western blotting.

This following Table is in reference to the number of animals per cell listed in Tables 1 and 2a.

Table 2b. Breakdown of the analysis that each muscle will be used for in Studies 1 and 2 (Table 1 and Table 2a)

Muscle and Analysis	***For cells with:***	
	15 mice	45 mice
TA-WB & IHC **L. EDL** - In vitro	10	10
L. EDL - IHC	5	5
TA- Ubiquination		30
Total	15	45

Note: Reference of cells is to Table 1 and Table 2a. Western blotting (WB); Immunohistochemistry (IHC); Left EDL (L. EDL). L. EDL muscles that undergo in vitro caffeine experiments cannot be used for immunohistochemistry.

Describe **in detail** the pre- and/or post-procedural care including drugs

➔

Justification of the number of animals required for this procedure using power analysis if possible (for help with power analysis see http://calculators.stat.ucla.edu/powercalc/

➔ Justification for the number of animals in study 1, 2, and 3 is based on preliminary reports from colleagues at Baylor College of Medicine. From these data, the investigator predicts a statistical power of 0.70 in the detection of 30% differences among groups (i.e., cell size of 10 mice) in TA or EDL muscle JP1 and JP2 protein content. Additionally, it is estimated that 5 muscles per cell are required in studies 1 and 2 to establish altered cellular location in longitudinal EDL muscle cryosections determined with immunohistology (Note: EDL muscles that perform the caffeine contracture test cannot be used for protein analyses). Moreover, in study 1 it is estimated that a total of 30 mice per time point (3 and 6 hr) will be required to assess ubiquination of JP1 and JP2 in injured and contralateral TA muscles. For technical reasons, in this analysis (i.e., SR isolation) 5 TA muscles will be pooled to form 1 sample. A power of 78.9 was calculated with a cell size of 6 and an effect size and coefficient of variation of 20% for this analysis. In study three, the number of animals required to perform the *in vitro* caffeine contracture test was based on previous research we have conducted (i.e., *J. Appl. Physiol.* 105:5, 2008). Based on these data, 4 mice per cell will achieve a power > 0.80 in detecting a 10% difference among groups.

In the past, we have experienced failure rates of approximately 10 and 30% for *in vivo* and *in vitro* experiments, respectively. To account for the experimental failure, the total number of animals needed for study 1 is increased from 180 to 198. The total number of animals needed for study #2 is increased from 75 to 83. The total number of animals needed for study 3 is increased from 56 to 73.

Possible/known side effects of the procedure:

➔ Muscle strength testing should not result in muscle injury. However, on rare occasions (approximately 1 out of 200 experiments), the common peroneal nerve is damaged by the placement of the sub-dermal stimulating electrodes. If the nerve is damaged, foot-drop is observed and the animal will be euthanized.

Procedure name: ➔*In vivo* exercise protocol – Single exercise bout

Infectious? ☐ yes **X** no (If yes, also see Question 6)
Radioactive? ☐ yes **X** no (If yes, also see Question 6)
Antibody production? ☐ yes **X** no
(If you are using intradermal or multiple injections of complete Freund's adjuvant (CFA), please state and justify)

Detailed description of <u>THIS PROCEDURE</u> including:
Will these animals be restrained during this procedure for any reason?
X Yes (If yes, explain below*)
☐ No

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above

Will animals be subjected to multiple survival surgeries (i.e. more than one penetration of a body cavity including the brain, or more than one procedure that impairs physical or physiologic function at different times)
☐ Yes (If yes, scientifically justify below*)
X No

The maximum number of times the treatment/procedure will be done to any animal is:

1

If anesthesia is used, please describe:

Anesthesia: Mice will be anesthetized as described above for "*In vivo* anterior crural muscle strength measurement". Please note that the exercise protocol is done immediately after strength testing, so the <u>induction</u> of anesthesia will NOT be performed. However, anesthetic supplements may be given as described in "*In vivo* anterior crural muscle strength measurement".

General description of procedure

***In vivo* eccentrically and concentrically biased exercise:** Induction of anesthesia, the preparation of the left hindlimb, and animal placement and restraint for *in vivo* testing is the same as described for the "*In vivo* anterior crural muscle strength measurement" protocol. All mice in study 1 and one group of mice in study 2 will perform a single injurious bout of exercise that consists of 150 eccentric contractions. In this contraction protocol, the left anterior crural muscles will be stimulated to contract (for 120 ms) while the foot is plantarflexed (i.e., eccentric contraction) over 40˚ at 2000˚/s; it takes 28 min to do the 150 contractions. The strength losses that result from the eccentric contraction-induced injury protocols are approximately 50% for tetanic contractions. This percent loss is typical of the functional loss a human experiences following exercise-induced muscle injury (*Med. Sci Sports Exerc.* 24: 512, 1992). The loss of strength has no observable effect on the ability of the mouse to ambulate, as the anterior crural muscles are not recruited heavily except at high running speeds.

In study 1, concentrically biased exercise will be accomplished by the performance of 150 concentric contractions. In this contraction protocol, the left anterior crural muscles will be stimulated to contract (for 120 ms) while the foot is dorsiflexed (i.e., concentric contraction) over 40˚ at 2000˚/s; it takes 28 min to do the 150 contractions. We have shown that maximum anterior muscle strength is unaffected after this concentric contraction protocol (150 concentric contractions at 2000°/s) (i.e., *J. Physiol.* 515: 609, 1999).

Describe **in detail** the pre- and/or post-procedural care including drugs

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above.

Justification of the number of animals required for this procedure using power analysis if possible

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above.

Possible/known side effects of the procedure:

➔ Known side effects of the eccentric and concentric exercise are temporary skeletal muscle damage, weakness and/or pain. In humans, mild-to-moderate muscle soreness has been reported 1-3 days after novel eccentrically biased exercise. Muscle soreness is known to be absent or greatly attenuated in subsequent eccentrically biased exercise bouts. Possible side effects of the eccentric and concentric exercise in certain genotypes are rhabdomyolysis, renal failure, and death. On rare occasions (approximately 1 out of 200 experiments), the common peroneal nerve is damaged by the placement of the sub-dermal stimulating electrodes. If the nerve is damaged, foot-drop is observed and the animal will be euthanized.

Procedure name: ➔In vivo exercise protocol – Two injury bouts

Infectious? ☐ yes **X** no (If yes, also see Question 6)
Radioactive? ☐ yes **X** no (If yes, also see Question 6)
Antibody production? ☐ yes **X** no
(If you are using intradermal or multiple injections of complete Freund's adjuvant (CFA), please state and justify)

Detailed description of THIS PROCEDURE including:
Will these animals be restrained during this procedure for any reason?
X Yes (If yes, explain below*)
☐ No

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above

Will animals be subjected to multiple survival surgeries (i.e. more than one penetration of a body cavity including the brain, or more than one procedure that impairs physical or physiologic function at different times)
☐ Yes (If yes, scientifically justify below*)
X No

The maximum number of times the treatment/procedure will be done to any animal is:

2

If anesthesia is used, please describe:

Anesthesia: Mice will be anesthetized as described above for "*In vivo* anterior crural muscle strength measurement". Please note that the exercise protocol is done immediately after strength testing, so the induction of anesthesia will NOT be performed. However, anesthetic supplements

may be given as described in "*In vivo* anterior crural muscle strength measurement".

General description of procedure

Induction of anesthesia, the preparation of the left hindlimb, and animal placement and restraint for *in vivo* testing is the same as described for the "*In vivo* anterior crural muscle strength measurement" protocol. In study 2, all but one group of mice will perform two bouts of injurious exercise identical to that described above for a single bout of eccentric contractions (Table 2). Mice will perform two bouts of 150 eccentric contractions with the left anterior crural muscles, which will be stimulated to contract (for 120 ms) while the foot is plantarflexed (i.e., eccentric contraction) over 40˚ at 2000˚/s. The two injury bouts will be separated by 14 days. In general, the strength loss observed immediately after a second injury bout is either similar to or lesser than strength loss after a single injury bout, while strength deficits 1-5 days after the second injury bout are significantly less than after a single injury bout. Therefore, the second injury bout has lesser adverse effects on ambulation and animal well-being than a single injury bout.

Describe **in detail** the pre- and/or post-procedural care including drugs

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above

Justification of the number of animals required for this procedure using power analysis if possible

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above

Possible/known side effects of the procedure:

Known side effects of the eccentric and concentric exercise are temporary skeletal muscle damage, weakness and/or pain. In humans, mild-to-moderate muscle soreness has been reported 1-3 days after novel eccentrically biased exercise. Muscle soreness is known to be absent or greatly attenuated in subsequent eccentrically biased exercise bouts. Possible side effects of the eccentric and concentric exercise in certain genotypes are rhabdomyolysis, renal failure, and death. On rare occasions (approximately 1 out of 200 experiments), the common peroneal nerve is damaged by the placement of the sub-dermal stimulating electrodes. If the nerve is damaged, foot-drop is observed and the animal will be euthanized.

Procedure name: ➔In vitro muscle testing

Infectious? ☐ yes **X** no (If yes, also see Question 6)
Radioactive? ☐ yes **X** no (If yes, also see Question 6)
Antibody production? ☐ yes **X** no
(If you are using intradermal or multiple injections of complete Freund's adjuvant (CFA), please state and justify)

Detailed description of THIS PROCEDURE including:
Will these animals be restrained during this procedure for any reason?
X Yes (If yes, explain below*)

☐ No

Please see description of protocol below

Will animals be subjected to multiple survival surgeries (i.e. more than one penetration of a body cavity including the brain, or more than one procedure that impairs physical or physiologic function at different times)
☐ Yes (If yes, scientifically justify below*)
X No

The maximum number of times the treatment/procedure will be done to any animal is:

1

If anesthesia is used, please describe:

Mice will be anesthetized with sodium pentobarbital (100 mg/kg ip). This dosage, which we have used for the last 14 years, is required to achieve the necessary plane of anesthesia needed for muscle dissection in mice. **Multiple toe pinches will be used to ensure proper plane of anesthesia prior to dissection.** If necessary, mice will be given supplemental doses of sodium pentobarbital to ensure that this plane of anesthesia (i.e., absence of leg withdrawal from toe pinch and reduction in ventilation rate) is maintained throughout the procedure (usually 45 minutes).

General description of procedure

Study 3 experimental design: We have previously reported that the magnitude of strength deficits and excitation-contraction coupling failure immediately after performing eccentric contractions *in vitro* is temperature dependent (Warren et al., *Am J Physiol Regul Integr Comp Physiol*, 282(4), R1122-1132, 2002), whereby the magnitude of excitation-contraction coupling failure and strength deficits increase from non-physiological (15˚C) to physiological temperatures (37˚C).

Study 3 (N=73) will characterize the effect of experimental temperature (i.e., 15, 25, 37˚C) during the performance of eccentric or isometric contractions *in vitro* on JP1 and JP2 protein content. As such, in study 3 dissected EDL muscles will undergo an *in vitro* "exercise" protocol in which the muscles will perform either 5 eccentric or 5 isometric contractions at one of these temperatures. (See table 3; This table identifies the various experimental groups in study 3 plus the measurements that are made on the mice in those groups. The number of mice needed for each group is also indicated; these numbers do not account for experimental failures).

On the final day of *in vivo* testing in studies 1 and 2 and in one of group of non-exercised (*in vivo*) mice in study 1, the left EDL muscle will be isolated and tested *in vitro* at 37˚C only. For all muscles of any study undergoing *in vitro* testing, the muscle will at least be subjected to a force-frequency and caffeine contracture test. Because caffeine directly stimulates the SR to release calcium and therefore by-passes upstream components of the excitation-contraction coupling process, the ratio of caffeine contracture force to maximal voltage-induced force may be compared between injured and uninjured muscle to assess the degree of excitation-contraction coupling failure in injured skeletal muscle.

***In vitro* contractile function measurement:** On the final day of *in vivo* testing in studies 1 and 2, in one group of non-exercised (*in vivo*) mice in study 1, and in all experiments in study 3, the left

EDL muscle will be dissected from anesthetized mice (sodium pentobarbital) and mounted in an organ bath for determination of contractile characteristics (e.g., force-frequency relationship and caffeine contracture test). Immediately after muscle dissection, a bandage is taped over the left hindlimb. Further muscle dissection and tissue collection is described below. Once the tissue necessary to perform this study has been collected, euthanasia will be achieved via sodium pentobarbital (200 mg/kg IP).

