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Functional and architectural complexity within and between muscles: regional variation and intermuscular force transmission

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Abstract:

Over the past 30 years, studies of single muscles have revealed complex patterns of regional variation in muscle architecture, activation, strain, and force. In addition, muscles are often functionally integrated with other muscles in parallel or in series. Understanding the extent of this complexity and the interactions between muscles will profoundly influence how we think of muscles in relation to organismal function, and will allow us to address questions regarding the functional benefits (or lack thereof) and dynamics of this complexity under *in vivo* conditions. This paper has two main objectives. First, we present a cohesive and integrative review of regional variation in function within muscles, and discuss the functional ramifications that can stem from this variation. This involves splitting regional variation into passive and active components. Second, we assess the functional integration of muscles between different limb segments by presenting new data involving *in vivo* measurements of activation and strain from the medial gastrocnemius (MG), iliotibialis cranialis (IC), and iliotibialis lateralis pars preacetabularis (ILPR) of the helmeted guinea fowl (*Numida meleagris*) during level running on a motorized treadmill. Future research directions for both of these objectives are presented.

1. Introduction

Animal locomotion is a field of central importance to research in biology and engineering [1-3]. In addition, how muscles actuate running in vertebrates has captivated the interest of scientists for hundreds of years [4-6], and there continues to be an ever broadening set of approaches to research on this topic. Over the past few decades, significant advancements in our understanding of muscle function have been accompanied by the discovery of considerable complexity within and between muscles. Perhaps a pertinent analogy to a muscle is an orchestra, which only functions appropriately when all of the instrumental components (string, brass, woodwind, and percussion) work in a synergistic fashion. Similarly, a muscle is comprised of many different components, all of which act in a coordinated fashion in order to execute a movement (Fig. 1). The overall goal of this manuscript is to address the complexity of muscle function, with specific foci on the regional variation in architecture and function within muscles, and the complex interactions that can occur between muscles.

A single muscle, or muscle fascicle, can exhibit variation in activation, strain, and architecture [7-17]. Many muscles exist within the limb of an animal, with muscles that work together as synergists or in opposition as antagonists across a common joint. Among functionally equivalent muscles (i.e. synergists), substantial variation can occur depending on the role of the muscle [18-21]. The muscles within a limb, however, are often connected, resulting in the potential for intermuscular force transmission [22-25] (Fig. 1). For example, recent work has highlighted the connections between muscles, whether they are within a single limb segment [26] or between adjacent limb segments [27]. Ultimately, muscle architecture and fibre type composition, *in vivo* recruitment patterns, activation history, and the way in which a muscle is recruited relative to other muscles ultimately determine the mechanical function of that muscle.

This hierarchical organization and complexity of muscle function is reviewed in this manuscript and new data is presented regarding the mechanical linkages between muscles of different limb segments in the helmeted guinea fowl, *Numida meleagris*. Given that a single cohesive analysis of regional variation within muscles does not exist, we seek to integrate the existing studies regarding passive and active regional variation and to propose common themes and possible avenues for future research. Regional variation and force transmission between muscles are key topics that are likely to drive a large portion of neuromuscular research over the coming decades. Thus, our contribution is timely and should assist those who will explore this interesting aspect of muscle function.

2. Functional heterogeneity within muscles:

It is common for muscles to exhibit regional variation in a number of important factors, including activation [14, 28-32], mechanical action [33], fibre type [34-36], architecture [37-39], and strain [8, 10, 11, 13, 40-42]. In fact, it is unlikely that many muscles actually exhibit homogeneous structure and function. The added level of complexity is something that will require future consideration when constructing musculoskeletal models [43, 44] or performing *in vivo* muscle experiments. Despite the apparent ubiquity of this regional variation, a complete understanding of the mechanisms underlying the dynamic variation and/or the functional ramifications of this heterogeneity is lacking.

(a) Regional variation in muscle activation patterns

Several aspects of neuromuscular function can vary with muscle region, whether the muscle is compartmentalized [45, 46] or not [15, 31]. For example, work by English [9]

highlighted the compartmentalization of the lateral gastrocnemius (LG) of the cat hindlimb with respect to activation. He found that more intense EMG activity was often observed in the distal compartments of the LG than the proximal compartments at slow locomotor speeds. However, activity in the proximal compartments equaled or surpassed that in the distal compartments at moderate to fast locomotor speeds. This not only highlights the functional complexity within a muscle, but also the context-dependent nature of this heterogeneity. More recent work by Wakeling [14] found that recruitment of different compartments within several human ankle extensors depends on the mechanics of movement. In this case, individuals were tested on a stationary bicycle at various pedaling frequencies and crank torques. Ultimately, this type of *in vivo* data will reveal how regional variation can change with demand, and whether there are commonalities among diverse groups of vertebrates.

Different regions of a muscle can be recruited based on their action at a specific joint. For example, the cat sartorius has two regions that control separate movements [47]. Based on activation patterns, the medial region provides the forces need to flex the hip and knee during the initial stages of the swing phase. However, the anterior region of the same muscle provides forces for hip flexion and knee extension. Thus, two regions of the same muscle can act in opposite ways at a single joint. This was also found by Higham and colleagues [8] in that a portion of the MG primarily exerts an extensor moment at the knee while another portion primarily exerts a flexor moment at the knee. This complexity in function has not received much attention, but highlights the potential for the division of labor within a given muscle.

Why would a single muscle exhibit regional variation in recruitment patterns? We discuss two possible explanations, including 1) regional variation in fibre type and 2) regional variation in branching patterns of motor neurons. Given that the ‘size principle’ states that slow oxidative (type Ia) fibres will be recruited prior to fast oxidative (type IIa) or fast glycolytic (type IIb) fibres, any region that is predominantly slow oxidative will be recruited in the absence of

activity in other regions under conditions of low demand (e.g. slow walking), setting up a situation of regional variation in activation. Several studies have examined the regionalization of fibre types within muscles, but few have correlated fibre type with differential activation patterns. In the rat medial gastrocnemius, for example, the proximal region contains predominantly fast-twitch oxidative fibres whereas the distal region is comprised of predominantly fast-twitch glycolytic fibres [48]. As highlighted in the next section, the regional gradient of slow oxidative fibres from deep to superficial areas of a muscle are common [49], leading to differential recruitment. In the pig masseter, histochemical fibre type was found to correlate with activation patterns [28]. Thus, it is likely that regional variation in activation will occur when there is variation in the distribution of fibre types.

