Myosin isoform fiber type and fiber architecture in the tail of the Virginia opossum (*Didelphis virginiana*)

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ABSTRACT

Myosin heavy chain (MHC) isoforms are the primary determinants of muscle fiber contractile properties, and therefore the distribution of slow and fast MHC fibers reflects functional specializations of muscles. Muscle architecture and fiber type are well studied properties in limb muscles, however, much less is understood about these properties in axial muscles, particularly those of tail musculature. Opossums are an interesting lineage in this context, as muscle fiber type has been studied in only two opossum marsupials and fiber typing of the tail in one species was limited to histochemical classification of fibers as either slow or fast. To expand on these previous studies, MHC isoform fiber type and their regional distribution (proximal/middle/distal) were determined in the tail of the Virginia opossum (*Didelphis virginiana*). Fiber type was determined by a combination of Myosin ATPase histochemistry, immunohistochemistry and SDS-PAGE. Results indicate a predominance of the fast MHC-2A and 2X isoforms in each region of the tail. The presence of two fast isoforms, in addition to the slow MHC-1 isoform, was confirmed by SDS-PAGE analysis. The overall MHC isoform fiber type distribution for the tail was: 25% MHC-1, 71% MHC-2A/2X hybrid, and 4% MHC-1/2A hybrid. Oxidative MHC-2A/2X isoform fibers were found to be relatively large in cross-section compared to slow, oxidative MHC-1 and MHC-1/2A hybrid fibers. A large percentage of fast MHC-2A/2X hybrids fibers may be suggestive of a evolutionary transition in MHC isoform distribution (slow-to-fast fiber type) in the tail musculature of an opossum with primarily a terrestrial locomotor habit vs. arboreal lifestyle.

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DEDICATION

I dedicate my Thesis to my parents who have worked very hard their entire lives for me and my family members.

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ATP - Adenosine tri-phosphate

CSA - cross-sectional area

GPD - α glycerol-1 dehydrogenase

IHC - immunohistochemistry

ms^{-1} - meters per second (velocity units)

Mabs - monoclonal antibodies

MHC - Myosin Heavy Chain

MyATPase - myosin ATPase

MyATPase histochemistry - classical technique of fiber typing

μm^2 - micrometers squared (CSA units)

NAD - Nicotinamide adenine dinucleotide

PCSA - physiological cross-section area (in cm)

SDS-PAGE – sodium dodecylsulfate-polyacrylamide gel electrophoresis

Introduction

Migration & Speciation

The Great American Interchange was an important geological event which allowed many species of animals to migrate between the two American continents. The occurrence of the interchange led to the joining of South America and North America via modern day Central America (Panama) to form the Americas 65 million years ago (Pascual & Jaurequizar, 1992; Cozzuol et al., 2006). Species migrating from North America to South America were able to establish themselves in large numbers and thus became very diverse. Radiation of these migratory species is often attributed to the extinction of many native South American species. However, invasion by species migrating from South America to North America proved to be more difficult. Many northwardly migrating animals were often unsuccessful at competing for resources with their native northern counterparts who already occupied and were successful in the same ecological niche. Therefore, southern species that survived in North America did not undergo much diversification (Simpson, 1980; Webb, 1991). Of these surviving species, opossums were among the most successful migrants allowed by the continental interchange, and their modern presence in both Central America and North America is explained entirely by the Great American Interchange (Webb, 1991). Opossums are part of an early class of mammals (marsupials) that evolved toward the end of the Mesozoic era (Cretaceous period) and were contemporaries of dinosaurs roaming the earth during that epoch. While most species of opossum still inhabit South America, the Great American Interchange allowed the successful spread of 13 species to Central America, and one species to North America, often referred to as the North American opossum or the Virginia opossum (*Didelphis virginiana*) (Nowak 1991; Hershkovitz, 1997).

Opossum are a specific order of marsupials known as Didelphimorphia. The family of American opossums is Didelphidae, which has 15 genera and more than 60 species ranging from North America to the southern-most regions of Argentina (Nowak, 1991; Hershkovitz, 1997). Figure 1 shows an abbreviated phylogeny of opossums. Regions where opossums are located coincide with their color. Species of opossum in northern territories have thick underfur that is white in color and has black tips. Overall this gives the animal a gray appearance because of pale guard hairs. Underfur is sparse in southern

species, and their fur is much darker in appearance than that of northern species and populations. The coloration of the feet also varies geographically. Predominantly, northern species show an area of white on their feet (Allen et al., 1901), while southern species typically have no variation of color on their feet as it matches the color of their dark fur. Despite variation in color patterns of body fur and feet, all species of opossum, regardless of geographic region, exhibit white cheek hairs (McManus, 1970) for acute facial sensory information.

As a group of mammals, marsupials are characterized as having distinct pouches to carry and nurture their young; however, only three genera of opossum have well-developed pouches: *Philander*, *Didelphis*, and *Chironectes*. The chironectes is an aquatic genus of opossum in which males and females both have a water-tight pouch. Opossum species belonging to the genera *Didelphis* and *Philander* are neither specifically arboreal nor terrestrial in their locomotor habit, but are all considered good climbers (i.e. scansorial habit). Similarly, species within these two genera overlap in diet, habitat, and the strata of the forest occupied (Hunsaker, 1977; Charles-Dominique, 1983; Emmons & Feer 1990). For example, one species of the genus Philander, *Philander opossum*, occupies a similar niche as several species of Didelphis in both South America (e.g. *Didelphis aurita*) and Central America (e.g. *Didelphis marsupialis*) (Streilein, 1982; Cerquwira, 1985; Emmons & Feer, 1990).

Despite occupying similar niches, there is notable size difference among genera of opossum, with species of Didelphis being much larger. In fact, two species of Didelphis have the largest body size of all opossums, *Didelphis aurita* and *Didelphis virginiana* (Grzimek, 1990; Nowak, 1991; Vieira, 1997; Hershkovitz, 1997). Moreover, members of Didelphis have longer forelimbs relative to their body size compared with species in other genera of opossum (Hildebrand, 1961; Vieira & Delciellos, 1997). This could be due to the fact that in certain marsupial lineages, longer and more developed forelimbs are needed by newborns in order to reach their mother's pouch shortly after birth. In newborn opossums the forelimbs are relatively well developed, but complete flexion at the joints is still not evident. The newborn opossum performs rhythmic and alternate movements of the forelimbs that help it maneuver to attach to the mother's nipple. Although, complete flexion is still underdeveloped at this time, slight flexion of one wrist

followed by flexion of the digits allows the newborn to grasp hair on the mother's belly to pull itself up to latch to the nipple. In contrast, its hindlimbs are immobile and have no visible articulations at this time in development. Newborn opossums are very immature and the entire animal is no more than an embryonic bud until about the third to fourth week of development. When detached from the mother after the fourth week, an opossum can lift the anterior part of its body with its forelimbs and can fully support the weight of its body. Not until the seventh week of development do the hindlimbs fully develop to where the opossum can bear its body weight on all four limbs (Pfieger et al., 1996). Fiber type data of opossum limb muscles (Hansen et al., 1987) support this developmental process, indicating different distributions of slow and fast muscle fibers between the forelimbs and hindlimbs in *D. virginiana*. A proximal-to-distal increase in the percentage of fast fiber types was observed in the Virginia opossum forelimb, while the reverse pattern of fiber type distribution was found in the hindlimbs (Hansen et al., 1987).

Fiber type distributions seen in the Virginia opossum hindlimb are typical of those seen in cursorial animals (i.e. running animals) that display elongated distal limbs with muscle-tendon specializations for fast and economical running over long distances (Hildebrand, 1960; Cavagna et al., 1977; Heglund et al., 1982; Biewener, 1983; Thomason, 1991; Butcher et al., 2009). Indeed, animals evolved for running have well developed hindlimbs rather than forelimbs. Furthermore, species of Didelphis are generally considered to be terrestrial walkers rather than terrestrial scamperers (e.g. smaller species), whom also require well developed hindlimbs rather than forelimbs (Hildebrand, 1988; Vieira & Delciellos, 1997). The forelimbs in all species of opossum are important for grabbing and vertically accelerating their body mass when climbing, and for maintaining balance on uneven or thin substrates such a branches during arboreal locomotion.

Members of the family Didelphidae also exhibit several features that are evolutionarily linked to other early (basal) mammals, and their more primitive reptilian predecessors. It has been inferred that the general body plan (i.e. shape) for opossums may be representative of an early stage in the evolution of mammals (Jenkins, 1971; Jenkins & Weijs, 1979). Perhaps this is well evidenced by the lack of specialization (i.e. functional compartmentalization) in the distal hindlimb muscles of *D. virginiana*. The

lateral gastrocnemius (LG) and soleus (SOL) are united as a single muscle compartment, much like the condition in some non-avian reptiles (Peters et al., 1984). The terrestrial iguanid lizard, *Dipsosaurus dorsalis,* is an example of a non-avian reptile with a single-headed gastrocnemius muscle, a more primitive analogue to more recent mammals where the gastrocnemius is compartmentalized into lateral and medial heads. It also interesting that the single-headed gastrocnemius muscle of this lizard has a similar histochemical profile to the single LG/SOL muscle compartment of the Virginia opossum hindlimb (Putnam et al., 1980). These two muscles became independent in more recent and derived mammals specialized for terrestrial locomotor function, such as running, which is strongly linked to fitness (survival) of those lineages. Therefore, as locomotor behaviors became more diverse, limb muscle (i.e. fiber type and muscle architecture) consequently became more specialized as mammals evolved. Contractile properties of muscles in the Virginia opossum may thus reveal patterns associated with body size and mode of locomotion (Peters et al., 1984).

Morphology

Each species (and genera) of opossum is uniquely characterized by the niche that they occupy and how each species maneuvers in their environment. Opossums exhibit an early and generalized body plan for mammals. A long snout, narrow braincase, and a prominent sagittal crest (similar to that in primates), are cranial features common to all opossums. Interestingly, opossum also have 52 teeth in their jaw, more than any other land mammal (Grzimek 1990; Hershkovitz, 1997). In contrast, features including body size (mass) and certain adaptations for specialized locomotor tasks and performance reflect speciation in opossum. Indeed, differences in body mass among species of didelphid marsupials are more evident than differences in body shape (Delciellos & Vieira, 2006). For example, the Virginia opossum can be characterized as a small-to-medium-sized marsupial. The smallest opossum are generally the size of a mouse, and the largest being the size of a domestic cat (*Felis domesticus*). Opossum range in body mass from 40 g to 4 kg, the latter being the average body mass of *D. virginiana*. These proportional differences are best exemplified in species with contrasting locomotor lifestyles: terrestrial *versus* arboreal *versus* semi-aquatic (Vieira, 1997). Specifically,

certain studies (Lemelin, 1999; Hamrick, 2001; Delciellos & Vieira, 2006) have linked smaller body size and other anatomical differences such as reduced claws and longer digits shown in arboreal species with better grasping ability for climbing, while terrestrial species have larger body mass and possess much broader feet with smaller digits, indicative of reduced grasping ability. Overall, terrestrial species are much larger and slower than their arboreal counterparts and occupy a different strata of the forest for locomotion (e.g. understory *versus* canopy) (Charles-Dominique 1983, Szalay 1994, Cunha & Vieira 2002, Delciellos & Vieira 2006, 2007). Interestingly, studies have also shown that arboreal species have well developed hindlimbs as compared to terrestrial species. Although bridging discontinuities between branch supports in trees is commonly achieved by way of their grasping ability and prehensile tail, arboreal didelphid marsupials also utilize jumping as an important part of their locomotory repertoire (Delciellos & Vieira 2009).

All species of opossum have a clawless, opposable first digit (*hallux*) on their hindfeet, which is analogous to the opposable great toe of arboreal primates. While the forefeet have five digits, all are clawed and the first digit is not opposable. The paws function in grasping and climbing, and aid in food gathering and its delivery to the mouth (Cutts & Krause, 1983). The feet of Didelphids also demonstrate a plantigrade foot posture, where their metatarsal (or metacarpal) bones are in contact with the substrate and they are supported by the entire plantar (or palmar) surface of the feet when walking (Peters et al., 1984). However, as they increase speed from a walk to a running gait, opossum may also assume a digitigrade foot posture and hold the ankle (or wrist) off the ground, leaving only the phalanx bones of the digits touching down. The preferred running gait of opossums is the quadrupedal trot, a gait that is mechanically similar to human running. The fastest recorded speeds for opossums are in the range of 1.8 m s^{-1} (Layne & Benton, 1954; McManus, 1970). For comparison, a similar-sized domestic cat can run at a top speed approaching 7.2 m s^{-1} (Van de Graaff et al., 1977). Part of the explanation for differences in top speed is the employment of a different running gait to achieve top speeds between opossums and cats (trot *versus* gallop).

Relating to locomotor habits, muscle fiber composition is another factor worthy of serious consideration. Previous studies of muscle fiber types in *D. virginiana* (Peters et

al., 1984; Hansen et al., 1987) revealed a large population of slow, fatigue resistant fibers (Type I) in hindlimb muscles. In one study, muscle fiber type distribution in the medial gastrocnemius (MG) was shown to be 53% slow fibers and 47% fast fibers. The LG/SOL was shown to have the opposite pattern, with an overall distribution of 39% slow fibers and 60% fast fiber types (Peters et al., 1984). Because of differential staining patterns and limitations of the myosin ATPase histochemical staining technique used, the presence of specific fast fiber types could not be identified (see below: section *Muscle architecture and fiber types*). In another study, a high percentage of fast fibers types (specific type also unidentified) were shown in several body regions including: shoulder muscles (68%), hip muscles (63%), and major muscles of both the upper (69%) and lower (77%) forelimb (Hansen et al., 1987). In that same study, the lower hindlimb muscles were shown to have an equal distribution of slow and fast fiber types (~50%). Similar studies of muscle fiber type in cats indicate hindlimb muscles (e.g. ankle extensors) are composed of larger populations of fast, high fatigability fibers (Type II 71%) (Burke et al., 1973; Burke & Tsairis, 1974; Stephens et al., 1975; Talmadge et al., 1995). Fiber type distributions in the limbs of *D. virginiana* are more comparable to those of the skunk (*Mephitis mephitis*), two animals with similar behavioral patterns of slow wandering and of scansorial habit (Van de Graaff et al., 1977). Selection on these traits are likely key pressures influencing phenotypic similarity in expression of slow, Type I fibers (and slow motor unit contraction velocities) in limb muscles of the Virginia opossum and skunk.

