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Muscles Stretch: factors that influence the active lengthening of skeletal muscles

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Muscles Stretch: factors that influence the active lengthening of skeletal muscles

Dissertation

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

Emily M. Abbott

Dissertation Committee:
Assistant Professor Manny Azizi, Chair
Professor Matthew McHenry
Professor Vince Caiozzo

2017
DEDICATION

To

My parents and sister, for inspiring me to explore further

To move things is all that mankind can do ... for such the sole executant is muscle, whether in whispering a syllable or felling a forest

Charles Sherrington
Linacre Lecture, 1924
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Skeletal muscles not only accelerate our bodies during movement, they also play a crucial role in deceleration. During decelerating tasks, such as landing and braking, muscles are stretched while actively producing force in order to dissipate mechanical energy. One negative consequence of energy dissipation is that active lengthening can cause muscle damage. Muscles are dynamic and complex— their ability to vary architecture and timing of activation allows for incredible variation in mechanical function. The presence of in-series tendons magnifies this diversity of motor functions by 1) decoupling the length changes of the MTU from the length changes of the fascicle and 2) providing geographical space for fibers to attach to beyond bone. In this dissertation, we look at muscle-tendon factors that may affect active lengthening on the level of the whole muscle-tendon unit. These factors include timing of activation, muscle-tendon kinetics and morphology. Using in situ and in vitro muscle preparations, we found that early timing of activation is an important risk management strategy, rate of fascicle stretch is driven by relaxation rate of muscle and that pennation of fascicles may provide a protective advantage during high impact eccentric contractions.
INTRODUCTION

Locomotion is the hallmark of the animal kingdom and animals are generally characterized by their modes of movement. Whether swimming, running, jumping or flying, organisms generate the mechanical energy needed to move by the displacements and forces produced by their muscles.

Most of our knowledge about muscle mechanics arises from isolated muscle studies. We understand that muscles exert force by shortening, or contracting. The basic unit that performs this muscular contraction is the sarcomere, which is the microscopic arrangement of contractile proteins. From isolated muscle studies on the sarcomere level, we know that muscles produce force related to the amount of overlap between contractile proteins that slide past each other (Gordon et al., 1966). This finding is called the sliding filament theory and it is highly informative because it connects the structural organization of a sarcomere with a muscle’s force-producing function. However, muscles have many structural organizations beyond the sarcomere. Hence, we are beginning to realize that studying muscles in isolation provides us with a limited understanding of how muscles actually function in a moving organism.

One potential limitation in isolated muscle studies is that most locomotor muscles act in series with elastic elements. Collagen fibers form transparent sheaths that wrap various levels of muscle structures similar to how a sausage casing wraps around ground meat. These collagenous structures are contiguous with the tendon.
that ultimately connects muscle to bone (Fig. 1). In fact, while we colloquially refer to a “muscle” as a “muscle”, it is really a muscle-tendon unit (MTU). Any force produced by muscle fibers will also be applied to the elastic elements. Therefore, it is critical to understand the contractile properties of muscle within a realistic mechanical context. How do muscles and their series elastic elements interact?

Muscle-tendon interactions give rise to the diversity of locomotor modes seen in the animal kingdom. Throughout the evolutionary history of animals, sarcomeres have remained highly conserved, especially in vertebrates (Desjardins et al., 2002; McGuigan et al., 2004). Consequently, vertebrate muscles function with a similar set of physiological constraints. However, series elastic elements, such as tendons, allow animals to overcome constraints set by properties of the sarcomere. Specifically, muscle-tendon interactions have three distinct functions 1) amplify muscle power, 2) minimize energy consumption, and 3) dissipate energy to limit injury (Roberts and Azizi, 2010).

Animals dissipate energy when they decelerate. For terrestrial animals, deceleration occurs during braking, running downhill, maneuvering, and landing. Thus, energy-dissipating tasks are common and important for survival. These events stretch muscles to absorb impacts. Unfortunately for the muscle, active lengthening is generally associated with muscle damage (Lieber and Fridén, 1993). However, tendons may function as mechanical buffers to protect muscles from environmental perturbation and thus overcome the injury constraints set by the sarcomere.
By understanding the interaction between the contractile properties of muscles and the mechanical properties of tendons we can begin to understand how these interactions have shaped muscles specialized for diverse mechanical functions. In addition we can begin to predict the muscles or external conditions with the highest propensity for muscle injury. The basic research proposed may also provide important insight to biomedical questions. Changes in the mechanical properties of muscles and tendons are associated with a wide variety of neuromuscular disorders such as muscular dystrophies and cerebral palsy, or following stroke, spinal cord injury and aging. Therefore, understanding the factors that alter muscle-tendon interactions may inform rehabilitation strategies aimed at those suffering neuromuscular deficiencies.

**Summary of work**

This study intends to improve the understanding of the link between muscle and tendon behaviors at the level of the isolated muscle. Muscles are dynamic and complex- their ability to vary architecture, force, strain and timing of activation allows for incredible variation in mechanical function. The presence of in-series tendons magnifies this diversity of motor functions by 1) providing geographical space for fibers to attach to beyond bone and 2) decoupling the length changes of the MTU from the length changes of the fascicle. Mechanical performance of both elastic and motor properties is determined by measurement of force generation and change of length, or strain.

Chapter 1 asks, are there mechanical benefits to pre-impact activation? In vivo, pre-activation is observed before braking and landing events across a wide range of
taxa. Using an in vitro muscle-tendon preparation, we test the prediction that muscle activation in anticipation of lengthening can reduce the rate and magnitude of stretch applied directly to muscle fascicles. This hypothesis was not supported, we found that the magnitude and rate of stretch applied directly to the fascicles increased when the muscle was stimulated in advance of lengthening (early-activation). However, during trials using early activation, muscles were able to dissipate more mechanical energy without being stretched to longer lengths. It has been shown that with the same amount of strain, fibers that start at shorter operating lengths are less likely to get damaged than fibers at longer operating lengths (Macpherson et al., 1996). Our results indicate that early activation with in-series compliance, allows muscles to dissipate more energy without extending into dangerous zones of the length-tension curve. Thus, pre-impact activation is an important risk management strategy.

Chapter 2 asks, what determines the rate of stretch on the muscle fascicle? During active lengthening of a muscle-tendon unit, the rate of stretch of the fascicle occurs after relaxation of the muscle. We hypothesized that the rate of stretch of the fascicle is driven by the rate of stretch on the muscle and not the recoil rate of the tendon. Muscles relax when their sarcoplasmic reticulum reuptakes calcium ions (Ca$^{2+}$) by active transport. Therefore, like all enzymatic functions, muscle relaxation rates increase with raised temperature until a maximal limit (Stein et al., 1982). Thus we predicted that the rate of fascicle stretch would display temperature sensitivity. We characterized the rates of tendon recoil and muscle relaxation at three temperature perturbations with in vitro preparations of free tendons and
MTUs. We verified that tendon recoil is faster than the rate of MTU and fascicle stretch. We found that slow rates of force development can limit loading of elastic elements. And, we observed that faster relaxation rates apply a faster stretch to muscle fascicles during tendon recoil, therefore these muscles may be at a higher risk of injury. This study indicates that while some muscle-tendon interactions, like power amplification, are thermally robust, the power-attenuating function of MTUs is temperature dependent. Thus, environmental and dynamic muscle properties may limit an animals’ ability to land and brake.

Chapter 3 asks what muscle properties underpin high-force damage? Both pennation and slow type fibers may provide better resistance to eccentric contractions. Thus we used the natural variation in muscle fiber type and architecture in rat muscles to investigate the link between muscle parameters and injury. Soleus is a slow, paralleled fiber muscle (20% fast fibers, 8.2° pennation) while plantaris is a fast, pennate fiber muscle (95% fast fibers, 17.42° pennation). Muscle injury can be characterized as the loss of the force producing capability of muscle. We observed that muscles with pennate architecture lost less stress than soleus across all the three magnitudes of eccentric contractions. These results indicate that in high-impact active lengthenings, the architecture of the muscle provides more protection than the differing ultrastructure of fiber-types.
**Figure 1**
Skeletal muscles and their associated elastic elements are organized in a stereotyped hierarchy.

CHAPTER 1

Timing of muscle activation alters series elastic function

INTRODUCTION

Terrestrial animals pounce on prey, run down declines, negotiate uneven terrain, maneuver and ultimately come to full and complete stop. These diverse behaviors involve rapid deceleration where an animal reduces the kinetic and potential energy of its body mass. To perform a controlled deceleration, an animal must absorb, or dissipate, mechanical energy in its skeletal muscles. This is achieved by eccentric contractions in which skeletal muscles actively produce force during an imposed stretch. Unfortunately for skeletal muscles, eccentric contractions are often associated with muscle fascicle damage (Lieber and Fridén, 1993). Muscle damage from eccentric contraction elicits soreness and stiffness and also reduces range of motion and strength (Armstrong et al., 1983; Clarkson and Tremblay, 1988; Morgan and Allen, 1999; Newham et al., 1983). Since the eccentric contractions are a necessary and ubiquitous aspect of terrestrial locomotion, mechanisms that limit or mitigate muscle damage may be critical for retaining locomotor performance.

Most locomotor muscles act in series with elastic elements, such as tendons and aponeuroses. Tendons behave like biological springs and have the capacity to temporarily store elastic energy (Ker, 1981). During jumping, a tendon can act to amplify mechanical power by storing the energy of muscular work slowly and then quickly releasing it’s stored energy to propel the body (Aerts, 1998; Alexander and Vernon, 1975; Bobbert, 2001; Roberts et al., 2011). While the function of elastic
elements during acceleration is well described, the role elastic elements play during deceleration is less well understood. Specifically, what factors affect muscle-tendon interactions during eccentric contractions and how do they influence the effective utilization of tendons?

Previous studies have highlighted the basic mechanisms of muscle-tendon interaction during active lengthenings (Reviewed in Roberts and Konow 2013). Isolated studies of muscle-tendon units during eccentric contractions have shown that the presence of series elastic elements decouples the length changes of the muscle fascicles from that of the MTU (Griffiths, 1991; Roberts and Azizi, 2011). During these contractions a muscle will produce force and stretch the tendon to temporarily store elastic energy. When the muscle relaxes the tendon recoils and releases the stored elastic energy thereby stretching the muscle. By temporarily storing elastic energy, tendons can slow the rate of stretch applied directly to muscles (Konow et al., 2012; Roberts and Azizi, 2011a). The role of tendons as mechanical buffers has been extended to in vivo behaviors like landing where the presence of series elastic elements has been shown to reduce the rate of stretch applied directly to muscle fascicles (Konow et al. 2011). Thus, in-series elastic elements have a demonstrated power-attenuating function and consequently may act as mechanical buffers to protect muscles during energy dissipating behaviors.

In this study, we seek to understand how variation in motor control strategies might affect muscle-tendon interactions during an imposed lengthening. To date, studies investigating in situ muscle-tendon interactions during eccentric contractions have used a single motor control strategy: simultaneous muscle
stimulation and imposed stretch to the muscle-tendon unit (Azizi and Roberts, 2014; Roberts and Azizi, 2011a). However, researchers investigating the motor control strategies associated with landing in humans and other animals have observed anticipatory pre-landing EMG activity (Azizi and Abbott, 2013; Gillis et al., 2010a; Santello et al., 2001). It remains unclear how different motor control strategies affect muscle-tendon interactions during an eccentric contraction.

