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Pushing Outward: The role of fiber architecture and radial constraint in fusiform muscle

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Pushing Outward: The role of fiber architecture and radial constraint in fusiform muscle

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Ecology and Evolutionary Biology

by

Amber Rae Deslauriers

Thesis Committee:
Assistant Professor Emanuel Azizi, Chair
Associate Professor Matthew McHenry
Professor James Hicks

2015

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DEDICATION

To

My mother

Terri Mason

Whose love of learning
Guided me to this pursuit
And whose bravery and wisdom
Has taught me
That fulfillment and passion
Are areas of research
In need of persistent exploration

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ACKNOWLEDGMENTS

I would like to express my gratitude to my committee chair, Assistant Professor Emanuel Azizi whose help and assistance during experiments and modeling were immensely important to the project's success. He taught me valuable and practical lab skills through several project attempts, the most important of which was perseverance. Without his guidance and persistent help this dissertation would not have been possible.

I would like to thank my committee members, Associate Professor Matthew McHenry and Professor James Hicks, whose feedback and wisdom on my work during committee meetings have helped me have realistic expectations in experimental design and whose voices at 210 meetings have helped physiologists in the Ecology and Evolutionary Biology Department remain competitive and productive in the field.

Special thanks to Dr. Natalie Holt, who assisted me during many of the experiments and thoughtfully addressed any issue I brought to her attention. Her presence in the lab is a gift of serenity as well as experience.

I also thank my cohorts, especially Beck Wehrle and Caitlin Looby for their hospitality and friendship for the last three years. I also thank Dr. Shea Garrison-Kimmel for help in revisions and graphics. The University of California, Irvine and NSF Grant 1051691 provided financial support.

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ABSTRACT OF THE THESIS

Pushing Outward: The role of fiber architecture
and radial constraint in fusiform muscle

By

Amber Rae Deslauriers

Master of Science in Ecology and Evolutionary Biology

University of California, Irvine, 2015

Assistant Professor Emanuel Azizi, Chair

Muscles act as motors that perform positive mechanical work when fibers, which are comprised of sarcomeres and are organized into bundles encased by connective tissue sheaths, shorten across a joint. Here, we examine the relationship between muscle structure and function in a fusiform muscle. We model the muscle as a constant volume barrel to predict regional strain patterns throughout the muscle, and test the model empirically with a fusiform muscle, *palmaris longus* (PL), in two species of frogs, *Rana pipiens (R. pipiens)* and *Rana catesbeiana (R. catesbeiana)*. We allowed the muscle to contract against a servomotor at submaximal force conditions while filming with a high-speed camera, and analyzed the images taken at the extremes of the muscle length to measure strain in the inner and outer fibers. Both species of frogs exhibited significantly less strain in the outer muscle fibers compared to the innermost fibers, in broad agreement with predictions from our model. We then added an external constrain to the *PL* muscle of *R. catesbeiana* and measured force and length output under otherwise identical conditions. Adding this radial constraint reduced the length the muscle could contract by 50%, resulting in a decrease in the amount of mechanical

work it could perform of 50%. We discuss how regional strain variations may similarly affect the positive work output and implications for these findings on natural constraints to radial expansion.

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INTRODUCTION

Muscles generate positive mechanical work by producing force while shortening. Given that mechanical work is the product of force and displacement (shortening), a change in either variable can alter the ability of muscles generate positive work. Both the magnitude of shortening and the rate of shortening have important implications for the amount of force muscles can produce. First, during isometric (constant length) contractions muscles can produce the largest force at intermediate sarcomere lengths, where actin and myosin have the optimal amount of overlap (Gordon et al.1966). When the sarcomere are too short, the actin filaments crowd themselves, and there is not enough overlap for the actin and myosin to work together effectively when the sarcomere are too long. Second, during isotonic contractions, the rate of muscle shortening has a large effect on force. In fact the force-velocity relationship is hyperbolic for all muscle fiber types (Hill, 1938). Given the familiar force-length and force-velocity relationships of skeletal muscles, it is clear that the length changes muscles undergo during a contraction significantly influence their ability to produce mechanical work.

As muscles shorten to produce mechanical work, they must also expand radially to maintain a constant volume. This expansion results from the fact that muscles primarily contain fluids with very low compressibility (Kier and Smith, 1985), implying that the total volume of the muscle must remain nearly constant (Baskins and Paolini, 1967). This isovolumetric behavior of muscles is likely to result in radial expansion of muscle cells or muscular hydrostats (Kier and Smith, 1985). However, isovolumetric

behavior of whole muscles has also been shown to have important implications. One important consequence of muscle shape changes is the potential for influencing the lengths changes of muscle fascicles and fibers (Azizi and Brainerd, 2007, Wakeling et al. 2011). Muscles with pennate architecture are composed of fascicles that are oriented at an angle (pennation angle) relative to the muscle's line of action. As a result, when the muscle shortens, the muscle fibers not only shorten, they also rotate. The functional significance of fiber rotation is that it decouples the magnitude of shortening in the fiber from that of the whole muscle. This results in muscles operating with a "gear ratio" that provides muscles with a velocity advantage (Brainerd and Azizi, 2005; Azizi et al., 2008). In pennate muscles, orthogonal shape changes are the basis for circumventing the velocity limits of the muscle force-velocity relationship (Azizi et al., 2008).

The functional consequences of radial expansion and muscle shape changes in fusiform fibers remain poorly understood. In fusiform muscles, fibers are oriented along the line of action, often tapering at either end. One potential consequence of fusiform architecture is regional heterogeneity in the length changes of fibers. Specifically, the inner fibers of fusiform muscles are slightly shorter and have less curvature than the outer fibers. It has been suggested in fusiform muscles the inner fibers create outward pressure on the surrounding fibers that cause the muscles to thicken and curve outward as they shorten (Daggfeldt, 2006). It has also been predicted that shape changes in fusiform muscles are likely to differentially affect outer fibers as the muscle expands radially to maintain a constant volume (Otten, 1988). To date however, no empirical study has examined how the dynamic shape changes in fusiform muscle are likely to affect strain (shortening) patterns of muscle fibers within the muscle.

This study aims to understand how shape changes in a fusiform muscle affect fiber length changes and the capacity of muscles to generate positive mechanical work. We use a simple model to develop predictions about regional strain patterns in a fusiform muscle. Here we treat the muscle as barrel that radially expands as it shortens (Otten, 1988). We assume the radius of the muscle-tendon junction of the proximal and distal end remain constant during contraction (Figure 1). This radial expansion induced by shortening causes the fibers on the outermost part to increase in curvature. The innermost fibers are modeled to lack any curvature as the muscle shortens. At the midline the fibers run straight from end to end. From this model we predict that the greatest strain will be in the innermost fibers whereas the outer fibers increase in curvature but shorten less. Our model also predicts that when the muscle shortens significantly, radial expansion of the muscle causes the outer fibers to lengthen as the muscle shortens. We predict that this regional variation in fiber strain may ultimately place an upper limit on muscle shortening and the capacity of the muscle to perform positive work.

