Hindlimb morphology in Eastern cottontail rabbits (*Sylvilagus floridanus*): Correlation of muscle architecture and MHC isoform content with ontogeny

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ABSTRACT

Rabbits have powerful hindlimb muscles that allow them to accelerate rapidly during locomotion. Moreover, juveniles may have performance advantages relative to adults that could increase their chances of survival to reproductive maturity. To investigate the ontogeny of power capacity in rabbit hindlimb extensors, muscle architectural properties and myosin heavy chain (MHC) isoform content were quantified in 9 juvenile and 5 adult eastern cottontail rabbits (*Sylvilagus floridanus*). It is hypothesized that musculoskeletal features of the hindlimb will be optimized in juveniles to promote increased locomotor performance similar to that of adults. We measured architectural properties including muscle mass (MM), belly length (ML), fascicle length (l^F), pennation angle, and physiological cross-sectional area (PSCA), known to provide functional estimates of maximum isometric force, joint torque, and power. MHC isoform composition was determined by SDS-PAGE and densitometry. Overall, the growth trajectories of MM and power increase faster (i.e., positive allometry) than body size with ontogeny and suggest that adults are more developed than juveniles. However, juvenile rabbits appear to have several compensatory features that may allow for increased locomotor performance (and fitness) including higher l^F/ML ratios, faster MHC isoform composition, and greater ankle joint mechanical advantage. Therefore, the findings only partially support the hypothesis. The development of the musculoskeletal system appears to provide rabbits with some advantages to evade predation by rapid acceleration; however, adult rabbits may be over-developed affecting their performance. Further studies are required to fully understand the locomotory development of rabbits through ontogeny.

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DEDICATION

I dedicate my Thesis to my family, especially my father Scott, my mother Brenda and my grandparents William and Joyce for their unfaltering support, both emotionally and financially, Finally, I would like to thank my brother Benjamin for always being their when I needed advice throughout my academic career and during the completion of this Thesis.

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θ - pennation angle (in deg)

AEI - ankle extensor index

AI - architectural index

CI - crural index

F_{max} - maximum isometric force

FRI - femur robusticity index

HMI - hip moment index

IHC - immuno-histochemistry

l^F - fascicle length

M - body mass

ML - muscle length

MM - muscle mass

MHC - myosin heavy chain

N - Newtons (unit of force)

PCSA - physiological cross-section area (in cm^2)

r_m - muscle moment arm

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

TL - tendon length

TM - tendon mass

TRI - tibia robusticity index

TSI - tibial spine index

V_{max} - maximum shortening velocity

W - Watts (unit of power)

INTRODUCTION

Young animals are most vulnerable to predation and hence have the greatest amount of pressure for survival. However, they must be able to escape predators and compete for the same resources as adults in order to reach reproductive maturity (Carrier, 1996). In particular, rabbits are altricial at birth, but develop rapidly in a way in which acceleration is essential for their survival, although young rabbits are incapable of sustaining their maximal speed for long intervals. This is because juveniles have a greater stride frequency than adults, which requires greater power generation to maintain a high velocity of running (Carrier, 1983, 1996). Despite this apparent disadvantage, several studies (e.g., Baker, 1983; Carrier, 1983; Heinrich et al., 1999; Torzilli et al.,1981; Young, 2005) have shown that the limbs of juveniles are often 'overbuilt', meaning they have musculoskeletal features that compensate for their lack of overall body size. For example, other studies show juveniles often have relatively high mechanical advantage about their hindlimb joints and relatively robust limb bones (i.e., cross-sectional area: CSA) as compared with adults (Carrier, 1983, 1996; Currey, 1984, 2001; Young, 2005). These features allow young animals to perform at near-adult levels with a reduced risk of skeletal injury (Main and Biewener, 2004, 2006, 2007). Impressively, immature jackrabbits are capable of achieving a take-off speed equivalent to that of adults when they are only about 25–30% of the adult body mass (Carrier, 1983).

Muscles are specialized to meet the performance needs of species in relation to the selective pressures experienced in their natural habitat (Hazimihalis et al., 2013). Therefore, locomotor habits directly influence the contractile properties of limb muscles. Muscle architectural properties (e.g., muscle mass, fascicle length, and pennation angle) are used to evaluate the functional capacity of limb muscles through estimates of peak isometric force (F_{max}) and power output (W) (Moore et al., 2013; Rose et al., 2013; Williams et al., 2007 a, b). In addition, the expression of myosin heavy chain (MHC) isoforms in muscle fibers determines contractile physiology; oxidative MHC fiber types (MHC-1 and MHC-2A) produce lower force and power over prolonged contractile periods, while more glycolytic fiber types (MHC-2X and MHC-2B) produce higher force and power for shorter durations (Shiaffino and Reggiani, 1996, 2011). The force and power that a muscle applies at a limb joint, however, are strongly influenced by the

architecture of the muscle fibers (Butcher et al., 2010; Lieber, 2009). Pennate muscles with short fibers have large physiological cross-sectional area (PCSA), and thus the ability to produce high force (Alexander et al., 1981, 1984). Alternatively, muscles with long fibers arranged in parallel (or close to parallel) with the axis of force production have a greater ability to shorten and produce force over a large range of joint motion (Zajac, 1989, 1992). A trade-off between these two general architectures indicates that a muscle is capable of performing appreciable work at potentially high power, depending in part on the distribution of fast MHC isoforms.

Analyses of locomotor performance are often related to quantification of muscle architecture, muscle fiber type, and limb bone properties in order to reconstruct the evolutionary function of the observed limb morphology. Far too often these analyses are conducted using solely adults, while few studies seek to understand how muscle contractile properties and locomotor performance change throughout ontogeny (see Carrier, 1996; Wang and Kernell, 2001; Werner and Gillian, 1984; Young, 2005). Because of their rapid development and high population density in Ohio (Huagen, 1942; Negus, 1958), eastern cottontail rabbits (*Sylvilagus floridanus* Allen, 1890) provide an excellent opportunity to study how limb muscle architecture and fiber type are correlated with both performance and survival. In particular, selection of musculoskeletal traits involved with their unique hindlimb morphology and accelerative performance, and how their morphological features develop quickly with ontogeny, make it possible to more clearly evaluate the biomechanical factors related to survival in this species.

While no morphological data exist for either adult or juvenile *S. floridanus*, muscle architectural properties have been previously quantified in the hindlimbs of adult laboratory rabbits, *Oryctolagus cuniculus*, (Lieber and Blevins, 1989) and wild hares, *Lepus europeus* (Williams et al., 2007b). From these studies it was observed that several massive hip extensor and knee extensor muscles have the functional capacity to provide a majority of the force and power required for rapid acceleration. Several of these muscles are pennate including all four heads of the quadriceps and parts of the biceps femoris m. (vertebral head). Other hip extensor muscles were observed to have parallel fiber architecture. For example, in hares, muscles of the gluteal complex have high fascicle length-to-muscle length ratios (~1.0) indicating a large capacity for muscle shortening at

high velocity (Williams et al., 2007a). In contrast, most muscles found in the distal segments of the hindlimb of both the rabbit and hare consistently show appreciable pennation and short fascicles compared to their muscle belly length (Lieber and Blevins, 1989; Williams et al., 2007a), thus indicating potential for high force production. Fiber type distributions in the hindlimb muscles of adult laboratory rabbits also have been evaluated, although the available data are conflicting (Korfage et al., 2009; Wang and Kernell, 2001), and fast MHC isoform composition has only been reported for the gastrocnemius m. and soleus m. (Korfage et al., 2009). Moreover, evidence of MHC isoform changes with ontogeny in rabbits is also very limited with only one previous study (Korfage et al., 2009) showing a fast-to-slow transition in MHC isoform expression in the soleus of laboratory rabbits during development. Distributions of MHC isoform fiber types have not been investigated in wild *S. floridanus*.

Our aim in this morphological analysis is to quantify muscle architectural properties and MHC isoform composition in the hindlimb extensor muscles of *S. floridanus* and compare ontogenetic differences between juvenile and adult rabbits. It is hypothesized that the morphological features of the hindlimbs in juvenile cottontail rabbits will be distinguished by 'compensatory' musculoskeletal growth trajectories that either approximate or exceed adult levels of performance. Specifically, we predict that in comparison with adults, the hindlimb extensors of juveniles will have higher relative percentages of muscle mass, larger PCSA, and a broader distribution of fast MHC isoforms (predominately MHC-2X). In addition, we predict that juvenile rabbits will have relatively higher muscle mechanical advantage, especially at the ankle joint, and hindlimb bones that are relatively more robust than adults. As a result of these differing musculoskeletal properties, *S. floridanus* should be able to achieve a high level of locomotor performance at a small body size, and their maximal running speed and acceleration capability will be directly related to the morphological features quantified.

MATERIALS AND METHODS

Study animals and Muscle samples

A total of N=15 rabbits (6 adults and 9 juveniles) were used for this study. Rabbits were classified as either adults or juvenile based on body mass (juveniles $\leq$ 800 g). Animals

were live captured in the field (Portage and Columbiana Counties, Ohio) during the Summer and Fall 2013 using wooden and metal rabbit traps (80 x 30 x 35 cm) and euthanized by an overdose IP injection of Fatal Plus (Vortech Pharmaceuticals, USA). Animal trapping (ODNR Wild Animal Permit: 10-252) and all experimental procedures comply with approved IACUC protocols (NEOMED 03-09, PI: J.W. Young; YSU, PI: M.T. Butcher). Specimens were stored at -20°C until observation, and allowed to thaw for 24–36 h at 4°C prior to hindlimb dissection and measurement from 10 hindlimb muscles: *gluteus medius m.* (GLM), *gluteus profundus m.* (GLP), *biceps femoris m.* [BF: pelvic (BFP) and vertebral (BFV) heads], *semimembranosus m.* (SM), *vastus lateralis m.* (VL), *rectus femoris m.* (RF), *gastrocnemius m.* [lateral (LG) and medial (MG) heads], and *flexor digitorum longus m.* (FDL) (Lieber and Blevins, 1989; Williams et al., 2007a). This work was conducted at Youngstown State University (YSU) between 2013 and 2014. Morphometric data from animals used in this study are presented in Table 1.

In a sample of N=5 adult and N=5 juvenile specimens, muscle tissue blocks were harvested from each muscle studied immediately post-mortem (left hindlimb). Briefly, muscle blocks (mid-belly) were dissected and mounted to cork with O.C.T. compound, frozen in isopentane cooled in liquid nitrogen, and stored at -80°C until analysis. Two additional small muscle blocks from each muscle were collected following dissection (see below) and prepared as a protein stock for electrophoresis by flash freezing in liquid nitrogen, grinding to powder, and homogenizing 50 mg of the muscle powder in 800 ml (ratio 1:16) of Laemmli buffer with 62.5 mM Tris (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 2.3% SDS (Laemmli, 1970). Protein samples were diluted (1:500) to a final protein concentration of ~0.125 μg/ml with gel sample buffer containing 80 mM Tris (pH 6.8), 21.5% glycerol, 50 mM DTT, 2.0% SDS, and 0.1% bromophenol blue (Mizunoya et al., 2008; Hazimihalis et al., 2013; Rupert et al., 2014). Samples were then heated (90ºC) for 5 min and loaded on gels or briefly stored at -20°C until analysis.

Muscle and Bone measurements

Hindlimb muscle architecture was quantified following the procedures of Rose et al. (2013), originally derived from the combined methods of Payne et al. (2005) and Williams et al. (2007a). Muscle names, origin, insertion, and action were previously described in detail (Lieber and Blevins, 1989; Williams et al., 2007a). The right

hindlimbs were skinned and n=10 extensor muscles were systematically dissected. Muscles were periodically moistened with phosphate buffered saline (PBS) to prevent desiccation during dissection and measurement. Muscle and tendon length *in situ* and muscle moment arm (r_m) were measured using digital calipers (Mitutoyo, Japan) with the hindlimb in its normal postural conformation. Specifically, r_m was measured (3x) as the perpendicular distance from an estimated line of muscle force action to the center of joint rotation (approximated with a pin), with each limb joint placed at an angle estimated to be a position in which antagonistic muscles could exert equal joint torque. Following removal of muscles and their free tendons, resting muscle belly length (ML) and tendon length (TL) were measured using digital calipers, and muscle mass (MM) and tendon mass (TM) were recorded using an electronic balance (Mettler-Toledo, USA: accurate to 0.01g). Muscles bellies then were incised under a dissection microscope to reveal the fiber fascicles and muscle pennation (Payne et al., 2005; Williams et al., 2007a). Incisions were made along the plane of the fascicles from origin to insertion for parallel-fibered muscles, and bisected at 90º to the internal tendon to visualize the fascicles of pennate-fibered muscles. Resting fascicle length (l^F) was measured from 5–10 random fascicles in muscle belly using digital calipers. Resting pennation angle (θ: Gans and De Vree, 1987) is defined as the angle between the fascicles and either the long axis of muscle or internal tendon. This angle (to the nearest degree) was measured at 5–10 random locations in the muscle belly using a goniometer.

With all muscles removed, hindlimb long bones were cleaned free of tissue and length and width parameters were measured using digital calipers: femur length (FemL), maximum length from the femoral head to distal condyles; proximal femur length ($FemL_P$), length from the femoral head to distal end of the greater trochanter; femur width (FemW), transverse width at mid-shaft; tibia length (TibL), maximum length from the tibial condyle to tip of the malleolus; proximal tibia length ($TibL_P$), length from the proximal articular surface of the tibia to the distal point of the tibial tuberosity; and tibia width (TibW), transverse width at mid-shaft. Length of the calcaneus (CalL) and metatarsal III (MT III) were also measured. Bone measurements were adapted for rabbits from Elissamburu and Vizcaíno (2004).

Architectural quantification and Functional indices

Muscle volume was calculated by dividing mean MM by a muscle density of 1.06 g cm^{-3} (Mendez and Keyes, 1960). Physiological cross-sectional area (PCSA) was calculated as (muscle volume/mean l^F) × cos θ, where θ is mean pennation angle (in deg) (Zarucco et al., 2004; Moore et al., 2013). Pennation angle was used in our calculations of corrected PSCA to permit more accurate estimates of isometric force (Rose et al., 2013). Maximum isometric force (F_{max}) was estimated by multiplying PCSA by a maximum isometric stress of 30 N cm^{-2} (Woledge et al., 1985; Medler, 2002). Joint torque was calculated as $F_{max} \times r_m$. Muscle power was estimated to be one-tenth of the product of F_{max} and V_{max} (Hill, 1938), where V_{max} is maximum fiber shortening velocity (in fiber lengths per second: FL s^{-1}). A value of 6.3 FL s^{-1} measured at 30ºC was used as V_{max} for rabbit MHC-2X fibers (Pate et al., 1995). Importantly, calculations of F_{max} and V_{max} are only estimates, and were used here to indicate muscle functional capacity (Williams et al., 2007a). Tendon volume was calculated by dividing mean TM by a tendon density of 1.12 g cm^{-3} (Ker et al., 1988). Tendon CSA was calculated as tendon volume ÷ mean TL. Tendon stress at maximum isometric force was estimated by dividing F_{max} by tendon CSA, whereas tendon strain was estimated by dividing tendon stress (in MPa) by 1.5 GPa, a value of Young's Modulus of mammalian tendon (Ker et al., 1988). Tendon length change then was estimated as resting TL × tendon strain (Williams et al., 2007b, 2008).

Muscle architecture was determined as both absolute and normalized values. Muscle and bone anatomy were assumed to scale isometrically with body mass (M), and thus were normalized accordingly (Alexander et al., 1981; Biewener, 2005): masses were normalized directly to $M^{1.0}$, areas to $M^{0.667}$, and lengths to $M^{0.333}$. Mass of each muscle studied was also normalized to muscle group mass. Muscles were categorized into three major functional groups for this analysis: hip extensors, knee extensors, and ankle extensors (FDL was evaluated separately from the ankle extensors in our analysis). Ratios of PCSA to muscle mass (PCSA/MM), fascicle length to muscle belly length (l^F/ML), and fascicle length to muscle moment arm length (l^F/r_m) (Moore et al., 2013; Rose et al., 2013) were calculated to assess muscle functional capacity, where each ratio is considered an important architectural index (AI). Specifically, PCSA/MM ratios (i.e., size-adjusted PCSA) were calculated using normalized muscle mass ($MM^{0.667}$) to

compare between adults and juveniles. The properties ML and l^F were also normalized to muscle mass ($MM^{0.333}$) and r_m was normalized to bone length (Table 1) in these calculations.

