THESIS

Anatomy and Muscle Fiber Types of Kangaroo Rat Hindlimb Muscles

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Anatomy and Muscle Fiber Types of Kangaroo Rat Hindlimb Muscles

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ABSTRACT

Kangaroo Rats (*Dipodomys spp.)* utilize a specialized bipedal hopping locomotion like that of kangaroos. In contrast to kangaroos that have elastic tendons capable of storing energy, kangaroo rats have inelastic tendons that are unable to store large amounts of energy. Thus, it is the musculature of the ankle joint that provides the greatest power contribution to kangaroo rat hopping. Skeletal muscle can be divided into slow twitch (type I) and fast twitch (type II) fibers. Fast fibers are found in higher concentration in muscles that perform quick, dynamic movements, whereas, slow fibers are found in higher proportion in muscles that perform slow, endurant movements. Using fiber type specific antibodies, I was able to identify four pure fibers (type I, IIA, IIB, and IIX) and two hybrid fibers (type I/IIA and IIA/IIX) in six hindlimb muscles from three kangaroo rats (*Dipodomys merriami*) to determine a relationship between fiber composition and hindlimb muscle function. Every hindlimb muscle studied, except soleus, was dominated by type IIB fibers, which are known to be best suited for rapid and explosive movements. Soleus was dominated by type I fibers, which are known for having a postural role. This high percentage of type IIB fibers found in nearly all muscles studied can be related to the explosive hopping behavior that the kangaroo rat exhibits in order to escape predators, such as rattlesnakes. Oxidative type IIA and type IIX fibers were found at moderate concentrations and are functional in maintaining a continual saltatory locomotion. Two hybrid fibers (type I/IIA and IIA/IIX) were observed in minimal amounts suggesting that there may be continual muscle regeneration occurring, which relates to the variable muscle functions.

INTRODUCTION

Kangaroo rats (*Dipodomys spp.)*, like kangaroos, have a specialized type of bipedal locomotion described as a continual saltatory movement in which the hindlimbs come in contact with the ground but the forelimbs do not (Bartholomew and Caswell, 1951). When preparing to leap, the rat flexes its ankle, knee, and hip joints and subsequently uses its hind limb extensor muscles to spring into the air and then land back on its hind feet with its body in an erect position (Fokin, 1978). Of extant animals, bipedal hopping is most frequently observed in mammals and has independently evolved in one marsupial family and five rodent families (Figure 1; McGowan and Collins, 2018). These groups have key morphological characteristics, such as elongated feet and modified tarsal bones, which allow the animal to stabilize the ankle joint for bipedal hopping (McGowan and Collins 2018). Though the selective pressures that drove bipedal evolution remain elusive, several hypotheses suggest that enhanced locomotor efficiency, greater forelimb specialization for foraging behaviors, and avoiding predation all could have been drivers for evolution (McGowan and Collins, 2018). Freymiller et al. (2019) found that the springing action involved in hopping must occur very quickly, between 38 and 150 milliseconds, in order for these animals to successfully escape predation. While the knee and hip joints are also involved in hopping, it is the musculature of the ankle joint that provides the greatest power contribution (Schwaner et al., 2018).

Small mammals that utilize bipedal hopping use less energy for movement at faster speeds than similarly sized quadrupedal mammals (Nikolai and Bramble, 1983). Biewener et al. (1981) investigated elastic energy storage in muscle tendons, and the role it plays in this

type of locomotion. In large bipedal hoppers, tendons are capable of storing elastic energy for later use, thus enabling the animal to reduce the cost of muscle contraction and overall metabolic cost during hopping. This mechanism works very well for kangaroos, allowing them to recover more than 54% of their energy in their tendons (Biewener et al., 1981). However, the study concluded that the energy recovered from elastic storage in kangaroo rats is no more than 14%. This reduction is due to kangaroo rats' thin muscle tendons that are incapable of storing the elastic strain energy, indicating that the majority of the power generated for hopping must come from kangaroo rats' muscle fibers.

Skeletal muscle fibers can be broadly divided into slow twitch (type I) and fast twitch (type II) fiber types based on physiological and functional characteristics (Rosser and George, 1986). Fast muscle fibers are found in higher concentration in muscles that perform moderate to rapid robust movements, whereas, slow fibers are found in higher proportion in muscles that perform postural or slow movements (Ryan et al., 1992; McFarland and Meyers, 2008). Muscle fibers can be classified based on oxidative capacity and contractile speed. The typical nomenclature for fiber type identification with ATPase histochemistry is slow oxidative (SO), fast oxidative glycolytic (FOG), and fast glycolytic (FG). Contractile speed is related to the myosin heavy chain (MyHC) classification (Smerdu and Soukup, 2009), typically described as type I (containing MyHC I), type IIA (containing MyHC IIA), type IIX (containing MyHC IIX), and type IIB (containing MyHC IIB). SO corresponds to type I fibers, FOG to type IIA fibers, and FG to type IIB fibers (Bandman and Rosser, 2000). Type I fibers have the slowest contraction speed and utilize the aerobic metabolic pathway to generate ATP, thus they are highly fatigue resistant (Hesse et al., 2010). Type IIA fibers are

relatively fast contracting and make use of both the aerobic and anaerobic metabolic pathways, thus having a moderate degree of fatigue resistance (Hämäläinen and Pette, 1993). Type IIB fibers have the fastest contraction speed and utilize the anaerobic glycolytic pathway to generate ATP, thus they fatigue rather quickly (Hämäläinen and Pette, 1993). Type IIX fibers have a similar contractile speed to IIA fibers (Pette and Staron, 2000), but they are fatigable like IIB fibers (Zhong et al., 2008). Hybrid fibers, which express two different MyHCs (Stephenson, 2001), are also common.

Williamson and Frederick (1977) examined the fiber type arrangements of four ankle extensor muscles in the kangaroo rat: medial gastrocnemius, lateral gastrocnemius, plantaris, and soleus, using myosin ATPase histochemistry to classify fibers based on oxidative capacity. The plantaris and medial and lateral gastrocnemius muscles were found to contain more than 90% fast fibers, which Williamson and Frederick (1977) relate to forceful contraction and rapid movements of the kangaroo rat ankle extensors muscles. In contrast, the soleus muscle was composed of 92% slow oxidative fibers. This suggests that some ankle joint extensor muscles have been adapted for the rapid movements that the kangaroo rat exhibits during hopping, while other extensor muscles are more endurant (Williamson and Frederick, 1977) relating to their postural role. Williamson and Frederick's (1977) study on kangaroo rat muscles relied on ATPase histochemistry, which is pH sensitive and not directly related to myosin heavy chain identity. The goals of my study are (1) to determine the fiber type composition of ankle extensor muscles using myosin heavy chain-specific antibodies that are specific to four fiber types and (2) to determine the fiber type composition of ankle flexor muscles (extensor digitorum longus and tibialis anterior) for

comparative analysis to expand on the data pertaining to the hind limb muscles of the kangaroo rat.

