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Gaining New Understanding of Sarcomere Length Nonuniformities in Skeletal Muscles

Li, Meng

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Gaining New Understanding of Sarcomere Length Nonuniformities in Skeletal Muscles

by

Meng Li

A THESIS

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Abstract

Sarcomere lengths are non-uniform on all structural levels of mammalian skeletal muscle. These non-uniformities have been associated with a variety of mechanical properties, including residual force enhancement and depression, creep, increased force capacity, and extension of the plateau of the force-length relationship. However, the nature of sarcomere length non-uniformities has not been explored systematically. The purpose of this study was to determine the properties of sarcomere length non-uniformities in active and passive muscle. Single myofibrils of rabbit psoas (n=20; with 412 individual sarcomeres) were subjected to three activation/deactivation cycles at short, intermediate, and long sarcomere lengths of 2.7, 3.2, and 3.6 µm respectively, and individual sarcomere lengths were measured at 4 passive and 3 active points during the activation/deactivation cycles. The primary results were that sarcomere length non-uniformities did not occur randomly but were governed by some structural and/or contractile properties of the sarcomeres and that sarcomere length non-uniformities differed greatly between the active and passive state. We propose that the mechanisms that govern the systematic sarcomere lengths non-uniformities observed in active and passive muscle are associated with the variable number of contractile proteins and the variable number and stiffness of titin filaments in individual sarcomeres.
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**List of Symbols, Abbreviations and Nomenclature**

<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>SL</td>
<td>Sarcomere Length</td>
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<tr>
<td>SLNU</td>
<td>Sarcomere length nonuniformity</td>
</tr>
<tr>
<td>SLNUs</td>
<td>Sarcomere length nonuniformities</td>
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<tr>
<td>FC</td>
<td>Force creep</td>
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<tr>
<td>FLR</td>
<td>Force length relationship</td>
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<td>RFE</td>
<td>Residual force enhancement</td>
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<td>PFE</td>
<td>Passive force enhancement</td>
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<td>FD</td>
<td>Force depression</td>
</tr>
<tr>
<td>Ig domain</td>
<td>Immunoglobulin domains</td>
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<tr>
<td>PEVK</td>
<td>proline (P), glutamate (E), valine (V) and lysine (K)</td>
</tr>
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Chapter One: Introduction

A sarcomere is the smallest functional unit for muscle force production. Sarcomere length is an important outcome measure for the understanding of functional properties of skeletal muscle. Given the muscle hierarchy from the whole muscle to the single sarcomere, studies have been carried out towards understanding the association of muscle structure and its functional properties. In 1954, Andrew Huxley and Hugh Huxley and collaborators independently postulated that muscle contraction is caused by the relative sliding of two sets of filaments: actin and myosin (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954). Andrew Huxley then derived a theory of how the relative sliding between actin and myosin could be occurring. This resulting so-called cross-bridge theory suggests that the relative sliding of the two sets of filaments is achieved by the cyclic attachment and detachment of myosin-based cross-bridges to specific binding sites on the actin filaments (Huxley, 1957). When a sarcomere is maximally activated, the active force produced varies as a function of the sarcomere length which is explained by changes in the overlap region between the thick (myosin) and thin (actin) filaments (Gordon et al., 1966b).

It has been known for a long time that sarcomeres in vertebrate muscles are non-uniform in length (Llewellyn et al., 2008; Moo and Herzog, 2018; Moo et al., 2017). Sarcomere lengths non-uniformities have not only been observed in passive (e.g., Huxley and Peachey, 1961; Johnston et al., 2016; Llewellyn et al., 2008; Pincheira et al., 2021) but also in active muscles (Moo et al., 2017) and in fully activated single myofibrils during isometric (Johnston et al., 2016) and after eccentric contractions (Johnston et al., 2019). Sarcomeres lengths also change when a muscle is activated or stretched and these length changes differ between sarcomeres (Edman, 1980; Edman & Flitney, 1982; Edman & Reggiani, 1984).

Sarcomere length non-uniformity has been associated with a variety of functional mechanical properties of skeletal muscle, such as force creep (FC) (Gordon et al., 1966a,b; Julian and Morgan, 1979 ; Günzel and Rathmayer, 1994), residual force depression (RFD) and residual force enhancement (RFE) (Morgan, 1994; Morgan, 1990). However, others have not been able to associate specific mechanical properties, such as RFE, RFD with the non-uniformity of sarcomere length (Granzier & Pollack, 1989; Herzog & Leonard, 1997; Johnston et al., 2016; Joumaa et al., 2008b; Lee & Herzog, 2003; Peterson, 2004). Despite the generally accepted observation that vertebrate skeletal muscles have large sarcomere length non-uniformities in the passive and active state, and the functional importance associated with these sarcomere length non-uniformities, the causes underlying sarcomere length non-uniformities remain unexplored. In fact, there is no evidence to indicate if sarcomere length non-uniformities are primarily caused by passive structural proteins, differences in contractile proteins, or the result of purely random processes. Understanding the cause of sarcomere length non-uniformity and identifying the possible functional implications for muscle
contraction may help understand how muscles work and how mechanical properties are affected by sarcomere length non-uniformity. Hence, the purpose of this thesis was to determine if sarcomere length non-uniformity occurs in a predictable, repeatable or in a purely random manner, and try to explain the mechanisms that may cause sarcomere length non-uniformities in active and passive muscles and myofibrils.

In chapter 2, I will provide some of the background of this study through a review of the relevant literature. In chapter 3, I will describe the methodology of the experimental study. In chapter 4, I will show the results of all experiments and in chapter 5, I will discuss the results, talk about the limitations of the study, and provide some direction for possible future research in this area of science. The thesis concludes with an attempt to draw general conclusions from the results of this work.

**Chapter Two: Literature Review**

**2.1 Structure of Muscle**

Skeletal muscles may be considered as consisting of layered structural units of decreasing size. Muscles are generally surrounded by connective tissue layers or fascia and the connective tissue layer closest to the muscle is referred to as the epimysium. The epimysium consists primarily of a collagenous, reticular and elastic fibre network, connective tissue cells and fat cells (Frontera and Ochala, 2015). Muscles are composed of muscle bundles or fascicles, which are separated by another layer of connective tissue called the perimysium. Fascicles in turn are made up of individual muscle cells, in muscles also called fibres, that are connected to each other by another connective tissue layer, the so-called endomysium. Muscle fibres are surrounded by a cell membrane, which in the case of skeletal muscles is called the sarcolemma, “sarco” from the Greek for “flesh” and “lemma” from the Greek for “sheath”. The arrangement of the fibres in a fascicle can influence the functional properties of a muscle (Herzog and Nigg, 2006).

The muscle fibre is made up of parallel-arranged myofibrils. Myofibrils are sub-cellular organelles, which have a striated pattern that is visible under light microscopy. The dark (anisotropic or A-) and the light (isotropic or I-) bands are areas covered by the thick (myosin) filaments and the thin (actin) filaments, respectively (Figure 2.2). Myofibrils are comprised of serially arranged sarcomeres, and individual sarcomeres are bordered by a dark connective tissue structure referred to as the Z-line (from the German “Zwischenscheibe”). There are two contractile proteins in the sarcomere, myosin and actin. Myosin filaments are typically centred in the sarcomere, while actin filaments are attached to the Z-lines at either end of the sarcomere and from there reach towards the centre of the sarcomere. At a given sarcomere length, actin and myosin filaments typically overlap and thus myosin based cross-bridges can attach to the actin filament and pull it past the myosin filament. In the centre of the A-band, there is a fine line called the
M line that separates the sarcomere into two “axisymmetric” halves. In a cross-section of a vertebrate sarcomere in the region of actin and myosin filament overlap, one can observe that each thick filament is surrounded by six thin filaments giving a ratio of 1 myosin filament for 2 actin filaments.

![Figure 2.1 Schematic image of muscle hierarchy. Adapted from Herzog and Nigg (2006) with permission of Wiley.](image)

The main constituents of thick filaments are myosin molecules, which have a diameter of approximately 16 nanometers (~16nm). Myosin molecules contain a long tail portion composed of light meromyosin and a globular head attached to the tail composed of heavy meromyosin (Figure 2.3A). The myosin filament is in the centre of the sarcomere and in mammalian species is about 1650 nm. The globular head of myosin has

![Figure 2.2 Schematic of sarcomeres (between Z-lines) and its proteins. The myosin filaments of rabbit psoas are 1.65 µm in length, and the actin filaments are 1.08 µm (Herzog et al., 1992). Adapted from Vedantu.com with permission.](image)

### 2.1.1 Thick filament

The main constituents of thick filaments are myosin molecules, which have a diameter of approximately 16 nanometers (~16nm). Myosin molecules contain a long tail portion composed of light meromyosin and a globular head attached to the tail composed of heavy meromyosin (Figure 2.3A). The myosin filament is in the centre of the sarcomere and in mammalian species is about 1650 nm. The globular head of myosin has
an enzymatic site for catalyzing the hydrolysis of ATP (adenosine triphosphate), and it is this head, typically referred to as cross-bridge, that can attach to the thin filament. Cross-bridges on the thick filament are offset longitudinally by about 14.3 nm and 60 degrees. Since cross-bridges come in pairs that are 180 degrees offset at each location on the thick filament, one can estimate that each half myosin (in a half-sarcomere) contains about 96 cross-bridges (Figure 2.3). Each side piece has two cross-bridge heads, and there are approximately 16 side pieces available for interaction with one of the six thin filaments that surround each thick filament (Figure 2.3C).