In our study we use an isolated muscle preparation to quantify the consequences of muscle shape changes in a fusiform muscle. In our first set of experiments we quantify fiber strain in the inner and outer regions of a muscle to test the hypothesis that inner fibers undergo greater shortening than outer fibers. In our second set of experiments we use a physical constraint to limit the ability of the muscle from expanding radially. These experiments will test the hypothesis that constraining radial expansion limits the ability of a fusiform muscle to shorten and therefore limit the ability

of the muscle to generate positive mechanical work. These experiments will provide fundamental insight into the consequences of radial expansion in fusiform muscles and provide a functional understanding of how natural physical constraints in a muscle may alter the function performance of muscle.

Model and predictions

We model fusiform muscles as isovolumetric barrels, which must necessarily radially expand as it shortens (Otten, 1988). We assume the radii of the tendomuscular junctions at the proximal and distal end remain constant during contraction (Figure 1). The radial expansion induced by shortening must therefore cause the fibers on the outermost part to increase in curvature. At the midline, however, the muscle fibers run straight from end-to-end, with the degree of curvature smoothly varying between the innermost and outermost fibers. This model naturally predicts that the greatest strain will occur in the innermost fibers.

Daggfeldt et al (2006) and others have postulated that in fusiform muscles the inner fascicles create outward pressure on the surrounding fibers that cause the muscles to thicken and curve outward as they shorten. We postulate that this intramuscular pressure will decrease the amount of strain that the outer fibers experience. The force-velocity curve states that muscles contracting at a slower rate produce a greater amount of force. Because the inner fibers experience a greater strain than the outer fibers in the same period of time, the inner fibers must contract faster. We therefore predict the existence of a negative force, which acts to prevent additional bulging and shortening, thereby decreasing the positive work of the entire muscle. We

imagine a resistive force prohibiting additional bulging because, as muscles slow down, force rises causing a resistance to the internal pressure. As if it were a shell, the outer fiber moving more slowly would be resisting fibers that were more inward and moving faster, and thus presumably with less force.

Adding a constraint to radial expansion

An additional implication of our isovolumetric barrel model applies to muscles that are restricted from radial expansion. In these cases, overall length change should be limited due to the constant volume properties of muscles. This could have implications for mechanical function, including the amount of work and power that the constrained muscle can produce. By adding a secondary sheath to the fusiform muscle that restricts its radial expansion, we tested the effect of limiting expansion as the muscle contracted, while collecting length and tension data. Though the sheath was fabricated from a nonbiological material, it mimics the biologic response of epimesial connective tissue sheaths, which can alter stiffness through fibrotic proteins when in disease and disuse. Fibrotic muscles are constrained by the addition of extra cellular matrix and rigid protein collagen.

Restricted expansion may also have implications for other spatial relationships in muscle architecture. Muscles act synergistically in groups and strain is an important consideration for how multiple muscles control groups. Muscles constrained radially may experience mismatching in the strain and thus mechanical function they were patterned to do and movement discordance may follow.

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Another implication of resistance to expansion could occur in muscles compartments, or muscle segments moving against tough fascia, which may create changes in the regional mechanical functioning of the muscle This could be relevant to the study of compartment syndrome, where impingement happens as a result of inflammation and structural hardening of the connective tissue.

Cascading chemical responses to protein degradation and regeneration make it difficult to study fibrosis in isolation without the concurrent processes of muscle atrophy, sarcopenia, etc. (Leiber and Ward, 2013). Our model may help to decouple the disease and disuse response of increased extracellular matrix and decreased muscle tone, and help to elucidate the effect of a constraining extracellular matrix on the mechanical function of muscle.

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1. MATERIALS & METHODS

The *palmaris longus (PL)* of leopard frogs (*R. pipiens*) was used for both the modeling and experimental aspects of the study. The PL is a biarticular muscle spanning the elbow and wrist and is primarily considered a wrist flexor. At both the origin and the insertion the muscle tapers into a narrow musculotendinous junction. The *PL* has a slender fusiform shape and lacks any internal tendinous inscriptions, making it an ideal muscle for testing our stated hypotheses.

Our model is based on an isovolumetric barrel that changes length M. We assume that the radius (R_1) of the proximal and distal muscle-tendon junction are the same and remain constant during shortening. We also assume the innermost fibers undergo the same strain as the muscle and take the most direct path from tendon to tendon. Figure 1 is a labeled schematic of the model elements. As stated in Azizi and Deslauriers 2014, our geometric model utilizes the geometric relationship for the midbelly of the muscle after contracting (eq. 1) to find the predicted length of outermost fibers L_{02} (eq. 2) and uses strain (eq3) to compare length changes in inner and outer fibers. For reference, $\boldsymbol{\varepsilon}_m$ is the strain of the whole muscle.

$$R_3 = \sqrt{\frac{2R_1^2 + R_2^2}{1 - \epsilon_m} - 2R_1^2} \qquad (1)$$

$$L_{O2} = \frac{2M(1-\epsilon_m)\arctan(\frac{2k_2}{M(1-\epsilon_m)})}{\sin(2\arctan(\frac{2k_2}{M(1-\epsilon_m)}))} \qquad (2)$$

$$\epsilon_{fO} = \frac{L_{O1} - L_{O2}}{L_{O1}} \qquad (3)$$

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The results of our simple geometric model are compared to empirical measurements of the regional fiber strains in the *palmaris longus (PL)*. To measure fascicle strains in various regions of the muscle during a contraction, we used an *in vitro,* isolated muscle preparation similar to those previously described in Azizi & Roberts (2010). We euthanized the frog with a double pithing protocol, then isolated the muscle from surrounding tissue, keeping both tendinous ends intact, and placed it in an aerated amphibian Ringer's solution (100mM NaCl, 2.5mM KCl, 2.5mM $NaHCO_3$, 1.6mM CaCl, 10.5mM Dextrose). Kevlar strands were tied to one end of the muscle and then attached to a fixed clamp controlled with a three-axis micromanipulator while the other end of the muscle was attached to the lever arm of a dual-lever servomotor (Aurora Scientific, 305C LR, 10N, Ontario, CA) as shown in Figure 3. In some experiments, the radial expansion of the muscle was physically constrained. In these experiments we constructed small perforated plastic sheaths, fitted to touch the muscle at the widest diameter during rest and tapered to cover approximately 75% of the muscle (Figure 5).

We maximally stimulated the muscle using a pair of platinum stimulating plates. The stimulation unit (S48 Stimulator, Grass, W. Warwick, RI) was connected via BNC cable to platinum field electrode plates placed on either side of the muscle. We then constructed a force–length curve by performing a series of twitches (0.1 ms duration, 50V) at varying lengths to determine the optimal fiber length for producing maximal force. This force was recorded as a wave in the Igor file and calibrated to the servomotor's force and length parameters. All subsequent contractions were done at

this optimal length, which produced maximal tetanus. We then performed an initial isometric tetanus contraction (train duration 400ms, Stim rate 100 pps, Frequency 50V). We measured the maximum isometric force (F_o) of the muscle during a full tetanic contraction. A series of isotonic contractions at loads corresponding to 25% and 50% of F_0, were used to quantify the shortening behavior of the muscle and fascicles against consistent loads. By having the muscle contract against a constant load, we remove the potentially confounding effect of in-series compliance. Care was also taken that stimulation could still occur in the constrained condition.

