Different Segments within Vertebrate Muscles Can Operate on Different Regions of Their Force–Length Relationships

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DIFFERENT SEGMENTS WITHIN VERTEBRATE MUSCLES CAN OPERATE ON
DIFFERENT REGIONS OF THEIR FORCE-LENGTH RELATIONSHIPS

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ABSTRACT

To relate in vivo behavior of fascicle segments within a muscle to their in vitro force-length relationships, we examined the strain behavior of paired segments within each of three vertebrate muscles. After determining in vivo muscle activity patterns and length changes of in-series segments within the semimembranosus muscle in the American Toad (Bufo americanus) during hopping and within the sternohyoid muscle in the rat (Rattus rattus) during swallowing, and of spatially separated fascicles within the medial gastrocnemius muscle in the rat during trotting, we measured their corresponding in vitro (toad) or in situ (rat) force-length relationships. For all three muscles, in vivo strain heterogeneity lasted for about 36 to 57% of the behavior cycle, during which one segment or fascicle shortened while the other segment or fascicle simultaneously lengthened. In the toad semimembranosus muscle, the proximal segment shortened from the descending limb across the plateau of its force-length relationship from 1.12 to 0.91 of its optimal length ($L_0$), while the distal segment lengthened (by $0.04 \pm 0.04 L_0$) before shortening down the ascending limb from 0.94 to 0.83 $L_0$. In the rat sternohyoid muscle, the proximal segment tended to shorten on its ascending limb from 0.90 to 0.85 $L_0$ while the distal segment tended to lengthen across $L_0$ (0.96 to 1.12 $L_0$). In the rat medial gastrocnemius muscle, in vivo strains of proximal fascicles ranged from 0.72 to 1.02 $L_0$, while the distal fascicles ranged from 0.88 to 1.11 $L_0$. Even though the timing of muscle activation patterns were similar between segments, the heterogeneous strain patterns of fascicle segments measured in vivo coincided with different operating ranges across their force-length relationships simultaneously, implying differences in force-velocity behavior as well. The three vertebrate skeletal muscles represent a diversity of fiber architectures and functions and suggest that patterns of in vivo contractile strain and the operating range over the force-length relationship in one muscle region does not necessarily represent other regions within the same muscle.
INTRODUCTION

The force-length relationship (FLR) remains one of the most fundamental and best studied properties of striated muscle (Blix, 1895; Gordon et al., 1966; Fig. 1). A muscle fiber’s optimal sarcomere length represents the length at which the muscle (fiber) generates maximum isometric force when tetanically stimulated (Gordon et al., 1966). At shorter lengths, stretch of a muscle fiber to longer length on the ascending limb leads to increased force (Ramsey & Street, 1940). Whereas, active isometric force decreases at lengths greater than the optimal length on the descending limb (Ramsey & Street, 1940; Altringham & Bottinelli, 1985; Granzier & Pollack, 1990; Allinger et al., 1996).

The range of lengths at which muscles operate in vivo has been estimated to span different regions along the FLR, including both ascending and descending limbs (for review, see Burkholder & Lieber, 2001). Historically, most muscles are thought to operate on the ascending limb of the FLR (e.g., cat triceps surae, Herzog et al., 1992; human triceps surae, Maganaris, 2001; roach leg extensors, Ahn et al., 2006; frog anconeus, Azizi, 2014) or on the plateau region where maximal force can be generated by the muscle (e.g. frog semimembranosus, Lutz & Rome, 1994). However, the operating ranges of some muscles extend onto the descending limb of the FLR (Lieber & Boakes, 1988; Azizi & Roberts, 2010; Gidmark et al., 2013), where active isometric force decreases at longer lengths (Altringham & Bottinelli, 1985; Allinger et al., 1996; Morgan, 1990; Rassier et al., 2003). Consequently, the in vivo operating range of skeletal muscles can vary over their force-length relationship (Burkholder & Lieber, 2001).

A simple view of muscle mechanics might be to scale a single sarcomere and its inherent properties up to a whole muscle. However, this ignores architectural features of whole muscles, and the sarcomeres of isolated fibers are sometimes of non-uniform, or “inhomogenous,” lengths along the fiber (Huxley & Peachey, 1961; for review, see Telley & Denoth, 2007). These non-uniform sarcomere lengths sometimes consist of central sarcomeres that shorten or strain less
how operating length, velocity, and activation affect muscle force generation (Morgan, 1985; Telley & Denoth, 2007).