Figure 2.3 Thick filament organization (Manuel Megías Pacheco@Pilar Molist García Manuel Ángel Pombal Diego with permission)

2.1.2 Thin filament

The thin filament is mainly made up of actin and it extends from the Z line towards the centre of the sarcomere (Figure 2.2). In contrast to the thick filament, which seems to be of a defined length for most vertebrate species, the thin filament length varies. For example, thin filament length is about 925nm in frog muscles (Page and Huxley, 1963) and about 1270nm in human muscles (Walker and Schrodt, 1974). A thin
filament is composed of two chains of serially linked actin globules of about 6nm diameter. Thin filaments also contain so-called regulatory proteins, tropomyosin and the tri-complex of troponin. Tropomyosin lies in the groove formed by the two actin filament chains (Figure 2.4). Troponin I, T and C appear in a semi-regular pattern about every 35 to 38 nm along the long axis of the actin filament. Troponin I blocks the attachment site for cross-bridges on actin in the relaxed state, troponin T is bound to the tropomyosin, and troponin C is the regulatory protein that binds calcium upon muscle activation, causing a configurational change in the troponin/tropomyosin complex that allows for cross-bridge attachment to the actin filament (Henderson et al., 2017; Farah and Reinach, 1995).

Figure 2.4 Organization of tropomyosin, troponin, and actin filaments. (Manuel Megías Pacheco@Pilar Molist García Manuel Angel Pombal Diego with permission)

2.1.3 Titin

At 3.0 - 3.7 MDa titin is the biggest protein in the natural world and is an important polypeptide protein chain in the sarcomere (Maruyama et al., 1977). Titin extends from the Z-line to the M-line and its length changes with changes in muscle/sarcomere length (Maruyama et al., 1977). Titin is thought to be rigidly connected to the thick filament, and thus is considered inextensible in the A-band region of sarcomeres (Figure 2.2) (Granzier and Wang, 1993). In the I band region of sarcomeres, titin is extensible and composed of immunoglobulin domains (Ig domain) and insertions with a characteristic sequence of proline (P), glutamate (E), valine (V) and lysine (K). This so-called PEVK domain is flanked by a proximal and a distal Ig region (Figure 2.5). Titin has different isoforms in different muscles that have different molecular weights and different mechanical properties (Neagoe et al., 2003; Prado et al., 2005; Tsiros et al., 2022). In rabbit psoas, the muscle used in this study, there are 70 tandem Ig repeats and about 1400 residues in the PEVK segment in the I band region of titin. In the human soleus, the I band titin is made up of 90 tandem Ig repeats and 2174 PEVK residues. The size of titin and its variation in the number of Ig domains is directly related to the slack length of titin and the corresponding slack length of sarcomeres. The differential splicing of the PEVK sequence is thought to directly relate to the passive stiffness of sarcomeres within their functional range (Linke et al., 1996).
There are two connected spring-like elements: a series of tandem immunoglobulin (Ig) segments and the PEVK domain, both separated by the N2A region.

### 2.1.4 Other proteins

Myomesin have been shown to connect to titin thus integrating the structural elements of the M-band (Agarkova et al., 2003; Lange et al., 2020). As a member of the Ig superfamily, myomesin is mainly made up of Ig-like and fibronectin type III domains. Proteins in the M-band have been shown to play dynamic roles in mediating interactions with myosin. In addition to the proteins in the M-band, nebulin is localized along the thin filament, myotilin and α-actinin exist in the Z disc, with α-actinin functioning as an actin filament cross-linker and a cross-linker for other proteins in the sarcomere (Agarkova et al., 2003.; Henderson et al., 2017; Lange et al., 2020).

### 2.2 The Sliding Filament and the Cross-bridge Theory

In 1954, H.E. Huxley and Hanson introduced the sliding filament theory (Huxley and Hanson, 1954). The idea was that muscle contraction occurred through the sliding of two sets of filaments relative to each other: the actin filaments moving past the myosin filaments when a muscle changes its length. In that same year, A.F. Huxley and Niedergerke suggested that the sliding of the thick and thin filaments may be caused by linkages from the myosin filaments that attach cyclically to the actin filament and pull actin past myosin (Huxley and Niedergerke, 1954). In 1957, A.F. Huxley proposed the structure of a sarcomere, and introduced a mathematical description of the interaction between the actin and myosin filaments that allowed for muscle force production and contraction, and allowed for precise predictions of force, stiffness, and metabolic cost of a muscle for defined contractile conditions. A.F. Huxley proposed that there were side-pieces of the thick (myosin) filament (cross-bridges) that interact cyclically with specific points of attachment on the thin (actin) filament. Cross-bridge attachment to actin was controlled via a set of attachment and detachment rate constants, and force was proportional to the cross-bridge’s distance from its neutral equilibrium position. This 1957 description of muscle contraction is considered the first attempt of defining muscle contraction on the molecular level. It is referred to as the cross-bridge theory. The main points of this theory are shown in Figure 2.6.
Huxley’s theory proposed that cross-bridges (M, Figure 2.3) attach to actin at designated attachment sites, A, and tend to pull actin towards the centre of the sarcomere. Attachment and detachment of the cross-bridges is defined by rate functions which Huxley called f and g, respectively. These rate functions were assumed to depend exclusively on Huxley’s “x” distance, a variable that was defined as the distance from the cross-bridge equilibrium position (O) to the nearest eligible actin attachment site (A). Note that the rate functions for attachment and detachment are defined asymmetrically around O so that a cross-bridge upon initial attachment can only produce a tensile force but not a pushing force.

*Figure 2.6 Illustration of the cross-bridge theory.* The rate constants of attachment and detachment between A and M are represented by functions f and g, respectively. Initially, because of thermal agitation, the cross-bridge M is thought to oscillate around its equilibrium position, O. Adapted from [Fukutani and Herzog, 2019](#) with permission.

In 1965, Reedy and his co-workers found that in insect flight muscle most of the cross-bridges were perpendicular to the actin and myosin long axes, but they became angled at about 45° to the myosin filament long axis in the rigor state [Reedy et al., 1965](#). Based on this observation, and work on the changing lattice spacing with muscle/sarcomere length, H.E. Huxley proposed that the force produced by cross-bridges is caused by cross-bridge rotation from a perpendicular (90°) to a 45° tilted position [Huxley, 1969](#). A.F. Huxley and Simmons added additional details to the rotating cross-bridge model suggested by H.E. Huxley based on quick shortening experiments of intact frog fibres. They also proposed a mathematical theory for the rotating cross-bridges based on their observations of a so-far unexplained rapid force recovery followed by a much slower force recovery observed in their quick release experiments [Huxley & Simmons, 1971](#). The new theory suggested multiple attachment states of the cross-bridges that were achieved by rotation of the cross-bridge head along the actin filament (Figure 2.7)
Figure 2.7 Multi-state model of cross-bridge attachment (Huxley and Simmons, 1971). $S$ (represents the number of possible attachment sites, $M$, on the cross-bridge: $M_1$, $M_2$ and so on). The number of stable attachment positions is $(S-1)$. There are corresponding attachment sites, $A$, on the actin filament ($A_1$, $A_2$ and so on). There is a difference in affinity between two neighboring MA sites (e.g., affinity: $M_1A_1 < M_2A_2 < M_3A_3 < M_4A_4$), which tends to rotate the head from the $M_1A_1$ to the $M_4A_4$ position. $\theta$, is the cross-bridge rotation angle indicating the amount of rotation of the cross-bridge in a given attachment cycle Adapted from Huxley and Simmons, 1971 with permission.

The 1971 cross-bridge model was further refined by Rayment et al. in 1993. Using high resolution x-ray diffraction descriptions of the atomic structure of the cross-bridge and the actin attachment site in different states, they proposed that part of the cross-bridge attached to actin in a fixed configuration, while only part of the cross-bridge rotated thereby producing filament sliding and force (Rayment et al., 1993). Rayment and colleagues also proposed a defined relationship between the mechanical states of the cross-bridge and the corresponding biochemical states associated with ATP hydrolysis. Briefly, they proposed that ATP was hydrolyzed (ATP – ADP· P) while the cross-bridge was in the detached state. Following ATP hydrolysis, the cross-bridge was thought to attach in a first (weakly) bound state to actin. Phosphate was then released allowing for the cross-bridge head to rotate and produce force and filament sliding, followed by ADP release leaving the cross-bridge in the (attached) rigor state. Attachment of a new ATP then allowed for detachment of the cross-bridge from actin and the cross-bridge cycle could start over again with ATP hydrolyzation (Rayment et al., 1993).

