

MINIMAL DISUSE MUSCLE ATROPHY AND SEASONAL ALTERATIONS IN THE
CALCIUM HANDLING SYSTEM IN SKELETAL MUSCLE OF HIBERNATING BROWN
BEARS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of
JOHN DAVIDSON HERSHEY find it satisfactory and recommend that it be accepted.

Chair

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Abstract

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Previous studies on wild black bears (*Ursus americanus*) have shown skeletal muscle morphology, composition and overall force generating capacity do not differ drastically between seasons despite prolonged inactivity during hibernation. The first goal of these studies was to compare the amount of muscle atrophy in captive brown bears (*U. arctos*) to that observed in wild black bears and measure seasonal differences in twitch characteristics. Samples from the biceps femoris muscle were collected during the summer and winter; protein concentration, fiber type composition and fiber cross-sectional area were measured along with twitch characteristics. Protein concentration of samples decreased 8.2% during winter compared to summer. Myosin heavy chain (MHC) isoforms I, IIa, and IIx were identified and the relative proportions did not change between seasons. Fiber cross-sectional area and the relative proportion of fast and slow fibers also remained unchanged between seasons. There were no significant changes in twitch contraction time or half-fall time between seasons while the half-rise time in the twitch contractions increased 53% in winter relative to summer samples. Increased half-rise time

without corresponding changes in the proportion of MHC isoforms during hibernation was unexpected given the amount of inactivity during hibernation and may indicate altered calcium handling during contractions in the myocyte. The second goal of these studies, therefore, was to determine if seasonal alterations were present in the calcium handling system of skeletal muscle in brown bears. We hypothesized that bears undergo seasonal alterations in the calcium handling system, specifically the elements related to the sarcoplasmic reticulum (SR) including the type 1 ryanodine receptor (RyR1), SR associated calcium ATPases (SERCA1 and 2) and calsequestrin (CSQ). In the biceps femoris muscle from summer to winter, RyR1 levels decreased by 52%, SERCA1 and 2 levels decreased by 25% and 15%, respectively; CSQ levels increased 16% during hibernation. These data are consistent with the seasonal changes observed with twitch dynamics. Furthermore, changes in calcium dynamics may impact the cell signaling pathways involved with phenotypic expression of contractile proteins during hibernation and could help minimize disuse muscle atrophy.

ATTRIBUTIONS

Charles T. Robbins - Dr. Robbins is the director of the Washington State University Bear Research, Education and Conservation Program and an active researcher on many aspects of bear physiology. His contribution to this dissertation included use of his equipment, facilities and overall expertise in bear handling. Additionally, Dr. Robbins contributed to the revision of both manuscripts comprising this dissertation.

O. Lynne Nelson - Dr. Nelson is a veterinary cardiologist at the Washington State University School of Veterinary Medicine. Dr. Nelson's previous research and surgical experience with the bears was invaluable with respect to the planning and completion of harvesting tissue; her early involvement in these studies prepared me to perform the various surgical procedures without any additional assistance. Additionally, Dr. Nelson contributed to the revision of both manuscripts comprising this dissertation.

David C. Lin - Dr. Lin is an associate professor at Washington State University with a joint appointment in the College of Veterinary Medicine and the College of Engineering and Architecture and has been my PhD advisor for the past six years. He has been integral in the design of these experiments, along with the writing, revising and subsequent publishing of the included manuscripts. In addition, grants he has written and procured have provided the funding for this research.

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GENERAL INTRODUCTION

GENERAL PHYSIOLOGICAL CHARACTERISTICS OF BEAR HIBERNATION

Hibernation in bears is often referred to as “carnivorean lethargy”, “winter dormancy” or “heavy sleep” (Hock 1957a; Lyman and Chatfield 1955) in order to distinguish it from the “deep” hibernation of other hibernating species, such as hamsters, chipmunks, marmots, hedgehogs and squirrels. Smaller hibernating species alternate between a state of profound torpor with no voluntary movement and a significant drop in body temperature, followed by periodic arousals (every 4-10 days) involving intense muscular activity and re-warming (Barnes 1989; Folk *et al.* 1976; Lyman and Chatfield 1955; Wassmer and Wollnik 1997; Wilz and Heldmaier 2000). Bears, however, hibernate in a very shallow torpor (*i.e.* they remain conscious and readily respond to external disturbances) and experience a decrease in body temperature of only a few degrees below the normal of 37°C (Brown *et al.* 1971; Harlow *et al.* 2001; Hellgren 1998; Hissa 1997; Hock 1957b; Watts *et al.* 1981). In addition to maintaining near-normal body temperature, bears maintain near-normal metabolic rates without food or water (Farley and Robbins 1995; Kleiber 1975; Watts and Cuyler 1988) and do not urinate or defecate while hibernating (Folk 1967; Nelson 1980; Nelson *et al.* 1973). During the overwintering period female bears will also gestate and lactate, further increasing the extreme metabolic demands of hibernation (Hock 1957a; Matson 1954; Nelson 1973; Wright *et al.* 1999).

Bears typically hibernate in dens not much larger than the dimensions of their own bodies for 6-8 continuous months every year as smaller dens help retain body heat which in part maintains normal denning metabolic function. The average dimensions of a brown bear den in Canada during hibernation is approximately 1x1x1 m (British Columbia Ministry of Water, Land

and Air Protection). The majority of wild black bears in the southern Appalachian region of the United States hibernate in elevated tree cavities, which severely restricts movement and weight bearing (Wathen *et al.* 1986). The limited space of the average den during hibernation implies that weight bearing activities are minimal, and in many cases not possible. For example, black bears hibernating in the Lower Mississippi River Valley demonstrated a 90% decrease in mean activity level during the hibernation period (Smith 1986). This minimal amount of weight-bearing activity and near euthermic body condition would normally cause significant disuse skeletal muscle atrophy in every mammalian species studied thus far (see next section). Hibernating bears, however, appear to be resistant to disuse atrophy as they experience a minimal loss of muscle strength (detailed later).

DISUSE SKELETAL MUSCLE ATROPHY

Disuse muscle atrophy occurs rapidly and is unavoidable during prolonged skeletal muscle unloading and includes conditions such as tenotomy, hypogravity, bed rest or limb casting (Bloomfield 1997; Boonyarom and Inui 2006; Edgerton *et al.* 2002; Michael 2000). While the specific histological and biochemical changes were unknown at the time, disuse atrophy was recognized and described by Hippocrates in the fourth century, B.C. (Chadwick and Mann 1950):

“For if the whole body is rested much more than is usual, there is no immediate increase in strength. In fact, should a long period of inactivity be followed by a sudden return to exercise there will be an obvious deterioration. The same is true of each separate part of the body. The feet

and limbs would suffer in the same way if they were unaccustomed to exercise, or were exercised suddenly after a period of rest.”

Disuse atrophy is traditionally characterized by two significant changes in the affected muscle. The first is a decrease in overall cross-sectional area of muscle fibers/myocytes (Bloomfield 1997; Edgerton *et al.* 2002; Kasper *et al.* 2002). The decrease in cross-sectional area results primarily from an increase in protein degradation (primarily those proteins related to the contractile machinery of the muscle). This process includes activation of lysosomal (primarily cathepsins), cytosolic calcium dependent (primarily calpains), caspase and ATP-dependent ubiquitin-proteasome pathways (Kandarian and Stevenson 2002; Ventadour and Attaix 2006). Activation of these pathways during disuse is reflected by an overall negative nitrogen balance, which can lead to significant increases in urinary nitrogen excretion in humans in as little as five days of bed rest (Bloomfield 1997). Because force output of muscle is correlated to muscle mass (which is reflected by fiber cross-sectional area) (Edgerton *et al.* 2002), loss of total protein within the muscle translates to an overall net decrease in maximal force production.

The second alteration in skeletal muscle as a result of disuse is the conversion of type I fibers to type IIx and type IIa (Bloomfield 1997; Bodine *et al.* 2001; Edgerton *et al.* 2002; Kasper *et al.* 2002; Pette and Staron 2000). One of the main contractile proteins in skeletal muscle is myosin, a ~500 kDa hexamere comprised of four light chains and two heavy chains, found on the thick filament. One portion of myosin known as the myosin heavy chain (MHC) has multiple isoforms and is the prime determinant of the shortening velocity of muscle and the rate of force development (Bottinelli *et al.* 1996; Harridge *et al.* 1996). MHC isoforms are subdivided into two main categories - MHC type I (slow) and MHC type II (fast). MHC type I is

more efficient at utilizing oxygen as a fuel source (aerobic, oxidative) and is suited for slower, prolonged contractions and is fatigue resistant. These fibers have been referred to as “red” fibers, due to the presence of myoglobin (the primary oxygen-carrying molecule of muscle), which imparts a red color to the fiber. MHC type II (sometimes referred to as “white” fibers) is more efficient under anaerobic conditions (glycolytic) and is better suited for short bursts of intense activity (fatigable). MHC type II is further divided into subtypes including MHC IIa, MHC IIx and MHC IIb (fastest). The various MHC isoforms create a spectrum of muscle phenotypes which defines the contractile properties of muscle and fatigability; the progression from slowest to fastest is :

MHC I → MHC IIa → MHC IIx → MHC IIb

It is important to note that the MHCs listed are those commonly found in adult, mammalian skeletal muscle; others exist in other species, neonatal animals, and certain specialized muscles. Most muscles contain a mixed population of fiber types although there are some exceptions such as the soleus muscle, which is comprised primarily of slow fibers (Falempin *et al.* 1990; Steffen *et al.* 1990; Thomason *et al.* 1987).

While all muscle experiencing disuse conditions will demonstrate the two hallmarks of atrophy, not all muscles are affected to the same degree and at the same rate (Boonyarom and Inui 2006). In general, atrophied muscle will be weaker, have faster contractile characteristics and fatigue more rapidly. Disuse muscle atrophy most severely affects muscle containing significant populations of slow fibers, including postural and antigravity muscles such as the extensors of the limbs and spine (Bloomfield 1997; Edgerton *et al.* 2002).

Practically every other species to date has demonstrated a susceptibility to disuse skeletal muscle atrophy. For example, seven days of hindlimb suspension, a common model for disuse atrophy, caused a reduction in mean fiber area in the soleus muscle of 37% in mice (Dapp *et al.* 2004). Three weeks of cast immobilization in the hindlimb of rabbits caused a 19% loss in the wet weight of the cranial tibial muscle along with a reduction in fiber cross-sectional area of 26% (Qin *et al.* 1997). Similar findings can be found in all species studied including rats (Mattiello-Sverzut *et al.* 2006; Yu *et al.* 2007), dogs (Bebout *et al.* 1993; Fung *et al.* 1995), horses (Booth and Clegg 2000), and humans (Akima *et al.* 2000; Akima *et al.* 2001; Kawakami *et al.* 2001). Even other mammalian hibernators are susceptible to disuse atrophy including bats (Kim *et al.* 2000), hamsters (Wickler *et al.* 1987) and ground squirrels (Wickler *et al.* 1991).

Disuse atrophy was induced in the above mentioned species using several accepted techniques. Hindlimb suspension/unweighting is commonly employed in small species such as rats and mice and involves suspending the hindlimbs of the animal by the base of the tail using a padded clamp or traction tape (for examples see Schulte *et al.* 1993; Wronski and Morey-Holton 1987). The tail is raised via a wire attached to a swivel suspended above the cage until only the forelimbs are touching the floor. In larger species such as cats, dogs and livestock, disuse atrophy is usually induced by splinting or casting of the limb whose muscles are to be studied (for examples see Fung *et al.* 1995; Qin *et al.* 1997). A third technique that is sometimes used includes complete transection of one of the tendons of attachment of the muscle that is to be studied (for example see Turner 2007). In humans, disuse atrophy is typically induced by prolonged bed rest although splinting and casting are occasionally employed (for examples see Kawakami *et al.* 2001; Rittweger *et al.* 2005; Thom *et al.* 2001).

LACK OF DISUSE SKELETAL MUSCLE ATROPHY DURING BEAR HIBERNATION

The study of the resistance to muscle atrophy in bears was pioneered by Dr. Henry J Harlow in wild black bears. Harlow *et al.* (2001) designed and implemented an apparatus which measured torque generated at the hock joint of a bear, in response to contraction of the hock flexors (primarily the cranial tibial and long digital extensor muscles). Utilizing percutaneous stimulation of the common peroneal nerve in sedated wild black bears, bears lost only 23% of strength in the hock flexors over a 130 day period (time period described as early and late winter), compared to a predicted loss of 90% in humans over the same time period and conditions. Additionally, the bears had no loss of skeletal muscle fiber size or number during the same time period. A more recent study on wild black bears using the same experimental apparatus determined that over a 110 day period, bears lost only 29% of muscle strength in the hock flexors, compared to human reports of a 54% decrease in the same muscle over a 90 day period of bed rest. Additionally, muscle contractile properties, including half-rise time, twitch contraction time and half-fall time remained unchanged (Lohuis *et al.* 2007).

