



Hindlimb muscle anatomical mechanical advantage differs among joints and stride phases in basilisk lizards

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ABSTRACT

The vertebrate musculoskeletal system is composed of skeletal levers powered by muscles. Effective mechanical advantage (EMA) and muscle properties influence organismal performance at various tasks. Anatomical mechanical advantage (AMA) is a proxy for EMA that facilitates the study of preserved specimens when many muscles or many species are of interest. AMA is the quotient of in-lever to out-lever length, and quantifies the force–velocity trade-off of a lever, where high AMAs translate into high force, low velocity levers. We studied AMAs, physiological cross-sectional areas (PCSAs), fiber lengths, and fiber widths for 20 hindlimb muscles of the lizard *Basiliscus vittatus*, moving the hip, knee, and ankle during both the stance and swing phases of the stride. We tested the hypotheses that muscles moving proximal limb joints, and those active during stance, would have characteristics that maximize force. We also tested whether adults had more force-optimized levers than juveniles to compensate for higher body mass. We found no differences between adults and juveniles, but found differences among joints and between stride phases. AMAs were lowest and PCSAs highest for the knee, and PCSA was higher for stance than swing muscles. Fiber width decreased distally, but did not differ between stride phases. Fiber length of stance muscles decreased distally and was highest for swing muscles of the knee. Our findings show that different muscle and lever characteristics allow the knee to be both force- and velocity-optimized, indicating its important role in locomotion.

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1. Introduction

Animals rely on their musculoskeletal system to enable them to accomplish various tasks (Hildebrand et al., 1985; Huijing, 1998). Many muscles are involved in multiple tasks and their properties frequently represent a compromise between those tasks (Gans and De Vree, 1987; Foster and Higham, 2014). The action of a given muscle depends on its moment arms and attachments to the skeleton (Huijing, 1998). The properties of muscles and the skeletal lever systems that they move determine locomotor capacity and reflect the animal's ecology (Biewener, 1989; Westneat, 1994; McHenry and Lauder, 2006; Scales et al., 2009; Higham et al., 2011; Holzman et al., 2011a). Ultimately, since different muscles move different joints, and may be active during different phases of a behavior (Gregersen and Carrier, 2004; Foster and Higham, 2014), they may have different demands placed on them depending on the size of the animal (Carrier, 1983, 1996; Herrel and Gibb, 2006). One would expect, therefore, that muscle and lever system properties

vary according to the functional demands they experience. Here we study anatomical properties of muscles and lever systems in the hindlimb of the basilisk lizard (*Basiliscus vittatus*) to test if they differ among joints and between stance and swing phase muscles.

Muscles are composed of dissectible fibers, which are bundles of myofibrils, containing sarcomeres, the serially arranged contractile units of the myofibrils (Hildebrand et al., 1985; Gans and De Vree, 1987). The contractile properties of muscles are affected by fiber lengths, widths, angles, and types. Longer fibers have more sarcomeres in series, and so have higher contraction velocities and displacements, but fiber length is independent of force (Sacks and Roy, 1982; Gans and De Vree, 1987; Azizi et al., 2008). Wider fibers have more sarcomeres in parallel and produce more force (Gleeson, 1983; Gans and De Vree, 1987). This pattern scales up to the whole muscle in that muscles with higher physiological cross-sectional areas (PCSAs) also produce more force (Sacks and Roy, 1982; Powell et al., 1984; Gans and De Vree, 1987). The angle of muscle fibers relative to their line of action influences both force and contraction velocity in the direction of action (Gans and De Vree, 1987). Pennate muscles have short fibers oriented at an angle to the line of action, reducing velocity but increasing total force because more fibers are placed in parallel (Sacks and Roy, 1982; Huijing, 1998).

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The contractile properties of muscles are also influenced by their fiber types (Gleeson et al., 1980; Bonine et al., 2005), but this is one factor not dealt with here. The physiology and architecture of muscles establish force–velocity trade-offs that have implications for organismal performance at various tasks (Gans and De Vree, 1987; Bonine et al., 2005).

Another way in which musculoskeletal systems vary is in the properties of their lever systems, influencing organismal task performance above the cellular level (Gans and De Vree, 1987; Biewener, 1989). A lever rotates around a fulcrum, and the in-lever and out-lever are the distances from the fulcrum to the in-force applied by a muscle and the out-force produced by the lever (Hildebrand et al., 1985). Lever systems are governed by the formulae

$$F_{\text{out}} = \frac{F_{\text{in}}L_{\text{in}}}{L_{\text{out}}} \quad \text{and} \quad V_{\text{out}} = \frac{V_{\text{in}}L_{\text{out}}}{L_{\text{in}}},$$

where F is force, L is the lever length, and V is velocity for the in- and out-levers (Hildebrand et al., 1985; McHenry and Lauder, 2006). From these equations, it is apparent that changing the relative lengths of the in- and out-levers affects out-force and out-velocity inversely. Conveniently, one can define the lever mechanical advantage (MA), the inverse of gear ratio (GR), as $MA = 1/GR = L_{\text{in}}/L_{\text{out}}$, where a lever with a low MA or high GR produces low-force, high-velocity movement (Gans and De Vree, 1987; Biewener, 1989, 1990; Carrier et al., 1998; McHenry and Lauder, 2006).