2.3 The Force-length Relationship

It has been known for a long time that force capacity in a muscle depends on muscle length (e.g., Blix, 1894). This so-called force-length relationship describes the maximal, active, isometric force a muscle can produce as a function of its length. Following the discovery of the cross-bridge theory, there was a molecular explanation for the force dependence on muscle length. In 1966, Gordon, Huxley and Julian quantified the relationship between the maximal, active, isometric force as a function of sarcomere length using intact single muscle fibres from frogs (Figure 2.5; note that Figure 2.5 is drawn for the actin and myosin filament length of rabbit psoas and thus differs slightly from that originally found for frog fibres). The force-length relationship contains a steep and shallow ascending segment (A-C Figure 2.8), a plateau region where force
is maximal and does not vary with changing muscle/sarcomere length (C-D, Figure 2.8), and a descending limb (D-E, Figure 2.8). Gordon et al. (Gordon et al., 1966b) associated the various segments of the force-length relationship with the overlap between the actin and myosin filaments, and thus, the number of cross-bridges that could attach to actin, which corresponds to the sarcomere length.

Starting at point E (Figure 2.8), there is no overlap between actin and myosin filaments, thus no cross-bridge formation is possible between the two filaments and active force is zero. The sarcomere length corresponds to twice the actin filament length (1.08 µm), plus the myosin filament length (1.65 µm), plus the Z-line width (0.1 µm), or, for rabbit psoas muscle, 2·(1.08) + 1.65 + 0.1 = 3.91 µm. Shortening of the sarcomere from E to D (Figure 2.8) is associated with a linear increase of the amount of overlap between actin and myosin filaments and a corresponding linear increase in the number of possible cross-bridge formations, and thus, force. The sarcomere length at point D corresponds to twice the actin filament length (2.16 µm) plus the Z-line width (0.1 µm), plus the width of the bare zone in the centre of the myosin filament (0.17 µm), the zone where there are no cross-bridges, or 2.16 + 0.1 + 0.17 = 2.43 µm. The plateau of the force-length relationship, sometimes also referred to as the optimal sarcomere/muscle lengths is the length between points C and D (Figure 2.8). The active isometric force in the plateau region is constant and maximal since the overlap between actin and myosin filaments allows for all myosin cross-bridges to attach to actin.

The ascending limb of the force-length relationship (points A to C, Figure 2.8) is associated with a decrease in active force capacity for reasons that are not fully clear. However, the following suggestions have been made that may explain the force decrease for lengths below the optimal sarcomere length: interference of cross-bridge attachment caused by the actin filament overlap from both sides of the half-sarcomere (Epstein, 1998), decreased calcium release at short length causing sub-maximal activation (Fabiato and Fabiato, 1977; Rüdel and Taylor, 1971), forces by structural proteins that tend to oppose cross-bridge forces (Gordon et al., 1966b; Herzog and Nigg, 2006), deformation of the myosin filament at sarcomere lengths below about 1.75 µm (Gordon et al., 1966b), and cross-bridge attachments to actin filaments from the opposing side of the sarcomere (Gordon et al., 1966b; TrombitÁs and Tigyi-Sebes, 1984).

The sarcomere force-length relationship depends on actin and myosin filament lengths, which vary across species. For example, in vertebrate skeletal muscles, myosin filament lengths are about 1.65 µm (Walker and Schrod, 1974), while in insect flight muscles, they are almost twice as long (about 3.0 µm) (Beinbrech and Ader, 2006). Similarly, actin filament lengths vary greatly, and even vary among vertebrate species. For example, actin filament lengths are about 0.925 µm in frog muscles, 1.09µm in rat muscles, 1.12µm in cat muscles, 1.27µm in human muscles, and 1.08µm in rabbit psoas muscles (Page and Huxley, 1963; Walker and Schrod, 1974; Herzog et al, 1992).
2.4 History Dependence of Muscle Force

Although the sliding filament and cross-bridge model well explain the force capacity during isometric and shortening muscle contractions, Huxley (1957) predicted that the maximal force during lengthening would be about 5 times greater than the maximal isometric force. However, experimental evidence observed that this force in eccentric (lengthening) muscle contraction is maximally about 2 times the maximal isometric force. Huxley realized that the predictions of his theory were not correct for eccentric actions, and suggested that this error could be corrected by assuming that cross-bridge attachments might be “mechanically broken” in eccentric actions of muscles (Huxley, 1957). Huxley also stated in later publications that there might be some as of yet unknown feature that allows muscle force during lengthening to be predicted accurately (Huxley, 1980). Prior to the development of the cross-bridge theory, extra force following lengthening muscle contractions had already been observed. For example, Abbott and Aubert described extra force following stretching of different muscles that could not be readily accounted for, either with theories available at the time, nor with the newly developed cross-bridge theory (Abbott and Aubert, 1952). This extra, isometric force at steady-state following stretching of an active muscle was later termed residual
force enhancement or RFE (e.g., Edman et al., 1982). Residual force enhancement was not only observed in whole muscle preparations, but also in single fibre (e.g., Edman and Tsuchiya, 1996; Edman et al., 1982; Herzog and Leonard, 2002; Rassier et al., 2003a) myofibril (e.g., Joumaa et al., 2008; Leonard et al., 2010; Rassier et al., 2003b) and isolated sarcomere preparations (first done by Leonard et al., 2010). Similarly, when an active muscle is shortened, its active, isometric, steady-state force following shortening is smaller than a purely isometric contraction at the corresponding lengths. This history dependent effect is typically referred to as residual force depression (e.g., Abbott and Aubert, 1952; Herzog and Leonard, 1997; Maréchal and Plaghki, 1979).

2.4.1 Residual force enhancement

Residual force enhancement is defined as the increase in steady-state, isometric force of a muscle following active stretching, compared to the corresponding (same length and same activation) force obtained during a purely isometric reference contraction (Edman et al., 1982). RFE is often (but not always) accompanied by an increase in passive force, referred to since its discovery in 2002, as passive force enhancement (Herzog & Leonard, 2002; Lee & Herzog, 2002).

The magnitude of the RFE increases with increasing stretch amplitude (Edman et al., 1982; Hisey et al., 2009; Morgan et al., 2000), depends on the region of the force-length relationship (Edman et al., 1982; Morgan et al., 2000), but is typically not found to be associated with the speed of stretch or a change in the stiffness of the muscle (Edman et al., 1978; Morgan et al., 2000). RFE can be abolished instantaneously by deactivating the muscle (Abbott & Aubert, 1952; Morgan et al., 2000) and is associated with a distinct decrease in metabolic energy required per unit of force (Joumaa and Herzog, 2013).

![Figure 2.9 Residual and passive force enhancement increase as the magnitude of stretch increases. Cat soleus muscle 35 deg C. (Herzog, 2019; Herzog & Leonard, 2005; Hisey et al., 2009 with permissions). Yellow line: + 0mm stretch (isometric); blue line: + 3mm stretch; red line: + 9mm stretch. 0 mm on the length axis indicates the optimal length of the muscle.](image-url)
2.4.2 Force depression

Force depression is defined as the decrease in active, isometric, steady-state force following active muscle shortening compared to the corresponding (same muscle length, same activation) purely isometric force (Abbott and Aubert, 1952; Sugi and Tsuchiya, 1988). The amount of force depression increases with increasing amounts of shortening, and increasing force during shortening (Abbott & Aubert, 1952; Herzog & Leonard, 1997; Maréchal & Plaghki, 1979), and thus is often thought to be directly dependent on the mechanical work performed by the muscle in the shortening phase (Herzog et al., 1998, 2000; Kosterina et al., 2009).

Several mechanisms have been proposed to explain residual force enhancement and force depression. In 1953, Hill introduced the notion of instability of muscle contraction on the descending limb of the force-length relationship. He suggested that the instability may cause non-uniformities in muscle segment lengths which causes the observed force depression following muscle shortening (Hill, 1953; Morgan et al., 2000). Granzier and Pollack (1989) later associated force depression with a fatigue-like process caused by free phosphate and hydrogen ion accumulation in the shortening phase of muscle contraction (Granzier and Pollack, 1989). In 1996, Edman proposed that force depression may be related to an inhibition of calcium binding to the regulatory proteins on actin during the shortening phase of muscle contraction resulting in an inhibition of cross-bridge attachment to actin (Edman, 1966). However, all these proposed mechanisms were shown to be untenable. The most acknowledged mechanism for residual force depression was proposed by Maréchal and Plaghki (Maréchal and Plaghki, 1979). These scientists suggested that residual force depression was caused by a stress-dependent inhibition of cross-bridge attachment. Research in which stiffness and force depression were measured simultaneously (e.g., Joumaa et al., 2012), or where cross-bridge binding to actin was directly quantified using x-ray diffraction (e.g., Joumaa et al., 2021) seemed to confirm that indeed, force depression was caused by a decrease in the proportion of attached cross-bridges. However, the mechanisms by which such cross-bridge “inhibition” occurs remain far from clear.