In addition, several functional indices from the hindlimb bones were calculated to further assess limb functional capacity. *Hip moment index* (HMI)=$FemL_P$/FemL: an index of the mechanical advantage of the gluteal muscles acting across the hip joint in hip extension (Vizcaíno and Milne, 2002). *Femur robusticity index* (FRI)=FemW/FemL: an indicator of the relative strength of the femur to support locomotor loading (Biewener and Taylor, 1996). *Crural index* (CI)=TibL/FemL: an index of the relative length of distal limb segment and extent to which the hindlimb is adapted for speed (Fleagle, 1979). *Tibial spine index* (TSI)=$TibL_P$/TibL: an indicator of the relative width available for attachment of the adductor and ankle extensor muscles; shorter tibial spine is indicative of higher speed of movement (Elissamburu and Vizcaíno, 2004). *Tibia robusticity index* (TRI)=TibW/TibL: an indicator of the relative strength of the tibia (Elissamburu and Vizcaíno, 2004). *Ankle extensor index* (AEI)=CalL/CalL+MT III: an index of the mechanical advantage of the extensor muscles acting across the ankle joint in ankle extension (Carrier, 1996).

MHC expression and Densitometry

MHC isoforms were separated on SDS-PAGE gels using established methods (Talmadge and Roy, 1993) performed with slight modifications (Mizunoya et al., 2008) as previously described (Rupert et al., in press). Briefly, the acrylamide-N,N'–methylenebisacrylamide (Bis) ratio of the gels was 50:1, with the total acrylamide percentage equaling 8% and 4% in the separating gel (35% glycerol) and stacking gel (30% glycerol), respectively. The basic formulation of the electrode buffer was 50 mM Tris (pH 8.3), 75 mM glycine and 0.5% SDS; upper buffer was 6X the concentration of the lower buffer and also contained 0.12% β-mercaptoethanol (Mizunoya et al., 2008). Approximately 1.0 μg of protein was loaded per gel lane, and electrophoresis was run on a mini-PROTEAN Tetra system (Bio-Rad) at constant low voltage (140 V) for 24–25 h at 4°C (Talmadge and Roy, 1993; Mizunoya et al., 2008). Gels were stained with silver (Bio-Rad) for visualization of MHC isoforms, and imaged using Pharos FX Plus (Quantity One software: Bio-Rad). MHC isoform identity was evaluated by resolution

and comparative migration patterns of the protein bands. MHC isoform content was quantified by densitometry in Image J (v.1.43: NIH) using a brightness area product (BAP) method similar to Toniolo et al. (2008). Band intensity values in each gel lane were summed and used to calculate a percentage for each MHC isoform band expressed in a single muscle. Percentages of the MHC isoforms for each muscle across individuals were averaged to provide a overall percentage composition of slow and fast MHC isoforms. Final MHC isoform percentages (%) represent weighted means from at least 2-3 independent gel experiments (Rupert et al., in press).

Statistical analysis

Descriptive statistics for all measurements are reported as mean±s.d. (standard deviation) unless otherwise specified. Body-size scaling analyses of hindlimb musculoskeletal features were performed using R (v. 3.1.0: http://www.r-project.org) on the following muscle properties and functional indices: MM, PCSA, F_{max}, power, and l^F/ML. Data for these variables were naturally log transformed and regressed against body mass (log-log transformed axes) then fit with curves to yield line equations in the form $Y=aM^b$, where M is body mass and b is the scaling exponent. Scaling relationships were assessed for relative increases or decreases (i.e., allometry: deviations for isometry) in morphometric variables as they relate to body mass in rabbits (between adults and juveniles). Significance for all regressions (differences from isometric scaling slope or differences from a slope of zero) was accepted at $p \leq 0.05$.

RESULTS

Functional distribution of muscle mass

Mean mass of the hindlimb muscles studied is 51.5±14.1 g for adults and 14.9±10.1 g for juveniles, accounting for 4.6% and 3.2% of their body mass, respectively. Overall, the hip extensors are the most massive functional muscle group of the hindlimb followed by the knee extensors and ankle extensors for both adults and juveniles. Of these functional muscle groups, the biceps femoris pelvic (BFP) and vertebral (BFV) heads (both hip extensors) are the two single largest muscles of the hindlimb, and together they account for 37.1% of the hindlimb muscle mass studied for adults and 37.6% for juveniles. The medial head of the gastrocnemius (MG) is also large, and combined with the lateral lead

(LG), the entire gastrocnemius muscle accounts for 17.2% of the hindlimb muscle mass for adults and 18.7% for juveniles.

The distribution of muscle group mass normalized to total body mass is shown in Figure 1. Again, the hip extensors are notably the largest functional group and they account for 2.8±0.4% and 1.8±0.5% of the body mass for adults and juveniles, respectively. The second largest functional group is the knee extensors, accounting for 0.8±0.1% (adults) and 0.7±0.1% (juveniles) of the body mass. The third largest functional group is the ankle extensors and they account for 0.8±0.1% and 0.6±0.1% of the body mass for adults and juveniles, respectively. Lastly, the smallest muscle group is the digital flexors containing only the FDL muscle, accounting for 0.14±0.03% (adults) and 0.11±0.03% (juveniles) of the body mass (Fig. 1).

Muscle architectural properties

The rabbit hindlimb has 10 major extensor muscles for which muscle architectural properties have been quantified. The absolute muscle measurements are presented in Table 2 while the normalized measurements are represented in Table 3. Proximal muscles acting at the hip joint all have long fascicles arranged in a parallel fiber architecture, whereas the distal muscles acting at the knee and ankle joints become progressively more pennate with shorter fascicles along the length of the limb. The muscles with the longest absolute fascicle lengths in both age groups are the BFP (6.3±0.7 cm adult; 4.4±1.0 cm juvenile) and BFV (5.9±0.8 cm adult; 3.9±1.2 juvenile) (Table 2). Other muscles acting at the hip joint, including the gluteus medialis (GLM), gluteus profundus (GLP), and semimembranosus (SM) also have relatively long fascicles, each with a mean fascicle length that approximates their muscle belly length. The remainder of the muscles studied have relatively short fascicles by comparison. The muscles with the shortest fascicles and highest degree of pennation include the ankle extensors and FDL with absolute fascicle lengths less than 1.5 cm for both adults and juveniles (Table 2).

Ratios of fascicle length (l^F) to muscle length (ML) are shown in Figure 2, where higher values are indicative of greater range of contraction and fascicle shortening capability. Nearly half of the muscles of the hindlimb have an l^F/ML ratio of 0.5 or greater. There is a consistent pattern among functional muscle groups in which the hip extensors have very high ratios, the knee extensors have moderate ratios, and the ankle

extensors and FDL have the lowest ratios. The BFP has the highest l^F/ML ratio of all muscles with means of 0.84±0.1 for adults and 0.77±0.1 for juveniles (Fig. 2). Of the remaining hip extensors the GLM, GLP, and SM each have values exceeding 0.7 for both adults and juveniles, while the BFV has mean l^F/ML ratios just under 0.7 for both adults and juveniles. Except for the unipennate vastus lateralis (VL), which has a moderate value, the knee and ankle extensors as functional groups have low l^F/ML ratios ranging between 0.15–0.34. In general, muscles acting at the knee and ankle joints are pennate and have ratios less than 0.35, with the bipennate MG and FDL having the lowest values of all muscles for both adults and juveniles (Fig. 2).

On average, pennation angles (θ) range from 0–34º, with most muscles displaying parallel and unipennate fiber architecture (Table 2). Muscles with the highest mean pennation angles are the MG (34±7º, adult; 31±4º, juvenile) and FDL (31±5º adult; 29±4º juvenile) (Table 2). Unipennate muscles including the BFV, VL, rectus femoris (RF), and LG have mean pennation angles 25º or greater. Corresponding with its relatively high values of θ and short fascicles, the muscle with the highest PCSA is the medial gastrocnemius (5.53 cm^2, adult; 1.72 cm^2, juvenile) (Table 2). The VL has the second largest PCSA, while the remaining muscles functionally grouped as hip extensors have moderate-to-low PCSA ranging from 0.8–2.0 cm^2 for adults to 0.4–1.0 cm^2 for juveniles.

Ratios of PCSA to muscle mass (MM) are shown in Figure 3, where values of 0.5 or higher are indicative of greater force production capability. No notable differences between adults and juveniles are observed with the exception of the MG. The ankle extensors (as a functional group) and FDL have relatively low mass, and correspondingly have the highest PCSA/MM ratios. The VL, RF, GLM, and GLP have moderate values ranging from 0.5–0.63 for adults and are all approximately 0.5 for juveniles (Fig. 3). In contrast, the major muscles that act to extend the hip joint (e.g., BFP, BFV, and SM), have the lowest PCSA/MM ratios. In relation to its exceedingly high mean PCSA/MM ratio of 1.6±0.6 in adult rabbits, the MG also has the highest estimated isometric F_{max} of 146 N kg^{-1} (Table 3), where muscle force is normalized to body mass. The MG in juveniles, which also has the large PCSA/MM ratio greater than 1.0, additionally has the highest normalized value of estimated isometric F_{max} of 104 N kg^{-1}.

Figure 4 shows the summed total isometric force that each functional muscle group is capable of producing. Again, no major differences between adults and juveniles are observed with the exception of the ankle extensors. The largest force producing groups are the hip and ankle extensors, for which adults have an average summed isometric F_{max} of 171 N kg^{-1} and 184 N kg^{-1}, respectively. These values are 2x greater than the total force of the knee extensors and nearly 4x that of the FDL. The hip and ankle extensors for juveniles have averages of summed isometric F_{max} of 162 N kg^{-1} and 149 N kg^{-1}, respectively (Fig. 4). Similar to adults, these values are approximately twice that of the knee extensors and four times that of the FDL in juvenile rabbits.

Figure 5A shows PCSA as a function of fascicle length, where higher values of both properties are indicative of power capability. No muscles of the rabbit hindlimb have the capacity for high power output, although some are capable of either high force (e.g., MG) or high range of shortening (e.g., BFP) (Fig. 5A). Similarly, no muscles have the capacity for high joint torque, although the MG is capable of appreciable torque (Fig. 5B). However, the majority of the muscles studied have the capacity for relatively high velocity of joint rotation (Fig. 5C). The muscles with the highest individual estimates of instantaneous power are the BFP (1.52 W kg^{-1}, adult; 0.97 W kg^{-1}, juvenile), BFV (1.34 W kg^{-1}, adult; 0.87 W kg^{-1}, juvenile), and VL (1.07 W kg^{-1}, adult; 0.89 W kg^{-1}, juvenile) (Table 3), where power is normalized to body mass. As a functional group, the hip extensors have highest summed total power of 4.79 W kg^{-1} for adults and 3.14 W kg^{-1} for juveniles. In both adults and juveniles, the knee and ankle extensors are capable of generating relatively low power. The FDL by itself is capable of generating 0.21 W kg^{-1} and 0.17 W kg^{-1} for adults and juveniles, respectively (Table 3).

Rabbit hindlimb muscle moment arms (r_m) and estimated joint torques are presented in Table 4. For biarticular muscles, only joint torques in which the muscle extends a limb joint are reported. The muscle that has the largest mean joint torque is the MG (198.7 N.cm kg^{-1}, adult; 106.8 N.cm kg^{-1}, juvenile). Other muscles with appreciable joint torque are the BFP and SM. With the exception of the VL (84.4 N.cm kg^{-1}, adult; 89.9 N.cm kg^{-1}, juvenile), all hindlimb muscles studied are capable of applying relatively higher joint torque in adult rabbits compared to juveniles. Additionally, the hip extensors adults (and juveniles) have l^F/r_m ratios ranging from 2–5 with the BFV having the largest ratio of all

muscles studied, where values greater than 2 are indicative of a muscle's ability to rotate a joint through a large range of motion (Table 4). Values for the knee extensors are lower and range from 1.25–2.05 for both adults and juveniles. Overall, the VL has a larger l^F/r_m ratio (2.05, adult; 1.65, juvenile) than the RF (1.33, adult; 1.25, juvenile). With the exception of the FDL, which has moderate l^F/r_m ratio of 1.70 and 2.24 for adults and juveniles, respectively, the distal limb muscles have low l^F/r_m ratios less than 1.12 (Table 4). These ratios correspond with the data shown in Figure 5C.

Lastly, tendon properties for MG, LG, and FDL are presented in Table 5. All other muscles studied had no discernable free tendons at either their origin or insertion. Tendons of insertion for the distal limb muscles have relatively low values of CSA, stress, strain, and locomotor length change. Estimates of stress *in vivo* were highest in the tendon of the MG (57.0 MPa, adult; 21.5 MPa, juvenile). Tendon stress of the LG is similar in both adult and juvenile rabbits (Table 5). The FDL tendon is the longest of the three tendons quantified and has both the largest mass (0.35 g, adult; 0.14 g, juvenile) and CSA (0.05 cm^2, adult; 0.02 cm^2, juvenile). Estimates of strain (%) across all tendons studied are relatively low and range from 0.51–3.80% for both adults and juveniles when compared to the hare (Williams et al., 2007a) (Table 5). The MG has the highest capacity for strain and length change, especially in adult rabbits.

Functional bone indices

The six osteological indices calculated for rabbit hindlimb bones are shown in Figure 6. Overall, limb bone proportions are similar between adults and juveniles. Average values of the bone robusticity indices are 0.08 (FRI) and 0.07 (TRI) for both adults and juveniles, respectively (Fig. 6A, B). The index that indicates the relative area for muscle attachment about the proximal tibia (TSI) is also the same with adults having a value of 0.24 and juveniles having a value of 0.25. The crural index (CI) that relates to the speed of movement of the limb is 1.11 in adults and 1.05 in juveniles on average (Fig. 6D). Lastly, the indices that relate to the mechanical advantage about the hip (HMI) and ankle (AEI) joints are the only ones that show disparity between adults and juveniles, with the juveniles having greater mechanical advantage at each joint. Average values of HMI are 0.21 for adults and 0.25 juveniles, while average values of AEI are 0.37 for adults and 0.45 for juveniles (Fig. 6).

Body-size scaling relationships

Regressions relating muscle properties to body size across individuals are shown in Figure 7. Regressions of muscle mass (MM) for all functional muscle groups are significantly different ($p<0.0001$) from isometry (slope = 1) and all show positive allometry (i.e., slope > 1) (Table 6; Fig. 7A, B). Regressions of F_{max} across functional muscle groups are not significantly different ($p=0.07–0.16$) from isometry with slopes not significantly different from 1 with the exception fo the ankle extensors ($p=0.005$) (Fig. 7C, D). For regressions of power (W), the hip extensors, knee extensors, and FDL are significantly different ($p=0.004–0.03$) from isometry (slope = 1.33). Both the hip extensors (Fig. 7E)and FDL show positive allometry while the knee extensors (Fig. 7F) show negative allometry (i.e., slope < 1.33) (Table 6). Power for the ankle extensors is not significantly different ($p=0.29$) from isometry across body mass. Regressions for l^F/ML for all functional muscle groups are significantly different ($p\leq0.001$) from isometry (slope = 0) and all show negative allometry (Fig. 7G, H).

MHC isoform composition and Fiber types

Rabbit hindlimb muscles show expression of four MHC isoform bands: MHC-1, 2A, 2X, and 2B. Fast MHC-2X and 2B bands are most clearly resolved in all muscles from each individual (N=5 adults; N=5 juveniles) with the exception of the adult FDL. Slow MHC-1 and fast MHC-2A bands are not identified in all muscles studied from *S. floridanus*, and bands for these isoforms are comparatively lighter in their resolution to that of MHC-2X and 2B. Minimal separation of the MHC-2A and 2X isoforms (similar molecular weight) is observed in all gels analyzed, while MHC-1 and MHC-2B isoforms show good separation.