Segments of intact, whole muscles also strain non-uniformly, where in-series or in-parallel segments exhibit differential strain. Non-uniformity among sarcomere lengths occurs in the passive tibialis anterior muscle of rats due to epimyscular myofascial force transmission (Tijs et al., 2015). Additionally, sarcomere length non-uniformity increases with activation of an intact whole muscle (Moo et al., 2017). Non-uniform strain also occurs in vivo in the human biceps brachii muscle during elbow flexion, with the distal region shortening less (3.7%) than the midportion (28.2%; Pappas et al., 2002). By contrast, in-series segments of proximal regions of the pigeon pectoralis muscle shorten by 2 to 17% less than distal regions during flight (Soman et al., 2005). Pennate muscles also show in-parallel non-uniformity between fascicles. Proximal fascicles of the medial gastrocnemius muscle in guinea fowl produce 4-times more positive work during running than in-parallel distal fascicles due to differences in strain magnitude (Higham et al., 2008). Proximal fascicles in the pennate lateral gastrocnemius muscle of turkeys shorten by ~30% less than in-parallel proximal fascicles during isometric contractions (Azizi & Deslauriers, 2014). Thus, both in-series and in-parallel segments within different vertebrate muscles can shorten non-uniformly, but not necessarily in predictable ways.

Different from non-uniform single fiber strain, heterogeneous strain occurs when one segment along a muscle fascicle shortens while its adjacent segment simultaneously lengthens (Ahn et al., 2003; Konow et al., 2010). In isometric contractions of single fibers, central sarcomeres shorten while end sarcomeres lengthen (Edman & Reggiani, 1984; Mutungi & Ranatunga, 2000; for review, see Telley & Denoth, 2007). Alternatively, central sarcomeres can lengthen while end sarcomeres shorten during fixed-end, “isometric” contractions of single fibers (Huxley & Peachey, 1961), especially at very long fiber lengths (Altringham & Bottenelli, 1985; Granzier & Pollack, 1990). In the intact semimembranosus muscle (SM) of a hopping toad, the
during feeding, such that the mid-belly (or central) segment usually shortens, while the end segments simultaneously lengthens (Konow et al., 2010).

In the present comparative study, we test the null hypothesis that muscle segments along the length of a single fascicle, as well as spatially separated fascicles, operate on the same region of their FLR, as commonly assumed when only one segment or region of a muscle is examined. Alternatively, different muscle segments can operate on different regions of their FLRs because they strain heterogeneously in vivo (Fig. 1). Assuming muscle segments contract to initiate motor behaviors at similar lengths with respect to their FLR, the proximal segment (or mid-belly) of the muscle may shorten across its plateau (Lutz & Rome, 1994; Azizi et al., 2010), while the end segments may actively lengthen to longer lengths with respect to their FLRs (Ahn et al., 2003; Konow et al., 2010; Fig. 1).

METHODS

Animals

All animals were housed at the animal care facility of the Concord Field Station in Bedford, Massachusetts. Adult American toads (Bufo americanus) were collected in and near Bedford, MA. Adult toads (n = 7; mass = 38.7 ± 16.8g; mean ± S.D.) were fed crickets dusted with calcium and vitamin powder and had free access to water. Adult male rats (Sprague Dawley strain) were purchased from Charles River and housed on commercially available rat chow and free access to water [n = 3; mass = 310.0 ± 20.0g for rat swallowing and n = 4; mass = 276 ± 66g for rat trotting]. The animal rooms were maintained at 20-22° C with a 12h:12h photoperiod. All experiments were performed at room temperature (22°C) and in accordance to with the guidelines of the FAS Institutional Animal Care and Use Committee of Harvard University.

The three skeletal muscles vary in anatomy and architecture (Fig. 2). The toad semimembranosus muscle (SM) is biarticular and spans hip and knee joints (Fig. 2A). However,
some fibers do not extend along the entire length of the muscle, extreme care was taken to
implant crystals and electrodes along fascicles that span the muscle from origin to insertion (Ahn
et al., 2003). By contrast, the rat sternohyoid (SH) is the main infrahyoid muscle and responsible
for hyoid retraction during swallowing and other intraoral behaviors in feeding and phonation
(Fig. 2B). The parallel-fibered SH runs from the deep aspect of the second rib to the caudal
aspect of the hyoid bone. An inscription delineates the neuroanatomical boundary between a
rostral or “distal” segment (innervated by XII) and a caudal or “proximal” segment (innervated
by XII and C1-3; Müntener et al., 1980). Finally, the rat medial gastrocnemius muscle (MG) is a
knee flexor and ankle extensor with a unipennate fiber architecture spanning from a proximal-
superficial to a distal-deep aponeurosis (Fig. 2C). The proximal fibers are shorter, rest at a more
obtuse angle, and have a higher oxidative capacity than the distal fibers (De Ruiter et al., 1995b;
Furrer et al., 2013).

In vivo recordings

Surgical Procedures

Toads and rats were anesthetized for surgery using standard methods for amphibians and
rats, respectively. American Toads were anesthetized by immersion in a buffered tricaine
methanesulfonate solution (MS-222; 1g l⁻¹) in order to implant sonomicrometry crystals
(Sonometrics Corp.) and fine-wire electromyography (EMG) electrodes into the
semimembranosus muscle (SM). Rats were anesthetized using isoflurane administered to effect
via a mask (4% induction, 1-2% maintenance). During surgery, rats were placed on a heating pad
to prevent hypothermia. Under anesthesia, an incision was made through the skin of the toad
dorsal upper leg to reveal the SM of the toads, or the ventro-lateral neck to expose the SH of the
rats, or the medial aspect of the hindlimb to expose the MG of the rats.