Since kangaroo rats are small bipedal mammals that are quick to escape predation and do not heavily rely on elastic energy storage in their tendons (Biewener et al., 1981), I predict that their ankle extensor muscles (excluding M. soleus) will have the highest proportion of type IIB fibers, which are known to be best suited for rapid and explosive movements (Ryan et al., 1992; Hämäläinen and Pette, 1993; Zhong et al., 2008). Furthermore, I predict that the flexor muscles will have a lower proportion of type IIB fibers due to the fact that they are not participating in this rapid extension.

MATERIALS AND METHODS

Tissue Collection

Three kangaroo rats (*Dipodomys merriami*; two males and one female) were used for fiber type analysis. Specimens were donated by Dr. Duke Rogers from Brigham Young University's Monte L. Bean Museum. Animals were wild caught in Southern Utah, stored in a freezer, and defrosted prior to dissection. The left and right hindlimbs of each animal were dissected and muscles were removed. The right leg was selected for immunohistochemical reactions. For one animal (kangaroo rat 2), the right limb muscle tissue quality was unsuitable for fiber quantification, therefore, muscles from the left limb were used for analysis. A third male specimen, whose hindlimb muscles remained intact, was fixed in 5% formalin and stored in 1% phenoxyethanol for anatomical analysis.

Muscles were selected based on their relative location, contractile properties, and previous anatomical analysis (Howell, 1932; Williamson and Frederick, 1977; Biewener et al., 1981). The muscles selected for analysis include the Mm. medial gastrocnemius, lateral gastrocnemius, soleus, and plantaris, which act as knee flexors and ankle extensors, as well as Mm. tibialis anterior and extensor digitorum longus, which are considered ankle flexors.

Tissue collection consisted of removing the entire muscle from both hindlimbs of each animal. A mid-belly portion of the muscle, approximately 1cm long, was mounted to a 2x2 cm piece of cork block using 5% gum tragacanth. I mounted these muscles in such a way that the fibers lay perpendicular to the cork. Samples were flash-frozen in isopentane, cooled to approximately -150°C by liquid nitrogen, and subsequently stored in an ultra-cold freezer at -80°C. Using a freezing microtome (Tissue-Tek II microtome/cryostat models 4551 and 4553 respectively) at -20°C, I cut 10 µm serial slices of the muscle and transferred them to microscope slides in order for antibody studies to be completed.

Immunohistochemistry

Following the protocol used by Meyers and Stakebake (2005), fiber-specific antibodies were used to identify the muscle fiber types of interest. Serial sections were reacted with the following primary antibodies: S-58 (DSHB, Iowa City, IA, USA), which binds to type I fibers, MY-32 (Sigma-Aldrich, St. Louis, MO, USA), which binds to type II fibers, SC-71 (DHSB), which binds to IIA fibers, BF-F3 (DHSB), which binds to IIB fibers, and BF-35 (DHSB), which binds to type I, IIA, and IIB, but does not bind to IIX fibers (Figure 2).

Muscle sections were incubated in a humidity chamber at room temperature for one hour, after which they were rinsed with phosphate buffered saline (PBS) solution (pH 7.2).

The muscle sections then underwent another (30 min) incubation period, this time using appropriate secondary antibodies: anti-mouse IgA (Vector Laboratories, Burlingame, CA, USA), anti-goat IgG (Life Technologies, Carlsbad, CA, USA), and anti-mouse IgM (Thermo Fisher Scientific, Waltham, MA, USA). Following another PBS rinse, slides were developed and stained using an AEC peroxidase kit (Vector Laboratories). Slides were cover-slipped and overlapping photographs of the muscle were taken with a Canon Rebel T1i (Ota, Tokyo, Japan) under a Zeiss Axioskop 40 microscope (Oberkochen, Germany). Using Adobe photoshop 2.0 (San Jose, CA, USA), photos were compiled and merged in order to create a photomontage of the entire muscle. The image was printed and taped together to allow for fiber type quantification.

Fiber Type Analysis and Statistics

Type I muscle fibers were counted using positively reacted cells in the S-58 reaction, with the exception of the *M. soleus* which was counted using negatively reacted cells in the SC-71 reaction, because it provided the best visualization of cell borders. The SC-71 reaction was used to count type IIA fibers, as well as hybrid fibers in every muscle. In order to differentiate hybrids from pure IIA fibers, I classified an intermediate positive reaction as a hybrid fiber and a strong positive reaction as a pure IIA fiber (Table 1; see Rupert et al. 2014, Spainhower 2018). Positively reacted cells in the BF-F3 reaction were counted and classified as IIB fibers. Type IIX fibers were counted using the negatively reacted cells in the BF-35 reaction. Total counts for each muscle were obtained by counting all fibers from the MY-32 reaction. The exception to this was the *M. soleus* which was totaled using the SC-71 reaction because, again, it provided the best visualization of cell borders. Following fiber

quantification, means, standard deviations, and percentages were calculated. A one-way ANOVA test was used to determine significance of fiber percentages within a muscle at $p<0.005$. Post-hoc T-tests were used to determine significance between the most numerous fiber types in each muscle at $p<0.05$.

RESULTS

For each of the kangaroo rat hindlimb muscles studied, a brief anatomical description is provided, and the fiber type percentages and arrangements are reported. Gross anatomical descriptions are derived from Howell (1932). Four pure fiber types (I, IIA, IIX, IIB) and two hybrid fibers (I/IIA and IIA/IIX) were identified during muscle analysis.

ANKLE EXTENSION

M. Lateral Gastrocnemius

The M. lateral gastrocnemius (LG) is a robust muscle that extends from the distal lateral femur to the lateral calcaneus. LG originates from the lateral epicondyle of the femur bone and the lateral sesamoid, with the center of the superficial belly being tendinous in origin. Some of the proximal fibers of LG have a close connection with Mm. medial gastrocnemius and plantaris. The distal LG tendon fuses with the tendons from Mm. medial gastrocnemius, soleus, and plantaris to become the calcaneal tendon that inserts onto the lateral aspect of the calcaneus bone (Figure 3B).