In a different study, the relative proportion of fast and slow fibers were quantified in the gastrocnemius of seven wild black bears and the biceps femoris of six wild black bears; samples were collected during the fall (October-November) and spring (March) (Rourke *et al.* 2006). Based on gel electrophoresis, the proportions of fast and slow fibers remained unchanged during hibernation in the gastrocnemius while the biceps femoris actually experienced a 50% increase in the relative proportion of slow fibers at the end of hibernation. Based on reverse-transcription polymerase chain reaction (RT-PCR) of relevant mRNA, there were no significant alterations in MHC isoform gene expression between collection periods. One possible reason for the 50% increase in the slow MHC isoform without an corresponding increase in mRNA could be due to

sampling; the biceps femoris muscle has a complex regional compartmentalization and slight deviations from the desired collection site could lead to drastic alterations in the results.

Tinker *et al.* (1998) examined the skeletal muscle of nine radio-collared, wild black bears during the hibernation period; late fall/early denning samples were collected in September-December while late winter/early spring samples were collected in March-April. Samples were harvested in the field by excisional biopsy from the lateral head of the gastrocnemius muscle and the biceps femoris. Overall body mass in these bears decreased between 15-33% during the hibernation period. The bears maintained skeletal muscle cell size and number in both the gastrocnemius and biceps femoris, based on histological analysis of sectioned samples stained for ATPase activity. Modest decreases in protein concentration of 4% and 10% (based on dry weight) were observed in the gastrocnemius and biceps femoris, respectively. The proportion of fast twitch fibers increased moderately (mean change 9.9%) in the biceps femoris and did not change in the gastrocnemius during hibernation; all these data suggest limited muscle atrophy over a four to six month period.

While the amount and extent of muscle atrophy varied between studies (even within the same muscle), these studies clearly demonstrate that bears have a remarkable resistance to disuse muscle atrophy, despite prolonged anorexia and minimal weight bearing activities during hibernation. This unique adaptation allows bears to emerge from hibernation with the immediate ability to hunt, gather food and protect themselves without having to spend time rebuilding lost muscle mass. A bear emerging from hibernation in an atrophied state would be at a severe disadvantage to acquire food and defend itself compared to other predators.

ROLE OF CALCIUM AS A CELL SIGNAL CONTROLLING MUSCLE GROWTH, BREAKDOWN AND TYPE

Disuse atrophy conditions, such as prolonged recumbency, quickly lead to alterations in the normal intracellular fluctuations of calcium in practically all studied mammalian species (see previous section). These fluctuations are vital to maintaining calcium sensitive signaling pathways, including those related to muscle atrophy and hypertrophy. Calcium serves a crucial role as a second messenger in the myocyte and a regulator of many key proteins including G proteins, IP₃ receptors, various calcium channels (including RyR1), calmodulin, troponin C, parvalbumin and others (Berchtold *et al.* 2000; Berridge *et al.* 2003). More specifically, calcium has a clear role in pathways regulating muscle hypertrophy and atrophy (reviewed in Bassel-Duby and Olson 2006; Schiaffino *et al.* 2007; Zhang *et al.* 2007). For example, calcineurin (CN) is a calcium/calmodulin-regulated protein phosphatase and is activated by sustained, low-amplitude calcium waves and serves as a sensor of calcium fluctuations (Crabtree 1999; Dolmetsch *et al.* 1998). CN induces the translocation to the nucleus and causes transcriptional activation of a family of transcription factors known as the nuclear factor of activated T cells (NFAT) (Rao *et al.* 1997; Schiaffino *et al.* 2007). NFAT transcriptional activity has been shown to be higher in slow muscles compared to fast and NFAT serves as a coregulator of several slow fiber type genes which promote MHC I expression and inhibits promoters of MHC IIb genes (McCullagh *et al.* 2004; Parsons *et al.* 2003).

Myocyte enhancer factor-2 (MEF2) is a family of transcription factors which activate multiple muscle specific genes involved with the transformation of myofibers in response to intracellular calcium fluctuations (Bassel-Duby and Olson 2006; Schiaffino *et al.* 2007). Both exercise and electrical nerve stimulation leads to activation of MEF2 and it is associated with

fast to slow fiber transformation (Bassel-Duby and Olson 2006; Wu *et al.* 2001). MEF2 has also been postulated to interact with NFAT in the regulation of slow phenotype genes (Black and Olson 1998; Wu *et al.* 2000) and can be regulated by calcium/calmodulin-dependent protein kinase II (CaMKII) via histone deacetylase (HDAC) (McKinsey *et al.* 2000).

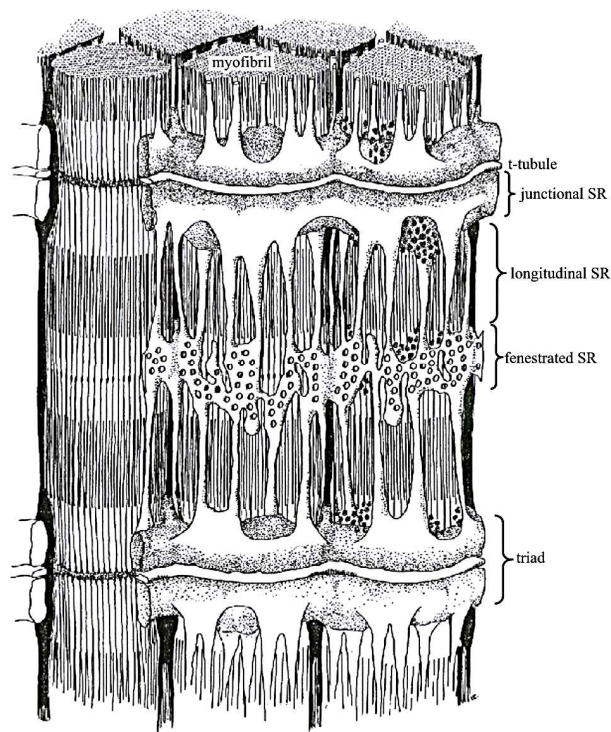
Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), a transcriptional co-activator, is a regulator of mitochondrial gene expression and is found in both brown fat and skeletal muscle (Puigserver *et al.* 1998; Vega *et al.* 2000). PGC-1 α is regulated by both CN and MEF2 (Czubryt *et al.* 2003; Handschin *et al.* 2003) and is expressed at higher levels in slow fibers compared to fast fibers and is inducible by exercise (Russell *et al.* 2003; Terada *et al.* 2002). In addition, overexpression of PGC-1 α in fast muscles causes an increase the proportion of type I fibers and makes the muscle more fatigue-resistant (Lin *et al.* 2002).

ROLE OF CALCIUM IN NORMAL SKELETAL MUSCLE CONTRACTION

In addition to a regulatory role in skeletal muscle hypertrophy and atrophy, calcium is vital to normal force generation. When a muscle contraction is initiated, action potentials are generated at the neuromuscular junction and travel deep within the myocyte along invaginations of the plasma membrane known as t-tubules; ultimately calcium is released into the cytosol and the muscle contracts. The calcium concentration within the myocyte normally fluctuates between the relaxed state (less than 10^{-7} M) and the contracting state (2×10^{-4} M) – a thousand-fold difference (Guyton and Hall 1997). Calcium cycling on this scale requires an isolated, intracellular storage area for calcium since sustained, elevated cytosolic calcium levels can quickly lead to cell death (Berchtold *et al.* 2000; Berridge *et al.* 2000). Additionally, two

separate systems are required for calcium release and uptake for a myocyte to function normally (MacLennan *et al.* 2002).

Calcium is stored in a complex, membranous system known as the sarcoplasmic reticulum (SR). The SR is intimately associated with the individual myofibrils and is also closely associated with the t-tubule network. It has a complex physical arrangement although this can vary both under normal and pathologic conditions (figure Peachey 1965):



The SR is not an empty space in which calcium is deposited, however. Contained within its membrane and lumen are many proteins, of which calsequestrin (CSQ) is of prime importance. CSQ functions as a calcium sponge, loosely binding many calcium ions in effect reducing the SR calcium concentration, allowing many more ions to be stored in a limited space.

In addition, CSQ is a complex regulator of calcium release from the SR (Beard *et al.* 2004; Szegedi *et al.* 1999; Wei *et al.* 2006).

The ryanodine receptor (RyR) is the primary channel responsible for the release of intracellular stores of calcium and is embedded within the membrane of the sarcoplasmic reticulum. Three isoforms of RyR are found in mammals – RyR1, found primarily in skeletal muscle and some areas of the brain and is also known as the α isoform; RyR2, found primarily in the cardiac tissue but also found the stomach, endothelial cells and areas of the brain and is also known as the β isoform; and RyR3 which is found in endothelial cells and in the striatum, thalamus and hippocampus of the brain. In skeletal muscle RyR1 is commonly found in close contact with the dihydropyridine receptor (DHPR), an L-type calcium channel. DHPR is found embedded within the membrane of the t-tubule system and serves as a voltage sensor (Timmerman and Ashley 1988). Depolarization of the plasma membrane and subsequently the t-tubule system causes a conformational change in the DHPR tertiary structure. Because the RyR1 is in close contact with the DHPR, this structural shift in DHPR in turn causes a subsequent change in the RyR1. It is believed that this alteration in RyR1 leads to the opening of the channel and rapid release of calcium; this interaction between the RyR1 and DHPR is critical to normal excitation-contraction coupling in skeletal muscle (Timmerman and Ashley 1988).

Once calcium is released in the myocyte, it is rapidly sequestered back into the SR to prevent undesired muscle activity and potentially damaging cytosolic calcium levels. This is accomplished via a family of proteins known as the sarco/endoplasmic reticulum calcium ATPases (SERCA). Because calcium is being moved against its ionic gradient, energy is required in the form of ATP. SERCA couples the dephosphorylation of one molecule of ATP to the influx of two calcium ions into the SR (de Meis *et al.* 2003). Several isoforms of SERCA

exist including SERCA1, found only in the SR of type II (fast) skeletal muscle. SERCA2 is subdivided into SERCA2a, found in type I (slow) skeletal muscle, cardiac and smooth muscle and SERCA2b, present in all other cell types of the body. SERCA3 is found in platelets, mast cells, lymphoid cells and epithelial cells.

Disuse atrophy conditions not only lead to histological alterations of muscle, but also profoundly affect the elements of calcium cycling within the myocyte outlined above. Atrophied muscle typically demonstrates faster contractions, in part due to alterations in calcium handling including the expression, or amount, of proteins involved in calcium release and uptake from the SR (Bloomfield 1997; Edgerton *et al.* 2002; Kim *et al.* 1982; Thom *et al.* 2001; Yoshioka *et al.* 1996). For example, rats experiencing tail suspension for two weeks demonstrate a 57% increase in levels of RyR1 in the soleus muscle (Kandarian *et al.* 1996), while rats tail suspended for four weeks demonstrate an 80% increase in levels of RyR1 in the soleus (Bastide *et al.* 2000). Hindlimb unweighting in rats led to a 300% increase (based on Western blotting) in SERCA expression and a concurrent decrease in the one-half relaxation time in the soleus after 28 days (Schulte *et al.* 1993). Additionally, disuse conditions lead to alterations in the expression of CSQ. Seven days of hindlimb unloading via tail suspension in rats led to a 68% decrease in CSQ mRNA levels and a 24% decrease in overall CSQ protein levels in the soleus (Hunter *et al.* 2001).

THE POTENTIAL ROLE OF CALCIUM IN MAINTAINING SKELELTAL MUSCLE PROPERTIES DURING URSINE HIBERNATION

While the role of calcium in bear hibernation has not been previously studied, the effects of hibernation on skeletal and cardiac muscle in respect to calcium handling have been examined

in rodent hibernators. Over thirty years ago T.H. Rosenquist noted a difference in myocardial cells in hibernating ground squirrels compared to active squirrels (Rosenquist 1970), including alterations in the thickness of the intercalated discs and an increase in SR surface protein density, both of which were related to altered calcium handling. An increase in calcium uptake by the SR in hibernating ground squirrels compared to active ground squirrels has been demonstrated although the mechanism of the increased uptake is unknown (Belke *et al.* 1991). A complete summary of the multiple changes that have been characterized in the cardiac intracellular calcium handling system in hibernating ground squirrels has been previously published (Wang *et al.* 2002).