In musculoskeletal systems, gearing occurs at multiple levels, including the levers of the myosin heads, the levers associated with the architecture of muscles (pennation), and the levers of the skeleton being moved by muscles (Azizi and Brainerd, 2007; Azizi et al., 2008). At the level of the skeletal levers, which is our focus here, MAs have been calculated in two ways. Effective mechanical advantage (EMA) calculates the in-lever as the perpendicular distance from the center of the joint to the line of action of the muscle, and the out-lever as the perpendicular distance from the joint to the ground reaction force (GRF) vector (Gans and De Vree, 1987; Biewener, 1989; Carrier et al., 1998; Gregersen and Carrier, 2004). EMA allows one to precisely model gearing during the behavior of an animal, accounts for the non-independence of EMA estimates among connected joints, and provides data on the dynamic nature of gearing as a joint moves (Biewener, 1989; Carrier et al., 1998; Gregersen and Carrier, 2004). However, the EMA cannot be applied to preserved specimens, and is labor-intensive, limiting its utility for assembling large comparative datasets. It is also typically applied on a joint-specific as opposed to muscle-specific basis (Carrier et al., 1998; Gregersen and Carrier, 2004; Azizi and Brainerd, 2007).

Anatomical mechanical advantage (AMA) is a static estimate of the actual MA whose accuracy depends on the behavior being studied (Young, 2009; Fellmann, 2012). AMA is more easily measured than EMA because it does not require live specimens and GRF or kinematic data. AMA calculates the in-lever as the distance from the center of the joint to the insertion of the muscle, and the out-lever as the length of the bone being moved by the joint (Carrier, 1983; Hildebrand et al., 1985). The AMA has the advantages of easily being applied on a muscle-specific basis, and to large numbers of species and specimens, facilitating evolutionary studies (Hildebrand et al., 1985; McHenry and Lauder, 2006). However, the AMA treats gearing as static and does not take kinematics, kinematic chains, or behavior into account (Young, 2005, 2009).

Here, we compare AMA among hindlimb muscles that rotate the hip, knee, and ankle during the stance and swing phases of the stride in the lizard *Basiliscus vittatus*. This species is a good

candidate for our study because individuals exhibit high levels of locomotor performance, being bipedal specialists that run quickly on both land and water (Snyder, 1949, 1962; Hsieh, 2003; Hsieh and Lauder, 2004). They need to generate large forces during these locomotor behaviors (Glasheen and McMahon, 1996; Hsieh and Lauder, 2004). They also increase in size by an order of magnitude (Glasheen and McMahon, 1996), allowing us to investigate whether AMAs change through ontogeny, something that has been shown to happen in various animals, possibly to compensate for decreased muscle strength and relative muscle mass of juveniles relative to adults (Goldspink, 1980; Carrier, 1983, 1996; Young, 2009).

Despite considerable work on mechanical advantage in vertebrate limbs, there are few studies that simultaneously consider a large number of muscles that differ functionally, allowing us to test several novel hypotheses. First, we test the hypothesis that muscle properties and AMAs differ among joints of the hindlimb. We expect that more proximal joints will be optimized for force output, while more distal joints will be optimized for velocity because proximal joints move a heavier proportion of the limb than distal joints. This is exacerbated by proximal muscles tending to be largest (Maloiy et al., 1979). Hence, we expect muscles powering proximal joints to have higher AMA and PCSA, and shorter, wider fibers than distal joints (Carrier et al., 1998; Azizi and Brainerd, 2007; Li et al., 2012). Second, we test whether muscles active during stance phase will be force-optimized, while those active during swing phase of the stride will be velocity-optimized. The force needed to propel an animal during locomotion is exclusively generated during stance (Higham et al., 2011), so stance phase muscles should be force-optimized. Third, we test whether muscle and lever system properties of juvenile animals differ from those of adult animals. Juvenile animals sometimes exhibit relatively higher locomotor performance than adults (Herrel and Gibb, 2006) and may have higher mechanical advantage to compensate for relatively weaker and smaller muscles (Carrier, 1996). However, adults may need to generate more force to compensate for their disproportionately greater weight (Biewener, 1989). Finally, we wish to highlight the utility of AMA as a viable approach to studying lever properties when the goal is to study large numbers of muscles in large numbers of specimens or species.

2. Materials and methods

2.1. Study animals

We used 15 *B. vittatus* specimens for limb muscle dissections, obtained from a commercial dealer. The size of the animals followed a bimodal non-overlapping distribution, with six juveniles (snout–vent length (SVL) mean = 70.5 mm, range: 62.4–80.5 mm) and nine adults (SVL mean = 119.0 mm, range: 104.7–138.9 mm). We euthanized the animals using an overdose of isoflurane gas (AVMA, 2000). We then fixed them for three days using 10% neutral buffered formalin in standardized position with the thigh perpendicular to the body, and the knee and ankle joints at 90° to ensure that muscles were comparable in terms of length and appearance (Pisani, 1973; Sacks and Roy, 1982; Herrel et al., 2008). Using this standardized orientation of the limb segments will facilitate comparison to species studied in the future. These joint angles fall within the range of angles that lizards use during locomotion (Irschick and Jayne, 1998; Foster and Higham, 2012), including basilisks running on water (Hsieh, 2003). Finally, we soaked specimens in water for 24 h to remove excess formalin and preserved them in 70% ethanol. All of these procedures are standard and were approved by Clark University's Institutional Animal Care and Use Committee.