The M. lateral gastrocnemius showed a heterogeneous composition of all four muscle fiber types as well as type IIA/IIX hybrid fibers. LG is primarily composed of type IIB fibers (63%), and type I fibers are the least numerous in the muscle (4%) (Table 2). Type I

fibers were concentrated in the deepest (most cranial) portion of LG, near the M. soleus (Figure 3C). Subsequently, type IIA, IIX, and IIA/IIX hybrid fibers were primarily located in the deep portion of LG but extended more superficially than the type I fibers (Figure 3E, G). Type IIB fibers were predominantly located in the lateral and superficial portions of LG (Figure 3F). There was a significant difference between fiber types within LG [F (4,10) = 137, p= 1.09E-8]. Post-hoc T-tests showed that type IIB fibers are more numerous than both the IIA fibers [df=4, T=11.5, p=1.64E-4] and the IIX fibers [df=4, T=14.2, p=7.13E-5], however, there was no difference observed between type IIA and IIX fibers [df=4, T=0.12, p=0.46].

M. Medial Gastrocnemius

The M. medial gastrocnemius (MG) is a robust muscle that extends from the distal medial femur to the lateral calcaneus. MG arises from an extensive superficial aponeurosis that originates from the distal medial shaft and the medial epicondyle of the femur bone. Some of the proximal fibers of MG are in close contact with Mm. lateral gastrocnemius and plantaris. The distal tendon of MG fuses with the other muscle tendons to insert onto the lateral aspect of the calcaneus bone via the calcaneal tendon (Figure 3A).

The M. medial gastrocnemius is very similar to that of M. lateral gastrocnemius with a heterogeneous fiber composition of all four pure fiber types as well as type IIA/IIX hybrid fibers. MG was primarily composed of type IIB fibers (62%), and type I fibers are the least numerous in the muscle (5%) (Table 2). The highest concentration of type I, IIA, IIX, and IIA/IIX hybrid fibers was observed in the deepest portion of MG (Figure 3C, E, G), and type IIB fibers were largely concentrated in the superficial portions of MG (Figure 3F). There was a significant difference between fiber types within MG [F (4,10) = 177, p= 3.12E-9]. Post-hoc

T-tests showed that type IIB fibers are more numerous than both the IIA fibers [df=4, T=12.6, p=1.14E-4] and the IIX fibers [df=4, T=20.9, p=1.54E-5], however, there was no difference observed between type IIA and IIX fibers [df=4, T=0.54, p=0.31].

M. Plantaris

The M. plantaris (PL) is a moderately robust muscle that is hardly separable from the medial and lateral gastrocnemii. The proximal tendon of PL originates at the lateral sesamoid, near the origin of lateral gastrocnemius. The distal tendon of PL passes medially and inserts onto the calcaneus bone via the fused calcaneal tendon (Figure 3B).

The M. plantaris showed a heterogeneous fiber type arrangement composed primarily of type IIA (28%), IIX (29%), and IIB fibers (33%) (Table 3). Type I and type IIA/IIX hybrid fibers were also observed in small quantities throughout the muscle (Table 2; Figure 3C, E). Type I fibers were concentrated in the deepest portion of the muscle near M. soleus (Figure 3C), similar to the location of type I fibers observed in the gastrocnemii. There was a significant difference between fiber types within PL [F (4,10) = 80.6, p= 1.44E-7]. Post-hoc T-tests showed that type IIB fibers were not more numerous than type IIA [df=4, T=2.34, p=0.08], but type IIB fibers were more numerous than type IIX [df=4, T=2.83, p=0.04]. Furthermore, there was not a difference between type IIA and IIX fibers [df=4, T=0.29, p=0.78].

M. Soleus

The M. soleus (SOL) is a small slender muscle located deep to Mm. lateral gastrocnemius, medial gastrocnemius, and plantaris. The proximal tendon of Sol originates from the fibular head. The distal tendon of Sol fuses with the common gastrocnemius

tendon near the middle of the gastrocnemii muscle bellies. SOL inserts onto the lateral calcaneus via the calcaneal tendon (Figure 3A).

The M. soleus was nearly homogeneous in composition with type I fibers dominating 88% of the muscle (Table 3; Figure 3C). The remaining 12% of SOL was shared between pure type IIA fibers (9%) and type I/IIA hybrid fibers (3%) located throughout the entire muscle (Table 2; Figure 3E). There were no type IIB or type IIX fibers observed in SOL (Table 2; Figure 3C, G). There was a significant difference between fiber types within SOL [F (2,6) = 9912, p= 2.77E-11]. Post-hoc T tests showed that type I fibers are significantly more numerous than IIA fibers [df=4, T=96.8, p=3.4E-8].

ANKLE FLEXION

M. Tibialis Anterior

The M. tibialis anterior (TA) is a robust muscle extending from the proximal tibia to metatarsal I. TA arises from the entire tibial fossa and the lateral aspect of the proximal tibia bone. The distal tendon of TA passes beneath the transverse ankle ligaments to insert onto the middle portion of the first metatarsal bone (Figure 4A).

The M. tibialis anterior showed a heterogeneous fiber arrangement predominantly composed of type IIB (51%) and type IIX fibers (31%) (Table 3). Small percentages of type I, IIA, and IIA/IIX fibers were primarily observed in the deepest (most caudal) portion of TA (Table 3; Figure 4B). There was a significant difference between fiber types within TA [F (4,10) = 135, p= 1.15E-8]. Post-hoc T-tests showed that type IIB fibers were more numerous than the IIX fibers [df=4, T=5.3, p=0.003].

M. Extensor Digitorum Longus

The M. extensor digitorum longus (EDL) is a slender muscle, with a long distal tendon, that extends from the femur to metatarsals II-V. The proximal tendon of EDL originates at the lateral epicondyle of the femur bone, near the patellar ligament. The long distal tendon of EDL arises near the mid belly of M. tibialis anterior and follows the tibialis anterior tendon beneath the transverse ankle ligaments. The EDL tendon then branches to insert onto the lateral aspect of metatarsal II, the middle aspect of metatarsal III, and the medial aspects of metatarsal IV and V (Figure 4A).