2.5 Sarcomere Length Non-uniformity

2.1.1 Description of sarcomere length non-uniformity

Sarcomere length non-uniformities (SLNUs) is the phenomenon observed in active and passive vertebrate skeletal muscles where neighbouring sarcomere lengths differ by a substantial amount. For example, in whole muscle, sarcomere length for a small sample of about 30 sarcomeres in series can easily differ by 1.0 \( \mu \text{m} \) or about 40% of the optimal sarcomere length (Llewellyn et al., 2008; Moo et al. 2016). Such sarcomere length non-uniformities are observed at all structural levels of muscle, ranging from single myofibrils (Bartoo et al., 1993; Johnston et al., 2016), single fibres (Edman & Reggiani, 1984; Iwazumi & Pollack, 1981; Julian & Morgan, 1979) to entire muscles (Llewellyn et al., 2008; Moo and Herzog, 2018; Moo et al., 2016; Moo et al.,
However, the factors underlying these sarcomere length non-uniformities, the functional significance of the non-uniformities, or whether these non-uniformities occur randomly or are deterministically defined, remains unknown. Noticing that sarcomere length non-uniformities have been associated with a variety of functional properties of muscle, but without compelling evidence, makes the quantification and study of sarcomere length non-uniformities important and intriguing.

2.1.2 The problem of sarcomere length non-uniformity

It has been widely accepted that the sarcomeres are non-uniform in vertebrate skeletal muscles (Bartoo et al., 1993; Gordon et al., 1966a; Huxley & Peachey, 1961; Julian & Morgan, 1979). However, it is also known that this is not the case for all muscles. For example, indirect flight muscles of insects have virtually perfect sarcomere lengths in the passive and active state, and also following active shortening and stretching (Blyakhman et al., 2001).

Sarcomere length non-uniformities occur in active and passive vertebrate skeletal muscles, but they seem to differ in the active and passive states (e.g., Johnston et al., 2016; Moo et al., 2017). Why some muscles have evolved with essentially uniform sarcomere lengths (insects) and others have evolved with great non-uniformities (vertebrate muscles) is not known and has received little attention, despite the presumed importance of sarcomere length non-uniformities for many mechanical properties.

To the best of our knowledge, there have been no systematic studies aimed at investigating how sarcomere length non-uniformities are caused. Furthermore, although it has been well-documented in single myofibril (Johnston et al., 2016; Rassier and Pavlov, 2010) and entire muscle preparations (Moo and Herzog, 2018; Moo et al., 2017) that sarcomere length non-uniformities increase with activation, there is no research available that presents an explanation for this observation. In fact, it is not known if sarcomere length non-uniformities are the by-product of an inherently unstable system (as has been proposed in the past – e.g. Gordon et al., 1966a; Hill, 1953; Zahalak, 1997), and thus are random, or if sarcomere length non-uniformities are determined by structural and contractile proteins in the sarcomere and higher structural levels.

2.6 Purpose

The purpose of this study was to explore if sarcomere length non-uniformities are a random, non-determinant property of skeletal muscles, or if they are predictable and repeatable for identical contractile conditions.

2.7 Hypothesis

We hypothesized that:

(i) Activation increases sarcomere length non-uniformities.
(ii) Sarcomere length non-uniformities are not random but occur deterministically/repeatably for given contractile conditions.

(iii) Sarcomere length non-uniformities in passive and active states are not related.

(iv) Active and passive components play interacting roles in the production of sarcomere length non-uniformities.

Chapter Three: Methodology

3.1 Specimen
Small strips of psoas muscle from eight female New Zealand white rabbits (-Charles River Labs Senneville QUEBEC, Canada; 6 months years old; approved by the Life and Environmental Sciences Animal Care Committee of the University of Calgary) were tied to wooden sticks (long: 2.5cm; thin: 2-3mm) and stored in a rigor solution (Tris: 50mM; KCl: 100mM; MgCl2: 2mM; EGTA: 1mM) with glycerol (50:50 v/v) for 2 weeks (pH 7.0, 4°C). Protease inhibitors (cOmplete®, Millipore-Sigma Inc., Oakville, ON, Canada) were used.

On the day of the experiment, a small strip of muscle tissue was removed from the storage tube and soaked for 1 hour in the rigor solution (14ml, 4°C, pH 7.0) on ice. Then a small sample of about 5mm in length and 1mm in diameter was cut from the initial sample and dropped into a blender tube (part#72-1327, Harvard Apparatus Inc., St-Laurent, Quebec, Canada) containing rigor solution. The blender (Model PRO250, Pro Scientific, Oxford, CT, USA) was used to blend the tissue into a homogenized suspension containing small pieces of myofibrils by turning it on at 1000rpm for 5s, 13200rpm for 5s, and 16000rpm for 5s. The homogenized solution was put onto ice at a temperature just below 0°C until further use.

3.2 Set-up
An inverted microscope (Zeiss Axiovert 200M, Zeiss, Germany) with three hydraulic joysticks and an adjustable x-y positioning stage was used and was placed on a pneumatic isolation table. The joysticks hydraulically manipulate a glass needle (pulled from a 5µL glass pipette by a pipette puller model 720, David Kopf Instruments, CA, USA), a set of cantilevers (pairs of nanofabricated silicon nitride cantilevers with a stiffness of 150nN/µm in this study, Cornell Nano-scale Facility of Cornell University, Ithaca, NY, USA) and a “jacuzzi” activating fluid delivery tube (outer diameter, OD: 1.5 mm; inner diameter, ID: 0.86 mm; Item #: BF150-86-15, Sutter Instrument Co., Novato, CA, USA). Two stainless steel, 18 Gauge tubes served to infuse and remove the different activating and relaxing solutions into a small bath that was placed on the x-y stage of the microscope which contained the mechanical setup for myofibril testing. A video camera (Rolera Bolt, Quantitative Imaging Corp., Surrey, BC, Canada) was connected to one of the camera ports of the
microscope to record the experiments. Videos were saved using StreamPix 5 Video Imaging software (NorPix Inc., Montreal, Canada) at 30 Hz. The objective used for all testing was 40x and used in conjunction with a 2.5x optovar magnifier (NA 0.75, Zeiss ImmersoLTM, 518F, Zeiss, Germany), and the theoretical optical resolution of 87.5nm/pixel (Figure 3.1A).

3.3 Protocol
On the day of the experiment, 3-4 drops of the homogenized muscle suspension (about 200µL) was placed into the chamber and allowed to stabilize for 10 minutes (room temperature, pH 7.0). Then the rigor solution was replaced by a relaxing solution 10 mM Imidazole, 3 mM MgCl2, 47.7mM Na2CrP, 2 mM DTT, 1 mM K2Cl2Na2MgATP, and 3 mM K4Cl2EGTA. A single myofibril with a clear striated pattern was then selected and attached at one end to the glass needle and the other end to a pair of stiff cantilevers (Dow Corning® 3145 and 3140 – 50:50 mixture, Midland, MI, USA) (Figure 3.1B). Myofibrils were manually brought to one of three starting lengths (P0) of the experiment: (i) short sarcomere length experiments (N=7, mean sarcomere length 2.7±0.17µm); middle sarcomere length experiments (N=6, mean sarcomere length 3.2±0.17µm); and long sarcomere length experiments (N=7, mean sarcomere length 3.6±0.24µm). After 3 minutes of relaxation, myofibrils were maximally and isometrically activated via the “jacuzzi” system by injecting activating solution into the experimental chamber 10 mM Imidazole, 3 mM MgCl2, 47.7mM Na2CrP, 2 mM DTT, 1 mM K2Cl2Na2MgATP, and 3 mM K4Cl2EGTA. Once the specimen had reached a fully activated steady state (Activate 1, A1), the relaxing solution was delivered into the experimental chamber to deactivate the myofibril and take it back to the fully relaxed/Passive state (Passive 1, P1). This cycle of activation/deactivation was repeated three times and sarcomere lengths were measured in each of the three active steady state conditions (A1, A2 and A3) and the corresponding passive conditions preceding the first activation (P0) and following each of the three activated conditions (P1, P2 and P3). Activation/deactivation cycles were separated by a 10 min rest period at a short length where there was no passive force (< 2.7 µm/sarcomere) (Figure 3.1C).

Length Measurement and Control
Individual sarcomere lengths for the three active, A1, A2, and A3, as well as the four passive, P0, P1, P2, and P3, conditions were measured as published before by our group (e.g., Joumaa et al., 2008a; Leonard and Herzog, 2010; Rassier et al., 2003b). In short, we used the intensity profiles created by the I- (light intensity) and A-bands (dark intensity) of sarcomeres. I-bands and A-bands were defined by the maximum slope of the intensity threshold change between A- and I-bands, as described previously (e.g., Leonard and Herzog, 2010; Rassier et al., 2003b; Schmidt et al., 2021; Tesi et al., 2002). Once I- and A-bands were demarcated, the centroid of the A-bands was determined through an area integration, and sarcomere lengths were calculated from one A-band centroid to the adjacent A-band centroids. Sarcomere lengths were then
calculated using custom written software (Schmidt et al., 2021) in MATLAB® (The Mathworks Inc., Natick, MA, USA). In cases where A- and I-bands were hard to identify automatically, some manual demarcation was performed. All sarcomere length measurements were made three times on different days to test reliability of the process. 1 standard deviation of the mean for reliability for all conditions was always equal or better than 0.09 µm.