Prehensile Tail

The most distinguishing evolutionary trait exhibited by all species of opossum is a long, prehensile tail. The term prehensile is defined as the ability to grasp or hold objects. Opossums utilize their tail as a fifth appendage for hanging, climbing and grasping objects. Interestingly, opossums can also employ the grasping capability of their tail to carry leaves and grass when constructing their nests (Gardner, 1973). The tail is equally important for arboreal and terrestrial species in that it is used as a balancing organ, although there are different degrees to which this balancing organ is employed. Tail use defines animals as either fully prehensile or partially prehensile. Fully prehensile tails are for those animals that use the tail to hold and manipulate objects, and more importantly,

to aid in climbing, thus allowing arboreal animals to thrive in trees. Partially prehensile tails are not used for climbing, but instead allow animals to hang from branches and to use the tail for balance when maneuvering through complex three-dimensional environments (e.g. tree branches) (Grizimek 1990; Nowak 1991). The distribution of opossums with fully prehensile tails *versus* partially prehensile tails varies. Predominantly, South American species (e.g. *D. aurita*) have fully prehensile tails whereas species found in Africa, Southeast Asia and North America (e.g. *D. virginiana*) have partially prehensile tails. This could be due to the fact that the forests in South America are very dense, and comprise thick rainforest climates compared to North America and other regions of the world inhabited by opossums (Grzimek, 1990; Nowak, 1991; Hershkovitz, 1997).

For most species of opossum, typically the length of the tail is roughly proportional to head and body length. In Didelphids, tail length is exactly equal to its head-body length and species of this genus have the shortest tail length relative to body size among the Didelphids (Vieira, 1997). This suggests that climbing costs (i.e. metabolic energy consumption) may be higher for animals with a larger body size, although many species of Didelphis exhibit arborealism. Specialized morphological features such as body size and length of digits (see section *Morphology*, pg. 4) that vary among species could be more predictive of arboreal *versus* terrestrial locomotor behavior. Furthermore, the niche occupied is reflective of tail use and evolutionary specialization for locomotor behavior. All species have characteristic long tails with varying degrees of prehensile ability, but occupy different habitats. Of these habitats, the majority of opossums have an arboreal/semi-arboreal lifestyle whereby they dwell, forage and locomote above the ground (Grizimek, 1990; Hershkovitz, 1997; Vieira, 1997; Delciellos & Vieira, 2006, 2007, 2009).

Arboreal species exhibit anatomical specializations such as longer digits and narrow feet, compared to the feet of terrestrial species. Arboreal species of opossum are typically smaller in body size to minimize energetic costs associated with climbing (Nowak, 1991; Delciellos & Vieira, 2006, 2007, 2009). Their smaller size also provides them advantages for maneuvering among 3-D structures, bridging discontinuities in trees, competing for resources and escaping predation (Alexander, 2003; Delciellos & Vieira, 2009). In

contrast, species of opossum that are terrestrial in their locomotor habit, for example *D. virginiana*, have a larger body mass, shorter digits and broader feet than opossums with an arboreal lifestyle. They can climb trees and even hang from branches using their tail, but typically do not often engage in these adaptive behaviors except to avoid predation or possible compete for food resources (Grzimek, 1990; Hershkovitz, 1997). There are also instances where particular species can occupy a locomotory lifestyle that is either semi-arboreal or semi-terrestrial (Hunsaker, 1977; Szalay, 1994; Delciellos & Vieira, 2006). The relatively large species *D. aurita,* which inhabits the eastern Atlantic Rainforest in regions of Argentina, Brazil and Paraguay, is an example of a semi-arboreal opossum that is deft climber and often forages in both the rainforest understory and canopy (Hershkovitz, 1997; Cunha & Vieira, 2005).

Muscle Architecture & Fiber Type

Muscles have evolved to meet a variety of functions demanding gross differences in performance. Skeletal muscle is a contractile tissue that when excited, produces force and performs mechanical work. Muscles produce movement by shortening and pulling on the bones of the skeleton to which they are attached. As such, muscles have traditionally been viewed as work and power generating machines, or actuators (McMahon, 1987; Bagshaw 1982; Alexander, 2003; Crook et al., 2008). However, recent literature is replete with examples of muscles that perform in unexpected ways during routine locomotor tasks such as walking, running and hopping, critical to the survival of many animals (e.g. Roberts et al., 1997; Daley and Biewener, 2003; Biewener et al., 2004; Gabaldon et al., 2004; Payne et al., 2005; Watson & Wilson, 2007; Butcher et al., 2007, 2009). Findings from these locomotor studies indicate that limb muscles perform little-to-no mechanical work during locomotion by contracting isometric, or by undergoing lengthening contractions. Certain distal limb muscles in the horse (*Equus caballas*) can even function to absorb large amounts of mechanical energy by eccentric contraction (Wilson et al., 2001; Butcher et al., 2009) and produce high force economically, while other limb muscles can modulate their contractile function and work performance to match the demands of locomotion (e.g. running uphill *versus* downhill) (e.g. Roberts et al., 1997; Gabaldon et al., 2004). Therefore, muscles can be specialized for a variety of

functions. The arrangement and orientation of muscle fibers and composition of slow and fast muscle fibers reflect functional specializations of muscle. Relating muscle architecture and fiber type with performance is essential to understanding muscle function.

Skeletal muscles show remarkable diversity in the architectural arrangement of their muscle fibers. Some muscles have long fibers arranged longitudinally (parallel fiber architecture) indicating a specialization for substantial shortening and force production over a large range of contraction. Other muscles have shorter fibers that are arranged at an angle to the longitudinal axis of the muscle (pennate fiber architecture) indicating a specialization for higher force production and less mechanical work output (Biewener & Roberts, 2000; Zarucco et al., 2004; Butcher et al., 2009). Yet other muscles, often associated with muscular hydrostats, can have fibers arranged in longitudinal, circular or radial fiber architectures, and these types of fiber arrangements may be expected in other extremity muscles such as those in the penis and possibly the tails of some animals.

Depending on the functions required for survival in their niche, muscle fiber architecture differs among species of mammals. Therefore, specializations of muscles reflect the relationship between evolution and functional behavior, understood as a structure-function relationship, and also implies the metabolic cost associated with a functional behavior (Biewener & Roberts, 2000; Zhong et al., 2008). Minimizing metabolic cost during movement is a direct consequence of evolution, as energy savings are critical to survival. Important architectural properties that are proportional to force production and power generation, and thus metabolic energy consumption, are physiological cross-sectional area (PCSA) and muscle volume, respectively. Peak force production capacity of a muscle is determined by the total cross-sectional area of the fiber fascicles. PSCA also takes into account the angle or pennation of muscle fibers, which effectively increases the total cross-sectional area (Hollinshead, 1974; Bagshaw, 1982; McMahon, 1987; Alexander 2003). Likewise, muscle fascicle (fiber) length and fiber orientation relative to the long axis of a muscle determine force and range of contraction. Parallel fiber muscles have low PCSA due to long fascicles. In cursorial animals, this muscle architecture is often found in more proximal, long muscles where the short tendons form an in-line relation to the fiber fascicles (Alexander, 1993). Additionally,

parallel fiber muscles may often contain faster muscle fiber types for higher work and power generation capacity (Woodburne, 1978; Zarucco et al., 2004). This is understood as longer muscle fibers have a larger number of sarcomeres in series resulting in a greater change in length during contraction. As power is equal to the rate of work performed, and mechanical work is the product of force and length, muscles with longer and faster fibers have an advantage for high power generation but lower force production.

Pennate, or feather-like, arrangement of fascicles results in muscle fibers attaching to their tendons at an angle. There are several levels of pennate fiber architecture observed in skeletal muscles: unipennate, bipennate, multipennate and circumpennate. Unipennate muscles have relatively long muscle fibers attaching at an angle to one side of both the tendon of origin and tendon of insertion. Bipennate muscles have shorter muscle fibers attaching at an angle to two sides of the tendon of insertion. To understand this fiber architecture, it is appropriate to visualize the structure of a feather, with two sets of angled vanes connected to either side of the central shaft. Multipennate muscles are highly complex and display multiple levels of pennation. Multipennate muscles often have very short muscle fibers attaching between numerous aponeurotic tendon inscriptions passing longitudinally throughout the muscle belly. Other complex muscles have fibers from all directions attaching to a centrally located tendon of insertion. This fiber architecture is defined as circumpennate (Bagshaw 1982, McMahon, 1987) and is typical of the human supraspinatus muscle. In general, the higher the degree of pennation (= higher PSCA) the more specialized a muscle is for high force production (and not mechanical work) due to progressive shortening and higher pennation angles of the muscle fibers. Therefore, pennate muscle architecture sacrifices range of contraction and power generation for more economical high force production, especially during locomotor behaviors (Biewener & Roberts, 2000; Payne et al., 2005; Butcher et al., 2009). The energetic benefit of recruitment of a lower total volume of muscle fibers for a given amount of force is an advantage of pennate muscles over parallel fiber muscles.

Muscle architecture alone may be a determinant of function or role in locomotion. However, inherent contractile properties, such as maximum shortening velocity, are determined by the muscle composition of slow and fast fiber types, which are in turn determined by the type of myosin protein expressed as a myosin heavy chain (MHC)

isoform in single muscle fibers (Reiser et al., 1985; Schiaffino & Reggiani, 1996; Pellegrino et al., 2003; Toniolo et al., 2005). MHC isoform determines the rate of myosin ATPase activity. ATP is catalyzed by the myosin ATPase (i.e. myosin heads) to power the movement of actin filaments bound to the myosin heads forming acto-myosin cross bridges (Bagshaw, 1982). By catalyzing ATP at different rates, isoforms of MHC determine how quickly cross bridges cycle, and thus determine the velocity of fiber contraction. Therefore, whole muscle performance is a functional extension of the molecular composition of slow and fast myosin isoform proteins of muscle fibers (Schiaffino & Reggiani, 1996).

Identification of muscle fiber type and their constituent MHC isoforms is critical for the study of contractile properties of muscle tissue. Muscles can be composed of predominately slow fibers or fast fibers, or varying proportions of both slow and fast fiber types. Slow muscles (i.e. composed of predominately slow MHC isoform fibers) utilize ATP at a slow rate and thus produce relatively low levels of force, and perform low mechanical work, but are fatigue resistant. Fast muscles (i.e. composed of predominantly fast MHC isoform fibers) utilize ATP rapidly and thus produce higher force, and generate high power, but fatigue more easily as ATP stores are depleted (Bagshaw, 1982; Zhong et al., 2001; Zhong et al., 2008). Mammalian skeletal muscle fibers are classified into four conventional fiber types based on their MHC isoform: MHC-1 or slow, and three types of fast MHC-2A, MHC-2X and MHC-2B. Each fiber type is unique in its structure, function, biochemistry, and immunohistochemistry (Hoy et al., 1992). At the same time, each fiber type functions differently in its velocity of contraction, power, and endurance performance. Properties of each fiber type as they relate to expression in marsupials are considered below.

Marsupials are an early class of mammals that retain expression of all four conventional MHC isoforms in their skeletal muscles (Lucas et al., 2000; Zhong et al., 2001). MHC-1 is the slow contracting fiber type, while the three remaining MHC isoforms are all fast, and listed in order of increasing shortening velocity: 2A, 2X and 2B (Zhong et al., 2008). MHC-1 fibers (slow, oxidative) are highly aerobic and fatigue resistant, and have an abundance of mitochondria and myoglobin (muscle oxygen binding protein) present in the muscle cell. These muscle fibers are historically and

histochemically classified as "slow" because they use aerobic or oxidative cellular pathways that produce a high *net* ATP, but metabolize ATP at slow rates (Bagshaw, 1982). In contrast, fast muscles use both oxidative and glycolytic metabolic pathways to generate ATP. The predominant metabolic pathway used is strongly dependent on the fast MHC isoform expressed in an individual fiber. Glycolysis produces far less ATP (*net* = 2), but fast MHC isoforms metabolize ATP at more rapid rates and thus fatigue more rapidly (Bagshaw, 1982; Miller et al., 1989). Consistent with this notion, fast muscle fibers types will be discussed in order of their velocity of shortening.