After exposing the muscle, sharp forceps were used to create three small pockets along
The output of either sonomicrometer unit was viewed during surgery on an oscilloscope (Tektronix 2235A 100Mhz) to ensure maximum signal strength when implanting and aligning the crystals. Closure of the pocket and anchoring of the lead wires prevented lead wire movement that might alter the orientation of the crystals embedded in the muscle. For the toad SM (Fig. 2A), the middle crystal emitted ultrasonic pulses that were received by the two adjacent (proximal and distal) crystals, allowing for the measurement of instantaneous muscle lengths from two adjacent in series portions of the fascicle (for details, see Ahn et al., 2003). For the rat SH and MG muscles (Fig. 2B, 2C), distance measurements were acquired from omnidirectionally operating crystals. Two pairs of bipolar electrodes (offset twist hook; Loeb & Gans, 1985) were constructed from insulated silver wire (0.1 mm diameter; California Fine Wire Co.) to record EMG activity from the respective muscle segments along the fascicle. In the rats, a monopolar ground electrode (AM-Systems) was also implanted. Skin incisions were then closed with 4-0 Vicryl suture.

Sonomicrometry recordings of muscle segment length change are based on measurements of the transit time of a 5 MHz ultrasonic pulse that travels from an emitting to a receiving piezoelectric crystal (for details, see Griffiths, 1987; Ahn et al., 2003). Both sonomicrometers (model 120-1001; Triton Technology, Inc., San Diego, CA, USA) used for toad SM experiments and for rat MG and SH experiments (Sonometrics TRX, London, Ontario, Canada) convert ultrasonic signals to a voltage signal calibrated to measurements of length. All negative strains represent shortening, while all positive strains represent lengthening of the in-series or in-parallel muscle segments.

For toads, the sonomicrometry and EMG wires were braided together external to the animal to avoid electrical crosstalk. The braided wires were sutured (4-0 silk) to the animal’s back to prevent the animal from tangling or pulling the wires. For rats, sonomicrometry and EMG lead wires from the MG or SH muscles were passed subcutaneously to a miniature
In vivo data collection

Toad semimembranosus

While hopping on a level treadmill (21 x 60 cm working space), proximal and distal segment lengths of the toad SM were recorded from 7 toads (84 hops total) (Fig. 2A). Simultaneously, EMG signals from 5 of the 7 animals (63 hops) were amplified 1000x at a bandwidth of 100 to 3000 Hz (Grass P511 series A.C. pre-amplifiers). Voltage changes representing muscle length changes and EMG signals were acquired (AxoScope 8.0) at 4 kHz on a PC computer. In vivo strain ranges were determined during series of consistently strong jumps.

Rat sternohyoid

The rat SH muscle is important for food bolus transport and swallowing. Data on other feeding behaviors, such as chewing and suckling, were recorded but not presented here. While subjects were engaged in feeding on a variety of foods, we recorded sonomicrometry and EMG data from the rostral (superior or “distal”) and caudal (inferior or “proximal”) segments of the sternohyoid muscle (Fig. 2B). EMG signals were acquired via DAM50 amplifiers (World Precision Instruments) and amplified 1000x with a band-pass of 3Hz-10kHz. Voltage differentials were acquired at 10kHz via a PowerLab 8/30 A/D converter to the disc of a PC running LabChart v.7.0 (AD instruments).

Rat medial gastrocnemius

Rats were trained to trot on a level treadmill (21 x 60 cm working space), during which MG fascicle length changes and EMG were collected from separate proximal and distal regions (Fig. 2C). EMG signals and voltage differentials were acquired as for the rat SH.

In vivo strain heterogeneity
during which the two segments strained in opposing directions was multiplied by 100 to determine %Heterogeneity. Additionally, we calculated %Heterogeneity during the period while both muscle segments were activated to allow a more direct comparison of locomotory and feeding muscles.