Because of the lightweight nature of the needle tip side and its immersion in the solution, there was on occasions a slight drift (a few nanometres) of the motor during activation and deactivation. Any such drift that affected the total length of the myofibril specimen was carefully corrected such that all sarcomere length measurements were made at the same total myofibril (or equivalently the same average sarcomere) length. Sarcomere lengths at the end of myofibrils where sarcomeres were attached to the mechanical testing system were not included in the analysis to prevent possible attachment artifacts affecting the results.

3.5 Statistical Analysis

Statistical analyses were performed using SPSS (version 29, SPSS Inc.). Since the data analyzed were interval data, non-parametric statistics were used. Sarcomere length distributions (Figure 4.1; SD and CoV of SLs) were analyzed for the seven steady-state conditions using the Kruskal-Wallis H test across groups, considering the independent nature, and the Friedman’s 2-way ANOVA by ranks within groups considering the correlated nature of the observations. To examine the relationships, non-parametric Spearman’s rank-order correlations were used for analyzing SLs among the passive states (Figure 4.2), active states (Figure 4.3), and neighboring states of passive and active conditions (Figure 4.4). All statistical tests were conducted using a two-sided approach with a significance level of α=0.01 to control for type I error.

In order to assess if sarcomere lengths for corresponding experimental conditions were the same or similar for repeat activation/deactivation cycles, best fitting straight-line approximations of the data were made. These best fitting lines were forced through the origin and similarity was assessed by checking the coefficient of determination (r²), and the slope of the best fitting line. A slope and an r²-value of close to 1.0 indicates good agreement of sarcomere lengths for repeated activation/deactivation cycles and the same condition (that is for the four relaxed/passive, or the three active conditions at a given length – short, medium, or long lengths). Due to the limited resolution of light microscopy, sarcomere length that differ by 200 nm (0.2 µm) or less were considered the same. In the figures, this is indicated by two lines parallel to the best fitting regression line that are offset by ±0.2 µm from the best fitting regression line. Sarcomere lengths within these parallel lines are considered the same.
B. 

Length (Initial mean sarcomere lengths: 2.7μm, 3.2μm, 3.6μm)

---

Force

\[ P_0 \quad A_1 \quad P_1 \quad A_2 \quad P_2 \quad A_3 \quad P_3 \]

C.
Figure 3.1 Experimental setup, protocol, and specimen record depicting the mechanical testing setup for the myofibrils. 
A: the experiments were conducted in a small chamber/bath. Two soft tubes were connected to the opposite edges of the chamber for adding and removing activating and relaxing solutions. Isolated myofibrils were fixed at one end to a glass needle for controlled length changes of the myofibril, and to a cantilever for force measurement at the other end. All experiments were performed on the stage of an inverted microscope. To activate the myofibril, an activating solution was added to the experimental chamber. To relax myofibrils a relaxing solution was added to the experimental chamber after the activating solution had been removed. B: Each myofibril was activated and deactivated for 3 cycles at each of the three experimental lengths. A 10-minute rest was given between activation/deactivation cycles. C: top: a relaxed/passive myofibril is attached to the glass needle at the left end and to the force cantilever at the right end. The same myofibril which was activated after the activation solution was changed in the chamber under the microscope.

Chapter Four: Results

4.1 Sarcomere lengths

Table 1. Individual myofibril data in each passive and active state

<table>
<thead>
<tr>
<th>Myofibril (µm)</th>
<th>n</th>
<th>P₀</th>
<th>P₁</th>
<th>P₂</th>
<th>P₃</th>
<th>A₁</th>
<th>A₂</th>
<th>A₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL avg 2.7µm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>2.89±0.13</td>
<td>2.80±0.15</td>
<td>2.78±0.18</td>
<td>2.73±0.30</td>
<td>2.64±0.37</td>
<td>2.61±0.43</td>
<td>2.56±0.58</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>2.49±0.12</td>
<td>2.44±0.08</td>
<td>2.43±0.08</td>
<td>2.44±0.10</td>
<td>2.35±0.56</td>
<td>2.30±0.16</td>
<td>2.27±0.80</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>2.85±0.06</td>
<td>2.77±0.08</td>
<td>2.76±0.08</td>
<td>2.71±0.12</td>
<td>2.62±0.38</td>
<td>2.54±0.50</td>
<td>2.52±0.47</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>2.76±0.06</td>
<td>2.70±0.04</td>
<td>2.70±0.06</td>
<td>2.62±0.13</td>
<td>2.45±0.48</td>
<td>2.48±0.39</td>
<td>2.46±0.47</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>2.63±0.09</td>
<td>2.53±0.16</td>
<td>2.49±0.15</td>
<td>2.42±0.20</td>
<td>2.33±0.41</td>
<td>2.38±0.30</td>
<td>2.41±0.16</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>2.69±0.05</td>
<td>2.64±0.10</td>
<td>2.52±0.15</td>
<td>2.50±0.16</td>
<td>2.26±0.36</td>
<td>2.35±0.39</td>
<td>2.41±0.34</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>2.59±0.15</td>
<td>2.59±0.17</td>
<td>2.54±0.24</td>
<td>2.53±0.30</td>
<td>2.49±0.72</td>
<td>2.55±0.58</td>
<td>2.51±0.54</td>
</tr>
<tr>
<td>Total/Mean</td>
<td>156</td>
<td>2.69±0.18</td>
<td>2.64±0.17</td>
<td>2.60±0.20</td>
<td>2.57±0.24</td>
<td>2.45±0.50</td>
<td>2.46±0.49</td>
<td>2.44±0.53</td>
</tr>
</tbody>
</table>

| SL avg 3.2µm   |     |     |      |      |      |      |      |      |
| 8              | 24  | 3.28±0.11 | 3.23±0.15 | 3.28±0.18 | 3.22±0.22 | 3.20±0.35 | 3.17±0.33 | 3.23±0.47 |
| 9              | 24  | 3.34±0.17 | 3.27±0.21 | 3.23±0.20 | 3.25±0.23 | 3.32±0.37 | 3.22±0.39 | 3.18±0.33 |
| 10             | 20  | 3.28±0.14 | 3.25±0.24 | 3.25±0.32 | 3.25±0.34 | 3.25±0.59 | 3.20±0.87 | 3.21±0.77 |
| 11             | 16  | 3.24±0.17 | 3.26±0.35 | 3.31±0.29 | 3.31±0.38 | 3.10±0.47 | 3.20±0.61 | 3.21±0.51 |
| 12             | 25  | 3.06±0.10 | 3.04±0.10 | 3.02±0.14 | 3.01±0.19 | 2.93±0.40 | 3.02±0.23 | 3.05±0.28 |
| 13             | 12  | 3.12±0.14 | 3.03±0.18 | 3.09±0.18 | 3.13±0.26 | 2.85±0.52 | 2.88±0.32 | 2.98±0.45 |
| Total/Mean     | 121 | 3.22±0.17 | 3.19±0.23 | 3.20±0.25 | 3.19±0.29 | 3.13±0.47 | 3.13±0.51 | 3.15±0.49 |

| SL avg 3.6µm   |     |     |      |      |      |      |      |      |
| 14             | 17  | 3.54±0.21 | 3.52±0.29 | 3.53±0.42 | 3.46±0.34 | 3.52±0.70 | 3.44±0.62 | 3.41±0.69 |
| 15             | 18  | 3.70±0.26 | 3.69±0.33 | 3.74±0.35 | 3.81±0.32 | 3.67±0.53 | 3.64±0.57 | 3.73±0.46 |
| 16             | 13  | 3.65±0.23 | 3.67±0.44 | 3.68±0.46 | 3.66±0.40 | 3.75±0.49 | 3.72±0.79 | 3.74±0.87 |
| 17             | 19  | 3.72±0.23 | 3.67±0.28 | 3.69±0.28 | 3.66±0.29 | 3.67±0.42 | 3.60±0.52 | 3.59±0.56 |
| 18             | 18  | 3.33±0.15 | 3.38±0.31 | 3.38±0.31 | 3.40±0.31 | 3.40±0.47 | 3.44±0.37 | 3.41±0.38 |
| 19             | 23  | 3.57±0.20 | 3.62±0.25 | 3.62±0.28 | 3.65±0.33 | 3.64±0.51 | 3.73±0.63 | 3.67±0.58 |
| 20             | 27  | 3.45±0.14 | 3.59±0.28 | 3.53±0.31 | 3.61±0.33 | 3.34±0.68 | 3.56±0.77 | 3.49±0.60 |
| Total/Mean     | 135 | 3.56±0.27 | 3.59±0.35 | 3.59±0.36 | 3.61±0.37 | 3.55±0.58 | 3.59±0.64 | 3.57±0.61 |
20 myofibrils with a total number of 412 sarcomeres underwent three isometric activation-deactivation cycles at nominal average sarcomere lengths of 2.7, 3.2 and 3.6 µm. There were 156 sarcomeres in the short group, 121 sarcomeres in the middle group and 135 sarcomeres in the long group. The mean sarcomere lengths (±SD) for each myofibril for the seven experimental conditions (P₀: initial reference passive state; A₁: first activation; P₁: passive condition after first activation; A₂: second activation; P₂: passive condition after second activation; A₃: third activation; P₃: passive condition after third activation) are shown in table 1. In addition, the individual sarcomere lengths for each of the myofibrils are shown in Appendix A (see Raw Data in Appendix A). The mean sarcomere lengths did not significantly change between any of the passive conditions for the repeat measurements (p>0.05). The mean sarcomere length was slightly smaller in the active compared to the passive/relaxed states for the short group sarcomeres (p<0.05) but remained the same (p>0.05) between passive and active states for the middle and long group sarcomeres.