Across all hindlimb muscles studied, densitometric quantification of MHC isoforms reveals that MHC-2X is the predominant isoform expressed with relative percentage compositions of 58.5% and 57.5% for adults and juveniles, respectively. Fast MHC-2B is next most abundant isoform and the mean percentage composition of juveniles (33.0%) is higher than that of adults (23.4%) (see Table 7 for individual muscle means). Overall, percentage compositions of the MHC-2A isoform between adults and juveniles were low, and slow MHC-1 has the lowest percentage composition of all isoforms expressed in rabbit hindlimbs. Figure 8 shows the relative composition of MHC isoforms among the

functional muscles groups in the hindlimbs of adult and juvenile rabbits. The hip extensors are composed of almost entirely of the fast MHC-2X and 2B isoforms. In both adults and juveniles, MHC-2X accounts for approximately half of the isoform composition of the knee extensors. The ankle extensors are predominately composed of MHC-2X with means of 77.0±3.0% and 60.0±7.8% for adults and juveniles, respectively (Fig. 8). Additionally, MHC isoform composition for the FDL shows a near homogeneous expression of fast MHC-2X in juveniles (94.7%) and a mixed composition of MHC-2A (33.6%) and 2X (62.58%) for adults (Fig. 8).

DISCUSSION

Selection of musculoskeletal features

Evaluation of hindlimb function (i.e., locomotor performance) between adult and juvenile eastern cottontail rabbits begins with detailed analyses of their musculoskeletal structure. Relative hindlimb proportions are characterized by several osteological indices that are indicative of strength of the bones, areas of locomotor muscle attachment, muscle mechanical advantage, and limb out-velocity (speed) capabilities. Bone indices used to relate the locomotor performance of *S. floridanus* across ontogeny are largely similar. This indicates the hindlimb bones of juveniles have equal ability to resist locomotor forces (bending loads) and have similar area available for the attachment of extensor and adductor muscles. However, juvenile rabbits have higher mechanical advantage about their ankle joints as compared with adults. The ankle extensor index (AEI) is slightly greater in juvenile rabbits and this is suggestive of the ability to apply a relatively larger extensor moment at the ankle joint. Similar findings were observed in wild cats (Carrier, 1983) and jackrabbits (Carrier, 1996). Higher mechanical advantage at the hindlimb joints is related to greater accelerative ability (Hudson et al., 2011a) and may be a selected trait that increases fitness in juvenile *S. floridanus*. Our future studies involving micro-CT scan measurements of hindlimb structure and bone mechanical properties and in cottontail rabbits will allow for more rigorous tests of this hypothesis.

Osteological indices do well to discriminate the functional abilities of the hindlimb between adult and juvenile rabbits. Although, it is important to consider the muscle properties that relate to accelerative ability and how they change with development. The

majority of muscle architectural properties quantified are also observed to be largely similar between adult and juvenile cottontail rabbits. The relative distribution of extensor muscle mass is the same except for greater development of the hip extensors in adults. However, the large investment of relative hip extensor muscle mass in both adults and juveniles suggests the importance for power generation at the hip joint for fast acceleration throughout ontogeny. Notably, the proximal hindlimb extensor muscles of both adults and juveniles have similar shortening capacity by their relatively high l^F/ML ratios, which relate to a large contractile range and appreciable velocity of shortening (Lieber and Blevins, 1989; Moore et al., 2013; Rose et al., 2013). The hip extensors are indicated to have the greatest capability to shorten, and thus perform mechanical work and generate power. Despite the power capacity (by their architectural properties) of this functional group, findings from our complimentary study of hindlimb joint powers determined from 3D kinematics and force platform recordings of acceleration in cottontail rabbits (Young et al., 2014) show that power output is highest at both the ankle and lumbopelvic joints. Correspondingly, the high l^F/ML ratio for the MG of juveniles indicates they have relatively greater shortening capacity for power at the ankle joint.

Differences in power (and torque) output between adults and juveniles may be related to the higher mechanical advantage that juveniles have at their ankle joint and the ability of the ankle extensors in young rabbits to supplement the hip extensors in generating power for the stride. This may be the main mechanism by which juveniles achieve increased locomotor performance (Carrier, 1983, 1996; Young, 2005) early in life to overcome growth-related limitations in their development of hip extensor muscle mass. Interestingly, the MG of adults has a large PCSA/MM ratio indicating high force production capability. In general, force production capacity increases proximal-to-distal along the hindlimb in both adults and juveniles reflecting a functional trade-off between force and shortening velocity. A similar trend was observed in both new Zealand white rabbits (Lieber and Blevins, 1989) and the hare (Williams et al., 2007a). The MG appears to develop disproportionately with ontogeny where it becomes relatively more massive in adults, is more highly pennate, and consequently has larger PCSA. Therefore, function of the MG in the adult form appears to be more specialized for spring-like behavior to save energy during running as opposed to generating higher power for acceleration early in

development. The different tendon properties measured for the MG of adults and juveniles match these functional interpretations. However, by our calculations, the tendons of the ankle extensors (and FDL) in *S. floridanus* undergo relatively low strain and do not store large amounts of elastic strain energy like those of jackrabbits (Carrier, 1996) and hares (Williams et al, 2007a).

The ability to generate power and apply torque at the hindlimb joints is critical to acceleration and achieving maximal running speeds to escape predation. Evaluation of Figure 5 allows for the best comparison of the functional capacity of the hindlimb musculature for speed between adult and juvenile rabbits. A similar analysis was performed for the limbs of the cheetah and greyhound (Hudson et al., 2011a) to indicate the muscles (or groups) capable of high power, high joint torque, and joint rotational velocity. Power is determined as the product of force and shortening velocity (Lieber and Ward, 2001), thus power capacity is indicated by the relation of normalized PCSA and fascicle length (Fig. 5A). Homologous extensor muscles in adult and juvenile rabbit hindlimbs share similar power output by their matching relative positions in the plot. The hyperbolic-shaped curve formed by the data points emphasizes the tradeoff between force and shortening velocity (i.e., fascicle length excursion) between proximal and distal muscle groups. The ankle extensors all fall within the high force quadrant (upper left) while the major hip extensors are mostly found in the high shortening capacity quadrant (lower right). The knee extensors and gluteal muscles have intermediate properties of force production and shortening capacity. Interestingly, no individual extensor muscle of the rabbit hindlimb (adult or juvenile) is capable of high power output by their measured architectural properties. Similar findings were reported for the hindlimb muscles of adult hares (Williams et al. 2007a); however, the adductor (an adductor and hip extensor) was included in their sample and this muscle indicated to be capable of high power.

The application of joint torque involves both high force and a sizable muscle moment arm. Large joint torque corresponds to a reduction in the capability of a muscle to produce high joint rotational velocities (Hudson et al., 2011a,b). Homologous hindlimb extensors in adults and juveniles share similar abilities to apply joint torque (Fig. 5B). The ankle extensors fall primarily in the area of higher force production (upper left) while the proximal hip extensors fall within the area of longer moment arms (lower right). No

muscles fall with in the high joint torque quadrant (upper right); however, the MG is the only muscle capable of appreciable torque as it falls near this region of the plot. In contrast, a number of muscles of the rabbit hindlimb are capable of generating high joint velocities (Fig. 5C). Specifically, the hip extensors (e.g., GLM and BFV) likely act as the primary source of power to retract the hindlimb and accelerate the animal. The knee extensors fall in the middle region of the plot and are capable of appreciable joint rotational velocities allowing for fast extension of the knee joint, thus assisting with power generation during the second-half of the stance phase. These interpretations are supported by the moderately high l^F/r_m ratios of gluteal muscles and high ratios for the BFV (adults and juveniles). By this ratio, the knee and ankle extensors (and FDL) have relatively low ability to rotate their respective joints, and these trends were consistent across development. Furthermore, our findings are consistent with previous studies of hindlimb muscle architectural properties of the hare (Williams et al. 2007a) and cheetah (Hudson et al., 2011a), in which more proximal muscles were capable of larger amounts of power and joint excursion and the distal limb muscles produced relatively greater amounts of force and joint torque.

Muscle fiber physiology also plays a role in determining the contractile performance of muscles, and the recruitment of faster MHC fiber types directly relates to the metabolic energy cost of locomotion. Fast MHC-2X is the predominant isoform expressed in the hindlimb extensor muscles of rabbits. This trait suggests that appreciable power is required to cycle the limbs rapidly during locomotion and is consistent with studies of hindlimb muscles from other high-speed cursors including the caracal (Kohn et al., 2011a), cheetah (Goto et al., 2012), and black wildebeest (Kohn et al., 2011b). The finding of an abundance of the 2X isoform is further consistent with fiber type data from the rabbit psoas m. (hip flexor) known to have a uniform composition of MHC-2X (Reiser et al., 1985). Additionally, this study is the first to report MHC isoforms from major extensor muscles other than those of the distal hindlimb [e.g., MG, LG, and FDL (Korfage et al., 2009; Wang and Kernell, 2001)]. MHC-2X isoform fibers have intermediate glycolytic and power properties (Schiaffino and Reggiani, 2011), and the overall distribution of this fast fiber type is nearly identical between adults and juvenile cottontail rabbits. For example, the LG and MG have distributions in which MHC-2X

comprises over 50% of their isoform composition. These findings are consistent with changes in fiber type with development (1–20 weeks) in the gastrocnemius m. of laboratory rabbits (Korfage et al., 2009).

The hip and knee extensors of cottontail rabbits are primarily composed of MHC-2X and 2B isoforms, which can contribute to high power generation. Both heads of the biceps femoris m. are almost exclusively MHC-2X and 2B in their isoform expression, with the latter isoform comprising over 50% of the MHC content. Expression of the MHC-2X isoform is predominant in the VL of adult rabbits, while the VL of juveniles expresses both fast MHC-2X and 2B suggesting greater power capacity of their knee extensors. Collectively, the MHC isoform composition matches the function of these muscles indicated by their fiber architecture, and suggest that knee extensors of juveniles may contribute relatively more power to the running stride than adults. A greater expression of fast MHC-2B while young, and thus a greater capacity of the both the knee and ankle extensors to supplement power, may be the adaptive strategy by which juveniles compensate for relatively lower hip extensor muscle mass. Higher overall limb muscle power would allow juveniles to achieve similar maximal speeds as adults. MHC isoform composition becomes slower in adults, namely by a transition of the 2B isoform to 2X. At the adult stage of development, there is less selective pressure for survival and the hindlimbs show a slightly different suite of musculoskeletal features for locomotor economy vs. high speed exclusively.

The hindlimb musculature of other high-speed running animals may also develop in a similar manner. For example, an abundance of the MHC-2X isoform in both the hip and knee extensors of adult rabbits is consistent with the MHC content of those muscles in adult springboks (Curry et al., 2012), medium-sized gazelles under similar selective pressure from pursuit predators. In these animals, MHC-2X isoform fibers are not only directly related to high running speed, but also fatigue resistance. High fatigue resistance in large distributions of MHC-2X fibers has also been show is several other African cursors capable of achieving high speeds (Kohn et al. 2011a, 2011b). Since MHC-2B fibers are glycolytic in their ATP metabolism and fatigue easily, this further emphasizes that speed is a major factor related to the fitness and survival of juvenile cottontail rabbits. The selective presence of MHC-2B in the medial gastrocnemius of the cheetah (Hyatt et

al., 2010) is also attributed to specialization for high speed. The broader distribution of MHC-2B may also explain while juvenile rabbits cannot sustain maximal speeds as long as adults. However, in most cases of escape from a predator, rabbits need to accelerate quickly and just long enough to reach safe refuge (Beckwith, 1954).

Locomotor advantages with ontogeny

Studies on locomotor performance through ontogeny may elucidate examples of compensatory mechanisms through development (e.g., Carrier, 1996; Rome et al., 1990; Curtin and Woledge 1988; Altringham et al., 1996; James et al., 1998). Scaling analyses on muscle architectural properties for each functional muscle group indicate advantages and disadvantages in cottontail rabbits during development. This form of analysis best provides information about performance related abilities as a function of their growth trajectory. Collectively, the growth trajectories evaluated suggest that adult rabbits (based on body mass) have a distinct advantage for generating power to be used for rapid acceleration and predator escape. Muscle mass for each functional group shows a trend of positive allometry indicating that as rabbits grow, their functional muscle group mass, increases faster than overall increases in body mass (see Fig. 7A, B). Power for hip and ankle extensors also shows positive allometry further indicating that adult rabbits are relatively more powerful than juveniles (see Fig. 7E). This suggests that adult rabbits are 'overbuilt' for their size, meaning that for their body mass and power generation capabilities, adults have relatively larger muscles than juveniles. However, this may be a disadvantage for adults as they have to be more powerful to accelerate during locomotion to overcome the constraint of having disproportionality large body mass.

Our assessment of disproportionate hindlimb muscle hypertrophy relating to increased muscle power logically follows the finding, that with the exception of the ankle extensors as a functional group, isometric F_{max} develops in proportion with increases in body mass (see Figs. 7C, D). This indicates that the relative amount of force production of the hip and knee extensors (and FDL) remains the same throughout ontogeny. These data correlate with the findings from our companion study of 3D kinematics and force platform analyses showing that these muscle groups maintain joint position during the stance phase via high force production and therefore, do not much generate much power despite the capability to do so by their architectural properties (Young et al., 2014).

Moreover, the negatively allometric scaling trends of l^F/ML for all functional muscle groups agrees with the locomotor data set by indicating that adults have relatively shorter muscle shortening excursions than juveniles. However, this may also suggest another adaptive strategy for younger rabbits whereby they have relatively longer fascicles that are capable of undergoing substantial shortening at higher velocities (see Fig. 7G, H). This feature directly corresponds to the greater expression of fast MHC-2B in younger rabbits and the inherently high shortening velocities of muscle fibers expressing the 2B isoform. A larger distribution of MHC-2B fibers may be required during the early stages of development in rabbits for high power generation to compensate for relatively lower muscle mass and similar force production abilities of the hindlimb extensor muscles.

The ankle extensors show positive allometry for the development of F_{max}, indicating that the force production of these muscles is greater in adults, potentially providing them with an performance advantage over juvenile rabbits (see Fig. 7D). This combined with growth trajectories of muscle mass is in agreement with findings in both pigs (Anapol and Herring 1989) and fish (Grubich, 2003), where notable hypertrophy of the masseter m. occurs during ontogeny and suggested to explain observed increases in bite force. However, the larger AEI in juveniles suggests an additional adaptive strategy by which young rabbits have higher mechanical advantage to produce large out-force and torque at the ankle joint that would facilitate acceleration. Similar functional interpretations were made for the hindlimbs of jackrabbits (Carrier, 1996), where it was observed that they have a relatively stronger gastrocnemius m. early in development (by a greater AEI) that allow them to accelerate rapidly by producing high out-force to leap forward. Lastly, high force (and torque) capability of the ankle extensors also agrees with the findings from our locomotor analyses showing that high ankle joint power is one of the main sources power output for acceleration in rabbits (Young et al., 2014). This may seem counterintuitive given that power for ankle extensors develops in proportion with increases in body mass (i.e., isometry). It is plausible that this finding emphasizes that when rabbits reach adult sizes, the ankle extensors become more important for economical high force production and elastic energy storage savings by spring-like function of these muscle-tendon units.