The M. extensor digitorum longus showed a heterogeneous composition of all four pure fibers as well as type IIA/IIX hybrid fiber. EDL is primarily composed of type IIB fibers (44%) and type I fibers are the least numerous in the muscle (1%) (Table 3). Type IIA, IIX, and IIA/IIX hybrids were also observed in small quantities throughout EDL (Table 2; Figure 4C). There was a significant difference between fiber types within EDL [$F(4,10) = 87.5$, $p = 9.71E-8$]. Post-hoc T-tests showed that type IIB fibers are significantly more numerous than IIA [$df=4$, $T=5.17$, $p=0.003$].

DISCUSSION

Kangaroo rats are small bipedal mammals that utilize hopping as their primary mode of locomotion (Bartholomew and Caswell, 1951). In contrast to larger bipedal hoppers, such as kangaroos and wallabies, kangaroo rats do not heavily rely on elastic energy storage in their tendons (Biewener et al., 1981), but rather they rely on muscle fiber energy for contraction. Based on previous kangaroo rat muscle fiber studies done by Williamson and

Frederick (2008), I hypothesized that the kangaroo rat's ankle extensor muscles (excluding M. soleus) would have the highest proportion of type IIB fibers, which are known to be best suited for rapid and explosive movements (Ryan et al., 1992; Hämäläinen and Pette, 1993; Zhong et al., 2008). In support of this hypothesis, I found that type IIB fibers were the most numerous in ankle extensor muscles MG (62%), LG (63%), and PL (33%), while type IIB fibers were absent in SOL (0%, Table 3); these coincide with the percentages of type IIB fibers reported by Williamson and Frederick (2008). This high percentage of type IIB fibers can be related to the explosive hopping behavior that the kangaroo rat exhibits in order to escape predators, such as rattlesnakes (Freymiller et al., 2019). Furthermore, I predicted that the ankle flexor muscles would have a lower proportion of type IIB fibers due to the fact that they are not participating in this rapid extension. Unexpectedly, I found that the flexors muscles TA and EDL were also primarily composed of type IIB fibers with 51% and 44% respectively (Table 3). This high percentage of type IIB fibers in flexor muscles could function to prevent rapid hyperextension and act to decelerate the joint angle of the ankle joint during landing. Additionally, during hopping, type IIB fibers in these muscles could act to rapidly flex the ankle joint in order to prepare for another rapid extension.

Type I fibers, which are best known for having a postural role (Ichikawa et al., 2018; Meyers, 2019), were found in very small percentages ranging from 0.13-5.3% in PL, MG, and LG, which is comparable to the percentages reported by Williamson and Frederick (2008). This minimal percentage of type I fibers suggests that the kangaroo rat's ankles may not require sustained postural support, which can be related to its small body size (Suzuki, 1990). The SOL muscle was composed of 88% type I fibers (Table 3), which is also similar to

what Williamson and Frederick (2008) reported. Soleus muscle is known to be predominantly composed of type I fibers in rats (Armstrong and Phelps, 1984), guinea pigs (Ariano et al., 1973), rabbits (Lobley et al., 1977), and house shrews (Suzuki, 1990). Furthermore, it is commonly reported that type I fibers are typically found in deeper muscle regions in mammals (Ryan et al., 1992). My results are consistent with this observation since type I fibers were restricted to the deep portions of PL, MG, and LG, while SOL (the deepest of the ankle flexor muscles studied) was almost entirely composed of type I fibers.

Type IIA fibers, which are best known to be suited for fast endurant actions (Hämäläinen and Pette, 1993), ranged in percentage from 7-28% in all muscles studied (Table 3). These percentages are considerably different than what Williamson and Frederick (2008) reported; however, they described type IIA and another highly oxidative fiber (likely type IIX) as FOG fibers in their study. By combining the percentages of type IIA and type IIX fibers reported in Table 3, the percentages would be similar to that reported by Williamson and Frederick (2008). With this fiber type grouping, the argument could be made that kangaroo rats require higher percentages of fast oxidative fibers in order to maintain a continual saltatory locomotion. However, if we consider that type IIX fibers are functionally similar to type IIB fibers, as suggested by Zhong et al. (2008), and group these fibers together, the percentages in the muscles studied would increase to 62%, 77%, and 77%, respectively. This high percentage of fast glycolytic fibers in three of the four ankle extensor muscles would enhance the argument that fast contracting fibers are responsible for the rapid explosive movement exhibited by the kangaroo rat during hopping.

Minimal type I/IIA hybrid fibers were observed in SOL (2.6%) but were not found in any other muscle. In contrast, type IIA/IIX hybrid fibers were observed in every muscle but SOL, ranging from 4-11% (Table 3). Hybrid fibers result from the co-expression of two different MyHC isomers (Stephenson, 2001) and are thought to arise during development and/or muscle regeneration (Korfage et al., 2005). The low percentage of hybrid fibers found suggest that there may be continual muscle regeneration occurring in these muscles relating to the variable muscle functions.

Comparison of muscle fiber types in hoppers

Bipedal hopping has independently evolved multiple times in both rodents and marsupials (McGowan and Collins, 2018). Previous muscle fiber type studies have been conducted on other hoppers such as jerboas (Jouffroy et al., 2003), and kangaroos and wallabies (Dennington and Baldwin, 1988).

Jouffroy et al. (2003) compared hindlimb muscles from a domestic rat (*Rattus rattus*) and a jerboa (*Allactaga elater*). Both animal's muscles contained four pure fibers (I, IIA, IIX, and IIB) and one hybrid fiber, though the authors did not state which MyHCs co-reacted to result in this hybrid fiber. The authors also neglected to provide percentages for all rat muscles, but they did report that the soleus muscle had an extremely high percentage of type I fibers in both the rat and jerboa (96%). All other ankle extensor muscles studied were dominated by type IIB fibers (Jouffroy et al., 2003). Furthermore, the authors reported that TA was primarily composed of type IIB fibers (85%), with a small amount of type IIA (11%) and IIX fibers (3%), and a negligible percentage (< 1%) of type I fibers. I described a similar fiber type composition in TA, except that I reported more IIX fibers (31%) and fewer IIB

fibers (51%) in the kangaroo rat compared to the jerboa. The only hybrid fiber reported by Jouffroy et al. (2003) was found at a low percentage (4%) in SOL. The authors didn't explicitly say which MyHCs co-reacted to result in this hybrid fiber, however, I also reported a hybrid fiber in SOL at a low percentage (3%) and determined that it was a type I/IIA hybrid. Thus, it is likely that this type I/IIA fiber is the hybrid fiber reported by Jouffroy et al. (2003).