4.2 Sarcomere length non-uniformity

Myofibril activation produced an increase in sarcomere length non-uniformities at each average sarcomere length that was tested (Figure 4.1); that is, sarcomere length non-uniformities were always greater in the activated compared to the corresponding relaxed/passive myofibril (p<0.05).

![Figure 4.1](image_url)

**Figure 4.1** Activation (going from the relaxed to the activated state) produced an increase in sarcomere lengths non-uniformities at each average sarcomere length that was tested but repeat activation/deactivation did not change sarcomere length non-uniformity in the passive conditions (P₀ to P₃; p>0.05) or the active conditions (A₁ to A₃; p>0.05). i: Sarcomere length non-uniformity expressed as mean standard deviations. ii: Sarcomere length non-uniformity expressed as mean coefficients of variation.
expressed as mean coefficients of variation. The absolute sarcomere length non-uniformities were greatest for the myofibrils in the longest average sarcomere length group (3.6µm) and the active conditions (i), while the coefficients of variation were greatest for the myofibrils in the shortest average sarcomere length group (2.7µm) and the active condition (ii).

4.3 Sarcomere lengths in the passive states
There was a strong linear relationship between the sarcomere lengths measured at the 0-state($P_0$) and those measured at $P_1$, $P_2$, and $P_3$ (Figure 4.2). Best fitting linear regressions forced through the origin had slopes of nearly 1.0 (0.99 in each case) and coefficients of determination, $r^2$, of 0.88, 0.86, and 0.83, respectively with $p$-values in all cases $p<0.001$.

4.4 Sarcomere lengths in the active states
There was a high linear relationship between the sarcomere lengths measured at the A$_1$-state and those measured at A$_2$ and A$_3$. The best fitting linear regressions forced through the origin had slopes close to 1.0 (1.0026 and 1.0003, respectively) and coefficients of determination of 0.84 and 0.81, respectively with $p$-values in both cases smaller than 0.001.

4.5 Sarcomere length changes when transitioning from passive to active states
Significant but weak relationships were found when myofibrils were activated and went from the passive to the active states ($p<0.01$, $0.22<r^2<0.66$). For the long average sarcomere tests (3.6 µm on average), sarcomere length predictions from passive to the active states became better than for the tests performed with middle (3.2 µm) and short average sarcomere lengths (2.7 µm). Slopes of the best fitting regression lines were always significantly greater than 1.0 (1.27 to 1.58), and regression lines always had negative y-intercepts ranging from -0.85 to -1.97. Best fitting regression lines forced through the origin (as done for the previous figures) did not result in feasible regressions with random residuals, and thus were not used.
Figure 4.2 Sarcomere lengths of the original passive state ($P_0 = \text{before the first activation}$) vs. sarcomere lengths in the passive/relaxed state after the first, second and third activation/deactivation cycles ($P_1 - \text{Figure A}; P_2 - \text{Figure B}; \text{and } P_3 - \text{Figure C, respectively}$). Purple squares are sarcomeres from the short group (nominal average sarcomere lengths of about 2.7 µm). Blue squares are from the middle group (average sarcomere length of about 3.2 µm). Green squares are from the long group (average sarcomere length of about 3.6 µm). Yellow squares: sarcomeres whose length changes are greater than the spatial resolution of measurement of 0.2 µm. Red dotted line and words: data of best fitting linear regression and equation. Solid black line and words: data of the best fitting linear regression that is forced through the origin (0/0) of the coordinate system. Black dotted lines: offset from the best fitting regression line by ±0.2 µm, which represents the spatial resolution of light microscopy. In other words, sarcomeres within these lines are considered to be of the same length.
Figure 4.3 Sarcomere lengths of the original active state ($A_1 = \text{first activation}$) vs. sarcomere lengths in the active state following the second and third activation/deactivation cycles ($A_2$ – Figure A; $A_3$ – Figure B, respectively). Purple circles are sarcomeres from the short group (nominal initial average sarcomere lengths of about 2.7 µm). Blue circles are from the middle group (average initial sarcomere length of about 3.2 µm). Green circles are from the long group (average initial sarcomere length of about 3.6 µm). Yellow circles are sarcomeres whose length changes are greater than the resolution of light microscopy of 0.2 µm. Red dotted line and words: data of best fitting linear regression and equation. The solid black line and words represent data of the best fitting linear regression that is forced through the origin (0/0) of the coordinate system and the equation. The black dotted lines are offset from the best fitting regression line by ±0.2 µm, which represents the spatial resolution of light microscopy. In other words, sarcomeres within these lines are considered to be of the same length.
Short: $y = 1.39x - 1.29$, $r^2 = 0.22$
dotted line: $y = 0.91x$, $r^2 = 0.19$

Middle: $y = 1.58x - 1.97$, $r^2 = 0.33$
dotted line: $y = 0.97x$, $r^2 = 0.28$

Long: $y = 1.49x - 1.73$, $r^2 = 0.38$
dotted line: $y = 1.00x$, $r^2 = 0.34$

Short: $y = 1.39x - 1.29$, $r^2 = 0.22$
dotted line: $y = 0.91x$, $r^2 = 0.19$

Middle: $y = 1.48x - 1.57$, $r^2 = 0.46$
dotted line: $y = 0.99x$, $r^2 = 0.41$

Long: $y = 1.36x - 1.29$, $r^2 = 0.47$
dotted line: $y = 1.00x$, $r^2 = 0.44$
Corresponding linear regression that is forced through the origin (0/0) of the coordinate system

**Figure 4.4** Sarcomere lengths of the original passive state (0 = before activation) vs. sarcomere lengths in the active state of the first activation (A₁ – Figure A). Sarcomere lengths of the passive state (P₁ = after the first activation/deactivation cycle) vs. sarcomere lengths in the active state of the second activation (A₂ – Figure B). Sarcomere lengths of the passive state (P₂ = after the second activation/deactivation cycle) vs. sarcomere lengths in the active state of the third activation (A₃ – Figure C). Purple symbols are sarcomeres from the short group (nominal average sarcomere lengths of about 2.7 µm). Blue symbols are from the middle group (average sarcomere length of about 3.2 µm). Green symbols are from the long group (average sarcomere length of about 3.6 µm). The solid lines of purple, blue, and green represent the best fitting linear regression for the short group, middle group, and long group myofibrils respectively. The dotted lines of purple, blue, and green represent the linear regression lines that were forced through the origin (0/0) of the coordinate system. The circles of purple, blue, and green represent the residuals of the linear regressions in the short, middle and long group.
4.6 Sarcomere length changes

Sarcomeres that were shortening when going from the initial passive/relaxed state (relaxed state “0”) to the first active state (A₁) also tended to shorten when myofibrils were activated the second (A₂) (Figure 4.5A) and the third time (A₃) (Figure 4.5B). Similarly, sarcomeres that elongated when going from the initial relaxed state to the first active state also tended to elongate when myofibrils were activated for the second (Figure 4.5A) and the third time (Figure 4.5B). The proportion of sarcomeres shortening or elongating consistently (indicated by the purple, blue and green numbers in the first and third quadrants of Figures 4.5A and B) was 0.91 for tests at an average sarcomere lengths of 2.7 µm (purple number in Figures 4.5A and B), was 0.87 and 0.84 for the tests at an average sarcomere length of 3.2 µm, (blue numbers in Figures 4.5A and B), and was 0.76 and 0.75 for the tests at an average sarcomere length of 3.6 µm (green numbers in Figures 4.5A and B). When accounting for the reliability of sarcomere length measurements of 0.2 µm, there only remained a handful of cases in which sarcomeres that were shortening in one activation cycle were lengthening in another.