We used an in vitro muscle-tendon preparation to mimic different motor control strategies. The plantaris longus (PL) muscle (Dunlap, 1960), often referred to as the gastrocnemius, was used to characterize the behavior of the muscle tendon unit during eccentric contractions with varying patterns of activation. The PL is the primary ankle extensor in frogs and provides a good system for in vitro studies as it is well-studied and relatively large (Olson and Marsh, 1998; Roberts et al., 2011). The PL is also a good system for studying muscle-tendon interactions due to the extensive aponeuroses and Achilles tendon (Robertson and Sawicki 2015). We use this experimental model to test the prediction that muscle recruitment in anticipation of lengthening may allow for more effective utilization series elastic elements. Specifically, we predict that muscle activation prior to a stretch will reduce the rate of lengthening applied directly to muscle fascicles.

METHODS

Animals

Adult American bullfrogs, Rana catesbeiana, were obtained from Rana Ranch (Twin Falls, ID, USA) and housed at The University of California, Irvine. Average
body masses were 320.5 ± 52.3g. Animals were maintained in small groups, in 10gal semi-aquatic tanks (12L: 12D) at room temperature (20°C) and ambient humidity (35-54%). Twice a week, bullfrogs were fed large vitamin-enriched crickets *ad libitum* and received clean water. All animal procedures were approved by UC Irvine Institutional Animal Care and Use Committee.

**In vitro preparation**

Post-mortem, we carefully isolated 2cm of the sciatic nerve from the thigh muscles and gently removed the thin connective tissue surrounding the nerve with glass probes in Ringer’s solution (20°C). We then dissected the plantaris longus (PL) and its associated tendon. The distal tendon was detached at the plantar fascia and the proximal origin of the PL was left connected to the isolated knee joint. The average plantaris longus muscle mass was 3.87 ± 0.68g which corresponded to approximately 1.2% body weight.

To measure muscle fascicle length, a pair of 1mm round sonomicrometer crystals (Sonometrics, London, Ontario, Canada) were implanted along the proximal fascicle of the PL (Azizi and Roberts, 2010). To implant, a small slit (1mm wide, 3mm deep) is made with a sapphire blade between two fascicles. The first crystal was implanted on the superior dorsal side of the muscle, distal to the knee. The second crystal was similarly implanted between the same two fascicles at least 5mm distal to the first crystal. Great care is taken to avoid any surrounding connective tissues such as the fascia surrounding the knee joint or the extensive distal aponeuroses. To limit any motion artifact, both incisions in the fascia were closed with 6-0 silk sutures, which were reinforced with the smallest dabs of cyanoacrylate...
adhesive. Throughout the experiment, the quality of the signal was confirmed by monitoring an oscilloscope (ADS 1102, ATTEN Instruments, Shenzhen, China).

Muscle activation was accomplished by electrical stimulation of the isolated sciatic nerve. A custom-made bipolar electrode nerve cuff was placed directly on the sciatic nerve. This cuff was composed of two silver wires that were threaded across the width of a small piece of plastic tubing (4mm length, 1mm inner diameter). The sciatic nerve was threaded through the cuff so that the cuff rested proximal to the knee. Wire leads from the cuff were connected to a Grass S88D stimulator (Grass Technologies, Warwick, RI, USA).

After instrumentation, the muscle-tendon unit (MTU) was fastened within an adjustable, non-compliant frame in order to interface with the dual-mode muscle servomotor (310C, Aurora Scientific, Cambridge, MA, USA). The knee joint was secured in a clamp that was attached to a vertical manual stage (Thor Labs, Newton, NJ, USA). For the distal end, the tendon was threaded through a lightweight custom-fabricated clamp. This distal clamp was secured to the servomotor with thin, lightweight stainless steel cable. In vitro preparations were kept in an acrylic tank with cycling Ringer’s solution (concentrations in g l⁻¹, 6.78 NaCl, 0.09 KCl, 0.11 CaCl₂, 0.23 NaHCO₃, 2.0 dextrose; pH 7.4) and a regular supply of oxygen. We acquired data with Igor Pro software (Wavemetrics, Lake Oswego, OR, USA) at 1000Hz. Measurements from the servomotor and sonomicrometer were collected using a 16-bit data acquisition system (National Instruments USB-6212).
**Muscle property measurements**

For each experiment, we determined optimal settings for stimulation voltage and muscle length. We determined supramaximal voltage by increasing the voltage of isometric twitch contractions by one volt increments until force ceased to increased with increasing voltage (9-12V). We constructed a twitch force-length curve for each individual muscle using sonomicrometry for length and the servomotor for force. During this step, twitch contractions were preferred over tetanic contractions in order to minimize fatigue. Optimal muscle length ($L_0$), the fascicle length at which the muscle produces the greatest force, was assessed from the twitch length-force curve. All subsequent length parameters were applied relative to this $L_0$. Additionally, we performed one tetanic contraction at $L_0$ prior to the eccentric trials in order to determine the muscle’s maximum isometric force ($P_0$). We also performed a tetanic contraction at the end of the experiment to ensure that force did not decline significantly during the eccentric trials. Experiments where $P_0$ was reduced by more than 15% during course of the experiment were discarded.

**Active Lengthening**

To perform an eccentric contraction we imposed a stretch on an active muscle. Prior to all contractions, we adjusted the passive tension on the MTU such that the fascicle started at $1.17 \pm 0.02 \ L_0$. This passive tension was chosen so that there was no slack in the MTU. The duration of the imposed stretch was fixed at 100ms because our stimulation lasted 50ms and force persisted for approximately 30-50ms after the end of stimulation. In our experimental procedure, we stretched
the muscle tendon unit at a rate of 2 L₀/s and 1 L₀/s for 100ms with the servomotor. We referred to the 2 L₀/s stretch as a fast stretch and the -1 L₀/s as a slow stretch. However, both stretch speeds are conservative and early pilot data at stretch rates faster than 5 L₀/s resulted in significant force depreciation due to muscle damage.

This study is unique in that it varies the timing of stimulation relative to the initiation of the stretch. We stimulated the muscle for 50ms in three ways; one set of trials was stimulated 50ms before the stretch (early-stimulation), one set was simultaneously stimulated with stretch (simultaneous-stimulation) and one set was stimulated 50ms after initiation of the stretch (late-stimulation).

Data analysis and statistics
Data were analyzed in Igor Pro (Wavemetrics corp., OR, USA). To simplify analysis, each trial was divided into two phases; the shortening and the lengthening phase of the muscle fascicle. We measured fascicle velocity, change in length and absolute length for both phases. Tendon length changes were not measured directly but rather calculated as the difference between the MTU length and the fascicle length. We also calculated work done by the muscle during the shortening phase (positive) and work dissipated by the muscle during the lengthening phase (negative). The sum of both of these values equals net energy absorbed, work (Jkg⁻¹ muscle), for each trial.

The results from the two phases were statistically analyzed in R with two-way ANCOVA statistical models. Stretch rate, timing of activation and their interaction effect were the covariates and bullfrog individual was included as a random effect.
**RESULTS**

We performed *in vitro* eccentric contractions by stretching an activated muscle-tendon unit (MTU). In this study, we manipulated the timing of activation and the speed of the stretch. The three timing of activations are termed “early”, for muscle activation 50ms before the stretch, “simultaneous”, for muscle activation concurrent with the stretch, and “late”, for muscle activation 50ms after a stretch. We imposed two stretch speeds; “slow”, for 1 L₀/s, and “fast” for 2 L₀/s. We measured peak force output (P₀) of the plantaris longus, 35.33N ± 6.98 N, during isometric tetanus, and normalized our force measurements to this value. For the muscle fascicles, we measured 6.55±1.98mm (this only represents the segment being measured by sonomicrometry) as the average optimal length (L₀) and normalized fascicle length changes to this value.

Figure 1.1 illustrates representative contractions with three different activation timing. Regardless of the timing of activation or the velocity of MTU stretch we observed an initial period of fascicle shortening in all eccentric contractions. Although the fascicles shortened against the stretch of series elastic elements, we still refer to these contractions as eccentric because the MTU experienced net lengthening and energy absorption. We partitioned the data into two phases; the shortening phase and the lengthening phase of the muscle fascicle (Fig. 1.1).

During the shortening phase (Fig. 1.2), the velocity of fascicle shortening varied significantly with the timing of activation (p<0.001). Muscles with early activation shortened more quickly than muscles with late activation (Fig. 1.2a).
Consequently, muscles with early activation shortened more (Fig. 1.2b) and therefore had shorter fascicle lengths (Fig. 1.2c) before the beginning of the lengthening phase (p<0.001). The speed of the imposed stretch also affected the amount of fascicle shortening (Fig. 1.2b) in that fast stretches were associated with less shortening (p<0.01).

Typically, fascicles lengthened when the muscle was deactivating and the MTU was isometric after the imposed stretch. During the fascicle lengthening phase, we did not observe a significant effect of timing or MTU stretch velocity on the velocity of fascicle lengthening (Fig. 1.3a). However, timing of activation did affect the magnitude of lengthening (p<0.001). Fascicles with early activation experienced more lengthening (Fig 1.3b). Since the duration and speed of MTU stretch was controlled across activation trials, fascicle length at the end of the eccentric contraction was not affected by the timing of activation (Fig. 1.3c). However, stretch speed did affect the amount of fascicle lengthening because the imposed stretches had the same duration but different speeds: fast stretches had a larger net length change both at the level of the MTU and the fascicle. As a result, fascicle length at the end of the eccentric contraction was longer for the fast stretches (Fig 1.3c).

The force developed in an eccentric contraction was dependent on the timing of activation, the speed of stretch and the interaction effect of these two factors. Eccentric contractions that had early activation had more time to develop higher forces (Fig 1.4a). Also, more force was developed with the fast stretch as fascicle shortening velocity was reduced thereby increasing force due to the effects of the force-velocity relationship (Fig 1.4a). We observed a similar pattern for the energy
dissipated by the muscle over the course of an entire contraction (Fig 1.4b). The net negative work done by the fascicles during the contraction was highest in early timing of activation and in fast stretches (p<0.001). While timing of activation affected the length profile of muscle fascicles, it did not have an effect on the fascicle length at the end of the contraction (Fig. 1.3c). Because fast and slow ramp perturbations had the same duration, fast ramp speeds resulted in longer fascicle lengths (Fig. 1.3c).
DISCUSSION

Animals decelerate by activating muscles while muscles are stretched. This absorbs energy in the “negative” direction. In vivo, the magnitude of these decelerations can vary depending on the task; some tasks require more energy absorption than others. For terrestrial animals, an example of a small deceleration would be the “braking phase” during the first half of limb support when an animal exerts decelerating force in the direction of travel (Alexander, 1991; Dickinson et al., 2000). We see the braking phase across taxa despite differences of anatomy, posture, stride length, and duty factor including cockroaches (Full et al., 1991), lizards (Chen, 2006), birds (Roberts, 1997) and mammals (Gillis and Biewener, 2002; Hoyt et al., 2005). A larger form of deceleration is landing in which animals must absorb the potential energy that was stored when the animal was elevated above the ground as seen in toads (Gillis et al., 2010b), turkeys (Konow et al., 2012), cats (Prochazka et al., 1977), goats (Carroll et al., 2008), monkeys (Dyhre-Poulsen and Laursen, 1984) and humans (Santello and McDonagh, 1998). During deceleration, researchers generally verify that extensor muscles on one side of a joint, absorb energy and control stiffness of the joint, whereas the antagonists stabilize the joint and position the limb (Burkhart and Andrews, 2013; Hobara et al., 2007; Iida et al., 2011; Minetti, 1998; Yeadon et al., 2010).