There have been a few studies which examined the calcium handling system in the skeletal muscle of hibernating ground squirrels. Hibernating ground squirrels experienced a 10% decrease in SERCA and a two-fold decrease in RyR1 at the end of hibernation (January-February) compared to the active period (June-July) (Malysheva *et al.* 2001a; Malysheva *et al.* 2001b). Hibernating ground squirrels also experienced a 43% decrease in the maximal rate of calcium uptake along with a 17% decrease in the calcium storage capacity of the SR at the end of the hibernation period (Agostini *et al.* 1991).

All of the above mentioned studies examined rodent hibernators, most likely due to the relative ease of handling and maintenance of these species in captivity. It is important to note that the changes seen in the myocardium of these smaller hibernators are compensatory to the drastically depressed body temperature during winter, unlike bears. This major shift in temperature drastically alters the kinetics of the intracellular calcium pools, and so the animal must adapt in order to maintain viable cardiac muscle (*i.e.* maintain productive contractions and prevent arrhythmias). Regardless, these studies are relevant in that they demonstrate a seasonable

adaptability of the calcium handling system to changes in the external environment and demonstrate that seasonal alterations in the calcium handling system in the skeletal muscle of hibernators are possible.

Minimal muscle atrophy in hibernating bears could be linked to seasonal alterations in calcium handling as the function of many important regulatory proteins such as calmodulin, CAM kinase, protein kinase C (PKC), adenylyl cyclase, nitric oxide synthase (NOS) and MAP kinases are extremely sensitive to cytosolic calcium levels. As outlined previously, calcium is also a key regulatory ion in many specific signaling pathways directly involved in muscle atrophy and hypertrophy (see above). In this way the calcium dynamics of the myocyte directly impact the two principal alterations associated with muscle atrophy – cross-sectional area of the fiber and specific contractile protein content.

The studies included in this dissertation are designed to test the hypothesis that bears alter the calcium handling system in the skeletal myocyte in a seasonal manner. While changes in the calcium handling system of the skeletal myocyte do not directly account for resistance to muscle atrophy, seasonal alterations in calcium handling likely impact the myriad of cell signaling pathways that are directly or indirectly regulated by calcium, many of which determine the muscle phenotype. Calcium handling in striated muscle is a plastic system, as hibernating rodent species undergo significant changes in calcium handling in both cardiac and skeletal muscle during the overwintering period. Perhaps bears alter the calcium handling system in skeletal muscle in a similar manner to maintain muscle mass.

SIGNIFICANCE OF WORK

Disuse muscle atrophy is an extremely common condition affecting tens of millions of people worldwide. In 2003, over eight million people were hospitalized in the United States for musculoskeletal disorders, with a total cost of \$215,000,000,000. Of these injuries, one-half were sprains, dislocations or fractures; injuries which usually require some form of splinting or casting (American Academy of Orthopaedic Surgeons). In addition, one in two American citizens will break a bone at some point in their life. Splinting and casting will both quickly lead to muscle atrophy and typically require prolonged physical therapy to rebuild lost muscle mass.

Sarcopenia is the term which describes the gradual loss of skeletal muscle mass as humans age. After the age of 45, people will lose on average 1% of their skeletal muscle mass every year due to protein deficiency, decreased exercise and overall increased frailty (United States Department of Agriculture). In the year 2000 it was estimated that the direct healthcare cost of sarcopenia in the United States in patients over 60 years of age was \$18,500,000,000 (Janssen *et al.* 2004). This condition gradually weakens those affected and makes individuals more prone to future musculoskeletal injuries, and has a psychological toll as well.

Disuse muscle atrophy affects a broad spectrum of people ranging from those suffering the loss of muscle mass when a broken limb is immobilized by splinting or casting, elderly and arthritic patients experiencing prolonged periods of bed rest, and even astronauts subjected to microgravity conditions encountered during space flight (Adams *et al.* 2003; Akima *et al.* 2000). Therapies developed from this research would aim to minimize, or potentially prevent, disuse skeletal muscle atrophy by utilizing the same signaling pathways employed by hibernating bears. These therapies could not only ease human suffering both physically and psychologically, but could also save millions of dollars in lost wages and hospital fees.

GENERAL OBJECTIVES AND SPECIFIC AIMS

Because the few studies documenting resistance to muscle atrophy were varied and performed on wild black bears, the first goal of this body of work was to validate our captive population of brown bears as a suitable model for this unusual phenomenon. Hibernation behavior in these bears has been previously studied and shown to be similar to that of wild hibernating bears (Rode *et al.* 2001). Once established that the captive bears at Washington State University were resistant to disuse muscle atrophy, the second goal was to determine if there were any seasonal changes in the calcium handling system in the skeletal muscle of hibernating bears, compared to the summer active period.

The general objective for this study involved examining the potential role of calcium in the resistance to muscle atrophy that has been observed in overwintering bears. Calcium is not only crucial to normal contraction in muscle, but is also a key regulator of many intracellular signaling pathways, many of which regulate contractile protein expression in myocytes. While seasonal alterations in calcium handling have been documented in the skeletal and cardiac muscle of rodent hibernators, little research has been performed in overwintering bears.

Washington State University possesses a captive bear facility, one of only a few in the world, managed by the Washington State University Bear Research, Education and Conservation Program under the direction of Dr. Charles T. Robbins. Through this facility our laboratory has continuous access to brown bears regardless of the season, along with the ability to observe the animals at any time of day or night.

The specific aims for this proposal focused on the calcium handling system in skeletal muscle that may be altered during hibernation. We proposed that bears alter the intracellular calcium handling system within skeletal muscle during the hibernating period which may help

reduce muscle atrophy; this would involve changes in calcium uptake, storage and release in order to prolong intracellular calcium. This phenomenon has yet to be investigated in bear skeletal muscle and is a novel approach to the phenomenon of bear hibernation and skeletal muscle physiology.

Specific Aim 1 – Characterize the overall amount of muscle atrophy in hibernating brown bears compared to summer active bears.

Although minimal atrophy has already been documented in wild black bears, the amount and degree varied between studies. We performed measurements to help clarify the existing literature and validate our captive population of brown bears focusing on three factors – percent MHC expression (fiber type), average myofiber cross-sectional area and protein concentration.

HYPOTHESIS: There will be no significant change in cross-sectional area of fibers or protein concentration along with minimal (<20%) change MHC isoform expression in hibernating bears compared to summer-active bears.

Specific Aim 2 – Examine the characteristics of skeletal muscle twitch in overwintering and summer active bears based on excisional biopsies.

Calcium was expected to be released in greater quantity by RyR1 and taken up more slowly by SERCA1 and 2 in winter. This experiment provided an integrated view of the various changes in calcium handling and how they relate to overall force output in hibernating bears.

HYPOTHESIS: There will be a steeper rising phase followed by a shallower falling phase in a single muscle twitch.

Specific Aim 3 – Quantify the amounts of RyR1, SERCA1 and 2 and CSQ found on the SR in overwintering and summer active bears.

Calcium-dependent signaling pathways were expected to be maintained during winter in part by increasing the amount of calcium released into the cytosol and prolonging its uptake.

HYPOTHESIS: The amount of RyR1 and CSQ will increase in hibernating bears (enhanced release of calcium) while the amount of SERCA1 and 2 will decrease in hibernating bears (prolonged uptake of calcium).

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Minimal Seasonal Alterations in the Skeletal Muscle of Captive Brown Bears

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ABSTRACT

Previous studies on wild black bears (*U. americanus*) have shown skeletal muscle morphology, composition and overall force generating capacity do not differ drastically between seasons despite prolonged inactivity during hibernation. However, the amount and characteristics of the seasonal variations were not consistent in these studies. The goals of this study were to compare the amount of muscle atrophy in captive brown bears (*U. arctos*) to that observed in wild black bears and measure seasonal differences in twitch characteristics. Samples from the biceps femoris muscle were collected during the summer and winter. Protein concentration, fiber-type composition and fiber cross-sectional area were measured along with twitch characteristics. The protein concentration of the winter samples was 8.2% lower than that of the summer samples; fiber cross-sectional area and the relative proportion of fast and slow fibers remained unchanged between seasons. Myosin heavy chain isoforms I, IIa, and IIx were identified by immunoblotting and electrophoresis, and the proportions did not change between seasons. The half-rise time in the twitch contractions increased in winter relative to summer samples, which is unexpected under disuse conditions. These results agreed with a study which showed minimal skeletal muscle atrophy between seasons in wild black bears.

INTRODUCTION

Hibernation in bears is characterized by a very shallow torpor in which they readily respond to external disturbances, a body temperature slightly below the normal of 37-38°C (31.2-36°C), and a resting metabolic rate approximately 73% of the interspecific basal metabolic rate (Farley and Robbins 1995; Hellgren 1998). Further, bears do not undergo substantial skeletal muscle atrophy during hibernation, which is unexpected because weight bearing activities are greatly reduced in wild, hibernating bears (Harlow *et al.* 2001; Lohuis *et al.* 2007; Rourke *et al.* 2006; Tinker *et al.* 1998). Such an unusual physiological adaptation would be advantageous in that bears could emerge from hibernation with the immediate ability to hunt, gather food and protect themselves without having to spend time rebuilding lost muscle mass.

Atrophy in limb muscles during prolonged unloading conditions is well documented in many mammalian species including mice (Dapp *et al.* 2004; Frimel *et al.* 2005), rats (Mattiello-Sverzut *et al.* 2006; Yu *et al.* 2007), rabbits (Kilic *et al.* 2004; Qin *et al.* 1997), dogs (Bebout *et al.* 1993; Fung *et al.* 1995), horses (Booth and Clegg 2000), and humans (Akima *et al.* 2000; Akima *et al.* 2001; Kawakami *et al.* 2001). Unloading in these studies was imposed by limb suspension, casting, orthopedic injury, bed rest, or decreased gravity. Other mammalian hibernators are also susceptible to disuse atrophy including bats (Kim *et al.* 2000), hamsters (Wickler *et al.* 1987) and ground squirrels (Wickler *et al.* 1991). Muscle atrophy results in a significant loss of strength and motor function due to several factors. Force generating capacity decreases due to reduced fiber cross-sectional area, overall protein loss, and reduced muscle mass (Baldwin and Haddad 2001; Bloomfield 1997; Edgerton *et al.* 2002). Changes in contractile protein expression often occur and include a decrease in the proportion of slow-oxidative type I fibers relative to fast-oxidative type IIa and fast-glycolytic type IIx and IIb fibers

(Booth and Baldwin 1996; Kasper *et al.* 2002; Vermaelen *et al.* 2005). Atrophy is most pronounced in antigravity postural muscles containing a significant proportion of MHC I such as extensors of the limbs (reviewed in Booth and Baldwin 1996). Relatedly, atrophy may not occur to the same degree in all muscles or even between the slow and fast fibers within the same muscle (Edgerton *et al.* 2002; Kasper *et al.* 2002). Based on the current literature, one would expect measurable atrophy to occur in mammalian muscles involved with postural function when prolonged periods of inactivity occur. In this way, bears should experience disuse atrophy in their limb muscles during hibernation.

Measurements to quantify the seasonal changes in the skeletal muscle of wild black bears show that the atrophy process is attenuated during hibernation although the extent of this attenuation has not yet been resolved. Ankle torque elicited by surface electrical stimulation of the common peroneal nerve, which innervates the cranial tibial and long digital extensor muscles, decreased by 23-29% from early to late hibernation (Lohuis *et al.* 2007; Tinker *et al.* 1998). Although it was concluded in these studies that the decrease was far less than the atrophy that would have occurred in other species, the decrease still represents a significant loss of strength. In addition, the temporal twitch parameters did not vary between seasons, further supporting an atypical response (Lohuis *et al.* 2007). In a different study, muscle samples were harvested in late fall (September-December) and early spring (March-April) from the biceps femoris and gastrocnemius (Tinker *et al.* 1998). Protein concentration, fiber number, relative amounts of slow and fast fibers and fiber cross-sectional area measurements changed slightly (less than 10% when statistically significant) and indicate only minor loss of strength and small changes in contractile properties. In a third study, the proportion of MHC I in the biceps femoris

was reported to increase by 50% from late fall to early spring (Rourke *et al.* 2006), which represents a major change in contractile properties.

The Washington State University Bear Research, Education and Conservation Program provides a unique opportunity to study the apparent resistance to disuse muscle atrophy in captive brown bears. The goals of our study were two-fold. First, we wanted to determine if captive hibernating brown bears maintain muscle properties in a way consistent with any of the existing studies in wild black bears; this may help resolve conflicting reports of the extent and characteristics of atrophy resistance. Secondly, we wanted to provide details on the seasonal differences in isolated skeletal muscle in hibernating bears by recording twitch contractions of living, excised skeletal muscle and more thoroughly quantify potential seasonal changes in MHC isoform expression.