Table 3. Study 3: Temperature dependency of *in vitro* eccentric-contraction induced force deficits, E-C coupling, and junctophilin disruption (N=56)

Treatment Group	Experimental Temperature (°C)		
	37	25	15
Isometric (n=14)	*In vitro* strength; protein analyses (n =14)		
Eccentric (n=42)	*In vitro* strength; protein analyses (n =14)	*In vitro* strength; protein analyses (n =14)	*In vitro* strength; protein analyses (n =14)

For all cells: Only 4 EDL muscles will perform caffeine contracture testing, while the remaining 10 muscles will be used for JP1 & JP2 Western blotting.

Describe **in detail** the pre- and/or post-procedural care including drugs

Mice will be checked for skeletal abnormalities, **by visually examining the animal's hindlimb morphology and gait**, and adequate body weight prior to the experiment.

Justification of the number of animals required for this procedure using power analysis if possible

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above

Possible/known side effects of the procedure:

This is a terminal procedure and thus presents no side effects

Procedure name: ➔Tissue harvest

Infectious? ☐ yes **X** no (If yes, also see Question 6)
Radioactive? ☐ yes **X** no (If yes, also see Question 6)
Antibody production? ☐ yes **X** no
(If you are using intradermal or multiple injections of complete Freund's adjuvant (CFA), please state and justify)

Detailed description of <u>THIS PROCEDURE</u> including:
Will these animals be restrained during this procedure for any reason?
X Yes (If yes, explain below*)
☐ No

Please see description of the "*In vitro* muscle testing" protocol above

Will animals be subjected to multiple survival surgeries (i.e. more than one penetration of a body cavity including the brain, or more than one procedure that impairs physical or physiologic function at different times)
☐ Yes (If yes, scientifically justify below*)
X No

The maximum number of times the treatment/procedure will be done to any animal is:
1

If anesthesia is used, please describe:

Please see description of the "*In vitro* muscle testing" protocol above

General description of procedure

Tissue dissection and animal euthanasia: Once the mouse is under a surgical plane of anesthesia (sodium pentobarbital, 100mg/kg ip), **which will be ensured with multiple toe pinches**, the left hindlimb TA muscle will be dissected and frozen. Then the left hindlimb EDL muscle will be dissected, functionally tested *in vitro*, and frozen. During the automated measurement of the left EDL muscle *in vitro* testing, the right hindlimb TA and EDL muscles will be dissected and frozen. Frozen muscle samples will be analyzed later via Western blotting and immunohistochemistry for JP1 and JP2 alterations.

Describe **<u>in detail</u>** the pre- and/or post-procedural care including drugs

Mice will be checked for skeletal abnormalities, **by visually examining the animal's hindlimb morphology and gait**, and adequate body weight prior to the experiment.

Justification of the number of animals required for this procedure using power analysis if possible

Please see description of "*In vivo* anterior crural muscle strength measurement" protocol above

Possible/known side effects of the procedure:

This is a terminal procedure and thus presents no side effects

*** **<u>COPY AND PASTE THE ABOVE HEADINGS FOR ADDITIONAL PROCEDURES IF NEEDED</u>** ***

USDA Classifications and Examples of Pain Categories:

Classification B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.

Examples:

- Breeding colonies - Includes parents and offspring.
- Animals held under proper captive conditions or wild animals that are being observed.

Classification C: Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.

Examples:

- Procedures performed correctly by trained personnel such as the administration of electrolytes/fluids, administration of oral medication, blood collection from a common peripheral vein per standard veterinary practice or catheterization of same, standard radiography, parenteral injections of non-irritating substances.
- Euthanasia performed in accordance with the recommendations of the most recent AVMA Panel on Euthanasia, utilizing procedures that produce rapid unconsciousness and subsequent humane death.
- Manual restraint that is no longer than would be required for a simple exam; short period of chair restraint for an adapted nonhuman primate.

Classification D: Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.

Examples:

- Surgical procedures conducted by trained personnel in accordance with standard veterinary practice such as biopsies, gonadectomy, exposure of blood vessels, chronic catheter implantation, laparotomy or laparoscopy.
- Blood collection by more invasive routes such as intracardiac or periorbital collection from species without a true orbital sinus such as rats and guinea pigs.
- Administration of drugs, chemicals, toxins, or organisms that would be expected to produce pain or distress but which will be alleviated by analgesics.

Classification E: Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

Examples:

- Procedures producing pain or distress unrelieved by analgesics such as toxicity studies, microbial virulence testing, radiation sickness, and research on stress, shock, or pain.
- Surgical and postsurgical sequela from invasion of body cavities, orthopedic procedures, dentistry or other hard or soft tissue damage that produces unrelieved pain or distress.
- Negative conditioning via electric shocks that would cause pain in humans.
- Chairing of nonhuman primates not conditioned to the procedure for the time period used.

Across all of the above, describe all painful and/or distressful procedures you plan to use during this protocol. If category E were selected above, please provide references indicating that both of the two major classes of pain-relieving medication (i.e. opiates and non-steroidal anti-inflammatory drugs) will interfere with your goals.
➔D

In vivo anterior crural muscle strength (described above)
In vivo exercise protocol – Single exercise bout (described above)
In vivo exercise protocol – Two injury bouts (described above)
In vitro muscle testing (described above)

Tissue harvest (described above)

If painful procedures must be performed, appropriate sedatives, analgesics or anesthetics must be used (unless withholding of these was justified above). List the drugs you will use to prevent pain or distress.

Name of Drug: ➔ fentanyl (F), droperidol (D), diazepam (DZ)
Dose: ➔ F: 0.3 mg/kg D: 16.7 mg/kg DZ: 5 mg/kg
Route of Administration: ➔ip

Name of Drug: ➔ Sodium pentobarbital
Dose: ➔100 mg/kg
Route of Administration: ➔ip

Name of Drug: ➔ Buprenorphine
Dose: ➔0.05 mg/kg
Route of Administration: ➔ ip

Who will be responsible for monitoring the animals for pain and/or distress during the experimental procedure(s)?
➔ The PI and/or GRAs will monitor the level of anesthesia in the animals.

How will the comfort level of the animals be determined?
➔ Mice will be checked by the PI and/or his GRAs daily after the exercise procedure. If a mouse is lethargic or limping on the first or second mornings after the in vivo exercise protocol, then 0.05 mg.kg bupreorphine will be administered ip twice. If pain and/or disability are not resolved by the third day and the mouse has lost more than 20% of its body weight, then 0.05 mg/kg buprenorphine will be re-administered twice. If pain and/or disability are not resolved by the fifth day and the mouse has lost more than 20% of its body weight, then the mouse will be euthanized after consultation with the university veterinarian.

In the event that an animal needs to be euthanized or removed from the experiment, please list the criteria for the decision:
➔ If an animal becomes ill (as evident by a loss of 20% of body weight and lack of grooming), then
it will be euthanized.

Who will determine this action?
➔ The PI in conjunction with the university veterinarian

At the termination of the experiment, describe in detail the method of euthanasia (if any) you will use? How will remains be disposed? Methods of euthanasia must be in accordance with the most current American Veterinary Medical Association Panel on Euthanasia http://www.avma.org/resources/euthanasia.pdf. References must be cited to justify deviations from this document, or to justify use of cervical dislocation or decapitation without anesthesia.

➔ Animals will be euthanized via an anesthetic overdose of either CO_2 or 200 mg/kg ip of sodium pentobarbital. These methods are approved for mice by the AVMA Panel on Euthanasia, 2000. After the animal ceases respiration, cervical dislocation will be performed to ensure death before placing the carcass in the freezer. Animal carcasses will be stored in the Muscle Biology Laboratory freezer until the time they are transferred to the animal care facility freezer.

5. **EXPERIENCE AND TRAINING OF PERSONNEL**
List the experience and/or training of the personnel that will conduct each Procedure.

If training has not been completed, visit http://www.gsu.edu/reseearch/iacuc.html and click on "How to Enroll in and Use the AALAS Learning Library". Complete the Required Education prior to submitting this protocol application.

All employees of Georgia State University who work with vertebrate animals must enroll in the Medical Monitoring Program for Vertebrate Animal Exposure, found at http://www.gsu.edu/images/vp_research/GSU_MMPVAE_Form_May06.doc

Principal Investigator:
Name: Christopher P. Ingalls, PhD
Procedure: All
Experience: The PI is a physiologist with 5 years of postdoctoral experience and 14 years experience in rodent handling, surgery, and exercise protocols. The PI has formal training in animal care techniques, having completed three Texas A&M University Lab Animal Research and Resource courses (i.e., Mice: Basic Handling and Techniques; Asepsis and Aseptic Techniques; Rat: Basic Handling and Techniques), as well one workshop on Mouse Handling Workshop at GSU, and has completed online training courses.
Has required training been completed? Xyes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? X yes ☐ no If "no", see above

Note: You can copy and paste the sections below as needed to list additional personnel.

Postdoctoral personnel:
Name:
Procedure:
Experience:
Has required training been completed? ☐ yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? ☐ yes ☐ no If "no", see above

Name:
Procedure:

Experience:
Has required training been completed? ☐ yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? ☐ yes ☐ no If "no", see above

Graduate Students:
Name: Benjamin Corona
Procedure: All
Experience: 4 years experience with rodent handling and exercise protocols
Has required training been completed? X yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? X yes ☐ no If "no", see above

Name:
Procedure:
Experience:
Has required training been completed? ☐ yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? ☐ yes ☐ no If "no", see above

Technicians:
Name:
Procedure:
Experience:
Has required training been completed? ☐ yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? ☐ yes ☐ no If "no", see above

Name:
Procedure:
Experience:
Has required training been completed? ☐ yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? ☐ yes ☐ no If "no", see above

Undergraduates:
Name: Jared Wagman
Procedure: *In vivo* anterior crural muscle strength; *In vivo* exercise protocol – Single exercise bout;
In vivo exercise protocol – Two injury bouts
Experience: Mr Wagman has no formal experience working with laboratory animals. He will be trained by the PI and/or Mr. Corona.
Has required training been completed? X yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? X yes ☐ no If "no", see above

Name:
Procedure:
Experience:
Has required training been completed? ☐ yes ☐ no If "no", see above
Is this person enrolled in the MMPVAE? ☐ yes ☐ no If "no", see above

6. **Biosafety and Radiation Safety Summary:**
a) Are you working with infectious agents and/or biologically-derived toxins?
X No
☐ Yes I have approval from the Biosafety Committee
Date Approval #

List and explain the precautions that will be taken to ensure the safety of the working environment for personnel). What special precautions are recommended for personnel who handle animals or do housekeeping? Should cage materials or animals from these experiments be autoclaved or decontaminated?
➔NA

b) Does the project use any recombinant DNA (i.e. cloning/expression systems, viral vectors, etc.) material in animals?
X No
☐ Yes I have approval from the Biosafety Committee
Date Approval #

List and explain the precautions that will be taken to ensure the safety of the working environment for personnel). What special precautions are recommended for personnel who handle animals or do housekeeping? Should cage materials or animals from these experiments be autoclaved or decontaminated?
➔NA

c) Are any carcinogenic compounds to be used in animals?

X No
☐ Yes I have approval from the Biosafety Committee
Date Approval #

List and explain the precautions that will be taken to ensure the safety of the working environment for personnel). What special precautions are recommended for personnel who handle animals or do housekeeping? Should cage materials or animals from these experiments be autoclaved or decontaminated?
➔NA

d) Will any radioactive compounds be administered to the animals?

X No
☐ Yes I have approval from the Radiation Safety Committee
Date Approval #

List and explain the precautions that will be taken to ensure the safety of the working environment for personnel). What special precautions are recommended for personnel who handle animals or do housekeeping? Should cage materials or animals from these experiments be autoclaved or decontaminated?
➔NA

7. **Animal Housing and Husbandry:**

a) Will you breed these animals?

X No
☐ Yes (Explain and justify below)
➔

b) Do the animals require housing other than standard caging/bedding?

☐ No
X Yes (List housing required and explain below)
➔ Due to minor allergies by the PI, the PI requests that hypoallergenic bedding (i.e., alpha dry) be used in the cages for animal housing.

c) Do the animals require special care?

X No
☐ Yes (List special care and explain below)
➔

d) Do the animals require diet other than the standard diet for this species used at GSU?

X No
☐ Yes (List diet and explain below)
➔

e) Will animals remain outside the Department of Animal Resources facility for more than 12 hours?