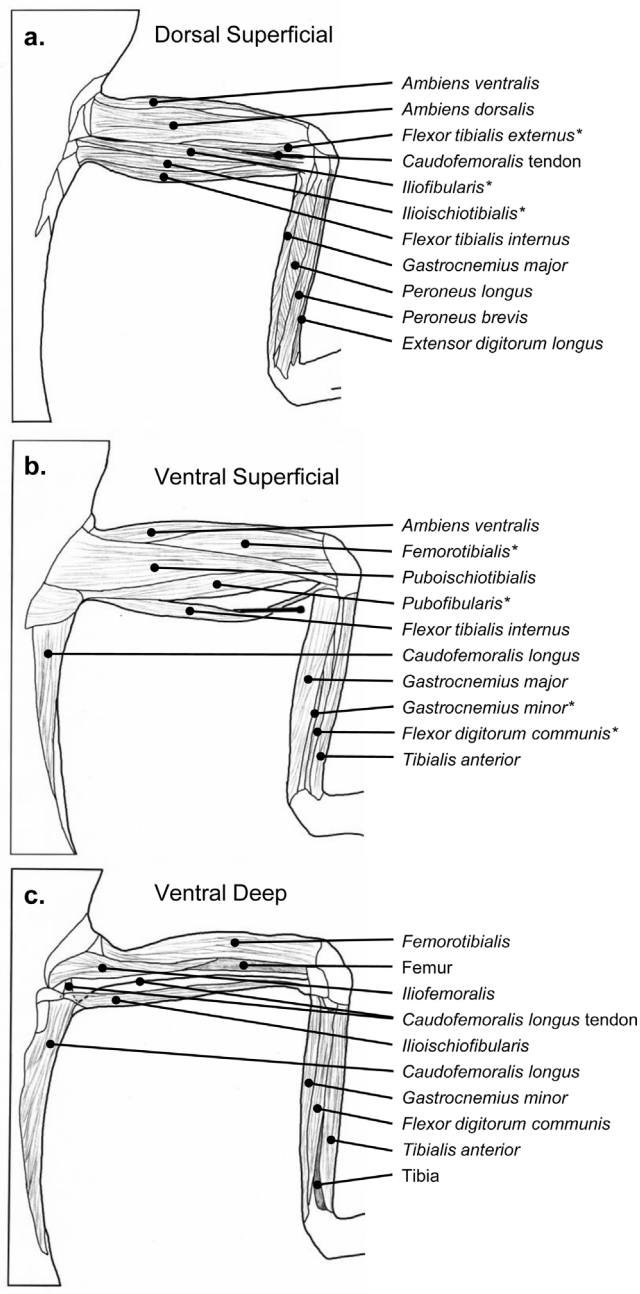


Fig. 1. Hindlimb muscles of *Basiliscus vittatus* that were dissected to study their properties. Superficial muscles of the (a) dorsal and (b) ventral limb are shown lightly spread apart, so as to reveal some of the deeper muscles (indicated with *), and making the thighs and crus appear more robust than in life. (c) Ventral deep muscles are revealed by removal of muscles that appear in (b) but not (c).

2.2. Muscle morphology and dissection

We skinned and dissected the hindlimbs of the specimens between 4 and 10 months after placing them in ethanol and excluded individuals that were either thin or that had muscles that were poorly preserved (leaving us with the 15 specimens mentioned above). We dissected 20 of the largest muscles of the hindlimb, using the detailed analysis of *Anolis* by Herrel et al. (2008) as a guide for nomenclature and muscle actions. We supplemented this information using Russell and Bauer (2008), using their published synonymy lists, muscle origins, insertions, and actions to help identify muscles in *B. vittatus* (Fig. 1). We classified hip adductors and retractors, knee extensors, and ankle extensors as

stance phase muscles, and we classified hip abductors and protractors, knee flexors, and ankle flexors as swing phase muscles. These classifications are consistent with those in the literature, based both on anatomical assessments (Herrel et al., 2008; Russell and Bauer, 2008) and electromyography (Reilly, 1998; Higham and Jayne, 2004; Higham et al., 2011). However, some muscles are multifunctional either because they are biarticular, spanning two joints (Gans and De Vree, 1987; Prilutsky et al., 1996; Kaya et al., 2005), or because they are activated multiple times during the stride (Foster and Higham, 2014). We classified clearly multifunctional muscles based on both functions. Specifically, the mm. flexor tibialis internus, flexor tibialis externus, iliofibularis, and puboischiotibialis move both the hip and the knee and the m. caudofemoralis longus has two insertions, moving those same joints (Gatesy, 1997; Reilly, 1998; Higham and Jayne, 2004; Herrel et al., 2008; Foster and Higham, 2014), so we included these muscles twice in our data, once for each joint or insertion. There are other biarticular muscles, including the mm. gastrocnemius and peroneus, that we did not include twice because the literature indicates that these muscles are predominantly active only in moving the ankle, while stabilizing the knee (Reilly, 1998; Foster and Higham, 2014). In total, we included nine hip muscles (two swing, seven stance), nine knee muscles (six swing, three stance), and seven ankle muscles (two swing, five stance), for a total of 25 muscle insertions/actions. Details are provided in Table 1.