MATERIAL AND METHODS

Population and Hibernation Characteristics. Six captive brown bears (two males, four females) ranging in age from 4-20 years were studied; two of the females were pregnant and had cubs during the winter collection period. All bears hibernated each winter from late October to late March. To prepare the bears for hibernation, they were fed excess food beginning in early August to achieve a targeted body fat content in excess of 30% (personal communication from Dr. Charles T. Robbins). In early October, food was gradually reduced until completely withdrawn in late October. Water was available *ad libitum* at all times, and straw was provided for bedding. Bears hibernated singly or in pairs in unheated pens (3 X 3 X 2.5 m) with continuous access through a small door to an outdoor area (3 X 5 X 5 m) that was covered on all sides to minimize external noise and stimulation. Because the pens were open to the outside, the

bears experienced daily light and temperature fluctuations. Monthly average temperatures for Pullman, WA, during winter 2004-2005 (November - February) ranged from 0.6°C to 9.7°C.

All dens were equipped with a video camera (model MGB600, Silent Witness, Surrey, BC, Canada) and three infrared illuminators (model SWIR, Silent Witness, Surrey, BC Canada). Video images were stored on a high definition digital recorder (model X240, Open Eye, Spokane, WA). From January 1 to February 15, the captive brown bears were recumbent for $98.2 \pm 0.5\%$ of each day. The remaining 26 minutes per day were spent standing or slowly walking as they gathered straw for their bed, altered their position on the bed, groomed or occasionally drank water. During the summer months, bears had access to a 0.56 ha yard for about eight hours each day and were released in small groups to allow social interactions. Summer activity in this population has been previously described and quantified (Rode *et al.* 2001). Briefly, bears spent about one-half of their time foraging, walking, running and playing and the other half lying quietly or resting in a pool; activity in this captive population was similar to that observed in wild populations (Stelmock and Dean 1985).

Muscle Sampling. Bears were darted with tiletamine HCl and zolazepam HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA) to induce anesthesia and were maintained on isoflurane inhalant anesthetic in oxygen via an endotracheal tube. Muscle biopsies from the superficial portion of the biceps femoris were collected mid-thigh as measured halfway from the greater trochanter of the femur to the stifle, just caudal to the shaft of the femur. We were careful in the exact location for sampling because the biceps femoris has a complex compartmentalization and slight deviations from the prescribed area could lead to significant differences in our measurements. Summer samples were collected between July 22-28, 2005 (active period) on the

left side and winter samples were collected between February 9-18, 2005 (hibernating period) on the right side. All experiments were approved by the WSU Institutional Animal Care and Use Committee (protocol #3257).

Live Muscle Measurements. Excisional biopsies with cylindrical dimensions of 3 cm x 5 mm were harvested by carefully cutting with a scalpel along a fascicle division, tying off the segment with suture, and cutting the segment free with scissors. The biopsies were immediately placed in a solution containing 118 NaCl, 3.4 KCl, 2.5 CaCl₂, 0.8 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11 glucose (all values mM, pH = 7.4) (Wappler *et al.* 1999). Two samples were harvested from each bear each season. The solution was continuously aerated with carbogen (95% O₂, 5% CO₂) and kept at room temperature. Samples were taken to the laboratory within 15 minutes of harvesting and mounted in a tissue bath (Radnoti Glass Technology, Monrovia, CA; Figure 1) equipped with field stimulation electrodes. One end of the sample was anchored with suture material to a glass hook in the bottom of the bath and the other end was connected to a force transducer (model FT03, Grass-Telefactor, West Warwick, RI) suspended above the bath. Tissue samples were continuously bathed with the fore-mentioned solution heated to 36°C. All samples were stretched to a length corresponding to a resting force of 50 mN which provided enough tension to remove any laxity from the anchoring sutures. Samples were stimulated at 100 V (1 ms stimulus width) at 0.5 Hz for 21 s with an electrical stimulator (model SD9, Grass, West Warwick, RI) and force was sampled at a rate of 2000 Hz with an A/D board (model 1102, dSpace, Paderborn, Germany). Typically, we recorded 10 twitches during a stimulation run. A total of three twitch trains were recorded every 5 minutes over a 15 minute period. Not all the samples harvested produced consistent forces throughout the protocol; if a sample did not

generate more than 20 mN of force above baseline, the sample was no longer considered viable for the electrical stimulation experiments. After recording was completed or the sample was judged not viable, the sample was saved by immediate freezing in liquid nitrogen and stored at -80°C until processed.

We analyzed the twitch data to measure half-rise time (time to half maximum force, rise), time to peak, half-fall time (time to half maximum force, decay) and total twitch time (end of twitch). The first twitch of each series of twitches was not analyzed because potentiation artifacts were possible. These analyses were averaged across individual twitch trains, across the three twitch trains for each sample, and for the two samples taken from each bear for each season. For display purposes, average twitch force versus time plots were generated by fitting the averages of the four measured time points to a double exponential function (Herbert and Gandevia 1999) and smoothed with a two pole Butterworth lowpass filter (50 Hz cutoff).

Protein Concentration. Protein concentration was measured using the Bradford technique (Bradford 1976). A portion of the muscle sample was weighed, freeze-dried for one week, reweighed, homogenized in glass tissue homogenizer with a 30:1 (v:w) 0.05 M KH_2PO_4 , 5% β -mercaptoethanol solution and spun (500 g, 5 min) to remove particulate material (Murphy *et al.* 1997). Protein concentration was measured by diluting the supernatant ten-fold with 0.05 M KH_2PO_4 buffer and mixing 30 μl of the diluent with 970 μl Bradford reagent. Colorimetry was performed using Bradford Dye Reagent (Bio-Rad Quick Start, Bio-Rad Laboratories, Hercules, CA) and measured on a photospectrometer (Genova, Jenway, Essex, UK) using bovine serum albumin as the standard. All samples were measured in triplicate. Results are reported as milligrams of soluble protein per gram of dry weight (DW).

Fiber Typing and Cross-Sectional Area. Fiber typing was performed using two methods. A portion of samples were differentially stained based on ATPase activity (Brooke and Kaiser 1970; Sakkas *et al.* 2003). Tissue was sectioned (10 μ m) on a cryostat (-20°C) and mounted on Fisher brand colorfrost/plus yellow slides. Slides were preincubated in 0.1 M sodium acetate and 0.1 M KCl (pH 4.6) for 10 minutes. Sections were then incubated for 30 s in 20 mM glycine and 20 mM CaCl₂ (pH 9.4) and then incubated for 25 min in 40 mM glycine, 20 mM CaCl₂ and 2.5 mM ATP (pH 9.4). Slides were washed 3 times/30 s each in 1% CaCl₂ (w/v), 3 min in 2% CoCl₂ (w/v) followed by 3 rinses/30 s each, in distilled water. Slides were then developed for 5 s in 1% ammonium sulphide (w/v) and finally rinsed 2 times/30 s each in distilled water. Slow, type I fibers stained dark brown/black while fast, type II fibers stained light beige (Figure 2). All steps were performed at room temperature. Sections were cover slipped and digitized using a microscope at low power (40x) equipped with a video camera.

A 500 x 500 μ m square sampling grid was overlaid onto the entire image (montaged) of the ATPase stained section. Individual squares were randomly selected until a minimum of 400 fibers for each sample were analyzed. Fast and slow fibers were outlined as two separate sets of objects and fiber cross-sectional areas were measured using NIH Image software.

Samples were also typed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a method that can quantify the four common MHC isoforms (I, IIa, IIx, and IIb) in adult mammalian skeletal muscle (Bamman *et al.* 1999; Widrick *et al.* 1997). Following the methods of Bamman *et al.* (1999), we used a 4% acrylamide stacking gel and 8% acrylamide separating gel. Briefly, a small piece from each biopsy was homogenized in sucrose buffer (1 ml buffer/10 mg wet tissue; sucrose buffer = 250 sucrose, 100 KCl, 5 EDTA, 20 Tris, all values mM), boiled in a loading buffer (1:1 homogenate: Laemmli buffer, 3% β -

mercaptoethanol) for 2 min, and frozen (-20°C) until assayed. Samples were later loaded onto the gel and run for 20 hours at 150 V and 5°C. Gels were silver stained using Silver Stain Plus Kit (Bio-Rad Laboratories, Hercules, CA). Staining lasted for 7-12 min, based on visual inspection as the gels developed. Stained gels were digitally photographed (Figure 3) and the densitometric profile analyzed using a Kodak image station (IS2000R, Kodak, Corning, NY). Electrophoretic analysis of MHC was repeated three times for each biopsy taken and the percentages averaged for all the samples belonging to a particular bear and season.

Western Blotting. Separation of the MHC isoforms was performed by SDS-PAGE as described previously. Bands were transferred to a polyvinylidene difluoride (PVDF) membrane for 5 hours at 350 mA in a transfer buffer consisting of 48 mM Tris and 39 mM glycine. Blots were incubated in a blocking solution consisting of 5% powdered milk, 0.1% Tween 20 in phosphate buffered saline (PBS-T) overnight at room temperature. The blots were rinsed the following morning 3x for 5 min each in 0.1% PBS-T and incubated with a primary antibody, either MHC IIa (sc-53094, Santa Cruz Biotechnology, CA; dilution 1:100) or fast MHC (all type II isoforms) (M 4276, Sigma-Aldrich, St. Louis, MO; dilution 1:1000) for 2 hrs at room temperature. Blots were washed again 3x for 5 min each in 0.1% PBS-T and incubated with secondary antibody (peroxidase linked sheep antimouse, RPN2124, dilution 1:25000, Amersham ECL Plus Western blotting reagent pack, GE Healthcare Bio-Sciences Corp, Piscataway, NJ,) for 1 hr at room temperature. Blots were washed 3x for 5 min each and incubated with visualization media (RPN2132, Amersham ECL Plus Western blotting detecting reagents, GE Healthcare Bio-Sciences Corp, Piscataway, NJ,) for 5 min; bands were visualized on a ChemiDoc™ XRS image station (Bio-Rad, Hercules, CA).

After visualization, blots were stained for 5 minutes in Coomassie Brilliant Blue R-250 and destained (5% methanol, 7.5% acetic acid) overnight and allowed to air dry (Figure 4). Air drying significantly decreased background staining, making bands much more visible. Blots were visualized again and aligned to the chemiluminescent images in order to determine the bands to which various antibodies were bound.

Statistical Analysis. Because the final goal for each measurement was to test significance between summer and winter values for the population, we pooled and averaged all the data for each specific measurement. For most statistical tests we had an $n=6$, although some data sets were incomplete and are so noted in Results. Because we could not adequately test for equal variance and normality in the data we had to assume unequal variance and lack of normality. For these reasons, we used a two-tailed, non-parametric, Wilcoxon Signed Rank statistic to test for significant seasonal differences. Statistical analysis was performed using NCSS (Number Crunching Statistical Software, Kaysville, UT). Statistical significance was accepted at $P<0.05$.

RESULTS

Muscle Atrophy Measurements. The average protein concentration was 194.2 ± 7.8 mg/g DW in the summer and decreased to 178.2 ± 7.6 mg/g DW during hibernation ($P=0.028$, Figure 5). In the ATPase stained cross-sections, the slow and fast fiber percentages were 48.0 and $52.0\pm9.9\%$ respectively in summer and 47.7 and $52.3\pm7.7\%$ in winter (Figure 6), indicating that there were no seasonal differences in fiber type. Cross-sectional area of slow fibers decreased slightly, although insignificantly, between summer ($10074\pm2618\ \mu\text{m}^2$) and winter ($9125\pm958\ \mu\text{m}^2$). Also,

cross-sectional area of fast fibers did not change between summer ($9450 \pm 1774 \mu\text{m}^2$) and winter ($9421 \pm 1870 \mu\text{m}^2$) (Figure 7).

In the SDS-PAGE analysis, we consistently separated three MHC isoforms (Figure 3) and positively identified IIa and fast MHC isoforms (Figure 4); similar results were observed in the rat with the exception of four bands instead of three. Because black bear skeletal muscle lacks the IIb isoform (Rourke *et al.* 2006), we determined that the commonly expressed MHC isoforms in the brown bear to be IIa, IIx and I in terms of electrophoretic mobility from slowest to fastest, respectively. The average percentage of MHC isoform expression for type I, IIx and IIa were 41.4, 25.1 and 33.6% in summer and 41.7, 24.8 and 33.5% in winter (Figure 8); there was no statistical difference in isoform expression between summer and winter.

Live Muscle Measurements. We successfully obtained twitch data in five of the bears (Table 1 and Figure 9). Maximal twitch force in the samples ranged from 50 to 350 mN above baseline. Although all values in winter were higher than the corresponding summer values, only the half-rise time was statistically different between seasons ($P=0.043$). Figure 10 schematically demonstrates the difference between a summer and winter twitch.