X No

☐ Yes (List where and explain below)
➔

f) Will animals undergo experimental manipulations outside the Department of Animal Resources facilities?
☐ No
X Yes (List where and explain below)
➔ Animas will be transported in cages placed in a ventilated carrier to the Muscle Biology Laboratory in the Sports Arena room G19 where all experiments will be performed.

g) Can your animals be provided environmental enrichment?
☐ No (Explain below)
X Yes (List enrichment requested below)
➔ Plastic domes

APPENDIX B

INDIVIDUAL SUBJECT DATA

Study One: Isometric Torque-Frequency (Nmm·kg^{-1}) – Pre Injury

Condition	Name	Weight 1 (g)	Wt 2 (g)	Weight 1 (g)	Wt 2 (g)	Pre-Exercise Isometric Torque-Frequency 20	40	60	80	100	125	150	200	250	300	350	400
Concentric-1mm	D1001	23.3		23.3		20.86	24.64	41.70	67.91	83.76	94.49	100.66	106.61	108.96	110.50	111.06	111.30
	D1002	23.2		23.2		17.94	17.41	23.34	39.28	61.38	78.31	83.81	88.91	91.68	93.44	93.87	93.49
	D1019	33.2		33.2		19.90	19.86	24.94	44.08	72.60	88.18	95.44	102.26	105.53	107.48	108.30	109.20
	D1020	31.6		31.6		23.88	21.80	25.15	38.37	68.09	89.72	97.41	104.74	108.31	110.00	110.61	110.97
	D1025	37.5		37.5		25.36	29.76	39.45	59.00	82.57	96.16	102.73	106.21	109.92	110.90	111.24	112.62
	D1032	34.9		34.9		18.40	19.99	24.46	45.96	70.69	85.20	90.63	96.07	99.44	99.69	99.54	99.87
Concentric-1d	D1016	30	29.1	30	29.1	23.66	21.83	26.54	43.01	69.46	90.51	97.35	103.89	105.74	105.89	106.68	106.66
	D1021	33.5	31.1	33.5	31.1	21.75	22.36	30.81	60.79	91.50	107.42	114.29	120.81	123.35	124.99	125.46	125.36
	D1023	34.3	32.6	34.3	32.6	28.31	29.73	39.10	63.60	87.46	100.63	106.52	112.71	115.90	117.71	118.44	118.70
	D1026	26.3	29	26.3	29	22.65	22.29	28.86	52.72	80.17	99.90	109.61	116.08	119.63	121.61	123.27	123.32
Eccentric-1mm	D1000	22.8		22.8		12.35	12.09	16.92	31.90	53.68	73.04	80.14	87.19	90.93	93.04	94.28	95.67
	D1004	22.8		22.8		18.81	19.80	27.51	45.91	69.86	87.74	93.75	99.56	102.64	104.34	105.66	106.21
	D1007	24.7		24.7		23.19	24.38	36.71	56.88	74.16	84.24	89.05	93.70	95.71	96.47	97.39	97.96
	D1022	36.4		36.4		21.23	19.39	22.83	35.57	65.28	84.87	91.54	96.97	98.93	100.73	101.12	100.38
	D1028	31.7		31.7		21.03	22.80	31.58	54.69	78.87	92.36	97.70	103.22	105.62	106.50	107.39	108.05
	D1029	31.2		31.2		26.34	29.39	38.39	64.57	87.90	101.12	106.41	111.69	113.82	113.42	111.86	111.74
	D1033	32.4		32.4		24.45	24.68	30.27	49.32	76.36	100.40	108.49	115.88	119.41	121.41	121.92	122.94
	D1047	23.7		23.7		20.65	21.27	28.47	50.41	77.64	96.36	104.11	110.90	113.70	115.22	115.11	115.56
	D1051	26.9		26.9		22.73	20.53	22.82	35.97	66.27	81.55	86.84	91.43	93.01	94.16	95.12	94.31
0																	
Eccentric-1d	D1006	22.8	23.6	22.8	23.6	24.79	30.80	54.37	79.04	100.73	114.61	121.19	127.83	130.74	131.97	131.89	131.55
	D1008	22	22	22	22	26.75	27.29	36.53	56.92	80.45	96.85	103.53	109.13	112.71	114.38	115.85	116.73
	D1015	26.7	27.1	26.7	27.1	25.14	28.00	40.54	63.37	85.06	100.62	108.16	115.33	118.69	120.46	121.25	122.31
	D1027	34.7	34.7	34.7	34.7	24.78	25.03	31.28	46.60	71.71	87.44	93.55	99.76	102.78	104.44	105.17	105.73
	D1030	34.7	34.4	34.7	34.4	20.94	20.99	27.40	46.01	82.66	97.36	103.32	108.80	111.28	111.23	112.37	111.97
	D1040	27.4	25.4	27.4	25.4	23.75	22.58	28.41	44.54	77.80	96.27	102.72	110.18	112.43	112.82	115.22	115.06
	D1045	26	26.2	26	26.2	19.62	20.42	29.36	51.70	76.72	94.99	101.65	107.99	112.42	115.97	115.26	116.02
	D1046	26	25.3	26	25.3	19.12	19.51	25.27	44.00	69.27	85.56	91.12	97.22	100.19	102.28	103.68	104.40
	D1049	25.4	24.2	25.4	24.2	27.40	30.88	48.83	79.65	104.82	120.35	126.67	134.70	137.95	139.81	140.35	141.19
0																	
Eccentric-3d	D1005	24.7	20.7	24.7	20.7	22.49	25.54	40.47	69.17	90.37	104.18	109.51	115.49	118.74	120.26	121.40	121.87
	D1012	31.6	32.1	31.6	32.1	26.57	24.36	30.20	46.62	70.09	85.84	91.97	97.24	99.95	101.22	101.80	102.33
	D1013	29	29.4	29	29.4	26.98	27.84	33.28	59.05	90.36	114.11	122.29	130.34	134.32	136.24	135.91	136.74
	D1014	30	29.7	30	29.7	21.67	20.11	24.61	41.60	70.64	89.21	95.61	101.65	104.78	105.30	105.52	105.61
	D1017	26.4	27.3	26.4	27.3	18.39	18.52	22.59	41.63	65.02	79.53	84.51	89.58	91.43	92.66	93.54	93.89
	D1024	35.9	34.4	35.9	34.4	17.52	16.25	22.99	38.20	59.43	74.50	80.28	85.66	88.67	90.73	91.57	91.85
	D1031	39	35.2	39	35.2	27.66	27.68	32.60	48.40	71.91	89.23	96.35	102.74	105.04	106.54	106.56	106.83
	D1042	24.9	24.9	24.9	24.9	26.35	27.42	37.33	63.32	88.51	106.37	113.67	120.61	124.63	127.50	128.72	131.54
	D1048	30.6	29.4	30.6	29.4	17.87	16.20	18.85	33.56	66.11	87.51	94.98	101.25	104.05	105.26	106.01	106.59
	D1050	31.3	29.9	31.3	29.9	23.03	27.17	47.92	77.50	91.40	100.90	104.75	107.74	109.80	111.13	112.11	112.61

Study One: Isometric Torque-Frequency (Nmm·kg^{-1}) – Immediate Post-Injury

Condition	Name	Immediate Post Exercise Isometric Torque-Frequency									
		20	40	60	80	100	125	150	200	250	300	350	400
Concentric-1mm	D1001	21.48	22.06	35.95	64.14	82.85	92.90	97.63	104.90	107.45	109.07	109.71	110.15
	D1002	16.09	17.10	25.55	43.70	64.85	79.04	84.55	89.87	92.55	94.45	95.32	95.93
	D1019	18.35	16.83	18.54	26.28	45.57	81.16	90.70	100.13	104.25	103.54	105.33	104.66
	D1020	20.48	17.28	21.84	33.77	55.98	76.72	83.96	88.15	91.84	93.15	95.16	96.03
	D1025	23.43	24.85	31.02	46.34	67.32	90.38	99.23	106.23	109.49	110.93	111.17	110.94
	D1032	20.04	18.89	22.36	37.43	60.23	85.23	92.84	99.84	103.07	103.68	104.26	105.48
Concentric-1d	D1016	20.75	19.83	24.06	42.49	70.05	91.21	98.04	104.48	107.82	109.73	109.93	110.05
	D1021	20.03	15.73	24.51	39.43	63.19	81.34	96.82	99.30	103.57	107.71	109.40	110.49
	D1023	24.55	22.90	26.77	41.33	68.11	88.08	94.80	100.74	103.72	105.27	107.04	108.47
	D1026	24.98	21.60	28.13	46.46	73.62	101.73	111.75	119.89	122.55	123.36	123.51	124.09
Eccentric-1mm	D1000	2.73	2.96	3.62	5.45	9.74	19.21	33.51	51.29	57.10	59.34	59.65	59.82
	D1004	4.20	4.87	7.71	13.42	23.36	40.77	57.22	67.68	70.61	71.16	71.07	69.37
	D1007	6.36	6.80	9.82	16.68	26.43	43.39	53.36	62.20	64.37	65.17	65.00	63.45
	D1022	6.27	6.16	8.17	12.78	19.90	33.57	49.13	62.58	65.33	64.35	64.82	64.25
	D1028	4.82	5.29	6.41	10.28	16.25	31.79	46.59	60.38	63.14	64.43	64.10	64.38
	D1029	6.94	6.89	8.40	14.03	22.48	43.40	60.29	81.99	78.20	79.35	79.35	78.50
	D1033	6.53	6.58	7.43	9.91	18.26	31.97	50.74	75.31	80.43	81.99	81.39	80.86
	D1047	5.16	4.89	6.39	10.20	18.12	30.58	46.65	68.05	74.41	75.88	75.94	75.41
	D1051	5.13	5.60	7.46	9.55	15.70	28.11	41.71	58.21	61.33	62.11	63.43	63.67
0													
Eccentric-1d	D1006	4.96	6.18	10.11	15.57	23.64	35.45	47.90	61.13	64.39	65.36	65.15	65.54
	D1008	5.45	5.27	7.45	11.72	20.21	36.97	51.88	66.82	70.95	72.76	73.90	73.88
	D1015	4.70	4.69	5.95	9.47	16.43	28.84	43.55	63.68	70.06	72.22	72.72	73.26
	D1027	4.32	4.86	8.15	11.40	18.18	36.25	49.14	61.73	65.19	65.80	63.93	63.90
	D1030	4.48	4.37	6.82	13.14	19.36	36.80	52.20	68.42	69.08	69.79	69.07	66.72
	D1040	5.76	6.53	8.44	12.49	19.81	36.51	51.14	64.17	67.32	68.79	69.22	68.85
	D1045	3.16	3.18	4.68	7.71	14.07	33.85	50.58	65.16	68.94	69.56	69.63	70.81
	D1046	3.97	4.34	5.64	9.97	17.07	36.76	55.82	70.79	75.28	76.98	77.24	77.04
	D1049	6.45	7.22	8.93	13.00	20.51	41.37	64.09	87.03	92.12	93.75	93.75	92.93
0													
Eccentric-3d	D1005	5.77	7.04	10.21	17.50	26.31	41.80	57.31	71.21	75.07	76.31	76.66	76.49
	D1012	4.14	4.33	4.57	10.20	17.28	28.46	41.84	56.56	60.50	61.53	62.47	62.09
	D1013	6.08	6.57	8.26	10.96	17.50	32.97	49.74	69.50	75.32	74.37	75.80	76.69
	D1014	4.40	4.54	5.71	9.45	16.94	30.31	41.97	55.15	56.75	59.06	60.27	59.40
	D1017	6.00	6.44	9.51	12.58	21.03	39.09	53.64	64.22	66.85	68.47	69.28	68.66
	D1024	4.12	4.20	5.54	8.65	14.97	27.51	39.26	54.04	58.20	59.40	59.82	59.63
	D1031	5.93	6.49	8.00	12.10	18.07	35.28	47.73	60.64	63.42	64.47	63.81	62.89
	D1042	4.97	6.38	9.17	14.13	22.37	37.18	54.80	75.01	80.30	83.09	84.61	85.19
	D1048	6.72	5.64	7.14	11.99	19.13	32.69	45.93	62.06	66.77	68.89	69.25	68.25
	D1050	5.42	6.47	9.35	13.37	21.78	36.23	48.03	61.79	65.07	66.41	65.51	64.63