Different parts, or compartments, of a muscle can receive input from motor neurons that are located in different regions of a motor nucleus. For example, the proximal compartment of the cat lateral gastrocnemius (LG) receives input from neurons that primarily occupy more rostral portions of the LG motor nucleus [50]. Whereas mostly large motoneurons innervate proximal compartments, the distal compartments receive input from both large and small motoneurons. Thus, the recruitment of a given area of muscle will depend on what region of the motor nucleus is activated, and the spatial pattern of motoneuron innervation in that region of muscle.

(b) Differential force generation and force-length relationships within muscles

Different parts of a muscle can vary in the way in which force is generated via several mechanisms. Different regions can exert different torques about a joint [33, 48, 51], but single muscles can also have multiple actions at a single joint [8, 47]. These differences can result from segregation of fibre types or segregation of motor units within vertebrate muscle (reviewed

in [49]). For example, it is relatively common to observe a decreasing gradient of slow-oxidative fibres from deep to superficial areas of a muscle. According to the size principal of recruitment, slow-oxidative fibres will be recruited prior to the faster fibres, which are located superficially. Thus, force will be transmitted from the active muscle fibers to the passive muscle fibers. The latter will therefore become a compliant structure that could be in parallel (as per the example just given) or in series (see below).

In addition, different parts of a muscle, if active at different times, can exert different torques at a given joint. For example, Carrasco et al. [33] studied the magnitudes and directions of torques exerted by four different compartments of the cat LG, and found that different compartments exerted significantly different pitch, yaw, and roll torques at the ankle joint. These compartments were located in different proximo-distal and medio-lateral regions. It was postulated by Carrasco et al. [33] that these neuromuscular compartments are important anatomical substrates that can be used by the nervous system to modulate the overall mechanical action produced by a muscle. How this mechanical regionalization relates to dynamic locomotor behavior is still unknown.

An interesting study by Turkawski and colleagues [52] determined whether individual motor units within the masseter muscle of the rabbit were capable of generating different force vectors, and whether different motor units types were distributed heterogeneously throughout the muscle. They found that the motor unit force decreased, on average, going from anterior to posterior in the muscle and from superficial to deep. The anterior region of the masseter produced the greatest forces. The torques produced by different regions of the muscle also differed. The largest torques, like forces, were produced by the motor units in the anterior superficial masseter, whereas relatively small torques were produced by the motor units in the posterior deep masseter. In terms of function, the distribution of torques and forces likely represents distinct roles within the masseter of rabbits. The superficial region of the muscle is

likely responsible primarily for generating large jaw closing moments, whereas the posterior deep masseter mainly functions in lateral jaw movements. Thus, a single muscle can exhibit functional segregation that corresponds with architectural and activation differences.

The medial gastrocnemius of rats is compartmentalized and exhibits considerable variation in function and architecture between these compartments. De Ruiter and colleagues examined the function and fibre type composition of the most proximal and most distal compartment of this muscle under *in situ* conditions [48]. The most proximal compartment is comprised of predominantly fast-twitch oxidative fibres whereas the distal compartment contained mainly fast-twitch glycolytic fibres. Each of these compartments was stimulated independently by isolating the branches of the sciatic nerve that served these regions. Interestingly, the force-length relationship of whole muscle was narrower when the proximal compartment was stimulated and maximum force was observed at shorter lengths for this compartment. As expected from fast-twitch glycolytic fibres, the maximum shortening velocity of the muscle was significantly higher when the distal compartment was stimulated. Although regional activation patterns have not been quantified for this muscle, it is postulated that the proximal compartment would be recruited under *in vivo* conditions when lower power outputs are required. In contrast, the distal compartment would become important during high power demanding activities. Taken together, these results highlight the variation in mechanical properties that can occur within single locomotor muscles. However, the functional importance of this regionalization is yet to be determined.

(c) Regional variation in strain within muscles: patterns and mechanisms

More recent work has highlighted the variable fascicle strain patterns that can occur within single muscle over a range of vertebrate and invertebrate taxa [8, 11, 12, 40, 42, 53].

Within the medial gastrocnemius of helmeted guinea fowl (*Numida meleagris*), the proximal region (closer to the knee) undergoes a stretch-shorten cycle when force is being generated during stance [7, 8]. In contrast, the distal region of the same muscle remains relatively isometric during the same period of time. It appears that these differences in muscle fascicle strain are not necessarily due to differences in activation intensity [8]. Instead, regional differences in stiffness and fiber type might drive differences in strain along the length of a muscle. The distal region of the MG of guinea fowl is associated with a broad aponeurosis, whereas the proximal region of the muscle lacks a significant external aponeurosis. Indeed, aponeuroses can act as stiff springs in both the longitudinal (parallel with the long axis of the muscle) and transverse (perpendicular to the long axis of the muscle) directions [54]. One potential explanation for heterogeneous fascicle strain within a muscle could be regional variation and prevalence of aponeuroses.

As highlighted by Blemker and Colleagues [44], variation in fascicle lengths and curvature of muscle fascicles can help explain heterogeneity in strain within the human biceps brachii. They used a 3D muscle model to interpret *in vivo* data obtained by Pappas and colleagues [11]. Although Blemker and Colleagues were able to explain the *in vivo* results using the model, they note that other factors, such as sarcomere popping, may contribute to strain heterogeneity. However, the latter normally occurs when muscles operate at extreme lengths on the descending limb of the force-length curve, rather than the ascending limb, which is where the biceps brachii typically operates [55]. Whatever the case, it is clear that the mechanisms underlying strain heterogeneity are multidimensional and require further investigation.