Kangaroos are large bipedal hoppers that are capable of storing elastic energy in their tendons for later use, allowing them to recover up to 54% of their energy (Biewener et al., 1981). On the contrary, kangaroo rats have remarkably tough inelastic tendons (Javidi et al., 2019), allowing them to recover no more than 14% of their energy. Dennington and Baldwin (1988) studied ankle extensor muscles of the Western grey kangaroo (*Macropus fuliginosus melanops*) and the Parma wallaby (*Macropus parma*) classifying muscle fibers as type I, IIA, and IIB. In both the kangaroo and the wallaby, SOL was homogeneously composed of type I fibers (100%), which is comparable to the high percentage of type I fibers reported in kangaroo rat SOL (88%, Table 3). The other three kangaroo and wallaby ankle extensor muscles studied (PL, MG, and LG) were dominated by type IIA fibers (43-67%), with moderate percentages of type IIB fibers (20-37%), and minimal percentages of type I fibers (8-19%). These results do not coincide with my results from kangaroo rat ankle extensors, which were dominated by type IIB fibers. This discrepancy can be explained by comparing the elasticity, or lack thereof, of the ankle tendons in these animals. Since kangaroo (and wallaby) tendons are able to store elastic energy (Biewener et al., 1981), they can reduce the cost of muscle fiber contraction during hopping. Rather than supplying

quick and powerful contractions, muscles used for storing elastic energy in tendons use isometric contractions, where the muscle doesn't change length, and are most effective with oxidative fibers that permit tendon stretch and recoil (Hilber et al., 1999). On the contrary, kangaroo rats do not have elastic tendons (Javidi et al., 2019) capable of storing large amounts of energy (Biewener et al., 1981). Instead, they require muscle fibers to supply the quick and powerful contractions required for hopping. Thus, kangaroo and wallaby ankle extensor muscles need to have a higher proportion of type IIA fibers and kangaroo rats need to have a higher proportion of type IIB fibers.

Functional division of labor

Animals can maximize muscle function by having heterogeneous muscle fiber type compositions (Zhong et al., 2008; for review see Meyers, 2019). This permits a broad range of force generation and fatigue resistance options within a muscle (Armstrong, 1980). For example, in fishes, superficial oxidative fibers are utilized for stable swimming, whereas deeper fatigable fibers are utilized for burst swimming (Johnston, 1981). Trout muscles, in particular, contain primarily oxidative fibers (73%) because they have to continually swim against the river current; however, they do have a moderate amount of fatigable fibers (27%) to assist in predatory avoidance and prey capture (Goldbogen et al., 2005). Fiber type distribution in bird muscles can be related to their typical flight duration (Bandman and Rosser, 2000). Migratory birds, such as warblers and sparrows, have pectoralis muscles entirely composed of oxidative (type IIA) fibers for sustained flight (Bandman and Rosser, 2000). Nonmigratory birds, such as pigeons, have primarily oxidative (type IIA) fibers, but also have a population of large fatigable (type IIB) fibers for landing and takeoff when more

power is needed (Welsford et al., 1991). Within mammals, the triceps muscle group of the horse forelimb showed high percentages of oxidative (type I or IIA) fibers in two of the three muscle heads (medial and lateral) to sustain posture for a long period of time, whereas the long head of the triceps muscle contained primarily fatigable (type IIB) fibers for dynamic activities such as running (Ryan et al., 1992). Kangaroo rat ankle extensor muscles are composed of both oxidative and nonoxidative fibers, with nonoxidative (type IIB) fibers being predominate in all muscles except SOL. It stands to reason that this large percentage (33-63%; Table 3) of nonoxidative fibers is responsible for explosive hopping whereas the moderate percentage (14-28%; Table 3) of oxidative fibers is responsible for steady hopping.

CONCLUSION

Based on the fact that kangaroo rats are small bipedal mammals that are quick to escape predation and do not heavily rely on elastic energy storage, I expected to find high percentages of type IIB fibers in ankle extensor muscles, excluding soleus, and lower percentages of type IIB fibers in ankle flexor muscles. I found that ankle extensor muscle had high percentages of type IIB fibers and I related this finding to the explosive hopping behavior that the kangaroo rat exhibits. Unexpectedly, I found that the ankle flexor muscles also had high percentages of type IIB fibers which could function to prevent rapid hyperextension and act to decelerate the joint angle of the ankle during landing.

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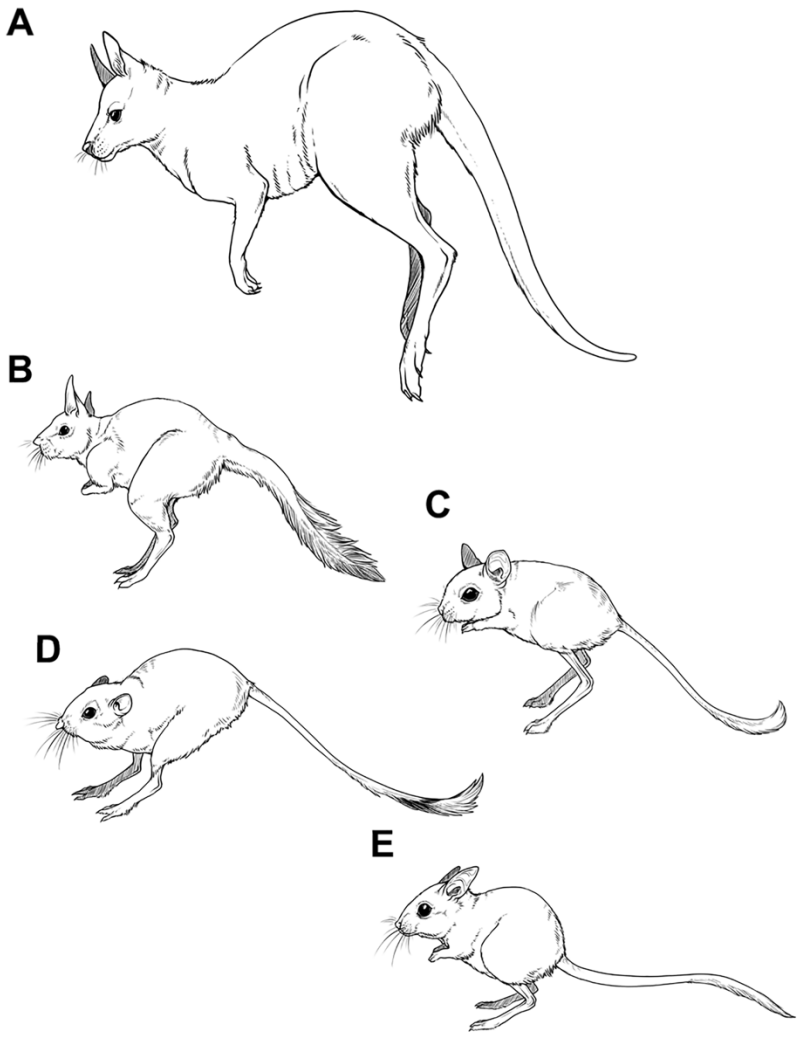