In vitro or in situ force-length relationships

Toad SM. All toads used for in vivo recordings during hopping were also used for in vitro preparations to determine the FLR of two in-series muscle segments. After a short rest to allow for recovery from hopping on the treadmill, toads were double-pithed to avoid effects of anesthesia on the neuromuscular system. In vivo muscle length was measured with the hip and knee joints set to 90°, which approximates the midpoint of the joints’ ranges of motion. The toad SM was carefully dissected without damage to the aponeuroses and tendinous insertions into the hip and knee joints. The proximal end of the musculo-tendon unit originates on the ischium, which was held fixed in the preparation by a surgical clip. The surgical clip was tied securely with a 2-0 multifilament polyamide (nylon) suture to a pin fixed within a 2.7 x 11.5 x 2.2 cm well that was machined in a 10 x 19 x 5 cm block of Plexiglas. The distal end of the muscle-tendon unit inserts onto the proximal tibia just below the knee. The proximal portion of the tibia to which the muscle inserts was clamped with a surgical clip. After the muscle was carefully aligned and positioned so that no twisting occurred during contractions, the surgical clip was then tied with the 2-0 nylon suture securely onto the lever arm of a servomotor (Aurora Scientific, Inc.; model 305B). The servo motor lever was positioned to be aligned with the pin, which anchored the proximal end of the muscle-tendon unit. The servomotor was bolted onto a linear manipulator (Velmex, Inc., Bloomfield, NY, USA; A25 series), which adjusted the length of the muscle to its resting length as determined in vivo. Oxygenated amphibian Ringer's solution (Carolina Biological Supply, Burlington, NC, USA) was maintained at 22° C and
whole muscle-tendon. Muscle-tendon length was monitored using a linear manipulator using a modified computer program written by RK Josephson & JG Malamud (Labview; National Instruments) that controlled whole muscle length and nerve stimulation parameters, while acquiring muscle force and whole muscle length data from the servo motor, as well as segment length signals from the sonomicrometry crystals (Ahn *et al.*, 2003).

The SM muscle was stimulated through the sciatic nerve with a suction electrode using 0.5 ms square-wave pulses at 200 Hz for 200 ms (supramaximal burst; Grass S48 Stimulator). A supra-threshold voltage was used for all trials. The stimulation pattern (200Hz for 200 ms) was chosen because 200 Hz exceeded the maximum *in vivo* EMG frequencies during hopping. Force, whole muscle length, segment length, and stimulation signals were acquired at 4 KHz (Labview DAQ system; NI PCI-MIO-16E-4 board).

Trials were separated by 2-3 minute intervals to minimize both potentiation and fatigue. The order of stimulation levels and muscle lengths was varied randomly. Trials were stopped when the amplitude of force generated by an isometric contraction declined by more than 10% of its original force. Passive measurements were obtained after the active properties recorded during the stimulation trials were completed. After removal of the SM muscle from the *in vitro* muscle bath, all non-muscle tissue was dissected away (e.g., nerve, tendon, bone) and the muscle weighed with an electronic balance (Sartorius ISO 9002).

**Rat SH.** All rats used for *in vivo* swallowing recordings were also used for measurements of *in situ* FLR for the proximal and distal in-series segments of the SH muscle. The incision over the SH muscle was reopened, the skin was separated from underlying suprahypoid and infrahypoid musculature using blunt dissection, and all other muscles acting upon the hyoid bone were transected. The hyoid bone was carefully dissected free and severed at the midline to remove the left SH belly from the preparation. The sternum was then clamped to the stereotaxic frame using an alligator clip, and a 4-0 silk suture was tied to the hyoid end of the muscle and to the lever.
muscle (see Popovic et al., 1991 for an evaluation of the equal efficacy of hook and nerve cuff electrodes in muscle stimulation). Care was taken to suspend and rig the stimulation electrodes from the stereotaxic rig so that they did not interfere mechanically with muscle length-changes during contractions. After determination of supramaximal voltage, delivered from a stimulator (Grass S48 West Warwick, RI; voltage range across the three preparations: 1.5-2.5V; with stimulation frequency held at 125Hz; pulse width at 200 μs, and duration at 350 ms), tetanic FLR curves for the supramaximally activated SH muscle were constructed. Isometric forces were again determined for short to long lengths (for only supramaximal stimulation). After the experiment, rats were euthanized using an overdose of intracardially-injected pentobarbital sodium.

Rat MG. All rats used for in vivo recording during trotting were also used for assessment of in situ FLR of proximal and distal fascicles of the MG muscle. The skin of the right hind limb was reopened and separated from the muscles via blunt dissection. The biceps femoris muscle was excised to expose the underlying belly of the MG and the sciatic nerve. The femur was exposed to allow attachment of a bone clamp. The distal tendons of the lateral gastrocnemius, soleus, and plantaris muscles were then cut leaving only the distal tendon of MG attached to the calcaneus. A bipolar cuff electrode was placed around the sciatic nerve and neural connections between the muscles and the central nervous system were isolated by cutting the sciatic nerve proximal to the cuff electrode. The calcaneus was cut with the MG tendon intact and attached to the lever arm of a servomotor (305B-LR; Aurora Scientific Inc., Aurora, ON, Canada) using Kevlar thread. The right hind limb was secured to the experimental set-up by clamping the femur and anchoring the foot to a plastic plate. The servomotor was then aligned with the line of action of the muscle-tendon unit. All exposed tissue was immersed in a pool of mineral oil to prevent desiccation and maintain temperature (35 °C), created by attaching and tenting up the skin with elastic bands. MG muscles were stimulated supramaximally (amplitude: 2-3V) through the
For the fixed-end contractions, the strain of the segments was always determined at the time of peak force generation at the end of the stimulation burst. Active force was determined by subtracting the passive force from the total force measured at the length reached during contraction. For each series of maximum isometric contractions on an individual muscle, 2nd or 3rd-order polynomial regressions were fit to the force-length relationship, depending on the R-value. Only R > 0.9 were accepted. The 2nd-order polynomial coefficients from the regression equations were averaged to determine an average force-length relationship for the SM muscle. The *in vivo* range of segment strains could be directly compared to the *in vitro* range of lengths in the FLR because the sonomicrometry crystals were left intact and used in both *in vivo* and *in vitro* experiments. All strains are presented relative to the optimal length \( L_0 \) as determined from the FLR of each in-series or in-parallel muscle segment.