Study One: Isometric Torque-Frequency ($Nmm \cdot kg^{-1}$) – Xday Post-Exercise

		X-day Post-Exercise Isometric Torque-Frequency										
Condition	**Name**	**20**	**40**	**60**	**80**	**100**	**125**	**150**	**200**	**250**	**300**	**350**	**400**
Concentric-1mm	D1001												
	D1002												
	D1019												
	D1020												
	D1025												
	D1032												
Concentric-1d	D1016	25.23	25.95	38.14	65.09	86.39	98.78	103.82	108.99	109.75	109.24	110.09	108.79
	D1021	22.95	26.35	42.59	68.99	88.56	98.59	103.76	108.54	110.43	111.90	111.24	111.38
	D1023	29.64	34.64	46.21	73.81	96.27	107.96	113.24	118.77	121.73	122.95	123.60	123.51
	D1026	24.08	24.96	30.85	46.27	68.01	83.77	88.11	90.89	97.67	99.41	99.52	100.28
Eccentric-1mm	D1000												
	D1004												
	D1007												
	D1022												
	D1028												
	D1029												
	D1033												
	D1047												
	D1051												
0													
Eccentric-1d	D1006	4.58	5.31	8.34	16.97	31.00	44.52	51.83	57.98	60.17	61.07	60.81	59.32
	D1008	8.98	11.01	16.41	25.99	34.54	56.22	69.11	81.01	86.48	89.26	90.30	90.62
	D1015	6.25	7.10	9.58	15.26	28.26	47.53	59.07	72.95	78.86	82.39	83.82	83.81
	D1027	6.78	7.91	10.69	19.47	37.53	57.51	69.14	78.70	82.69	84.98	86.07	84.45
	D1030	4.37	4.97	7.28	10.71	21.23	39.90	55.23	68.89	74.22	76.29	75.64	76.86
	D1040	6.26	6.65	9.59	15.92	34.28	52.99	63.28	73.82	78.06	79.79	79.76	77.98
	D1045	6.53	6.25	8.12	13.83	28.39	49.53	60.79	71.66	76.07	78.47	79.16	78.60
	D1046	7.15	7.73	13.11	22.19	38.26	52.28	58.09	67.10	70.46	72.40	73.03	73.14
	D1049	4.52	3.95	3.96	7.74	18.49	32.29	45.71	59.31	65.12	66.38	64.74	60.93
0													
Eccentric-3d	D1005	14.55	21.05	23.83	40.25	59.36	73.65	79.88	86.31	87.72	85.72	82.25	82.25
	D1012	11.97	11.68	14.59	22.67	36.72	58.59	68.95	76.23	79.03	80.49	81.24	81.19
	D1013	11.32	11.80	15.32	25.44	44.94	74.92	88.21	98.48	102.50	104.26	104.99	104.20
	D1014	7.56	7.80	10.05	17.43	32.22	54.89	65.12	72.85	75.81	76.95	77.30	76.31
	D1017	14.82	15.04	19.78	31.18	51.52	63.61	68.72	73.07	74.77	75.65	76.12	75.70
	D1024	8.35	8.67	11.23	21.20	37.75	59.87	69.05	76.04	78.90	80.48	80.68	79.88
	D1031	10.91	11.02	14.88	24.12	39.86	63.86	73.49	81.78	84.82	87.76	86.99	84.93
	D1042	19.92	20.73	31.41	51.02	75.76	93.62	101.94	109.60	112.56	112.30	111.26	111.66
	D1048	8.79	10.15	14.60	24.82	42.08	64.13	72.74	79.73	83.44	84.30	85.38	84.20
	D1050	9.77	10.70	24.80	53.92	73.84	86.17	93.40	96.00	97.88	98.02	97.40	96.00

Study One: Concentric or Eccentric Torque ($Nmm \cdot kg^{-1}$)

				Contraction #					
Condition	**Name**	**Weight 1 (g)**	**Weight (kg)**	**1**	**10**	**20**	**30**	**40**	**50**
Concentric-Imm	D1001	23.3	0.0233	113.652361	108.11588	112.424893	110.88412	109.961373	109.652361
	D1002	23.2	0.0232	93.4396552	91.2758621	91.2758621	90.9698276	91.5862069	91.2758621
	D1019	33.2	0.0332	103.51506	100.493976	100.060241	99.1987952	98.9819277	98.9819277
	D1020	31.6	0.0316	104.901899	98.7753165	97.8670886	96.7341772	96.278481	94.2373418
	D1025	37.5	0.0375	101.778667	97	97.1893333	96.616	96.0426667	95.6613333
	D1032	34.9	0.0349	103.813754	104.020057	104.226361	104.842407	104.636103	104.842407
Concentric-1d	D1016	30	0.03	101.176667	99.7433333	102.61	102.61	102.85	102.61
	D1021	33.5	0.0335	117.78209	111.576119	110.722388	110.292537	109.223881	108.152239
	D1023	34.3	0.0343	113.991254	109.183673	108.346939	107.093294	107.303207	106.676385
	D1026	26.3	0.0263	121.680608	119.771863	119.771863	119.224335	118.680608	118.680608
Eccentric-Imm	D1000	22.8	0.0228	185.635965	170.54386	140.986842	118.662281	112.372807	114.574561
	D1004	22.8	0.0228	196.95614	178.403509	149.162281	133.754386	129.350877	128.407895
	D1007	24.7	0.0247	195.736842	179.773279	153.651822	137.688259	131.303644	128.109312
	D1022	36.4	0.0364	200.571429	175.755495	148.774725	128.489011	121.005495	114.700549
	D1028	31.7	0.0317	210.634069	189.829653	159.977918	139.170347	129.44795	123.791798
	D1029	31.2	0.0312	226.647436	208.035256	182.532051	161.163462	151.282051	144.391026
	D1033	32.4	0.0324	235.734568	214.712963	176.657407	154.08642	147.228395	141.694444
	D1047	23.7	0.0237	248.763713	221.540084	193.105485	166.185654	153.489451	149.244726
	D1051	26.9	0.0269	188.256506	175.464684	153.877323	133.356877	122.431227	116.565056
Eccentric-1d	D1006	22.8	0.0228	242.548246	205.758772	161.109649	140.04386	133.754386	129.666667
	D1008	22	0.022	223.340909	199.881818	177.072727	163.386364	154.259091	149.368182
	D1015	26.7	0.0267	232.625468	213.831461	175.973783	154.494382	145.363296	141.067416
	D1027	34.7	0.0347	204.40634	186.227666	154.204611	133.956772	120.319885	113.089337
	D1030	34.7	0.0347	233.123919	210.605187	186.432277	163.086455	150.48415	146.559078
	D1040	27.4	0.0274	196.857664	184.29927	163.105839	144.788321	133.277372	125.689781
	D1045	26	0.026	244.957692	217.657692	194.223077	174.646154	163.915385	158.1
	D1046	26	0.026	198.634615	182.642308	165.546154	149.276923	141.834615	134.111538
	D1049	25.4	0.0254	282.637795	244.251969	218.003937	199.094488	188.366142	180.744094
Eccentric-3d	D1005	24.7	0.0247	241.88664	214.311741	183.255061	167.004049	159.45749	154.522267
	D1012	31.6	0.0316	199.958861	180.218354	150.727848	132.123418	123.278481	119.873418
	D1013	29	0.029	263.372414	235.931034	196.37931	168.444828	155.096552	148.668966
	D1014	30	0.03	225.676667	209.903333	186.486667	163.066667	150.88	142.756667
	D1017	26.4	0.0264	167.109848	152.44697	139.685606	127.734848	127.462121	123.390152
	D1024	35.9	0.0359	188.587744	171.615599	146.651811	130.67688	122.490251	116.899721
	D1031	39	0.039	213.671795	186.097436	158.707692	144.371795	135.364103	131.135897
	D1042	24.9	0.0249	256.927711	229.289157	202.514056	182.361446	171.995984	164.799197
	D1048	30.6	0.0306	240.464052	210.006536	181.660131	163.620915	153.545752	82.7973856
	D1050	31.3	0.0313	223.86262	192.942492	158.357827	136.597444	125.146965	120.335463

Study One: Isometric Torque-Frequency (Nmm·kg^{-1}) Estimated Parameters

		Pre-Injury					Immediate					X-day				
Condition		min	amplitude	max	EC50	Hill	min	amplitude	max	EC50	Hill	min	amplitude	max	EC50	Hill
Concentric-1mm	D1001	18.8186077	91.7532255	110.571833	79.1976641	-3.6259457	18.8581941	89.6910196	108.549214	81.9231149	-4.0159787					
	D1002	16.798831	75.8887801	92.6876112	94.4041622	-4.9645277	15.1124097	79.2470103	94.35942	90.5966981	-4.3380895					
	D1019	18.2722874	88.6551122	106.9274	94.439257	-4.8596291	17.2632957	86.4989854	103.762281	110.136269	-6.7091407					
	D1020	21.641459	87.4728842	109.114343	100.301972	-5.5760134	18.1794633	75.1705353	93.349986	100.77416	-5.4220632					
	D1025	25.8344184	85.1301407	110.964559	87.3448961	-4.3420800	23.5357313	87.2438973	110.779629	99.3224293	-4.7455093					
	D1032	17.7833673	81.1788138	98.9621811	90.458499	-5.107409	18.6876968	85.0681674	103.75864	100.86704	-5.4238017					
Concentric-1d	D1016	22.0343229	83.8510996	105.885423	96.4213173	-5.6647929	19.0965021	89.5809268	108.677429	96.4319097	-5.3077792	24.13158	85.2412288	109.372809	82.0468051	-4.9109240
	D1021	20.2039509	103.728057	123.932008	87.6231421	-5.0428829	16.9431417	90.8594141	107.802556	101.048646	-4.4922604	21.8800742	89.1011825	110.981257	78.535323	-4.194736
	D1023	26.8904669	90.2817016	117.172169	87.5466728	-4.3621201	22.6276022	82.7122607	105.339863	98.4221902	-5.3771657	29.3536393	93.1504964	122.504136	82.3860765	-4.2460329
	D1026	20.6940624	100.871958	121.566021	94.2961126	-4.6863963	22.6800688	100.55728	123.237349	99.4907436	-5.4042873	23.2127507	98.3260235	93.9571273	-4.5439434	
Eccentric-1mm	D1000	10.9153637	82.4015534	93.3169171	99.8699061	-4.5458657	3.08392476	57.1320918	60.2160165	146.962819	-5.4490125					
	D1004	18.0537598	86.3551485	104.408728	93.0186612	-4.595234	5.20533816	66.019813	71.2251511	119.538724	-5.3340311					
	D1007	21.7693598	74.9904384	96.7597982	83.0317254	-4.0783518	6.786342	58.2771613	65.0635033	112.955805	-4.9226568					
	D1022	19.5636118	79.9000853	99.4636971	97.489551	-6.2041440	6.94707821	58.5487449	65.4958231	126.835842	-5.5120625					
	D1028	20.296152	86.1162991	106.412451	87.1622585	-4.6720560	5.44609212	59.2390965	64.6851886	129.677052	-5.6095687					
	D1029	26.4112755	86.1176234	112.528899	84.1720794	-5.0010755	7.70649383	72.5329944	80.2394442	125.328565	-6.1330703					
	D1033	23.4108733	97.6275551	121.038428	97.4921096	-4.9616734	6.99272269	75.4174811	82.4102038	140.37263	-5.7480506					
	D1047	19.6074199	94.9634309	114.570851	92.6715801	-4.9462818	5.48079288	71.5814288	77.0622217	139.839166	-5.0271027					
	D1051	20.7320071	72.3513548	93.083362	94.9654678	-6.7741459	5.89867312	57.84232	63.7409931	136.076875	-5.2849825					
Eccentric-1d	D1006	23.0829555	109.056544	132.1395	78.3631249	-3.5513118	6.06521896	60.7357237	66.8009426	124.010483	-4.1608657	4.49789214	56.0791103	60.5770025	103.229055	-4.7306574
	D1008	25.3724983	89.288777	114.661275	91.439992	-4.3431054	5.4567577	68.5800465	74.0368042	129.29957	-4.891698	9.83428613	81.3866737	91.2209598	118.233485	-3.8826756
	D1015	23.9012679	97.1698862	121.071154	88.5604226	-3.8180869	4.91162974	69.0796039	73.9912336	141.822259	-4.8161793	5.83478341	78.1662422	84.0010256	124.120433	-4.115401
	D1027	23.8173631	80.2635338	104.080897	94.8764458	-4.7834442	5.52104025	59.7159288	65.236969	124.381942	-5.5499311	6.60456632	78.3780709	84.9826373	110.61572	-4.6471165
	D1030	20.482424	90.1519396	110.634364	90.8426584	-6.177445	5.48222691	64.2030335	69.6852604	124.916419	-5.6215735	4.68804052	71.9168485	76.604889	126.697246	-4.9778665
	D1040	22.2891346	90.7524929	113.041628	95.0981025	-5.5918405	6.56129874	62.7503525	69.3116512	127.006692	-5.2591102	5.82973366	73.3880205	79.2177542	111.918205	-4.779455
	D1045	18.1168908	96.4217975	114.538696	92.17425	-4.3209516	3.47139442	66.6782263	70.1496107	129.520915	-6.0014232	5.69794338	72.737412	78.435354	117.371164	-4.9135339
	D1046	17.866652	84.1514527	102.018105	93.4679811	-4.7592759	4.485674	72.7382663	77.2239403	129.793971	-5.7040016	6.34413617	66.6969025	73.0410387	104.51479	-3.8772837
	D1049	25.5797385	114.140003	139.719741	82.8797633	-3.9335016	7.60000784	86.5285956	94.1286034	134.50521	-5.8358254	3.73167722	61.1191172	64.8507944	127.707302	-5.2331494
Eccentric-3d	D1005	20.9062262	99.3340819	120.240308	82.3869171	-4.0201912	6.81122978	70.8690988	77.6803286	123.479428	-4.4277876	17.0954888	67.9657019	85.0611907	90.9166678	-5.1560094
	D1012	24.4826252	76.3009399	100.783565	94.5094737	-5.0779805	4.15134859	58.8776267	63.0289753	132.516675	-4.7016884	11.673905	68.9104261	80.5843311	110.05615	-5.2523476
	D1013	25.6541318	109.519339	135.173471	94.2715478	-4.9960234	6.88354897	69.7287315	76.6122805	137.070388	-5.4970732	11.3922328	92.643631	104.035864	110.10824	-5.3487780
	D1014	19.931269	84.5999265	104.531196	95.173695	-5.6494203	4.42567072	55.4771404	59.9028111	128.485187	-4.9220504	7.54318832	69.046293	76.5894813	110.94521	-5.5261402
	D1017	17.2586523	74.987995	92.2466473	91.7231017	-5.2579992	6.82947698	61.9720721	68.8015491	123.212681	-5.4667223	14.3988784	60.8356419	75.2345203	94.153499	-5.2007451
	D1024	15.7874081	74.5685376	90.3559458	94.9087634	-4.5943725	4.31435408	56.0722376	60.3865917	134.341085	-4.9331478	8.13518029	71.8254592	79.9606395	106.345486	-5.2780708
	D1031	26.6995877	79.2827617	105.982349	96.1900424	-4.9905551	6.65257172	57.5535793	64.206151	126.147674	-5.5641758	10.8867277	75.3394005	86.2261282	108.186275	-5.1481635
	D1042	24.328283	103.528026	127.856309	90.8703521	-4.1143142	6.26047203	79.5045465	85.7650186	136.126603	-4.4751931	19.2594323	92.943381	112.20277	91.7601118	-4.5222026
	D1048	15.8792241	88.5914701	104.470694	97.9691982	-6.0782868	6.17888155	63.5202241	69.6991056	133.754237	-4.7467902	9.24019016	75.1829736	84.4321638	104.46755	-4.8952000
	D1050	21.3501474	89.3974391	110.747586	72.6703971	-4.1288158	6.46103476	60.0009092	66.4619439	125.040815	-4.7564966	8.54036456	88.7128039	97.2531684	80.4487885	-4.8114226