In vivo, muscle instrumentation like electromyography (EMG) is a powerful tool to understand the complexity of motor control. To study muscle function, it is important to measure both activation and fascicle strain with sonomicrometry or ultrasound because estimating muscle strains from joint kinematics can often be
unreliable due to the compliance of series elastic elements (Biewener et al., 1998; Hoyt et al., 2005). While there are many EMG datasets few studies link motor control with muscle function by measuring in vivo fascicle length. For the studies that make this link, researchers observe that animals activate their muscles before decelerating events of all magnitudes in which muscles are actively stretchee. It may be tempting to hypothesize that similar motor patterns may be evolutionarily constrained, yet it is more probable that comparable motor patterns are due to similar solutions to facilitate the immediate functional demands of the activity (Herrel et al., 2008).

In cyclical locomotion, like walking or running, the magnitude of energy required to absorbed increases with speed or with slope decline. Within an individual, timing of activation occurs earlier, or what is referred to as an increase in phase advance. Hoyt et al describe that the correlation of phase advance and speed could be attributed to Taylor’s ‘economical force hypothesis’. The force hypothesis stipulates that as an animal increases speed, the stance time of each limb decreases. Thus, to produce the same net work (body mass times distance traveled) the muscles must produce more force in a shorter time (Taylor, 1994). This force hypothesis is supported by data that show increases in myoelectrical signal which are often correlated with the number of motor units recruited (Solomonow et al., 1990). Therefore, changes in timing of activation may be due to the need for more time to develop larger forces.

In singular decelerating motions, like landing, the magnitude and timing of muscle activation can vary with the amount of energy absorptions much like in
cyclical tasks. For example, goat elbow extensors activated significantly earlier and more intensely when animals land from a downward jump in comparison to an upward jump (Carroll et al., 2008). And, toad hopping shows “tuned” activation pattern in which extensor muscles activate earlier with hop length (Azizi and Abbott, 2013; Gillis et al., 2010a). However, when humans were dropped at different heights, the timing of landing appears consistent (Santello and McDonagh, 1998). A possibly reason for the discrepancy between toads and humans could be that the tasks depend on different sensory cues.

Because pre-activation is observed before decelerating events of various magnitudes and across a wide range of taxa, we hypothesized that pre-activation offers a mechanical benefit to animals. One potential mechanical benefit of pre-activation arises from the utilization of in-series tendons. Tendons are thought to play a protective role during decelerating events because tendons slow the rate of lengthening of muscle fascicle compared to the rate of lengthening of the muscle-tendon unit (Roberts and Konow, 2013). Since animals activate their muscles and thus stretch their tendons before a landing, we wondered if early activation provided a mechanical benefit regarding the use of energy storage in tendons. We hypothesized that during pre-activation if force develops prior to MTU stretch, then the muscle fibers will resist a stretch more. Thus, after pre-activation, muscle fascicles would be stretched at a slower rate compared to other motor control strategies. To test our hypothesis, we used an in vitro experimental method that would allow us to recreate different motor control strategies and measure fascicle strains.
In this experiment, we changed the timing of activation during active lengthening of an isolated frog plantaris muscle tendon unit (MTU). We activated the muscle 50ms before, simultaneously and 50ms after an applied stretch. We analyzed our data in two phases. First, there was a shortening phase where the muscle fascicle shortened against the elastic element. Energy is stored in the elastic element until the muscle begins to relax. This phase is followed by a lengthening phase in which the recoil of the tendon stretches the muscle fascicle. Analyzing our data in two phases, fascicle shortening and fascicle lengthening, allowed us to better understand how motor control strategies affect the mechanics of braking.

In vitro muscle studies provide us a cleaner, though limited, way to understand the link between timing in motor control and muscle function. For example, in vivo data have a lot of individual variability. Ruiter et al, demonstrated that humans have significant variability between maximal contractions between voluntary control and electronically activated knee extension (de Ruiter et al., 2004). In our methods, we use supramaximal activation which diminishes variability in the rate of force development. In vitro experimentation also cuts out motor control complications since in vivo muscle activation patterns can change if there is planned subsequent activity (Ambegaonkar et al., 2011). Additionally, in vitro methods allow us to measure the amount of energy input by the system (servomotor length) and output by the muscle (sonomicrometry).

Under the conditions of our experiment, our hypothesis was supported during the shortening phase but not during the lengthening phase. During the shortening phase, we were correct that the early activation allowed the muscle fiber
to shorten more and thus produce more force (Fig. 1.4a). During the lengthening phase, however, our hypothesis was not supported. The early timing of activation did not reduce the lengthening velocity of the fascicle. In fact, we observed that during the early timing of activation fascicles lengthened more quickly. Our hypothesis was not supported during the lengthening phase due to several challenges presented by experimentally controlling muscle.

We acknowledge that changing the timing of activation presents several challenges and limitations. First, muscle physiology experiments are often complicated by nonlinear properties and co-varying factors. We controlled for temperature, the amount lengthened, motor units, fascicle optimal length and the lengthening speed relative to optimal length. Regarding co-varying factors, any changes in fiber length will change the maximal force due to the length-tension relationship. And, any changes in force will change the rate of fiber strain rate due to the force-velocity relationship. This highlights that, even in a controlled setting, changes in the timing of activation have many down stream effects on biomechanical measurements.

Because we changed the timing of activation, the duration of the shortening and lengthening phases was not consistent across treatments. Early activation treatments had longer shortening periods than simultaneous and late activation treatments. If a muscle has a longer shortening duration, then it produces more work- stretching the tendon more and storing more elastic energy. Thus, while the ramp stretch inputted a nearly consistent amount of energy, the total work varied across treatments. Second, the amount the muscle shortening during the initial
phase determines the speed of recoil of the tendon. Due to the non-linear elasticity of tendons, a tendon that is barely stretched recoils slowly while a tendon stretched a lot recoils quickly. Early timing of activation means that muscles have more time to do work and store energy into the tendon (energy stored). Therefore, even though the applied MTU stretch is held constant across trials, (energy input), the energy absorbed by the fiber (total energy) is more during early activation trials.

It would be very difficult to control for total energy input to the system. To do so, we require a feedback controller system that stops the stretch applied to an MTU based on instantaneous total energy. This hypothetical experimental system necessitates instantaneous readings of both sonomicrometry data and servomotor data with a servomotor length output. With our current experimental set up, we are unable to do this. Computer modeling is another experimental approach that could avoid these limitations. Unfortunately, computer models tend to do poorly when muscles work eccentrically. This is because the Hill model does not explain the eccentric force-velocity relationship well. Despite limitations, our experiment allowed us to make unexpected conclusions.

Under limited conditions, we saw that motor control strategies may not decrease the rate of strain of the fiber. Therefore, timing of activation may not change the effectiveness of power attenuation by the tendon. However, our results are interesting in the context of the length-tension curve. We saw that for the same amount of MTU stretch, fascicles in pre-activation perturbations were able to dissipate more energy (Fig. 1.4b) while ending at the same length on the length tension curve (Fig. 1.5). These results have important implications regarding muscle
damage. First, it has been shown that with the same amount of strain, fibers that start at shorter operating lengths are less likely to get damaged than fibers at longer operating lengths (Macpherson et al., 1996). Second, fascicles that shorten onto the ascending limb of the length tension curve may be able to resist a stretch more as the strain stretches the fascicles onto the plateau. Third, the magnitude of strain also corresponds to the likelihood of damage (Lieber and Fridén, 1993). Therefore, under conditions where the amount of strain is constant, an early timing of magnitude would place fascicles at safer initial lengths. While our expected mechanical advantage of early activation was not supported, we did measure a mechanical benefit of early activation—muscles activated early absorb more energy without being stretched to longer lengths.

In situ, pre-activation may provide greater benefits than what we saw in vitro. In vivo, pre-activation is nearly ubiquitous. Animals encounter a variety of fascicle strains where small strains are seen in small decelerations and large strains are seen in big decelerating tasks. In both instances muscles are preactivated. In fact, instances of energy dissipating events where animals do not pre-activate their muscles require extreme experimental manipulation. For example, Watts did not see preactivation in cat falling after. In a complex environment, ground perturbations are often unpredictable. Animals may miscalculate jump distances or navigate uneven substrate and consequently experience unexpected fascicle strains. Therefore, if early activation allows animals to dissipate more energy but not experience dangerous fascicle lengths, then pre activation is an important risk management strategy.
In summary, early activation allows greater force development, therefore a muscle may resist an applied stretch better. However, under our experimental conditions this did not lessen the velocity of fascicle lengthening. Therefore, motor control strategies may not necessarily reduce injury via decreased fascicle velocity. Yet, our findings do show that early timing of activation is a risk management strategy that allows animals to function safely in unpredictable environments.
**Figure 1.1 Representative Active Lengthening Contractions**

Representative data for fast eccentric contractions with early (a), simultaneous (b), and late (c) timing of activation. The top panels show the force developed by the muscle-tendon unit (MTU), normalized to peak isometric tetanic force (Po). The bottom panels show the change in length of the MTU (solid black line), change in length of the muscle fascicle (solid gray line) and change in length of the tendon (dashed gray line). The dark solid bars between the panels indicate the 50ms period of stimulation. Eccentric contractions were analyzed in two phases. The first phase was designated as the period of fascicle shortening (solid background color) while the second phase was the period of fascicle lengthening (hatched background).
**Figure 1.2 Summary Data for Fascicle Shortening**

Summary data for the fascicle shortening phase of the eccentric contractions. All data were normalized to optimal fascicle length (Lo). (a) Velocity of shortening varied significantly by timing of activation (t.o.a.; $p<0.001$): the later the timing of activation, the slower the velocity of fascicle shortening. (b) Total amount of fascicle shortening significantly varied with timing of activation (t.o.a.; $p<0.001$) and the speed of stretch ($V_{mtu}$; $p<0.01$) applied during the eccentric contraction: total amount of shortening decreased as the timing of activation advanced and at faster...
stretch speeds. (c) At the end of the shortening phase, the fascicle length significantly varied with the timing of activation (p<0.001): the later the stimulus, the longer the fascicle length. This trend is most likely explained by the velocity of fascicle shortening (a) which determined the total amount of fascicle shortening (b). Error bars are the S.E.M.
Figure 1.3 Summary Data for Fascicle Lengthening

Summary data for the fascicle lengthening phase of the eccentric contractions.

All data were normalized to optimal fascicle length (Lo). (a) Velocity of lengthening did show any significant trends. (b) The total amount of fascicle lengthening significantly varied with timing of activation (t.o.a.; p<0.001): as timing of activation advanced, the amount of total fascicle lengthening decreased. (c) The fascicle length at the end of the lengthening period varied with the speed of stretch (Vmtu; p<0.001). This is because each eccentric stretch was applied for the same amount of
time but at different speeds. Thus, a fast stretch imposes larger length changes on the MTU than a slow stretch. Error bars are the S.E.M.