MHC-2A fibers (fast, oxidative) have contractile properties similar to MHC-1 fibers. They are rich in mitochondria and myoglobin, which give them a characteristic "red muscle" appearance commonly associated with slow muscles (Bagshaw, 1982). Despite having similar or higher oxidative capacity, MHC-2A fibers shorten at higher velocities and consequently generate more power than MHC-1 fibers. MHC-2X fibers are more glycolytic and contract with greater force, shorten at higher velocities and generate more power compared with either the slow or fast oxidative fiber type (Zhong et al., 2001; Zhong et al., 2008). However, MHC-2X fibers fatigue more quickly (medium-to-low oxidative capacity) because their anaerobic cellular pathways make limited amounts of ATP that only allow for shorter duration bursts of muscle activity. MHC-2B fibers (fast, glycolytic) exclusively utilize glycolysis to generate ATP rapidly, but also fatigue very quickly. These fibers have comparatively few mitochondria and less myoglobin than the other fiber types, which give them a characteristic "white muscle" appearance (Bagshaw, 1982). MHC-2B fibers contract with the greatest force, shorten at highest velocity and generate the most power. Interestingly, marsupials of a variety of body sizes appear to maintain expression of the fast MHC-2B isoform (Lucas et al., 2000; Zhong et al., 2008) in their limb muscles. This is uncommon in lineages of eutherian mammals (i.e. placental mammals) where animals with increasingly larger body sizes (e.g. dog, humans, horse) fail to express all four isoforms and lack MHC-2B fibers (Smerdu et al., 1994; Toniolo et al., 2007; Zhong et al., 2008).

Usually, only one MHC isoform is expressed in a single muscle fiber (Reiser et al., 1985). However, co-expression of MHC isoforms (called hybrid fibers) can occur in a single fiber. Hybrid fibers are suggested to occur during MHC transitions. Fiber type

transitions occur in conjunction with physiological parameters such as functional load, patterns of use, and hormonal levels (Lucas et al., 2000). Hybrid fibers showing co-expression of MHC-1/2A are most common, while 2A/2X and 2X/2B hybrids are also known to occur (Miller et al., 1989).

Determination of the slow and fast properties of muscle fibers can be accomplished by three well established techniques. The first technique, myosin ATPase histochemistry (MyATPase), involves both alkaline and acid incubation of muscle tissue to determine histochemical fiber type (Brooke & Kaiser, 1970; Hermanson & Cobb, 1992; Hermanson et al., 1998; Rivero et al., 1999b; Smerdu et al., 2009). Briefly, slow fibers are acid stable (stain dark) while fast fibers are either acid labile or mildly acid stable. Conversely, all fast fibers are alkaline stable while slow fibers are alkaline labile (do not stain) (Brook & Kaiser, 1970). The second technique, immunohistochemistry (IHC), involves staining muscle tissue with monoclonal antibodies (Mabs) specific to MHC isoforms (Lucas et al., 2000; Rivero et al., 1996; Zhong et al., 2001, 2008). The third technique, SDS-PAGE, involves using electrophoresis to separate the myosin isoform proteins by their molecular weight (Talmadge & Roy, 1993; Stienen et al., 1996; Rivero et al., 1999a; Lucas et al., 2000; Toniolo et al., 2005, 2007). These three techniques compliment one another by: 1) evaluating the overall distribution of slow and fast fiber types and their oxidative/glycolytic capacities, and 2) identifying the exact MHC isoforms present in single fibers that ultimately determine the slow or fast contractile properties of whole muscle. A combination of muscle fiber architecture such as fiber cross-sectional area, in addition to fiber typing by each of the three techniques, provides a powerful analysis tool for fundamentally understanding structure-function relationships of muscles.

Muscle Studies in Opossum

Architecture and fiber type are well-studied properties in limb muscles from a diversity of vertebrate lineages, but these are not well understood for axial muscles such as tail musculature. Moreover, muscle physiological studies involving mammals other than small eutherians such as mouse, rat or rabbit models are highly underrepresented in the literature. Opossum marsupials therefore make an interesting model of study for muscle fiber architecture and fiber types. In addition to the paucity of physiological data for most

species of opossum, only two studies (Peters et al., 1984; Hansen et al., 1987) are known to have evaluated muscle fiber types in the Virginia opossum, and only one of these studies (Hansen et al., 1987) examined fiber type in the axial muscle of the tail. The tail of *D. virginiana* was reported to be composed of predominately fast fibers with fast fiber type distributions specified by the following regions: proximal (90%), middle (60%) and distal (71%). However, the analysis was limited to MyATPase histochemistry, which shows only the presence of slow and fast fiber types, but does not identify specific MHC isoforms of fast fiber types (Zhong et al., 2008), and has been shown to be inconsistent when used on marsupial limb muscles (Peters et al., 1984; Sciote & Rowlerson, 1998). Knowing the specific identity of fast MHC isoforms provides a clearer understanding of the intrinsic patterns of muscle structure-function in the tail. In addition, Hansen et al. (1987) also reported peculiar staining patterns in the distal region of the tail, further suggesting the inadequacy of MyATPase histochemistry alone to correctly identify fiber types in the tail of opossums. To date no further studies have been done to explain the functional significance of these findings or identify the MHC isoforms present in the tail of the Virginia opossum.

Objectives & Hypotheses

The primary objective of the study is to determine MHC isoform fiber type and fiber architecture in the axial tail musculature of the Virginia opossum (*D. virginiana*). The specific goals of the analyses presented in this thesis are: (i) determine specific MHC isoform fiber types present in the proximal, middle and distal regions of the tail and (ii) measure the cross-sectional area and diameters of each MHC fiber type present in the tail in *D. virginiana*. MHC fiber type will be determined by a combination of MyATPase histochemistry and IHC staining techniques. Constituent slow and fast MHC isoforms identified by reaction with Mabs against specific MHC isoforms will be verified by electrophoresis techniques (SDS-PAGE). Muscle fiber architecture will be assessed by precise microscopic measurement of muscle fiber morphology.

It is hypothesized that the population of fast muscle fibers identified will contain a majority of the fast, MHC-2A isoform. Despite being a scansorial or climbing animal, *D. virginiana* prefers a terrestrial locomotor habit, where the tail is used to support transport

of young on the dorsal body surface, and carry objects to aid in constructing ground nests. These tasks would require periods of prolonged force production typical of highly oxidative fibers. Alternatively, fast muscle fibers identified in the tail of *D. virginiana* may contain a majority of the fast, MHC-2X isoform or fast hybrid fibers. These findings would be suggestive of an evolutionary change in patterns of usage of the tail by *D. virginiana* and a consequent transition in muscle fiber type to faster isoform expression because of apparent disuse. The later results would suggest and support an evolutionary view that ecology and anatomy are closely linked. Species evolve anatomical specializations to increase their fitness in their respective habitats. Inherent to these hypotheses is the evolution of certain tail muscle specializations for energy conservation during adaptive behaviors seen in opossums.

Materials & Methods

Animals

Five (*N*=5) Virginia opossums (*Didelphis virginiana* Kerr) were used in this study (Table 1). Male and female opossums were live captured in the field (Mahoning County, Ohio). Opossums were captured using Havahart easy catch traps and transported to YSU for euthanasia by overdose IP injection of a sodium pentobarbital solution (Euthasol® Virbac AH, Fort Worth, TX USA). Animal trapping (ODNR Wild Animal Permits: 10-252, 11-292) and all experimental procedures followed approved protocols (YSU, IACUC protocol: 03-09; PI: M.T. Butcher).

Muscle Tissue Harvesting & Sectioning

Tail length was measured (in cm) with a tape measure and the entire tail was removed for dissection post-mortem. The tail was divided into proximal, middle, and distal thirds for muscle dissection. Small sections of muscle tissue were harvested from longitudinal bands of muscle positioned laterally (two lateral muscle bands) and ventrally (one ventral muscle band) along the tail vertebrae. Harvested muscle tissue sections were prepared for freezing and storage by mounting the fascicles to 1x1 inch cork blocks in Tragacanth (Sigma-Aldrich, St. Louis, MO USA) using methods of Hermanson et al. (1998). Tail muscle fascicles were mounted vertically in the orientation of cranial-to-caudal, where

cranial was the end of the fascicle not completely embedded in the mounting media. Fascicles were also mounted by aligning superficial and deep (aligned to the left) regions of the fascicles consistently for muscle tissue sectioning and imaging purposes. Upon completion of mounting, muscle tissue samples were immediately snap-frozen in isopentane (2-methylbutane) cooled by liquid nitrogen (Hermanson & Cobb, 1992; Hermanson et al., 1998). Samples were allowed to dry for at least 30 min on dry ice (-70ºC) in a styrofoam cooler after removal from isopentane. This was done to minimize freezing artifact, which is caused by tissue undergoing repeated freeze-thaw cycles while preparing tissue for deep freezing. Muscle tissue samples were finally wrapped in parafilm (to seal in moisture), placed in small plastic bags and quickly removed to a freezer for storage at -80°C.

Muscle tissue was cut in serial sections at a uniform thickness (10 μm) using a cryostat (Model 1850: Leica Microsystems, Wetzlar, Germany) at Northside Hospital in Youngstown, OH. Before cutting, frozen tissue samples were allowed to temperature equilibrate in the cryostat (-20ºC) for 1 hr. This was done to ensure even thickness of frozen sections. Sections of muscle tissue were mounted to either 75x25mm glass microslides (GOLD SEAL® micro slides; Becton Dickinson & Co., Portsmouth, NH USA) for metabolic and MyATPase staining procedures or 75x25mm charged Superfrost® slides (12-550-15; Fisher Scientific, Fairlawn, NJ USA) for IHC analyses. Muscle tissue was cut and mounted to slides for metabolic and MyATPase staining and placed in glass coplin jars at room temperature. Slides mounted for IHC were retained at -20ºC until 5 mins prior to the time of monoclonal antibodies (Mabs) application.

Histochemistry

Fiber type staining was performed using established methods (Hermanson & Cobb, 1992; Hermanson et al., 1998; Soffler & Hermanson, 2006) derived from the original MyATPase staining protocols of Padykula and Herman (1955), Guth and Samaha (1970), and Brooke and Kaiser (1970). Oxidative and glycolytic properties of fibers were first estimated by metabolic staining procedures on continuous serial tissue sections. Serial sections of muscle tissue were treated with either Nicotinamide adenine dinucleotide (NAD) to assess oxidative potential of muscle tissue or α-glycerol phosphate

dehydrogenase (α-GPD) to assess glycolytic potential of muscle tissue. Sections of muscle tissue tested with α-GPD were incubated for 35 min at 35°C (water bath) in a solution containing 0.2 M phosphate buffer (pH 7.4), 90mg α-GPD, 12mg Nitro Blue Tetralzoium (NBT) and 12mg menadione, while tissue tested with NADH were incubated for 10 min at 35°C in a solution containing PBS buffer (pH 7.4), 15mg NADH and 30mg NBT.

A second set of serial tissue sections were stained by a series of MyATPase reactions. Slides were initially incubated at 35ºC in either alkaline (pH 9.4, 10.1, 10.2, 10.3, 10.4; 10 min) or acidic (pH 4.3, 4.4, 4.5, 4.6; 5 min) pre-incubation solutions. The alkaline preincubation medium contained glycine buffer, 0.15 M NaOH, and 0.175g $CaCl_2$ (Hermanson & Cobb, 1992). The acidic preincubation medium contained 0.2 M Barbital Acetate buffer, 0.1 M HCL, and dH_2O (Hermanson et al., 1998). After 10 min (total pre-incubation time), all samples were removed from the water bath and alkaline slides were rinsed in dH_2O ("superwater", dH_2O (pH 8.6-9.0)) while acid slides were rinsed in a Sodium Barbital buffer with the following formulation: 4.12g sodium barbital, 1.98g $CaCl_2$ and 1L dH_2O (Hermanson & Cobb, 1992). Next, all slides were then reacted in an ATP incubation medium (pH 9.4; 100ml sodium barbital buffer and 0.15g ATP) for 30 min at 35ºC (body temperature for opossum). Following ATP incubation, all slides were again removed from the water bath and rinsed in 'superwater' before being alternately washed in 1% $CaCl_2$ and 2% $CoCl_2$ solutions between dH_2O rinses. Finally, all slides were reacted in a 1% ammonium sulfide solution and washed with running tap water (5 min) before being dehydrated in a series of ethanol dilutions (70%, 80%, 90%, 100%) and lastly dried in Xylenes (Hermanson et al., 1998). Dehydrated tissue slides were mounted with glass covers (22x22-1) using Permount® (Fisher Scientific, Fairlawn, NJ USA).

Immunohistochemistry

MHC isoforms in tail muscle fibers were identified by IHC analyses using a combination of methods as described by Hermanson et al. (1998), Lucas et al. (2000) and Zhong et al. (2001, 2008). Briefly, freshly cut and mounted serial sections of muscle tissue were removed from the cold (-20ºC) and allowed to warm to room temperature for 5 min

before a PAP pen (Liquid Blocker: Newcomer Supply, Middleton, WI USA) was used to encircle sections of muscle tissue, providing a hydrophobic well for application of Mabs (anti-mouse). A blocking solution (Histostain Plus Kit; Invitrogen Corp., Carlsbad, CA USA) was first applied to all muscle sections for 10 min. Next, Mabs specific to one of the four MHC isoforms (see Table 2) were applied to tissue sections and reacted for 12-16 hours in a humid chamber at 4°C. Reaction to Mabs was followed by reaction against a polyclonal secondary antibody (anti-mouse) for 1 hour at room temperature, and then DAB staining (3-6 min) in the dark using a Histostain Plus kit (Invitrogen). After a final dH_2O wash (10 min), all slides were secondarily stained with Hematoxylin (Mayer modified; Newcomer Supply) to visualize fiber morphology and nuclei. Briefly, slides were reacted in hematoxylin (1% aqeous) for 15 min (Hermanson et al., 1998), washed in dH_2O (3 min), dehydrated in a series of ethanol dilutions (70%, 95%, 100%), and finally dried in Xylenes to be mounted with glass covers (22 x 22-1) using Permount®.