Figure 1. Drawings of representative species of five bipedally hopping mammalian lineages (adapted from McGowan and Collins, 2018). (A) Tammar wallaby (*Macropus eugenii*, mass=5kg). (B) South African springhare (*Pedetes capensis*, mass=2.5kg). (C) Desert kangaroo rat (*Dipodomys merriami*, mass=100g). (D) Lesser Egyptian jerboa (*Jaculus jaculus*, mass=120g). (E) Spinifex hopping mouse (*Notomys alexis*, mass=32g).

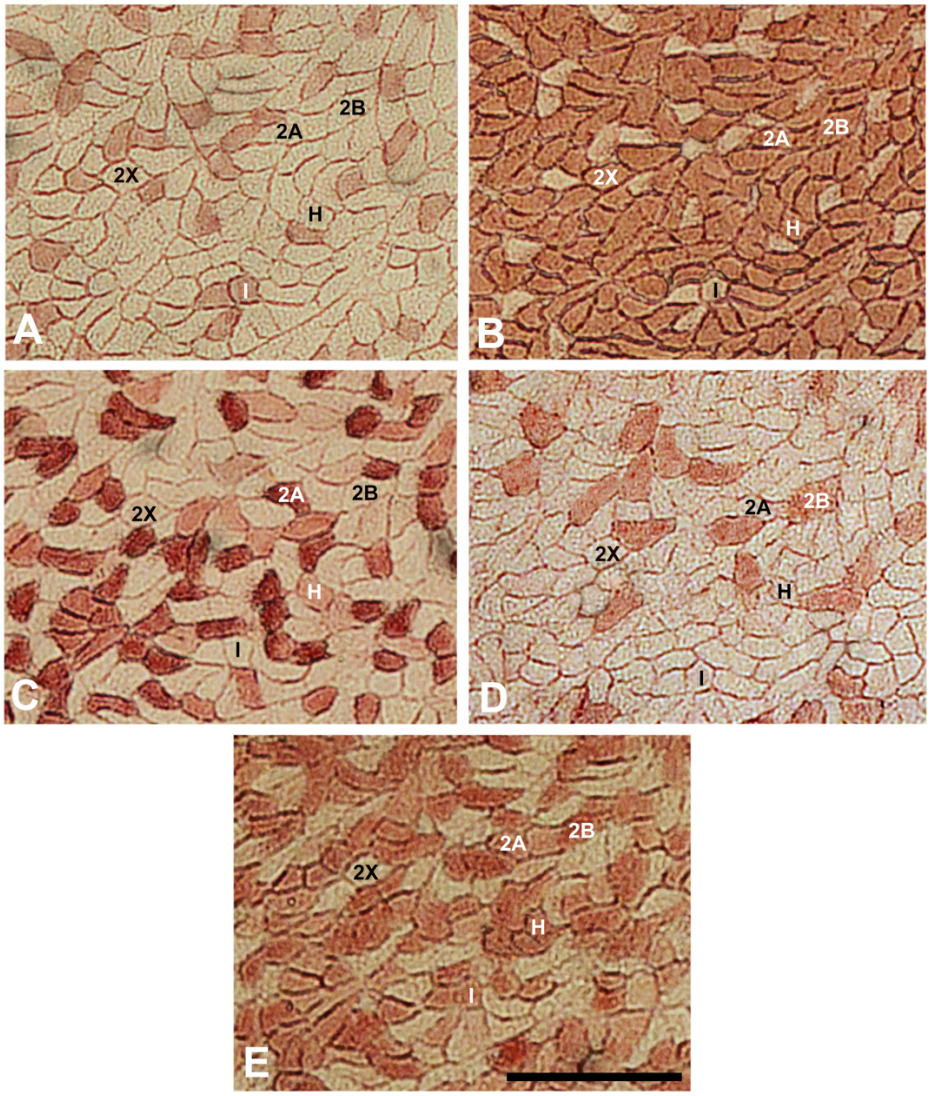

Figure 2. Serial sections of M. medial gastrocnemius from kangaroo rat 3 (*Dipodomys merriami*) were reacted with antibodies for fiber typing. (A) S-58 antibody, which reacts with type I fibers. (B) MY-32 antibody, which reacts with type II fibers. (C) SC-71 antibody, which reacts with type IIA and hybrid fibers. (D) BF-F3 antibody, which reacts with type IIB fibers. (E) BF-35 antibody which reacts with type I, type IIA, type IIB, and hybrid fibers, but not type IIX fibers. In each image, type I fibers are denoted with a 'I', type IIA with '2A', type IIB with '2B', type IIX with '2X', and hybrid IIA/IIX fibers with 'H'. Red cells are representative of positive reactions, light red/pink cells represent intermediate reactions, and white cells are representative of negative reactions. Scale bar: 0.05 mm.