(d) Regional variation within muscles in relation to muscle fatigue

Given that muscles can exhibit regional variation in architecture and physiological properties, it is likely that muscle fatigue (or whole-body fatigue) will influence single muscles in complex ways. Indeed, De Ruiter and colleagues [48] found that the distal compartment of the rat medial gastrocnemius (MG) fatigued faster than the proximal compartment. This was likely due to the fact that the distal region was comprised of fast-glycolytic fibres whereas the proximal compartment contained fast-oxidative fibres. How this regional variation in the effects of fatigue influence the overall mechanics of the muscle under *in vivo* conditions is not fully understood. If a muscle is compartmentalized, with compartments in series responding differently to exercise-induced fatigue, then it is likely that the fatigued compartment will be become a passive element that can be pulled on from other, non-fatigued, compartments. This could significantly influence the overall length of the muscle in relation to its force-length curve, which might then lead to a sub-optimal active length. Whether muscles operate in different regions of their force-length curve during fatigue would be worthwhile to investigate in future work.

In a recent study, Higham and Biewener [53] examined the *in vivo* responses of different regions within a muscle to fatigue, finding that fascicle shortening in the proximal region of the MG of guinea fowl, but not the distal region, decreased significantly with fatigue. This is the first evidence that *in vivo* mechanical changes due to fatigue can vary between muscle regions. It is quite possible that this differential effect of fatigue is related to fibre type regionalization in the MG of guinea fowl given that recent work, using immunohistochemistry, indicates that the proximal region of the MG contains 100% fast-twitch fibres compared to 58% fast-twitch in the distal region (J.W. Hermanson, T.E. Higham & A.A. Biewener, unpublished data). However, Higham and Biewener [53] did not find a difference in EMG activity between the two regions as a result of fatigue, suggesting that factors downstream of the neuromuscular junction in the muscle fibres became impaired as a result of fatigue.

(f) Functional benefits of regional variation within muscles

It is important to note that the functional benefits of regional variation are not known, but will likely become apparent over the next few decades. However, it is likely that the benefits are multidimensional and that, in many cases, a functional benefit may not exist. Here we propose several possibilities that might suggest functional benefits of regional variation in activation, architecture, and contractile properties. These possibilities, of course, depend ultimately on the mechanism of the variation. For example, if the variable stiffness of aponeuroses results in stiffness differences across the muscle under *in vivo* conditions, then the effect of an aponeurosis on a muscle's regional contractile behavior first needs to be identified. In the case of the guinea fowl medial gastrocnemius, the distal region of the muscle is associated with a sheet of connective tissue, which increases the stiffness in that region [8]. Thus, the distal region remains relatively isometric, enhancing force generation while limiting work output. The increased stiffness in the distal region also enhances the muscle-tendon unit's ability to resist tensile forces, analogous to a tie rod.

Another functional benefit to heterogeneity is the ability of the nervous system to recruit different parts of a muscle that then might exert different torques about a given joint [33]. This could potentially give an animal an increased level of control over joint mechanics and an increased diversity of movements. Vertebrates can execute a number of dynamic locomotor movements, including jumping, turning, hopping, running, gliding, flying, swimming, and many others. Thus, it might be beneficial for an animal to have fine control over joint mechanics via differential recruitment of compartments that can produce different torques about a joint.

Finally, architectural diversity within a muscle might yield beneficial functions. For example, differences in fiber and/or fascicle length will potentially result in different force-length relationships between fibers. If this is the case, then different fibers will reach their optimal

length for force generation at different overall muscle lengths, which would effectively increase the plateau of the muscle force-length curve. This would lead to a more 'generalized' muscle in that it could operate more effectively over a variety of lengths and thus locomotor behaviors. Alternatively, muscles that are architecturally homogeneous would be more 'specialized' and would only be able to produce force effectively over a narrow range of lengths and ultimately conditions.

(g) Future directions

Given that motor units can be distributed in a non-random fashion within a muscle, and the fact that locomotion can vary (with respect to intensity and kinematics) drastically depending on the situation, it is not surprising that heterogeneity is a feature of muscle function. The main question that remains unanswered is whether this heterogeneity has adaptive significance or whether it is merely a byproduct of architecture and/or motor unit distribution. It is true that regional variation in other factors, such as the distribution of connective tissue, might suggest benefits to heterogeneity. If patterns of regional variation prove to be adaptive, then future work assessing the origins and consequences of regional variation across diverse taxa will yield important information regarding how complex systems evolve.

It is clear after more than 30 years of research that regional variation in architecture and function is a common feature of muscle biology. Now that the prevalence of this phenomenon is recognized, we now must work to understand regional variation in the context of natural dynamic locomotor behavior. Recent work has taken a step in this direction by quantifying activation and strain patterns within muscles under dynamic conditions that vary in demand [7, 14]. However, much like the work by Hoffer and colleagues [47], understanding how motor units are recruited under dynamic *in vivo* conditions will yield important information regarding how a

single pool of motor neurons can be used to control functional disparate regions of a muscle. This would lead to defining motor units based on their function *and* morphology, not just the latter. This will be particularly important for interpreting the role of multifunctional muscles that contain regions that might be more important for specific tasks.

Incorporating regional variation in architecture into three dimensional muscle models will provide a more sophisticated way of analyzing muscle injury [56]. The distribution of aponeurosis tissue throughout a muscle has a large impact on the strain distribution [8]. To link variation in aponeurosis with potential for injury, Rehorn and Blemker [56] constructed a finite element model of a human hamstring muscle, the biceps femoris longhead (BFLH), using magnetic resonance (MR) images. They discovered that muscles with one wide and one narrow aponeurosis are more likely to get injured than muscle with two wide aponeuroses. In areas where the aponeurosis is relatively narrow (proximal region near the myotendinous junction), BFLH strains are likely higher, which then increases the incidence of injury. Future work assessing *in vivo* strains in relation in aponeurosis width would confirm this.