Conclusions and Future directions

In conclusion, adult cottontail rabbits appear to be overbuilt for their body size throughout ontogeny. In particular, muscle mass, force, and power show trends of positive allometry with development relating to high levels of locomotor performance. Moreover, the rabbit hindlimb appears to develop in a manner that increases the mass and power properties of the proximal hindlimb muscles and force production properties of the distal hindlimb muscles. Therefore, our findings partially support the hypothesis that juveniles could perform at or near adult levels of locomotor performance by revealing several compensatory musculoskeletal features: (1) equally robust hindlimb bones that can resist high locomotor bending forces; (2) greater mechanical advantage at the ankle joint (and stiffer tendons) that allows for higher force and torque by the ankle extensors; (3) relatively longer fascicle lengths correlating with increases in shortening velocity; and (4) inherently more powerful hindlimb extensor muscles by greater expression of the fast MHC-2B isoform. However, the question remains whether adult rabbits are powerful enough to overcome the developmental constraint of having relatively larger body mass and do juveniles benefit by being relatively less massive in their hindlimbs? Future directions for this study include evaluation of muscle architectural properties of the vertebral column extensor muscles that may aid in rapid acceleration. In addition, a thorough study of MHC isoform fiber type of the hindlimb extensor by immuno-histochemistry (IHC) is currently being performed. This analysis will determined the distribution of MHC isoform fibers across the entire muscle cross-section and better characterize the physiological specialization of rabbit hindlimb musculature for power generation and fatigue resistance. Finally, this study is the first to link both muscle architectural properties and MHC isoform composition across ontogeny, and it improves our understanding of the relationship between functional morphology and fitness. Adaptive strategies observed in juvenile rabbits are musculoskeletal features likely common to other taxa to reduce selective pressures across ontogeny.

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APPENDIX

Introduction

Environmental conditions strongly influence both the morphology and physiology of animals. Selection for (or against) traits related to an increase in the chances of survival of a species is generally defined as fitness. Fitness is a key principle of **Evolution** and is directly related to the success of a species to acquire resources in their native habitat and survive to a reproductively mature age (Kennedy and Lindsay, 1984; Nevo, 1979). Because of their flexibility to inhabit a broad range of climates and their high reproductive success, Eastern cottontail rabbits (*Sylvilagus floridanus*: Allen, 1890) make an excellent model species to study the fundamentals of natural selection and survival. In particular, selection of musculoskeletal traits involved with their unique hindlimb morphology and running performance, and how these morphological features and behaviors develop quickly with ontogeny, make it possible to more clearly evaluate the biomechanical factors related to survival in this species. In addition, muscle performance requirements will vary with the constraints of the niche that a species occupies. Therefore, specializations of muscles reflect the relationship between evolution and locomotor behavior, understood as a *structure-function relationship*, and further implies the metabolic cost associated with locomotion (Biewener, 1998). The ability to minimize the metabolic cost of muscle work is an important factor for survival and is also related to the fitness of a species.

Evolution, Ecology, and Behavior

S. floridanus belong to the order Lagomorpha and family Leporidae (including rabbits and hares) (Halanych, 1996). Despite much debate over the evolution and phylogenetic ancestry of Lagomorphs, it is generally accepted that they shared a common ancestor with ungulates over 85 million years ago, and their order separated into the families Leporidae (rabbits and hares) and Ochotonidae (pikas) approximately 40 million years ago. Moreover, rabbits and hares, which share a similar morphology, appear to have diverged in the late Miocene (Matthee, 2009; Williams et al., 2007b). Today, *S. floridanus* inhabit a broad range of climates and microclimates spanning the Americas. Specifically, they range throughout Eastern North America (as far north as Southern

Canada), Central America, and Northwestern South America (Chapman et al., 1980; Olcott, 2000). Within these broad geographical home ranges a number of contrasting environmental conditions are present including, harsh winters at the northern range boundary, arid conditions in the central range, and temperate climates along the southern range border.

Body size is an important feature that allows these rabbits to survive disparate environmental conditions. Throughout their entire home range, *S. floridanus* exhibit the largest body size in the northeastern North America environments, particularly in areas around Lake Ontario, and the smallest body size in areas of Northern Mexico (Olcott, 2000). On a more regional scale, *S. floridanus* tend to be smallest along coastal regions where the winter is not as harsh and a larger body size is not required. Topographical elevation also influences body size, where the body size of rabbits tends to be larger in areas of high elevation with cool rainy summers and predictable winter conditions (Olcott, 2000). Other species of rabbits also display variability in body size due to environmental conditions, but are often more limited by the availability of resources resulting in increasing and decreasing population cycles. The generalist nature of *S. floridanus*, however, allows them to be more resistant to these sudden shifts in population cycles through the annual availability and variation of food resources (Dalke and Slime, 1941; Olcott, 2000). Furthermore, compared with other species of rabbits, *S. floridanus* have a relatively small body size, and yet they are not limited to being defenseless against predation, and exhibit several means of predator evasion. When threatened, rabbits always attempt to flee from a predator by using a series of short jumps while rapidly changing directions (Chapman et al., 1980, Schnurr, 1984). During these evasive maneuvers, Eastern cottontails are capable of achieving speeds of about 29 km h^{-1} (Linzey, 1998) and if cornered, they will attempt to use their powerful hind legs to kick and claw at a predator (Haugen, 1942).

Eastern cottontails are primarily nocturnal, leaving their burrows when the time is close to dusk and remain active throughout the late morning (Linzey, 1998). They are solitary animals that are typically not found in groups, with the exception of the mating season and when offspring are present with their adult parents. *S. floridanus* go through two molts per year; the Spring molt begins in March and lasts through August (Dalk,

1942), and the Fall molt begins in September and full Winter pelage is achieved within the first week of November (Negus, 1958). Their mating season takes place annually in the Spring and typically starts at the beginning of May (Baker, 1983). The gestation period has been observed in captive rabbits to last 30-34 days (Haugen, 1942). At birth, rabbits are altricial (i.e., born with eyes closed and incapable of moving) but, they develop quickly and become independent of their mothers in as early as three weeks. Females commonly have between one-to-four litters during the mating season with an average litter size of 5 from a total of 12 nests (Haugen, 1942). Lastly, the diet of *S. floridanus* is largely composed of various herbaceous plants (e.g., bluegrass and clover) and woody plants (e.g., apple trees, sumacs, and berries) (Chapman et al., 1980). In Ohio, herbaceous plants are the primary diet of Eastern cottontails throughout most of the year except for times of heavy snow cover (Chapman et al., 1980).

Limb Morphology and Function

S. floridanus are distinctive by their long ears, reduced forelimbs, and disproportionately large hindlimbs and long hindfeet. In many ways their anatomy is similar to that of closely related hares; however, whereas rabbits generally have hindfeet 2–3 times longer than their forefeet, hares are more extreme in their limb proportions by having hindfeet that are 4–5 times longer than the forefeet. Notably, the forelimbs of *S. floridanus* exhibit more generalized features than those of their hindlimbs. As in hares, it is expected that forelimb muscles will primarily play a role in stabilizing (i.e., braking action) the stance phase of locomotion, withstanding high forces during activities such as changes in direction at high speeds (Williams et al., 2007b). In general, rabbits, much like kangaroos and wallabies, jump using both hindlimbs to propel them forward in a synchronous manner known as saltatory locomotion. Specifically, their fast running gait is a type of gallop called a half-bound (Hildebrand, 1985). It involves a repeated series of jumps at high frequency where the hindfeet land simultaneously ahead of the forefeet, which then land alternately in succession (e.g., 1-2-3 beat gait pattern) before the entire limb cycle is repeated. This locomotor behavior resulted in the selective development of robust hindlimbs having hypertrophied hindlimb muscles that allow rabbits to accelerate quickly, and perform agile turning maneuvers for evading predators. Interestingly, it has been

reported that relative to their body size, juvenile hares (average mass = 600–1500 g) tend to have a more powerful *gastrocnemius m.* than adults of the same species, which allows them to accelerate quickly to speeds comparable to that of the adults (Carrier, 1996). Similar muscle contractile performance differences between juveniles and adults have yet to be evaluated in species of rabbits.

a. Hindlimb Morphology:

The hindlimb, or pelvic limb, of hares and rabbits contains a substantial volume of proximal hip extensors and abductor/adductor muscles, which are required for power generation and stability during rapid turning maneuvers, respectively (Lieber and Blevins, 1989; Williams et al., 2007a). The investment in large muscles about the hip joint is offset by a proximal-to-distal reduction in muscle mass (and volume) and fascicle length (i.e., due to increased muscle fiber pennation) similar to that observed in many other cursorial quadrupeds (e.g., Brown et al., 2003; Panye et al., 2005; Williams et al., 2008; Hudson et al., 2011). A reduction in distal limb mass via the replacement of muscle volume by long distal limb tendons allows for storage and recovery of elastic strain energy, thus reducing the metabolic cost of locomotion (Alexander, 1984). This muscle-tendon unit (MTU) morphology is prevalent in muscles spanning the ankle joint (e.g., ankle extensors) and all joints distal to the ankle (e.g., digital flexors and extensors) (Lieber and Blevins, 1989; Williams et al., 2007a).

Relating the insertion of tendons to the center of rotation of the joints they cross (i.e., muscle moment arm) creates of mechanism of limb muscle gearing (Carrier, 1993, 1996). This is most simply understood as changes in muscle moment arm (and joint torque) with changes in joint position. The majority of hindlimb muscle moment arms vary with joint position giving them the ability to modulate muscle function with changes in limb posture, and presumably perform a range of locomotor roles (Williams et al., 2007a). Also important to the muscle gearing mechanism is the function of biarticular muscles that perform both hip extension and knee flexion. For example, in hares, muscles of the hamstrings group (*biceps femoris m.*, *semimembranosis m.*, and *semitendinosis m.*) have high fascicle length-to-muscle length ratios (close to 1.0) indicating a large capacity for muscle shortening (Williams et al., 2007a). Long muscle fibers are required for a muscle to perform actions at two joints (Eng et al., 2008). When activated, biarticular muscles

cause rotation at one joint, which shortens the muscle, while rotation at the second joint lengthens the muscle. By this mechanism, fascicle length can remain relatively isometric (same length) during running to produce a high amount of force instead of high velocities of shortening. This type of muscle contractile function may be particularly important for evasive maneuvering in rabbits.

The biarticular *gastrocnemius m*. is specialized for generating high force economically (i.e., high force at a reduced metabolic energy cost). Muscle specialized for economical high force production have long tendons that allow the MTU to undergo large strain (change in length) while the fascicles in the muscle belly contract nearly isometric. This optimizes the elastic performance of the MTU (functioning like a biological spring) in terms of stretching the tendons for storage and recovery of elastic strain energy (Biewener et al., 2004; Williams et al., 2007a; Butcher et al., 2007, 2009). Energy recovered from elastic recoil of limb tendons helps power the stride at no additional metabolic cost to the animal. Limb muscles contracting isometrically (or eccentrically by a lengthening contraction) reduces both the consumption of ATP and the volume of active muscle needed to produce high force required for spring function (Roberts et al., 1997, 1998; Biewener and Roberts, 2000). Integrated spring-like functions of the muscle and tendon in long MTU's are key to metabolic energy savings in the limbs of cursorial mammals (Alexander, 2003). For example, by this mechanism kangaroos and wallabies save nearly 50% on metabolic energy consumption during high-speed hopping, and horses save nearly 40% during fast trotting (Biewener, 1998). Rabbits would be expected to save a moderate amount of metabolic energy during high-speed galloping by spring-function of their distal hindlimb muscles.

Because of their locomotor habits, rabbits also need to be able to resist high forces at the ankle joint for either storage of elastic strain energy or extension of the joint. The need for increased elastic energy storage and rapid ankle extension are both likely to be important for acceleration. The major ankle extensor of the rabbit is the *gastrocnemius m.,* and in several running animals it has been shown *in vivo* that the power generated by the large proximal muscles is likely to be transferred to distal joints via the biarticular *gastrocnemius m*. (Jacobs et al., 1996; Aerts, 1998; Dutto et al., 2004; McGowan et al., 2005) that spans both the knee and ankle joints. The transfer of power from the proximal

extensors (e.g., *gluteus superficialis m.*, *biceps femoris m.*, and *vastus lateralis m.*) to the ankle joint may also occur via the same mechanism in *S. floridanus*. This could assist in modulating power output during unsteady-state activities such as lateral maneuvering to evade predators. The long, elastic calcaneal tendon might also aid in modulating power output of all ankle extensor muscles during high-speed locomotion (Galantis and Woledge, 2003).

b. Muscle Physiology and Hindlimb Architecture:

Skeletal muscles are mechanical actuators in which the amount of force produced is dependent on the length of muscle fibers and the velocity at which fibers contract (Lieber and Ward, 2011). Moreover, the amount of force that a muscle produces is a direct function of sarcomere length, in which total force is the aggregate force of all active actomyosin cross-bridges within a muscle. This is known is the length-tension relationship (Lieber and Ward, 2011). At optimal sarcomere lengths (2.2–2.8 μm), actin and myosin interactions are optimized (by their degree of overlap) allowing muscle fibers to produce maximum force. At sarcomere lengths either greater or shorter than optimal (i.e., plateau region of the length-tension curve), force decreases due to a decreased number of cross-bridges or double interdigitation of actin with both myosin and actin filaments on the opposite sides of the sarcomere, respectively (Lieber and Ward, 2011). Changes in muscle force production with changes in sarcomere length that occur during joint rotation is accommodated in the design of the hindlimb system, thus sarcomere length can remain near optimal length *in vivo* during high-speed locomotor events.

The velocity at which a muscle fiber changes length also affects the force it can produce. Active force declines (relative to maximum isometric force: F_{max}) in a hyperbolic fashion as the fiber shortening velocity increases, eventually reaching zero at maximum shortening velocity (V_{max}). This is known is the force-velocity relationship (Lieber and Ward, 2011), and it has strong implications for the rate at which muscles perform mechanical work (W_{mech}=force × shortening length), and thus generate power (W=work ÷ time). Since power is derived as product of force and velocity, muscles generate no power at either F_{max} (due to zero velocity) or V_{max} (due to zero force). Therefore when muscles are contracting at peak power, active force and shortening velocity is 0.3–0.5 F_{max} and V_{max}, respectively (Hill, 1938). The reverse holds true for

when a muscle fiber is lengthened; force increases above isometric and a muscle may reach an absolute maximum of 1.8 times F_{max} while being actively lengthened (Hill, 1953). In circumstances where muscles undergo lengthening contractions (i.e., negative velocity), work and power are absorbed (negative power) because work is done on MTU. Therefore, muscles are not serving as actuators to rotate a distal limb joint, but instead they act as energy dampers or biological springs, as is often the case for distal limb muscles with long, compliant tendons.

Whereas length-tension and force-velocity relationships are normally determined by mechanical tests on single muscle fibers or small fascicles *in vitro*, the architecture of a whole muscle estimates force production capacity by the number and orientation of muscle fibers within the muscle belly in relation to the line of muscle action (force axis) (Gans, 1982; Sacks and Roy, 1982; Lieber and Friden, 2000). Muscles with long fascicles that span the length of the muscle belly are defined to have *parallel fiber architecture*, while muscles with shorter fascicles that extend at an angle along the length of muscle are defined to have *pennate fiber architecture* (Lieber and Ward, 2011). Pennate muscles typically have flat (aponeurosis) or internal tendons that attach to the insertion tendon. The fascicles attach to the aponeurosis and form a pennation angle relative to the force axis of the muscle. If all fascicles are on one side of the aponeurosis, the muscle architecture is *unipennate*. If fascicles are on both sides of a central tendon, the muscle architecture is *bipennate*. Lastly, if the central tendon branches within the muscle belly and muscle fibers attach between tendon inscriptions, the muscle architecture is *multipennate*.

Muscles with parallel fiber architecture have low force production capacity, but have high shortening capability (at high shortening velocity) that allows for high mechanical work and power generation (Zajac, 1989). Parallel fiber muscles also allow for torque production over a greater range of joint rotation. Conversely, muscles with pennate fiber architecture are capable of producing high force, but have low capacity for work and power generation (Zajac, 1992). Short-fibered pennate muscles have little ability to rotate a distal limb joint and produce high force over a small range of motion. The functional performance contrasts between parallel and pennate muscles indicates a trade-off between fascicle length and muscle volume, which are used to calculate physiological

cross-sectional area (PCSA). PCSA is directly proportional to how much isometric force (F_{max}) a muscle can produce (Alexander, 1984). Pennate muscles have large PCSA because of high numbers of shorter muscle fibers (i.e., sarcomeres in parallel) per unit volume of muscle. A parallel-fibered muscle with fewer long fascicles (i.e., sarcomeres in series) would require as substantial volume to have similarly large PCSA, and thus would be too large for any limb system (Ward and Lieber, 2005). Therefore, pennation is viewed as an adaptive strategy for distal limb muscles to become smaller without their sacrificing force capabilities, and this is critical to metabolic energy savings by minimizing the muscle volume recruited to meet functional force requirements. This feature further indicates that the most important muscle architectural property is fiber length; at a given mass, the relationship between muscle fiber length and isometric force is inversely proportional (Lieber and Ward, 2011).