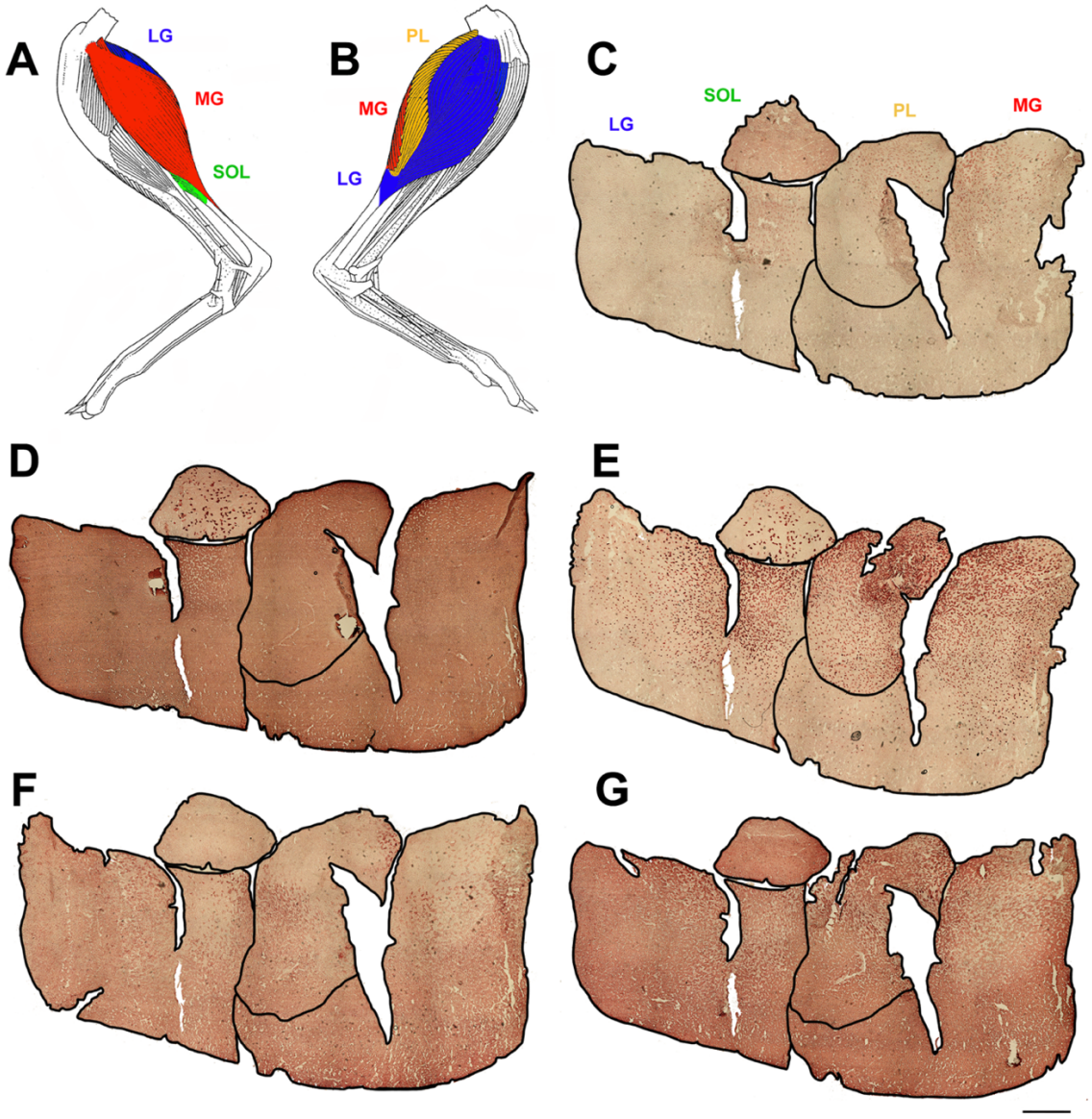

Figure 3. (A) medial view and (B) lateral view of the muscular anatomy of a right lower leg and foot of the kangaroo rat (*Dipodomys merriami*) highlighting the triceps surae muscle group. (modified from Howell 1932). Muscle abbreviations: SOL, soleus; PL, plantaris; MG, medial gastrocnemius; LG, lateral gastrocnemius. Five serial sections of these muscles are shown reacted with five different antibodies. (C) Antibody S-58 reacts with type I fibers; (D) MY-32 antibody reacts with type II fibers; (E) SC-71 antibody reacts with type IIA and hybrid fibers; (F) BF-F3 antibody reacts with type IIB fibers; (G) BF-35 antibody reacts with type I, IIA, IIB, and hybrid fibers, but not IIX. Red cells are representative of positive reactions and white cells are representative of negative reactions. Cranial is to the top of the page and medial is to the right of the page. Scale bar: 1mm, referring to the muscle cross-sections only.