Functional heterogeneity within muscles has been revealed for a limited number of vertebrate taxa, including cats [9], rats [41], pigs [28], guinea fowl [8], pigeons [10], desert iguanas [15], toads [13], and humans [14, 40]. Future work that focuses on exploring the diversity in heterogeneity will provide important information regarding the evolution of complex function within muscles. In addition, examining multiple species within a genus or family would facilitate linking relatively subtle differences in heterogeneity to differences in ecology, biomechanics, or limb morphology. By understanding the functional ramifications of heterogeneity, we will be better equipped to apply this to musculoskeletal models [43, 44] and *in vivo* experiments.

(i) A cautionary note for *in vivo* studies?

We propose that the questions being addressed in a given study will dictate the importance of the regional variation outlined in this paper. It is true, however, that determining if and how regional variation exists can only provide additional information, even if to highlight the lack of regional variation within a muscle [57]. We highlight three scenarios where quantifying regional variation will be important in future work. First, if the questions forming a study are related to *how* muscles work under *in vivo* conditions, then addressing regional variation in architecture and/or function will be important. For example, if one wishes to determine how much work a muscle does while an animal runs, it is increasingly evident that regional strain should be addressed. As highlighted by Higham et al. (2008), using only strain measurements in the proximal region of the MG of guinea fowl would result in an over-estimation of whole-muscle work, whereas a single measurement of strain in the distal region would result in an under-estimation. Thus, combining strain measurements in two or more locations would likely yield a more accurate measure of whole-muscle strain. A second situation in which regional variation will be important is when a study wishes to link limb kinematics with muscle strain [58]. It is possible for a part of a muscle to exhibit very little strain while another region undergoes a considerable amount of shortening or lengthening [8]. If *in vivo* measurements were taken only from the region that remained relatively isometric, and there were significant changes in joint angle, then one might conclude that a decoupling exists between joint movement and muscle strain. However, the conclusions would be quite different if measurements had only been obtained from the region that underwent a considerable amount of length change. A third scenario in which regional variation should be quantified is in studies that wish to use EMG signals to determine the recruitment of various fibre types. As highlighted above, muscles can exhibit considerable degrees of regional variation in fibre type composition. Thus, the signals obtained from a given EMG electrode will be linked to the regional variation within the muscle. In this case, it would be beneficial to understand the distribution of fibre types within the muscle of interest, and then sample from different regions under *in vivo* conditions.

In many cases, quantifying the patterns of activity (using EMG) that are recorded from many muscles simultaneously can provide a detailed picture of the relative activation patterns and hence muscle use [59-62]. In these cases, it is likely not feasible to assess variation within a single muscle given space, surgical, and data acquisition limitations. In addition, the *question* in these studies is predominantly focused on the inter-muscular or even inter-specific relationships rather than the specific functioning of a single muscle. Thus, while heterogeneity is likely prevalent in almost all terrestrial vertebrates, it is not always pertinent to a given study.

3. Inter-segmental connections between muscles: A case study using the helmeted guinea fowl, *Numida meleagris.*

(a) Introduction

Apart from the dynamic coupling of different limb segments that arises naturally from the multiarticular nature of a body [63], hindlimb muscles of vertebrates are often connected to others via several different mechanisms [22, 26, 27, 64]. First, synergists can join at a common tendon, thus exerting force at a common insertion [8]. Second, synergists can be connected in parallel via common aponeuroses along the length of the muscles [23-26], resulting in the transmission of forces via connections of the intact inter-muscular connective tissue network. Third, muscles can be connected in series across adjacent limb segments by fleshy connections or via connective tissue networks. This aspect of inter-muscular force transmission has arguably received the least amount of attention, yet, to the extent that it exists, likely has substantial effects on the *in vivo* function of muscles.

In guinea fowl, more than one of these in-series (and in-parallel) connections exist. As highlighted by Ellerby and Marsh [27], the flexor cruris lateralis pars pelvica (FCLP), flexor cruris lateralis pars accessoria (FCLA), and the gastrocnemius intermedia (GI) form a triarticular

346 complex. However, an additional complex exists between the iliotibialis cranialis (IC), iliotibialis
347 lateralis pars preacetabularis (ILPR), and medial gastrocnemius (MG) (Fig. 2). The latter
348 receives insertions from both the IC and ILPR. However, the MG itself is divided into sections
349 that act to flex the knee and a section that exerts an extensor moment at the knee [8]. The
350 latter section actually wraps around the lower limb and the knee, and this part of the MG is
351 where the IC and ILPR insert (see Fig. 2). The goal of this study was to explore the activation
352 and strain of these three muscles under *in vivo* conditions to assess potential functional
353 interactions (i.e. periods of co-activation) during running. We hypothesized that, while a period
354 of co-activation might occur, there would be tractable strain patterns that relate to the activation
355 of the muscles. In other words, if one muscle is active and shortening, then the other muscle in
356 series (if not active) will be lengthened by the in-series connection.

357 **(b) Methods and materials**

358 ***(i) Experimental subjects***

359 Four helmeted guinea fowl (*Numida meleagris* L.) of comparable size (average mass:
360 2.3 ± 0.2 kg) were used. This species is ideal for studies of animal locomotion as individuals
361 are easily trained to run on a treadmill and are capable of maintaining a high level of running
362 performance [7, 8, 65, 66]. All surgical and experimental protocols were approved by the
363 Harvard University Institutional Animal Care and Use Committee.

364 ***(ii) Surgical protocol***

365 The birds were anesthetized using an intramuscular injection of ketamine (20 mg/kg)
366 and xylazine (2 mg/kg). During the surgical procedures, subsequent anesthesia was
367 maintained at 1-2% isoflurane while monitoring the animal's breathing rate. Recording
368 electrodes and transducers were passed subcutaneously to the shank from a 1-2 cm dorsal
369 incision over the synsacrum. A second 4-5 cm incision was then made over the anterior and

distal portion of the upper limb. This exposed the IC and ILPR, and the electrodes and transducers were pulled subcutaneously through using this incision. A third 4-5 cm incision was then made on the lateral side of the right shank, overlying the division between the anterior and posterior muscular compartments, which exposed the lateral gastrocnemius. This incision was used to pull the electrodes and transducers down to the lower limb from the synsacrum. A fourth 4-5 cm incision was then made on the medial side of the right shank to expose the MG.