![Chart](chart.png)

**Figure 1.4 Active Lengthening Force and Work**

Summary force and work data during the contractions. (a) Eccentric contractions with fast stretch speeds generally developed higher peak forces (Vmtu; p<0.001) while eccentric contractions with late activation developed the lowest peak forces (t.o.a; p<0.001). (b) The net amount of mechanical work dissipated by fascicles at the end of the contraction also varied with the timing of activation (t.o.a; p<0.001), stretch speed (Vmtu; p<0.001) and the interaction of these two factors (p<0.01). Eccentric contractions with fast stretch speeds generally dissipated more energy since the MTU was stretched to longer lengths. Also, the later the timing of activation, the less energy dissipated by the fascicle. Error bars are the S.E.M.
**Figure 1.5 Timing of Activation and Operating Lengths**

Summary operating lengths, normalized to optimal fascicle length (Lo). The fascicle lengths during the eccentric contractions are plotted on a representative force-length curve of a muscle. Arrows correspond to length changes during early (green), simultaneous (blue), and late (orange) timing of activations. Arrows only correspond to the length axis (x-axis). Arrows with a solid color represent the fascicle shortening phase of the eccentric contraction while arrows with a hatched pattern represent the fascicle lengthening phase of the eccentric contractions. All arrows have a SE of +/- 0.02Lo. All fascicles, for a given stretch speed, started and ended at the same relative lengths. However, early activation resulted in greater shortening during force development prior to the eventual stretch during deactivation. Thus, early activation experienced more length change, higher forces, and more work dissipated while reaching the same final length.
CHAPTER 2:

Muscle relaxation rate alters the rate of fascicle lengthening

INTRODUCTION

When we imagine impressive locomotion, we might envision a cheetah sprinting. It is renown for its speed and consequent hunting prowess. Components of this cheetah’s body, skeletal muscles and their associated elastic elements, power movements like running and leaping to catch prey. Yet, producing motion is only half the story. On land, animals are subject to gravity- that which rises falls. Ideally, that fall is either cushioned by a forgiving substrate, such as a frog diving into protective waters, or cushioned internally by the musculoskeletal components of the animal, such as a show horse clearing a fence and landing on it’s forelimbs. Thus, while we acknowledge a cheetah for its speed, an animal’s ability to slow down and stop is an important aspect of locomotion that gains far less attention in the research literature.

Limbs often act like shock absorbers in decelerating events like landing, decline running or braking. An exception to this are the most primitive living frogs in the genus *Ascaphus*. These frogs live their lives on the edges of riverbanks and cannot manage better than a “belly-flop” even onto discouragingly hard surfaces (Essner et al., 2010). Thus, developing the mechanics for a controlled terrestrial landing may be advantageous for species radiation into new terrestrial niches (Reilly et al., 2016). Thus, deceleration is functionally common and beneficial for successfully living on land.
It is important to define the mechanisms underpinning the interactions between muscles and tendons that decelerate a body mass. Until the early 1970’s, researchers thought tendons primarily transfer force from muscles to the skeleton. However, more mechanical functions of tendons were discovered with the descriptions of “spring-like” properties (Ker, 1981). As a biological spring, tendons can store and release energy at different rates than what muscles can produce (Alexander and Bennet-Clark, 1977). The three major muscle-tendon unit (MTU) interactions that have been described are 1) amplify muscle power 2) minimize energy consumption and 3) limit injury susceptibility (Roberts and Azizi, 2011b). Decelerating tasks tend to lengthen active muscles so that they absorb energy and act like brakes (Dickinson et al., 2000). If the rate and magnitude of the applied stretch is high enough, the muscle may be damaged (McCully and Faulkner, 1985; Proske and Morgan, 2001).

The presence of in-series tendons is thought to limit the risk of stretch-induced injury. When an animal decelerates muscles must ultimately be stretched to dissipate mechanical energy and slow the body. Before impact, an extensor muscle activates to brace the joint against impact; this contraction produces force and stretches the tendon and temporarily stores elastic energy. As the muscle relaxes, the tendon recoils and releases the stored elastic energy thereby stretching the muscle (Konow et al., 2012). This mechanism, called “power attenuation”, is thought to reduce the rate of stretch directly applied to a muscle fiber and may function to protect the muscle from stretch-induced damage (Konow et al., 2012).
Consequently, tendons may function as mechanical buffers to protect muscles from environmental perturbations.

Because muscle relaxation precedes tendon recoil, we hypothesized that relaxation rate of the muscle could predict the active lengthening of a fascicle. Our null hypothesis is if the muscle can drop force instantaneously then the rate of tendon recoil is solely determined by the tendon’s mechanical properties such as the length of stretch or stiffness of the. Our alternative hypothesis is that the relaxation rate of the muscle drives the recoil of the tendon. We will test our main hypothesis by investigating whether factors that affect muscle relaxation also affect the rate of stretch imposed on the fascicle. Specifically, we predict that muscles that have faster relaxation rates will experience faster fascicle stretch rates.

Muscles relax when Ca\(^{2+}\) disassociates from cross-bridges and is sequestered into the sarcoplasmic reticulum (SR) by active transport. It is possible to change the concentration of SR-Ca\(^{2+}\) pumps and thus the relaxation rate of whole muscles with changing fiber type composition (Rome and Klimov, 2000), or unloading conditions (Schulte et al., 1993). Fast muscles may also have a Ca\(^{2+}\) binding protein, parvalbumin, that shuttle the calcium ions to the SR more quickly (Hou et al., 1992). Relaxation rate may also increase with temperature due to the thermodynamics of biochemical reactions, like Ca\(^{2+}\) dissociation or sequestration (Hou et al., 1992; Stein et al., 1982). Muscle contractile properties are sensitive to changes in temperature (Bennett, 1985). Thus, movements powered by muscle are similarly susceptible to temperature changes, as seen in a wide range of ectotherms (Bennett, 1990). It is unclear if the “shock absorber” behavior of muscle-tendon interactions display
thermal dependence.

We chose temperature treatments because it is easier to change temperature rather than modify the molecular composition of a biological tissue. Also, the thermal effects on muscle are well described in the literature. In cold muscles, we predict to see lower rates of force generation (Herrel et al., 2007; Wilson et al., 2000) less fascicle shortening (Coughlin et al., 1996; Hill, 1938; Johnston and Gleeson, 1984), and consequently lower power output (Herrel et al., 2007; James et al., 2012; Ranatunga, 1998; Renaud and Stevens, 1983; Swoap et al., 1993)

Maximum force production is thought to be independent of temperature as it is a function of the physiological cross sectional area (PCSA). However, some studies do report lower force production in cold muscles (James et al., 2012; Rall and Woledge, 1990). Regardless of the value of P0, if the rate of force development is limited, activities that must happen in a fixed amount of time, such as the braking phase of running, will likely be greatly affected by muscle kinetics.

We also aim to measure the recoil rate of tendon. Historically, tendon material tests measure the tension developed to resist a given strain and strain rate (CITE). These studies help define the proportion of energy lost during cyclically loads. For example, tendon is thought to lose 10% (Ker, 1981). However, these tests do not define the limits of tendon recoil speed. Therefore, for a given MTU stretch, it is unclear whether it is the recoil rate of the tendon or the compressive properties of the muscle that attenuate the rate of energy dissipation. We predict that the temperature treatments will not affect the tendon dynamics.
METHODS

Animals

Adult American bullfrogs, *Rana catesbeiana*, were obtained from Rana Ranch (Twin Falls, ID, USA) and housed at The University of California, Irvine. Average body masses were 84.71 ± 28.64g (n=8). Animals were maintained in small groups, in 10gal semi-aquatic tanks (12L: 12D) at room temperature (20°C) and ambient humidity (35-54%). Twice a week, bullfrogs were fed large vitamin-enriched crickets *ad libitum* and received clean water. All animal procedures were approved by UC Irvine Institutional Animal Care and Use Committee #2011-3008-1.

In vitro preparation

Post-mortem, we carefully isolated 2cm of the sciatic nerve from the thigh muscles and gently removed the epineurium with Teflon covered tweezers and glass probes in Ringer’s solution (20°C). Next we dissected the plantaris longus (PL) and its associated tendon. The distal tendon was detached at the plantar fascia and the proximal origin of the PL was left connected to the isolated knee joint. The average plantaris longus muscle mass was 1.31 ± .55g which corresponded to approximately 1.54% body weight.

To measure muscle fascicle length, a pair of 1mm round sonomicrometer crystals (Sonometrics, London, Ontario, Canada) were implanted in the PL (Azizi and Roberts, 2010). To implant, a small slit (1mm wide, 3mm deep) is made with a sapphire blade between two fascicles. The first crystal was implanted on the superior dorsal side of the muscle, distal to the knee. The second crystal was similarly implanted between the same two fascicles at least 5mm distal to the first
crystal. Great care is taken to avoid any surrounding connective tissues such as the fascia surrounding the knee joint or the extensive distal aponeuroses. To limit any motion artifact, both incisions in the fascia were closed with 6-0 silk sutures, which were reinforced with the smallest dabs of super glue. Throughout the experiment, we monitored an oscilloscope (ADS 1102, ATTEN Instruments, Shenzhen, China) to confirm that the sonomicrometry crystals were generating a robust signal.

We used direct electrical stimulation on the isolated sciatic nerve to activate the muscle with a custom-made bipolar electrode nerve cuff. This cuff was composed of two silver wires that were threaded across the width of a small piece of plastic tubing (4mm length, 1mm inner diameter). The sciatic nerve was threaded through the cuff so that the cuff rested proximal to the knee. Wire leads from the cuff were connected to a Grass S88D stimulator (Grass Technologies, Warwick, RI, USA).

After instrumentation, the muscle-tendon unit (MTU) was fastened within an adjustable, non-compliant frame in order to interface with the dual-mode 50N servomotor (310C, Aurora Scientific, Cambridge, MA, USA). The knee joint was secured in a clamp that was attached to a vertical manual stage (Thor Labs, Newton, NJ, USA). For the distal end, the tendon was threaded through a lightweight custom-fabricated clamp. This distal clamp was secured to the servomotor with thin, lightweight stainless steel cable. In vitro preparations were kept in an acrylic tank with cycling Ringer’s solution (concentrations in g l⁻¹, 6.78 NaCl, 0.09 KCl, 0.11 CaCl₂, 0.23 NaHCO₃, 2.0 dextrose; pH 7.4) and a regular supply of oxygen.
Muscle property measurements

We acquired data with Igor Pro software (Wavemetrics, Lake Oswego, OR, USA) at 1000Hz. Our servomotor collected length, velocity and force signals through a 16-bit data acquisition system (USB-6212, National Instruments Corporation, Austin, TX, USA). For each experiment, we determined optimal settings for stimulation voltage and muscle length. We gauged supramaximal voltage by increasing the voltage of isometric twitch contractions by one volt. When the twitch force ceased to increase, we kept this stimulation voltage (5-7V) for all subsequent muscle contractions. We then constructed a twitch length-force curve for each individual muscle from sonomicrometry measurements. During this step, twitch contractions were preferred over tetanic contractions in order to minimize fatigue. Optimal muscle length \((L_0)\), the fascicle length at which the muscle produces the greatest force, was assessed from the twitch length-force curve. All subsequent length parameters were applied relative to this \(L_0\). Additionally, we performed one tetanic contraction at \(L_0\) prior to the eccentric trials in order to determine the muscle’s maximum isometric force \((P_0)\). We also performed a tetanic contraction at the end of the experiment to ensure that force did not decline significantly during the eccentric trials. Experiments where \(P_0\) was reduced by more than 15% during course of the experiment were discarded.

Muscle Active Lengthening

To replicate a decelerating event we imposed a controlled stretch on an active muscle. Prior to all contractions, we adjusted the passive tension on the MTU such that the fascicle started at 1.2 \(L_0\). This passive tension was chosen so that there
was not any slack in the MTU and, due to in-series compliance, the fascicle could shorten onto the plateau of the length-tension curve. The duration of the imposed stretch was fixed at 100ms because our stimulation lasted 50ms and force persisted for approximately 30-50ms after the end of stimulation. In our experimental procedure, we stretched the muscle tendon unit at a rate of -2 L₀/s for 100ms with the servomotor. However, this stretch speed is considered conservative because early pilot data at stretch rates faster than -5 L₀/s resulted in significant force depreciation due to muscle damage.