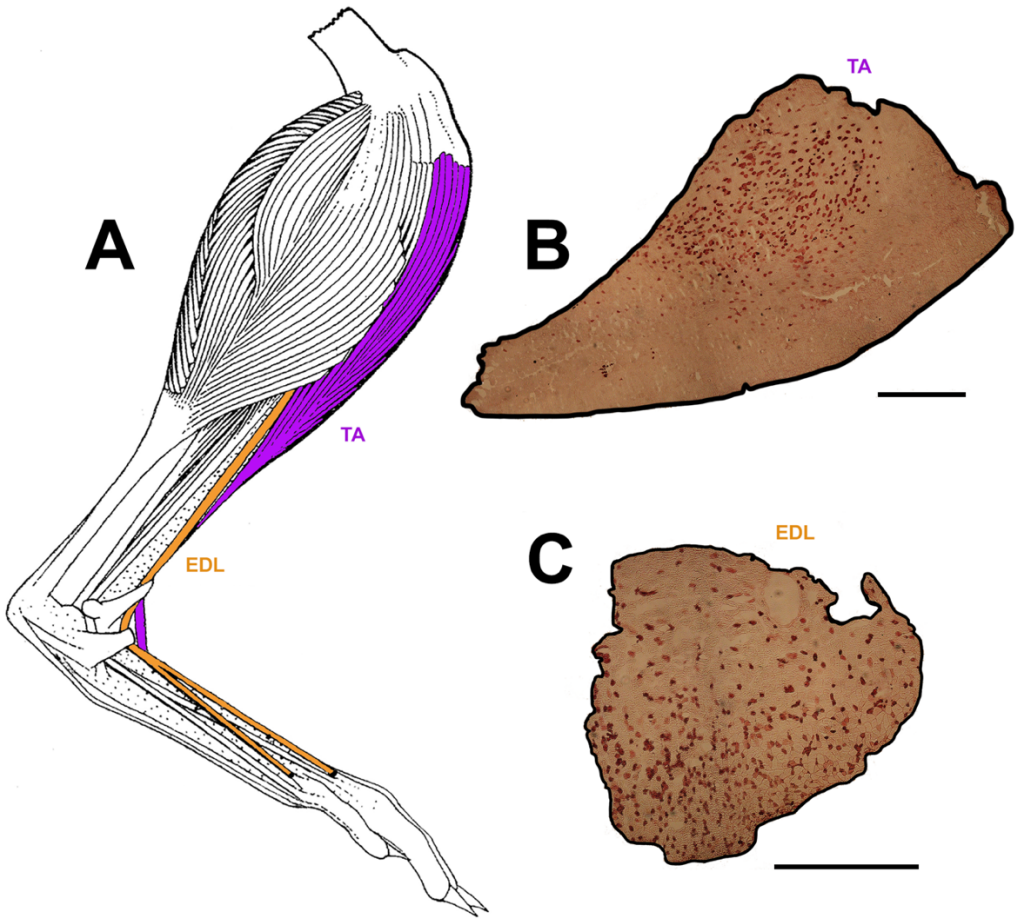

Figure 4. (A) Lateral view of the muscular anatomy of a right lower leg and foot of the kangaroo rat (*Dipodomys merriami*) highlighting muscles that preform dorsiflexion of the ankle joint (modified from Howell 1932). TA, tibialis anterior muscle; EDL, extensor digitorum longus muscle. Note that only the tendon of EDL is visible. Muscle cross sections for (B) TA and (C) EDL show SC-71 antibody reactions where type IIA fibers have a strong positive reaction and appear dark red and hybrid fibers have an intermediate reaction and appear light red/pink. Cranial is to the bottom of the page and medial is to the right of the page for (B) and (C). Scale bar: 1mm, referring to the individual muscle cross-sections only.

Table 1. Fiber type identification was accomplished using antibody reactions. An intermediate reaction is indicated by (+), a positive reaction is indicated by (++), a strong positive reaction is indicated by (+++), and a negative reaction is indicated by an empty cell.

	FIBER TYPE						
ANTIBODY	I	II	IIA	IIB	IIX	I/IIA	IIA/IIX
S-58	++					+	
MY-32		++	++	++	++	++	++
BF-F3				++			
SC-71			+++			+	+
BF-35	++	++	++	++		++	++

Table 2. Number of fibers from hindlimb muscles of kangaroo rats (*Dipodomys merriami*). Total fiber counts were obtained by using the MY-32 reaction unless otherwise noted. Fiber numbers denoted with (*) were counted using the unreacted cells from the SC-71 reaction. Fiber numbers denoted with (‡) were totaled by counting all cells in the SC-71 reaction. M denotes male and F denotes female.

		# OF FIBERS						
MUSCLE	Kangaroo Rat	I	IIA	IIX	IIB	I/IIA	IIA/IIX	Total
M. TIBIALIS ANTERIOR	1 M	5	422	1245	2464	0	213	5030
	2 F	8	204	1645	2546	0	190	4594
	3 M	2	405	1381	2102	0	228	4203
M. EXTENSOR DIGITORUM LONGUS	1 M	25	350	166	453	0	119	1168
	2 F	15	202	157	446	0	113	943
	3 M	6	212	152	383	0	101	854
M. SOLEUS	1 M	1119*	103	0	0	39	0	1261‡
	2 F	1238*	148	0	0	32	0	1418‡
	3 M	1537*	159	0	0	44	0	1740‡
M. PLANTARIS	1 M	126	872	771	1145	0	248	3047
	2 F	258	905	1192	1004	0	248	3612
	3 M	311	1206	1151	1231	0	310	3840
M. LATERAL GASTROCNEMIUS	1 M	97	404	589	2829	0	89	4037
	2 F	170	632	684	2828	0	299	4764
	3 M	371	1405	906	4491	0	543	7407
M. MEDIAL GASTROCNEMIUS	1 M	226	962	841	4085	0	188	6468
	2 F	384	868	1068	4543	0	204	7015
	3 M	522	1685	1263	4461	0	490	7730

Table 3. Percentages of different fiber types from six hindlimb muscles of kangaroo rats (*Dipodomys merriami*). The average and standard deviation of the three kangaroo rat percentages are included as well. M denotes male and F denotes female. (*) denotes significance at p<0.005 and (‡) denotes no significance between the fiber types.

		% OF FIBERS						MEAN % FIBERS ± SD					
MUSCLE	Kangaroo Rat	I	IIA	IIX	IIB	I/IIA	IIA/IIX	I	IIA	IIX	IIB	I/IIA	IIA/IIX
M. TIBIALIS ANTERIOR	1 M	0.1%	8%	25%	49%	0%	4.2%	0.13 ± 0.6%	7.3 ± 3.1%	31 ± 5.8%	51 ± 3.2%	0%	4.3 ± 0.6%
	2 F	0.2%	4%	36%	55%	0%	4.1%			*	*		
	3 M	0.1%	10%	33%	50%	0%	5.4%						
M. EXTENSOR DIGITORUM LONGUS	1 M	2%	30%	14%	39%	0%	10%	1.4 ± 0.7%	25 ± 4.5%	16 ± 2.1%	44 ± 4.2%	0%	11 ± 1.2%
	2 F	1.6%	21%	17%	47%	0%	12%		*		*		
	3 M	0.7%	25%	18%	45%	0%	12%						
M. SOLEUS	1 M	89%	8%	0%	0%	2.5%	0%	88 ± 1.0%	9.0 ± 1.0%	0%	0%	2.6 ± 0.2%	0%
	2 F	87%	10%	0%	0%	2.9%	0%	*	*				
	3 M	88%	9%	0%	0%	2.5%	0%						
M. PLANTARIS	1 M	4.1%	29%	25%	38%	0%	8.2%	6.4 ± 2.1%	28 ± 3.1%	29 ± 4.0%	33 ± 5.0%	0%	7.7 ± 0.8%
	2 F	7.1%	25%	33%	28%	0%	6.8%		*	‡	* ‡		
	3 M	8.1%	31%	30%	32%	0%	8.1%		‡	‡			
M. LATERAL GASTROCNEMIUS	1 M	2.4%	10%	15%	70%	0%	2.2%	3.7 ± 1.3%	14 ± 4.6%	14 ± 1.5%	63 ± 5.9%	0%	5.3 ±2.7%
	2 F	3.6%	13%	14%	59%	0%	6.3%		* ‡	‡	*		
	3 M	5.0%	19%	12%	61%	0%	7.3%			*	*		
M. MEDIAL GASTROCNEMIUS	1 M	3.5%	15%	13%	63%	0%	2.9%	5.3 ± 1.7%	16 ± 5.1%	15 ± 1.5%	62 ± 1.5%	0%	4.0 ± 1.9%
	2 F	5.5%	12%	15%	65%	0%	2.9%		* ‡	‡	*		
	3 M	6.8%	22%	16%	58%	0%	6.3%			*	*		