Study One: Isometric Specific Force at 37˚C

		Muscle Weight (mg)				Muscle Length		FL	Wet Wt.	Active Specific Force (N·cm^{-2})										
Condition	Animal	L-TA	L-EDL	R-TA	R-EDL	inches	cm	cm	Grams	10 Hz	20 Hz	40 Hz	60 Hz	80 Hz	100 Hz	125 Hz	150 Hz	200 Hz	250 Hz	300 Hz	Caffeine!
Concentric-1mm	D1001	42.59	9.2	49.54	8.77	0.55	1.41	0.62	0.01	3.26	3.49	4.17	5.62	9.05	12.72	16.02	18.31	21.03	21.03	21.03	
	D1019	54.99	11.3	58.72	11.47	0.59	1.50	0.66	0.01	2.70	2.83	3.25	4.28	6.63	10.41	14.00	16.63	20.14	21.28	21.61	6.21
	D1020	55.15	10.87	54.56	8.63	0.62	1.58	0.70	0.01	2.73	2.65	3.29	4.20	5.97	9.34	12.91	15.58	19.18	20.53	20.76	7.61
	D1032	63.85	12.11	64.46	10.95	0.63	1.59	0.70	0.01	3.00	3.17	3.15	5.03	7.76	11.24	15.02	16.39	19.03	19.56	19.32	7.49
Concentric-1d	D1016	52.16	10.33	51.7	9.31	0.60	1.53	0.67	0.01	3.46	3.63	4.17	5.31	8.94	11.53	15.06	18.69	21.64	22.69	21.34	7.41
	D1021	61.5	12.23	61.6	11.21	0.62	1.58	0.69	0.01	2.83	2.99	3.42	4.33	6.72	10.45	14.04	16.01	19.18	20.48	20.92	7.75
	D1023	64.02	12.18	60.13	11.83	0.64	1.63	0.72	0.01	2.58	2.72	3.06	3.78	5.27	8.60	12.74	15.37	18.74	19.66	20.05	7.23
	D1026	58.28	10.96	56.18	10.16	0.59	1.51	0.66	0.01	3.32	3.47	4.00	5.17	7.95	12.11	15.50	17.67	19.73	19.73	19.73	
Eccentric-1mm	D1000	44.7	10.02		7.47	0.52	1.31	0.58	0.01	1.12	1.17	1.31	1.59	2.33	3.53	5.25	6.70	9.55	11.42	12.38	5.25
	D1007	46.04	9.25	43.91	9.56	0.55	1.41	0.62	0.01	1.78	1.83	2.08	2.61	3.64	5.61	8.19	10.08	13.91	15.69	16.44	6.23
	D1022	60.07	16.34	58.05	14.34	0.64	1.63	0.72	0.02	1.09	1.13	1.21	1.46	2.01	2.91	3.94	5.12	7.59	9.37	9.94	5.63
	D1028	60.17	11.43	54.15	9.47	0.60	1.53	0.67	0.01	0.85	0.86	1.06	1.13	1.60	2.39	3.76	5.17	7.76	9.03	9.96	5.45
Eccentric-1d	D1006	52.56	9.17	45.24	7.06	0.54	1.37	0.60	0.01	1.23	1.35	1.67	2.04	3.39	5.50	7.94	9.64	11.81	12.67	13.26	6.77
	D1008	48.13	8.89	42.18	7.12	0.52	1.32	0.58	0.01	1.20	1.42	1.71	2.24	3.24	4.98	6.79	8.44	10.98	12.63	13.13	6.46
	D1015	66.11	12.18	56.95	9.58	0.57	1.45	0.64	0.01	1.11	1.16	1.27	1.57	2.20	3.07	4.22	5.18	7.45	8.10	8.41	5.54
	D1027	63	13.22	59.33	11.14	0.64	1.63	0.72	0.01	1.48	1.54	1.66	1.94	2.53	3.44	5.21	6.10	7.68	8.95	9.01	5.49
Eccentric-3d	D1012	57.01	12.98	46.52	9.82	0.61	1.55	0.68	0.01	1.99	2.02	2.38	2.91	4.00	6.08	8.77	11.15	14.46	16.15	16.61	6.35
	D1013	59.54	12.28	57.05	10.26	0.63	1.60	0.70	0.01	2.37	2.50	2.85	3.38	5.18	8.24	11.20	14.01	16.93	17.74	18.20	7.02
	D1014	66.73	11.87	60.5	9.74	0.61	1.55	0.68	0.01	1.70	1.82	2.00	2.60	3.84	5.80	8.37	10.78	13.01	13.73	13.83	5.70
	D1024	61.7	12.17	60.34	10.86	0.61	1.55	0.68	0.01	2.10	2.25	2.64	3.27	4.80	7.30	10.18	12.32	14.27	14.58	14.30	6.87
Control	D1003-L	48.32	7.63	51.42	8.24	0.56	1.43	0.63	0.01	2.66	2.76	3.00	3.57	5.72	8.80	12.49	14.13	17.61	19.39	21.56	7.62
	D1003-R		8.24			0.56	1.43	0.63	0.01	3.08	3.25	3.90	5.41	8.68	12.03	15.25	17.54	20.45	21.63	21.82	7.68
	D1009	50.59	9.96	48.91	8.93	0.58	1.48	0.65	0.01	3.11	3.31	3.70	4.68	7.46	12.05	16.31	18.95	21.82	22.87	23.68	7.68
	D1010	54.82	10.22	50.66	9	0.60	1.52	0.67	0.01	2.47	2.54	2.97	3.96	6.55	10.05	13.19	16.00	19.29	21.22	21.52	7.62

Study One: Isometric Specific Force Predicted Parameters

Condition	Animal	min	amplitude	max	EC50	Hill
Concentric-Imm	D1001	3.41	18.21	21.62	99.44	-3.79
	D1019	2.74	19.52	22.27	114.92	-3.66
	D1020	2.76	18.80	21.56	120.05	-3.66
	D1032	2.98	16.83	19.81	100.87	-3.93
Concentric-1d	D1016	3.62	19.20	22.81	108.43	-3.74
	D1021	2.85	18.55	21.39	113.16	-3.59
	D1023	2.72	17.67	20.39	118.54	-4.23
	D1026	3.49	16.57	20.06	99.26	-4.40
Eccentric-Imm	D1000	1.11	13.00	14.11	162.83	-3.08
	D1007	1.80	15.99	17.79	143.52	-3.33
	D1022	1.11	10.56	11.68	172.35	-3.11
	D1028	0.88	10.01	10.89	161.99	-3.57
Eccentric-1d	D1006	1.29	12.27	13.55	120.72	-3.65
	D1008	1.30	13.19	14.49	140.63	-2.96
	D1015	1.16	7.99	9.15	143.71	-3.36
	D1027	1.50	8.09	9.60	136.65	-3.45
Eccentric-3d	D1012	2.05	15.52	17.57	135.25	-3.56
	D1013	2.46	16.17	18.62	118.45	-3.93
	D1014	1.82	12.44	14.27	120.26	-4.10
	D1024	2.31	12.47	14.79	109.60	-4.41
Control	D1003-L	2.45	19.91	22.36	131.02	-3.03
	D1003-R	3.09	19.51	22.60	106.67	-3.26
	D1009	3.16	20.54	23.71	109.09	-3.99
	D1010	2.40	20.11	22.51	119.04	-3.31

Study Two: Isometric Torque-Frequency ($Nmm \cdot kg^{-1}$)

Pre1

Name	Weight_g	Weight_kg	Temp (°C)	20	40	60	80	100	125	150	200	250	300	350	400
D1080	31.8	0.032	35.7	28.36	30.36	37.93	78.82	93.12	107.58	113.93	120.91	124.05	125.56	126.54	127.04
D1081	31.6	0.032	36.4	22.31	19.35	24.74	42.65	71.09	88.11	94.12	99.56	101.06	101.45	101.14	103.42
D1082	30.1	0.030	36.3	19.58	17.90	23.86	46.61	72.49	88.17	94.41	100.09	102.81	104.05	103.20	102.43
D1084	30.5	0.031	35.2	32.91	37.75	55.53	78.86	98.00	108.42	113.54	119.27	121.88	123.32	124.38	124.53
D1085	34.5	0.035	36	18.74	19.12	25.75	42.41	69.99	87.60	93.63	99.48	100.57	101.64	102.23	102.68
D1086	29.7	0.030	35.5	23.66	24.42	34.73	60.91	80.12	89.79	94.73	99.46	101.79	101.69	101.52	100.74
D1088	32	0.032	36.5	19.18	19.41	27.43	53.80	80.04	93.00	98.63	104.02	105.95	106.82	107.28	106.46
D1089	32	0.032	35.7	23.33	27.73	33.51	53.39	88.46	110.42	118.74	125.77	128.48	130.16	131.00	131.36
D1090	28.8	0.029	35.1	21.96	20.04	22.83	38.04	75.80	102.84	112.04	120.82	124.40	126.80	127.66	128.14
D1092	33.4	0.033	36.7	22.59	21.10	23.35	36.42	71.68	96.79	104.95	111.46	114.64	115.78	116.17	115.58
D1093	34.8	0.035	35	23.20	25.97	34.50	59.40	82.28	95.59	101.47	107.54	110.60	111.78	112.12	113.10
D1094	30.6	0.031	36.1	23.78	23.43	29.74	47.95	78.67	93.02	99.20	105.26	107.99	109.58	110.23	110.69
D1095	35.9	0.036	35.3	23.06	20.94	27.21	42.13	74.32	92.56	99.16	105.89	109.25	111.24	112.30	113.06
D1096	32.3	0.032	36.5	23.25	23.42	28.41	45.18	74.73	94.47	101.80	108.56	112.15	113.74	114.78	115.27
D1097	32.4	0.032	35.7	19.61	16.11	18.02	28.98	62.63	96.26	104.50	110.86	111.97	117.24	117.50	117.67