Previous studies have quantified muscle architectural properties in the hindlimbs of New Zealand white rabbits (*Oryctolagus cuniculus*: Lieber and Blevins, 1989) and the European hare (*Lepus europeus*: Williams et al., 2007b), however, no study has evaluated similar properties in the hindlimb musculature of *S. floridanus*. Using data reported for *O. cuniculus* and *L. europeus*, generalized and specialized features of lagomorph hindlimb muscles can start to be discerned. It was observed that the massive hip extensor and knee flexor muscles have the functional capacity to provide a majority of the force and power required for rapid locomotion. For example, the long *biceps femoris m*. is the largest muscle of the hindlimb in hares with a combined mass (both pelvic and vertebral heads) of nearly 59.0 g, and yet has relatively high PCSA, and force and power properties due to its high mass and volume (Williams et al., 2007a). Other proximal muscles of the hare that have considerable mass and force capability include the *vastus lateralis m*. (20.1 g), s*emimembranosis m*. (19.0 g) and the *rectus femoris m*. (12.7 g) (Williams et al., 2007a). Several muscles about the hip and knee joints are pennate (i.e., pennation angles >15º) including all four heads of the quadriceps and *biceps femoris m*. (vertebral head). Most muscles found in the distal segments of the hindlimb of both the rabbit and hare are consistently shown to have appreciable pennation, with pennation angles ranging from 21–42º, and relatively short fascicles compared to their muscle belly length (Lieber and Blevins, 1989; Williams et al., 2007a). Muscles observed to have parallel fiber

architecture include the pelvic head of the *biceps femoris m.*, *semimembranosus m*., and gluteal complex. These muscles have relatively long fascicle lengths that span nearly the entire muscle belly (Lieber and Blevins, 1989).

c. Muscle Fiber Type:

Fiber typing characterizes muscles according to the metabolic properties of individual muscle fibers. Muscle fibers are generally classified as slow, oxidative or fast, glycolytic based on their inherent rate of ATP hydrolysis and mechanism of ATP synthesis (Wattenburg and Leong, 1960; Novikoff et al., 1961; Brooke and Kaiser, 1970). The rate at which ATP is hydrolyzed by the myosin ATPase (myosin heads) correlates with the V_{max} of a muscle fiber, which in turn is determined by the specific myosin heavy chain (MHC) isoform expressed (Schiaffino and Reggiani, 1996, 2011). Many studies have traditionally relied on mATPase histochemistry to classify 'fiber types', but using this technique alone limits the ability to identify MHC isoform expression. Analyses using mATPase histochemistry classify four fiber types: Type I, Type IIa, Type IIx(d), and Type IIb (old nomenclature). These traditional fiber types have been reclassified based on the MHC isoform expressed, where Type I fibers relate to MHC-1, Type IIa to MHC-2A, Type IIx(d) to MHC-2X, and Type IIb to MHC-2B. MHC-1 fibers have the slowest shortening velocity and are oxidative; MHC-2A fibers have a faster shortening velocity than MHC-1, and are highly oxidative with some glycolytic properties; MHC-2X fibers have a faster shortening velocity than MHC-2A, and are moderately oxidative/glycolytic; and MHC-2B fibers have the fastest shortening velocity and are glycolytic (Schiaffino and Reggiani, 1996, 2011). Skeletal muscles may also express special forms of MHC isoforms. For example, muscles of mastication (in carnivores) express MHC masseter (MHC-m), eye muscles express MHC extraocular (MHC-eo), and laryngeal muscles express MHC laryngeal (MHC-l). Identification of MHC isoform fiber types is done using a combination of techniques including gel electrophoresis (SDS-PAGE), Immuno-Blotting (Western Blot), and immuno-histochemistry (IHC) (Rivero et al., 1999; Kohn et al., 2007, 2011).

The four conventional MHC isoforms are commonly expressed in mammalian limb muscles: MHC-1, MHC-2A, MHC-2X and MHC-2B (Toniolo et al., 2004, 2005, 2007, 2008; Kohn et al., 2011). However, body size plays an important role in the functional

demands of skeletal muscles and strongly influences MHC expression and their V_{max} (Schiaffino and Reggiani, 1996, 2011). The limb muscles of small eutherian mammals (e.g., mice and rats) have predominantly fast MHC-2X and MHC-2B fibers with abundant oxidative enzymes (high mitochondrial volume), while the same muscles from large mammals like horses are mainly composed of slower MHC-1 and MHC-2A fibers and have lower levels of oxidative enzymes (low mitochondrial volume). Therefore, V_{max} is inversely related to body mass. This relationship has been repeatedly shown for each MHC isoform fiber type across a 20,000-fold difference in body size (Rome et al., 1990; Seow and Ford, 1991; Pellegrino et al., 2003; Toniolo et al., 2007). For example, a slow MHC-1 fiber shortens significantly faster in mouse limb muscle than a slow MHC-1 fiber in a horse limb muscle. This is directly related to the physiological principle that cellular energy metabolism per unit body mass is inversely related to body size; mass-specific metabolic varies with the negative one-fourth power of body mass ($M^{b\text{-}0.25}$: Kleiber, 1947). The metabolic activity of limb muscles is higher in smaller mammals than in large species, and this matches their requirements for high power output to cycle their limbs at high frequencies during running. Lastly, expression of the fast MHC-2B isoform in limb muscles has only been shown to occur in small rodents and lagomorphs (Mascarello et al., 2004), pigs (Toniolo et al., 2004), and marsupials (Lucas et al., 2000). Though is not clear why this MHC isoform does not strictly follow the body size relationship, it is clear that most medium-to-large mammals do not express a functional MHC-2B isoform protein, although they may retain an inactive gene that codes for this fiber type (e.g., humans and horses: Chikuni et al., 2004).

Muscle fiber type is generally not well-studied in rabbits, with most studies focusing on individual fibers from two muscles (*psoas m*. and *soleus m*.), or broad histochemical classifications of slow and fast fibers in various limb muscles of mature *O. cuniculus* only. Wang and Kernell (2001) showed that several rabbit hindlimb muscles including the *gastrocnemius m.*, *plantaris m.*, and *flexor digitorum longus m*. are predominately slow 'Type I' fibers, while the *soleus m*. is entirely composed of fast 'Type II' fibers. This study made no distinction between the sub-divisions of fast MHC-2 fiber types. However, one recent study (Korfage et al., 2009) evaluated development (1 week postnatal to 20 week old juveniles) and expression of MHC isoforms in ankle extensor

muscles and found the *gastrocnemius m.* contained 30% of MHC-2A and 60% MHC-2X fibers. Early in development, the *soleus m*. contained 32% MHC-2A and 38% MHC-2X fibers, however, the MHC isoform composition transitioned to predominately MHC-I fibers (~90%) throughout ontogeny (Korfage et al., 2009). These data suggest that there is a change in MHC isoform expression in the hindlimbs of juvenile rabbits as they develop into adults. Currently, no ontogenetic study of MHC isoform fiber types exists for wild *S. floridanus.*

Other examples of MHC isoform fiber types studied in rabbit hindlimb muscles include an almost homogeneous composition of MHC-2X fibers in the *extensor digitorum longus m*., *psoas m*., and *tibialis anterior m.*, but none of these muscles contain pure MHC-2B isoform fibers (Reiser et al., 1985; Hämäläinen and Pette, 1993; Drzymala-Celichowska, 2012). Two studies indicated that fast, glycolytic MHC-2B fibers are present in the *adductor magnus m.*, and the major hindlimb extensors *gastrocnemius m.* and *vastus lateralis m.* (Hämäläinen and Pette, 1993; Drzymala-Celichowska, 2012), each of these muscles are overall very fast in their MHC isoform fiber type distribution by primary expression of the 2B isoform in the superficial regions of the muscle bellies (Hämäläinen, 1993). Moreover, slow, oxidative MHC-1 fibers do not appear to be present, or more generally, are found in small percentages (deep regions of muscle bellies) in the hindlimb muscles of young rabbits compared with adults (Sartorius, 1998; Korfage et al., 2009).

Ontogeny

For most vertebrate animals, locomotor activity begins a few hours after birth. The juvenile period of life is a time when an individual is ecologically independent, but still reproductively immature, and can be particularly perilous for many young mammals (Carrier, 1996). It is also during this time that young animals are most vulnerable to predation and have the greatest amount of pressure for survival. They must endure the same environmental conditions as adults and be able to escape the predators in order to reach adolescence and reproductive maturity (Carrier, 1996). Due to their smaller body size, juveniles have less energy reserves and they have different metabolic needs (Lindsey, 1966). This results in a decreased amount of stamina and agility. Also, due to

the lack of energy reserves that juvenile rabbits are capable of storing, they consume their oxygen reserves much faster, thus limiting the amount of time that they can sustain high locomotor speeds (Heglund et al., 1982; Heglund and Taylor, 1988; Kram and Taylor, 1990).

Because juveniles do not have a great amount of stamina compared to their adult counterparts, *rabbits primarily develop in a way in which quick acceleration and rapid movements are essential for survival and they come at the expense of endurance*. In addition, juveniles are still growing and developing, thus a lot energy is used for growth and developmental processes. This means that adults are capable of maintaining their maximal speed for much longer periods of time than juveniles. An important factor in determining why running for extended periods of time is insufficient in young jack rabbits, is that juveniles have a much greater stride frequency than adults, which requires more energy and mechanical power generation maintain a high velocity of galloping (Carrier, 1993, 1996).

Several studies (e.g., Baker, 1983; Carrier, 1983; Heinrich, 1999; Torzilli, 1981; Young, 2005) have shown that the limbs of juveniles are often 'overbuilt', meaning they have features of their musculoskeletal structure that compensate for their lack of overall body size. For example, precocial species (i.e., born with eyes open and capable of movement) such as muskoxen, have femoral bones that are more highly mineralized compared to altricial species like rabbits (Heinrich, 1999). Within a few months after birth, the mineral bone density of muskoxen femora increases markedly with complete mineralization by 18 months (Heinrich, 1999). Juvenile muskoxen also have femora that are relatively longer, have a greater cortical cross-sectional area (CSA), and have relatively higher mechanical strength than femora of the adults. These structural properties allow young muskoxen to keep pace with the herd with a low risk of femoral fracture.

Juveniles often have relatively high mechanical advantages about the limb joints (allowing them to produce a greater amount of joint torque for a given muscle force) and have relatively robust limb bones (Carrier, 1983; Currey, 1984; Currey, 2001; Main and Biewener, 2004, 2007; Young, 2005). These morphological features allow young animals to perform at near-maximal levels that would be required during predator evasion, with a

reduced risk of skeletal injury (Main and Biewener, 2004, 2006, 2007). Notably, immature jack rabbits have relatively weaker bone tissue (compared with adults) in the early stages of life, but their hindlimb bones tend to be more robust with a greater CSA to compensate for a relatively high amount of force production by their hindlimb muscles (Carrier, 1993, 1996). Greater cortical CSA is necessary to resist high bending stresses and strains (i.e., bone loading) and keep loads within safe limits (i.e., limb bone safety factors) (Alexander 1981; Biewener and Bertram, 1993). Adults tend to assume a more upright limb posture to maintain loads within a limb bone safety factor limit of 2–4 (ratio of peak load to functional load), that is common for most mammalian limb bones (Biewener, 1990, 1993). A more upright limb posture results in a greater effective mechanical advantage of the limb muscles, thus reducing the amount of loading a bone experiences (Biewener, 1989) without sacrificing the accelerative abilities of adults (Carrier, 1996). Impressively, immature jack rabbits are capable of achieving a take-off speed equivalent to that of adults when they are only about 25–30% of the adult mass. Juveniles have a much higher mechanical advantage about their joints (by relatively larger muscle moment arms), which aids in their acceleration by allowing hindlimb muscles to produce a greater amount of torque (i.e., extensor moments) at the hip, knee and ankle joints (Carrier, 1996). It would be interesting to discover if similar limb mechanical advantages are present in the hindlimbs of Eastern cottontail rabbits during their early stages of life.

Objectives and Hypotheses

Muscles are specialized to meet the performance needs of species in relation the selective pressures experienced in their natural habitat. Therefore, locomotor habits directly influence the contractile properties of limb muscles. The expression of MHC isoforms in muscle fibers is the main determinant of muscle contractile properties and directly reflects functional specialization of muscles (Hazimihalis et al., 2013). Oxidative fiber types (MHC-1 and MHC-2A) produce low force and power over a long contractile period, while glycolytic fiber types (MHC-2X and MHC-2B) produce high force and power for short durations. Specialization also involves properties of muscle architecture including muscle mass, fascicle length, pennation angle, and PCSA. These properties are used to

evaluate the functional capacity of limb muscles for mechanical work by estimating peak isometric force (F_{max}), maximum shortening velocity (V_{max}), and maximum power (W).

Analyses of locomotor performance are often related to quantification of muscle architecture, muscle fiber type, and limb bone properties, in order to reconstruct the evolutionary function of the observed limb morphology. Far too often these studies are conducted using adults and fail to understand how muscle contractile properties and locomotor performance change throughout ontogeny, and how these factors have major consequences for the survival of animals. Juveniles are often distinguished by 'compensatory' musculoskeletal growth trajectories that either approximate or exceed adult levels of musculoskeletal performance. Evidence has shown a significant positive correlation for selection on locomotor performance traits in juveniles (Irschick et al., 2007), and yet only a few studies have examined the relationships between juvenile locomotor performance and fitness. The proposed research will help remedy gaps in our understanding of how juveniles survive and develop into mature adults by explicitly testing the links between *structure* (muscle architecture and fiber type) and *function* (production of mechanical work for locomotor performance).

The main objective of this study is to quantify muscle architectural properties and MHC isoform fiber type in the hindlimb extensor muscles of *S. floridanus* and compare ontogenetic morphological differences between juveniles (<1 year-old) and adult rabbits. Comparative analyses of muscle architecture and fiber type in the hindlimbs of rabbits will provide insight into muscle development and changes engendered by developing locomotor demands. This will also allow further insight into the means by which juveniles survive under equivalent environmental conditions as adults. It is hypothesized that musculoskeletal features of hindlimb will be optimized in juvenile cottontail rabbits to promote increased locomotor performance. We predict that in comparison with adults, juvenile *S. floridanus* will have higher relative percentages of muscle mass, larger PCSA, and a broader distribution of fast MHC isoforms (predominately MHC-2X) in the hindlimb extensors. In addition, we predict that juvenile *S. floridanus* will have relatively higher limb muscle mechanical advantages and hindlimb bones that are relatively more robust than adults. As a result of their differing musculoskeletal morphology, juvenile rabbits will be able to achieve higher levels of locomotor performance with a smaller

body size, and their maximal galloping speed and acceleration capacity will be directly related to the morphological features quantified.

Table 1. Morphometric data for study animals.