**Temperature control**

This study varies the temperature of the muscle during active MTU measurements. We set three temperature 10, 20 and 30°C. Each trial started at 20°C but the subsequent trials were randomized with a coin flip. We controlled the temperature of the ectothermic muscle by setting the temperature of a digital water bath (A&C series, Anova, Stafford, TX, USA). The water bath circulated temperature controlled water through a brazed plate heat exchanger. Likewise, a water pump (Masterflex L/S ™, Cole-Parmer, Vernon Hills, IL, USA) circulated the Ringers solution by through the same heat exchanger. The water and Ringers solution equilibrated in temperature without mixing. Temperature of the Ringers solution was confirmed with a digital thermometer (model HH23, Omega Engineering Inc., Stamford, CT, USA) that was placed in the acrylic in vitro tank. After 5 minutes of equilibrating we assumed that the temperature of the muscle was the same as the temperature of the Ringers solution. This was confirmed with pilot studies where a small temperature probe of the digital thermometer was placed in the muscle.
During our experiments though, we did not place the probe in the muscle.

**Muscle data analysis and statistics**

Data were analyzed in Igor Pro (WaveMetrics, Portland, OR, USA). To simplify analysis, each trial was divided into two phases; the shortening and the lengthening phase of the muscle fascicle. We measured fascicle velocity, change in length and absolute length for both phases. Tendon length changes were not measured directly but rather calculated as the difference between the motor lever length and the fascicle length. We also calculated work done by the muscle during the shortening phase (positive) and work absorbed by the muscle during the lengthening phase (negative). The sum of both of these values equals net energy absorbed, work (Jkg⁻¹ muscle), for each trial.

The results from the two phases were statistically analyzed in R with one-way ANCOVA statistical models, with a variable as a function of temperature with individual as a random effect.

**Tendon preparation**

After the in vitro muscle preparations, we isolated the tendon tissue from the plantaris muscle-tendon unit. To do this, we scraped the muscle tissue of the plantaris away with a sharp #10 blade leaving only tendon tissue. This tendon tissue was then cut to into a rectangle approximately 20mm long by 8mm wide. The top of the tendon sample was wiped clean with a cotton tip applicator and we placed a fine line of super glue along the edge to prevent tearing. We designed a custom tendon hook out of two #000 insect pins bent at the tips at 90* fixed with a drop of 5 minute
epoxy to a thin non-elastic Kevlar fiber. These hooks punctured the distal 2mm of the tendon and a thin strip of superglue was allowed to dry in this section to distribute tension. The base of the tendon was fastened in a C-clamp (CC-1, Siskyou, Grants Pass, OR) lined with sandpaper fixed into a custom acrylic tank with anuran Ringer’s solution. A scale bar was placed in line with the tendon with a piece of dental wax. The hooks for each tendon were tied to the lever arm of a 1N servomotor (Aurora Scientific, Cambridge, MA, USA).

**Tendon mechanical testing**

Resting tendon length ($L_r$) was defined as the length of the tendon just prior to producing any resistive force. We stretched each tendon to $1.025\, L_r$ and $1.05\, L_r$ or, 2.5 and 5% strain over a constant 30ms. Then, with a novel experimental set up, we allowed the lever arm of the servomotor to release and thus induce recoil of the tendon. Tendon length was tracked with a high-speed camera (Phantom, Vision Research, NJ, USA) at 1000 frames per second. Video data were synced with force data by an electrical post-trigger signal. Each tendon’s recoil was measured three times at 10, 20 and 30°C. Average length (between clamp and hook fixture) of the free tendons was $10.25\pm0.35$mm. Stiffness was characterized by quantifying the slope of the curve stretch prior to tendon recoil and normalizing for the shape of each tendon.
RESULTS

In this study, we aimed to change the relaxation rate of the muscle through temperature perturbations. The three temperatures are 10, 20 and 30°C. We measured peak twitch force output (P₀) of the plantaris longus, at 10, 20 and 30°C as 3.95N±1.35, 5.41N±0.66, 4.47N±1.21 respectively. We normalized our force measurements to the maximum force at each temperature. We characterized the length-tension curve of each muscle and measured an average optimal length (L₀) of 6.22±1.07mm. This measurement only represents the segment being measured by sonomicrometry. We used L₀ to normalized fascicle length changes. However, to apply consistent length changes to the entire MTU, we applied length changes that were calculated by the measured fascicle length from the aponeuroses to the proximal MTU head. Initial lengths of each muscle was set to 1.2L₀ so that the muscles would shorten onto the plateau of the force.

First, we characterized muscle kinetics at different temperatures (Fig. 2.1) by measuring half-rise and half-relaxation times. Half-rise time is the time for the muscle contraction to develop to half of its peak tension at 1.2L₀ (Azizi and Roberts, 2010). Half-relaxation time is the time for the force to decay to half of its peak value. We use half relaxation time because the tail of the force trace often creates ambiguity about when the muscle has returned to its initial state. Muscle twitches at 30°C reached half of their maximum force more quickly than muscles at 20°C and 10°C for both half-rise and half-relaxation times (Fig 2.1b,c). We calculated the average force decay rate during the parts of the force trace that had consistent slopes (Fig. 2.2). Warm muscles have faster relaxation rates than colder muscles.
We performed *in vitro* active lengthening contractions by stretching an activated muscle-tendon unit (MTU) at our three temperature treatments (Fig. 2.3). Figure 2.3a & 2.3b illustrate representative active lengthening contractions at three different temperatures. We analyzed the data into two phases; the shortening phase and the lengthening phase of the muscle fascicle. During the shortening phase (Fig. 2.3c) the amount of shortening increases from 10-20°C and then decreases at 30°C. During the fascicle lengthening phase, the rate of stretch on the fascicle increases.

This study also investigated tendon mechanical properties. Figure 2.4 portrays representative tendon recoils at three different temperatures. With the servomotor, we imposed two strain magnitudes on the tendon. 2.5% or 5% \( L_r \) (Fig. 2.4a) then released the tendon which recoiled back to \( L_r \). Digitized video data measured the stretch and recoil of each trial (Fig. 2.4b). Tendon recoil rate was affected by the strain of the tendon. Tendons that were stretched to 5% of their resting length recoiled faster than tendons that were stretched half that amount. This pattern is observed in both average (Fig. 2.4c) and maximal recoil speeds (Fig. 2.4d). We saw that tendons at warm temperatures tend to resist strains less (Fig. 2.5).
**DISCUSSION**

We used a temperature perturbation protocol in an *in vitro* muscle-tendon unit (MTU) preparation to relate the rates of muscle relaxation and fascicle stretch during lengthening contractions. We measured muscle length and force in the plantaris muscles of bullfrogs (n=8) while applying controlled stretch to the MTU. We also performed tendon mechanical tests to better relate muscle and tendon properties. First, we confirmed that the rate of force development and relaxation increased with increasing temperature. The MTU was then actively lengthened at the various temperatures to quantify the rate of fascicle stretch. In support of our hypothesis, we found that the rate of fascicle stretch correlated positively with rising temperature during lengthening contractions. Tendon recoil rates did not appear to change significantly with temperature. However, stiffness of the tendons decreases from 20-30°C.

**Mechanism of muscle-tendon shock absorption**

During an active lengthening event, like landing or the braking phase of a stride, a joint extensor muscle activates before impact. This early onset activation is seen across taxa in toads (Gillis et al., 2010b), turkeys (Konow et al., 2012), cats (Prochazka et al., 1977), goats (Carroll et al., 2008), monkeys (Dyhre-Poulsen and Laursen, 1984) and humans (Santello and McDonagh, 1998). This contraction shortens fascicle fibers against an elastic element while the entire MTU is lengthened during impact. When the muscle relaxes the tendon recoils and releases energy at a slower rate than the impact. If the dynamics of a muscle or tendon change, this may affect the “shock absorber” function of muscle-tendon interactions.
**Temperature sensitivity of muscle & tendon**

We used temperature to change the kinetics of muscle activity. As expected, a muscle at 30°C has faster force production and relaxation rate than a muscle at 10°C. We saw less difference between muscles at 20 and 30°C. During isotonic shortening contractions, other researchers also saw less temperature effects at high temperatures and low forces (Olberding and Deban, 2017). They conclude that small movements are not as affected by temperature (and by proxy, muscle kinetics) as much as large movements. Our experiment may be characterized as “small movements” since we used conservative magnitudes and rates of stretch to avoid muscle damage. However, the affect of temperature during lengthening MTU contractions is poorly understood.

Before this study, we assumed that temperature changes would not affect the properties of tendons. Generally, elastic tissues, such as the hinge ligaments in bivalves, have low thermal sensitivity (Alexander, 1966; Denny and Miller, 2006; Trueman, 1953). Thus, biomechanists have used temperature manipulation as a method to identify the prevalence of elastic-recoil mechanisms in animal movements as seen in chameleons (Anderson and Deban, 2010), toads (Deban and Lappin, 2011) and salamanders (Anderson et al., 2014; Deban and Richardson, 2011; Deban and Scales, 2016; Scales et al., 2016). These studies generally portray muscle-powered movements, such as tongue retraction, to have high temperature sensitivity while elastically powered tongue projection (for some but not all species) is mostly temperature independent.
Upon closer inspection of the literature, several studies cite some temperature sensitivity in tendons. Rigby describes no change in the load-strain relationship of rat tail tendons across a range of 0-37°C, yet increased stiffness at 41°C and an interesting “thermoelastic contraction” at 60-70°C (Rigby et al., 1959). With canine medial collateral ligament, Woo and colleagues found increased hysteresis that was especially pronounced at 2-6°C (Woo, 1987). Therefore, it is not remarkable that our frog tendons present slight temperature sensitivity.

**Integration of muscle-tendon unit properties- estimating tendon recoil speed**

The amount of stored elastic energy is determined by the force capacity of a muscle and the stiffness of the series elastic elements (Galantis and Woledge, 2003). We can estimate the amount of stored elastic energy if we assume the tendon is a Hookean spring (equation 1):

\[ E = \frac{1}{2} K \varepsilon^2 \]

Where \( E \) is the potential elastic energy or mechanical work loaded into the tendon, \( K \) is the spring constant (stiffness) and \( \varepsilon \) is the change in tendon length. Since the tendon can be considered a spring acting in series with the muscle (equation 2), the length change, \( \varepsilon \), will depend on the force produced by the muscle (\( F_m \)):

\[ \varepsilon = \frac{F_m}{K} \]

Therefore, the amount of energy stored in the tendon is inversely related to the tendon’s spring constant (equation 3) and positively correlated with the square of the force a muscle can produce.

\[ E = \frac{1}{2} F_m^2 K^{-1} \]
Since tendons, like all biological materials, are not perfect springs, some of the elastic energy loaded into tendons is lost as heat. Thus, tendons are about 85-90% efficient. We call this feature of the tendon, resilience, which describes the proportion of energy recovered following a stretch. If we assume that the recoil of the tendon is moving the mass of the muscle (equation 4) with 15% hysteresis:

\[
\frac{1}{2} M_m V_t^2 = .85E
\]

Where \( V_t \) is the theoretical recoil speed of the tendon, \( M_m \) is the mass of the muscle and the constant .85 is correcting for energy lost to hysteresis. We can approximate the speed of the tendon recoil (equation 5):

\[
V_t = \left( \frac{1.7E}{M_m} \right)^{-\frac{1}{2}}
\]

We can use these simple equations to estimate the boundaries of tendon recoil and compare them to our measured results from tendon recoil experiments (Table 2.1).