**Statistics**

Comparisons of strain magnitudes or timing of muscle activation patterns were tested with either paired or unpaired t-tests. When comparing between two segments within a muscle, we used paired t-tests. However, unpaired t-tests were used to evaluate comparisons made between groups that consisted of data obtained from different animals. Differences were considered to be statistically significant when \( P \leq 0.05 \). All reported values represent mean ± S.D.

**RESULTS**

**Animals**

Toad SM

Although seven toads were used to compare *in vivo* and *in vitro* strain patterns (73 hops), muscle activity patterns were measured for only the first five toads (mean mass = 40.9 ± 20.0 g). For each of these five animals, 14 ± 5 hops were analyzed with the total of 63 hops. For the
Rat SH

A total of 124 swallows were analyzed to quantify rat SH muscle activity and length patterns. SH muscle masses averaged 0.62 ± 0.03 g, muscle lengths were 28.4 ± 0.53 mm, and the cross-sectional area of this fusiform muscle was 0.023 ± 0.001 cm$^2$.

Rats MG

A total of 24 strides were analyzed for regional rat MG in vivo strain patterns, which included EMG recordings for 21 strides (1 animal excluded). MG muscle mass averaged 0.703 ± 0.217 g, with an average muscle length of 27.25 ± 5.93 mm and average physiological cross-sectional area of 0.69 ± 0.14 cm$^2$.

In vivo muscle activity and strain patterns

Timing of muscle activity patterns

In the SM muscle of a hopping toad, the durations of muscle activity patterns did not differ between the proximal (51.8 ± 4.8 ms) and distal segments (56.9 ± 8.6 ms; P = 0.5; paired t-test; N = 5 animals). When compared to the beginning of shortening of the proximal segment, onset times of the muscle activity patterns did not differ between the proximal and distal segments (P = 0.6).

In the rat SH, muscle activity was variable across swallows for both the distal (176 ± 135 ms) and the proximal (178 ± 133 ms) regions. For a given swallow, the lag between regions in onset-timing (2 ± 10 ms) as well as the difference in duration (2 ± 21 ms) were small and not different for activity onset (P = 0.97) or duration (P = 0.94).

In the MG muscle of a trotting rat, the durations of muscle activity patterns did not differ between the proximal (147.2 ± 8.7 ms) and distal fascicles (138.2 ± 3.5 ms; P > 0.05; paired t-test). The onset times of the muscle activity patterns also did not differ between the proximal and
In general, the proximal segments of the SM only shortened during hopping, while the distal segments first lengthened (by $0.037 \pm 0.037 L_o$; $P < 0.05$; $N = 7$ animals) before shortening (by $-0.087 \pm 0.064 L_o$; Fig. 3A). Additionally, proximal segments always shortened (by $-0.181 \pm 0.063 L_o$) more than distal segments ($N = 7$ animals; $P < 0.05$). The total strain, from peak shortening to peak lengthening, experienced by the proximal segments (by $0.171 \pm 0.063 L_o$) always exceeded that by the distal segments (by $0.124 \pm 0.051 L_o$; $P < 0.05$; $N = 7$ animals).

Strain patterns for the rat SH during swallowing

Although the contractile strain patterns for the rat SH were variable during swallowing, the proximal segment tended to shorten (by $-0.040 \pm 0.044 L_o$), sometimes with subsequent re-lengthening (Fig. 3B). Meanwhile, the distal segment mostly lengthened whilst activated (eccentric contraction; by $0.057 \pm 0.057 L_o$; Fig. 3B). Despite the differing strain directions, there was no statistically significant difference in total strain with respect to segment region (paired t-test, $P=0.2$).

Strain patterns during trotting in the rat

During level trotting, no differences were found in net shortening between fascicles in the proximal and distal region of the rat MG (by $-0.298 \pm 0.103 L_o$ and $-0.232 \pm 0.057 L_o$, respectively; $P=0.302$; $N=4$; Fig. 3C). However, during the initial period of muscle activation at the end of swing phase, the proximal fascicles shortened more (by $-0.157 \pm 0.045 L_o$) than the distal fascicles (by $-0.022 \pm 0.014 L_o$; $P=0.038$, $N=3$).

In vivo strain heterogeneity

The two segments or fascicles within each muscle all exhibited opposing strains in vivo during toad hopping, rat swallowing, and rat trotting (Figs. 3 and 4). Adjacent segments of the
vertebrate muscles, in-series or in-parallel segments strained heterogeneously during hopping, swallowing, or trotting (Fig. 4).