Monoclonal antibodies specific to slow and fast MHC were applied in combination to serial muscle tissue sections (2 serial sections per slide). Specifically, an anti-slow MHC antibody was commonly applied to one tissue section, while an anti-fast MHC antibody was applied the other. This was done to provide a reaction standard (+/-) for direct comparison of serial sections to correctly identify numbers of slow and fast MHC isoform fiber types on each slide. A slide mounted with muscle tissue from rat lateral gastrocnemius/soleus (LG/SOL) or tibialis anterior (TA) that was reacted against the same combination of Mabs to provide a specificity standard for slow and fast MHC isoform reactivity. Additionally, one slide of opossum tail muscle tissue was <u>not</u> reacted against Mabs but instead, treated only with blocking solution or PBS, and served as a control slide for experiments.

A diverse set of monoclonal antibodies specific to MHC isoforms in a number of eutherian and marsupial species, were used to thoroughly identify MHC isoform fiber types in tail of *D. virginiana* (Table 2). Mabs against slow myosin (MHC-1) were S58 (DSHB, University of Iowa, Iowa City, IA USA) and MHC-s (Vector Laboratories, Burlingame, CA). These antibodies were specific to MHC-1β in numerous mammalian species (Miller et al., 1985). Mabs against fast myosins were MY32 (Sigma-Aldrich, St. Louis, MO USA), F18 and F59 (DSHB). These antibodies were specific to all fast MHC

in numerous eutherian mammals ranging from rat (Naumann & Pette, 1994) to horse (Butcher et al., 2010). Additionally, we used three anti-fast Mabs specific to rat (Schiaffino et al., 1998): SC71 (ATTC, Raleigh, NC USA) to identify the fast MHC-2A fibers, BF-35 (DSHB) to identify all fast MHC except fast MHC-2X fibers, and BF-F3 (DSHB) to identify the fast MHC-2B fibers (Table 2). Lastly, we used two Mabs specific to marsupial fast MHC (Lucas et al., 2000): 6H1 (DSHB) to identify pure MHC-2X fibers, and 10F5 (DSHB) to identify pure MHC-2B fibers (Table 2).

Electrophoresis

Identity of MHC isoforms in opossum tail muscle fibers was verified by SDS-PAGE analysis using a combination of methods described by Talmadge & Roy (1993) and Mizunoya et al. (2008). MHC isoform composition was analyzed in fascicles adjacent to muscle tissue harvested for histo/immunohistochemical experiments in *D. virginiana*. Muscle bundles were dissected from the lateral and ventral bands of muscle in the proximal, middle, and distal regions of each opossum tail (n=5) with a systematic method of sampling from 3-5 proximal-to-distal locations. Three bundles from each tail region were placed in microcentrifuge tubes and snap-frozen in liquid nitrogen for 1 min and stored at -80ºC.

MHC isoform proteins were identified by evaluating migration patterns of bands of myosin extracted from the small muscle bundles. Briefly, frozen muscle bundles were ground to a powder using a mortar and pestle in a bath of liquid nitrogen (Mizunoya et al., 2008). Weighed frozen muscle samples (50 mg) were solubilized for 2 hrs at room temperature in Laemmli solution (Laemmli, 1970) containing 62.5 mM Tris (pH 6.8), 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, with 0.1% dithiothreitol (DTT) and 0.1% leupeptin as anti-proteolytic factors (Toniolo et al., 2007). Aliquots of protein stocks (20 µl) were prepared in separate microcentrifuge tubes and diluted with 2X gel sample buffer (100 mM DTT, 4.0% SDS, 0.16 M Tris (pH 6.8), 43% glycerol, and 0.2% bromophenol blue) and dH_2O (Mizunoya et al., 2008) to give final protein concentrations of approximately 0.125 µg/µl. Denatured protein samples were stored overnight at -20°C.

Prior to SDS-PAGE, protein samples were fully mixed (by vortexing) and boiled for 5 min at 95ºC. The acrylamide-*N, N'*–methylenebisacrylamide (Bis) ratio of the gels was

50:1 (Talmadge & Roy, 1993), with total acrylamide percentage equaling 8% and 4% in the separating and stacking gels, respectively. The separating gels consisted of 35% glycerol, 0.2 M Tris (pH 8.8), 0.1 M glycine, and 0.4% SDS. The stacking gels consisted of 30% glycerol, 70 mM Tris (pH 6.8), 4 mM EDTA, and 0.4% SDS (Mizunoya et al., 2008). The gel apparatus was filled with electrode buffer with a basic formulation of 0.05 M Tris (base), 75 mM glycine and 0.5% SDS (Mizunoya et al., 2008). The upper chamber electrode buffer was set at 6X the concentration of the lower buffer with an added 0.12% β-mercaptoethanol (Mizunoya et al., 2008). Typically 1-3 µl of each protein sample were loaded into each gel lane (20 lanes total; 2 mini gels). A high molecular weight standard was loaded into lane 5 of each gel. Electrophoresis was performed using a mini-gel protein system (mini-PROTEAN Tetra; Bio-Rad, Hercules, CA USA) at constant low voltage (140 V) for 22 hrs in a refrigerator maintained at 4°C (Talmadge & Roy, 1993; Mizunoya et al., 2008). Gels were silver stained (ProteoSilver™ Silver Stain Kit; Sigma Chemical Co., St. Louis, MO USA) photographed, and lastly wrapped (moist) in plastic wrap for storage at 4ºC.

Data Analysis

Images of serial sections of opossum tail muscle tissue were obtained with a 5MP digital camera (SPOT Idea: Diagnostic Instruments, Sterling Heights, MI USA) mounted to a compound light microscope (CX31: Olympus Microscopes, Center Valley, PA USA). Images were commonly captured at magnifications 4-10X using SPOT software (v.4.6.1; Diagnostic Instruments) run on a Macintosh mini computer (Apple Inc., Cupertino, CA USA). Percentage MHC fiber type from MyATPase and IHC analyses was quantified in numerous whole sections of muscle fibers with a minimum of ~1000 fibers counted from each animal (n=5). Percent distribution of MHC isoforms for the overall population of fibers was also quantified. For SDS-PAGE analysis, comparisons with protein band migration patterns of rat LG/SOL and TA (muscle prepared by the same methods as for opossum tail) were used to confirm the exact location of fast MHC isoforms bands in tail muscle from *D. virginiana*. MHC isoform identity was additionally verified by using published reports (rat: Bottinelli et al., 1991, 1994; marsupial: Zhong et al., 2001) as the different MHC isoforms have a specific migration sequence in the gel lanes (i.e. slow

MHC-1 isoform migrates fastest to lowest position in gel lane, followed by MHC-2B, then MHC-2X and MHC-2A for rat and marsupial skeletal muscle). The high molecular weight standard additionally confirmed protein bands evaluated were in the MW region for myosin (~200 kDa).

Fiber cross-sectional area (CSA) and diameter were determined using the software program Image J (v.1.43; NIH, Washington D.C. USA). Images of serial sections of opossum tail muscle tissue were initially calibrated for length in μm (in SPOT software) by use of a length calibration slide (500 μm; image captured under 4X, 10X and 20X magnifications). Images were then imported into Image J and recalibrated for a known length in μm to pixels. In Image J, fiber CSA was measured using the freeform tool to trace the outer perimeter of fibers followed by use of an area function to calculate CSA in μm^2. Fiber diameter was measured using the freeform tool in Image J to span fiber outer diameter in μm. Fiber CSA and diameter was determined in a subset of n=250 fibers minimum for each MHC isoform fiber type correctly identified in *D. virginiana*. All measured data were organized and descriptive statistics tabulated in MS EXCEL (MS Office 2008; Microsoft, Redman, WA USA) on a Macintosh laptop computer (MacBook Pro; Apple Inc.).

Statistics

All muscle fiber type data are presented as whole percentages (not average percentages). Fiber CSA and diameter data are presented mean±SD (standard deviation). No statistical tests were performed to assess significance on these measurements. The end goal was to present muscle fiber type and fiber architecture measurements representative for the entire tail of *D. virginiana*.

Results

Metabolic Activity of Opossum Tail Muscle Fibers

Strong NAD activity was evident in all muscle fibers from each tail region (Fig. 2D), indicating high oxidative capacity in Virginia opossum tail muscle. Moderately dark NAD staining was uniform across all sections of muscle tissue analyzed. Furthermore, NAD activity was consistently high for each tail region and animal studied irrespective of

incubation time and temperature (both varied in some experiments). Conversely, α-GPD activity was weak in all muscle tissue sections analyzed from each tail region (Fig. 2E), indicating low glycolytic potential in the tail muscle *D. virginiana*. Muscle sections consistently stained lightly in reaction to α-GPD verifying the high oxidative capacity of the tail muscle fibers. The low glycolytic activity also indicated a lack of pure MHC-2B fibers that show high glycolytic potential and have low oxidative capacity. No differences in both NAD and α-GPD activity were observed regardless of gender or body mass of opossums used for this study *(N*=5).

MyATPase Activity of Opossum Tail Muscle Fibers

Three fiber types in the tail of *D. virginiana* could be distinguished based on reactions in an alkaline (10.3) and acidic incubations (4.4, 4.5). Fast MHC fibers retained their MyATPase activity during alkaline incubations, and each muscle section analyzed showed large numbers of darkly stained fibers, while slow MHC-1 fibers were unstained or labile (data not shown). After acid incubations, three different staining intensities of the MyATPase activity were commonly observed across sections muscle fibers from each tail region. Images of histochemically stained (in acid incubation pH 4.4) tail muscle tissue are shown in Figs. 2A, 3A and 4A. Fibers darkly stained in acid incubations pH 4.4 and 4.5 (highly acid stable) were classified as slow, MHC-1 fibers. Fibers lightly stained at these acidic pH values were best classified as fast MHC-2A/2X hybrids (Rivero et al., 1996, 1999b). A third group of moderately stained fibers (moderately acid stable: see Figs. 2A, 3A) were classified as slow/fast hybrid fibers and these fibers were matched to reactivity against anti-slow and anti-fast MHC Mabs in IHC analyses (Fig. 4C, D). However, fibers that stained moderately in acid incubation could not be clearly identified as pure fiber types. This was in part due to inconsistent staining intensities of fibers that could be classified as either pure MHC-2X or fast hybrids over the range of acid incubations, and the inability to clearly distinguish pure MHC-2A fibers (normally unstained or acid labile at pH 4.4) based on acid MyATPase histochemistry alone (see Peters et al., 1984; Scoite & Rowlerson, 1998). At all pH values for acid incubations, fiber staining intensified only slightly with lower pH (range 4.3-4.6). The best resolution for MyATPase histochemistry for all animals studied occurred at acid pH 4.4.

Immunohistochemical Determination of MHC Isoforms

Three distinct MHC isoform fiber types were clearly identified using monoclonal antibodies specific for MHC isoforms, matching well with results of MyATPase histochemistry. Table 2 shows reaction specificity of Mabs with tail muscle in *D. virginiana*. Slow MHC-1 fibers were identified by strong reactivity against the S58 antibody (Fig. 2B: Fig. 3B, E: Fig. 5B, E, H; see figure legend for panel descriptions). S58 consistently identified slow fibers in each tail region and animal studied. By the principle of exclusion, fibers of serial muscle sections that did not react against S58 were fast fiber types, composed of either the MHC-2A or MHC-2X isoforms. An abundance of MHC-2A/2X hybrid fibers were identified by reaction against the antibody SC71 (Fig. 2C: Fig. 3C, F: Fig. 4B: Fig. 5C; see figure legend for panel descriptions), which is specific to the MHC-2A isoform in rat, and now shown to recognize both MHC-2A and 2X isoforms in Virginia opossums (this study) and Australian marsupials (Zhong et al., 2008). The third fiber type was identified as MHC-1/2A hybrids by matching fibers with moderate acid stability and reactions against S58 and SC71 (Fig. 4C, D). A relatively small number of these slow/fast hybrid fibers were found in all three regions of the tail, but reaction intensities against S58 and SC71 for the same fiber varied with tail region.

Reaction against 6H1, an antibody of known specificity for the MHC-2X isoform in marsupial limb muscle, showed weak ability to identify pure MHC-2X fibers in the Virginia opossum tail (Fig. 5A). 6H1 was found to react against either all or some of the same fibers reactive with SC71, verifying the presence of fast MHC-2A/2X hybrid fibers. However, often it was difficult to distinguish between positive reactions and background staining, while at other times, 6H1 did not appear to react with any fibers. Furthermore, the antibody BF-35, specific for all isoforms except MHC-2X (old formulation; Schiaffino et al., 1989; Toniolo et al., 2007), moderately reacted against all muscle fibers in each tail section studied (Fig. 5D). Reaction with all fibers also indicated a lack of pure MHC-2X fibers and validated the presence of the 2X isoform in fibers classified as MHC-2A/2X hybrids. Antibodies MY32, F18, and F59 were also used to identify any additional fast MHC isoform fiber type. Each of these three anti-fast Mabs showed weak reaction against fibers (particularly MY32) that did not react against antibodies specific for slow MHC-1 fibers (Fig. 5G), and thus were only effective in isolating the same fast

fibers reactive against SC71. Lastly, Mabs BF-F3 and 10F5 specific for the fast MHC-2B isoform, showed no reaction against muscle fibers from any section of the tail (Fig. 3D) confirming a lack of MHC-2B fibers. This finding further verifies the low glycolytic capacity of muscle fibers in the tail of *D. virginiana*.