We dissected muscles from the left hindlimb using fine forceps, scissors, size #3 entomological pins, and with the aid of a dissecting microscope (Wild Heerbrugg AG, Heerbrugg, Switzerland). We dissected superficial muscles first and worked deeply as they were removed. We first separated the belly of each muscle from underlying and neighboring muscles, so that it was attached only at its insertion and origin. We then measured the in-lever as the distance from the insertion to the middle of the joint that the muscle rotated, and the out-lever as the distance from the middle of the same joint to the distal end of the bone that the muscle inserted on using digital calipers (Mitutoyo, Kawaskai, Japan) to the nearest 0.01 mm. We used the distal end of the fourth toe as the end of the out-lever for muscles acting on the ankle because many of these muscles inserted distal to the metatarsals (Herrel et al., 2008). We then photographed each muscle in plane with its action using a 6 megapixel digital camera (Nikon D70; Nikon Corp., Tokyo, Japan). Finally, we cut each muscle at its insertion and origin, weighed it on an analytical balance (Mettler AJ100; Mettler-Toledo Inc., Columbus, OH, USA) to the nearest 0.0001 g, and stored it individually in 95% ethanol.

2.3. Muscle fiber data

Since the force that a muscle can generate is proportional to its physiological cross-sectional area (PCSA), we collected data to allow us to calculate PCSA for the muscles that we dissected. Muscle PCSA can be calculated as:

$$\text{PCSA} = \frac{m \times \cos \theta}{d \times L}$$

where m is a muscle's mass, θ is muscle fiber angle relative to the line of action, d is fiber density, and L is fiber length (Powell et al., 1984; Biewener, 2003). We measured these variables and calculated PCSA for all 20 muscles of three juvenile and three adult individuals. We used muscle masses obtained at time of dissection, and the published value of 1060 kg m^{-3} for muscle density (Mendez and Keys, 1960; Clarys and Marfell-Jones, 1986; Biewener, 2003). We measured fiber angles from our photographs using the "Angle" tool in ImageJ (Rasband, 1997–2014) from the surface of each muscle at three places along its length and used the mean in calculations (Gans and De Vree, 1987). Finally, we measured muscle fiber length

Table 1
Hindlimb muscles dissected from *Basiliscus vittatus* lizards, and the joints they move, their action, and the phase during which they are active, as inferred from the literature. Also presented are the anatomical mechanical advantage (AMA), physiological cross-sectional area (PCSA; $\text{mm}^2 \times 10^{-6}$), muscle fiber length (mm), and muscle fiber width (mm). Values presented are means \pm standard deviations.