**Methodological limitations**

When we stretched and released isolated tendons we measured recoil rates that were much slower than recoil rates we would expect based on our calculations of elastic energy storage. This discrepancy may be due to several factors. First, our servomotor is an imperfect latch with a lag of about 3ms. Any delay in a latch release may result in smaller yields of power amplification in an elastic system- the shorter the release time the quicker energy can be transferred (private correspondence with Army grant researchers). Second, we could be over estimating tendon efficiency. Nearly frozen tendons have higher rates of hysteresis (Woo, 1987).
Therefore, equation 5 may be overestimating the theoretical recoil speed of our cold tendons.

We could also be overestimating theoretical rate of tendon recoil because of our assumptions about the behavior and magnitude of the in series elastic elements. Our equations assume Hookean properties of tendons. However, tendons often characterize non-Hookean properties as exemplified by the J-shaped stress-strain curve with an initial “toe region” where the slope of the stress-strain curve is low (Wang, 2006). This toe region represents the stretching of the “crimp-pattern” of collagen fibers (Diamant et al., 1972). Thus, our use of the stiffness outside the toe region (~tangent modulus) may overestimate elastic energy stored (Roberts, 2016). We attempted to avoid this variation in stiffness by always initiating an active lengthening contraction after the MTU unit was lengthened to a passive resistance corresponding to 1.2L0.

On the other hand, we could be underestimating our theoretical rate of tendon recoil. Our calculations assume the stiffness of free tendon is the same stiffness of the MTU. However, the free tendon inserts onto the muscle via the thin sheet-like aponeuroses. Studies show that the aponeuroses play a critical role in a muscle’s series elasticity (Fukashiro et al., 1995; Huijing and Ettema, 1988; Maganaris and Paul, 2000b; Monti et al., 2003; Scott and Loeb, 1995). Frogs especially rely on aponeuroses in-series compliance to produce any fascicle shortening (Kawakami and Lieber, 2000). Yet, it is unclear if the amount of strain in tendon is equivalent to the amount of strain in the aponeuroses(Finni and Komi, 2002; Maganaris, 2002; Maganaris and Paul, 2000a; Muramatsu et al., 2001). MTU
compliance is further complicated by the observations that aponeuroses can be
loaded in the longitudinal and transverse direction (Roberts and Azizi, 2009).
Hence, MTU stiffness may be more compliant than free tendons but possibly not as
compliant as longitudinal load-strain tests would indicate. In the context of our
equations, an overestimate of stiffness in the denominator of Eq.3 would mean that
we underestimate stored elastic energy. Thus our theoretical tendon recoil
calculations may be conservative. Clearly, more work is required to understand the
rate of tendon recoil.

**Rate of fascicle stretch is driven by relaxation rate of the muscle**

Even with approximated calculations and limited measurements, it is clear
that the rate of stretch of the fascicle is not driven by the recoil of the tendon. As the
temperature increases from 10-20°C the amount of force developed increases but
the Young’s modulus, normalized stiffness, is consistent. Thus, the tendons are
storing more energy at warmer temperatures. However, as temperature rises from
20-30°C we see that force producing capacity of the muscle and stiffness of the
tendon decline. Therefore the amount of elastic energy stored declines (Table 2.1).
Yet, in all treatments, both theoretical and measured tendon recoil rates are greater
than the rate of stretch on the fascicle by one to three orders of magnitude.
Therefore, we conclude that the rate of muscle relaxation determines the rate of
fascicle stretch (Figure 2.5).

This conclusion is further supported by our calculated temperature
coefficients ($Q_{10}$). Temperature coefficients are unitless values that describe a
phenomena’s sensitivity to temperature. A value near 1 indicate temperature
insensitivity while biological processes are generally around 2-3. We see that $Q_{10}$ for the rate of fascicle stretch, 2.58, is approximately the $Q_{10}$ for muscle relaxation rate, 2.48. Meanwhile, both temperature coefficients for the measured and calculated tendon recoil speeds indicate that tendon recoil speeds are temperature independent, 1.26 and 1.08 respectively. The calculated speed of tendon recoil appears more temperature sensitive because its equations use elastic energy storage that is slightly temperature dependent (Table 2.2).

**Implications for injury**

Faster stretch velocities due to faster relaxation rates may explain the increased risk of muscle damage in fast twitch fibers. While there is a great body of literature addressing how muscle fiber composition affects muscle mechanics, there is little understanding of how fiber type composition affects muscle-tendon interactions. For example, in previous MTU studies, researchers report that some muscle fiber compositions are more prone to injury than others (Choi et al., 2011). While it is clear that muscle fiber type is associated with injury susceptibility, researchers are unsure which characteristics of fiber type contribute to muscle damage. Perhaps we may find answers to the quandary of fiber type specific injury if muscle damage is studied in the context of its in-series elastic elements. Since muscle fiber composition can change the rate of relaxation, and the rate of stretch on the fascicle varies with relaxation rate, we would predict that muscles composed of mostly fast fibers would be dissipate injury at riskier speeds than muscles composed of slow fiber types.
Conclusions

While we are impressed with the speeds and heights terrestrial animals reach, an animal's decelerating ability also contributes to its performance and overall survival. To successfully capture prey and survive, a cheetah needs to maneuver, change speeds and stop, all of which are decelerating tasks. Yet little focus has been given to how animals decelerate. Thus, it is unclear how the muscle traits that make animals move at impressive speeds may affect how they slow down. The presence of in-series tendons is beneficial because the tendons decouple the time of initial impact (stretch on MTU) from the time a fascicle begins to stretch thereby allowing the kinetic energy to be imparted to the fascicle at a rate less than the rate of stretch on the MTU.

We used the effect temperature has on muscle dynamics to test the hypothesis that muscle relaxation rate determines the speed of fascicle stretch during active lengthening. We found that slow rates of force development can limit loading of elastic elements. Therefore slow muscles may not derive the benefits from an in-series tendon during high intensity stretches. We also observed that faster relaxation rates apply a faster stretch to muscle fascicles during tendon recoil, therefore these muscles may be at a higher risk of injury. Lastly, this is one of the first studies to characterize recoil speeds in a biological material and we verified that the speed of fascicle stretch is less than the rate of tendon recoil.

Our results indicate that the ability of an individual to effectively attenuate power is dependent on the relative properties of muscles and tendons. An ideal MTU that dissipates energy 1) produces force quickly enough to store elastic energy
2) has a tendon compliant enough to stretch and 3) relaxes slowly enough to dissipate the energy at a safe rate.

**ACKNOWLEDGEMENTS**

Frog in vitro preparations were supported by NSF grant 1051691. Many thanks to Dr. Angela Horner (Cal State San Bernadino), she lent the servomotor used for our tendon mechanical tests. Thank you Dr. Greg Sutton, Dr. Mark Ilton, Elizabeth Mendoza for conversations about tendon recoil speeds. Isolated tendon preparations are based upon work supported by the U.S. Army Research Laboratory and the US Army Research Office under contract/grant #W911NF-15-1-0358.
Figure 2.1 Representative Twitches and Summary Kinetics

(A) Example isometric twitches at three temperature treatments for a single muscle (Tukey-HSD p<0.01, all temperatures) (B) Comparison of half-rise times across temperature treatments (C) Comparison of half-relax times across temperature treatments (Tukey-HSD p<0.05, all temperatures)
Figure 2.2 Temperature and Relaxation Rates
Comparison of normalized relaxation rates across temperature treatments.

(TukeyHSD 10-30°C p<0.05)
Figure 2.3 Representative Active Lengthenings and Fascicle Movement

(A) Representative length data for active lengthening contractions at different temperatures. Top line is the stretch applied to the muscle-tendon unit (MTU). Bottom lines are the change in fascicle length (B) Representative data for force exerted by the MTU during active lengthening contractions at different temperatures. (C) Total amount of fascicle shortening across temperature treatments (TukeyHSD 10-20° p<0.05) (D) Fascicle stretch rate across temperature treatments (TukeyHSD 10-30° p<0.05)
Figure 2.4 Tendon Recoil

(A) Representative length data for tendon stretch-recoil trials at different temperatures and strain amounts. (B) Representative length data for tendon stretch-recoil trials at different temperatures and strain amounts. 5.0% strains are dotted lines; 2.5% strains solid lines (C) Average speed of tendon recoil 5.0% strains are outlined in black; 2.5% strains are outlined in grey (ANOVA temperature p=no
sig., strain p < 0.001) (D) Maximum speed of tendon recoil (ANOVA temperature
p = no sig., strain p < 0.001)

Figure 2.5 Fascicle Stretch Corresponds to Relaxation Rate
Mean fascicle stretch rate plotted against mean relaxation rate. Error bars are
standard errors. Plot points are colored by temperature treatment: blue; 10°C,
purple; 20°, and red; 30°
Table 2.1 Calculated and Measured Tendon Recoil Dynamics

Values are means ± standard error. $F_m$ is force produced by the muscle, $dL_m$ is the amount shortened by the fascicle, $K_t$ is the stiffness of the tendon, $E$ is the calculated amount of energy stored in a tendon, $V_{tc}$ is the calculated rate of tendon recoil, $V_{tm}$ is the measured amount of tendon recoil and $dF_m/dt$ is the rate of relaxation of the muscle.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>$F_m$ (N)</th>
<th>$dL_m$ (mm)</th>
<th>$dL_m$ (N/m)</th>
<th>$E$ (J)</th>
<th>$V_{tc}$ (m/s)</th>
<th>$V_{tm}$ (mm/s)</th>
<th>$V_m$ (mm/s)</th>
<th>$dF_m/dt$ (N/s)</th>
</tr>
</thead>
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<tr>
<td>10</td>
<td>12.11 ± 1.26</td>
<td>0.038 ± 0.028</td>
<td>5126.15 ± 588.52</td>
<td>0.014</td>
<td>4.22</td>
<td>58.37 ± 2.36</td>
<td>0.72 ± 0.21</td>
<td>12.41 ± 2.16</td>
</tr>
<tr>
<td>20</td>
<td>21.65 ± 1.80</td>
<td>0.15 ± 0.034</td>
<td>4601.55 ± 1888.13</td>
<td>0.051</td>
<td>7.14</td>
<td>65.83 ± 11.83</td>
<td>2.94 ± 0.52</td>
<td>66.80 ± 12.43</td>
</tr>
<tr>
<td>30</td>
<td>17.15 ± 1.11</td>
<td>0.12 ± 0.035</td>
<td>3258.92 ± 118.01</td>
<td>0.045</td>
<td>6.8</td>
<td>67.81 ± 9.68</td>
<td>4.81 ± 0.50</td>
<td>76.09 ± 9.48</td>
</tr>
<tr>
<td>$F_m$</td>
<td>$dL_m$</td>
<td>$dL_m$</td>
<td>$E$</td>
<td>$V_{tc}$</td>
<td>$V_{tm}$</td>
<td>$V_m$</td>
<td>$dF_mdt$</td>
<td></td>
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<tr>
<td>1.19</td>
<td>2.93</td>
<td>0.80</td>
<td>1.51</td>
<td>1.26</td>
<td>1.08</td>
<td>2.58</td>
<td>2.48</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 Temperature Coefficients ($Q_{10}$) 10-30°C

A value near 1 indicate temperature insensitivity, values > 1 indicate physiological processes increase rate with temperature while values < 1 indicate rates decrease with temperature.
CHAPTER 3:

Comparative injury in rat hindlimb muscles

INTRODUCTION

Muscles act as “brakes” to prevent joints from collapsing under the body mass. When a force is applied to a muscle that exceeds the force produced by the muscle, the muscle will lengthen while activated. This is the hallmark of what muscle physiologists call eccentric contractions (Faulkner, 2003). Eccentric contraction is an odd name since they were originally, and appropriately, named ‘excentric’ for ‘away from the center’ (Asmussen, 1953). One negative consequence of muscles ends moving ‘away from the center’ is that, when active, the stretch applied to a muscle can physically disrupt the muscle structures that produce or transmit force.