Post1

Name	20	40	60	80	100	125	150	200	250	300	350	400
D1080	4.53	4.90	6.92	11.08	16.68	30.82	43.57	60.94	64.30	66.18	66.57	66.64
D1081	5.06	5.36	6.67	9.77	16.60	26.59	43.47	60.11	64.28	66.05	67.25	67.01
D1082	3.89	3.68	4.27	7.21	15.52	31.81	45.95	63.52	68.01	68.87	69.68	69.67
D1084	6.81	7.78	9.76	13.85	21.66	37.68	51.06	68.77	73.04	75.20	75.39	74.97
D1085	4.47	4.20	5.57	8.24	13.77	32.07	44.78	62.66	66.81	68.48	69.13	68.92
D1086	5.64	6.19	7.85	13.07	21.33	36.24	49.91	63.68	67.08	68.19	69.39	69.05
D1088	6.10	6.35	8.76	13.44	24.03	44.76	60.21	70.87	74.17	75.15	75.12	74.76
D1089	6.65	6.83	8.20	12.23	20.74	42.68	62.22	82.04	88.22	89.63	90.77	89.77
D1090	6.96	6.82	7.96	11.63	17.70	39.38	60.44	81.97	88.41	91.69	92.55	92.43
D1092	3.61	3.98	4.96	7.35	13.10	27.54	43.24	62.82	66.25	68.45	67.31	66.36
D1093	4.94	5.91	7.55	12.09	20.42	36.82	50.16	64.12	67.51	68.87	69.38	69.12
D1094	4.89	5.27	8.04	12.43	19.19	37.58	50.72	61.22	63.85	64.75	65.07	64.31
D1095	6.23	6.35	7.44	9.63	15.01	31.96	46.28	67.58	73.05	75.36	76.53	77.48
D1096	6.64	6.86	8.76	12.08	20.27	39.79	58.30	73.09	76.63	77.61	77.98	76.41
D1097	6.08	6.26	6.35	9.37	15.17	31.40	50.90	69.45	73.73	75.52	75.43	75.30

Study Two: Isometric Torque-Frequency ($Nmm \cdot kg^{-1}$)

Pre2/14d

Name	20	40	60	80	100	125	150	200	250	300	350	400
D1080	23.76	23.28	34.55	57.52	84.47	98.43	103.83	109.65	112.49	113.74	115.10	114.57
D1081	14.09	16.35	20.57	34.28	50.73	61.82	67.13	71.96	73.88	74.15	73.74	73.45
D1082	23.30	22.30	29.84	53.51	77.64	94.04	100.14	105.08	107.31	110.15	109.32	108.67
D1084	22.93	24.03	32.43	51.04	71.60	82.42	87.36	92.98	94.75	95.11	97.19	97.19
D1085	16.68	15.95	20.42	36.30	64.54	79.27	82.86	86.21	89.68	88.74	93.25	93.32
D1086	24.05	22.08	27.23	39.67	69.76	85.27	91.41	97.29	100.28	101.81	102.96	103.22
D1088	15.15	14.48	19.87	40.67	63.24	75.50	79.15	83.58	85.22	86.90	87.51	87.20
D1089	22.01	19.86	24.99	39.75	73.70	90.42	96.18	101.45	103.95	105.67	106.47	106.86
D1090	22.44	22.87	27.76	48.40	84.53	106.38	114.14	121.28	124.44	124.44	124.43	124.97
D1092	24.41	26.34	38.33	59.26	79.68	91.52	96.22	101.26	102.72	103.31	105.82	104.01
D1093	19.43	21.19	32.02	51.58	73.41	89.05	94.70	100.75	103.44	104.95	105.77	106.55
D1094	20.92	25.06	39.73	66.23	81.55	88.49	92.13	95.87	96.58	94.94	95.16	94.27
D1095	27.60	26.13	37.19	62.02	85.40	100.20	105.90	112.02	115.04	116.61	116.94	117.53
D1096	20.00	21.56	27.01	55.23	78.84	90.05	93.76	98.44	101.14	102.16	102.31	101.57
D1097	21.15	19.98	26.06	51.82	79.67	91.43	97.15	102.83	106.05	108.45	106.90	107.37

Post2-0d

Name	20	40	60	80	100	125	150	200	250	300	350	400
D1080	6.97	7.47	9.58	13.90	22.73	45.06	60.56	76.77	80.80	81.75	81.71	80.74
D1081												
D1082	6.29	5.69	7.01	10.09	18.04	39.38	57.77	74.83	79.07	81.06	82.35	82.92
D1084												
D1085												
D1086	7.50	9.01	10.28	14.59	22.33	47.00	60.59	70.12	73.60	75.09	76.12	76.29
D1088	8.46	8.93	9.59	16.15	30.17	46.54	58.21	68.31	71.63	73.35	74.06	74.45
D1089	5.87	5.78	6.97	11.23	23.33	51.47	69.66	83.41	87.86	89.70	90.42	90.27
D1090	10.49	10.32	12.05	17.60	29.42	59.91	79.98	96.11	100.28	102.10	102.59	102.34
D1092												
D1093												
D1094	4.15	4.68	7.80	13.55	23.56	41.60	53.17	61.80	63.86	65.23	65.67	65.87
D1095	6.38	6.95	9.98	16.57	24.63	45.99	60.68	76.73	80.88	81.59	82.35	82.52
D1096	5.57	5.31	7.69	11.69	19.08	38.96	53.90	70.32	74.29	76.26	75.99	76.25
D1097	7.09	6.24	6.86	12.83	21.64	44.00	57.43	75.52	80.61	81.11	81.15	79.38

Study Two: Isometric Torque-Frequency (Nmm·kg^{-1}) and Predicted Parameters

Name	Post2-3d												
	20	40	60	80	100	125	150	200	250	300	350	400
D1080	27.63	33.32	49.19	75.06	93.24	101.56	105.79	109.88	109.85	110.27	110.59	111.67
D1081												
D1082	20.96	22.65	35.54	58.93	74.54	82.78	87.81	92.27	94.79	95.65	95.57	92.93
D1084												
D1085												
D1086												
D1088												
D1089												
D1090												
D1092												
D1093												
D1094												
D1095												
D1096	24.48	27.86	42.15	65.67	85.01	95.49	99.88	104.21	106.38	108.13	108.70	108.31
D1097	20.58	22.69	30.60	47.84	74.02	90.32	96.09	101.86	104.66	106.10	106.94	106.88

Name	Pre-1		Post-1		Pre-2		Post-2		Post2-3d	
	min	max	min	max	min	max	min	max	min	max
D1080	28.36	127.04	4.53	66.64	23.28	115.10	6.97	81.75	27.63	111.67
D1081	19.35	103.42	5.06	67.25	14.09	74.15				
D1082	17.90	104.05	3.68	69.68	22.30	110.15	5.69	82.92	20.96	95.65
D1084	32.91	124.53	6.81	75.39	22.93	97.19				
D1085	18.74	102.68	4.20	69.13	15.95	93.32				
D1086	23.66	101.79	5.64	69.39	22.08	103.22	7.50	76.29		
D1088	19.18	107.28	6.10	75.15	14.48	87.51	8.46	74.45		
D1089	23.33	131.36	6.65	90.77	19.86	106.86	5.78	90.42		
D1090	20.04	128.14	6.82	92.55	22.44	124.97	10.32	102.59		
D1092	21.10	116.17	3.61	68.45	24.41	105.82				
D1093	23.20	113.10	4.94	69.38	19.43	106.55				
D1094	23.43	110.69	4.89	65.07	20.92	96.58	4.15	65.87		
D1095	20.94	113.06	6.23	77.48	26.13	117.53	6.38	82.52		
D1096	23.25	115.27	6.64	77.98	20.00	102.31	5.31	76.26	24.48	108.70
D1097	16.11	117.67	6.08	75.52	19.98	108.45	6.24	81.15	20.58	106.94

Study Two: Isometric Specific Force-frequency ($Nmm \cdot kg^{+1}$)

				Muscle Length		Fiber Length	Wet Wt.	Active										
Condition	Animal	L-TA	L-EDL	inches	cm	cm	Grams	10 Hz	20 Hz	40 Hz	60 Hz	80 Hz	100 Hz	125 Hz	150 Hz	200 Hz	250 Hz	300 Hz	Caffeine!
14d	D1081	51.68	10.42	0.61	1.55	0.68	0.01	2.24	2.20	2.84	4.14	6.43	10.18	14.31	17.88	20.88	21.61	21.73	6.79
	D1084	56.67	9.7	0.60	1.53	0.67	0.01	2.63	2.79	3.07	3.84	5.15	7.81	11.64	14.25	17.31	18.10	18.88	7.56
	D1085	69.41	10.92	0.62	1.57	0.69	0.01	2.51	2.64	3.06	3.99	5.80	10.01	14.39	17.50	20.02	20.90	21.29	7.09
	D1092	57.26	9.93	0.62	1.57	0.69	0.01	5.35	5.96	6.03	7.94	11.72	13.48	15.68	17.32	18.52	18.87	19.05	7.84
	D1093	67.22	10.61	0.61	1.56	0.68	0.01	2.45	2.59	2.81	3.38	4.74	7.63	12.25	15.60	18.71	19.62	20.16	7.41
Bout 2 0d	D1086	56.23	10.21	0.60	1.53	0.67	0.01	1.55	1.71	1.81	2.21	2.97	4.38	6.42	8.37	11.29	12.19	12.34	6.45
	D1088	73	11.74	0.60	1.51	0.67	0.01	1.68	1.84	2.11	2.54	3.39	5.07	7.65	10.11	12.40	13.44	13.63	6.28
	D1089	72.06	12.6	0.63	1.60	0.70	0.01	1.73	1.80	2.19	2.85	3.80	5.44	8.01	10.52	14.36	26.48	15.11	5.89
	D1090	70.22	10.83	0.61	1.54	0.68	0.01	1.74	1.86	1.95	2.29	2.80	3.66	5.18	7.14	11.36	13.38	13.34	7.08
	D1094	59.13	10.69	0.61	1.56	0.68	0.01	1.80	1.94	2.08	2.56	3.64	5.51	8.50	11.22	14.76	15.40	15.63	6.64
Bout 2 3d	D1082	66.58	10.6	0.64	1.61	0.71	0.01	2.41	2.77	3.24	3.83	5.41	8.22	13.08	15.43	19.28	20.81	22.97	8.65
	D1080	62.79	11.33	0.62	1.57	0.69	0.01	4.05	4.30	4.78	5.48	7.60	12.85	18.00	20.68	22.87	23.51	24.12	
	D1095	70.1	11.3	0.64	1.61	0.71	0.01	2.65	2.75	2.97	3.37	4.57	7.10	11.16	14.86	18.29	19.40	20.48	7.40
	D1097	62.03	11.42	0.60	1.53	0.67	0.01	3.22	3.37	3.71	4.08	5.65	9.42	13.76	17.30	19.70	20.55	20.81	7.60

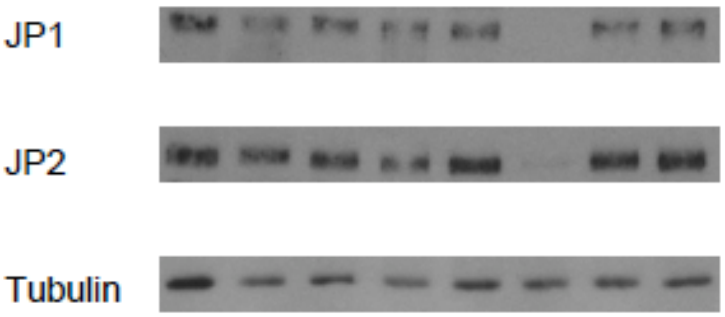
Study Three: Isometric Specific Force ($N \cdot cm^{-2}$)