Rabbit	Sex	Limb	Body mass (kg)	Femur length (cm)	Tibia length (cm)	Transverse Femur width (cm)	Transverse Tibia width (cm)	Calcaneus length (cm)	Metatarsal III length (cm)
Adult									
Sf 0603	M	L	1.3	8.2	9.2	0.7	0.6	2.1	4.0
Sf 0624	F	L	1.2	8.8	9.6	0.7	0.7	2.4	4.0
Sf 0711A	M	L	1.1	8.1	9.3	0.7	0.7	2.3	4.1
Sf 0808	F	L	1.3	8.2	9.1	0.6	0.6	2.6	4.0
Sf 0918	F	L	0.9-	8.2	9.0	0.6	0.6	2.6	4.0
Sf 1009	M	L	0.8	7.2	8.0	0.6	0.5	2.0	3.5
			1.1±0.2	**8.1±0.5**	**9.0±0.5**	**0.6±0.1**	**0.6±0.1**	**2.4±0.3**	**3.9±0.2**
Juvenile									
Sf 0610	F	L	0.57	7.0	7.1	0.7	0.5	1.9	2.7
Sf 0723	F	L	0.23	6.7	7.2	0.5	0.5	2.2	2.7
Sf 0725	F	L	0.65	4.5	4.6	0.4	0.4	1.7	2.0
Sf 0711B	F	L	0.77	6.7	7.1	0.6	0.5	2.1	2.8
Sf 0806	F	L	0.27	4.7	4.9	0.4	0.4	1.8	1.7
Sf 0813	F	L	0.36	5.7	6.0	0.4	0.4	2.0	2.1
Sf 0909	F	L	0.61	7.4	7.7	0.6	0.5	2.3	3.7
Sf 0923	F	L	0.13	3.5	3.6	0.3	0.3	1.3	1.4
Sf 0930	M	L	0.56	6.3	6.6	0.6	0.5	2.1	2.7
			0.46± 0.2	**5.8±1.3**	**6.1±1.4**	**0.5±0.1**	**0.4±0.1**	**1.9±0.3**	**2.4±0.7**

In bold are mean±s.d. (standard deviation).

Table 2 Architectural properties data for adult and juvenile rabbit hindlimb muscles.

Muscle group	Age	*n*	Muscle mass (g)	Belly length (cm)	Fascicle length (cm)	Pennation angle (°)	Volume (cm^3)	PCSA (cm^2)	F_{max} (N)	Power (W)	Fiber architecture
Hip extensors											
Gluteus medius (GLM)	A	6	2.13±1.0	2.70±0.5	2.35±0.7	0	2.01	0.92	27.5	0.38	Parallel
	J	9	0.95±0.7	2.21±0.6	1.85±0.5	0	0.90	0.47	14.0	0.17	
Gluteus profundus (GLP)	A	6	4.57±3.2	3.15±0.4	2.35±0.5	0	4.31	1.73	52.0	0.81	Parallel
	J	9	0.87±0.7	22.2±0.6	1.61±0.4	0	0.82	0.47	14.0	0.15	
Biceps femoris - *pelvic head* (BFP)	A	6	9.72±2.6	7.47±0.8	6.26±0.7	0	9.17	1.47	44.1	1.73	Parallel
	J	9	2.79±1.9	5.45±1.6	4.37±1.0	0	2.63	0.56	16.8	0.50	
Biceps femoris - *vertebral head* (BFV)	A	6	9.40±2.4	8.65±0.8	5.91±0.8	25±3	8.86	1.36	40.8	1.51	Unipennate
	J	9	2.79±1.8	6.06±1.5	3.99±1.2	25±4	2.63	0.59	17.6	0.45	
Semimembranosis (SM)	A	6	5.71±1.7	7.07±1.4	5.34±1.2	0	5.38	1.04	31.3	1.01	Parallel
	J	9	1.89±1.2	4.99±1.2	3.89±0.9	0	1.78	0.44	13.1	0.34	
Knee extensors											
Vastus-*lateralis* (VL)	A	6	7.92±2.2	6.30±0.5	3.28±0.6	30±5	7.47	1.98	59.3	1.21	Unipennate
	J	9	2.77±1.6	4.80±1.1	2.26±0.7	26±4	2.61	1.00	29.9	0.44	
Rectus femoris (RF)	A	6	1.62±0.4	5.81±0.4	1.84±0.4	26±6	1.52	0.77	23.0	0.25	Unipennate
	J	9	0.52±0.3	4.04±1.1	1.43±0.4	25±3	0.49	0.31	9.2	0.08	
Ankle extensors											
Gastrocnemius - *lateral head* (LG)	A	6	2.68±1.3	5.18±0.7	1.47±0.5	31±6	2.53	1.44	43.3	0.40	Unipennate
	J	9	0.87±0.5	3.66±1.2	1.02±0.2	29±5	0.82	0.67	20.1	0.13	
Gastrocnemius - *medial head* (MG)	A	6	6.20±1.6	6.52±0.6	1.00±0.6	34±7	5.84	5.53	166	0.90	Bipennate
	J	9	1.91±1.3	4.09±1.2	0.88±0.2	31±4	1.80	1.72	51.7	0.29	
Digital flexors											
Flexor digitorum longus (FDL)	A	6	1.56±0.5	5.79±0.7	1.01± 0.3	31±6	1.47	1.28	38.3	0.23	Unipennate
	J	9	0.57±0.4	3.69±1.2	0.83± 0.6	29±4	0.53	0.59	17.8	0.09	

Table 3 Normalized architectural properties data for adult and juvenile rabbit hindlimb muscles.

Muscle group	Age	*n*	Muscle mass (g/kg)	Belly length ($cm/g^{.333}$)	Fascicle length ($cm/g^{.333}$)	PCSA ($cm^2/g^{.667}$)	F_{max} (N/kg)	Power (W/kg)	$*r_m$
Hip extensors									
GLM	A	6	1.95±0.9	2.14±0.2	1.75±0.4	0.58	24.5	0.35	0.14
	J	9	1.86±0.6	2.39±0.3	1.95±0.3	0.50	29.8	0.33	0.12
GLP	A	6	3.88±2.4	2.07±0.36	1.51±0.3	0.63	44.7	0.69	0.12
	J	9	1.75±1.0	2.50±0.3	1.80±0.3	0.54	30.9	0.31	0.10
BFP	A	6	8.58±0.9	3.55±0.5	3.07±0.4	0.32	38.8	1.52	0.31
	J	9	5.47±1.9	4.11±0.3	3.27±0.4	0.29	36.2	0.97	0.27
BFV	A	6	8.30±1.0	4.15±0.4	2.85±0.4	0.30	36.1	1.34	0.15
	J	9	5.43±1.6	4.63±0.5	3.00±0.7	0.30	36.5	0.87	0.15
SM	A	6	5.01±0.8	3.99±0.6	2.89±0.5	0.32	27.3	0.89	0.35
	J	9	3.75±1.0	4.30±0.4	3.30±0.7	0.29	27.5	0.66	0.36
Knee extensors									
VL	A	6	7.00±1.1	3.20±0.2	1.73±0.3	0.50	56.6	1.07	0.21
	J	9	5.65±1.1	3.58±0.2	1.65±0.4	0.52	66.2	0.89	0.23
RF	A	6	1.44±0.2	5.03±0.7	1.28±0.4	0.55	20.2	0.23	0.18
	J	9	1.05±0.3	5.25±0.4	1.80±0.4	0.50	20.7	0.17	0.19
Ankle extensors									
LG	A	6	2.30±0.8	3.92±0.9	0.93±0.3	0.76	37.7	0.35	0.16
	J	9	1.76±0.7	4.01±0.6	1.14±0.2	0.74	43.7	0.27	0.17
MG	A	6	5.47±0.7	3.58±0.1	0.54±0.3	1.63	146.3	0.80	0.16
	J	9	3.74±1.3	3.53±0.5	0.78±0.3	1.08	103.9	0.56	0.17
Digital flexors									
FDL	A	6	1.38±0.3	5.05±0.6	0.69±0.2	0.95	34.0	0.21	0.05
	J	9	1.11±0.3	4.67±0.5	1.07±0.9	0.81	34.82	0.17	0.07

$*r_m$ normalized to bone length: femur length for hip and knee muscles; tibia length for ankle extensors and FDL.

Table 4 Muscle moment arms (r_m), joint torques, and functional muscle ratios for adult and juvenile rabbit hindlimb muscles.

Muscle	Age	Joint	Mean r_m (cm)	Joint Torque (N.cm kg^{-1})	l^F/r_m	l^F/ML
GLM	A	Hip	**1.2±0.3**	27.74	2.12	0.79
	J		**0.7±0.3**	10.0	2.90	0.79
GLP	A		**1.0±0.3**	46.0	2.56	0.74
	J		**0.5±0.2**	8.3	3.00	0.71
BFP	A		**2.5±0.7**	99.63	2.69	0.84
	J		**1.6±0.5**	28.8	2.84	0.77
BFV	A		**1.2±0.2**	43.0	5.11	0.69
	J		**0.8±0.2**	16.0	4.84	0.65
SM	A		**2.7±0.1**	60.2	1.94	0.76
	J		**2.0±0.7**	22.1	2.01	0.78
VL	A	Knee	**1.6±0.1**	84.4	2.05	0.52
	J		**1.4±0.2**	42.9	1.65	0.48
RF	A		**1.4±0.2**	28.8	1.33	0.32
	J		**0.5±0.2**	10.4	1.25	0.34
LG	A	Ankle	**1.3±0.1**	50.4	1.12	0.28
	J		**1.0±0.3**	44.6	1.05	0.30
MG	A		**1.3±0.1**	198.7	0.79	0.15
	J		**1.0±0.3**	106.8	0.93	0.23
FDL	A	Digit	**0.6±0.1**	19.6	1.70	0.18
	J		**0.4±0.1**	13.9	2.24	0.23

In bold are mean±s.d.
L_f is mean fascicle length
r_m is mean moment arm
Joint torque normalized to body mass in kg
L_f/r_m >2.0 indicate a high ability of the muscle to move a joint through a large range of motion

Table 5 Tendon properties for adult and juvenile rabbit hindlimb muscles**.**

Muscle group	Age	Tendon mass (g)	Volume (cm^3)	Rest length (cm)	CSA (cm^2)	Stress (MPa)	Strain (%)	Length change (cm)
Ankle extensors								
LG	A	0.19±0.09	0.17	3.76±0.90	0.05	10.8	0.72	0.03
	J	0.06±0.05	0.06	2.27±0.48	0.02	9.9	0.66	0.02
MG	A	0.09±0.02	0.08	2.62±0.12	0.03	57.0	3.80	0.10
	J	0.06±0.04	0.05	2.12±0.58	0.02	21.5	1.43	0.03
Digital flexors								
FDL	A	0.35±0.08	0.32	7.06±0.32	0.05	8.7	0.58	0.04
	J	0.14±0.1	0.13	5.20±1.04	0.02	7.6	0.51	0.03

Tendon mass and length data are mean±s.d.

Table 6 Scaling regression variables and body-size scaling exponents.

Regression	*n*	Allometry	*b*	H_0	r^2	*p*	Lower limit	Upper limit
Mass								
Hip extensors	15	+	1.46		0.98	<0.001	1.35	1.58
Knee extensors	15	+	1.28	1	0.99	<0.001	1.20	1.35
Ankle extensors	15	+	1.39		0.99	<0.001	1.29	1.49
FDL	15	+	1.33		0.97	<0.001	1.19	1.49
Fmax								
Hip extensors	15		1.09		0.95	0.15	0.96	1.25
Knee extensors	15		0.91	1	0.95	0.16	0.79	1.04
Ankle extensors	15	+	1.24		0.95	0.005	1.08	1.42
FDL	15		1.20		0.89	0.07	0.98	1.46
l^F/ML								
Hip extensors	15	-	-0.12		0.00	<0.001	-0.21	-0.07
Knee extensors	15	-	-0.16	0	0.02	<0.001	-0.28	-0.09
Ankle extensors	15	-	-0.32		0.48	<0.001	-0.49	-0.21
FDL	15	-	-0.43		0.59	0.001	-0.62	-0.29
Power								
Hip extensors	15	+	1.46		0.98	0.03	1.35	1.58
Knee extensors	15	-	1.26	1.33	0.99	0.03	1.19	1.32
Ankle extensors	15		1.38		0.29	0.29	1.29	1.48
FDL	15	+	1.31		0.96	0.004	1.16	1.48

P values <0.05 are significantly different from the H_0
H_0 Statistical null hypothesis for isometry
Groups marked with + show positive allometry; - show negative allometry
All regressions in the form $y= aM^b$; *M*, body mass; a, intercept; y, dependent variable; *b*, scaling factor

Table 7 Mean percentage MHC isoform content for adult and juvenile rabbit hindlimb muscles.

Muscle (abbreviated)	Age	MHC isoform composition (%)			
		1	2A	2X	2B
GLM	A	0.38±0.7	0.00	93.7±3.5	5.96±3.8
	J	0.21±0.5	0.00	56.3±22.8	43.5±22.5
GLP	A	0.00	0.00	34.1±19.0	65.9±19.0
	J	9.48±9.1	15.6±19.0	59.0±18.4	16.0±11.8
BFP	A	0.00	0.00	39.4±10.1	60.6±10.1
	J	0.40±0.7	0.00	43.7±11.9	54.3±14.6
BFV	A	0.00	0.36±0.80	43.6±12.2	56.1±12.8
	J	0.00	0.00	32.2±10.1	67.7±10.1
SM	A	0.52±0.9	22.0±30.3	58.3±19.2	19.1±18.5
	J	0.00	0.00	53.0±5.9	47.0±5.9
VL	A	0.19±0.4	27.8±31.3	62.8±19.5	14.3±13.7
	J	0.00	0.00	47.1±10.9	52.9±10.9
RF	A	39.0±48.1	24.2±25.8	36.3±39.0	0.50±1.1
	J	9.62±10.8	27.9±29.5	68.6±25.9	4.43±2.4
LG	A	7.21±12.4	10.5±20.9	79.2±32.0	3.14±6.3
	J	2.37±1.5	12.7±19.6	65.5±19.0	19.4±8.9
MG	A	5.99±11.8	10.7±23.9	74.9±31.0	8.42±15.8
	J	3.31±2.9	17.9±19.7	54.4±21.4	24.3±15.9
FDL	A	3.81±1.7	33.6±29.3	58.5±27.7	0.00
	J	3.71±1.8	0.79±1.4	94.7±2.32	0.76±1.3

All data are mean±s.d. across N=5 individuals per age group

Figure 1. Distribution of functional group muscle mass of rabbit hindlimb muscles. Functional group masses were summed and averaged, and then normalized to body mass. Proximal-to-distal muscle group mass is expressed as a percentage, with bars representing means for each functional group. Error bars represent the SD (standard deviation). Muscles with synergistic functions are combined in one functional group. In all data figures, blue represents adults and red represents juveniles.