DISCUSSION

Muscle Atrophy Measurements. We chose to sample the biceps femoris for several reasons. Because part of this study was to determine if our population of captive bears was experiencing the same phenomenon as wild bears in regards to skeletal muscle atrophy, we wanted to be able to compare our results directly to published studies such as Tinker *et al.* (1998) and Rourke *et al.* (2006). More importantly, the biceps femoris in bears serves a postural function and as such,

should be prone to disuse atrophy. Through dissection of several brown bear hind limbs, we have determined that the origin and insertions of the biceps femoris in the brown bear is similar to that of the dog. The biceps femoris in the dog is a major contributor to hip extension and is active during the stance phase of locomotion (Wentink 1976) and during quiet standing (Tokuriki 1979), similar to other muscles considered having an anti-gravity role. In addition, the biceps femoris in bears contains 40-50% slow fibers (this study, Tinker *et al.* 1998), indicating postural function. In bipeds and quadrupeds undergoing muscle unloading, the biceps femoris shows signs of disuse atrophy. For example, rats exposed to microgravity experienced a 9.6% decrease in muscle mass of the biceps femoris after 17 days (Lalani *et al.* 2000), while the long head of the biceps femoris in humans exposed to 20 days of head-down bed rest decreased in volume by 10%, similar to the decreases in the vastus lateralis and soleus muscles (Akima *et al.* 2007). In dogs and sheep, orthopedic injury was observed qualitatively to cause atrophy in the biceps femoris (Lewis *et al.* 1988; Lindboe *et al.* 1985; Millis *et al.* 2000). For these reasons, the biceps femoris would be expected to undergo disuse atrophy in this captive population of bears, which has such low amounts of daily activity for prolonged periods of time (see Methods).

A comparison of the studies documenting skeletal muscle atrophy (two of which studied the biceps femoris) following hibernation in wild black bears shows differing amounts and characteristics of muscle atrophy reported in each study. Our study showed the most similar seasonal changes to the study of Tinker *et al.* (1998). For example, the 8% decrease in protein concentration from summer to winter in brown bears in the current study corresponds well to the 10% decrease that occurred in the biceps femoris of wild black bears. Average cross-sectional area of fast and slow fibers in the biceps femoris remained unchanged from summer to winter in both captive brown bears and wild black bears. Tinker *et al.* measured a 10% increase of fast

twitch fibers relative to slow twitch in the biceps femoris before and after hibernation. We observed no significant change in fiber type composition between summer and winter in either our ATPase histochemistry or SDS-PAGE results (Figures 6 and 8). Harlow *et al.* (2001) and Lohuis *et al.* (2007) reported a 23-29% loss of electrically stimulated ankle torque between early and late winter. One possibility for the relatively large loss of muscle strength is the two ankle flexors may atrophy to a greater degree than the biceps femoris. Another possibility which cannot be ruled out is that stimulation of the common peroneal nerve is less effective in the winter due to changes in the neuromuscular junction or related excitation-coupling systems.

Using SDS-PAGE, we resolved three bands of MHC isoforms in brown bears (Figure 3). Compared to our standard of the rat diaphragm, which shows four isoforms (one slow and three fast), the electrophoretic mobility of the bear isoforms was slightly less. We observed no increase in MHC type I expression, a result consistent with our measurements by ATPase staining. In comparison to a previous study, our winter measurements were similar to theirs (both found ~40% of type I isoforms in the biceps femoris), but we did not observe such a dramatic change from summer to winter (Rourke *et al.* 2006). They cite that the differences in the sampling period and/or location within the muscle may account for the discrepancy with their earlier studies using histochemical fiber typing (Tinker *et al.* 1998). We were careful in choosing a consistent sampling site in order to avoid this problem. Also, unlike the previous study, which could only resolve two isoforms by electrophoresis, we observed three bands in most samples. The appearance of a second fast MHC isoform is consistent with the identification of two fast isoforms by reverse-transcription PCR (Rourke *et al.* 2006). We are unsure why we were able to resolve three bands even though we used similar methodology as the previous study. One possibility is minor differences in gels, buffers, or homogenization, which could influence the

migration rate of the proteins. Perhaps subtle species differences exist between MHC isoforms in brown and black bears.

Some differences between our results and those of the earlier studies may be due to the sampling periods. Our summer dates were chosen because late July represents the height of the bears' active period, before they physiologically prepare for hibernation. The winter dates were chosen because this time is immediately following the deepest portion of hibernation, before there is any increase in activity as they prepare to arouse for spring. By sampling at these two times, our measurements reflect the greatest change between physiological states, compared to previous studies which have had as little as two to three months between sampling and may have inadvertently sampled in more transitional periods. In addition, even though we collected our winter data first, we assumed that we accurately measured the effects of hibernation on skeletal muscle in this population because bears alternate between active and inactive states in an annual pattern. The variations in the reported literature regarding muscle atrophy in bears may also reflect limited sample size of all the studies, with seven as the largest sample size. Because our results in a captive population of bears are most consistent with those of Tinker *et al.* (1998) from wild bears, we believe that in the biceps femoris, morphology, composition and strength are conserved between summer and the prolonged inactivity of hibernation.

Twitch Measurements. The second objective for this study was to determine if there were differences between the twitch characteristics of summer and winter brown bear skeletal muscle. We opted to use excisional biopsies, a technique which is a valid estimate for temporal properties of whole muscle (Faulkner *et al.* 1982; Moulds *et al.* 1977). Because our measurements were purely temporal (as opposed to maximal force measurements), all measurements were made

relative to the time of maximum force generated for each individual sample. Half-rise time, twitch contraction time, and half-fall time are commonly measured isometric parameters in skeletal muscle and indicate potential changes in both contractile and calcium cycling protein composition (Carvalho *et al.* 1997; Gallo *et al.* 2006; Koryak 1998; Seki *et al.* 2001; Shields 1995). Half-rise time and twitch contraction time are primarily determined by the contraction speed (and MHC composition) (Gallo *et al.* 2004; Gordon and Stein 1985; Stein *et al.* 1982) while half-fall time is determined largely by sarco/endoplasmic reticulum calcium ATPase (SERCA) 1 and 2a expression in fast and slow fibers, respectively (Hamalainen and Pette 1997; Schwaller *et al.* 1999). It is interesting to note that we measured a significant change in half-rise time without concurrent changes in MHC isoform composition. The significance of this will be discussed later in this section.

We observed an increase in all the temporal measurements from summer to winter (Table 1), although statistical significance was achieved only in the half-rise time measurement. Statistical significance in the other measures was not achieved possibly due to the small sample size (n=5). The slower twitch time course in winter samples is in stark contrast to skeletal muscle undergoing disuse atrophy. For example, twitch contraction time decreased by 20 ms and half-relaxation time was 25% shorter in the soleus of chronically paralyzed humans (Shields 2002).

The measurements of twitch characteristics in this study support the trends in previous studies that skeletal muscle during hibernation not only maintains its summer-active properties, but can even show changes which are in the opposite direction of atrophied muscle. While Lohuis *et al.* (2007) also observed no changes in twitch parameters, their results are difficult to interpret because the study utilized the peroneal nerve for stimulation and the results could be confounded by changes in the neuromuscular junction. In addition, the peroneal nerve innervates

two almost equally sized ankle flexors, the cranial tibial and long digital extensor muscles. The direct stimulation of the muscle in this study compared to cutaneous stimulation through a variable amount of fat provides more unambiguous results that the contractile properties of one muscle do not vary much seasonally.

Fiber typing of our samples showed that there was no significant difference between the fiber type composition of summer and winter samples (Figures 6 and 8) and cross-sectional area of both slow and fast fibers also remained unchanged between seasons (Figure 7). These two observations rule out the obvious causes for changes in twitch contraction time. One possibility could be seasonal alterations in calcium cycling within the fibers. While seasonal changes in the calcium handling system have not been documented in bears, they have been measured in hibernating ground squirrels. Changes in the cardiac myocytes of hibernating rodents include enhancement of calcium pumps and downregulation of release channels found within the sarcoplasmic reticulum (Belke *et al.* 1991; Wang *et al.* 2002). While these adaptations in rodents are primarily to compensate for altered calcium kinetics at low temperatures, alterations in calcium handling could, in part, help maintain skeletal muscle properties because of calcium's role as a signal for the synthesis, expression, and degradation of contractile proteins. Changes in intracellular calcium levels and its temporal characteristics greatly influence muscle hypertrophy and atrophy (Berchtold *et al.* 2000; Berridge *et al.* 2003) and so a seasonal shift in calcium dynamics (i.e., different seasonal calcium responses to equal neurological inputs) could help maintain the original fiber type profile of the muscle despite reduced activity.

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Table 1. Live Muscle Twitch Measurements

<u>Season</u>	<u>HRT*</u>	<u>TCT</u>	<u>HFT</u>	<u>TT</u>
<i>summer</i>	0.019±0.004	0.067±0.016	0.126±0.032	0.392±0.088
<i>winter</i>	0.029±0.008	0.081±0.025	0.132±0.036	0.429±0.122

HRT = half-rise time (time to half force), TCT = twitch contraction time (time to maximal force), HFT = half-fall time (decay), TT = total twitch time; * = statistically significant, P = 0.043, n=5. All values reported in seconds as mean ±1 standard deviation.

FIGURES

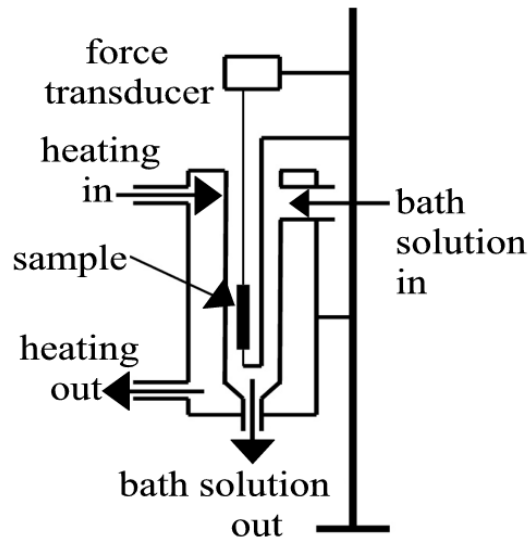


Figure 1. Live muscle setup. The muscle sample was hung between a force transducer and a stationary hook. Pre-heated, aerated carbogen was continuously flushed through the muscle chamber. The muscle chamber was maintained at 36°C by continuous flow of heated water.

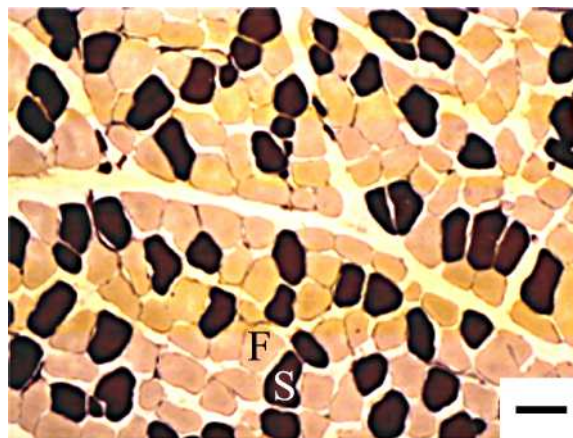


Figure 2. Cross-section of biceps femoris stained for ATPase activity. Slow fibers stain dark brown/black while fast fibers stain light tan. Magnification = 40x, scale bar = 200µm.

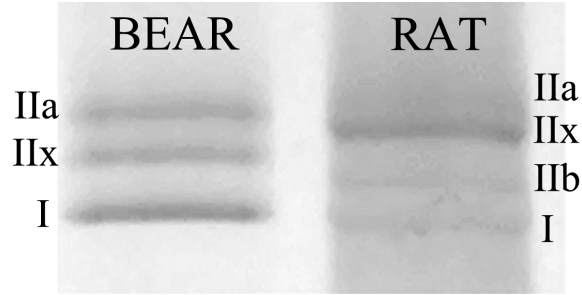


Figure 3. Gel electrophoresis of biceps femoris muscle from a brown bear and the diaphragm muscle of a Sprague-Dawley rat (representative samples). Bears lack the IIb isoform. The rat diaphragm standard shows that four isoforms can be identified with the method. The lower edge of figure represents the leading edge of gel.

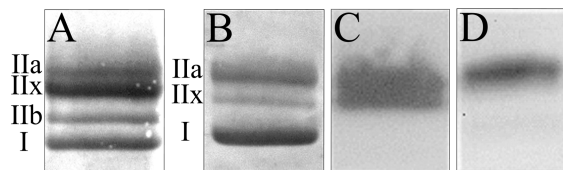


Figure 4. Western blot of biceps femoris muscle from a brown bear (representative images). Panel A = rat (Sprague-Dawley) diaphragm, Coomassie stained PVDF membrane, demonstrates four isoforms can be identified with the method. Panels B-D = brown bear; B = Coomassie stained PVDF membrane demonstrating three common MHC isoforms, C = reverse chemiluminescent image of blot stained for all fast MHC isoforms, D = reverse chemiluminescent image of blot stained with MHC IIa specific antibody.