Muscle	Joint	Action	Phase	AMA	PCSA	Fiber length	Fiber width
Adductor femoris	Hip	Adductor	Stance	0.598 \pm 0.136	2.72 \pm 0.21	14.93 \pm 4.81	0.064 \pm 0.024
Caudofemoralis longus	Hip	Retractor	Stance	0.130 \pm 0.024	6.20 \pm 3.41	33.11 \pm 18.27	0.063 \pm 0.014
Flexor tibialis externus	Hip	Adductor	Stance	0.940 \pm 0.030	4.02 \pm 1.84	22.14 \pm 5.07	0.083 \pm 0.022
Flexor tibialis internus	Hip	Adductor	Stance	0.919 \pm 0.024	2.80 \pm 0.61	12.56 \pm 2.36	0.073 \pm 0.022
Ilioschiotibialis	Hip	Adductor	Stance	0.908 \pm 0.044	2.35 \pm 0.92	15.58 \pm 5.75	0.073 \pm 0.015
Pubofibularis	Hip	Adductor	Stance	0.908 \pm 0.039	3.01 \pm 1.42	12.73 \pm 4.34	0.070 \pm 0.008
Puboischiotibialis	Hip	Adductor	Stance	0.949 \pm 0.033	6.88 \pm 2.59	16.65 \pm 4.33	0.094 \pm 0.039
Iliofemoralis	Hip	Abductor	Swing	0.366 \pm 0.032	2.47 \pm 0.63	8.11 \pm 2.08	0.062 \pm 0.004
Iliofibularis	Hip	Abductor	Swing	0.899 \pm 0.105	2.43 \pm 0.59	18.78 \pm 4.92	0.058 \pm 0.014
Ambiens pars dorsalis	Knee	Extensor	Stance	0.116 \pm 0.030	8.81 \pm 0.48	8.49 \pm 2.67	0.075 \pm 0.016
Ambiens pars ventralis	Knee	Extensor	Stance	0.098 \pm 0.027	11.29 \pm 3.19	5.07 \pm 1.36	0.063 \pm 0.019
Femorotibialis	Knee	Extensor	Stance	0.109 \pm 0.027	1.65 \pm 0.14	7.90 \pm 2.68	0.061 \pm 0.020
Caudofemoralis longus	Knee	Flexor	Swing	0.111 \pm 0.019	6.20 \pm 3.41	33.11 \pm 18.27	0.063 \pm 0.014
Flexor tibialis externus	Knee	Flexor	Swing	0.080 \pm 0.010	4.02 \pm 1.84	22.14 \pm 5.07	0.083 \pm 0.022
Flexor tibialis internus	Knee	Flexor	Swing	0.123 \pm 0.024	2.80 \pm 0.61	12.56 \pm 2.36	0.073 \pm 0.022
Iliofibularis	Knee	Flexor	Swing	0.119 \pm 0.021	2.43 \pm 0.59	18.78 \pm 4.92	0.058 \pm 0.014
Ilioschiotibialis	Knee	Flexor	Swing	0.099 \pm 0.036	3.74 \pm 0.86	12.36 \pm 2.79	0.063 \pm 0.018
Puboischiotibialis	Knee	Flexor	Swing	0.127 \pm 0.020	6.88 \pm 2.59	16.65 \pm 4.33	0.094 \pm 0.039
Flexor digit. communis	Ankle	Extensor	Stance	0.522 \pm 0.106	4.65 \pm 2.25	6.90 \pm 1.77	0.065 \pm 0.022
Gastrocnemius major	Ankle	Extensor	Stance	0.353 \pm 0.094	8.07 \pm 2.57	5.43 \pm 0.57	0.063 \pm 0.014
Gastrocnemius minor	Ankle	Extensor	Stance	0.379 \pm 0.071	13.63 \pm 7.50	4.04 \pm 1.46	0.063 \pm 0.012
Peroneus brevis	Ankle	Extensor	Stance	0.207 \pm 0.048	2.44 \pm 1.22	5.97 \pm 1.53	0.049 \pm 0.012
Peroneus longus	Ankle	Extensor	Stance	0.177 \pm 0.017	1.89 \pm 0.73	6.28 \pm 1.01	0.054 \pm 0.012
Extensor digit. longus	Ankle	Flexor	Swing	0.175 \pm 0.024	4.29 \pm 1.82	6.64 \pm 2.42	0.061 \pm 0.013
Tibialis anterior	Ankle	Flexor	Swing	0.145 \pm 0.028	1.87 \pm 0.85	7.80 \pm 1.65	0.064 \pm 0.019

and width after nitric acid digestion of whole muscles using an ocular micrometer and dissecting microscope. To do the digestion (following the protocol of [Timson and Dudenhoefler, 1990](#)), we rinsed muscles with distilled water and immersed them in 15% nitric acid for 8–12 h. We then rinsed the muscles again with distilled water and immersed them in glycerol for 1–2 h before rinsing them a final time and placing them on a microscope slide. Finally, we used fine forceps and entomological pins to tease individual muscle fibers apart. We mounted and measured three unbroken muscle fibers to 0.01 mm on each slide and preserved them using a coverslip and permount ([Sacks and Roy, 1982](#); [Powell et al., 1984](#)).

2.4. Statistical analysis

We were interested in testing whether our response variables (AMA, PCSA, fiber length or fiber width) differed among age groups, the joints that the muscles moved, or whether a muscle was active during the stance or swing phase of the stride. To analyze our data, we first calculated AMA, as described in Section 1 ([Gans and De Vree, 1987](#); [Carrier et al., 1998](#)). We then conducted all of our statistical analyses in R v. 2.14 ([R Development Core Team, 2012](#)). Since all of the explanatory variables (age, joint, and phase) were fixed factors, we used a fully crossed three-factor ANOVA. We also included muscle nested in individual as a random factor to account for individual variation.

Since the assumption of normality was violated for all of our response variables and \ln -transformation did not ameliorate this issue, we conducted randomization ANOVAs, which rely on a null F -distribution derived from randomizations of the data rather than the tabulated F -distribution, relaxing the distributional assumptions of the ANOVA ([Petraitis et al., 2001](#)). To do this, we first calculated our standard three-factor ANOVA with muscle nested in individual, retaining the F -statistics for each test. We then randomized the order of the response variable 10,000 times, ran the same ANOVA design on each randomized dataset, and retained the F -statistics for each randomization and each test. These randomized F -statistics formed the null F -distribution for each test. We calculated the p -value for each test as the number of F -statistics from each null distribution that was greater (more extreme) than the

empirical F -statistic obtained from the unrandomized data. With this approach, the sum of squares, mean squares, and F -statistic for each test is the same as for a standard ANOVA, but the p -value is calculated from the null distribution obtained from the 10,000 randomized data sets.