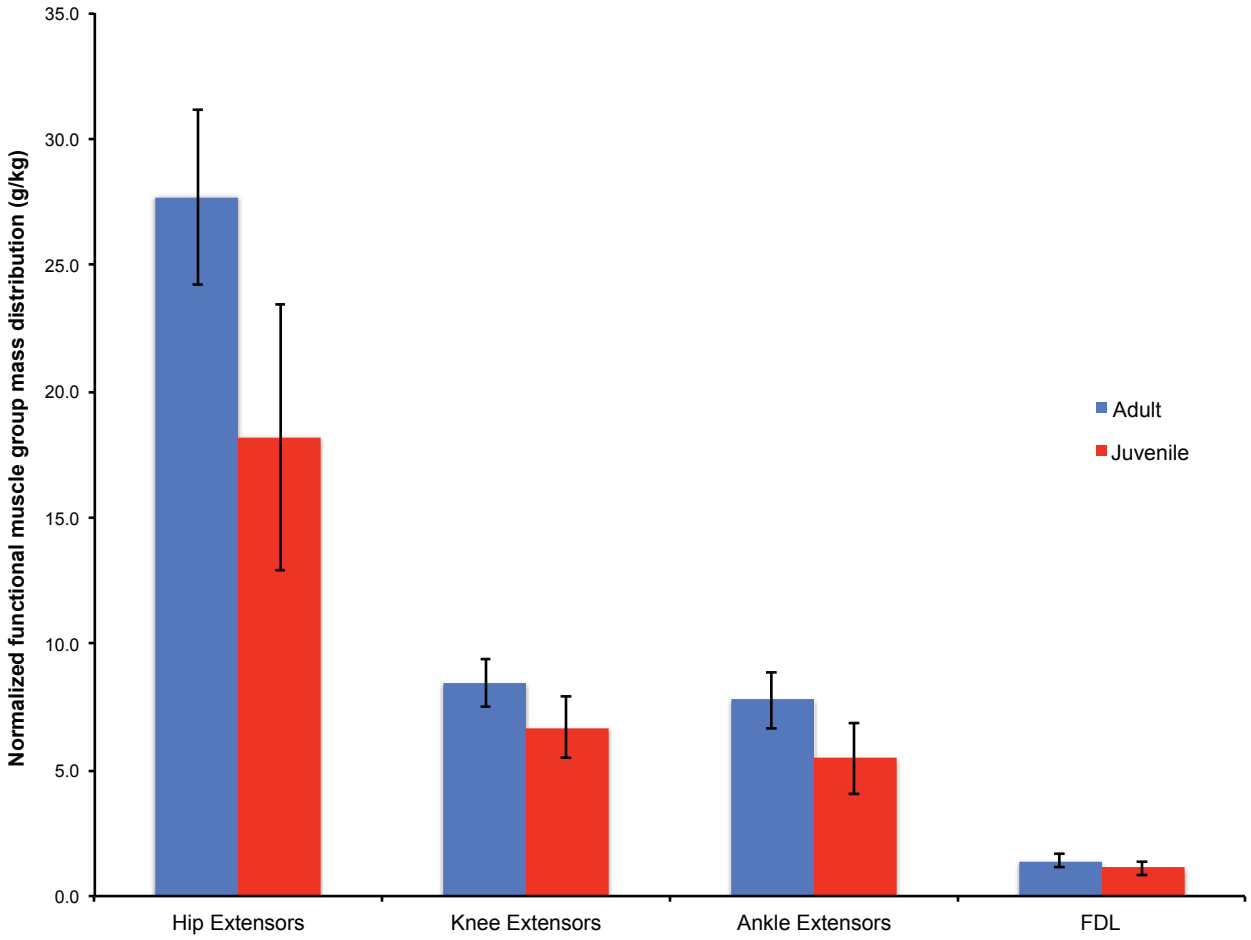

Normalized functional muscle group mass distribution (g/kg)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Hip Extensors
Knee Extensors
Ankle Extensors
FDL
Adult
Juvenile

Figure 2. Fascicle length (l^F) to muscle length (ML) ratios of rabbit hindlimb muscles. Higher l^F/ML values indicate a greater range of contraction and shortening capability. Bars are the means for each muscle and error bars represent the SD (standard deviation). Muscle abbreviations: GLM, gluteus medialis; GLP, gluteus profundus; BFP biceps femoris pelvic head; BFV, biceps femoris vertebral head; SM, semimembranosus; VL, vastus lateralis; RF, rectus femoris; LG lateral gastrocnemius; MG, medial gastrocnemius; FDL, flexor digitorum longus.

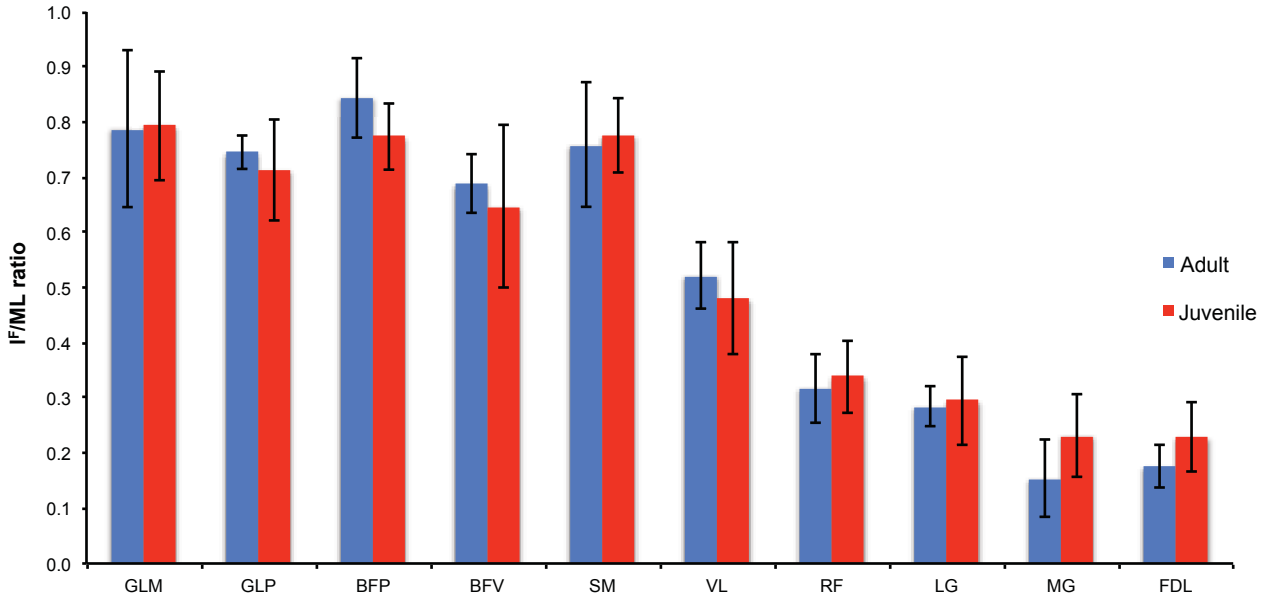

IF/ML ratio
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
GLM
GLP
BFP
BFV
SM
VL
RF
LG
MG
FDL
Adult
Juvenile

Figure 3. Physiological cross-sectional area (PCSA) to muscle mass (MM) ratios of rabbit hindlimb muscles. Muscle mass is normalized to $MM^{0.667}$. Higher PCSA/MM values (i.e., normalized PCSA) indicate either higher degrees of pennation and force production capability. Bars are the means for each muscle and error bars represent the SD (standard deviation). The combination of both higher PCSA/MM and l^F/ML ratios (see Fig. 2) indicates that a muscle is capable of performing appreciable mechanical work. Abbreviations are the same as those in Figure 2.

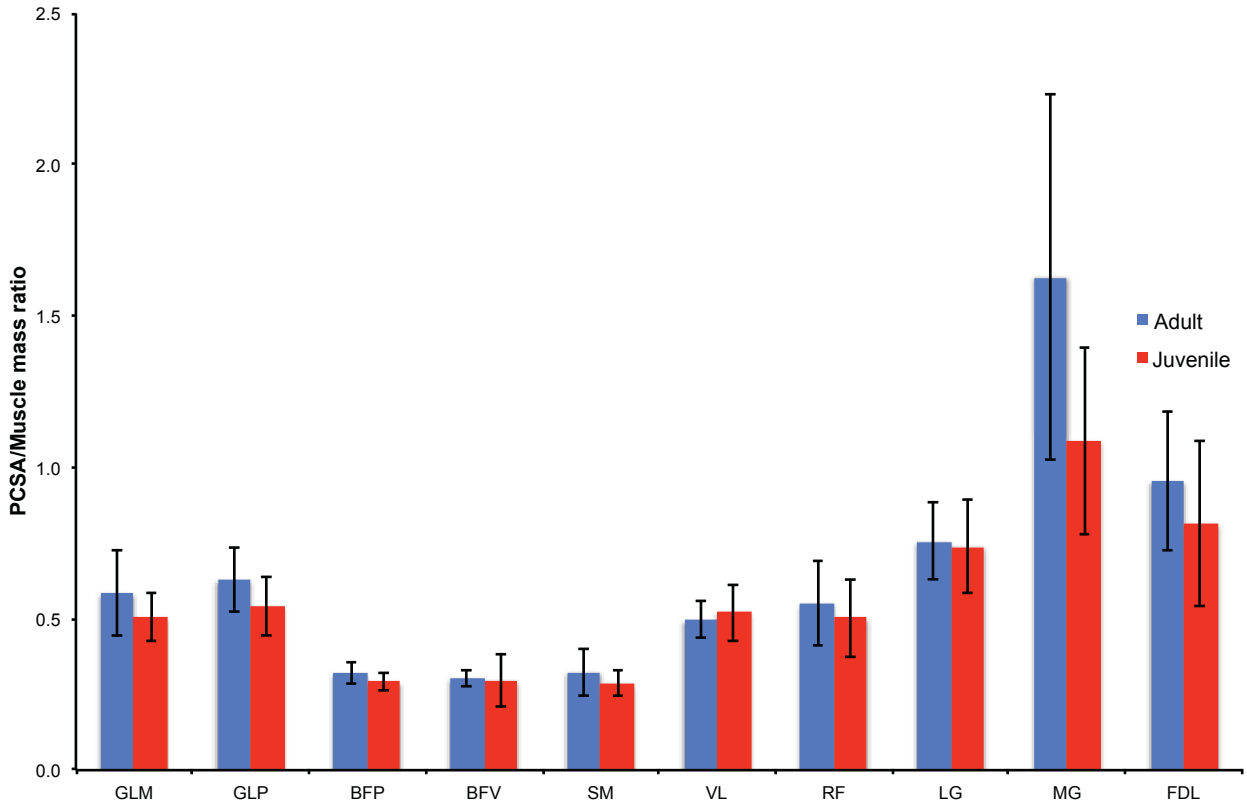

PCSA/Muscle mass ratio
2.5
2.0
1.5
1.0
0.5
0.0
GLM
GLP
BFP
BFV
SM
VL
RF
LG
MG
FDL
Adult
Juvenile

Figure 4. Mean summed isometric force (F_{max}) across the functional muscle groups in the rabbit hindlimb. Force is normalized to body mass (in kg). Bars are the means for each muscle and error bars represent the SD (standard deviation). The functional muscle groups are subdivided by their actions at each limb joint or segment and include the hip extensors (n=5 muscles), knee extensors (n=2 muscles), ankle extensors (n=2 muscles), and digital flexors (n=1 muscle).

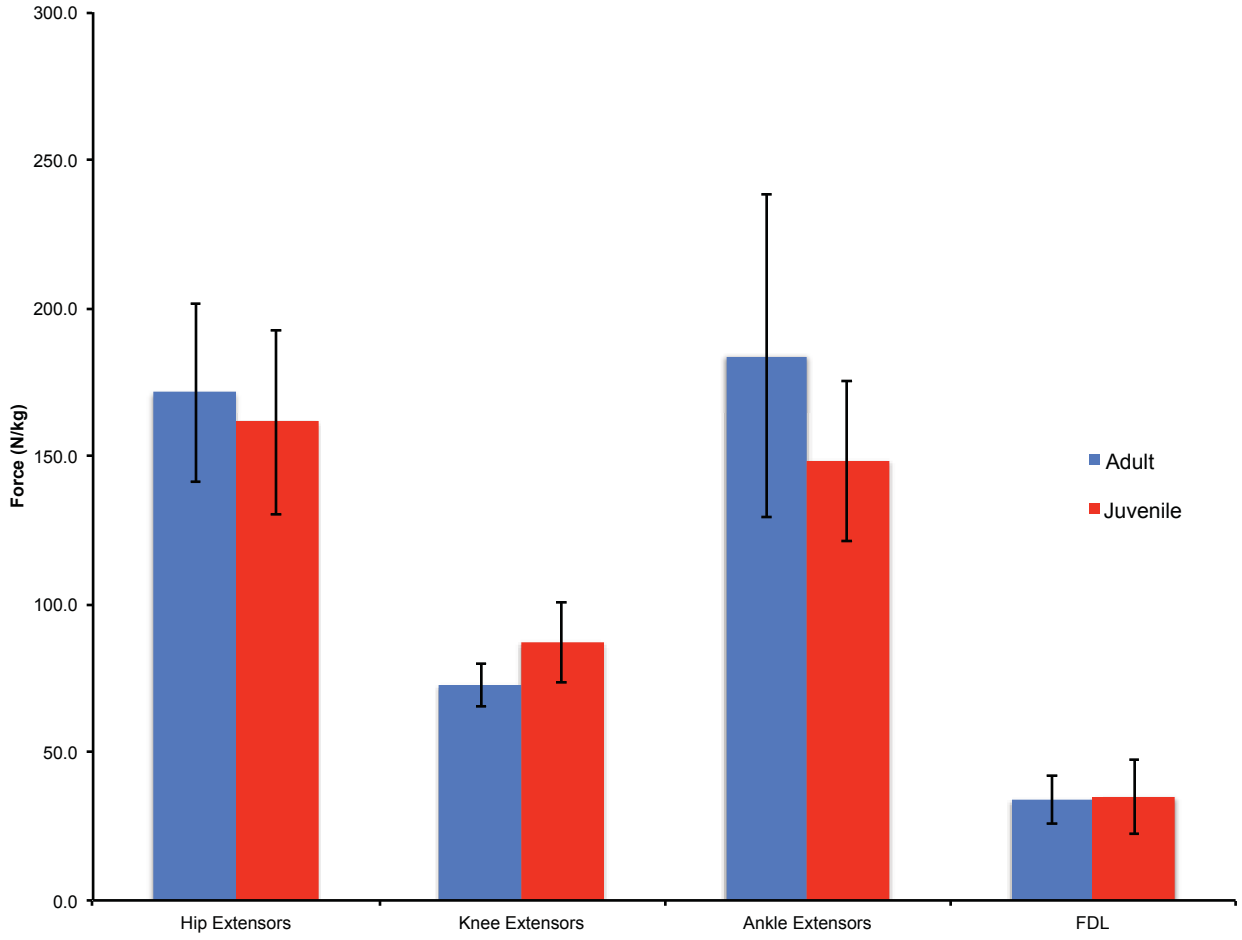

300.0
250.0
200.0
150.0
100.0
50.0
0.0
Force (N/kg)
Hip Extensors
Knee Extensors
Ankle Extensors
FDL
Adult
Juvenile

Figure 5. Muscle indices of relative power (A), joint torque (B), and joint rotational velocity (C). **A.** Normalized PCSA as a function of fascicle length for rabbit hindlimb muscles. PCSA is normalized to $MM^{0.667}$ and fascicle length to $MM^{0.333}$. **B.** Normalized PCSA as a function of r_m. Muscle moment arm is normalized to femur length for hip and knee extensors, and tibia length for ankle extensors and FDL. **C.** Normalized fascicle length as a function of normalized r_m. Data points are means, with no error bars shown. Selected muscles with high and low values of each index are labeled. Abbreviations are the same as those in Figure 2.

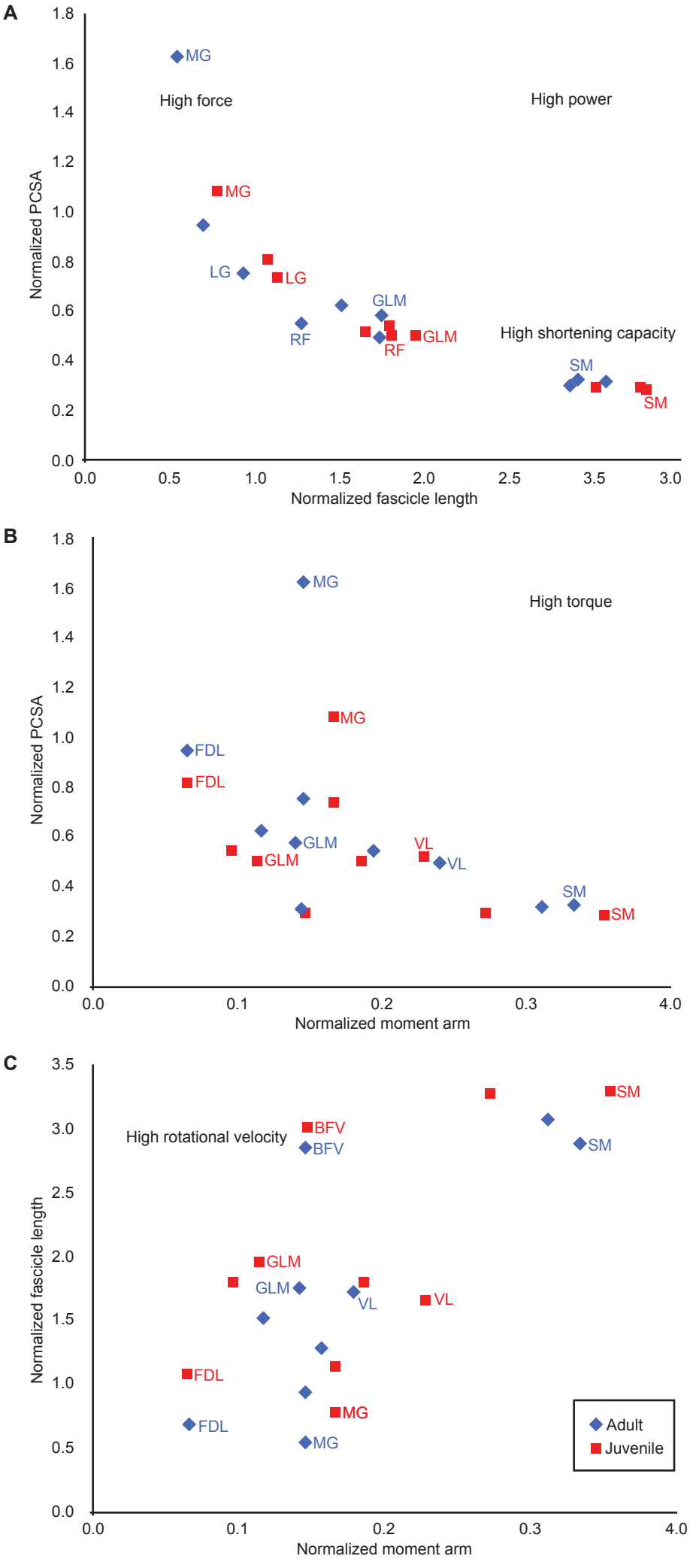

A
Normalized PCSA
Normalized fascicle length
High force
High power
High shortening capacity
MG
LG
GLM
RF
SM
B
Normalized PCSA
Normalized moment arm
High torque
MG
FDL
GLM
VL
SM
C
Normalized fascicle length
Normalized moment arm
High rotational velocity
BFV
GLM
VL
FDL
MG
SM
Adult
Juvenile

Figure 6. Box and whisker plots for functional bone indices in rabbit hindlimbs. Boxes show the range of each index (whiskers), mean (horizontal bars), and data quartiles (boxes). Indices are defined in the Materials and Methods section. Blue boxes are adults and red boxes are juveniles.