To measure the fascicle shortening and radial expansion of the muscle during the each contraction, the muscle was imaged from above using a Phantom MiroM120 high-speed camera. The camera captured each contraction at 100 frames per second. Each frame of the high-speed videos was examined to find the frame consisting of the longest (rest) and shortest muscle length. Muscle fascicle strains were measured from the video of the contracting muscle using the Image J software. The frames were spatially calibrated using a ruler in the video frame. Using the segmented line function, the internal fibers were analyzed by drawing a line through the center of muscle from the tendinous transcription of the origin to the tendinous transcription of the insertion. In fusiform muscles the innermost fibers run from these two points in a straight line. For the outermost fibers, we used the segmented line function to trace the curvature on the outside of muscle (left side), beginning and ending at the same two points. These calibrated measurements thus provide regional fascicle lengths during both the relaxed and fully contracted muscle. An example frame is shown in Figure 4 where the inner measurement in traced in red and the outer in blue

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The force and displacement of the muscle was measured with the servomotor and used to calculate muscle work during constrained and unconstrained conditions and used to quantify the effect of constraining radial expansion during shortening on muscle work. The force and length of the muscle during each contraction were recorded at 4000 Hz using a DAQ (National Instruments USB 6212, Austin, TX) using Igor Pro software program (IGOR Pro v. 6.1.2.1; Wavemetrics, Beaverton, OR).

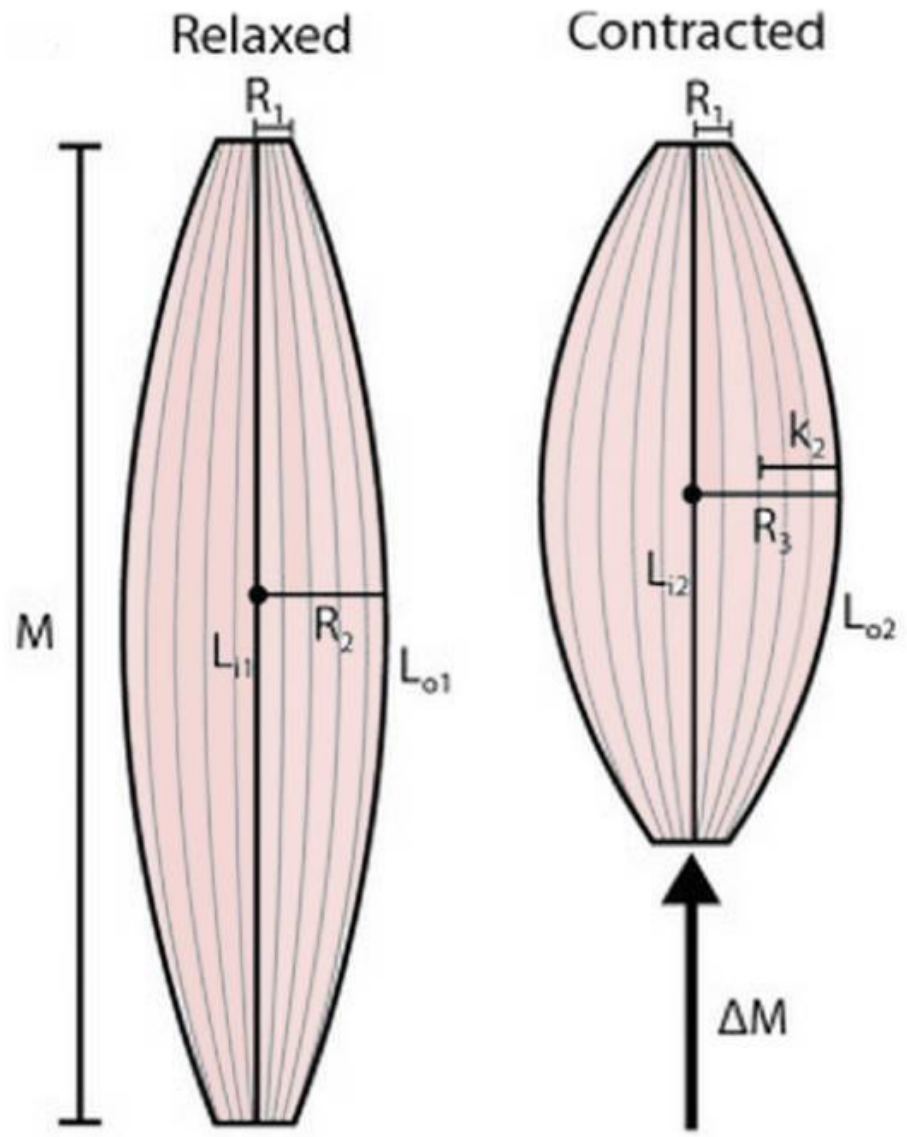

Figure 1. Schematic of the isovolumetric barrel model for a fusiform muscle. Schematic represents a fusiform muscle during relaxed and contracted conditions. Distance M is the length of the muscle. L_{I1}, the initial length of the inner fiber, is the same distance as M and is compared to $L_{O1,}$ the initial length of the outer fiber. After contraction there is a change in muscle length (**Δ**M). . L_{I2}, the final length of the inner fiber is compared to $L_{O2,}$ the final length of the outer fiber. In the model R1, the radius at the muscle tendon plate is the same for both proximal and distal ends and remains the same from relaxed to contracted. R_2 is the radius of the muscle belly at its thickest point and it smaller than R_3, the same point after contraction.
$K_2 = R_{3-} R_{2.}$

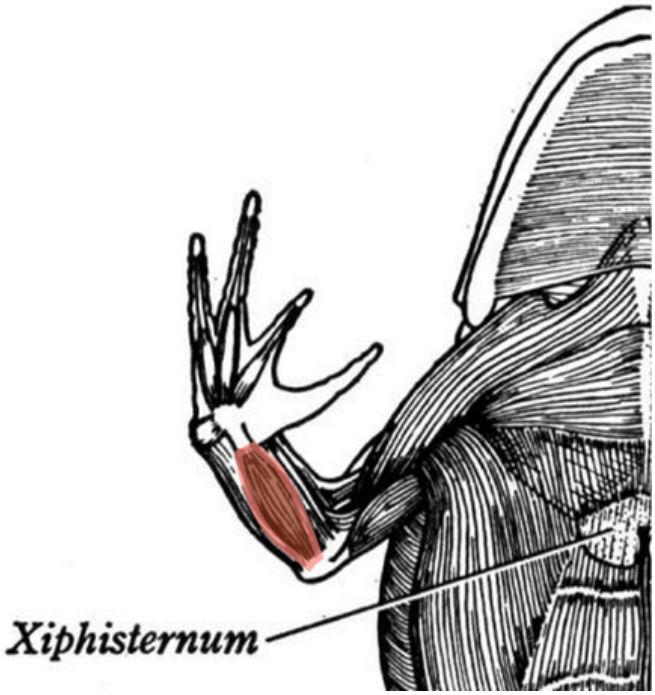

Figure 2. Anatomical placement of *Palmaris longus* (PL) in *R. catesbeiana*. The PL muscle is located on the ventral lower arm. The muscle is biarticular, crossing over the elbow joint through a narrow tendon at the proximal end and the wrist joint at the distal end to insert through a tendon onto a sheath on the ventral hand. The muscle was chosen for its fusiform shape.