Sonomicrometry crystals (2.0 mm, Sonometrics Inc., London, Ontario, Canada) were implanted in the proximal region of the MG, which we will now refer to this as the pMG given that this region of the muscle has been shown to function differently from other parts of the same muscle [7, 8]. We also implanted the same sized crystals into the distal regions of the IC and ILPR (Fig. 2). Small openings in the muscle (approximately 3mm deep) were made using fine forceps, and the crystals were placed in these openings such that each crystal pair was aligned along a fascicle axis. The crystals were secured using 4-0 silk suture to close the muscle opening. In all muscles and locations, crystals were spaced approximately 10 mm apart.

Fine-wire (0.1 mm diameter, California Fine Wire, Inc., Grover Beach, California, USA) twisted, silver bipolar electromyographic (EMG) hook electrodes (0.5 mm bared tips with 1 mm spacing) were implanted using a 24 gauge hypodermic needle immediately adjacent to each pair of sonomicrometry crystals and secured to the muscle's fascia using 4-0 silk suture. Electrodes were implanted into the proximal and distal regions of the LG and MG.

All lead wires (from EMG and sonomicrometry) were pre-soldered to an insulated connector (Newark, Chicago, Illinois, USA). The connector was wrapped in duct tape and sutured to the skin of the back using 4-0 vicryl. Vetwrap™ (3M, St. Paul, Minnesota, USA) was then used to surround the lead wires and connector.

(iii) Experimental protocol

Following at least one night of recovery, animals ran on a level motorized treadmill at a speed of 2.0 m s^{-1}, which represents a run [21, 67, 68]. Each sequence was recorded in lateral view using a digital high-speed camera (Photron Fastcam 1024PCI, Photron USA Inc., San Diego, California, USA) at a rate of 250 frames s^{-1}. A trigger (post) stopped the camera recording and the voltage pulse from the trigger was used to synchronize the video with the *in vivo* muscle data.

Lightweight shielded cable (Cooner Wire, Chatsworth, USA) attached to the connector on the bird's back was attached to a Triton 120.2 sonomicrometry amplifier (Triton Technology Inc., San Diego, USA) and EMG amplifiers (Grass, P-511, West Warwick, USA). EMG signals were amplified 2000x and filtered (60 Hz notch, 100-3000 Hz bandpass) before sampling. Voltage outputs from these amplifiers were sampled by an A/D converter (Axon Instruments, Union City, USA) at 5000 Hz. Lengths recorded by the Triton sonomicrometer were adjusted by 2.7% to correct for the faster speed of sound in muscle versus water. Also, because the Triton filters introduce a 5 ms phase delay, all length measurements were corrected for this offset, as well as an offset (+0.82 mm) introduced by the faster speed of sound through the epoxy lens of each sonomicrometry crystal (see [48] for details). Following experiments, animals were euthanized with an intravenous (brachial) injection of sodium pentobarbital (120 mg/kg). Each muscle was dissected free to confirm placement of sonomicrometry crystals and EMG electrodes and to verify origins and insertions.

(iv) EMG analysis

EMG recordings for each stride cycle analyzed were first baseline-corrected. Several timing variables were quantified including onset, offset and duration. Determination of the onset and offset followed previous methods [69]. These timing variables were related to other key events, such as the time of force generation (measured for the MG previously).

418 ***(v) Sonomicrometry***

419 Sonomicrometry techniques and analyses followed previous studies [7, 8, 21, 57, 70].
420 Fractional length changes ($\Delta L_{seg}/L_o$) of the muscle's fascicles were calculated based on segment
421 length changes measured between the crystals (L_{seg}) relative to the resting length (L_o), which
422 was measured while the animal stood at rest. As a convention, shortening strains are negative,
423 and lengthening strains are positive.

424 ***(vi) Statistical Analyses***

425 We used a two-factor analysis of variance where individual and muscle were the
426 independent variables and factors related to muscle function (e.g. fascicle strain) were the
427 dependent variables. To account for multiple observations within each individual, the *F*-values
428 were calculated by dividing the main effect (e.g. muscle) by the interaction term involving
429 individual and the factor of interest (e.g. muscle x individual). Further details of this calculation
430 can be found in [71]. $P<0.05$ was used as the criterion for statistical significance in all tests.
431 SYSTAT version 9 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Unless
432 stated otherwise, all values are mean ± S.E.M.

433 **(c) Results**

434 ***(i) General patterns***

435 As highlighted in previous work [7, 8], pMG activity began within the 50 ms preceding
436 footfall. Following footfall, the pMG lengthened and then shortened (Fig. 3). For the remainder
437 of the stance phase, the pMG remained relatively isometric. Similarly, the IC and ILPR often
438 lengthened immediately following footfall, although this lengthening period was longer for the IC
439 than the other muscles. Muscle EMG patterns differed considerably between the three muscles
440 (Fig. 3). The IC was active primarily during the swing phase of the stride, whereas the ILPR

441 was commonly active during the latter half of the stance phase of the stride. The pMG was
442 active for the very last portion of the swing phase and then the first 50-70% of the stance phase.

443 ***(ii) Overlap in activity patterns and resulting length changes***

444 The pMG and the IC did not exhibit any overlap in EMG activity apart from a brief period
445 during mid-swing. The average overlap of EMG activity between the ILPR and the pMG was
446 34.4 ± 2.3 ms, and this occurred during the latter half of stance. During this period of
447 overlapping activity, the ILPR shortens by approximately 6%, whereas the pMG remains
448 essentially isometric (less than 1% change in length) (Fig 4). This difference in strain was
449 significantly different (ANOVA, $P<0.05$). Overlap in activity between proximal muscles and the
450 pMG did not occur during the initial part of stance (Fig. 3), indicating that these muscles are
451 relatively independent during this phase.