SDS-PAGE Identification of MHC

Electrophoresis on muscle protein homogenates from each region of the tail showed two to three MHC bands (Fig. 6). In all samples analyzed, MHC-1 and prominent band for MHC-2A were detected after silver staining, correlating with the IHC analyses. The slow MHC-1 isoform was identified as the myosin band that was lowest in molecular weight and migrated the furthest and fastest. In gels clearly showing three MHC isoform bands, the MHC-2X isoform occupied the position intermediate to the slowest migrating MHC-2A isoform. MHC expression in opossum tail muscle was confirmed by band migration patterns of MHC isoforms in rat TA muscle (Fig. 6). The presence of the MHC-2X isoform (irrespective of tail region) and the inability to identify pure MHC-2X fibers in MyATPase histochemistry and IHC analyses, further verified the presence of the 2X isoform in fast MHC-2A/2X hybrid fibers. Hybrid fibers types are not evident in any gel experiment due to the limitations of SDS-PAGE which cannot provide information of the intramuscular distribution of hybrid fibers (Nguyen & Stephenson, 1999).

Fiber Type Distributions

MHC isoform fiber type was identified in a total of n=6640 fibers counted from numerous sections of muscle tissue sampled from the proximal, middle and distal regions of the tail. In all five animals studied there was an abundance of fast, oxidative MHC-2A/2X hybrid fibers in each tail region compared to slow, oxidative MHC-1 fibers (Table 3). In the proximal tail region, nearly 84% of the muscle fibers were positively identified as MHC-2A/2X hybrids by the panel of Mabs. The distribution of MHC-2A/2X hybrid fibers declined in the middle tail region to 61%, but increased in distal tail musculature to 73%. Overall, 70.6% of muscle fibers in the tail of *D. virginiana* were found to be composed of the MHC-2A/2X isoforms (Table 4). MHC-1 fibers were the only pure isoform fiber type to be identified with Mabs. Slow MHC-1 fibers also varied in their percent distribution with tail region in each animal, though the percentages of slow fibers

were very consistent in each tail region across each animal studied (Table 3). In the proximal tail, just over 10% of MHC-1 fibers were found, compared to the middle & distal tail regions which showed percentage distributions of 36% and 23%, respectively. Collectively, the tail of *D. virginiana* was composed of 25.4% MHC-1 fibers (Table 4). Finally, fibers identified by Mabs S58 and SC71 as MHC-1/2A hybrids were always present in low percentages and showed slightly different distributions in each tail region (Table 3). The percentage distribution of slow/fast, oxidative MHC-1/2A hybrid fibers was just over 4% for the entire tail (Table 4).

Fiber Architectural Measurements

Means of fiber cross-sectional area (CSA in μm^2) across all individuals and those representative of MHC isoform fiber types of the tail as whole are presented in Table 5 and Table 6, respectively. Measurements for CSA were consistently larger for fibers identified as MHC-2A/2X hybrids across all animals studied (Table 5). In the direction cranial to caudal, fiber CSA (for each fiber type identified) progressively decreased (Table 6), yet the relative order of fiber types by their mean CSA never changed. MHC-2A/2X hybrid fibers always had the largest CSA in each tail region. Fiber type CSA was in following order: MHC-2A/2X >MHC-1 >MHC-1/2A. Overall, MHC-2A/2X hybrids (population mean: 4888.1 μm^2) were substantially larger than fibers composed of the MHC-1 isoform (population mean: 3596.6 μm^2), and nearly two-fold larger in cross-section than MHC-1/2A hybrids (population mean: 2699.3 μm^2) (Table 6).

Means of fiber diameter (in μm) across all individuals and those representative of MHC isoform fiber types of the tail as whole are presented in Table 7 and Table 8, respectively. Architectural measurements for diameter of muscle fibers were consistent with those of fiber CSA, indicating a good correlation between fiber CSA and diameter. MHC-2A/2X hybrid fibers had largest fiber diameter, followed by MHC-1 fibers and MHC-1/2A hybrid fibers. However, unlike fiber CSA, population means for MHC-2A/2X hybrids (81.4 μm) and MHC-1 fibers (74.6 μm) were more similar, and both were substantially larger than the overall mean diameter of MHC-1/2A hybrid fibers (55.3 μm) (Table 8). Statistical differences in fiber type architecture were not a priority in this study.

Discussion

The major findings of this study are the identification of three MHC isoform fiber types (MHC-1, MHC-2A/2X, MHC-1/2A) and their regional distributions in the tail of *D. virginiana*. Results indicate a predominance of relatively large, oxidative MHC-2A/2X hybrid fibers in the proximal and distal regions of the tail. Conversely, the middle region of the tail had a more similar distribution of fast MHC-2A/2X hybrids and slow MHC-1 isoform fibers, and each tail region showed a low percentage of relatively small and highly oxidative MHC-1/2A hybrid fibers. Determination of MHC isoform fiber type by a combination of histochemistry, immunohistochemistry and gel electrophoresis is significant in two ways: (i) for the first time, MHC isoforms and fiber architecture were investigated in axial tail muscle, and (ii) fiber types in a second opossum species were identified by their isoform-specificity of myosin representing a marked improvement over earlier fiber typing studies in opossum marsupials. Overall, our findings for muscle fiber type and fiber architecture in the Virginia opossum improve understanding of structure-function relationships in the tails of didelphid marsupials.

Previous studies of muscle fiber type in the Virginia opossum (Peters et al., 1984; Hansen et al., 1987) relied solely on MyATPase histochemistry to broadly classify fibers as either Type I (slow) or Type II (fast) (old nomenclature). However, identification of subdivisions of fast MHC isoform fiber types by this technique is not clear, as difficulties are encountered with the use of acid-stable histochemistry due to differing pH sensitivities of MyATPases in fast fiber types (Zhong et al., 2008). Difficulties in determining fast fiber types (and hybrid fibers) by MyATPase histochemistry have been particularly noted in studies of marsupials including hopping species (Dennington & Baldwin, 1988), Virginia opossum (Peters et al., 1984; Hansen et al., 1987), and gray short-tailed opossum [*Monodelphis domestica* (Sciote & Rowerlson, 1998)]. Similarly, identification of pure fast fiber types by MyATPase histochemistry in this study was not clear. Despite the technique limitations, 'slow' and 'fast' fiber type percentages reported for the tail of *D. virginiana* (Hansen et al., 1987) are consistent with distributions of MHC isoforms found in our study. The tail of *D. virginiana* was previously determined to be composed of nearly 25% Type I and 75% Type II fibers (Hansen et al., 1987). These data match well with MHC isoform fiber type distributions of 25.4% slow MHC-1 fibers,

70.6% fast MHC-2A/2X hybrid fibers and 4.1% slow/fast MHC-1/2A hybrid fibers (Table 4). The latter hybrid fiber type would have been classified as Type II fibers by the previous histochemical analysis. The findings our study further demonstrate accurate identification of fiber types in opossums by MyATPase alone is impractical, and place increasing reliance on fiber typing analyses combining histochemistry with SDS-PAGE and IHC, using Mabs that have specific immunoreactivity against marsupial MHC isoforms (Lucas et al., 2000; Zhong et al., 2001, 2008).

Antibody Specificity in Opossum Tail Muscle

Immunoreactivity against the MHC-1 isoform using the monoclonal antibody S58 consistently identified the slow MHC-1 fiber type in the Virginia opossum. The MHC-1 isoform is highly conserved across several taxa of vertebrates spanning fish (e.g. zebrafish), birds (e.g. quail, chicken), and small and large eutherian mammals (e.g. mouse, rat, and horse) (Miller et al., 1985). Likewise, monospecific reactivity of S58 with the MHC-1 isoform across a diversity of vertebrate species is high (Crow & Stockdale, 1986), and thus can be used to reliably identify MHC-1 isoform fibers in mammals. We showed that S58 unambiguously identifies slow MHC-1 isoform fibers (Figs. 2-4) and verified its monospecific reactivity against marsupial MHC-1 in an opossum species.

In contrast to the slow MHC-1 isoform, fast MHC isoforms show stronger species-dependent immunoreactivity or antigenic specificity. MHC-2A and 2X were the predominant isoforms found in the tail, primarily identified by positive reaction against SC71, and further verified by metabolic analyses (NAD) and SDS-PAGE. The only other study to use IHC to identify MHC isoforms in opossum muscle (*M. domestica*) also showed SC71 reactivity with fibers initially assumed to be pure MHC-2A (Sciote & Rowlerson, 1998) and later interpreted to be a mix of MHC-2A and 2X isoform fibers or fast hybrids (Zhong et al., 2008). Interestingly, the same fibers identified by SC71 as MHC-2A (or 2X) in *M. domestica* also showed high NAD activity (Sciote & Rowlerson, 1998). Immunoreactivity of SC71 against two fast MHC isoforms reported herein for tail muscle of *D. virginiana*, as with that for limb muscles in *M. domestica*, suggests cross-reactivity of SC71 for both 2A and 2X isoforms in South American opossums, but limited specificity for identification of pure MHC-2A fibers as found when applied in

small eutherian mammals [rat, mouse, guinea pig (Schiaffino et al., 1989)]. Our findings for SC71 are also supported by fiber typing studies in species of Australian marsupials such as the bandicoot, brushtail bettong, and tammar wallaby (Zhong et al., 2008), where SC71 reacted distinctly with both MHC-2A and MHC-2X isoforms. Furthermore, cross-reactivity for SC71 has been reported for relatively large eutherian species [goat, humans (Arguello et al., 2001; Lefauceur et al., 2002)], and most recently in bears (Smerdu et al., 2009), where SC71 strongly reacted with the MHC-2X isoform in MHC-2A/2X hybrid fibers.

Variation in immunoreactivity across species requires additional fiber typing methods be employed to verify specific MHC isoform fiber type. Although metabolic staining techniques cannot reliably distinguish fiber types based on their metabolic capacity (Reichman & Pette, 1982), our metabolic analyses showed uniform staining of muscle tissue for oxidative potential (NAD) with little-to-no glycolytic potential (α-GPD) (Fig. 2). Accordingly, the high oxidative capacity of opossum tail muscle verifies a large distribution of slow, oxidative MHC-1 fibers and fast, oxidative MHC-2A/2X hybrid fibers, and potentially indicates the presence of either pure MHC-2A (highly oxidative) or MHC-2X (moderately oxidative) fibers. The inability of metabolic analyses to rule out either pure fast fiber type in opossum tail muscle is indicated by SDS-PAGE experiments showing three protein bands in the region of MHC-1, 2A, and 2X (Fig. 6). However, in no gel experiment was a fourth band present in the region typical of the MHC-2B isoform (see lane 1, rat TA: Fig. 6). Low glycolytic potential in tail muscle tissue is consistent with a lack of MHC-2B fibers. Additionally 10F5, a Mab with known specificity against the MHC-2B isoform in eutherians (Lucas et al., 2000) and several Australian marsupials including the ringtail possum (*Pseudocheirus peregrinus)* and red kangaroo (*Macropus rufus*) (Zhong et al., 2001, 2008), did not react in Virginia opossum tail muscle, thus confirming the lack of MHC-2B fibers (Table 2; Fig. 3).

Clear identification of pure MHC-2A and MHC-2X isoform fibers was difficult despite the combination of fiber typing techniques used to verify our panel of Mabs as monospecific against MHC in Virginia opossums. 6H1, specific for MHC-2X fibers in a number of eutherians and Australian marsupials (Lucas et al., 2000), was weakly reactive against muscle fibers in tail of the Virginia opossum (Fig. 5). In some experiments, 6H1

either partially reacted with some fibers that also reacted with SC71, or reacted with all fibers that reacted with SC71, albeit less intensely. The large distribution of MHC-2A/2X hybrid fibers, and possible cross-reactivity of both SC71 and 6H1 in *D. viginiana*, may also be indicated by moderate reactivity of BF-35 (Fig. 5). Our formulation of BF-35 revealed pure MHC-2X fibers by the principle of exclusion, that is reacting with all conventional MHC isoforms except 2X (Schiaffino et al., 1989; Schiaffino et al., 1998). Similar patterns of moderate intensity reaction with BF-35 for all fibers in a tissue section were observed in the limb muscles of *M. domestica* (Sciote & Rowlerson, 1998), although in that study, positive reaction with BF-35 could not be confirmed because results control experiments did not produce the expected specificity. Therefore, identification of pure MHC-2A and 2X fibers in opossum skeletal muscle has been challenging by both histochemistry (Peters et al., 1984; Hansen et al., 1987) and IHC (Sciote & Rowlerson, 1998), even when the latter is done with a broad panel of Mabs and verification of MHC isoforms by SDS-PAGE as in this study (Table 2; Fig. 6).

The mixed reaction specificity of 6H1 and BF-35 raises interesting questions about the presence of the MHC-2A and 2X isoforms purely in the form of MHC-2A/2X hybrid fibers, and the seemingly unusual hybrid fiber composition of the tail. High numbers of hybrid fibers are atypical in eutherian limb muscles (Toniolo et al., 2007), but relatively little is known about muscle fiber types in marsupials, and even less regarding muscle fiber distributions in axial tail muscle. High numbers of hybrid fibers in the tail of *D. virginiana*, especially in the distal region, may partially explain the incomplete staining patterns for MyATPase histochemistry that were previously observed (Hansen et al., 1987). High numbers of fast hybrid fibers may also represent a transition (shift in isoform) from a fast 2A/2X hybrid to a pure MHC-2A isoform fiber. This phenomenon has been shown to correlate with age in several studies of large mammals (Roneus et al., 1991; Rivero et al., 1993; Smerdu et al., 2009). For example, in bear limb muscles, MHC-2X fibers transitioned to the MHC-2A isoform via MHC-2A/2X hybrid fibers with maturation. Percentages of hybrid fibers in bear muscles decreased with age and were relatively low in mature adults, suggesting a possible functional role of hybrid fibers during natural muscle maturation (Smerdu et al., 2009). The ages of opossums used in this study were not known and correlation of fiber type with age of the animals is beyond

the scope this investigation. However, no systematic differences in fiber type or architecture in any tail region were observed across individual opossums (Tables 3, 5, 7).