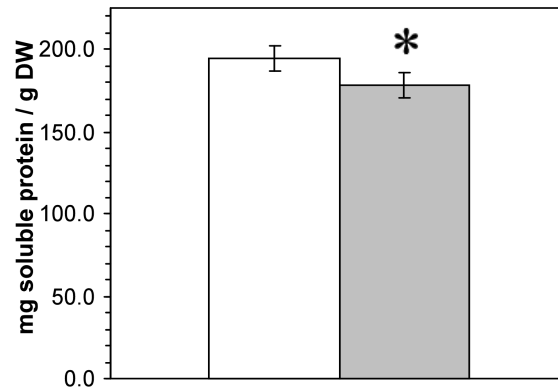


Figure 5. Total protein concentration in summer and winter. Open bar = summer, shaded bar = winter. Error bars represent one standard deviation. Average protein concentration decreased from summer to winter (*=statistically significant, $P=0.028$, $n=6$).

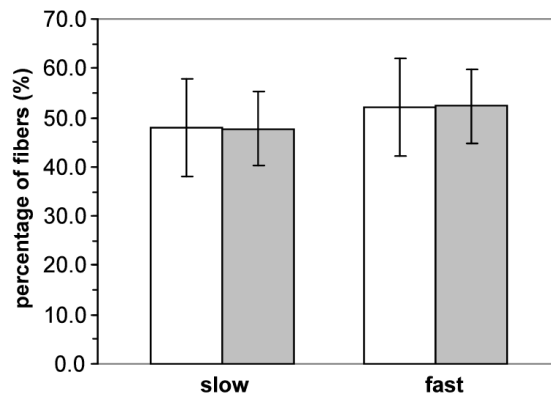


Figure 6. Percentage of slow and fast fibers in summer and winter as measured by ATPase staining. Open bar = summer, shaded bar = winter. Error bars represent one standard deviation. There were no significant differences in fiber type content between seasons.

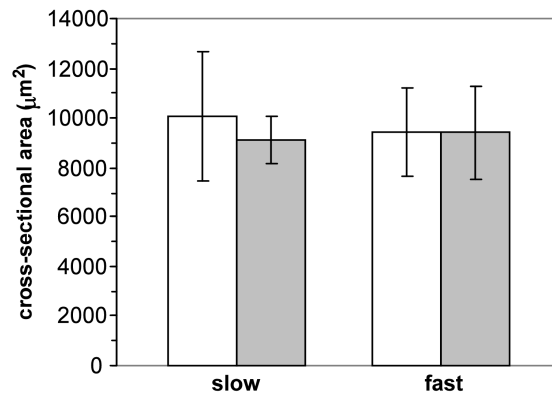


Figure 7. Cross-sectional area of slow and fast fibers in summer and winter. Open bar = summer, shaded bar = winter; results are reported as square microns. Error bars represent one standard deviation. Cross-sectional area of slow fibers decreased slightly, although insignificantly, between summer and winter. Cross-sectional area of fast fibers did not change between summer and winter.

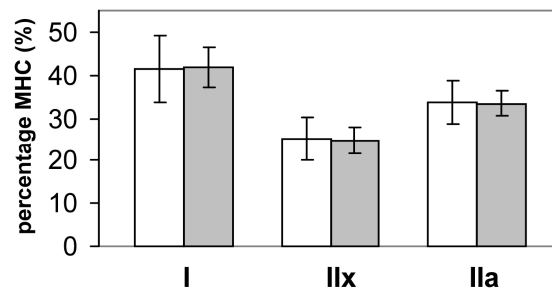


Figure 8. Seasonal comparison of MHC type I, IIx and IIa isoforms as measured by SDS-PAGE. Open bar = summer, shaded bar = winter; results reported as percentage of each MHC isoform present. Error bars represent one standard deviation. There was no difference in the percentage of any isoform between summer and winter.

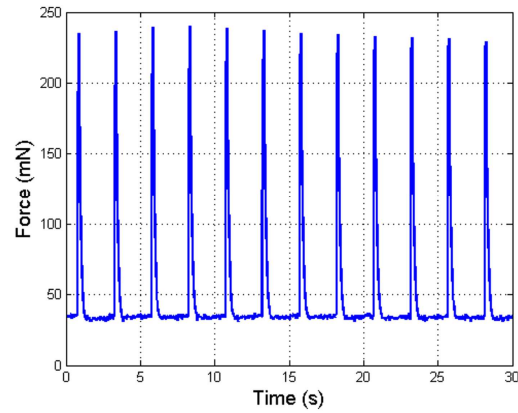


Figure 9. Example of typical twitch train. The muscle sample was stimulated at 0.5 Hz for 1 ms stimulus width and 100 V amplitude using field stimulation electrodes.

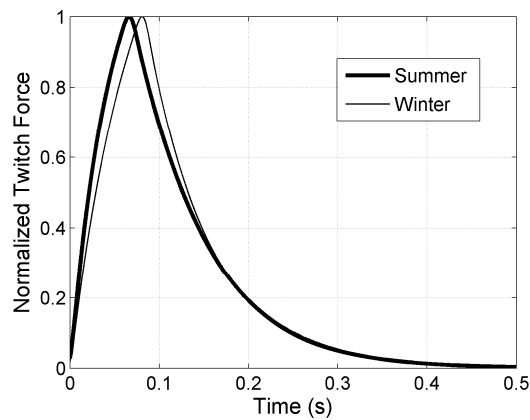


Figure 10. Schematic representation of summer and winter single twitch. Thick line represents summer data while thin line represents winter data. Force was normalized to its maximum value. Curves were generated from a fit to two exponential functions and smoothed by lowpass filtering. Half-rise time was greater in winter as compared to summer ($P=0.043$, $n=5$); no other changes were statistically significant.

**Seasonal Alterations in the Sarcoplasmic Reticulum Proteins within the Skeletal Muscle of
Brown Bears**

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ABSTRACT

Measurements of the seasonal changes in the skeletal muscle of overwintering bears have shown that the disuse atrophy process is attenuated during hibernation. This is unexpected because weight-bearing activities are greatly reduced during this prolonged bout of inactivity. These observations include minimal changes in the proportion of myosin heavy chain (MHC) isoforms and increased half-rise time for isometric twitches in biopsies collected during hibernation compared to summer. In the context of these two results, we hypothesized that bears undergo seasonal alterations in the calcium handling system of skeletal muscle, specifically the elements related to the sarcoplasmic reticulum (SR). By using Western blotting to measure protein amounts in the biceps femoris muscle, we found that from summer to winter: type 1 ryanodine levels decreased by 52%; type 1 and 2 SR calcium ATPase (SERCA) levels decreased by 25% and 15%, respectively; calsequestrin levels increased 16%. The changes are consistent with previous twitch measurements which demonstrate prolonged half-rise time during hibernation and likely lead to alterations in calcium dynamics within the myocyte. These alterations could impact cell signaling for phenotypic expression of contractile proteins during hibernation and in part curtail the skeletal muscle atrophy process.

INTRODUCTION

Mounting evidence supports the concept that bears do not undergo substantial disuse skeletal muscle atrophy during hibernation, which is unexpected because weight bearing activities and food intake are greatly reduced during this period (Harlow *et al.* 2001; Hershey *et al.* 2008; Koebel *et al.* 1991; Lohuis *et al.* 2007; Rourke *et al.* 2006; Tinker *et al.* 1998). Muscles experiencing disuse atrophy conditions demonstrate diminished strength and almost all have faster contractile properties, resulting from a decrease in fiber cross-sectional area, protein loss and a shift from slower, oxidative type I MHC contractile protein isoforms to faster, glycolytic type II MHCs (Booth and Baldwin 1996; Kasper *et al.* 2002; Vermaelen *et al.* 2005). Muscle samples obtained pre- and post-hibernation from the biceps femoris and gastrocnemius of wild black bears showed slight changes (less than 10% when statistically significant) in protein concentration, fiber number, relative amounts of slow and fast fibers (*i.e.*, relative proportions of MHC types 1 and 2) and fiber cross-sectional area measurements (Tinker *et al.* 1998). Hershey *et al.* (2008) determined fiber cross-sectional area, the relative proportion of fast and slow fibers, and the ratio of MHC isoforms remained unchanged in the biceps femoris of captive brown bears between mid-summer and mid-winter.

The altered contractile properties of muscle following disuse can be also observed as faster temporal measurements during an electrically stimulated twitch (Carvalho *et al.* 1997; Koryak 1998; Shields 1995). The faster twitch parameters not only reflects changes in MHC isoforms, but also concurrent alterations in the muscle's calcium handling system (Hunter *et al.* 2001; Li *et al.* 2002; Yoshioka *et al.* 1996). In skeletal muscle, calcium ions are stored and released from an extensive, intracellular, membranous system known as the sarcoplasmic reticulum (SR). The SR contains calsequestrin (CSQ), a protein which allows the SR to store

calcium at a much higher concentration than that of cytosol (Beard *et al.* 2004). Release of calcium in skeletal muscle is mediated primarily by the type 1 ryanodine receptor (RyR1), a calcium channel found in the SR membrane. Opening of RyR1 is initiated most likely via a physical interaction with the dihydropyridine receptor (DHPR), a membrane-spanning voltage sensor contained in deep invaginations of the plasma membrane of the myocyte known as t-tubules (Adams and Beam 1990; Schneider and Chandler 1973; Tanabe *et al.* 1990). Calcium uptake into the SR is achieved primarily by the sarco/endoplasmic reticulum calcium ATPase (SERCA) (Figure 1). Skeletal muscle SR contains two isoforms of SERCA; SERCA1 occurs mainly in fast twitch fibers and SERCA2 occurs mainly in slow twitch fibers (Brandl *et al.* 1987; Periasamy and Kalyanasundaram 2007). Expression and activity of the proteins associated with the calcium handling system are dependent on the past activity of the muscle; in general, under disuse conditions RyR1 and SERCA1 and 2 levels increase in the myocyte (Bastide *et al.* 2000; Kandarian *et al.* 1996; Schulte *et al.* 1993), while CSQ levels typically decrease (Hunter *et al.* 2001).

If bears undergoing long periods of inactivity experience the normal changes associated with disuse atrophy, there should be a shift in all components of the calcium handling system toward faster calcium kinetics and faster twitches. However, Lohuis *et al.* (2007) observed that twitch contraction time, half-maximum time, time to peak, and half-relaxation time from ankle torque measurements following percutaneous electrical stimulation of the common peroneal nerve did not change during the hibernation period in wild black bears. Our previous study demonstrated that during direct electrical stimulation of viable biopsies from the biceps femoris of captive brown bears, twitch contraction time and half-fall time also remained unchanged between mid-summer and mid-winter while the time to half maximal force (half-rise time)

increased 53% in winter relative to summer samples (Hershey *et al.* 2008). We may have detected a significant increase in half-rise time while Lohuis did not due to differences in technique and muscles; Lohuis stimulated all muscles innervated by the common peroneal nerve (primarily the cranial tibial and long digital extensor muscles) via cutaneous stimulation, while we directly stimulated a portion of the biceps femoris. Prolonged half-rise rise time in the biceps femoris without changes in MHC expression during hibernation in bears is unusual, and may be explained by alterations in calcium handling.

The purpose of this study was two-fold: to assess if there were seasonal changes in the proteins associated with the SR in bears; and, if changes were evident, to determine if they were consistent with the disuse muscle atrophy studies which show faster twitch characteristics, or with the bear studies documenting slower twitch characteristics. Additionally, for comparative reasons, we wanted to contrast any potential seasonal changes in bears to the protein alterations associated with the SR in other hibernating species such as rodents. We hypothesized that bears undergo seasonal alterations in the calcium handling system, specifically the elements involved with calcium storage, release and uptake. Moreover, we hypothesized that these alterations are clearly different than the changes during disuse muscle atrophy, and consistent with slower twitch measurements in winter muscle samples. These hypotheses were addressed by measuring the seasonal amounts of the proteins of the calcium handling system.

MATERIAL AND METHODS

Population and Hibernation Characteristics. Five captive brown bears (two males, three females) ranging in age from 4-20 years were studied at the Washington State University Bear Research, Education, and Conservation Program; pen and hibernation conditions have been described in

detail previously (Hershey *et al.* 2008). To summarize, bears hibernated from late October to late March singly or in pairs in unheated pens with continuous access through a small door to a small outdoor area. The outer area was covered on all sides to minimize external noise and stimulation, although bears experienced daily light and temperature fluctuations. Monthly average temperatures for Pullman, WA, during winter 2004-2005 (November - February) ranged from 0.6°C to 9.7°C. Video records of these captive bears from previous years showed that they were recumbent for $98.2 \pm 0.5\%$ of each day from January 1 to February 15.