*Fixed-End Contractions in Relation to In Vivo Strains*

*Muscle stress*

During maximally stimulated “isometric,” or fixed-end contractions at rest length, the toad SM generated 48.8± 11.8 N cm⁻² (N=7), the rat SH generated 35.0 ± 2.0 N cm⁻² (N=3), and the rat MG generated 13.0 ± 5.8 N cm⁻² (N=4).

*Toad SM strains*

In the toad SM, both proximal and distal segments always shortened non-uniformly during *in vitro* “isometric,” fixed-end contractions used to determine the muscle’s FLR. Even though the muscle-tendon unit was held isometric, the proximal muscle segment (by -0.173 ± 0.035 $L_0$) shortened more than the distal segment (by -0.011 ± 0.15 $L_0$; N=7; P<0.05).

For the toad SM, the *in vivo* strain range of the proximal segment spanned the middle of the *in vitro* FLR while the distal segment spanned only the region of shorter lengths of its FLR (Figs. 5A and S1A). The proximal segment strain ranged from 0.912 ± 0.080 $L_0$ to 1.12 ± 0.068 $L_0$ during hopping; whereas, the distal segment strain ranged from 0.827 ± 0.094 $L_0$ to 0.944 ± 0.076 $L_0$ during hopping. The proximal segment also operated over greater minimum and maximum lengths compared with the distal segment (P < 0.05; paired t-tests). During hopping, the magnitude of strain across the muscle’s FLR was 80% greater for the proximal segment compared with the distal segment.

*Rat SH strains*

During *in situ*, fixed-end contractions to measure the muscle’s FLR both rat SH segments
than the distal segment (0.96±0.02 \( L_0 \); \( P<0.05 \)). Moreover, the proximal segment tended to shorten to lengths below \( L_0 \) (0.85±0.01 \( L_0 \)), while the distal segment tended to lengthen across \( L_0 \) (1.12±0.12 \( L_0 \)). Thus, the operating length ranges reached by the two segments at the end of swallowing also differed (\( P<0.0001 \)).

**Rat MG strains**

No differences were observed in fascicle strains between proximal (-0.286 ± 0.105) and distal (-0.229 ± 0.084) regions of the rat MG during “isometric” contractions that were recorded to determine the muscle’s FLR (\( P=0.378 \); Figs. 5C and S1C).

*In vivo* locomotor strains of proximal rat MG fascicles ranged from 0.720 ± 0.061 \( L_0 \) to 1.018 ± 0.122 \( L_0 \), while distal fascicles ranged from 0.876 ± 0.037 \( L_0 \) to 1.108 ± 0.084 \( L_0 \) (Fig. 5C). Average strain in the proximal (0.856± 0.123 \( L_0 \)) and distal (1.002 ± 0.053 \( L_0 \)) regions did not differ between regions (\( P=0.073 \)). Maximum strain also did not differ between regions (\( P=0.216 \)). However, minimum strain in proximal fascicles was lower (\( P=0.031 \)) than in distal fascicles.

**DISCUSSION**

During hopping, swallowing, and trotting, adjacent segments (toad SM and rat SH) or different regions (rat MG) within a muscle strain heterogeneously *in vivo* and can operate on different regions of their force-length relationships (FLRs) (Figs. 3 and 5). These findings reject the null hypothesis that in-series or in-parallel segments within a muscle operate similarly on their FLRs. In the toad SM during hopping, the central (blue in Fig. 3) segment shortens from the descending limb to the plateau region of the muscle’s FLR, while the adjacent distal (red) segment lengthens and shortens across the ascending limb of the FLR. In the rat SH during swallowing, the proximal (blue) segment shortens on the ascending limb while the adjacent
5). During locomotion or feeding, therefore, the pairs of muscle segments operated on different regions of their FLRs. For these three muscles, *in vivo* segment shortening *versus* lengthening and operating length range with respect each muscle’s respective FLR also did not show a predictable pattern.

In addition to variable active operating lengths, in-series and in-parallel muscle fascicle segments may initiate contraction at non-uniform lengths (Huxley & Peachey, 1961; Moo et al., 2016; Figs 3 and S1). Before a hop, proximal central segments of the toad SM shorten from longer lengths on the descending limb, while distal segments contract from much shorter lengths on the ascending limb of the muscle’s FLR (Fig. 3A). In the rat SH, the distal segment tends to be held passively at longer lengths between swallows compared to the proximal segment (Fig. 3B). Thus, these muscle segments not only operate actively, but are also held passively on different regions of their FLRs.

Our studies compare three muscles from vertebrate locomotor and feeding systems, used for a range of routine motor behaviors that include weight-support, propulsion, and food transport. The three muscles range in fiber architecture from simple a parallel-fibered biarticular muscle (SM) to a parallel-fibered strap-like muscle (SH) that retracts the jointless hyoid, to a biarticular pennate muscle (MG). The in-series and in-parallel muscle segments studied here operate broadly across the three regions of their respective FLRs (Figs. 5 and S1).