Conversely, a large distribution of fast hybrid fibers may indicate a transition from slower, highly oxidative (MHC-1 or 2A) fibers to faster, moderately oxidative MHC-2X fibers. As evidence of this, a third fiber type was identified as MHC-1/2A hybrid fibers by distinct reactivity of fibers against both S58 and SC71 (Fig. 4). Fibers co-expressing slow and fast MHC isoforms are the earliest examples of MHC hybrids reported to occur in normal mammalian skeletal muscle (Pierobon-Bormioli et al., 1981). Transitions from slower-to-faster fiber types have been shown in numerous studies involving limb immobilization, suspension and disuse (e.g. Oishi et al., 1998; Grossman et al., 1998; D'Antona et al., 2003). It is now accepted that the distribution of MHC hybrids and their molecular complexity (i.e. pattern of co-expression) is higher in muscles undergoing molecular and functional transformation (see review by Pette et al., 1999). Perhaps the terrestrial locomotor habit, and thus lower dependence on the tail for climbing and hanging in adult Virginia opossums, explains the large distribution of fast MHC-2A/2X hybrid fibers seen in the tail musculature (see section below, *Evolutionary Structure-function in Opossum Tails*).

Finally, several factors related to the IHC protocol used in our study may have contributed to the inability to identify pure MHC-2A and MHC-2X isoform fibers: 1). the concentration of primary antibodies may have been too high in the working aliquots, 2). muscle tissue may have been reacted too long against Mabs, or 3). goat serum blocking solution, instead of a synthetic compound, was used to prevent non-specific protein binding in muscle tail tissue. The first two factors increase background staining (i.e. IHC reaction in prozone) and limit detection of positive Mabs reactivity, while the third factor increases species cross-reactivity (i.e. opossum muscle + mouse Mabs + goat serum) and decreases monospecific reactivity. SDS-PAGE experiments also were not coupled with Western blotting (Toniolo et al., 2005, 2007; Zhong et al., 2008) to validate monospecific reactivity of Mabs against opossum MHC. Despite these potential sources of error in our analyses, we find the tail of *D. virginiana* to be primarily composed of relatively large (CSA and diameter) and fast, oxidative hybrid fibers (MHC-2A/2X). At this time,

identification of MHC-2A and MHC-2X isoform fibers remains inconclusive, though percentage distribution of these pure fast fiber types in the tail is expected to be low.

Evolutionary Structure-function in Opossum Tails

The results of this investigation support our second hypothesis: muscle fibers identified in the tail of *D. virginiana* contain a majority of the MHC-2X isoform co-expressed in fast, oxidative hybrid fibers (see section *Objectives & Hypotheses*). The findings are suggestive of an evolutionary change in functionality of the tail and a consequent shift in muscle fiber phenotype with faster MHC isoform expression. It is believed that the common ancestor of *D. virginiana* was an arboreal animal, and that the tail evolved to function as an additional appendage for climbing and scansorial habit. All opossum marsupials retain long tails with varying degrees of prehensility, but occupy a diversity of niches. Although scansorial by nature, *D. virginiana* has adapted a terrestrial locomotor habit whereby function of the tail in climbing (or hanging) is reduced in the adult, and grasping ability is diversified for other adaptive behaviors such as manipulation of objects. In fact, locomotor gait patterns (White, 1990) and distinct morphological features including epipubic bones, epaxial muscle compartments (Reilly & White, 2003) and rigid axial skeleton (Argot, 2003), indicate *D. virginiana* is derived and more cursorial than other opossums (Reilly et al., 2009), and a good representative of the primitive terrestrial locomotor condition. It may therefore be expected that changes in patterns of tail function throughout the evolution of *D. virginiana* would lead to changes in MHC isoform composition and fiber architecture.

Peculiar MyATPase staining patterns in the distal tail region of *D. virginiana* were previously hypothesized to represent changes in muscle fiber structure-function from a primitive condition (Hansen et al., 1987). The large distribution of fast hybrid fibers found throughout the tail in this study provide evidence of an evolutionary transition in fiber phenotype that may be related to a primarily terrestrial lifestyle. Hybrid fibers enable a muscle to fine tune its efficiency for a wide range of forces, velocities and levels of resistance to fatigue (Bottinelli et al., 1994a, b; Pette et al., 1999; Pette & Staron, 2000). Intermediate contractile properties of hybrid fibers may be important for object manipulation (e.g. nest building) where fine motor movements are required. Though high

percentages of MHC hybrid fiber types are not typically found in limb muscles (Stephenson, 2001; Toniolo et al., 2007; Smerdu et al., 2009; Butcher et al., 2010), relatively large distributions of fast, oxidative hybrid fibers are expressed in other skeletal muscles such as laryngeal thyroarytenoid muscles in rats (%90: Stephenson, 2001) and dogs (30-57%: Toniolo et al., 2007). Thus a high percentage of fast hybrid fibers may not be atypical for axial tail muscle. Unfortunately, we are not aware other studies of MHC isoform fiber type in tail muscles of small mammals for comparison.

Transitions in MHC isoforms also result in changes in fiber size. In eutherian muscle, highly oxidative MHC-2A fibers have the smallest cross-sectional area and diameter, while MHC-2X fibers are of intermediate size between MHC-2A and MHC-2B (largest) isoform fiber types. These general size characteristics are functionally meaningful for evaluating the relative size of fast MHC-2A/2X hybrid fibers in the tail of *D. virginiana*. Limited data for fiber architecture in the tails of basal eutherian mammals [mouse, rat, tree shrew (Schilling et al., 2005)] indicate large fiber CSA relative to their long tail length and small body size. Muscle fibers in the tail of *M. domestica* are purported to be relatively large in their cross-section, although no CSA values have been reported (Schilling, 2009) and these data are not known to have been previously measured in *D. virginiana*. Similar to MHC isoforms in the tail, there is no current model of comparison for tail fiber architecture in other species of opossum. That said, mean fiber CSA for fast MHC-2A/2X hybrid fibers (4881 μm^2: Table 6) in the tail of *D. virginiana* is similar to average fiber CSA of fast hybrids reported for small limb muscles with long fascicles [e.g. dog tibialis cranialis (Toniolo et al., 2007)].

Functionally fiber size correlates with oxidative *vs*. glycolytic properties that influence capacity for muscle mechanical work. Important to muscle fiber physiology is oxygen diffusion distance, from capillaries into the interior of a muscle fiber (Hepple et al., 2000). A shorter diffusion distance allows more efficient uptake of oxygen for aerobic cellular respiration to generate high *net* ATP. MHC-1 and MHC-2A fibers typically have smaller CSA and diameters, correlating with their high oxidative capacity (Hepple et al., 2000). On the other hand, a larger diffusion distance for cellular gas exchange implies a greater dependence on anaerobic cellular pathways to generate ATP. In opossum tail muscle, large CSA and diameter of fast, oxidative MHC-2A/2X hybrids maybe less

indicative of shifts in oxidative *vs*. glycolytic capacity (see section *Metabolic Activity of Opossum Tail Muscle Fibers*) but instead, more representative of a structural feature common to axial tail muscle in basal mammals that retain long tails (Schilling, 2009). Further studies in opossum species will help clarify if larger fiber CSA and diameter are features normal to tail musculature, or if fiber architecture is dependent on MHC isoform fiber type. Additionally, the latter assertion raises interesting questions about diversity in MHC isoform fiber types and fiber architecture across species of opossum with differences in locomotor habit. Most species of opossum are arboreal (or semi-arboreal), thus they dwell, forage and maneuver complex (3-D) rainforest habitats in Central and South America. Differences in habit between terrestrial and arboreal species (Delciellos & Vieira, 2007) and use of the tail in locomotion, suggest MHC fiber type and architecture may be also different. The results of this study allow us to begin testing this hypothesis, and muscle data from species with contrasting locomotor habits are needed.

In conclusion, a thorough investigation of MHC isoform fiber type and fiber architecture in axial tail muscle of the Virginia opossum revealed a composition of three MHC isoform fiber types: MHC-1, MHC-2A/2X and MHC-1/2A. Fiber type and architecture varied slightly by tail region and consistently showed a large distribution of fast, oxidative MHC-2A/2X hybrid fibers. These results are suggestive of a evolutionary transition in MHC isoform distribution (slow-to-fast fiber type) in the tail musculature of an opossum with terrestrial locomotor habit *vs*. arboreal lifestyle. Future studies are aimed at refining fiber typing techniques for studies of tail muscle and comparative analyses with tail muscle tissue from arboreal opossum species.

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Table 1. Morphometric data from Virginia opossums.

Opossum	Sex	Mass (kg)	Tail Length (cm)
Op5-17	M	2.0	28
Op6-25	M	2.7	32
Op7-6	F	1.5	31
Op7-7	M	2.7	30
Op9-8	F	2.1	27
mean±SD		**2.2±0.5**	**29.6±2.1**

Table 2. Monoclonal antibodies (Mabs) used for IHC analyses and their MHC isoform reaction specificity against tail muscle from the Virginia opossum (*D. virginiana*).

Antibody	Slow, MHC-1	Fast (MHC)	MHC-2A	MHC-2X	MHC-2B
MHC-s	+/-				
S58	++				
F18		+/-			
F59		+/-			
MY32		+/-			
SC71			+	+	
6H1				+/-	
BF-35	+		+		
BF-F3					-
10F5					-

MHC – myosin heavy chain
IHC – immunohistochemistry
(+) positive reaction against muscle MHC antigen
(++) strong positive reaction against muscle MHC antigen
(+/-) weak reaction against muscle MHC antigen
(-) no reaction against muscle MHC antigen

Table 3. Regional distributions (%) of known MHC isoform fiber type in the tails of individual Virginia opossums.

Animal	Proximal			Middle			Distal		
	MHC-1	**MHC-2A/2X**	**MHC-1/2A**	**MHC-1**	**MHC-2A/2X**	**MHC-1/2A**	**MHC-1**	**MHC-2A/2X**	**MHC-1/2A**
Op5-17 (*n*=970)	11.2	82.7	6.1	34.4	62.1	3.4	20.5	72.7	6.8
Op6-25 (*n*=1773)	10.6	83.7	5.6	35.4	62.4	2.2	28.1	67.8	4.1
Op7-6 (*n*=1287)	8.7	85.8	5.5	38.6	58.7	2.7	20.2	75.3	4.5
Op7-7 (*n*=1252)	11.1	82.4	6.5	35.6	61.9	2.4	21.9	74.5	3.6
Op9-8 (*n*=1358)	10.1	84.2	5.7	38.1	58.0	3.9	21.7	74.7	3.6

In parentheses is the total number of fibers counted for calculation of percentage MHC isoform fiber type.

Table 4. Percentage distributions (%) of known MHC isoform fiber types in the tail of the Virginia opossum (*D. virginiana*).

Tail region	MHC-1	MHC-2A/2X	MHC-1/2A	*n*
Proximal	10.3	83.7	5.9	2244
Middle	36.3	60.9	2.8	3304
Distal	23.3	72.8	3.9	1092
Population %:	**25.4**	**70.6**	**4.1**	

n= total number of fibers counted for each tail region across all animals
Percent fiber type data are presented a whole percentages

Table 5. Regional means of CSA (μm^2) of known MHC isoform fiber types in the tails of individual Virginia opossums.

Animal	Proximal			Middle			Distal		
	MHC-1	**MHC-2A/2X**	**MHC-1/2A**	**MHC-1**	**MHC-2A/2X**	**MHC-1/2A**	**MHC-1**	**MHC-2A/2X**	**MHC-1/2A**
Op5-17	4297.2 (1254)	5925.3 (1343)	2066.7 (794.9)	3059. (1139)	4246.4 (1047)	2459.2 (616.7)	3731.3 (860)	5327.8 (1369)	2091.9 (528.1)
Op6-25	3810.3 (1383)	5274.0 (1196)	2006.7 (806.8)	3930.7 (1390)	5225.8 (1690)	2347.1 (619.7)	3491.7 (865)	3794.5 (961)	2232.1 (710.5)
Op7-6	3916.3 (1261)	5799.6 (1645)	3622.7 (629.6)	3387.9 (749)	5031.8 (1672)	2723.2 (673.4)	3312.9 (891)	3545.4 (884)	1659.4 (412.8)
Op7-7	4130.0 (1090)	3406.2 (1396)	3000.4 (1277)	3197.7 (1375)	3798.6 (815)	2043.8 (320.4)	3334.9 (786)	3657.7 (767)	2465.9 (455.6)
Op9-8	4042.1 (732)	5658.1 (1379)	3799.1 (980.9)	3249.2 (1272)	6019.4 (1379)	2782.2 (755.9)	3167.5 (867)	4350.2 (984)	2241.6 (685.4)

CSA: fiber cross-sectional area
In parentheses is standard deviation, SD

Table 6. Mean fiber CSA (μm^2) of known MHC isoform fiber types in the tail of the Virginia opossum (*D. virginiana*).

Tail region	MHC-1	MHC-2A/2X	MHC-1/2A	*n*
Proximal	4028.2±1171.8	5284.0±1635.0	2996.2±1202.7	633
Middle	3375.3±1243.3	4933.9±1570.4	2502.7±661.6	602
Distal	3403.4±869.3	4281.8±1261.5	2210.9±638.4	490
Population mean:	**3596.6 ± 1144.7**	**4881.0 ± 1566.2**	**2699.3 ± 1009.9**	

n= total number of fibers measured for each tail region across all animals
Fiber CSA are mean±SD

Table 7. Regional means of fiber diameter (μm) of known MHC isoform fiber types in the tails of individual Virginia opossums.