452 **(d) Discussion**

453 Our discussion focuses on the interactions between the ILPR and the pMG as this was
454 the only muscle combination to exhibit overlapping activity. Also, the connective tissue linking
455 these two muscles is more substantial than the connective tissue between the pMG and the IC.
456 During the overlap in activity in the latter half of stance, the ankle and knee are both being
457 extended [27, 67]. In accordance with this, previous studies indicate that there is an extensor
458 moment at the knee during this part of stance in guinea fowl [72] and turkeys [73]. Combined
459 with the fact that both of these muscles exert extensor moments at the knee, it is predicted that
460 shortening will occur in both the ILPR and the pMG. In addition, ankle extension would result in
461 shortening of the MG. Despite both of these kinematic predictors, the pMG remains relatively
462 isometric. What can explain the isometric behavior of the pMG? One explanation, which is
463 supported by our results, is that the shortening of the ILPR during this period is preventing the
464 pMG from shortening due to the connection between the muscles. This might help maintain an

optimal length of the MG while it is generating force. However, future work would be required to validate this explanation.

Although we predicted that the initial period of lengthening in the pMG might result from interactions with the ILPR or IC, this does not appear to be the case. Instead, the flexion of the knee that occurs during the initial half of stance in guinea fowl [67] likely results in stretching of this region while it is active given that the proximal region exerts a knee extensor moment. Thus, the strain patterns in the MG throughout a stride cycle are driven by multiple factors, including regional differences in architecture, interactions with other muscles, activation patterns, and joint kinematics. The relative importance of each factor is time-dependent, with intermuscular interactions being important during the latter half of stance.

Our study only examined locomotion on a level surface at 2 ms^{-1}. It is quite possible that the linkage between the ILPR and pMG provides functional flexibility under diverse conditions. Thus, we have only begun to understand how these muscles can interact. Under certain circumstances, for example, the overlap in activity might differ from that observed in the current study, which might be related to changes in functional demand. As suggested by Ellerby and Marsh [27], the presence of inter-segmental muscles complexes suggests that dividing a limb into segments might not be functionally relevant.

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Figure captions:

Figure 1. Schematic showing the control and feedback associated with terrestrial locomotion. Sensory input is integrated in the central nervous system, which then controls the pool of motor units in a given muscle. However, regional variation in motor unit (MU) recruitment (e.g. proximal or distal) will result in regional patterns of muscle work (force x fascicle strain). The dashed red lines highlight one scenario that would result in regional variation within a muscle. Collectively, the regional patterns of work will result in net work and net muscle force, which will

685 drive limb movement. However, work and force from other muscles can act to move the limb
686 (black arrow) or act on regions of other muscles (dashed blue arrow), highlighting inter-
687 segmental connections or the lateral transfer of force between muscles.
688
689 Figure 2. Schematic showing a lateral view of the left hindlimb of a helmeted guinea fowl. The
690 proximal portion of the medial gastrocnemius is shown wrapping around the leg and receiving
691 insertions from the ILPR and IC.
692
693 Figure 3. Representative fascicle length change patterns (A) and muscle activity patterns (B, C,
694 & D) for two consecutive strides of a guinea fowl running steadily at 2 m s^{-1} on a level motorized
695 treadmill. The pMG (blue), IC (black), and ILPR (red) are all shown. The initial footfall occurs at
696 0 ms and the stance phases are represented by the shaded areas.
697
698 Figure 4. Average fascicle strain (% of resting length) for the pMG (left) and ILPR (right) during
699 the period of co-activation during the latter half of stance. There was a significant difference in
700 strain between the two muscles (ANOVA; $P<0.05$).