Strains measured in these three muscles tend to be smaller in magnitude and span narrower ranges on their FLRs compared to other muscles used for jumping. Most frog hindlimb muscles operate at longer lengths across the plateau or descending limb of their FLRs (averaging 1.02 to 1.36 $L_o$; Lutz & Rome, 1994; Lieber & Brown, 1992; Azizi *et al.*, 2010). However, the anconeus muscle of the anuran forelimb, which actively lengthens to absorb energy at the end of the jump, operates on the ascending limb of its FLR (0.81 to 0.96 $L_o$; Azizi, 2014).

Unlike to the narrower *in vivo* operating range spanned by anuran muscles, sarcomere
on ankle position, the variation in sarcomere lengths in stimulated mouse TA muscle corresponded to 10-60% of the sarcomeres operating on the ascending limb, 10-30% on the plateau, and 20-60% on the descending limb of the muscle’s theoretical FLR (Moo et al., 2017). Like the nonuniform sarcomere lengths along the TA muscle that suggest sarcomeres of a single fiber may operate on all three regions of their theoretical FLR simultaneously, our comparative study shows similar in vivo strain ranges across the three regions of each muscle’s FLR.

Heterogeneous patterns of in vivo strain indicate that not only can in-series and in-parallel segments operate on different regions of their FLRs, but these segments can operate on significantly different regions of their force-velocity relationships (FVRs). Although we did not directly measure FVRs of these muscles, simultaneous shortening and lengthening between adjacent in-series or in-parallel segments require that these segments operate on different regions of their FVR. Differential FL and FV behaviors of muscles might arise from differential expression of myosin and titin isoforms along the length of single fiber in a parallel-fibered muscle, as well as compartmentalization of pennate-fibered muscle (Edman et al., 1988; Lutz & Lieber, 2000; Wilkins et al., 2001; Rosser & Bandman, 2003; Cornachione et al., 2015; Cutler et al., 2018). Given that different fascicle segments within a single muscle, depending on where in vivo strain measurements are made, can simultaneously operate on different regions of their FLR and FVR, sarcomere behavior cannot be reliably scaled up to a whole muscle level to understand muscle mechanics (Morgan, 1985; Winters et al., 2011)

The organization of fibers to generate a muscle, or its architecture, may introduce compliance in series and parallel elastic elements that results in strain heterogeneity of the muscle segments (Zuurbier & Huijing, 1993; Kawakami & Lieber, 2000; Ahn et al., 2003; Higham et al., 2008). However, the patterns of strain heterogeneity in single fibers (Edman & Reggiani, 1984; Mutungi & Ranatunga, 2000) match our measurements of in-series and in-parallel heterogeneous strains in intact muscles during locomotion and feeding (Fig. 3). The
Unlike the toad SM, the two rat muscles may both have neuroanatomical differences between their in-series or in-parallel segments that could permit the central nervous system to recruit different fascicle segments or regions of a muscle. The proximal segment of the SH muscle belly receives efferent fibers from C1-3 in addition to branches from XII, which also innervates the distal segment (Müntener et al., 1980). There is also evidence of distinct sciatic motor efferents supplying the distal versus proximal and mid-belly compartments of the rat MG (Rijkelijkuizen et al., 2003; de Ruiter et al., 1995a,b; Prodanov et al., 2005; Taborowska et al., 2016). Differing fiber-type compositions also exist within these compartments of the rat MG, with the proximal compartment having a higher oxidative capacity than that of the distal compartment (de Ruiter et al., 1995b; Furrer et al., 2013). Additionally, within the proximal MG compartment, fibers are shorter (Holewijn et al. 1984) and pennation angle is greater (proximal: 21 ± 7°; distal: 15 ± 4° measured at resting length, mean ± S.D., N= 4). Differences in pennation angle have been found to increase at shorter muscle lengths (Zuurberi & Huijing, 1993).

The idea that muscle bellies can contain functionally distinct regions with different contractile patterns due to different fiber type composition, fiber types with different origin-insertion architecture, or differences in innervation has so far received support from several studies. Using wavelet decomposition of EMG recorded from the proximal and distal compartments of the rat medial gastrocnemius, Wakeling & Syme (2002) found regional differences in how the nervous system controls fiber recruitment. Similarly, using measurements of muscle power from the same muscle in the Guinea fowl, Higham et al. (2008) calculated a 4-fold higher production of power by the proximal than the distal compartment.

Nevertheless, as our results demonstrate, the same timing of in vivo muscle activation patterns can result in pronounced strain heterogeneity with a muscle segments shortening on the plateau of its FLR, while the in-series or in-parallel segment lengthens on the ascending limb of its FLR or vice versa. Although differential activation patterns in some cases may produce
the muscle-tendon unit does not necessarily allow one to predict the dynamics of \textit{in vivo} force generation (Ahn \textit{et al.}, 2003; Ahn \textit{et al.}, 2006).