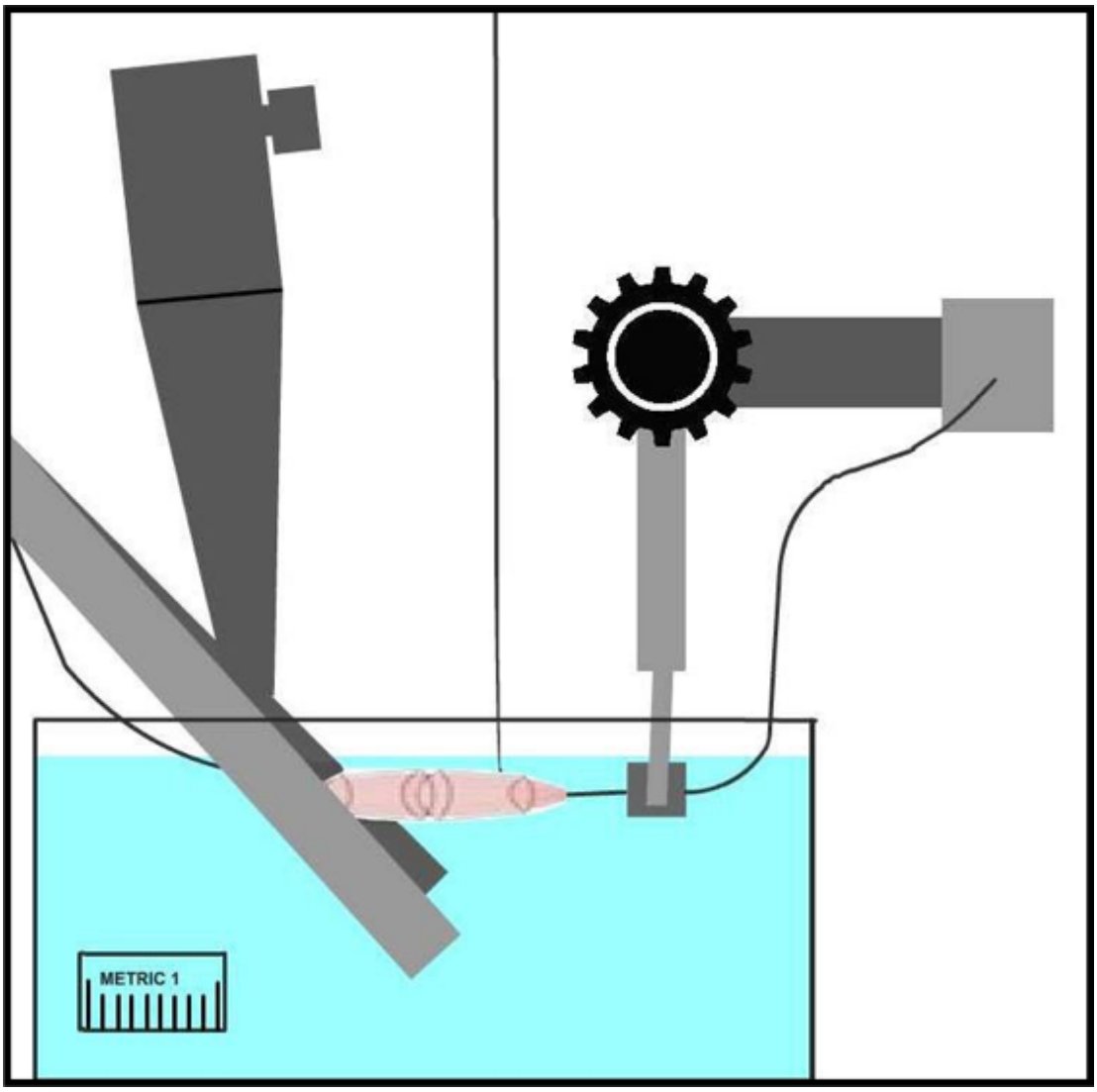

Figure 3. Schematic of muscle *in vitro.* The muscle was tethered between the 10 N servomotor and the fixed end of a micromanipulator using Kevlar strands. Field stimulation was used to fully stimulate muscles placed in Ringers solution. Each frame was calibrated using a millimeter scale bar on the side of the bath.

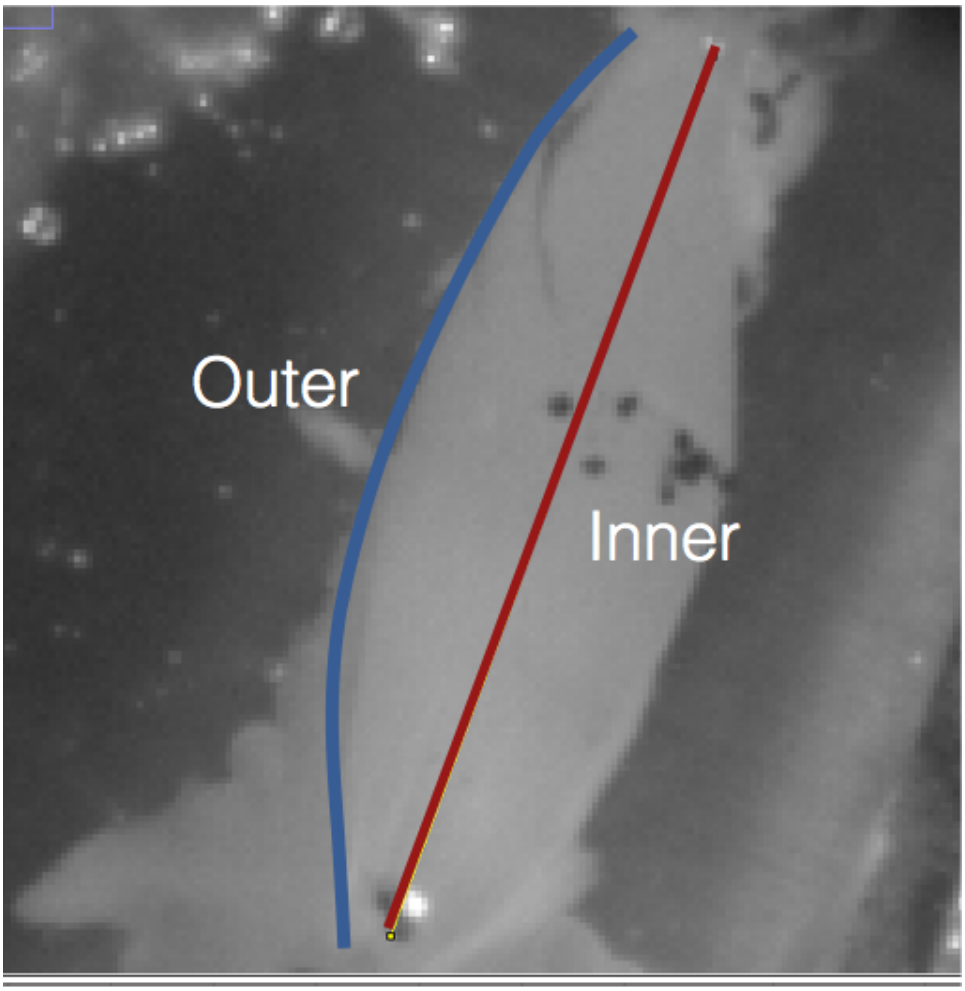

Figure 4: Representative analyzed video frame. Frames from resting and fully contracted (shortest) periods in the high-speed video were saved and analyzed using Image J. Inner Fibers (in red) were assumed to take the shortest distance from tendon plates. Outer fibers (in blue) were assumed to have the trajectory of the outer most edge of the muscle. Distances were calibrated using an in frame millimeter scale bar (not shown).