Eccentric contractions produce more force at a reduced energetic cost compared to isometric and concentric contractions (Abbott et al., 1952). Thus, active lengthening may be an optimal way to exercise or “condition” muscles (Komi and Buskirk, 1972). Exercise researchers generally find that increasing the negative workload of athletes elicits greater amounts of hypertrophy compared to positive work (Norrbrand et al., 2008). While negative work is also an essential part of daily movement, it is important to note that we are not constantly accruing damage. It is only the large-impact (high-force), long duration (repetitions), or novel eccentric contractions that are destructive.
Given that eccentric contractions have both negative and positive consequences, it is important to ask: what skeletal muscle factors underpin injury? From the literature, we identified two possible factors that may determine the likelihood of muscle damage; muscle architecture and fiber type.

Muscle architecture refers to the diversity of muscle shape. Two classical categories are pennate and parallel-fibered muscles. In pennate muscles the fibers are attached to the tendon at an angle. This arrangement provides the benefit that more fibers can fill a certain muscle volume, and thus produce greater force than a parallel muscle of equal volume. On the other hand, fibers in a parallel muscle can run the whole length of the muscle and can thus produce larger velocities. In response to an active stretch, a muscle with pennate fibers may experience lower strains than a muscle with parallel fibers (Azizi and Roberts, 2014). This is because in a pennate muscle a small stretch applied on the muscle can be accommodated purely by the rotation of the fibers. Whereas in a parallel muscle, any length change applied to the muscle will also stretch the fibers. While there are functional benefits to both categories of muscle shape, fibers in parallel muscles are thought to be more prone to injury than fibers in pennate muscles. Strain relates to injury because when stress on the muscle cell (fiber) is so great, the functional unit of the fiber is disrupted or ‘popped’ (Morgan and Proske, 2004). Consequently, the muscle produces less force.

The cellular unit of a muscle is the muscle fiber. Individual muscles are composed of a collection of distinct fiber types that substantially affect the muscle’s mechanical function. Fiber types can be categorized by their contractile protein
isoforms, specifically myosin heavy chain (MHC) isoforms. At the level of the fiber, we can generalize these fiber types as “slow” and “fast” due to their significant influence on the maximum contraction velocity (Bárány, 1967; Bottinelli et al., 1991; Reiser et al., 1985; Sweeney et al., 1986). At a larger scope, the pooled fiber force-velocity curves determine the force-velocity curve of the whole muscle (Hill, 1970). And furthermore, variation in fiber type also influences contractile force, time to fatigue and relaxation rate (Bodine et al., 1987; Caiozzo, 2002; Lutz et al., 2002).

Given that fiber type composition can strongly explain whole muscle performance, it is possible that fiber type can also explain a muscle’s susceptibility to injury.

There is growing evidence that fast-type fibers are more prone to damage than slow-type. Researchers biopsied human athletes after a bout of eccentric contractions and found that in mixed fiber muscles, injury was regionally specific to fast fibers (Friden et al., 1983; Jones et al., 1986). This pattern was also observed in single fiber comparisons under comparable conditions that controlled for force and strain of stretch (Macpherson et al., 1996). Some molecular hypotheses for preferential damage have been suggested. First, slow-type fibers have more costameric proteins which form structural complexes that physically link the sarcomeres with the cell membrane (Chopard et al., 2001). Additionally, fast muscle fibers appear to have less heat shock proteins. These proteins interact with the z-discs and may maintain structural integrity of the sarcomere under loads (Koh, 2002). Whether in a mix of fibers or singular, slow-type fibers appear to have better infrastructure to resist applied strains.

Rat muscles are an excellent model system because, besides the mouse
(Burkholder et al., 1994), they are the only species for which the complete fiber type composition and hindlimb muscle architecture have been concurrently measured (Eng et al., 2008). We used this natural variation of rat hindlimb muscles to investigate whether muscle architecture or fiber type composition determine the likelihood of injury. We chose soleus as the slow muscle as it consists of 20% fast muscle fibers and its fibers are arranged nearly parallel with a low pennation angle of 3.9± 2.4. Conversely, we chose gastrocnemius as the fast muscle since it has a 94% majority of fast fibers with a pennation angle reported of 16.4±3.2 (Eng et al., 2008).

We hypothesize that if large-impact muscle injury is due to architecture, we would predict that the soleus would incur more damage. However, if muscle injury is due to fiber type composition, the plantaris would be injured more.
METHODS

Animals

Trials were conducted on the soleus and plantaris muscles of young (n=8, age ~6months) Sprague Dawley rats (*Rattus norvegicus*; 410-600g) (Charles River, Hollister, CA, USA). At UC Irvine, rats were housed and treated in accordance with the US Public Health Service Policy for the humane care and use of laboratory animals. UC Irvine Institutional Animal Care and Use Committee approved all procedures under protocol number: 2014-3137.

In situ preparation

In situ, we determined the contractile properties of the muscles. We anesthetized and maintained rats with a closed anesthesia system at 1.5-2.5% isoflurane (Parkland Scientific, Coral Springs, FL, USA). After shaving the entire right leg, we exposed the sciatic nerve in the dorsal thigh and placed a nerve cuff on it. To prevent reflex loops, the nerve was cut as proximally to the hip as possible. We created an insulating pocket by filling the thigh compartment with mineral oil and suturing the skin closed around the nerve cuff wire.

To access the muscles, we made an incision on the skin from the heel halfway up the calf, removed the Achilles tendon and reflected back both medial and lateral heads of the gastrocnemius. This revealed the deeper ankle extensor muscles. We identified the ankle extensor muscles of interest by their insertions; the soleus distal tendon inserts onto the calcaneus while the plantaris distal tendon inserts onto the plantar fascia of the foot (Fig. 3.1). The plantaris tendon was tied off with a single cord of 3-cord size 207 Kevlar thread (The Thread Exchange, Weaverville, NC, USA).
The soleus tendon was kept attached to the calcaneus that we snipped with a Bone Rongeur. Each tendon was fastened in a custom laser etched acrylic clamp as close as possible to the distal end of the muscle.

Rats were placed prone below a custom stereotaxic frame (Thorlabs Inc., Newton, NJ, USA). We blunt dissect along the lateral thigh between the planes of the vastus lateralis and biceps femoris to expose the femur. This can be done with minimal blood loss. We modified a 2.5cm three-prong clamp (Fisher Scientific) by removing the vinyl sleeves and sharpening the ends. This clamp fixes around the femur and is attached to the stereotaxic frame that is anchored in two locations to prevent rotation under tension. A singular tendon in a clamp was attached to the lever arm of a 50N servomotor (310 B-LR, Aurora Scientific Inc., ON, Canada). The servomotor measured force, length and velocity of the muscle. In a randomized order, we either attached the soleus or the plantaris tendon to the servomotor and the other muscle was kept warm and moist.

Rat and muscle temperature were monitored with an infrared thermometer. To prevent dehydration, the rats’ eyes are sealed with a small amount of petroleum jelly. Exposed muscles and femur are covered with a saline-moistened cotton gauze with saran wrap on top. For thermoregulation, the rat is placed on a heat pad and a heat lamp is directed onto these exposed areas from above. This approach allowed us to keep the muscles moist and at 37°C. After experiments, the rat was sacrificed with an overdose of sodium pentobarbital.
Cautionary anecdote about instrumenting soleus muscles

Originally, we intended to instrument sonomicrometry crystals in both the plantaris and soleus. However, the soleus is superficially innervated by branches of the sciatic nerve; the soleus axons of the lateral gastrocnemius nerve and some rats may have supplementary innervation by aberrant axons of the plantar nerve (Favero et al., 2012; Thompson and Jansen, 1977). These superficial innervations and the thinness of the soleus muscles (2.07 ± 0.06mm) made it problematic to insert 2mm sonomicrometry crystals without damaging crucial components of the motor system. We also attempted to insert crystals onto the deep surface of the soleus. However, the manipulations required to access this surface also seemed to impair soleus function. Furthermore, from the deep surface the sonomicrometry crystal wires rubbing against the tibia bone caused rotation of the crystals and consequently unreliable measurements. Thus, we abandoned plans to measure fascicle length with sonomicrometry. Alternative methods that may be considered are embedding small tantalum beads for XROMM (Brainerd et al., 2010) or using high speed videography with fixed surface markers.

Muscle contractile properties

We elicited contractions with supra-maximal square wave pulses (3-5V, frequency 100Hz, duration 0.ms) with an electric stimulator (Grass Technologies, Warwick, RI, USA). Each muscle was characterized by a twitch force-length curve so that all subsequent contractions were performed at the same passive force (Holt and Azizi, 2014). We used tetanic isometric contractions to determine the muscle’s maximum force $P_0$. We planned three eccentric contraction magnitudes by
calculating 1.3, 1.5 and 1.7P₀. Eccentric contractions were performed by first stimulating the muscle for 300ms, enough time to reach P₀, then the servomotor was asked to apply an isovelocity stretch until the force hit the targeted force magnitude. Once the servomotor reached the targeted force, it controlled for constant force (Fig. 3.2). After each eccentric contraction, we performed an isometric contraction to monitor stress loss after high-force eccentric contractions. Between each contraction we allowed five minutes of recovery for the muscles.

**Muscle data analysis and statistics**

After the experiment, each treated muscle and its contralateral control were carefully dissected away at their proximal origins to be weighed and measured. We measured muscle mass, muscle length, fascicle length, muscle thickness and the angle of pennation. We calculated peak isometric stress, or specific tension from P₀, pennation angle and physiological cross-sectional area (PCSA). Physiological cross-sectional area was calculated as the following (Méndez, 1960; Powell et al., 1984; Sacks and Roy, 1982):

\[
(1) \text{PCSA} = \frac{(M_m \cdot \cos \theta)}{(\rho \cdot L_i)}
\]

Where \( M_m \) is the muscle mass (g), \( \theta \) is pennation angle, \( \rho \) is the density of muscle (1.056g cm\(^{-3}\)) (Méndez, 1960) and \( L_i \) is fascicle length.

We used the change in stress from peak isometric stress as a proxy to measure muscle injury. To test if eccentric contraction magnitude and muscle had an effect on the loss of stress, we performed a two-way ANOVA with post-hoc Tukey Honestly Significant Difference (HSD) tests to compare the different treatments (R; v3.3.1, The R Foundation for Statistical Computing)
**RESULTS**

Morphologically, soleus and plantaris differ significantly (Table 3.1). Plantaris muscles weigh more, are longer, more pennate and have a larger PCSA. Soleus muscles weigh less, are shorter, more parallel and have a smaller PCSA. We did not measure a significant difference between muscles that experienced the eccentric contractions and the contralateral control muscles (TukeyHSD, p<0.05). Peak isometric stress (mean ± standard deviation) of soleus was 24.86 ± 1.11 (N/cm²) and 22.35±0.51 (N/cm²) for plantaris. Three soleus muscles demonstrated complete failure at 1.7P₀. Consistently the failure occurred at the interface of the superficial aponeuroses and muscle belly.