The contractile units, or sarcomeres, of vertebrate skeletal muscles are hierarchically arranged in fibers and fascicles, in series and in parallel. Although vertebrate skeletal muscles share conservative contractile properties and may not seem diverse, variation in how architectural features are organized within a muscle can result in the diverse contractile patterns in muscles. Our findings suggest that strain heterogeneity within muscle provides a mechanism that influences muscle performance to the demands of movement. At different hierarchical levels, fibers and fascicles can be organized differently across muscles and can exhibit different \textit{FL} and \textit{FV} contractile behavior, even when activated simultaneously. Modelling a muscle as a “scaled up” sarcomere with homogenous mechanical properties, therefore, is not an appropriate tool for predicting whole muscle performance. Rather, the determinants of mechanical output of a whole muscle include its activation pattern and time-varying strain patterns in the context of regional force-length and force-velocity behavior as well as history-dependent effects (Edman, 1975; Herzog, 1998; Josephson, 1999; Nishikawa \textit{et al.}, 2016). Arguably, continued work at multiple levels of organization is needed to to understand how passive and active properties of in-series and in-parallel elements within a whole muscle are integrated to generate the diverse mechanical outputs of vertebrate skeletal muscle.

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FIGURE LEGENDS

Figure 1. An idealized force-length relationship (FLR). We test the null hypothesis that all in-series and in-parallel segments shorten similarly across the plateau region (hatched gray bar) of their FLRs (blue arrow). Alternatively, we hypothesized that different segments can operate on different regions of their FLR because they strain heterogeneously in vivo. For example, the proximal segment might shorten across the plateau of its FLR (blue arrow), while the distal segment lengthens from the same rest length, then shortens (red arrow).

Figure 2. Diagrams of the three muscles with placement of sonomicrometry crystals (circles) and EMG electrodes (hooks) to measure muscle segmental strain and activation, respectively. Bold blue lines represent the central/proximal segments or fascicle while bold red lines represent the distal segments or fascicle examined. (A) The toad semimembranosus muscle (SM) is a parallel-fibered hip extensor and knee flexor. (B) The rat sternohyoid muscle (SH) is the main infrahyoid muscle and responsible for hyoid retraction, e.g. during swallowing. The black dotted line indicates the approximate location of an inscription that delineates the neuroanatomical boundary
superficial to a distal-deep aponeurosis. The proximal fibers (blue line) are shorter and rest at a more obtuse angle than the fibers of the distal fascicle (red line).

Figure 3. Representative segment length changes and electromyographic activity during a single behavioral bout. Shaded bars represent periods of strain heterogeneity. (A) During toad jumping, the proximal segment (blue) of the semimembranosus muscle (SM) shortens during the hop, while simultaneously, the distal segment (red) lengthens first before shortening. (B) During swallowing, the proximal segment (blue) of the rat sternohyoid (SH) shortens or remains isometric while the distal segment (red) actively lengthens. Periods of strain heterogeneity are dispersed throughout the swallow. (C) During trotting, proximal fascicles (blue) of the rat medial gastrocnemius (MG) begin shortening around the onset of stance (black bar), whereas distal fascicles (red) start shortening earlier and show a brief period of active lengthening during stance, resulting in periods of heterogeneous contraction being dispersed throughout stance.

Fig. 4. *In vivo* strain heterogeneity in three vertebrate muscles. Strain heterogeneity is expressed as percentage mean ± S.D. for the period of muscle activity (EMG-on) and for the entire cycle. All three muscles experience substantial *in vivo* strain heterogeneity.

Fig. 5. *In vivo* operating ranges for the two segments of each muscle in relation to their force-length relationships. Parabolas represent the average FLR measured *in vitro* or *in situ*. Horizontal bars represent mean ± S.D. *in vivo* strain experienced by the proximal (blue) and distal (red) segments of each muscle. (A). For the toad SM, the proximal segment (blue bar) shortens from the descending limb across the plateau of the FLR, while the distal segment (red bar) first lengthens from the ascending limb, then shortens further down the ascending limb. FLRs for proximal and distal segments of the toad SM were similar and can be represented by a single
gastrocnemius muscle (MG) shorten, and then lengthen on the ascending limb of its FLR (blue curve). Simultaneously, the distal fascicles (red bar) lengthen across the plateau to the descending limb, and then shorten across its FLR plateau (red curve; average $R^2$ for both curves = 0.99).

Fig. S1. Individual segment *in vivo* operating lengths (horizontal bars) with respect to segment-specific force-length relationships (parabolas). For the (A) toad semimembranosus, (B) rat sternohyoid, and (C) rat medial gastrocnemius muscles, blue represents the proximal segment while red represents the distal segment. Black parabola indicates both proximal and distal force-length relationships were similar for proximal and distal segments in the toad SM. Each pair of matched blue and red horizontal bars represent the *in vivo* range of operating strains determined for each individual (N = 7 for toad SM, N = 3 for rat SH, and N = 4 for rat MG). Lighter blue and pink horizontal bars represent the *in vivo* operating strain for each muscle during EMG activation rather than the entire behavior cycle. For the lowest two toad SM and one rat MG individuals, EMG recordings are not available.
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