Figure 4.5 (A) Sarcomere length changes in the second activation cycle (going from the initial relaxed state “0” to the second active state “A₂”) as a function of the sarcomere length changes occurring during the first activation cycle (going from the initial relaxed state “0” to the first active state “A₁”), and (B) the corresponding sarcomere length changes going from the initial relaxed state “P₀” to the third active state “A₃” as a function of the length change in the first activation cycle (going from “P₀” to “A₁”). Data points in the first and third quadrant indicate that sarcomeres were shortening/elongating in both activation cycles, while data points in the second and fourth quadrant indicate that sarcomeres were elongating/shortening or shortening/elongating in the first and subsequent activation cycle. The proportion of sarcomeres falling into the four quadrants are indicated in the purple, blue, and green numbers for the short (2.7 µm), middle (3.2 µm) and long (3.6 µm) average sarcomere length tests. The vertical and horizontal lines through the
zero values indicate zero length change of a sarcomere in the activation cycle going from the relaxed/passive to the active state. The corresponding vertical and horizontal lines at ±0.2 µm from the zero lines indicate the reliability of our sarcomere length measurements. In other words, sarcomere length changes within the ± 0.2 µm lines are considered zero length change, or length changes that cannot be said to be greater than zero with a degree of certainty.

Chapter Five: Discussion

5.1 Objective
The primary results of this study were (i) that sarcomere length non-uniformities do not appear to occur randomly but are governed by some structural and/or contractile properties of the sarcomeres (Figures 4.2 and 4.3), (ii) that sarcomere length non-uniformities differ greatly between the passive and active state. Specifically, sarcomere length non-uniformities consistently increase from the passive to the active state at all average sarcomere lengths (Figure 4.1) and for each myofibril tested. However, despite the differences in sarcomere length non-uniformities between passive and activated sarcomeres, there was a predictability about how sarcomere lengths were changing upon activation. Sarcomeres that tended to be short in the passive myofibril tended to remain short upon activation and sarcomeres that tended to be long, tended to remain long (Figure 4.4). (iii) Furthermore, when sarcomeres shortened when activated the first time, they tended to shorten in subsequent activation cycles, and vice versa, if sarcomeres elongated when activated the first time, they tended to do so in subsequent activation cycles as well (Figure 4.5).

The results of this study confirm two previously published results: first, sarcomere lengths are non-uniform in active and passive muscles. This finding has been made for all structural levels of muscle ranging from whole muscles (Moo and Herzog, 2018; Moo et al., 2016; Moo et al., 2017; Pincheira et al., 2021) to single fibres (Edman & Reggiani, 1984; Iwazumi & Pollack, 1981; Julian & Morgan, 1979), and myofibrils (Bartoo et al., 1993; Johnston et al., 2016; Johnston et al., 2019). Second, sarcomere length non-uniformities increase substantially when a muscle is activated (Figure 4.1), that is, when a muscle, fibre or myofibril goes from the passive state to an active state (Bartoo et al., 1997; Horowits, 1992; Johnston et al., 2016; Johnston et al., 2019). Novel to the literature is the result that sarcomere lengths non-uniformities do not occur randomly, neither in passive nor in active myofibrils, but they appear to be “governed” by some rules or constraints. This interpretation is supported by the tight proportionality of the best fitting linear regression lines (forced through zero) that have slopes close to 1.0 (range from 0.989 to 1.003) and coefficients of determination ranging from 0.81 to 0.88 for measurements of sarcomere lengths obtained repeatedly for the same contractile conditions (Figures 4.2 and 4.3).

When relating the sarcomere length non-uniformities in the passive conditions to the sarcomere length non-uniformities in the active condition that immediately followed the passive condition, attempting to fit linear regression lines that were forced through zero produced unacceptable results, that is, regression lines, that in
contrast to those obtained in Figures (4.2 and 4.3), were far from the best fitting regression lines. This result suggests that the sarcomere length non-uniformities in the passive and active state are caused by different mechanisms. Best fitting regression lines between the passive and active state, calculated independently for the three length conditions, gave regression lines with slopes substantially greater than 1.0 (range from 1.27 to 1.58) and negative Y-value intercepts ranging from -0.85 to -1.97 (Figure 4.3). A slope of greater than 1.0 and a negative Y-intercept indicates that when a myofibril was activated, sarcomeres that were short in the passive state tended to become even shorter with activation, and sarcomeres that were long in the passive state tended to become even longer with activation. Sarcomeres that were of medium length would tend to stay at about the same length. In fact, for the 9 regression lines in Figure (4.3), one can calculate the sarcomere length that, on average, remained unchanged from the passive to the active state. These unchanged sarcomere lengths ranged from 3.14 µm to 3.66 µm, with an average of 3.39 µm. These results suggest that sarcomere length non-uniformities in the active state increased because of a dispersion of sarcomeres at the long and short end of sarcomere lengths.

An interesting observation was that the proportion of sarcomeres that shortened or lengthened upon activation depended crucially on the myofibril (or equivalently the average sarcomere) length. For example, for the shortest average sarcomere length (2.7 µm), the proportion of sarcomeres that tended to shorten repeatedly upon myofibril activation was 0.71 and 0.72 for the situations shown in Figures (6A and B), while the proportion that lengthened was 0.20 and 0.19, respectively. Since the average sarcomere length remained the same for the relaxed and activated myofibrils, this result implies that the average distance shortened by the “shortening sarcomeres” was smaller than the average elongation of the “lengthening sarcomeres”, as can be seen qualitatively in Figures (4.5A and 4.5B). In contrast, for the longest average sarcomere length (3.6 µm), the situation was reversed; that is the proportion of sarcomeres that tended to shorten repeatedly when going from the relaxed to the activated state was smaller (0.34 and 0.32, Figures 4.5A and 4.5B) than the proportion of sarcomeres that tended to elongate (0.42 and 0.43, respectively).

Furthermore, when going from the passive to the active state, the coefficient of determination increased with increasing myofibril (or equivalently increasing average sarcomere) lengths. The coefficients of determination at the shortest testing length (average sarcomere length of about 2.7 µm) was 0.22, 0.24, and 0.22 when relating the initial \( P_0 \) state to \( A_1 \), \( P_1 \) to \( A_2 \), and \( P_2 \) to \( A_3 \), respectively (Figure 4.4). The corresponding coefficients of determination at the longest myofibril testing conditions were 0.38, 0.46 and 0.66, respectively, indicating that predicting the length of sarcomeres in the active state from the passive state was better when the average sarcomere lengths (or myofibril lengths) were long. We propose that this result may be because at the different test lengths, the contribution of passive and active elements to sarcomere length non-uniformities changes systematically. At the shortest test length of about 2.7 µm/sarcomere, passive force in rabbit psoas
myofibrils is essentially zero (Bartoo et al., 1997; Horowits, 1992; Leonard and Herzog, 2010), at the intermediate testing length (about 3.2 µm/sarcomere) passive forces reach about 10-20% of the maximal isometric force at optimal sarcomere length (Bartoo et al., 1993, 1997; Rassier et al., 2003), and finally, at the longest testing length (about 3.6 µm/sarcomere), the passive forces in rabbit psoas myofibrils are similar or even exceed the active forces at that length (Bartoo et al., 1997; Joumaa et al., 2007). With increasing passive force and decreasing active force contributions at increasing sarcomere lengths, the structures determining sarcomere length non-uniformities in the active myofibril also change from a dominance of the active structures at the short testing length, to a dominance of the passive structures at the long testing length.

In single myofibrils, the exclusive provider of passive force is titin (e.g., Bartoo et al. 1997). Therefore, it is reasonable to propose the hypothesis that the sarcomere length non-uniformities in passive myofibrils are largely (maybe even exclusively) caused by the non-uniformities either in the number or the stiffness of titin filaments in adjacent sarcomeres. Although not systematically measured, sarcomere diameters (and thus presumably cross-sectional areas) of sarcomeres in myofibrils tend to differ, suggesting that the serially arranged sarcomeres may have different amounts of contractile proteins and titin filaments. A small (diameter/area) sarcomere comprised of fewer titin filaments thus would be expected to be longer, and individual titin forces greater, than a large (diameter/area) sarcomere that contains more titin filaments than the small sarcomere. Also, rabbit psoas titin isoforms are of two distinct types with different stiffness (Neagoe et al., 2003; Prado et al., 2005). If the distribution of these different isoforms differs between sarcomeres, the average stiffness of titin may differ as well, thus the sarcomere with the “softer” average titin isoform would be stretched to a longer sarcomere length than a sarcomere with “greater” average titin stiffness, thus accounting for the sarcomere length non-uniformities. Finally, titin filaments have a non-linear stiffness, and stiffness can be changed “instantaneously” by the unfolding of segments in the PEVK and immunoglobulin regions (Eckels et al., 2018; Kellermayer et al., 1998; Linke et al., 1996). Differences in segment unfolding of titin in one sarcomere compared to the next could be due to the random nature of protein folding/unfolding or could be achieved by differences in the short-term history of sarcomere elongation caused, for example, by an actively stretched muscle/myofibril. If a sarcomere during a dynamic contraction was stretched more than another sarcomere, and thus, more protein unfolding would have taken place, it might take minutes, before a comparable resting state between the two sarcomeres might be achieved (Herzog et al., 2012).