Finally, we were interested in whether AMA was related to PCSA, fiber width, fiber length, or muscle length, and whether PCSA was related to fiber width. To test this we used Pearson correlations ([Sokal and Rohlf, 1995](#)). For all of our analyses, we used the Benjamini–Hochberg method for multiple comparisons because it also accounts for false discovery rate, and Bonferroni corrections have been demonstrated as being overly conservative ([Benjamini and Hochberg, 1995](#); [Williams et al., 1999](#)).

3. Results

There was considerable variation between muscles in AMA, PCSA, fiber length, and fiber width ([Table 1](#)). Our first hypothesis, that muscles powering more proximal joints would be more force-optimized, was partially supported by our data. Although AMAs, fiber lengths, and fiber widths differed among joints, only some of these differences were predicted ([Table 2](#)). AMAs were highest for the hip, as expected, but lowest for the knee ([Fig. 2a](#)). Counter to expectations, PCSA was highest for the knee but not significantly ([Fig. 2b](#)), and fiber length was highest for the hip and lowest for the ankle, but mainly for stance muscles, as indicated by a significant joint \times phase interaction ([Table 2](#) and [Fig. 2c](#)). Fiber widths for stance muscles followed expectations, with hip muscles having the widest fibers and ankle muscles having the thinnest fibers ([Fig. 2d](#)).

Our second hypothesis, that stance phase muscles would be force-optimized, was strongly supported by our PCSA data ([Table 2](#) and [Fig. 2b](#)) and was supported by our AMA data for the hip and ankle ([Fig. 2a](#)). It was partially supported by fiber length data, with stance phase muscles of the knee and ankle having shorter fibers than swing phase muscles ([Fig. 2c](#)). There were no significant differences between stance and swing muscles in fiber width ([Table 2](#)).

Our third hypothesis, that juveniles and adults would differ in muscles and lever system characteristics, was not supported. There were no significant differences between age groups in AMA, PCSA,

Table 2

Randomized three-factor repeated-measures ANOVA results for age, joint and stride phase fixed effects on four response variables from *Basiliscus vittatus* hindlimb muscle dissections. *P*-values in bold are significant after correction for multiple comparisons.

Adjusted <i>R</i> ²	Effect	df	AMA 0.6917			PCSA 0.0790			Fiber length 0.5050			Fiber width 0.1383		
			MS	<i>F</i>	<i>P</i>	MS	<i>F</i>	<i>P</i>	MS	<i>F</i>	<i>P</i>	MS	<i>F</i>	<i>P</i>
	Age	1	2E–04	0.2	0.6443	2E–09	3.1	0.1839	1025.0	7.3	0.0766	1.5E–02	2.8	0.1890
	Joint	2	13.9	370.8	<0.0001	2E–10	1.8	0.1695	1213.2	39.1	<0.0001	1.9E–03	6.4	0.0014
	Phase	1	0.5	13.1	0.0003	5E–10	5.0	0.0239	346.3	11.2	<0.0001	6.9E–07	0.0	0.9617
	Age × joint	2	6E–03	0.2	0.8452	5E–11	0.4	0.6803	137.0	4.4	0.0133	8.8E–06	0.0	0.9704
	Age × phase	1	2E–03	0.0	0.8324	2E–10	1.5	0.2261	2.0	0.1	0.7990	2.2E–04	0.7	0.3866
	Joint × phase	2	0.2	4.7	0.0082	2E–11	0.2	0.8481	532.1	17.2	<0.0001	8.6E–04	2.9	0.0593
	A × J × P	2	8E–03	0.2	0.8129	5E–14	1.0	0.9994	32.0	1.0	0.3562	2.7E–04	0.9	0.3975
	Residual	^a	4E–02			1E–10			31.0			3.0E–04		

^a Residual degrees of freedom are 341, 132, 132, and 132, respectively, for each analysis.