We analyzed the data in two sets. In the first set we included the muscles that were torn (Fig 3.3). In this dataset, we reported that the loss of stress was the same as the peak isometric stress. Because the large change in stress may bias our analyses, we also analyzed the eccentric data without muscles that experienced complete failure (Fig. 3.4).

Regardless of the dataset, as the magnitude of eccentric contractions increased from 1.3-1.7P₀ both muscles lost more stress (two-way ANOVA, p<0.05). Comparing the loss of stress in both muscles, we saw a significant effect of muscle with the soleus losing more force producing capability than plantaris (two-way ANOVA, p<0.05).
DISCUSSION

In this study, we compared the fast, pennate plantaris muscle with the slow, parallel soleus to determine the predominate effect in large-force muscle injury. We predicted if large-force injury is due to architectural features, soleus would be injured more. If injury were due to fiber type composition, we expected that the plantaris would sustain greater damage. We observed that as the magnitude of eccentric contractions increased, both muscles lost more normalized force. For each eccentric contraction magnitude, the soleus muscle lost more normalized force. This suggests that higher pennation angles provide protection during large-force eccentric contractions.

Limitations

There are many methodological limitations in muscle injury studies, ours not excluded. We sought to induce injury via large stresses meant to disrupt structural features. Therefore, our experimental framework would be negated if fast-type fibers do not have a structural propensity for injury. In isolated fiber studies, the skinning process may remove protective enzymatic or structural proteins (Choi, 2014). Therefore the reason single fast-type fibers may show a higher propensity for damage on a minuscule scale may be entirely different than the mechanism that fast-type fibers may be damaged in vivo. Specifically, in vivo studies that demonstrate preferential damage to fast-type fibers apply many repetitions (Friden et al., 1983). Therefore fast-type fibers, which are not fatigue resistant, may be susceptible to injury via fatigue mechanisms such as the creation of actin-myosin cross-bridges in the rigor state. Additionally, in vivo whole muscle studies may be
complicated by differential fiber type recruitment (Henneman et al., 1965). Metabolic theories of injury have been dismissed (Choi and Widrick, 2009; Patel et al., 1998). Nevertheless, given the multitude of differences between fast and slow fibers, it is not unreasonable to suspect that factors other than ultrastructure could elicit damage of specific fiber-types.

Lastly, we measured the loss of specific stress as a proxy for injury. However, function can be compromised in conditions not associated with injury such as atrophy or fatigue. Our in situ approach allows natural blood flow to supply muscles with oxygen and glucose and each contraction is followed by a 5-7 minute break. Similar experimental methods that use over 10 isometric contractions do not present evidence of fatigue (Holt and Azizi, 2014; Holt et al., 2016; Swoap et al., 1997). Therefore, we are confident that the changes in stress are due to disruption of contraction structures.

**Comparison with other studies**

In vivo, most rat injury models run rats on decline surfaces in order to intensify the braking phase of cyclical locomotion (Armstrong et al., 1983; Gillis and Biewener, 2002; Lynn and Morgan, 1994; Lynn et al., 1998; Schwane and Armstrong, 1983). During stance, the knee extensor muscles strain approximately to 25% which is extensive enough to cause injury (Lieber et al., 1991; McCully and Faulkner, 1985). In their rat running experiment, Gillis et al (2002) observed that among the antigravity vastus muscles, the vastus intermedius (25.7% slow, 6.9° pennation) sustained more damage than the vastus medialis (6.19% slow, 24.7° pennation) and lateralis (1.24% slow, 10.0° pennation) (muscle values from: (Eng et
These in situ results parallel our findings: muscles with more parallel and slow-type fibers (vastus intermedius; soleus) have a higher propensity for damage than muscles with pennate and fast-type fibers (vastus medialis, vastus lateralis; plantaris).

Muscles alter their structural and functional properties to tailor to the functions they serve. In rats that run downhill, exercise training lessens the extent of muscle injury (Schwane and Armstrong, 1983). Muscle damage may be mitigated by the degeneration and regeneration of fibers susceptible to injury (Armstrong et al., 1983) or by increasing the number of sarcomeres in series (Lynn and Morgan, 1994; Lynn et al., 1998). However, these studies did not measure pennation angle. In longitudinal human studies, researchers have shown that pennation angle increases with eccentric exercise (Aagaard et al., 2001; Blazevich et al., 2007; Duclay et al., 2009). Thus, it is possible that muscles increase pennation angle to limit stretch-induced damage.

**Re-analysis of hindlimb morphometrics and fiber type**

After eccentric exercise training, muscles change their pennation angle. Since adaptations can happen on a temporal scale within an individual or, on an evolutionary scale within a species we asked: do muscles that primarily do negative work have larger pennation angles? This question prompted a re-analysis of available rat hindlimb data ((Eng et al., 2008) (Fig. 3.5). Pennation angle is higher in antigravity muscles (Fig. 3.5b) and ankle joint muscles (Fig. 3.5a) (p<0.05). A multivariate analysis of fiber type did not show any correspondence to muscle
function. Thus, muscle architecture varies more with function than fiber type composition.

**Considerations for eccentric contractions**

From this study, we infer that muscle architecture plays a larger role in predicting a muscle’s susceptibility to injury than a muscle’s fiber type composition. Under different conditions though, eccentric contractions make excellent models to study the infrastructure of fibers. After stretching active muscles, researchers have observed an unexpected increased in force (residual force enhancement). This has lead to the generation of new ideas that the sliding filament model should include a calcium activated molecular spring (Herzog, 2014; Nishikawa et al., 2012).

LaStayo cautions that the conceptual link between eccentric exercises and muscle damage should not be emphasized so readily (LaStayo et al., 2014). The properties that distinguish eccentric contractions 1) higher forces and 2) lower energy make these exercises great options for rehabilitation and maintenance (Komi and Buskirk, 1972). For example, in frail populations of older adults, small increases in eccentric activity within an experimental group saw greater improvements in balance, strength and braking activities compared to a physically active control group (LaStayo et al., 2003). Therefore, it is possible to gain functional improvement from eccentric contractions without experiencing harm- all gain no pain. Yet, frequent association of eccentric contractions and muscle damage may discourage health practitioners from prescribing eccentric training to vulnerable patient populations. We hope that this study does not discourage health professionals from utilizing eccentric training but highlights that eccentric
contractions can be applied at a range of magnitudes, some of which cause injury and some of which may not.

CONCLUSIONS

In this broad comparison, we saw that plantaris muscles have a lower propensity for injury even though these muscles have a large proportion of fibers that are thought to have a higher propensity for injury. Therefore the pennation of muscle fibers may mitigate large-force eccentric contractions. This result is further illustrated by the finding that antigravity muscles tend to be more pennate.

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Figure 3.1 Distal Muscles of the Rat Hindlimb

Distal muscles of the medial surface of the lower left leg. Plantaris (PL) is shaded pink and soleus (SOL) is shaded red. (Adapted from Figure 101 in Greene, E. C. (1935). The Anatomy of the Rat. New York: Hafner Pub.)
Example eccentric contraction measured by a servomotor. During time duration a) the muscle is activated 300ms to reach peak isometric force ($P_0$) and remains active for 500ms more. During time duration b) once $P_0$ is achieved, the servomotor applies a stretch until the muscle reaches a force of a predetermined magnitude. In this specific example the magnitude of the eccentric contraction is $1.5P_0$. During time c) the servomotor acts as a feedback controller and holds force steady by adjusting the length of the muscle.
### Table 3.1 Comparative Soleus and Plantaris Muscle Morphology

**Mean values ± standard error.** Both plantaris and soleus differed significantly by muscle (**p<0.001; *p<0.05; Tukey HSD**). Morphological measurements do not differ between muscles that have been eccentrically contracted and control muscles that were not manipulated. $M_b$ is the body mass of the rat, $M_m$ is the muscle mass, $L_m$ is the muscle length, $L_{fasc}$ is the length of the fascicle, $L_{th}$ is the thickness of the muscle belly, $\theta$ is the pennation angle and PCSA is the physiological cross sectional area.

<table>
<thead>
<tr>
<th>muscle</th>
<th>tx</th>
<th>$M_b$ (g)</th>
<th>$M_m$*** (g)</th>
<th>$L_m$*** (mm)</th>
<th>$L_{fasc}$* (mm)</th>
<th>$L_{th}$*** (mm)</th>
<th>$\theta$*** (°)</th>
<th>PCSA*** (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plantaris</td>
<td>ecc</td>
<td>497 ± 28.81</td>
<td>0.54 ± 0.03</td>
<td>33.94 ± 1.03</td>
<td>16.90 ± 0.64</td>
<td>5.02 ± 0.18</td>
<td>17.42 ± 0.77</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>Plantaris</td>
<td>ctrl</td>
<td>482 ± 28.58</td>
<td>0.53 ± 0.02</td>
<td>32.85 ± 0.85</td>
<td>17.27 ± 1.14</td>
<td>5.26 ± 0.18</td>
<td>18.15 ± 1.19</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Soleus</td>
<td>ecc</td>
<td>507 ± 31.10</td>
<td>0.23 ± 0.02</td>
<td>22.80 ± 0.66</td>
<td>14.74 ± 1.26</td>
<td>2.07 ± 0.06</td>
<td>8.21 ± 0.63</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Soleus</td>
<td>ctrl</td>
<td>475 ± 25.76</td>
<td>0.21 ± 0.01</td>
<td>23.57 ± 0.92</td>
<td>14.75 ± 0.58</td>
<td>2.33 ± 0.19</td>
<td>9.28 ± 0.94</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 3.3 Stress Loss After Eccentric Contractions with Torn Muscles

Comparison of stress loss across three magnitudes of eccentric contractions of 1.3, 1.5 and 1.7$P_0$. These data include soleus muscles that tore after 1.7$P_0$. Plantaris (PL) is shaded pink and soleus (SOL) is shaded red. (A) Muscles are grouped by magnitude of eccentric contractions (ANOVA muscle $p<0.05$, ecc $p<0.05$) (TukeyHSD 1.7-1.3 and 1.7-1.5 $p<0.05$) (B,C) Plantaris and soleus plotted separately.
Figure 3.4 Stress Loss After Eccentric Contractions without Torn Muscles

Comparison of stress loss across three magnitudes of eccentric contractions of 1.3, 1.5 and 1.7\(P_0\). These data do not include soleus muscles that tore after 1.7\(P_0\). Plantaris (PL) is shaded pink and soleus (SOL) is shaded red. (A) Muscles are grouped by magnitude of eccentric contractions (ANOVA muscle p<0.05, ecc p<0.05) (TukeyHSD 1.5-1.3, 1.7-1.3, and 1.7-1.5 p<0.05) (B,C) Plantaris and soleus plotted separately.
Figure 3.5 Re-analysis of Hindlimb Morphometrics and Fiber Type

(A) Comparison of pennation angle and muscle function across joints where color of the bar indicates muscle function (pink= antigravity; teal= other) (hip-ankle TukeyHSD p<0.05, no significant effect of function). (B) Comparison of pennation angle between anti-gravity and other muscle function (AOV p<0.05) (C) Comparison of pennation angle and across joints (hip-ankle TukeyHSD p<0.05).
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