During the summer, bears had access to a 0.56 ha yard for about eight hours each day in small groups to allow social interactions. Summer activity in this population is described in Rode *et al.* (2001). Bears spent about one-half of their time being active and the other half lying quietly or resting in a pool; activity in this captive population is similar to that observed in wild populations (Stelmock and Dean 1985).

Muscle Sampling. Bears were darted with tiletamine HCl and zolazepam HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA) to induce anesthesia and maintained on isoflurane inhalant anesthetic. Muscle biopsies were collected from the superficial portion of the biceps femoris mid-thigh as described in Hershey *et al.* (2008). We were careful in the specific location for the biopsies as slight deviations from the prescribed area could lead to significant differences in our measurements. Winter samples were collected the second week of February 2005 (hibernating period) and summer samples were collected the third week of July 2005 (active period). All protocols were approved by the WSU Institutional Animal Care and Use Committee (protocol #3257).

Western Blotting. Western blotting was based on the following protocols and modified by our laboratory as needed for optimal results: Brittsan *et al.* 2000; Kandarian *et al.* 1996; Terentyev *et al.* 2003. Samples collected for Western blotting were immediately flash-frozen in liquid nitrogen and stored at -80°C until processed. Frozen samples were coarsely minced with a scalpel blade and ground 12:1 (w:v) in homogenization buffer (5 mM HEPES, 250 mM sucrose, 0.2% NaN₃) using a conical tissue grinder for 5 min at room temperature. The homogenate was spun for 60 s at 500 g and the pellet was discarded. The total soluble protein concentration of the supernatant was quantified using the Bradford technique (Bradford 1976). Colorimetry was performed using Bradford Dye Reagent (Bio-Rad Quick Start, Bio-Rad Laboratories, Hercules, CA) and measured on a photospectrometer (Genova, Jenway, Essex, UK) using bovine serum albumin in 5 mM HEPES, 250 mM sucrose and 0.2% NaN₃ as the standard. All samples were measured in triplicate and the results averaged.

The supernatant was mixed 1:1 with loading buffer (Laemmli buffer, 2.5% β-mercaptoethanol) and boiled for 5 min. Twenty micrograms of protein (based on supernatant concentration) were loaded per lane onto pre-cast gradient gels (4-15% Tris-HCl, Bio-Rad Laboratories, Hercules, CA). A visible standard (Pierce Chemiluminescent BlueRanger, Thermo Fisher Scientific, Rockford, IL, or Precision Plus Protein Standards, Bio-Rad Laboratories, Hercules, CA) and a chemiluminescent standard (MagicMark XP, Invitrogen, Carlsbad, CA) were run on each gel. It is important to note that we did not assay for typical loading controls such as actin, as we could not assume that any proteins (especially those related to the contractile apparatus) remained at a constant level throughout the year. For the seasonal comparisons, summer and winter samples for an individual bear were run in triplicate on the same gel, which eliminated any variability between seasons introduced by the electrophoresis or transfer process.

All electrophoresis was performed using a Mini-Protean 3 Electrophoresis Cell (#165-330, Bio-Rad Laboratories, Hercules, CA).

Four different antibodies were used for Western blotting: RyR1, SERCA1 and 2, and CSQ; each required a unique protocol. Gels were run for 60 min (CSQ) or 120 min (RyR1 and SERCA1 and 2), 100 V at room temperature. Bands were transferred (CSQ, SERCA1 and 2 = 48 mM Tris, 39 mM glycine; RyR1 = 48 mM Tris, 39 mM glycine, 0.01% SDS, 10% methanol) onto a polyvinylidene fluoride (PVDF) membrane for 5 hrs at 300 mA (CSQ) or 350 mA (RyR1, SERCA1 and 2) (#170-3930, Mini-Trans Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, Hercules, CA). PVDF membranes were blocked overnight at room temperature (CSQ, SERCA1 and 2) or 4 hours (RyR1) with 5% w:v powdered milk solution (Dulbecco's phosphate buffered saline (PBS), 0.1% Tween; PBS-T).

Membranes were rinsed 3 times for 5 min each rinse (3x/5 min) with PBS-T. The membranes were then incubated with primary antibody (RyR1 = 1:500, 14 hrs, 4°C, R129; SERCA1 = 1:5000, S-1064; SERCA2 = 1:2500, S-1439 both for 2 hrs, room temperature; Sigma-Aldrich Co., St. Louis, MO; CSQ (cardiac/skeletal) = 1:2500, 3 hrs, room temperature, PA1-913; Affinity Bioreagents, Golden, CO). Membranes were rinsed again 3x/5 min with PBS-T and incubated with either HRP-conjugated sheep anti-mouse secondary antibody (SERCA1 and 2 and RyR1) or HRP-conjugated donkey anti-rabbit (CSQ) (RPN2124, Amersham, GE Healthcare Bio-Sciences Corp, Piscataway, NJ) at room temperature either for 1 hr (CSQ 1:12500; SERCA1 and 2, 1:25000) or 2 hrs (RyR1, 1:12500). All antibodies were diluted with PBS. Membranes were rinsed again 3x/5 min, with PBS-T before visualization.

Bands were visualized using an ECL Plus detection kit (RPN2132, Amersham, GE Healthcare Bio-Sciences, Piscataway, NJ). Images were captured on a ChemiDoc XRS image

station (Bio-Rad Laboratories, Hercules, CA) and image analysis of bands was performed using Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA). Protein amount was based on peak intensity of the bands and averaged across the three samples for an individual bear for each season.

Statistical Analysis. For all the Western blotting data, we assumed unequal variance and lack of normality as we could not adequately test for equal variance and normality with the small sample size in this study. For these reasons we used a two-tailed, non-parametric, Wilcoxon Signed Rank statistic to test for significant seasonal differences. Statistical analysis was performed using NCSS (Number Crunching Statistical Software, Kaysville, UT).

RESULTS

The antibodies used in this study were known to cross-react with the following species (based on the product description) and had not been previously used for Western blotting in brown bear muscle until this study; RyR1: human, cow, sheep, dog, mouse, rabbit; SERCA1: dog, mouse, rat, guinea pig, rabbit, SERCA2: human, dog, rat, pig, rabbit; CSQ: human, dog, mouse, rat, and sheep. All four commercially available antibodies bound to their prescribed proteins and were visualized on the membranes, based on the location each specific band (Figure 2). SERCA1 and 2 (molecular weight=105-110 kDa) bands were clearly visible between the 100 and 120 kDa standards and CSQ (MW=60-65 kDa) was visible between the 60 and 80 kDa (much closer to the 60 kDa band). We did not have standards with large enough molecular weights close to that of RyR1 (MW=400-450 kDa) but a distinct band much larger than the 220 kDa standard (the

largest standard in our ladders) was visible. These results indicate that much of the structures of the calcium handling proteins are well conserved across species.

Both SERCA1 and 2 levels decreased from summer to winter, 25% ($P=0.043$) and 15% ($P=0.080$), respectively (Figures 3, 4). RyR1 levels also decreased from summer to winter by 52% ($P=0.080$) (Figure 5). CSQ levels increased by 16.4% ($P=0.043$) from summer to winter (Figure 6). A pictorial representation summarizing these changes is included in Figure 7.

DISCUSSION

The first purpose of this study was to determine whether there were any significant seasonal changes in the basic components of the sarcoplasmic reticulum of hibernating bears. One inherent difficulty with studies on bears is limited sample size, which can make statistical significance difficult to achieve. SERCA1 and CSQ had seasonal changes that were statistically significant compared to $P=0.050$; RyR1 and SERCA2 did not. However, both of these values were $P=0.080$, only slightly higher the generally accepted value of $P=0.050$. A primary cause of this is that values from one bear (bear 4 in Figures 4 and 5) increased slightly in RyR1 and SERCA2 measurements from summer to winter (7% and 0.3%, respectively), opposite of the trend of the other four bears. Due to the sensitivity of the signed rank statistical test to this one opposite sign in the seasonal changes, the P value for the sample population became greater than the accepted standard of $P=0.05$. Considering this and the small sample size ($n=5$), we deem the collection of all the seasonal changes recorded in the Western data in this study, including RyR1 and SERCA2, as significant.

The second purpose of this study was to determine if the changes in the calcium handling system were similar to that of the atrophy literature, or to the bear twitch data which

demonstrated slower kinetics. Under disuse atrophy conditions, skeletal muscle demonstrates faster contractions, in part due to alterations in calcium handling including the expression, or amount, of proteins involved in calcium release and uptake from the SR (Bloomfield 1997; Edgerton *et al.* 2002; Kim *et al.* 1982; Thom *et al.* 2001; Yoshioka *et al.* 1996). For example, rats experiencing tail suspension for two weeks demonstrate a 57% increase in levels of RyR1 in the soleus muscle (Kandarian *et al.* 1996), while rats tail suspended for four weeks demonstrate an 80% increase in levels of RyR1 in the soleus (Bastide *et al.* 2000). Hindlimb unweighting in rats led to a 300% increase (based on Western blotting) in SERCA expression and a concurrent decrease in the one-half relaxation time in the soleus after 28 days (Schulte *et al.* 1993). Additionally, disuse conditions also lead to alterations in CSQ expression. Seven days of hindlimb unloading via tail suspension in rats led to a 68% decrease in CSQ mRNA levels and a 24% decrease in overall CSQ protein levels in the soleus (Hunter *et al.* 2001). Compared to these studies, it is clear bears do not undergo muscle atrophy during disuse conditions like other mammalian species, in that the characteristic shifts in the calcium handling system toward faster contractile properties associated with disuse are not evident.

While contraction speed is determined primarily by MHC isoform expression (Gallo *et al.* 2004; Harridge 2007; Pette and Staron 2000), the release rate of calcium via RyR1 also has a significant impact on the rate of force development, *i.e.* half-rise time and twitch contraction time (reviewed in Reggiani and te Kronnie 2006). There are multiple instances of disease processes that are characterized by alterations in twitch contractions due to altered RyR expression without changes in MHC expression. For instance, chronic sepsis in rats led to a significant decrease in the half-rise time and time to peak force in the long digital extensor muscle with minimal changes in fiber type; this change was attributed to a 2.4-fold increase in

the expression of RyR1 mRNA (Rossignol *et al.* 2008). In cardiac muscle, which only expresses slow MHC isoforms, decreases in RyR expression and subsequent decreases in the speed of contraction and prolongation of the contraction cycle are also associated with diabetic cardiomyopathy (Netticadan *et al.* 2001; Reuter *et al.* 2008; Yaras *et al.* 2005). For example, a 37% decrease in protein levels of RyR in diabetic rat hearts led to a 74% decrease in the rate of rise of calcium in the myocyte and a 65% decrease in the speed of contraction (Choi *et al.* 2002). Therefore, the 52% decrease in RyR1 expression in hibernating bears is consistent with the previously documented increase in half-rise time.

The sequestration of calcium into the SR (*i.e.* the decay phase of the calcium transient) is achieved via the various isoforms of SERCA, and is the primary determinant of the rate of relaxation, or half-fall time, of contracting muscle (Hamalainen and Pette 1997; Inesi *et al.* 2008; Periasamy and Huke 2001; Schwaller *et al.* 1999). For example, short interfering RNA (siRNA) was used to down regulate SERCA2 expression in rodent cardiac myocytes. An approximate 30% slowing of the decay phase of the calcium transient was observed after incubation with siRNA for 2 days (Seth *et al.* 2004). In another study, rat cardiac myocytes exposed to phenylephrine demonstrated a reduction in SERCA2 protein levels with a concurrent 50% decrease in calcium transport activity (Prasad *et al.* 2007). It is likely, therefore, that the 20% decrease in SERCA expression we measured impacts the calcium kinetics in the myocyte, although perhaps not to the extent which could be statistically detected in the half-fall time with the small sample sizes used in previous bear studies.

We measured a 16% increase in CSQ in hibernating bear muscle. Because CSQ enhances the calcium storage ability of the SR (Beard *et al.* 2004; Damiani and Margreth 1994; Rios *et al.* 2006; Szegedi *et al.* 1999), we believe these data indicate an increased capacity of hibernating

SR to store calcium compared to summer. The role of CSQ is not limited to calcium storage, however; CSQ has a role as a luminal regulator of calcium release, primarily through interactions with RyR (Beard *et al.* 2002; Kasai *et al.* 1999; Ohkura *et al.* 1998; Szegedi *et al.* 1999). While this regulation is variable and complex, direct binding of CSQ to RyR appears to suppress RyR. Beard *et al.* (2002) demonstrated a ten-fold increase in the duration of channel open time upon dissociation of CSQ from RyR. Therefore, the 16% increase in CSQ documented in hibernating bears not only indicates enhanced storage of calcium by the SR, but potentially slows the release of calcium from the SR.