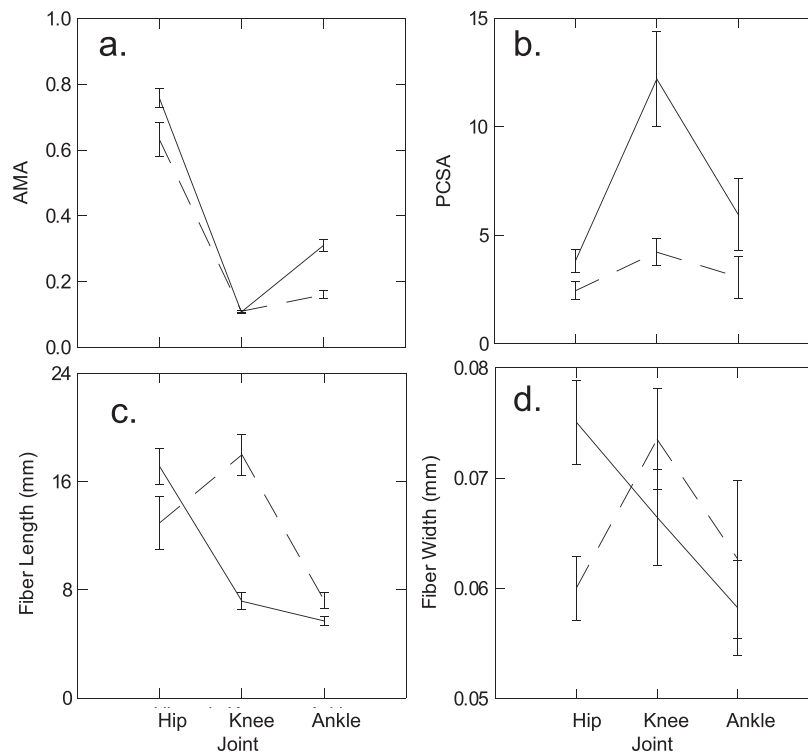


Fig. 2. (a) AMA, (b) PCSA (×10⁻⁶), (c) fiber length, and (d) fiber width for muscles that move the hip, knee, and ankle during stance (solid line) and swing (dashed line) phases of the stride in the hindlimb of *Basiliscus vittatus*. Mean values with error bars indicating standard errors of the means.

Table 3

Pearson correlations among pairs of continuous muscle variables for *Basiliscus vittatus* hindlimbs. None of the correlations are significant after correction for multiple comparisons.

X	Y	<i>R</i>	<i>P</i>
AMA	PCSA	–0.152	0.0646
AMA	Fiber width	0.163	0.0480
AMA	Fiber length	0.157	0.0563
AMA	Muscle length	–0.079	0.3528
PCSA	Fiber width	0.009	0.9095

or fiber width (Table 2). A significant interaction between age group and joint for fiber length (Table 2) indicated that muscles serving distal joints had shorter fibers, and that this pattern was less pronounced in juveniles (graph not shown).

We found no significant relationships after correction for multiple comparisons between AMA and PCSA, fiber width, fiber length, or muscle length (Table 3). In all cases, very little variation in one variable was explained by the variation in the other.

4. Discussion

4.1. Do muscle and lever properties differ among joints and stride phases?

We expected to find that muscles and their lever systems associated with more distal joints and the swing phase in the hind limb would be more velocity-optimized, while more proximal ones and those involved in the stance phase would be more force-optimized. A force-optimized muscle would have a higher AMA and PCSA, as well as shorter, thicker fibers (Carrier et al., 1998; Azizi and Brainerd, 2007; Li et al., 2012). We found some support for our predictions, particularly with AMA across joints, and PCSA between stance and swing (Fig. 2), but some of our other predictions were not well supported.

PCSA, which is a good predictor of force production capacity of a muscle (Gans and De Vree, 1987; Biewener, 2003), showed the expected pattern particularly well. Stride frequency changes are the primary mechanism of steady-state locomotor velocity

modulation in lizards (Irschick et al., 2003; Reilly et al., 2007), and so selection for high stride frequency will act on traits that increase the speed with which the limb is reset. However, as stride frequency increases, the duration of stance decreases, resulting in a need for stance phase muscles to generate more power (Irschick et al., 2003). These traits have been found to be more closely related to muscle characteristics than skeletal morphology (Vanhooydonck et al., 2002), and this is consistent with our results.

A major exception to our expectations was the knee, whose muscles exhibited low AMAs and high PCSAs (Fig. 2). Others have noted that the knee is of particular importance in lizard locomotion. In *Callisaurus draconoides*, a small cursorial lizard, knee extensor muscles do most of the work during running (Li et al., 2012). In *Anolis* lizards, knee extensor muscle mass is the main predictor of acceleration, an aspect of locomotor performance that is particularly force-dependent (Vanhooydonck et al., 2006; Higham et al., 2011). During water running in another basilisk species (*Basiliscus plumifrons*) the knee moves through a wider range of angles during the stride cycle than any of the other joints of the hindlimb (Hsieh, 2003), suggesting that it must be able to move quickly during this behavior. The knee also has a higher mediolateral excursion than the ankle, and so may make a considerable contribution to the large mediolateral forces generated during water running (Hsieh, 2003; Hsieh and Lauder, 2004). However, some of the distinct characteristics of the knee may be basilisk-specific, as the metatarsophalangeal joint (which we did not look at in the current study) is an important predictor of unsteady performance in *Sceloporus woodi* (McElroy et al., 2012). There is also evidence that the knee is used differently during water running than terrestrial running in basilisks because knee flexion does not continue at the beginning of stance (Snyder, 1949; Hsieh, 2003). Our findings suggest that the knee, with its low muscle AMAs and high PCSAs, can move both quickly and powerfully to enable high locomotor performance. This may also be evidence of different phenotypic traits being selected for different aspects of performance, possibly ameliorating some functional trade-off in locomotion (e.g., Alfaro et al., 2005; Holzman et al., 2011b).

Finally, some of our predictions may be affected by factors that we did not examine, such as tendons that store elastic energy and muscle fiber types. Tendons associated with more distal limb joints tend to have more capacity for the elastic storage of energy (Carrier et al., 1998; Li et al., 2012). Elastic enhancers can decrease the force–velocity trade-off of a lever system because velocity becomes independent of MA (McHenry and Lauder, 2006). Also, fast glycolytic fibers generate more force than other fiber types, and their diameter correlates with acceleration in *S. woodi* (Higham et al., 2011). However, not accounting for fiber types may not bias our results because fiber type proportions do not differ substantially among hip and knee flexors and extensors in the desert iguana, *Dipsosaurus dorsalis* (Putnam et al., 1980).