Condition	Animal	L-TA	L-EDL	Muscle Length inches	Muscle Length cm	Fiber Length cm	Wet Wt. Grams	Pre-37 °C Pt	Pre-37 °C Po	Pre-X °C Pt	Pre-X °C Po	Post-X °C Pt	Post-X °C Po	Post-37 °C Pt	Post-37 °C Po
35°C - ECC	D1059	60.94	11.23	0.62	1.58	0.69	0.01	2.88	24.36	2.87	23.97	2.45	20.85	2.46	20.87
	D1060	60.23	10.23	0.62	1.58	0.70	0.01	3.14	21.84	2.97	21.45	2.27	17.93	2.31	17.89
	D1061	49.05	10.62	0.61	1.55	0.68	0.01	3.60	24.95	3.30	23.58	1.93	19.76	2.01	19.66
	D1063	54.58	10.39	0.60	1.51	0.66	0.01	3.03	22.40	2.92	22.08	1.97	18.05	2.00	18.03
	D1064	57.5	11.3	0.62	1.58	0.70	0.01	2.78	22.08	2.72	21.57	1.85	15.76	1.86	16.13
	D1073-C	56.82	11.03	0.60	1.52	0.67	0.01	2.89	21.96	2.82	21.87	1.95	18.36	1.96	18.29
	D1074-C	53.32	10.98	0.61	1.56	0.69	0.01	3.23	24.22	3.11	24.23	1.93	19.63	1.86	19.20
	D1076-C	58.8	11.47	0.61	1.56	0.68	0.01	3.18	24.16	2.97	23.78	1.85	19.91	2.07	19.90
	D1100	52.22	9.64	0.58	1.46	0.64	0.01	2.66	25.03	2.15	24.83	2.18	20.24	2.18	20.47
	D1101	47.9	9.03	0.57	1.44	0.63	0.01	2.95	20.17	2.96	20.20	1.79	15.08	1.83	15.06
15°C ECC	D1067	58.07	10.23	0.60	1.54	0.68	0.01	3.10	23.97	3.70	11.73	1.50	5.27	2.80	21.81
	D1068	53.94	10.69	0.62	1.58	0.69	0.01	2.98	23.26	3.07	10.08	1.64	4.78	2.88	21.06
	D1069	55.01	10.56	0.61	1.56	0.69	0.01	3.52	26.30	4.30	12.86	3.44	8.93	3.37	24.01
	D1070	56.32	9.63	0.60	1.52	0.67	0.01	3.70	25.28	3.42	8.61	1.89	5.37	3.04	22.34
	D1071	54.47	11.33	0.61	1.54	0.68	0.01	3.24	23.84	4.01	13.84	3.44	10.91	3.05	21.03
	D1079-C	52.08	10	0.58	1.47	0.65	0.01	3.64	26.36	4.37	13.23	3.18	9.28	3.40	22.92
	D1083-C	62.7	10.52	0.59	1.49	0.65	0.01	2.99	21.31	3.90	10.15	2.10	4.18	2.90	18.05
	D1087-C	54.84	11.22	0.59	1.51	0.66	0.01	3.13	23.38	3.36	11.24	2.84	8.75	2.76	20.34
35 °C ISO	D1057	61.3	11.03	0.62	1.58	0.70	0.01	2.86	20.82	2.62	20.91	2.61	19.33	2.64	18.89
	D1058	55.52	9.56	0.61	1.56	0.69	0.01	3.70	23.83	3.51	24.13	3.44	22.50	3.40	22.22
	D1062	60.44	11.38	0.62	1.58	0.70	0.01	3.51	24.19	3.01	24.36	3.25	23.81	3.13	23.69
	D1065	56.95	10.63	0.62	1.58	0.69	0.01	3.42	23.19	3.19	23.06	3.33	22.55	3.36	22.63
	D1066	58.27	10.76	0.62	1.58	0.69	0.01	3.62	27.17	3.58	26.69	3.58	25.68	3.42	25.41
	D1072-C	58.67	11.12	0.58	1.47	0.65	0.01	3.25	24.33	2.78	23.76	2.93	22.54	2.89	22.64
	D1077-C	58.63	12.12	0.60	1.54	0.68	0.01	3.01	24.31	2.96	24.21	3.14	22.56	2.82	22.56
	D1091-C	70.83	11.88	0.58	1.48	0.65	0.01	3.21	22.93	3.04	22.34	2.72	19.51	2.92	21.32

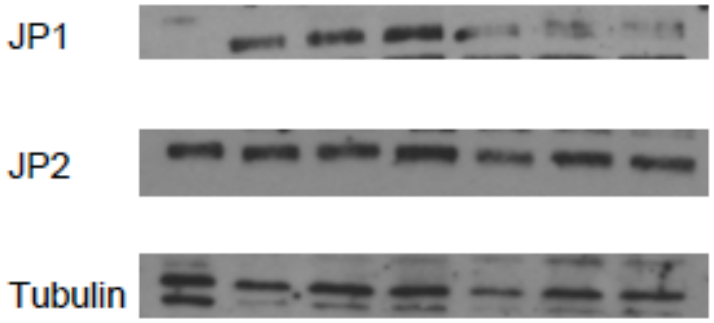
Study Three: Eccentric or Isometric and Caffeine Specific Force ($N \cdot cm^{-2}$)

Condition	Animal	Ecc/Iso	1	2	3	4	5	6	7	8	9	10	Caffeine (5(
35°C - ECC	D1059	33.82	32.72	31.47	30.64	30.40	30.05	29.74	29.61	31.64	31.16		
	D1060	31.65	29.87	26.33	25.72	26.07	26.18	25.88	27.55	27.09	27.13		
	D1061	36.15	33.95	33.66	32.40	31.86	31.03	30.56	30.41	29.44	27.82		
	D1063	31.47	32.08	27.74	27.20	27.46	27.38	27.06	26.74	26.13	25.99		
	D1064	30.75	27.75	25.44	25.17	25.65	25.13	25.03	24.65	24.62	24.03		
	D1073-C	31.47	32.05	27.54	25.95	25.98	26.12	25.95	25.81	25.51	25.24	5.82	
	D1074-C	35.67	35.21	32.94	32.38	31.93	31.72	31.26	30.77	30.28	29.90	6.26	
	D1076-C	34.28	33.41	31.01	30.34	30.00	29.64	29.20	28.77	28.37	27.97	6.12	
	D1100	36.90	35.48	32.09	31.38	31.23	31.04	30.56	30.63	30.60	30.33		
	D1101	31.29	29.92	25.44	25.48	25.44	25.44	25.21	24.93	24.42	23.95		
15°C ECC	D1067	30.88	29.22	28.48	27.67	26.93	25.64	24.16	22.67	22.01	20.94		
	D1068	27.50	26.67	26.55	26.44	26.05	25.14	26.08	25.39	25.28	25.47		
	D1069	34.32	33.56	32.95	32.62	31.71	30.44	29.02	29.57	29.53	28.80		
	D1070	25.82	24.62	24.16	23.34	22.72	22.41	22.18	21.44	21.75	21.25		
	D1071	36.63	35.83	34.78	34.16	33.59	33.06	32.62	32.19	31.72	31.35		
	D1079-C	36.86	35.45	34.94	34.40	33.93	33.38	32.59	32.41	31.65	31.25	6.24	
	D1083-C	28.85	25.10	23.46	22.21	21.37	21.37	19.39	17.76	16.51	15.88	5.90	
	D1087-C	32.61	30.95	30.49	29.99	29.20	29.07	28.44	27.84	27.44	27.44	6.14	
35 °C ISO	D1057	18.12	17.91	17.80	17.93	17.68	17.49	17.57	17.13	17.18	17.36		
	D1058	21.40	21.40	21.22	21.08	20.93	21.11	20.85	20.70	20.65	20.54		
	D1062	19.71	19.64	19.67	19.51	19.93	20.07	19.94	19.81	19.71	19.65		
	D1065	18.82	18.90	18.78	18.85	18.67	18.44	18.46	18.33	18.32	18.23		
	D1066	21.45	21.56	21.48	21.18	21.51	21.34	20.98	21.06	20.88	20.76		
	D1072-C	19.83	19.67	19.44	19.15	18.90	18.94	18.86	18.69	18.56	18.67	6.36	
	D1077-C	19.25	19.06	18.90	18.75	18.49	18.34	18.21	17.79	17.87	17.93	6.12	
	D1091-C	17.66	17.41	17.16	16.73	16.31	16.01	15.78	15.53	15.38	15.25	6.47	