Animal	Proximal			Middle			Distal		
	MHC-1	**MHC-2A/2X**	**MHC-1/2A**	**MHC-1**	**MHC-2A/2X**	**MHC-1/2A**	**MHC-1**	**MHC-2A/2X**	**MHC-1/2A**
Op5-17	74.74	102.4	54.93	80.46	93.2	51.41	69.41	76.9	45.48
	(14.5)	(13.4)	(11.7)	(13.1)	(11.0)	(11.0)	(14.2)	(14.0)	(13.6)
Op6-25	76.05	98.9	44.76	81.54	72.8	52.75	73.37	69.7	52.61
	(15.0)	(16.4)	(9.9)	(21.2)	(15.1)	(11.4)	(15.6)	(17.5)	(8.4)
Op7-6	79.44	80.2	60.85	75.36	72.1	60.87	66.84	69.0	45.67
	(10.7)	(21.7)	(11.9)	(16.0)	(8.9)	(12.5)	(10.6)	(17.7)	(2.1)
Op7-7	85.23	89.0	61.34	74.87	93.4	59.06	80.36	68.3	55.90
	(10.1)	(15.5)	(12.3)	(18.1)	(17.9)	(5.0)	(14.8)	(14.5)	(13.9)
Op9-8	72.70	82.7	56.46	65.75	82.9	60.88	63.57	60.3	48.34
	(15.0)	(14.1)	(9.9)	(15.0)	(20.5)	(16.0)	(12.9)	(12.4)	(10.6)

In parentheses is the standard deviation, SD

Table 8. Mean fiber diameter (μm) of known MHC isoform fiber types in the tail of the Virginia opossum (*D. virginiana*).

Tail region	MHC-1	MHC-2A/2X	MHC-1/2A	*n*
Proximal	77.3±13.9	89.9±18.6	56.3±12.7	658
Middle	75.6±17.6	82.2±18.0	56.2±12.5	604
Distal	70.7±14.8	71.7±17.6	50.3±10.6	565
Population mean:	**74.6 ± 15.8**	**81.2 ± 19.5**	**55.3 ± 12.5**	

n= total number of fibers measured for each tail region across all animals

Figure 1. Hypothesized phylogenetic genera of South American Didelphid marsupials. Branch lengths are proportional to time of divergence. Modified after Kirsch et al. (1997).

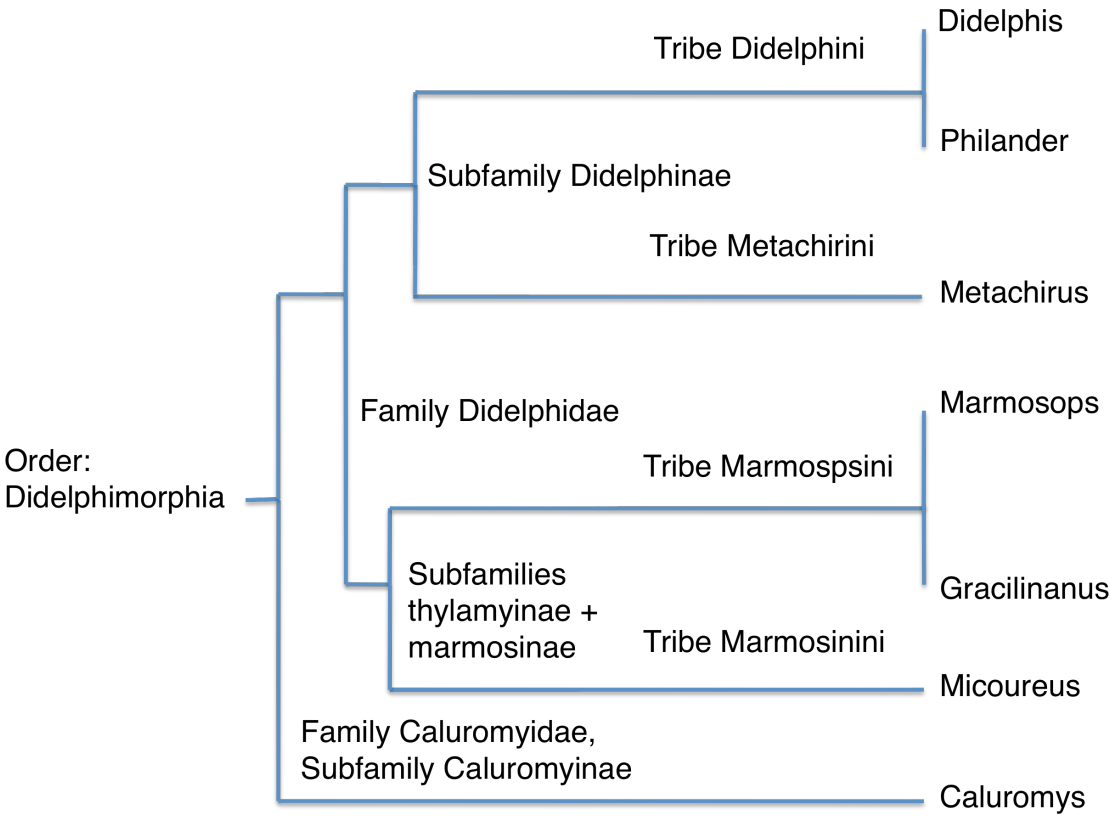
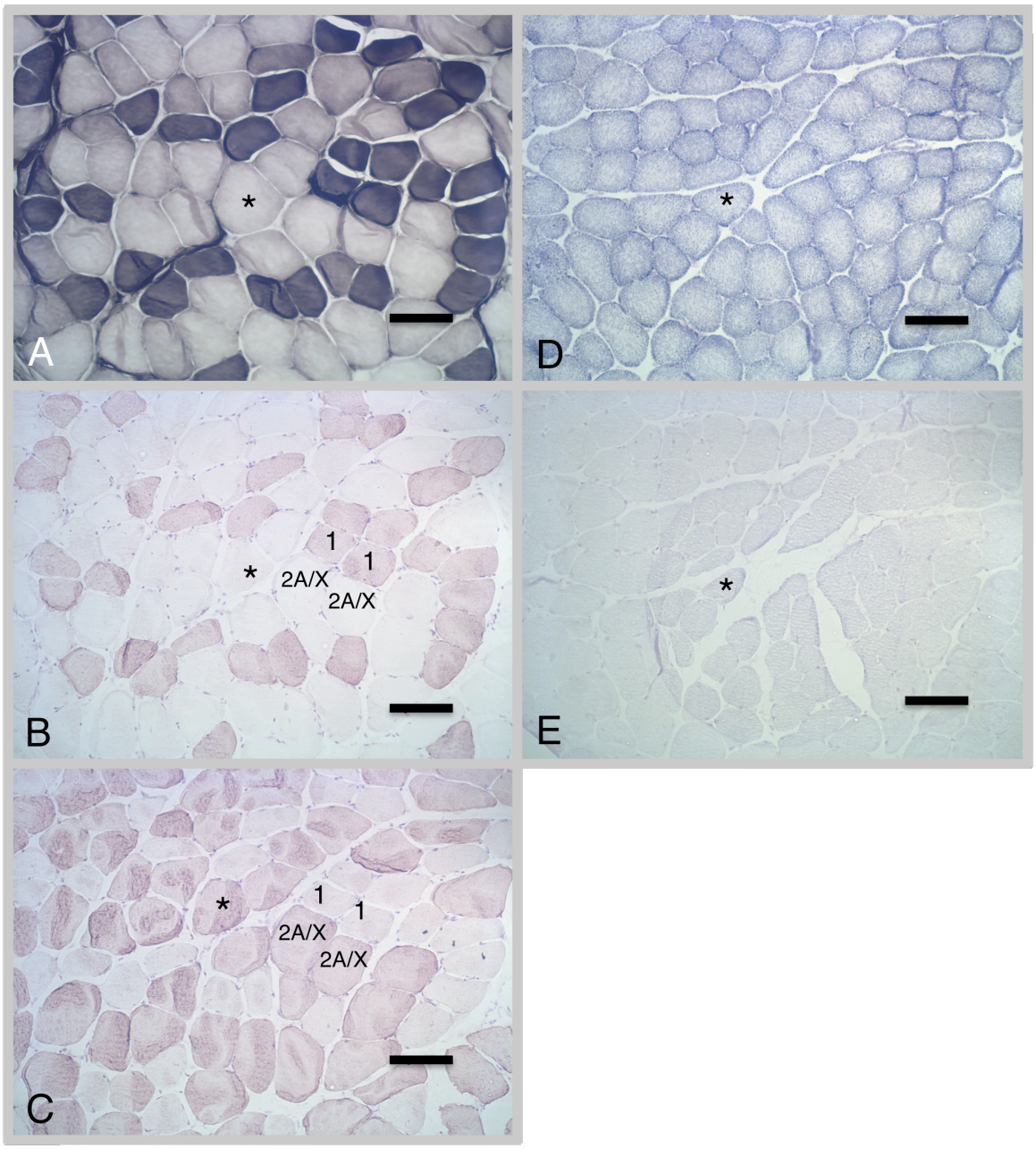

Order:
Didelphimorphia
Family Didelphidae
Subfamily Didelphinae
Tribe Didelphini
Didelphis
Philander
Tribe Metachirini
Metachirus
Subfamilies
thylamyinae +
marmosinae
Tribe Marmospsini
Marmosops
Gracilinanus
Tribe Marmosinini
Micoureus
Family Caluromyidae,
Subfamily Caluromyinae
Caluromys

Figure 2. Representative fiber type reactivity for MyATPase histochemistry and IHC in the proximal region of the tail in *D. virginiana*. Serial cryostat cross-sections of tail muscle reacted (**A**) after acid incubation **(**pH 4.4) and with a panel of monoclonal antibodies (Mabs) specific to MHC isoforms: (**B)** S58 (anti MHC-1) and (**C)** SC71 (anti MHC-2A, 2X). Fibers that stain darkly (highly acid stable) after acid incubation are slow, MHC-1fibers. Fast MHC-2A/2X hybrid fibers are lightly stained and slow/fast MHC-1/2A hybrids are moderately stained at acid incubation pH 4.4. MyATPase reactions were largely confirmed by Mabs S58, SC71, 6H1, BF-35 and 10F5. Panels (**D)** NAD and (**E)** α-GPD are additional serial sections from a separate experiment showing representative metabolic profiles of tail muscle tissue. Fibers exhibited high NAD activity, while α-GPD activity was very low in all fibers. Fibers labeled with an asterisk (*) are the same fiber of reference in each set of serial sections. Scale bar = 100 μm.


A
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D
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B
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1
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2A/X
E
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C
1
1
*
2A/X
2A/X

Figure 3. Representative fiber type reactivity for MyATPase histochemistry and IHC in the middle region of the tail in *D. virginiana*. Serial cryostat cross-sections of tail muscle reacted (**A**) after acid incubation (pH 4.4) or with monoclonal antibodies **(B)** S58 (anti MHC-1) or (**C)** SC71 (anti MHC-2A, 2X). Panels (**D)** 10F5 (anti MHC-2B), (**E)** S58 and (**F)** SC71 are additional serial sections from a separate experiment showing representative reactivity an antibody with MHC-2B specificity in marsupial muscle. The Mabs reaction against the MHC-2B isoform was negative. Fibers labeled with an asterisk (*) are the same fiber of reference in each set of serial sections. Scale bar = 100 μm.