Figure 1

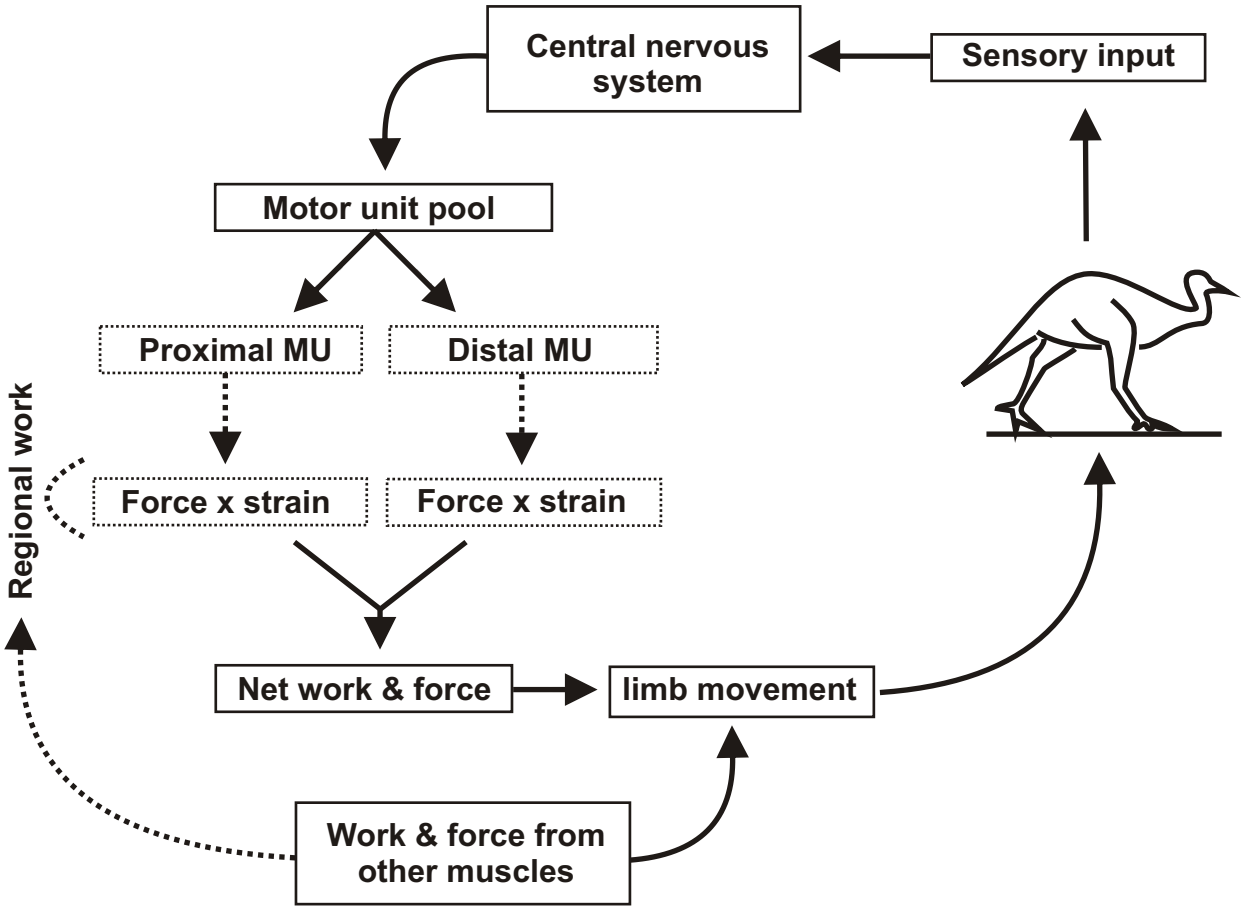

Central nervous system
Sensory input
Motor unit pool
Proximal MU
Distal MU
Regional work
Force x strain
Force x strain
Net work & force
limb movement
Work & force from other muscles