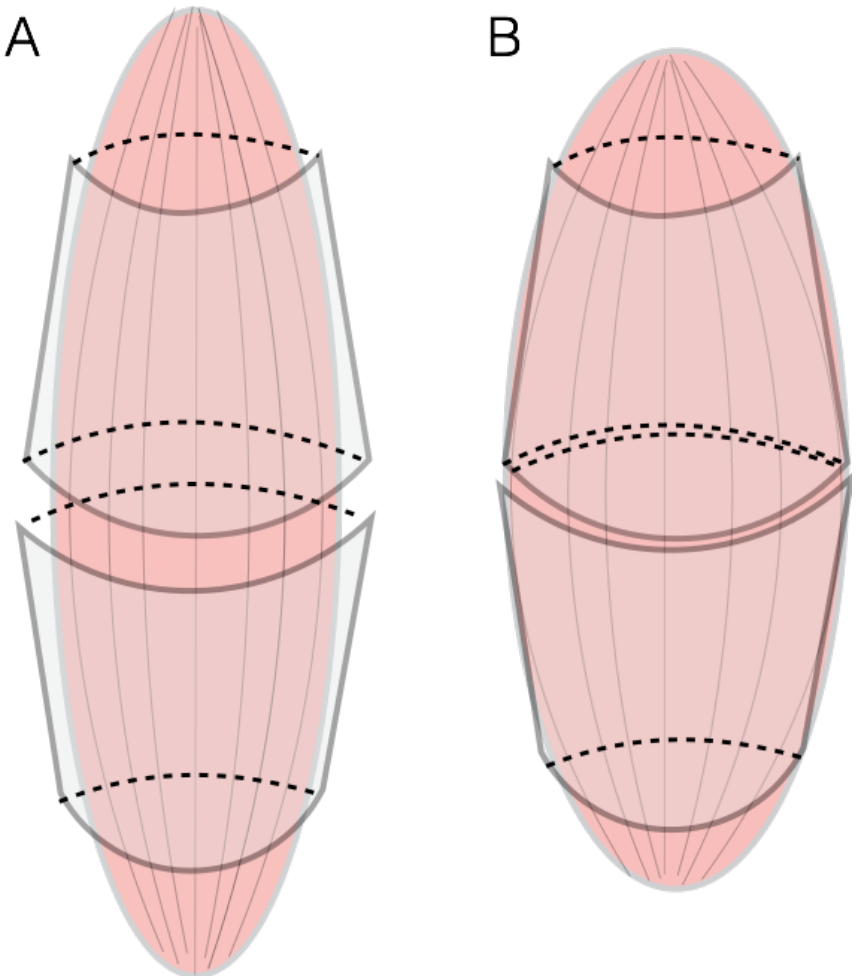

Figure 5. Schematic of length change in constrained and unconstrained muscles A custom muscle constraint was made for each muscle trial that would engage with the muscle as it shortens. Muscles were measured after being dissected out and an appropriately sized constraint that would engage with the muscle after minimal shortening (~80-70% of submaximal force) was placed on the muscle. The constraint was fashioned as two connecting hard plastic pieces that tapered at the ends with the same radius at the midbelly. As the muscle contracted the ends would join together at the midbelly and prevent radial expansion.

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2. RESULTS

Regional variation in fiber strain

We quantified the magnitude of radial expansion in the *Palmaris longus* muscle, a fusiform muscle, as a function of muscle strain and compared these measures to predictions of a simple geometric model (Figure 7). Our empirical measures of fiber strain in a fusiform muscle generally support the predictions of the isovolumetric model. We consistently observe less fiber shortening in the outer fibers of the muscle compared to the inner fibers (Figure 7).

In *R. pipiens* (N=4), we found we found that fiber strain increases significantly with muscle strain ($p < 0.001$) using a Two-Way ANOVA. Regional differences in fiber strain followed the predicted trend but were not statistically significant ($p = 0.089$). See Figure 7a.

In *R. catesbeiana*, The PL was found to produce an average maximum isometric force (F_o) of 1.65± 0.378 N (±SEM). At 25% F_0, Inner fibers had an average strain of 0.36±0.01 and outer fibers had an average strain of 0.33±0.03. Fiber strains measured at 25% of F_0 were not significantly different between inner and outer fibers (p= 0.24, n=7). using a Two-Way ANOVA.

For contractions at 50% F_0, average inner fiber strain was measured to be 0.31± 0.04 and average outer fiber strain was measured to be 0.27±0.05. Fiber strains measured at 50% of F_0 was nearly significantly different between inner and outer fibers (p= 0.06, n=4) Figure 7b visualizes these predicted trends and the resulting empirical data.

Constrained Radial expansion in fusiform muscle measurements

Using the same protocol as the unconstrained muscles, we also measured how physically constraining radial expansion affects muscle performance. Maximum isometric force (F_0) was compared before and after the addition of the physical constraint. Maximum isometric force for the unconstrained muscle was 1.66 ±0.19N (SEM) whereas the constrained muscle produced 1.68 ± 0.20 N. Maximum isometric force did not differ significantly between constrained and unconstrained muscle (p= 0.9347).

The effect of the physical constraint was greatest on the length changes that occurred during isotonic (constant load) contractions (Figure 8). During contractions at 50% F_0 the muscle shortened less after the application of the physical constraint nonsignificantly, but close (p=0.0688, n=3). However, during contractions at 25% F_0 the muscle shortened significantly less after the application of the physical constraint (p= 0.03, n=4). The mean length change for unconstrained muscles was 6.9 ± 0.37mm (SEM), while the length change for constrained muscles was 4.3±0.54 mm (Figure 8).

The mechanical work produced during isotonic contractions was also compared with and without the presence of the physical constraint. Mechanical work was found to be significantly lower in constrained versus unconstrained muscles operating at 25% F_0 (n=4). These results are shown in Figure 9. Using a two-way ANOVA the difference in constraint vs nonconstraint was significant (p=0.002) with no significant interaction between variables(p=0.539). The mean work for unconstrained muscles operating at 25% was 6.9±0.37 N mm (SEM), while the mean work for constrained muscles was

4.3±0.54 N mm (SEM). For unconstrained muscles operating at 50% F_0, the mean work was 3.6±0.83 N mm (SEM), while the mean work for constrained muscles was 1.4 ±0.34 Nmm (SEM). Figure 6 is a sample trace demonstrating the impact of decreased length change in constrained muscles.