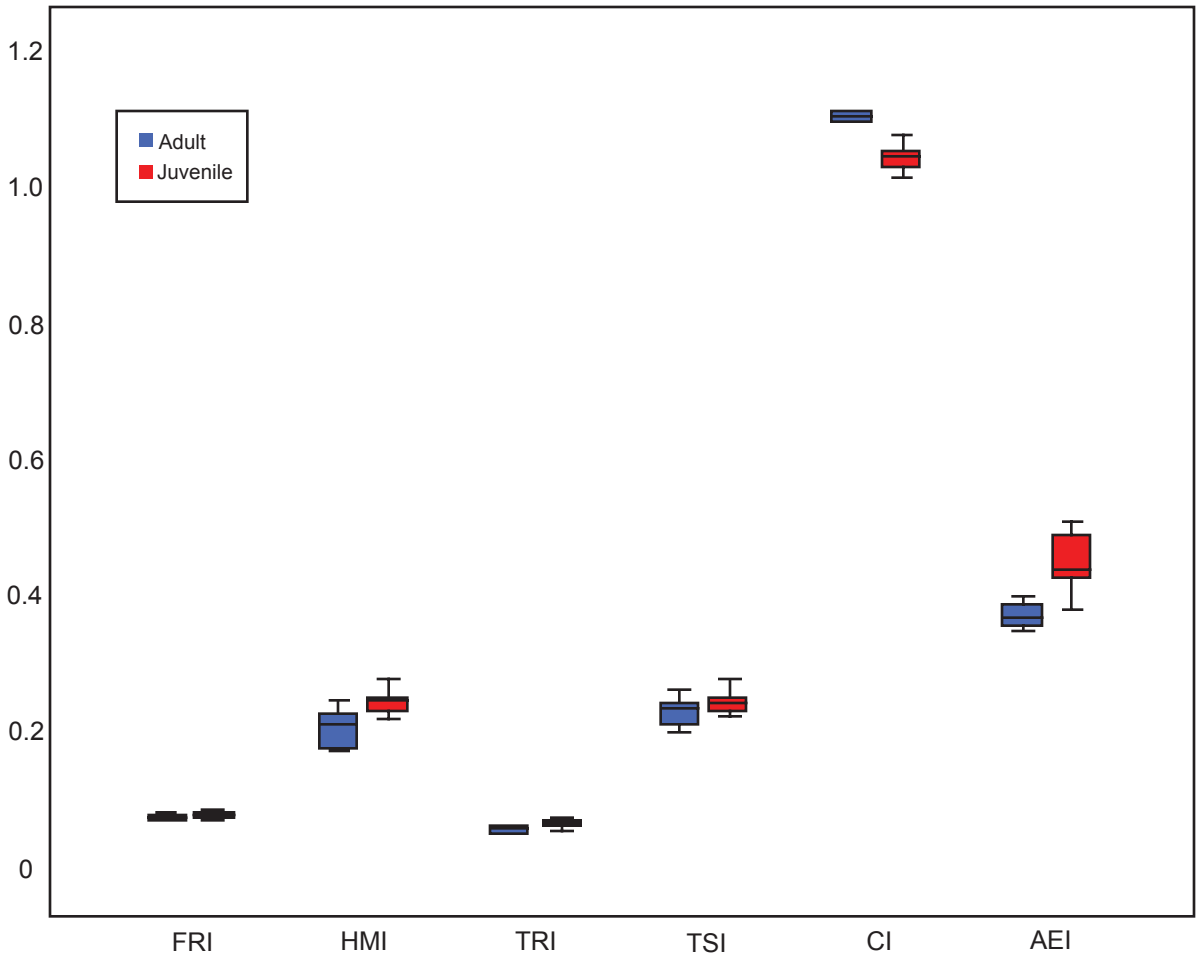

1.2
1.0
0.8
0.6
0.4
0.2
0
Adult
Juvenile
FRI
HMI
TRI
TSI
CI
AEI

Figure 7. Body-size scaling regressions for muscle properties in rabbit hip extensor muscles. Regressions of mass-related (and muscle force) muscle properties scale isometric with a slope=1. Regressions of area-related muscle properties scale isometric with a slope=0.66. Regressions of length-related muscle properties scale isometric with a slope=0.33. Regressions of power scale isometric with a slope=1.33. Blue data points are adults and red data points are juveniles.

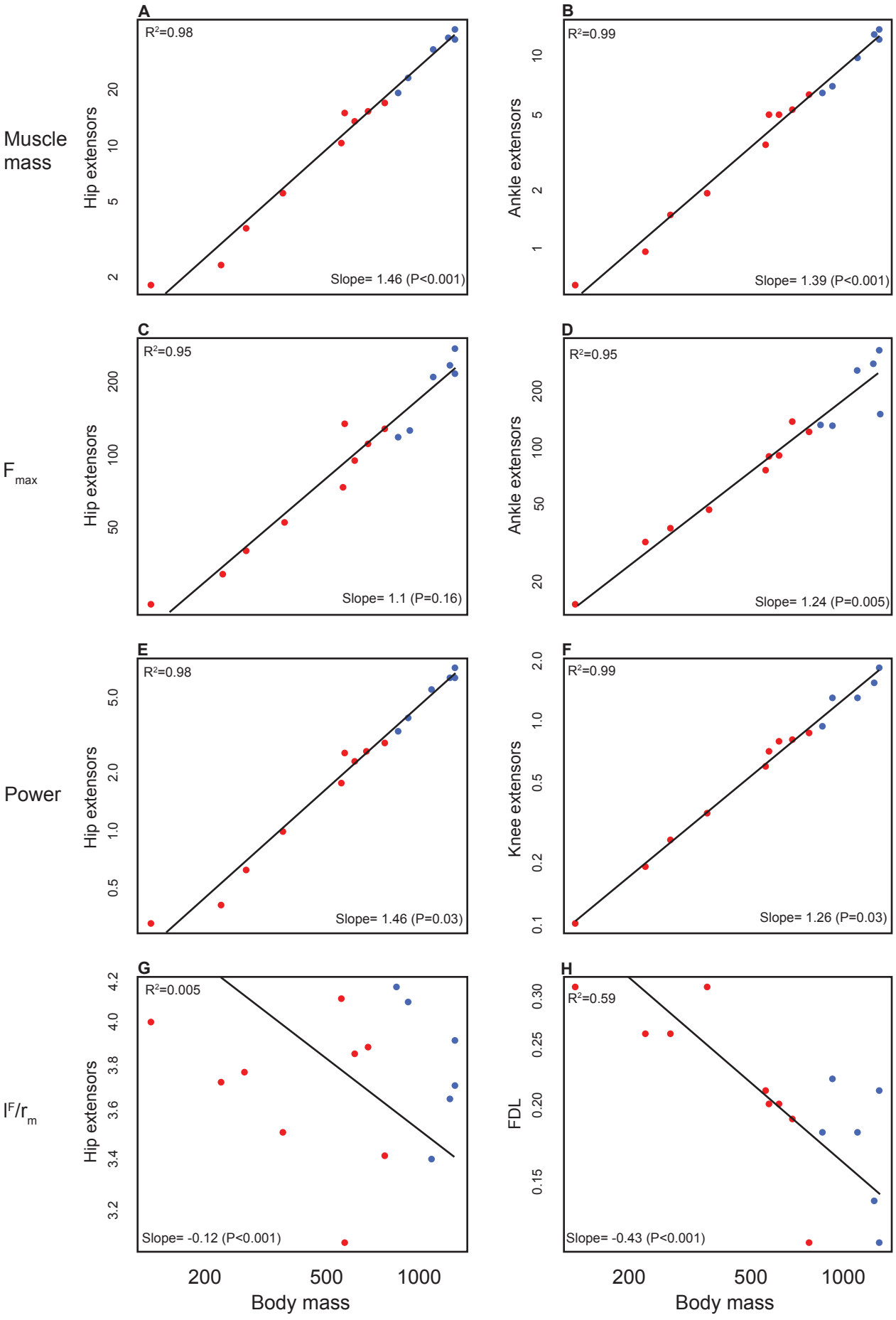
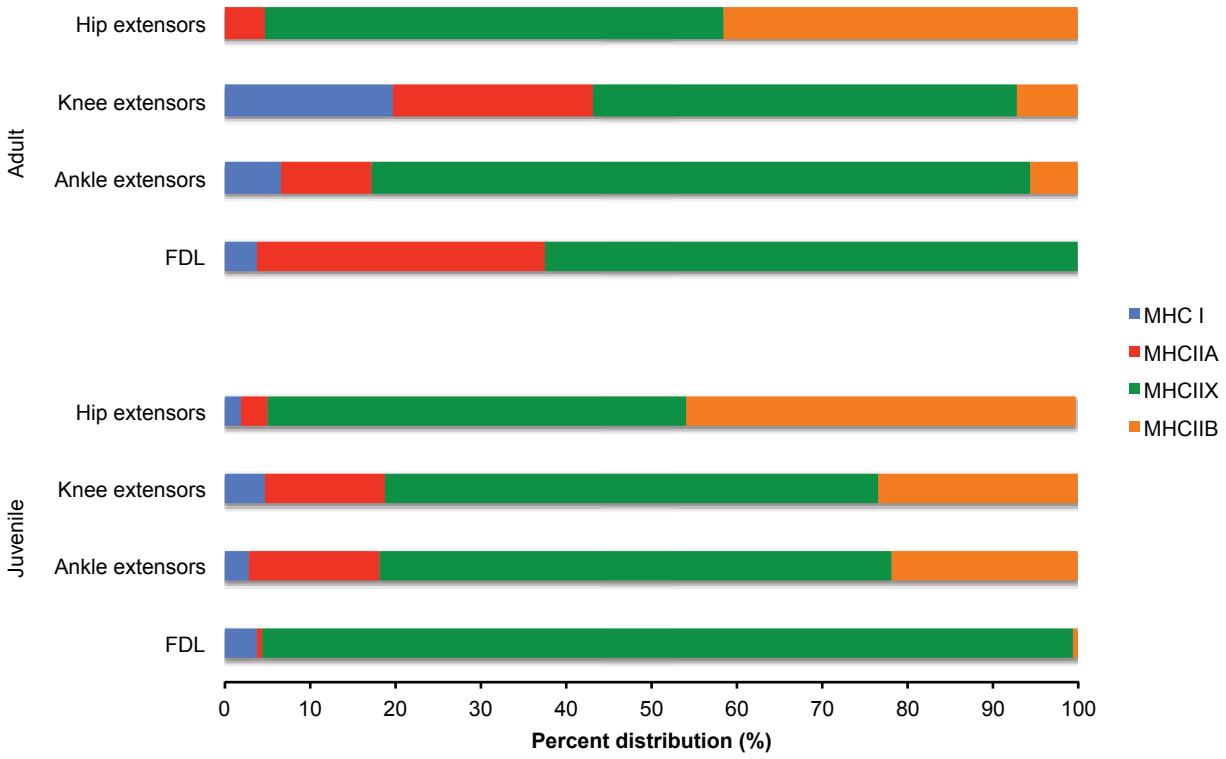

A
B
C
D
E
F
G
H
Muscle mass
F_{max}
Power
l^F/r_m
Hip extensors
Ankle extensors
Knee extensors
FDL
Body mass
R^2=0.98
Slope= 1.46 (P<0.001)
R^2=0.99
Slope= 1.39 (P<0.001)
R^2=0.95
Slope= 1.1 (P=0.16)
R^2=0.95
Slope= 1.24 (P=0.005)
R^2=0.98
Slope= 1.46 (P=0.03)
R^2=0.99
Slope= 1.26 (P=0.03)
R^2=0.005
Slope= -0.12 (P<0.001)
R^2=0.59
Slope= -0.43 (P<0.001)
200
500
1000

Figure 8. Myosin heavy chain (MHC) isoform composition in rabbit hindlimb muscles. Percentage composition of MHC isoforms is shown for functional muscle groups: hip extensors, knee extensors, ankle extensors, and FDL (digital flexor illustrated separate from ankle extensors but indicated to act in ankle extension). Means for each muscle were computed from densoimetric measurements on 2–3 independent gels experiments and then averaged for each functional muscle group in both adults and juvenile rabbits.


Adult
Hip extensors
Knee extensors
Ankle extensors
FDL
Juvenile
Hip extensors
Knee extensors
Ankle extensors
FDL
MHC I
MHCIIA
MHCIIX
MHCIIB
0
10
20
30
40
50
60
70
80
90
100
Percent distribution (%)

Northeast Ohio
MEDICAL UNIVERSITY

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

TO: Jesse W. Young, Ph.D.
Assistant Professor, Anatomy & Neurobiology

[redacted]

FROM: Christopher J. Vinyard, Ph.D.
Chairperson, NEOMED Institutional Animal Care and Use Committee

SUBJECT: Protocol Approval

DATE: October 30, 2013

The following Northeast Ohio Medical University (NEOMED) animal use protocol was reviewed and approved by this Institution's Animal Care and Use Committee (IACUC) on October 30, 2013. The protocol is approved for a three (3) year period of time; however, you must submit an annual renewal to the IACUC each year for review.

Any serious or adverse events regarding the use of animals approved in this study must be reported immediately to the IACUC Chairperson or the Attending Veterinarian. Protocols involving the use of human tissues require Institutional Review Board (IRB) approval.

NEOMED Protocol No.:	**#13-026**
Title of Proposal:	**Natural Selection on Growth and Locomotor Development in Eastern Cottontail Rabbits (*Sylvilagus floridanus*)**
Type of Vertebrate:	**Rabbits**
Funding Agency(ies):	**National Science Foundation**
Protocol Expiration Date:	**October 29, 2016**

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is A3474-01. This institution is also registered with the United States Department of Agriculture (USDA). The USDA registration number is 31-R-0092.

The Comparative Medicine Unit (CMU) at the Northeast Ohio Medical University (NEOMED) has been accredited with the Association for Assessment for Accreditation of Laboratory Animal Care (AAALAC) International since June 8, 1982. Continued Full Accreditation was last renewed on July 1, 2011.

Thank you.

CJV:lkn

Cc: Walter E. Horton, Jr., Ph.D.
Vice President for Research
NEOMED Institutional Official