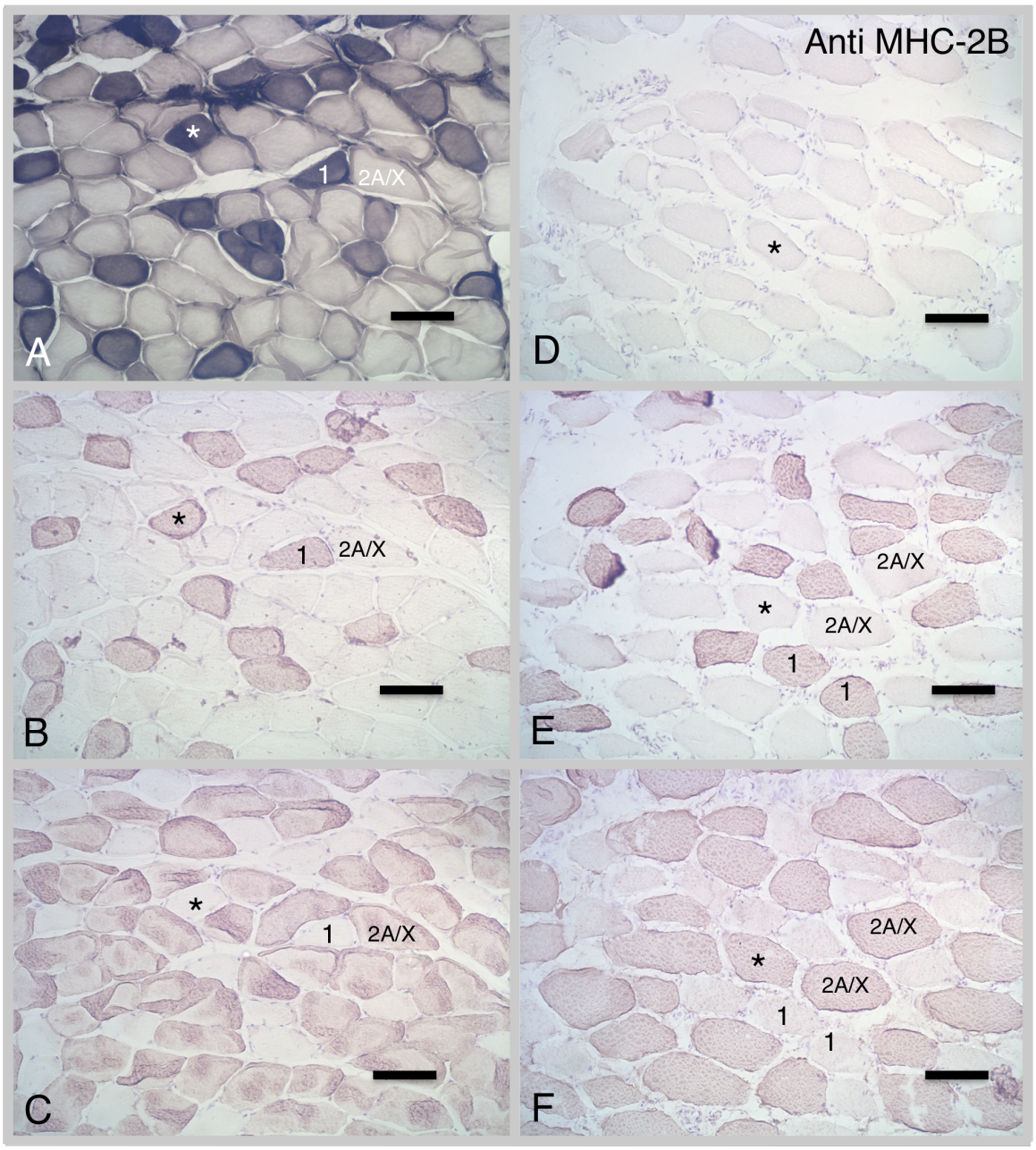
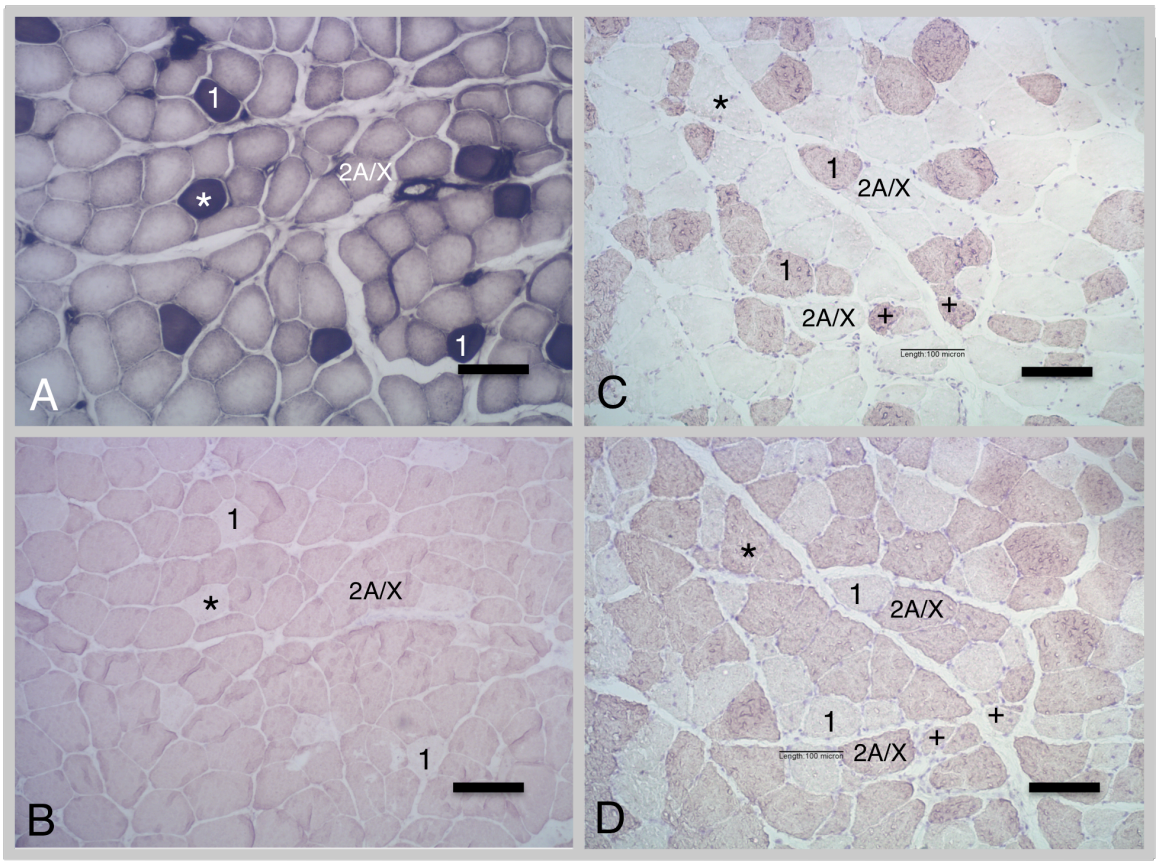

Anti MHC-2B
A
B
C
D
E
F
*
1
2A/X

Figure 4. Representative fiber type reactivity for MyATPase histochemistry and IHC in the distal region of the tail in *D. virginiana*. Serial cryostat cross-sections of tail muscle reacted (**A**) for MyATPase after acid incubation (pH 4.4) or with monoclonal antibody (**B)** SC71 (anti MHC-2A, 2X). Panels (**C)** S58 (anti MHC-1) and (**D)** SC71 are additional serial sections from a separate experiment showing hybrid fibers (+) that reacted with both S58 and SC71. Fibers labeled with an asterisk (*) are the same fiber of reference in each set of serial sections. Scale bar = 100 μm.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+
Length:100 micron
D

Figure 5. Fiber type reactivity for IHC in three sets of serial sections of tail muscle in *D. virginiana*. In the first column, tissue sections were reacted against Mabs (**A)** 6H1 (anti MHC-2X), (**B)** S58 (anti MHC-1) and (**C)** SC71 (anti MHC-2A, 2X). In the second column, (**D)** BF-35 (anti MHC-1, 2A, 2B), (**E)** S58 and (**F)** SC71. In the third column, (**G)** MY32 (anti MHC-2A, 2X, 2B), (**H)** S58 and (**I)** SC71. Reaction against 6H1 was weak or negative and indicative of no pure MHC-2X fibers. BF-35 works on the principle of exclusion by reacting against all MHC isoforms except 2X. All fibers reacted against the BF-35 antibody further indicating no pure MHC-2X fibers. MY-32 is shown to react weakly against all fast isoforms in tail muscle of *D. virginiana*; similar findings in limb muscles of *M. domestica* (Sciote & Rowlerson, 1998). Fibers labeled with an asterisk (*) are the same fiber of reference in each column of serial sections. Scale bar = 100 μm.

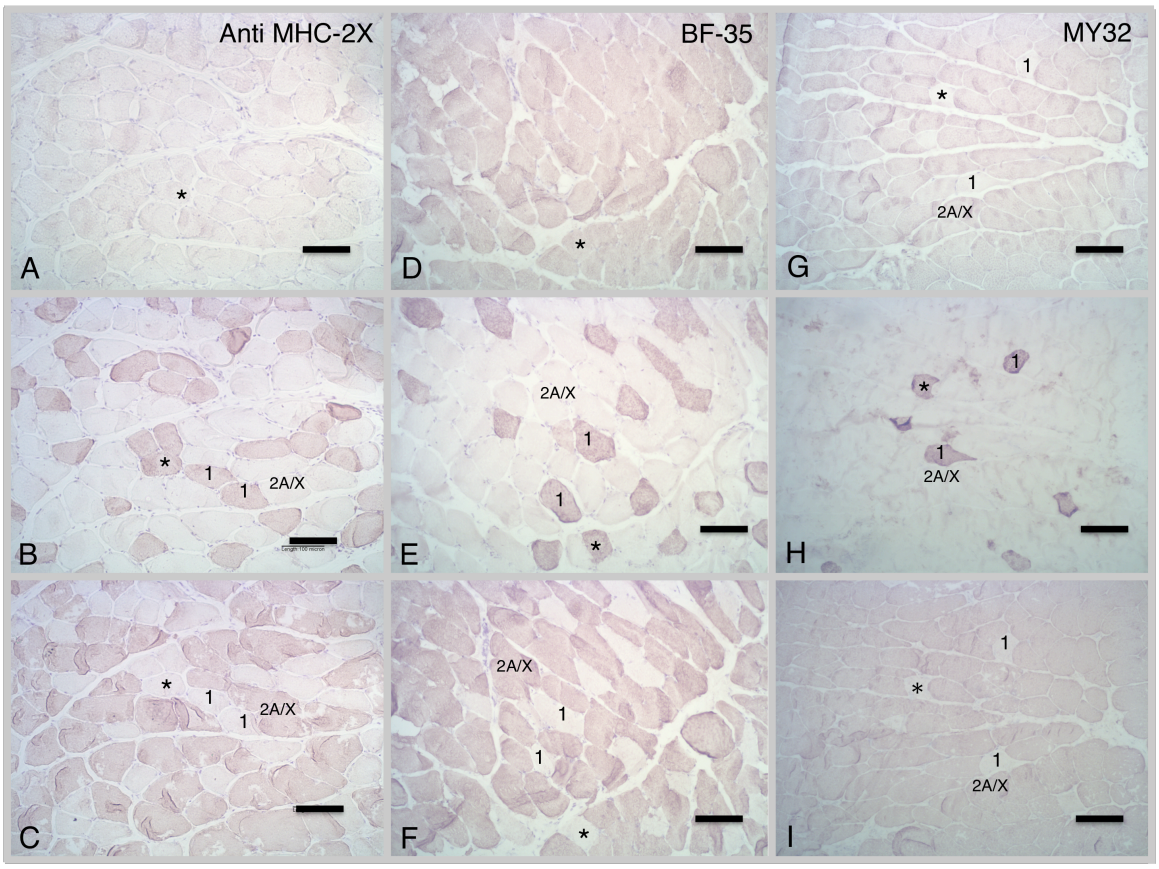
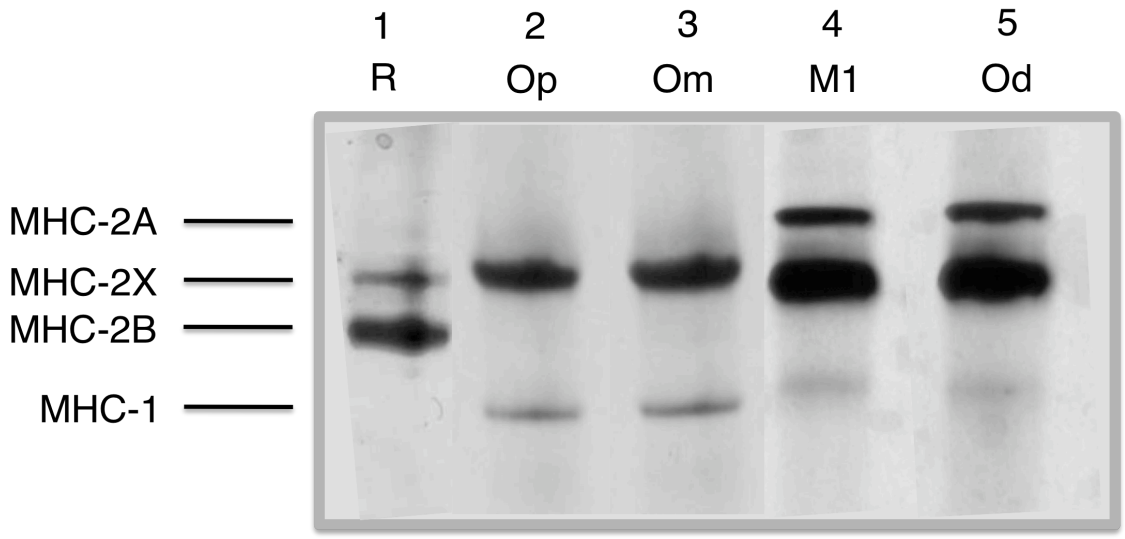

Anti MHC-2X
BF-35
MY32
A
D
G
B
E
H
C
F
I
*
1
2A/X

Figure 6. Representative SDS-PAGE gel results identifying MHC bands in tail muscle of the Virginia opossum, *D. virginiana*. MHC isoform identity and band migration patterns were confirmed by comparison with muscle homogenates from rat SOL (not shown) and TA (shown). Left to right: Lane 1 (**R**) Rat TA, Lane 2 (**Op**) MHC isoforms present in proximal tail region, Lane 3 (**Om**) MHC isoforms present in middle tail region showing two distinct protein bands, Lane 4 (**M1**) MHC isoforms present in middle tail region from a second individual showing three distinct protein bands, Lane 5 (**Od**) MHC isoforms present in distal tail region showing also showing three distinct protein bands.


1
R
2
Op
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5
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MHC-2A
MHC-2X
MHC-2B
MHC-1

Friday, January 29, 2010

Dr. Michael Butcher
Biology Department
UNIVERSITY

Re: IACUC Protocol # 03-09
Title: Muscle architecture and fiber type in the tail of the opossum: specializations for arboreal versus terrestrial locomotion

Dear Dr. Butcher:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled **"Muscle architecture and fiber type in the tail of the opossum: specializations for arboreal versus terrestrial locomotion"** and determined it should be unconditionally approved for the period of **August 19, 2009** through its expiration date of **August 19, 2012.**

This protocol is approved for a period of three years; however, it must be updated yearly via the submission of an Annual Review-Request to Use Animals form. These Annual Review forms must be submitted to the IACUC at least thirty days ***prior*** to the protocol's yearly anniversary dates of August 19, 2010 and August 19, 2011. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

Dr. Peter J. Kasvinsky
Associate Provost for Research
Dean School of Graduate Studies and Research

PJK:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Biological Sciences
Dawn Amolsch, Animal Tech., Biological Sciences