Figure 2

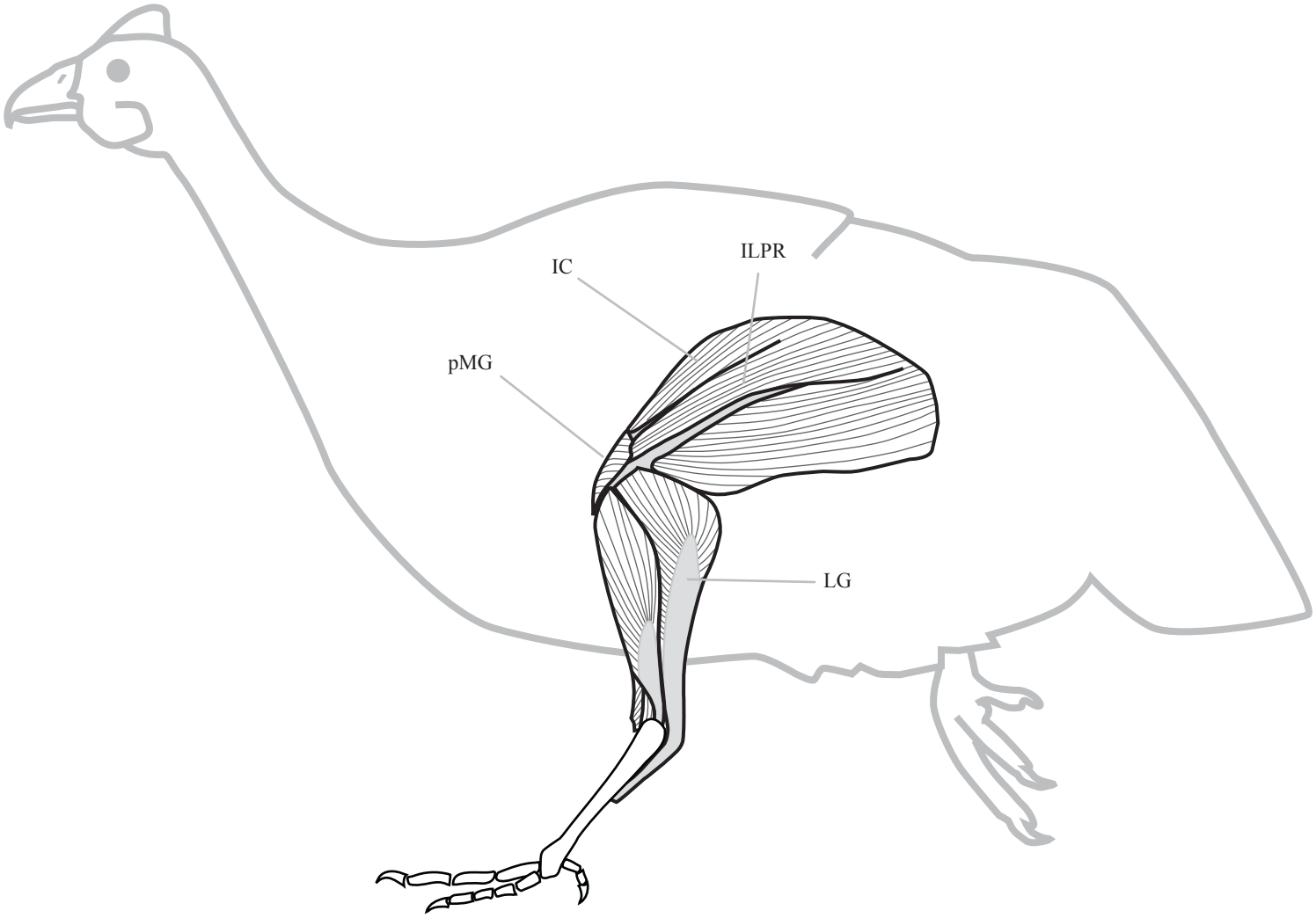


Figure 3

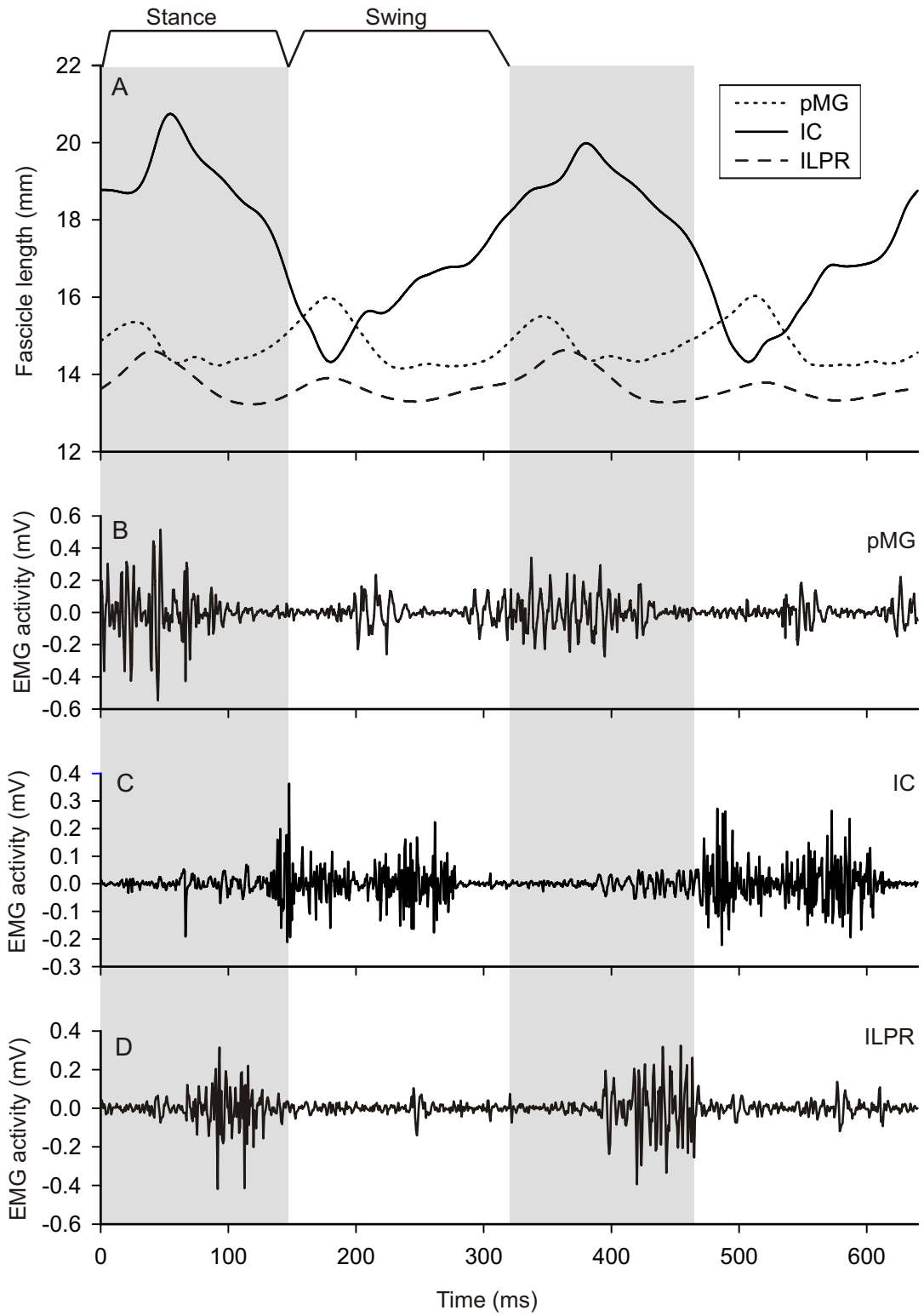
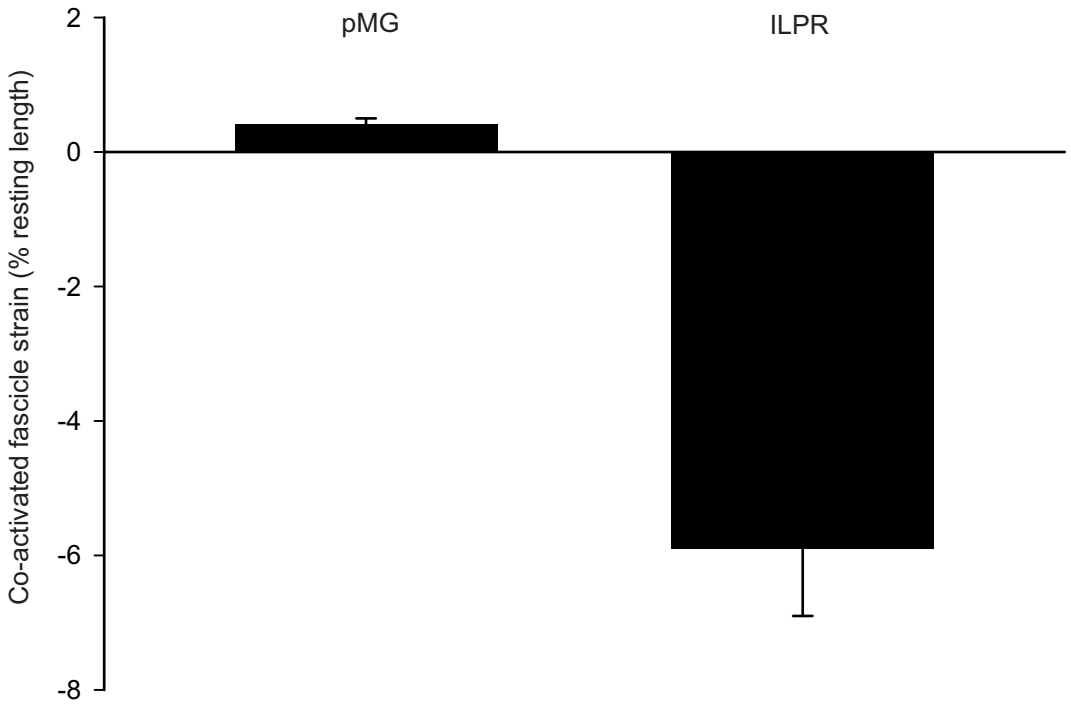

Stance
Swing
A
pMG
IC
ILPR
Fascicle length (mm)
22
20
18
16
14
12
B
pMG
EMG activity (mV)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
C
IC
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
D
ILPR
0.4
0.2
0.0
-0.2
-0.4
-0.6
0
100
200
300
400
500
600
Time (ms)

Figure 4


pMG
lLPR
2
0
-2
-4
-6
-8
Co-activated fascicle strain (% resting length)