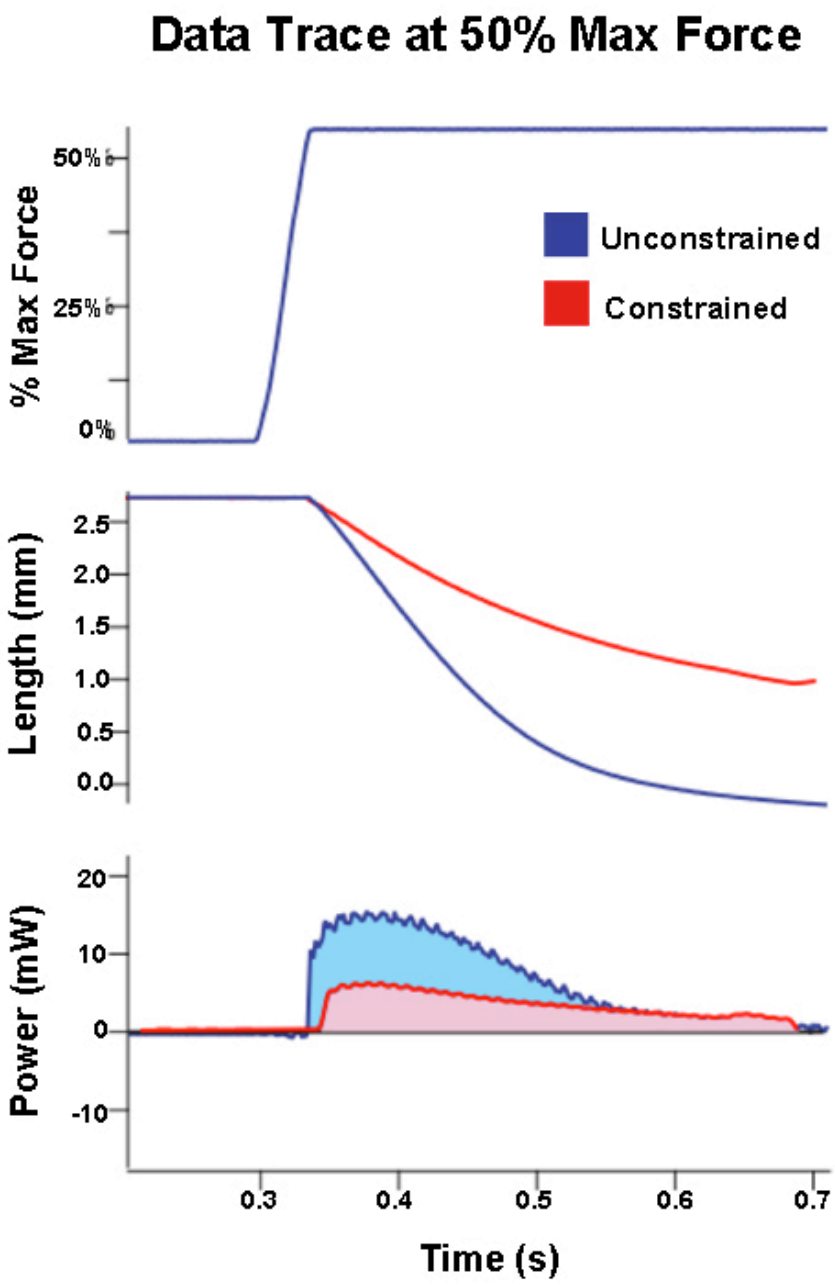
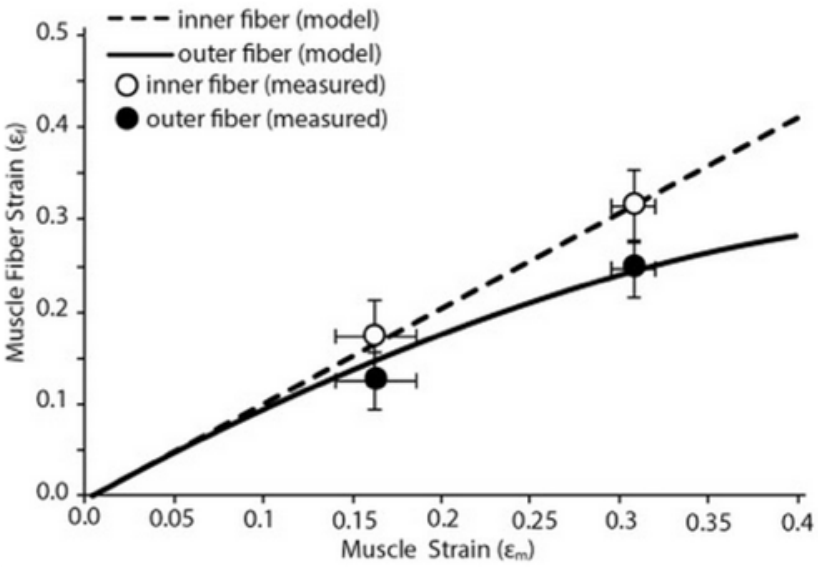


Figure 6: Data Trace of muscle performance variables at 50% Max Force. Two trials were performed were on the same muscle during a constrained and unconstrained trial at 50% of max force. Traces in blue represent unconstrained muscle and traced in red represent constrained muscle. **Top graph** is percent of max force (Fo) plotted against time, in which the two traces overlap. **Middle graph** is length change over time. Traces start at same initial length. As the muscle begins to contract, both muscles shorten with the constrained muscle shortening at a slower rate resulting in less length change in the constrained muscle. **Bottom graph** shows the power function of time with the area shaded in representing work for each condition.

A



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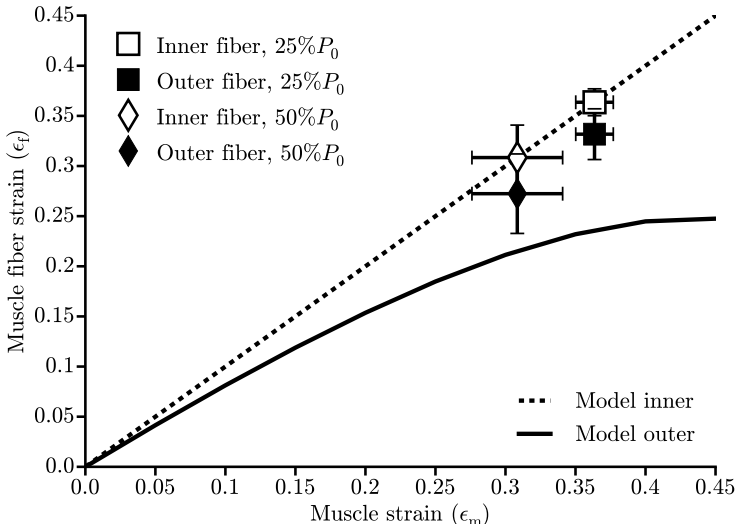

Figure 7: Inner and outer fiber strain as a function of muscle strain in R. Pipiens and R. Castesbeiana. Solid and dashed lines are the predicted values of fiber strain as a function of muscle strain for inner (dashed) and outer (solid) fibers. The model predicts variation in the strain of the innermost and outermost muscle fibers as whole muscle strain increases. The inner fibers undergo relatively higher strains than the outer fibers. Closed and open symbols represent mean strain measured in the outermost and innermost fibers, respectively. Data are collected from the frog palmaris longus muscle. Error bars represent the standard error of the mean. A)shows data from R. pipiens (Azizi and Amber 2014, N=4, p=0.002,two-way ANOVA strain by fiber) and B shows R. catesbeiana.(N=7 for 25% and N=4 for 50%, p=0.27 ,two-way ANOVA strain by fiber)