In activated myofibrils, sarcomere length non-uniformities are likely governed by the amount of active force that allows for a force equilibrium between neighbouring sarcomeres of a myofibril. As discussed above, sarcomeres in myofibrils appear to have different diameters, and thus presumably different amounts of contractile proteins and different strength capacities for identical contractile conditions. We observed that activation of myofibrils in our tests resulted in a dispersion of sarcomeres around some mean length of about
3.4 µm. Sarcomeres longer than 3.4 µm in the passive state tended to become longer with activation, and sarcomeres shorter than 3.4 µm tended to become shorter (Figure 4.4). This result makes sense because the testing lengths used in this study (2.7 to 3.6 µm) were on the descending limb of the force-length relationship for rabbit psoas muscles. Therefore, increasing sarcomere lengths is associated with a decrease in actin-myosin filament overlap, and thus a decrease in the capacity to produce active force (Gordon et al., 1966b; Huxley, 1957; Huxley, 1969b). Therefore, the following scenario seems feasible to explain the dispersion of sarcomere lengths upon activation in our experiments.

(i) Sarcomeres have non-uniform length in the passive state, with small diameter sarcomeres likely being longer than large diameter sarcomeres because of a smaller number of titin filaments.

(ii) Upon activation, the small diameter passively long sarcomeres also have less contractile proteins than the large diameter passively short sarcomeres, and the overlap in actin-myosin filament is reduced, both factors contributing to a reduced capacity for active force production.

(iii) For the reasons mentioned in (ii), the passively long sarcomeres are stretched further upon activation, while the passively short sarcomeres shorten.

(iv) In activated myofibrils, stretching of the sarcomeres increases the force due to the increased average cross-bridge force, the increased contribution of titin to force, and the residual force enhancement property, while shortening of activated sarcomeres decreases the force due to a reduction in the average cross-bridge force, the decreased contribution of titin to force, and the residual force depression property of muscles (Abbott & Aubert, 1952; Edman & Tsuchiya, 1996; Herzog, 2014; Herzog et al., 2010; Herzog & Leonard, 1997; Joumaa et al., 2007; Lee & Herzog, 2002; Maréchal & Plaghki, 1979; Rassier & Pavlov, 2012).

5.2 Limitations

The main purpose of this study was to explore the sarcomere length nonuniformity for isometric contractions. In this study, the single myofibril was attached to a needle at one end and to an optical force transducer at the other end. A force transducer of the greatest stiffness was used for all experiments, thus shortening of the myofibrils, and thus changes in the average sarcomere length were negligible for the two tests with average sarcomere lengths of 3.2 and 3.6 µm. However, at the shortest average sarcomere test condition, 2.7 µm, the average sarcomere length shortened by about 3% from the relaxed to the active state, likely because these contractions were the strongest (closest to the plateaus region of the force-length relationship) and passive forces were smallest. This small average shortening upon activation for the 2.7 µm condition may have affected the results in an unknown manner. However, there were no conceptual differences between the results obtained for the three lengths conditions, so we assume that any such effect did not influence the conceptual results and conclusions of this study.
Length changes in some sarcomeres across the three activation/deactivation cycles were excessive, up to ~0.7 µm for the passive and ~1.4 µm for the active condition. These outliers were double checked and indeed were found to be correct. It was also found that these large length changes typically did not occur in a single sarcomere, but in two or three neighboring sarcomeres, suggesting that there might have been some damage of the myofibril in that region. Myofibrils are beautiful but also fickle preparation that require months of training before reliable results can be obtained. However, excluding these outliers from the analysis did not change the general results of this study, and thus, we did not see a need for eliminating them from the analysis.

As has been observed in previous studies, we also observed A-bands shifts of sarcomeres in activated myofibrils (Joumaa et al., 2008a; Rassier & Pavlov, 2012; Telley et al., 2006). Therefore, measuring sarcomere lengths, as was done here from the centroid of the A-band to the centroid of adjacent A-bands does not necessarily reflect the traditional definition of sarcomere length: from Z-line to Z-line. However, previous work showed that non-uniformities in sarcomere and half-sarcomere lengths are well correlated, thus giving support to the idea that the measured sarcomere length non-uniformities in this study reflect well non-uniformities of sarcomere lengths defined by the Z-lines (e.g., Joumaa et al., 2008b; Rassier and Pavlov, 2012). Since the A-band is much easier identified than Z-lines in myofibril experiments, because of the greater change in intensity profile, A-band to A-band measurements are more reliable than Z-line to Z-line, making this the preferred method for quantifying the lengths of individual sarcomeres (e.g., Llewellyn et al., 2008; Moo et al., 2016; Moo et al., 2017).

5.3 Future directions
Joumaa et al. (2018) showed that partial titin degradation caused an increase in SL non-uniformities after active stretching, thereby supporting the idea that titin is a crucial component for the regulation of sarcomere length in active and passive/relaxed myofibrils. Titin deletion experiments should be used in a quantitative and systematic manner to explore the detailed roles of titin in determining sarcomere lengths and lengths changes for different contractile conditions. Such experiments could be made in a variety of ways including by degrading titin, actin, or other contractile or structural components of sarcomeres. Li and colleagues (Li et al., 2019) used the tobacco etch virus and HaloTag to compare passive forces in sarcomeres with precisely controlled functional deletion of titin. They found that their model was reliable and could be used to quantify titin-based passive force and active forces in muscle fibers.

Leonard and Herzog (Leonard and Herzog, 2010) investigated non-cross-bridge based forces by inhibiting cross-bridge attachment to actin using butanedione monoxime. It may also be interesting to perform experiments with myofibril preparations in which sarcomere length non-uniformities are negligible. For example, indirect flight muscles of many insects have been shown to have virtually perfect uniformity in
sarcomere lengths. These muscles are undergoing up to 100 stretch-shortening cycles per second, with an amplitude of just about 5% (Reedy & Beall, 1993; Swank, 2012; Vigoreaux, 2007). The uniformity of sarcomere lengths in these muscles seems to be achieved by extreme longitudinal stiffness, likely caused by the dense arrangements of structural proteins that limit excursion (Reedy and Beall, 1993). Therefore, one might speculate that muscles who undergo rapid stretch-shortening cycles with minimal excursion favour stiff sarcomeres that work almost elastically, and as a consequence, do not allow for great sarcomere length non-uniformities. In contrast, mammalian skeletal muscles often undergo large excursion at relatively slow speeds, thus sarcomeres that do not have much natural (passive) stiffness are preferred, but this may come at the expense of sarcomere length non-uniformities as small structural and size differences that exist between adjacent sarcomeres are not minimized by a strong passive sarcomeric framework. It might be revealing to study the stiffness properties of sarcomeres in different species and relate them to the natural occurring sarcomere length non-uniformities in the active and passive/relaxed myofibril, fibre, and muscle.

5.4 Conclusion
Sarcomere length non-uniformities are not occurring randomly but are deterministic for given contractile conditions. In passive rabbit psoas myofibrils, sarcomere length non-uniformities may be caused by differing numbers of titin and the different distribution of titin isoforms with different stiffness in serially arranged sarcomeres. Activation causes a dispersion of sarcomere lengths with passively long sarcomeres tending to elongate and passively short sarcomeres tending to shorten. This dispersion is likely caused by the different amounts of contractile proteins and titin between sarcomeres and the amount of overlap between contractile proteins in the passive state, which is thought to be decreased in small compared to large diameter sarcomeres.


Iwazumi, T. and Pollack, G. H. (1981). The effect of sarcomere non-uniformity on the sarcomere length-


Appendix A: Raw Data

Each figure shows the individual sarcomere lengths for each myofibril. There were 7 myofibrils in the short group (avg SL: 2.7µm), 6 myofibrils in the middle group (avg SL: 3.2µm) and 7 myofibrils in the long group (avg SL: 3.6µm) respectively. The x-axis shows the different conditions, and the y-axis shows the individual sarcomere lengths. The seven conditions were: 0: the initial passive reference state; A1: first activation; R1: passive steady state after first activation; A2: second activation; R2: passive steady state after second activation; A3: third activation; R3: passive steady state after third activation.
1. Short group (avg SL: 2.7µm)

2020-11-17-03

2021-01-08-03

2021-01-09-02

SL (µm)

States

P0, P1, P2, P3, A1, A2, A3

P0, P1, P2, P3, A1, A2, A3

P0, P1, P2, P3, A1, A2, A3
2021-01-22-03

SL (µm)

States

P₀  P₁  P₂  P₃  A₁  A₂  A₃
2. Middle group (avg SL: 3.2µm)
3. Long group (avg SL: 3.6µm)
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Proposed Mechanism of Force Generation in Striated Muscle

Author: A. F. HUXLEY et al
Publication: Nature
Publisher: Springer Nature
Date: Oct 22, 1971

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The variation in isometric tension with sarcomere length in vertebrate muscle fibres

Author: F. J. Julian, A. F. Huxley, A. M. Gordon
Publication: Journal of Physiology
Publisher: John Wiley and Sons
Date: May 1, 1966

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