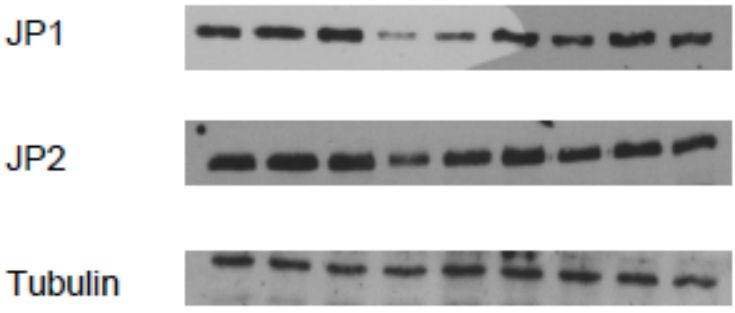
Study One: Western Blots



1. D1009-RTA
2. D1004-LTA
3. D1006-LTA
4. D1005-LTA
5. Old Sample
6. Old Sample
7. D1007-LTA
8. D1008-LTA

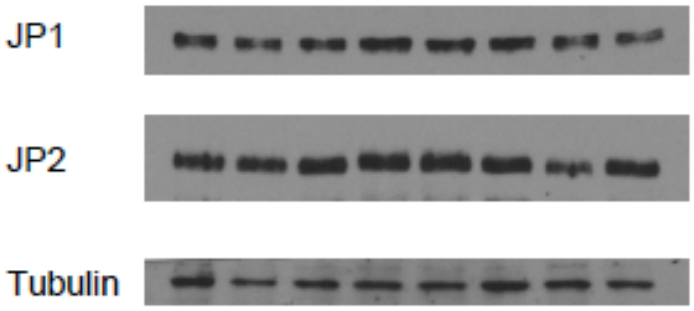


1. Heart
2. D1003-RTA
3. D1004-RTA
4. D1016-LTA
5. D1015-LTA
6. D1014-LTA
7. D1013-LTA

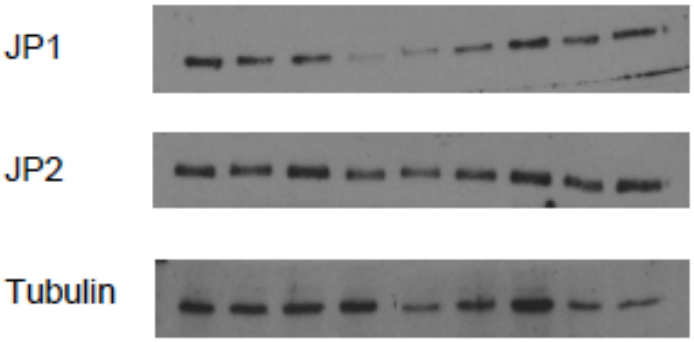


1. D1020-RTA
2. D1019-LTA
3. D1023-LTA
4. D1028-LTA
5. D1027-LTA
6. D1024-LTA
7. D1025-LTA
8. D1026-LTA
9. D1013-RTA

Study One: Western Blots



1. D1021-RTA
2. D1020-LTA
3. D1021-LTA
4. D1022-LTA
5. Loading error
6. D1012-LTA
7. D1023-RTA
8. D1016-RTA

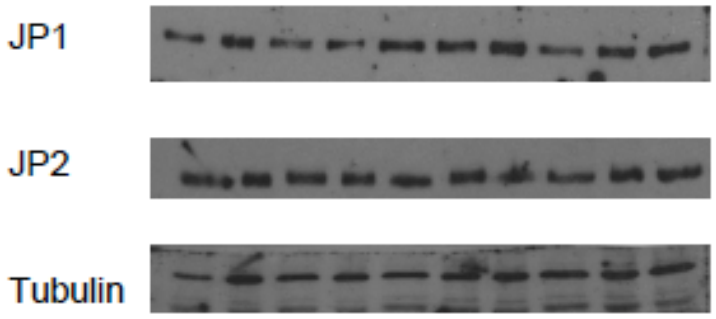


1. D1048-RTA
2. D1044-LTA
3. D1045-LTA
4. D1048-LTA
5. D1051-LTA
6. D1040-LTA
7. D1042-RTA
8. D1032-RTA

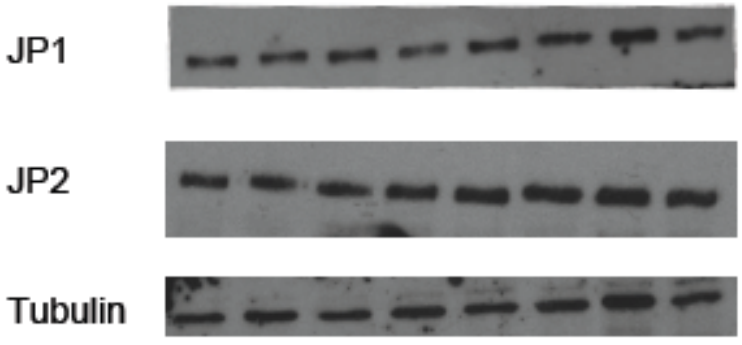
Study One: Western Blot Data

Condition	Animal	JP1	JP2	Tubulin	JP1:Tubulin	JP2:Tubulin
TA-Control	1085	13251	27892	6436	2.05888751	4.33374767
	1089	17512	23071	7825	2.23795527	2.94837061
	1010	15814	19775	7758	2.03841196	2.5489817
	1006	15752	17311	7211	2.18444044	2.40063791
	1003	17730	19075	8494	2.08735578	2.24570285
	1020	16096	26497	9555	1.68456306	2.77310309
	1013	16606	21377	8083	2.05443523	2.64468638
	1021	21197	22059	13173	1.60912472	1.6745616
	1016	20155	24870	10963	1.83845663	2.26853963
Concentric-0d	1032	14209	16319	7612	1.86665791	2.14385181
	1002	17105	19939	14989	1.14117019	1.33024218
	1019	16902	31313	9728	1.73745888	3.2188528
	1025	15478	20873	10556	1.4662751	1.97735885
	1020	19856	20475	7784	2.55087359	2.63039568
Concentric-1d	1016	21094	23158	13273	1.58924132	1.74474497
	1023	18931	27396	8847	2.13982141	3.09664293
	1026	20772	25473	9356	2.22017956	2.72263788
	1021	20936	24428	11552	1.81232687	2.11461219
Ecc-0d	1004	4780	5890	7469	0.63997858	0.78859285
	1007	4206	10314	14204	0.29611377	0.72613348
	1028	3499	14677	8236	0.42484216	1.7820544
	1022	27457	27287	16036	1.71221003	1.70160888
	1044	14707	15145	9619	1.52895311	1.57448799
	1051	6559	11376	9658	0.67912611	1.17788362
Ecc-1d	1015	8478	16393	5291	1.6023436	3.0982801
	1006	7334	10151	7267	1.00921976	1.39686253
	1008	9328	7334	13890	0.67156228	0.52800576
	1027	6896	23324	12441	0.55429628	1.87476891
	1045	16367	21194	12195	1.34210742	1.73792538
	1040	12206	15421	13606	0.89710422	1.13339703
Ecc-3d	1014	12214	19777	10667	1.14502672	1.85403581
	1013	2788	20002	11334	0.24598553	1.76477854
	1005	4727	8030	4915	0.96174975	1.63377416
	1024	17717	24619	9721	1.82254912	2.53255838
	1012	23330	22604	17418	1.33941899	1.29773797
	1048	3596	13982	13096	0.27458766	1.06765425
	1042	20913	22670	20963	0.99761485	1.08142918

Study Two: Repeated Bout of Eccentric Contractions



1. D1085-RTA
2. D1092-LTA
3. D1088-LTA
4. D1082-LTA
5. D1085-LTA
6. D1089-LTA
7. D1095-LTA
8. D1090-LTA
9. D1093-LTA
10. D1080-LTA

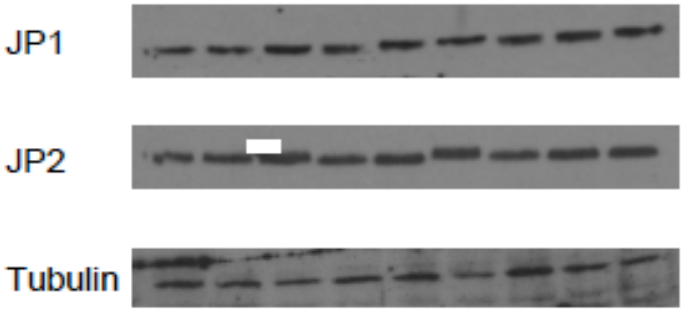


1. D1081-LTA
2. D1094-LTA
3. D1096-LTA
4. D1088-RTA
5. D1084-LTA
6. D1086-LTA
7. D1097-LTA
8. D1089-RTA

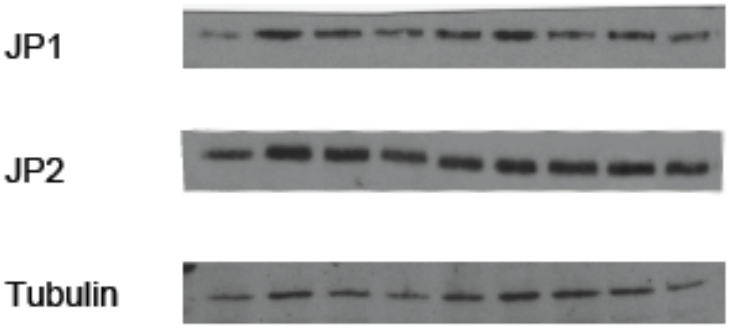
Study Two: Western Blot Data

Condition	Animal	JP1	JP2	Tubulin	JP1:Tubulin	JP2:Tubulin
Ecc-14d	1092	18123	20492	12556	1.44337369	1.63204842
	1085	21358	23326	9484	2.25200337	2.45951075
	1081	15302	17966	8092	1.89100346	2.2202175
	1084	20378	24886	8057	2.52922924	3.08874271
Ecc-B2-0d	1088	12770	25590	12152	1.05085583	2.1058262
	1089	18273	23811	13117	1.39307769	1.81527788
	1093	17746	22059	13559	1.30879858	1.62688989
	1094	14037	18516	9719	1.44428439	1.90513427
	1086	17023	25980	10316	1.6501551	2.51841799
Ecc-B2-3d	1082	11129	20616	10087	1.10330128	2.04381878
	1095	21324	24360	13503	1.57920462	1.80404355
	1080	18928	25459	11603	1.63130225	2.19417392
	1096	16504	20659	7611	2.16844042	2.71436079
	1097	23834	27650	14213	1.6769155	1.9454021

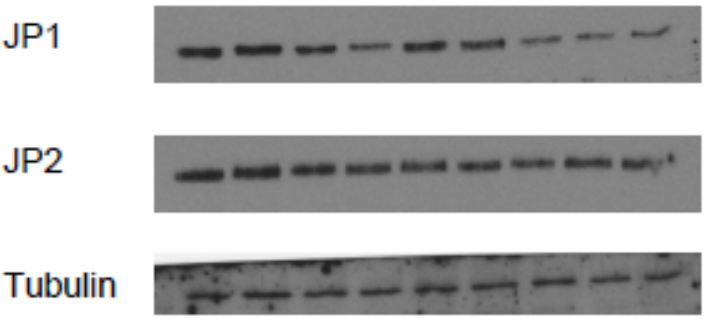
Study Three: Temperature Dependency of Muscle Injury



1. D1078-REDL
2. D1091-LEDL
3. D1087-LEDL
4. D1061-LEDL
5. D1040-REDL
6. D1077-LEDL
7. D1069-LEDL
8. D1064-LEDL



1. D1076-LEDL
2. D1065-LEDL
3. D1067-LEDL
4. D1063-LEDL
5. D1056-REDL
6. D1062-LEDL
7. D1068-LEDL
8. D1060-LEDL
9. D1034-REDL



1. D1099-REDL
2. D1058-LEDL
3. D1071-LEDL
4. D1101-LEDL
5. D1080-REDL
6. D1072-LEDL
7. D1057-LEDL
8. D1059-LEDL
9. D1000-LEDL

Study One: Western Blot Data

Condition	Animal	JP1	JP2	Tubulin	JP1:Tubulin	JP2:Tubulin
TA-Control	1085	13251	27892	6436	2.05888751	4.33374767
	1089	17512	23071	7825	2.23795527	2.94837061
	1010	15814	19775	7758	2.03841196	2.5489817
	1006	15752	17311	7211	2.18444044	2.40063791
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	1021	21197	22059	13173	1.60912472	1.6745616
	1016	20155	24870	10963	1.83845663	2.26853963
Concentric-0d	1032	14209	16319	7612	1.86665791	2.14385181
	1002	17105	19939	14989	1.14117019	1.33024218
	1019	16902	31313	9728	1.73745888	3.2188528
	1025	15478	20873	10556	1.4662751	1.97735885
	1020	19856	20475	7784	2.55087359	2.63039568
Concentric-1d	1016	21094	23158	13273	1.58924132	1.74474497
	1023	18931	27396	8847	2.13982141	3.09664293
	1026	20772	25473	9356	2.22017956	2.72263788
	1021	20936	24428	11552	1.81232687	2.11461219
Ecc-0d	1004	4780	5890	7469	0.63997858	0.78859285
	1007	4206	10314	14204	0.29611377	0.72613348
	1028	3499	14677	8236	0.42484216	1.7820544
	1022	27457	27267	16036	1.71221003	1.70160888
	1044	14707	15145	9619	1.52895311	1.57448799
	1051	6559	11376	9658	0.67912611	1.17788362
Ecc-1d	1015	8478	16393	5291	1.6023436	3.0982801
	1006	7334	10151	7267	1.00921976	1.39686253
	1008	9328	7334	13890	0.67156228	0.52800576
	1027	6896	23324	12441	0.55429628	1.87476891
	1045	16367	21194	12195	1.34210742	1.73792538
	1040	12206	15421	13606	0.89710422	1.13339703
Ecc-3d	1014	12214	19777	10667	1.14502672	1.85403581
	1013	2788	20002	11334	0.24598553	1.76477854
	1005	4727	8030	4915	0.96174975	1.63377416
	1024	17717	24619	9721	1.82254912	2.53255838
	1012	23330	22604	17418	1.33941899	1.29773797
	1048	3596	13982	13096	0.27458766	1.06765425
	1042	20913	22670	20963	0.99761485	1.08142918

APPENDIX C

WESTERN BLOT PROTOCOL

Primary Antibodies
Junctophilin 1: Rabbit Anti-mouse JP1 (C-terminus), Invitrogen (40-5100)
Junctophilin 2: Rabbit Anti-mouse JP2 (C-terminus), Invitrogen (40-5300)
Tubulin (multiple isoforms): Rabbit Anti-mouse, Sigma (T 3526)

Secondary Antibody
Goat Anti-rabbit HRP conjugated, Invitrogen (G21234)

Protocol

SDS-PAGE
- 7% 37.5:1 Acrylamide : Bis 1mm gel
- Load 25 µg of protein (Protein estimated from Bradford Assay)
- Run for 30 minutes at 100 V
- Run for 45 minutes at 150 V

Membrane Transfer
- Transfer to nitrocellulose membrane for 60 minutes at 100 V (peak current 360 mV)
- Be sure to equilibrate membrane and gel for 10 – 15 minutes in transfer buffer.

Treatment of Transferred Membrane
- Stain membrane briefly with Ponceau and scan the membrane
- Rinse membrane with TBS-T to remove most of Ponceau staining
- Block membrane in 5% Non-Fat Dried Milk TBS-T (0.1%) overnight at 4˚C
- Incubate membrane in primary antibody for 2 hours at room temperature with shaking
    - JP1 & JP2 1˚ Ab; 1: 20,000 (1 µL Ab in 20 mL TBS-T)
    - Tubulin 1˚ Ab; 1: 2,500 (6 µL in 15 mL TBS-T)
- Rinse 3 x 15 minute in TBS-T
- Incubated membrane in secondary antibody for 1 hour at room temperature with shaking
    - 2˚; 1 : 25,000 (1µL in 25 mL of TBS-T)
- Rinse 3 x 15 minute in TBS-T
- Blot dry and apply chemilumiscent substrate (Thermo Scientific ECL solution)
- Blot dry and place in plastic sheeth
- Expose x-ray film to membrane
    - JP1 – 3 minutes
    - JP2 – 1.5 minutes
    - Tubulin – 4 minutes
- Develop and Fix Film
- Allow film to dry and then scan for optic densitometric analysis.