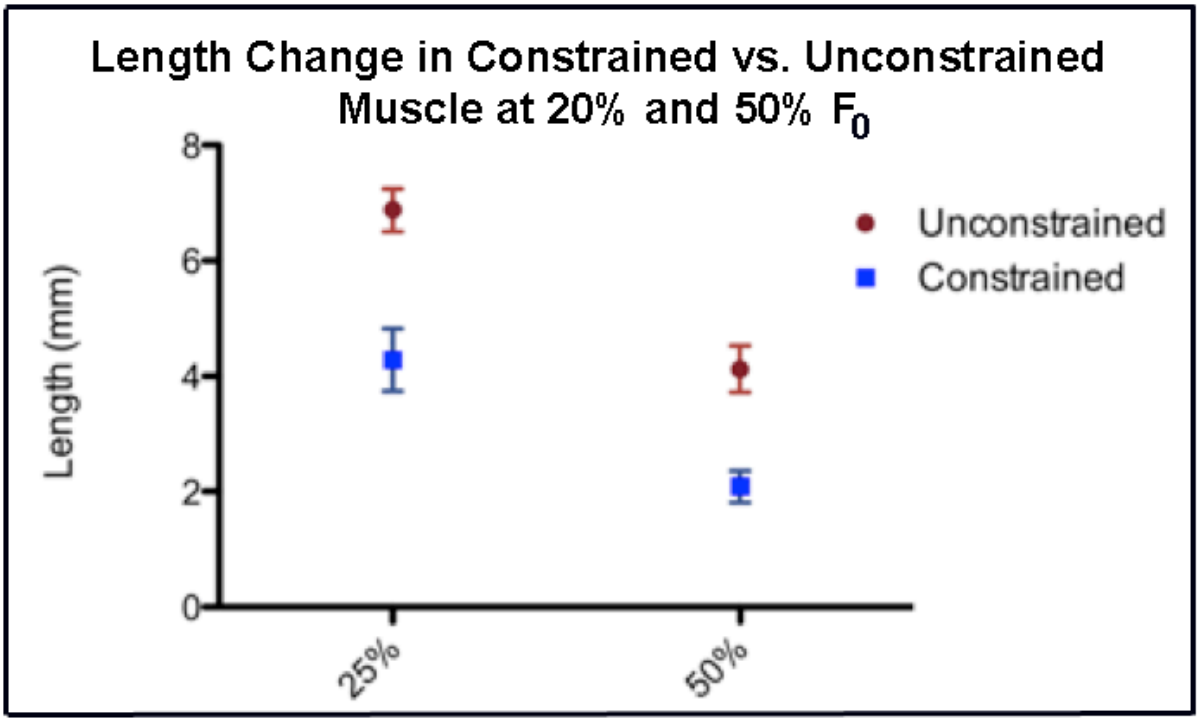

Figure 8. Constrained muscle results (length). On the left is paired tests of constrained and unconstrained muscle length change averaged and compared at 25% of submaximal force (N=7,)On the right is paired tests of constrained and unconstrained muscle compared averaged and compared at 50% of submaximal force (N=4). There is a significant difference between constrained and unconstrained data (ANOVA, p=0.002). Error bars represent SEM.

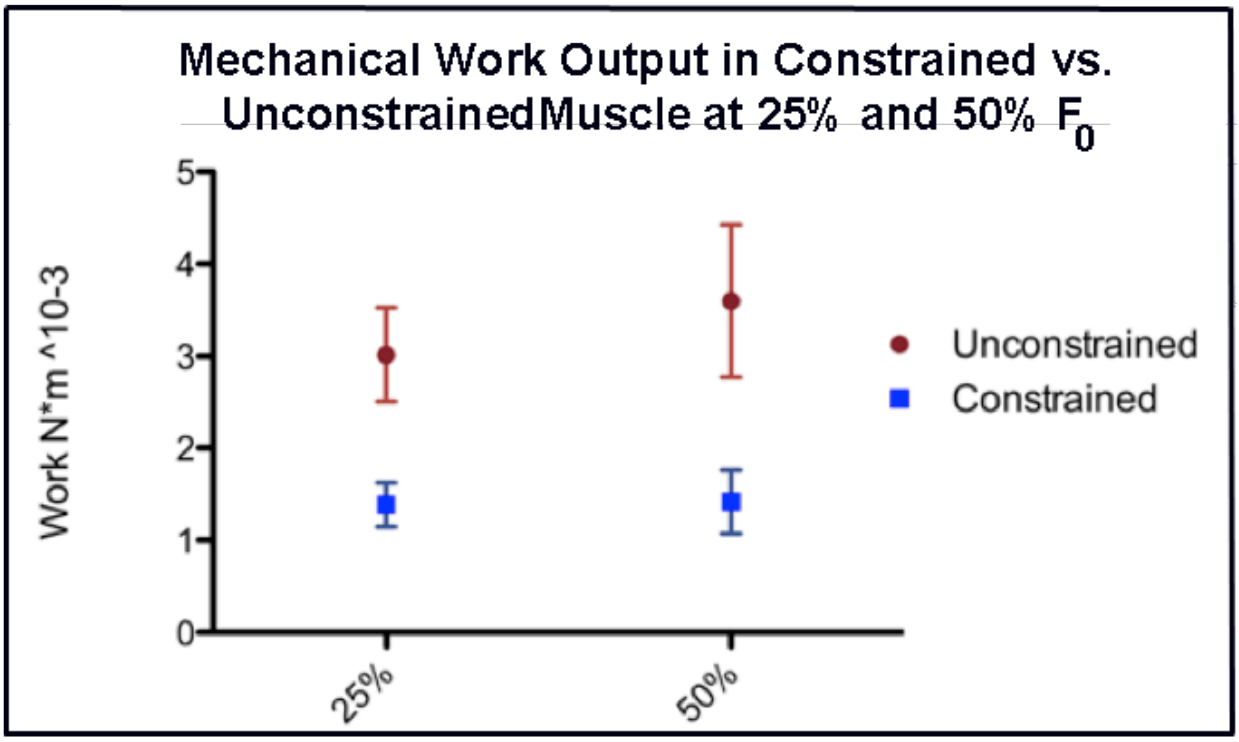

Figure 9. Constrained muscle results (work). Constrained muscle results length. On the left is paired tests of constrained and unconstrained muscle length change averaged and compared at 25% of submaximal force (N=7,)On the right is paired tests of constrained and unconstrained muscle compared averaged and compared at 50% of submaximal force (N=4). There is a significant difference between constrained and unconstrained data (ANOVA, p=0.002). Error bars represent SEM.

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3. DISCUSSION

In this work, we used fusiform muscles to explore the impact of muscle architecture on regional variation of strain and the limitations on the mechanical work that muscles can perform when radial expansion is constrained. We first found that the inner fiber strain is greater than the strain experienced by the outer fibers when the muscle operates at submaximal force conditions of both 25% and 50% in *R. pipiens*. We then showed that adding an external constraint to the radial expansion of a fusiform muscle restricts the amount that the muscle can shorten under submaximal conditions, which leads to a decrease in the amount of positive work that the muscle could perform.

Regional Heterogeneity in Parallel Fibers, spatially separated from the midline

Based on our "isovolumentric barrel" model of a fusiform muscle, we predicted a decrease in fiber strain as a function of distance from the sagittal midline, implying that the greatest difference in fiber strain should exist between the innermost and outermost fibers. Specifically, we predicted the muscle would thicken as it shortened and, due to the fixed narrow muscle-tendon junctions at both the proximal and distal ends, the greatest amount of thickening would occur at the transverse midline (i.e., that R_3 would increase as the muscle shortened). This thickening should lead to an internal pressure, resulting in an increased curvature in the outermost fibers.