In this study, SERCA and RyR1 expression decreased by 20% and 52% respectively, from summer to winter. In hibernating ground squirrels, there is a 10% decrease in SERCA and a two-fold decrease in RyR1 at the end of hibernation (January-February) compared to the active period (June-July) (Malysheva *et al.* 2001a; Malysheva *et al.* 2001b). Another study in hibernating ground squirrels documented a 43% decrease in the maximal rate of calcium uptake along with a 17% decrease in the calcium storage capacity of the SR (Agostini *et al.* 1991). While rodents hibernate in a manner different from bears, they may experience some similar seasonal changes in calcium handling and perhaps share some common physiological features.

The changes in the calcium handling system of bears most likely leads to an overall slowing of calcium kinetics during the hibernation period (Figure 7), and potentially impacts cell signaling pathways regulating muscle hypertrophy, atrophy and phenotype expression. Transient and steady-state calcium levels are transduced by activation of mitogen-activated protein kinase (MAPK), calmodulin-dependent protein kinase (CaMK), and/or calcineurin (CN) (Bassel-Duby and Olson 2006). In particular, CN is a Ca^{2+} /calmodulin-dependent protein phosphatase involved in the regulation of slow muscle fiber genes, and is activated by sustained increases in

intracellular calcium levels (Costelli *et al.* 2007; Miyazaki *et al.* 2006; Semsarian *et al.* 1999). In addition, the levels of MAPK and CaMK control the expression of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) (Bassel-Duby and Olson 2006; Schiaffino *et al.* 2007). PGC-1 α enhances the expression of slow contractile proteins (Bassel-Duby and Olson 2006; Schiaffino *et al.* 2007) and inhibits the transcriptional activity of forkhead-related (FoxO) transcription factors, which in turn suppresses the expression of atrogin-1 and Muscle-specific RING finger 1 (MuRF1) (Sacheck *et al.* 2007; Sandri *et al.* 2006). Atrogin-1 and MuRF1 are two key ligases in the ubiquitin proteasome system and are upregulated during many types of pathologies inducing muscle atrophy (Bodine *et al.* 2001; Gomes *et al.* 2001). Therefore, slower calcium cycling during hibernation in bears could potentially lead to changes in these signaling pathways, and in part maintain muscle mass and the expression of slow MHC isoforms during winter.

The data recorded in this study are the first of its kind in bears. We believe that the calcium handling system within ursine skeletal muscle changes in a seasonal manner that explains the previous findings for slower twitches. These changes are clearly different from those encountered under prolonged atrophy conditions and are consistent with the assertion that bears are resistant to disuse muscle atrophy. It is important to note that other modifications of the calcium handling system of bears could occur during hibernation, such as alterations in the phosphorylative and oxidative states of RyR and SERCA, which will alter the single pump/channel activity. Additionally, the relative density of these proteins has yet to be determined and could influence this system. Experiments on isolated SR vesicles or with *in situ* calcium imaging could further elucidate the extent and nature of these additional changes.

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FIGURES

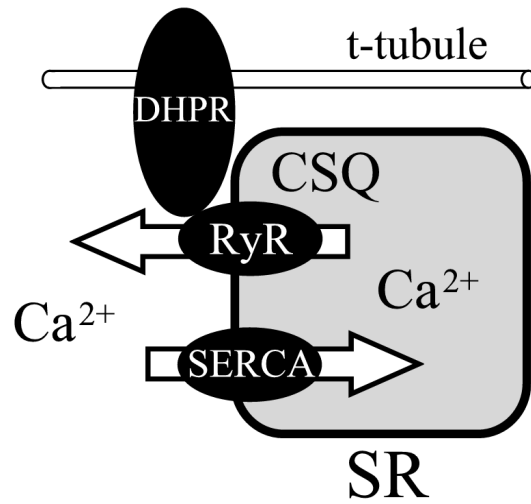


Figure 1. Overview of calcium cycling in the sarcoplasmic reticulum of the myocyte. DHPR = dihydropyridine receptor, RyR = ryanodine receptor, SERCA = sarco/endoplasmic reticulum calcium ATPase, CSQ = calsequestrin

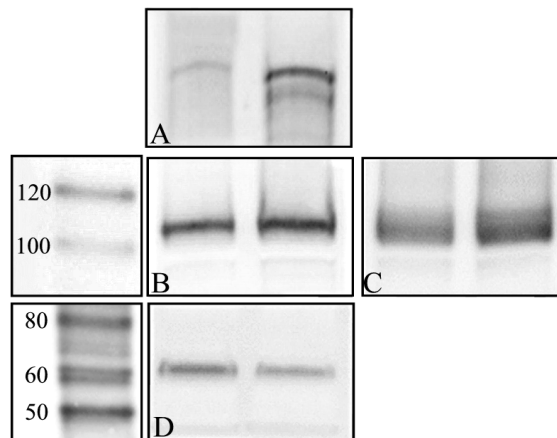


Figure 2. Representative Western blots of RyR1, and SERCA1 and 2 and CSQ with standards. The two panels in the left column are standards denoted by molecular weights in kDa. Panel A=RyR1, Panel B=SERCA1, Panel C=SERCA2, Panel D=CSQ; A-D, left lane = winter, right lane = summer.

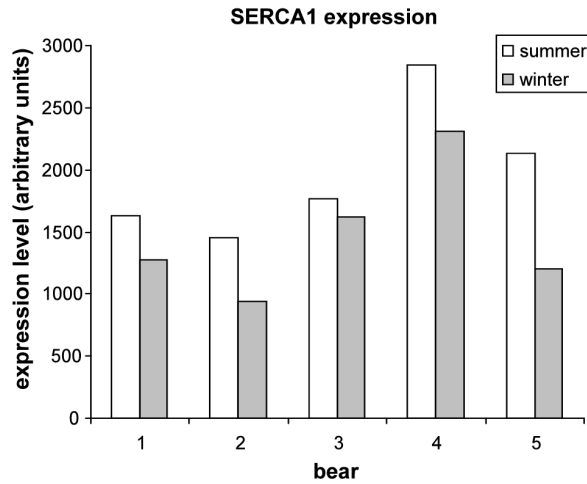


Figure 3. Relative expression of SERCA1 in summer and winter in five bears. Open bar = summer, shaded bar = winter; SERCA1 levels decreased on average by 25% ($P=0.043$).

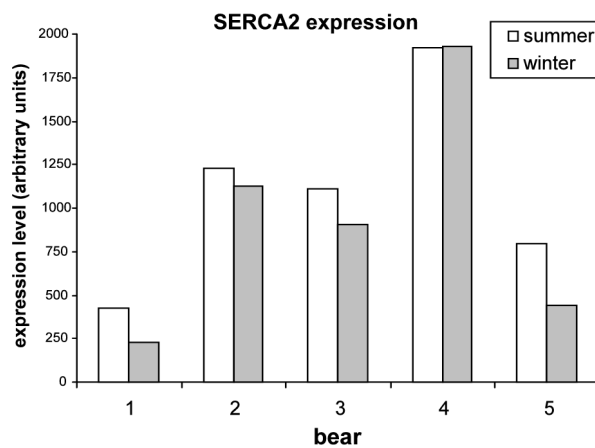


Figure 4. Relative expression of SERCA2 in summer and winter in five bears. Open bar = summer, shaded bar = winter; SERCA2 levels decreased on average by 15% ($P=0.080$).

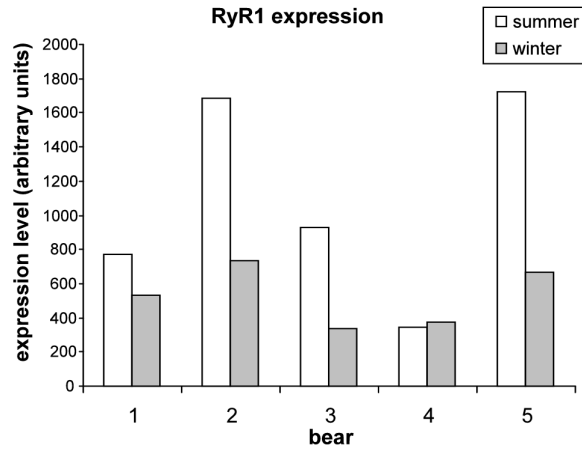


Figure 5. Relative expression of RyR1 in summer and winter in five bears. Open bar = summer, shaded bar = winter; RyR1 levels decreased on average by 52% ($P=0.080$).

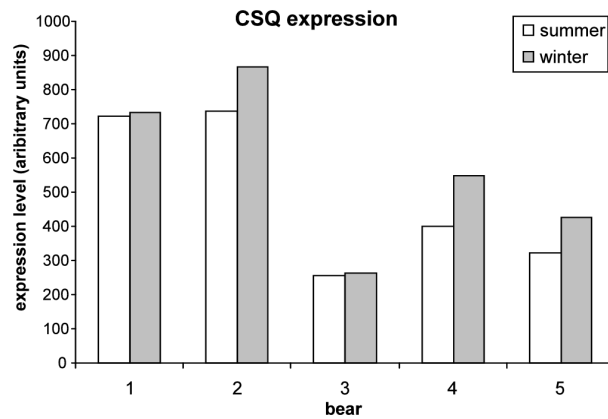


Figure 6. Relative expression of CSQ in summer and winter in five bears. CSQ levels increased on average by 16.4% ($P=0.043$). Open bar = summer, shaded bar = winter.

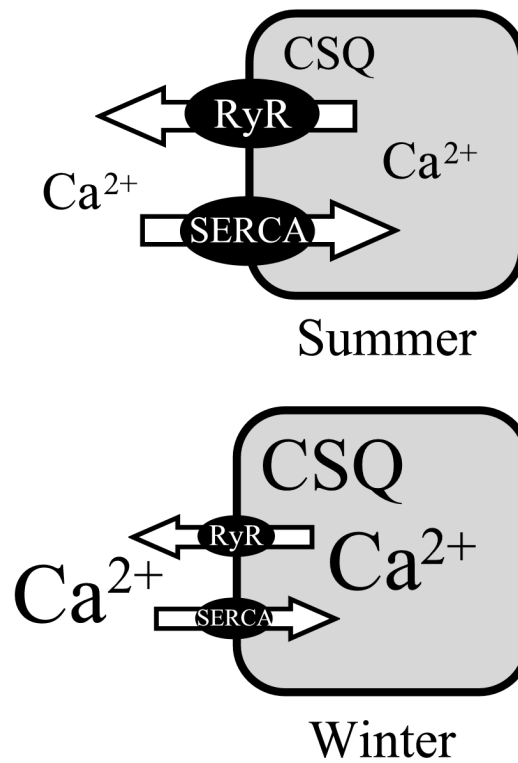


Figure 7. Summary of calcium system changes between summer and winter. Shaded area = SR (sarcoplasmic reticulum), inward arrow = SERCA1 and 2 (sarco/endoplasmic reticulum calcium ATPase), outward arrow = RyR1 (ryanodine 1 receptor), CSQ = calsequestrin. Size of font indicates relative amount of protein related to calcium release, uptake and storage.

CONCLUDING REMARKS

The research described in this dissertation is a first step toward understanding the complex physiology involved with the unusual phenomenon of resistance to disuse muscle atrophy observed in hibernating black and brown bears. While changes in the calcium handling system of the skeletal myocyte do not directly explain the effect observed, seasonal alterations in calcium likely impact the myriad of cell signaling pathways that are directly or indirectly regulated by calcium. Many of these pathways determine the muscle phenotype through regulation of the proteins related to the contractile apparatus of the myocyte. Continued research on the role of calcium in hibernation would first concretely establish that seasonal levels of calcium, be it release, uptake or resting cytosolic levels, are altered in bears. This could be accomplished by measuring the rates of uptake and release through various spectrophotometric techniques or calcium imaging. Once established, studies would examine the most logical pathways affected that impact muscle atrophy and hypertrophy, such as Muscle RING Finger 1 (MuRF1), a sarcomere-associated protein involved in the ubiquitin/proteasome pathway.

This work is only one potential mechanism that might explain resistance to muscle atrophy in bears. Other possibilities exist, such as altered neural input to the muscle or the ambiguous role of uncoupling proteins in hibernation; some of which are currently being investigated at Washington State University. The final solution is most likely going to be a combination of effects, starting at the myocyte level extending to higher systems. The legacy of this work will not only be the purely scientific contribution to the understanding of muscle physiology and the hibernation process, but also the application of the gained knowledge to medicine and humanity.