4.2. The multi-functionality and context dependency of muscles

A key finding in the study of locomotor muscles is that a single muscle can have multiple functions. Biarticular muscles can move and transfer mechanical energy between the joints they span (Prilutsky et al., 1996; Huijing, 1998; Kaya et al., 2005). Some muscles contract multiple times during the stride cycle, suggesting that they play multiple roles (Gatesy, 1997; Higham and Jayne, 2004; Foster and Higham, 2014). For example, the m. ambiens pars dorsalis in the lizard *Anolis carolinensis* contracts primarily at the beginning of stance, but also has a smaller contraction during the swing phase (Foster and Higham, 2014). Muscle activation patterns can also change with context, such as incline or behavior (Foster and Higham, 2014).

We accounted for the multi-functionality of certain muscles by analyzing them based on both of their functions. However, doing this indiscriminately could also bias results, as some of these muscles have a primary locomotor function and a secondary joint-stabilization function. For example, the m. ambiens pars dorsalis primarily extends the knee during stance and likely stabilizes the same joint during swing (Foster and Higham, 2014). Although electromyography (EMG) is the definitive approach to determining muscle function, existing EMG work (Gatesy, 1997; Reilly, 1998; Higham and Jayne, 2004; Foster and Higham, 2014) closely matches primary muscle functions that are inferred from morphology during lizard running (Herrel et al., 2008; Russell and Bauer, 2008; this study), while secondary functions are not.

However, muscle function is also context dependent. The recent work of Foster and Higham (2014) indicates that muscle activation can change with substrate incline and, to a lesser degree, perch diameter, and this further clouds muscle function inferences based on morphology alone. Basilisks are excellent at running on land, on water, climbing up trees, jumping, and swimming (Snyder, 1949; Hsieh, 2003; P.J.B., pers. obs.), and it is likely that the same hindlimb muscles are used differently in these different modes of locomotion. For example, during terrestrial running, lizards further flex their already flexed knee at the beginning of stance prior to extension (Irschick and Jayne, 1998, 1999), but this additional flexion does not happen when basilisks run on water (Hsieh, 2003). Our classifications of muscle function seem to be consistent with the kinematics of terrestrial and aquatic running (Snyder, 1949; Hsieh, 2003), but may not be appropriate for other behaviors. A detailed kinematic and EMG study would be necessary to resolve the issue of how muscle function changes for these different behaviors.

4.3. Do muscle and lever properties change with the size of the animal?

Since the force that muscles can generate is proportional to PCSA, which itself is proportional to the square of body length, and since body mass scales to the cube of body length, force production increases more slowly than mass as an animal increases in size, leading to an expectation of force limitation (Biewener, 1989, 2003). Performance differences between juveniles and adults are variable, with juveniles of many ectotherms performing at a higher level relative to body mass than adults (Herrel and Gibb, 2006), but with juveniles of endotherms frequently being clumsy and having weaker muscles than adults (Carrier, 1996). In basilisks, adults can potentially generate about half of the relative force of juveniles (Glasheen and McMahon, 1996) and are at a disadvantage when running on water on account of having a higher mass, relatively smaller feet, and sinking deeper in the water than juveniles (Rand and Marx, 1967; Hsieh, 2003).

We expected adults to compensate for the relative handicap of size by having lever and muscle properties that would allow them to generate more force than juveniles. A similar form of compensation is seen interspecifically in mammals, where larger species adopt more erect postures, which result in higher EMAs (Biewener, 1989; Reilly et al., 2007). Intraspecifically, findings for mammals are contradictory. Macaque monkeys exhibit positive allometry of AMA for the elbow (Fellmann, 2012), matching interspecific findings. In contrast, the ankles of jackrabbits and elbows of capuchin monkeys exhibit negative allometry of AMA, suggesting that juveniles compensate for low muscle strength with high mechanical advantage (Carrier, 1983, 1995; Young, 2005, 2009). We found little evidence of differences between age/size groups in our sample.

4.4. Anatomical mechanical advantage for studying function

Over the years, various studies have used both AMA (Dodson, 1975; Carrier, 1983; Young, 2005, 2009; Fellmann, 2012) and EMA (Biewener, 1989, 1990; Carrier et al., 1998; Gregersen and Carrier, 2004) to study limb mechanics. Although AMA is static, and an estimate of EMA (Young, 2009; Fellmann, 2012), we argue that it has a place in the study of limb mechanics. Here we used AMA to study how a large number of muscles and their lever systems differ across joints and stride phases, obtaining meaningful results. Using this approach has several advantages. AMA can be quantified using preserved specimens, and this provides an important opportunity for comparative studies of the evolution of lever systems because sampling many species is often easier with preserved than living specimens. AMA is also more easily quantified on a muscle-specific basis, so can provide more information than a joint-specific approach. The study of biological lever systems more broadly provides for a formal model of studying force–velocity trade-offs, which likely constrain the evolution of functional morphology.

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