We tested our model using the fusiform *Palmaris longus* muscle from two species of frogs in the same genus, *R. pipiens* and *R. catesbeiana*. We compared the

regions with the greatest predicted variation, the innermost deep fibers that take a direct path between the muscle-tendon junctions and the curved outermost superficial fibers. In both species of frogs, we found a lower strain in the outer fibers than in the inner fibers, clearly indicating regional heterogeneity. Moreover, our measurements of the outer fiber strain in *R. pipiens* closely matched the model predictions when scaled to that muscle.

However, the outer fibers of *R. catesbeiana* experienced more strain than predicted by our model. The discrepancy between our model and our data may be due to the two-dimensional analysis employed throughout. As a muscle shortens, the outer muscle fibers, which experience a greater strain than our model predicts, may undergo torsion as well as the bending that our model accounts for. This torsion would be undetectable by a camera with a lateral view, as in our experiments, but would increase muscle fiber strain.

Our results help illustrate that regional heterogeneity must be considered when attempting to measure muscle strain. Other recent studies (e.g. Pappas et al. 2002, Ahn et al. 2003, Azizi et al. 2008, Higham and Biewener 2008, Llewellyn et al. 2008) have also shown that muscles can experience strain heterogeneity at varying levels, including in fascicles enervated by the same nerve and the same fiber type. Nonetheless, these strain data were often presumed to be representative of the strain experienced by fibers throughout the entire muscle. As mentioned, muscle strain has implications for the mechanical function of the muscle, and thus these measurements may only represent the mechanical function of the most superficial fibers.

The importance of muscle architecture and its role in muscle performance has long been postulated in muscle physiology (e.g. Gans and Bock 1965), while the role of spatial orientation of fibers within muscle has also been modeled and empirically explored within the last decade (see Hingham and Biewener 2011 for review of current findings). For example, recently in has been shown by Ahn and others, that sarcomeres aligned in series can be operating at different lengths (Ahn et al., 2003) and that strain segments of parallel fibers that are adjacent to each other can also operate at different lengths (Ahn et al., 2004). This has clarified that intramuscular strain can occur between different fascicles even when the muscle is enervated by the same nerve, and thus activated simultaneously. Variation in strain can lead to different mechanical functions across a joint in different regions of the muscle (Higham et al, 2008, Pappas, 2002).

Our results further support the growing understanding that regional heterogeneity is critical to the study of muscle function. Other authors have similarly found regional heterogeneity in different parts of muscle. For example, Ahn et al. (2004) found the greatest variance in strain at the tapered end of a toad's semimembranosus muscle, which has a broad proximal origin on an aponeurosis and a tapered distal insertion onto a tendon. They predicted a symmetrical strain heterogeneity pattern in a muscle with two tapered ends, such as the *Palmaris longus*, which we explore here. Similarly, Thompson et al. (2014) demonstrated the existence of regional strain variation in the circular muscles of a squid mantle. Together, these findings confirm that regional heterogeneity in muscle strain must be considered in experimental design.

Constraining the radial expansion of fusiform muscles

Due to their constant volume properties, fusiform muscles must expand radially while contracting (as demonstrated explicitly above). However, there are many ways that this expansion could be limited or even eliminated, included pressure from surrounding muscle groups, pressure from other regions of that same muscle, or material changes in the protein composition of the extracellular matrix, specifically the addition of collagen. We therefore explored the impact of constraining radial expansion in fusiform muscles by fitting the muscle with a plastic sheath and stimulating under submaximal conditions *in vitro*.

We showed that constrained muscles (i.e., those that are not free to expand radially) shorten significantly less than their unconstrained counterparts when subjected to submaximal force conditions, in agreement with predictions from the constant volume model. This decrease in muscle strain directly translates into a decrease in the work that the muscle is able to perform in a single isotonic contraction at both 25% and 50% of the maximum force. This could have implications for variation in regional mechanical function, and the regional contribution of muscle fibers to positive work being done on the joint. In fact, fusiform muscles may have an upper limit to the mechanical work they can do if the positive work performed by inner fibers is counteracted by negative work of outer fibers. We demonstrated this in our experiment by manipulating the initial length and radius of the muscle and stimulating substantial shortening, and found the outer fibers could actually be stretched.

Additionally, variation in strain between adjacent fibers that are performing a contraction over the same time will lead to inner and outer fibers operating at different

velocities, and thus operating over differing regions of the force-velocity curve. The region that fibers operate on this curve has a profound impact on the mechanical role of the muscle, specifically in relation to whether it is optimized for force, power or velocity, (Rome et al., 1988). Regional differences in mechanical role within fiber groups have previously been characterized in other muscle architectural types (e.g. Dean et al., 2007, Azizi et al., 2008, Hingham and Biewener, 2011, Thompson et al., 2014). However, regional mechanical implications of fiber placement in fusiform muscle remained largely unknown. The effect of these regional differences may have implications for activation pattern, depending on the conditions the muscle is operating within. How this regional variation in the effects of fatigue influences the overall mechanics of the muscle under *in vivo* conditions is not fully understood. Regional heterogeneity may also influence the fatigability of a muscle, with regional "compartments" becomes passive elements as they fatigue and becoming a mass that must be moved by still-active regions (Higham and Biewener, 2011).

Likewise, constraint to radial expansion may also have implications to mechanical performance due to spatial relationships in muscle architecture. Muscles act synergistically in groups to perform actions across a joint. Muscles acting in unison are spatial close to one another and, as they shorten, thicken and push against each other. Muscle strain is therefore an important consideration for how multiple muscles control movement across a joint. Radially constrained muscles in a group may experience mismatched strain, altering the mechanical function they were patterned to do and resulting in movement discordance. Furthermore, muscle compartments, or muscle segments moving against tough fascia, may create changes in the regional mechanical

functioning. Therefore, radial constraints may be relevant to the study of compartment syndrome, wherein impingement occurs as a result of inflammation and structural hardening of the connective tissue. Our experimental constraint model may help to decouple the disease and disuse response of increased extracellular matrix from decreased muscle tone, thereby elucidating the effects of a constraining extracellular matrix on the mechanical function of muscles.

Further directions

There are several possible ways that fusiform muscles can counteract regional variations in fiber strain. On the smallest scales, there may be a difference in the resting sarcomere length, especially in the region of the outer fibers undergoing the most curvature around the transverse midline. Because of the curvature of the muscle, the resting length of the complete fiber may also differ, such that the outermost fibers are longer and possibly contain more sarcomeres in series. The muscle could also compensate on large scales by activating the outer fibers before the inner fibers, effectively causing the strain to be the same overall even if it is changing at different rates.

Many of these hypotheses could be tested experimentally. A dissection of the muscle would allow for direct measurements of the inner and outer fiber lengths, and may also reveal any regional variations in the number of sarcomeres in a fiber. Similarly, restricting the muscle to have equal strain throughout, regardless of the rate the muscle contracts, could reveal whether there is a regional difference in the fiber activation time. The sarcomere length may be determined with X-ray diffraction, with

measurements on the inner fiber performed on a cross-section frozen in liquid nitrogen. Further directions could also include physical measurements of strain in the innermost fiber. These experiments, comparing the inner and outer fibers of fusiform muscles, will result in a more complete understanding of fusiform muscle architecture.

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