Comparing fatigue induced by isometric and shortening contractions in skeletal muscle

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Abstract

The intramuscular mechanisms causing greater fatigue-induced reduction in force during shortening compared with isometric contractions is not completely understood. The focus of this thesis was to compare fatigue in repeated shortening contractions and isometric contractions and investigate if greater force loss during repeated shortening contractions is attributed to more severe reductions in sarcoplasmic reticulum Ca^{2+} release. Single intact flexor digitorum brevis muscle fibres from C57Bl/6 mice were mechanically dissected and loaded with indo-1 AM fluorescent dye to assess myoplasmic free [Ca^{2+}]. Fibres were either placed in an isometric or shortening protocol which were both stimulated at 70Hz of 600ms duration and 4 seconds rest for 50 tetani. The results of this thesis did not show that repeated shortening contractions cause greater fatigue-induced reductions in force compared with repeated isometric contractions, and there were no differences in the fatigue-induced reductions in SR Ca^{2+} release during the repeated contractions.

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Abbreviations

ADP:	Adenosine diphosphate
AMP:	Adenosine monophosphate
ATP:	Adenosine triphosphate
Ca^{2+}:	Calcium
DHRP:	Dihydropyridine receptors
ECC:	Excitation contraction coupling
FDB:	Flexor digitorum brevis
H^{+}:	Hydrogen ion
Mg^{2+}:	Magnesium
P_i:	Inorganic phosphate
SERCA:	Sarcoplasmic reticulum Ca^{2+} - ATPase
SR:	Sarcoplasmic reticulum

Chapter 1: Introduction

One of the primary functions of skeletal muscle is to generate contractile force and power to allow for locomotion (Frontera & Ochala., 2015; Cheng & Westerblad., 2017). Generally, there are three types of muscle contractions that allow for movement including isometric, concentric (shortening), and eccentric (lengthening) contractions (Fang et al., 2001). Isometric contractions generate force at a fixed muscle length, which is important for joint stability and for maintaining posture. On the other hand, shortening and lengthening contractions involve force generation while the muscle undergoes length change (Devrome & Macintosh., 2018; Fang et al., 2001), which allows us to interact with our environments and to perform locomotory tasks. Of the three contractions, shortening and lengthening contractions have greater relevance to locomotion (Devrome & Macintosh., 2018; Fang et al., 2001).

Muscle contractile force generation is an energy consuming process (Barclay et al., 1993) and when the energy demand of the muscle contractile activity through exercise exceeds the rate at which energy can be provided to the muscle, fatigue ensues (Cheng et al., 2018). Muscle fatigue is typically quantified as impaired force, velocity, or power generation (Allen et al., 2008; Sargeant AJ., 1994; Wan et al., 2017). The functional consequences of muscle fatigue is that it can decrease athletic performance as well as challenge the functional independence of aging, and diseased individuals by impairing the ability to carry out the tasks of daily living (Wan et al., 2017). In contrast to muscle weakness, which is defined by a chronic impairment in muscle force generation typically observed with aging or in chronic diseases, the acute force loss resulting from muscle fatigue is reversible by brief rest (Fitts RH., 2008; Sargeant AJ., 1994). It is well known that the mechanisms causing muscle fatigue are task-dependent (Enoka & Stuart., 1992) with one such task-dependent factor of fatigue being contraction-type. Traditionally, muscle

fatigue has been studied using isometric contractions because the equipment required to perform isometric contractions is more readily available and there are fewer variables to control when muscle fibres are held at a fixed muscle length. In comparison, shortening contractions must consider changes in contraction velocity as well as the amount of muscle length change, which requires specialized equipment. Nonetheless, there are a few studies which have compared muscle fatigability induced by isometric compared with shortening contractions, and these studies have shown that greater fatigue-induced force loss is typically observed during repeated shortening contractions versus isometric contractions (Newham et al., 1995; Seow et al., 1988; Vedsted et al., 2003; Ameredes & Clanton., 1990).

To understand the potential cellular mechanisms involved in the fatigue-induced decline in muscle force generation during dynamic and isometric contractions, we need to understand the processes involved in regulating muscle force generation, which is referred to as excitation-contraction coupling (ECC). To generate a contraction through ECC, an action potential is propagated along the sarcolemma of the muscle into the transverse tubular system. This action potential then activates the voltage-sensing dihydropyridine receptors (DHRP) which then mechanically interacts with the ryanodine receptor 1 (RyR1) to cause Ca^{2+} to be released from the sarcoplasmic reticulum (SR), which is the main storage site for Ca^{2+} in the muscle fibre. Once released, Ca^{2+} then binds to the troponin complex allowing for contraction via cross bridge cycling. After a contraction, Ca^{2+} is then pumped back into the SR via sarcoplasmic reticulum Ca^{2+} - ATPase (SERCA) allowing for relaxation (Periasamy & Kalyanasundaram., 2007). Muscle fatigue can be caused by impairment(s) in any step of ECC but decreased SR Ca^{2+} release has been shown to be a predominant cause for the force decline during fatigue-induced by repeated isometric contractions.

The current literature has shown that dynamic contractions cause greater fatigue when compared to isometric contractions (Ameredes & Clanton., 1990; Seow et al., 1988; Vedsted et al., 2003). However, there is limited understanding of the intramuscular mechanisms that are responsible in explaining the greater fatigue-induced reduction in contractile force during Shortening compared to isometric contractions. Therefore, the purpose of this thesis is to elucidate the intramuscular mechanism responsible for the greater fatigue-induced reduction in force during shortening contraction when compared to isometric contractions. In the next section I will discuss the existing evidence that may allude to the potential mechanisms explaining greater fatigue seen in shortening contractions when compared to the isometric contractions.

Chapter 2: Literature review

Fatigue can be defined as an exercise-induced decline in muscle performance (Allen et al., 2008) which can be related to multiple intramuscular mechanisms along the ECC cascade that regulate muscle contractile force and power generation. One of the fundamental causes of fatigue is that the rate of intramuscular energy consumption exceeds the rate at which energy is supplied within skeletal muscle (Wan et al., 2017), leading to ECC failure and skeletal muscle contractile impairments. In fact, the very process of fatigue is believed to prevent critical energy depletion within skeletal muscle by downregulating energy consumption in order to prevent the most serious consequences of substantial ATP depletion, which ultimately could result in rigor mortis and rhabdomyolysis with extreme fatigue. Furthermore, the intramuscular mechanisms causing fatigue are potentially contraction-type dependent, and the associated mechanisms involved may be directly or indirectly related to increased ATP consumption, i.e., decreased ATP concentration can directly inhibit various ECC processes, or increased metabolite accumulation as a by-product of increased ATP breakdown can cause increased fatigue. The purpose of the following sections is to elucidate potential intramuscular factors involved in the contraction-type dependency of fatigue. To understand the processes related to fatigue in shortening vs. isometric contractions, we will discuss 1) existing evidence comparing fatigue induced by repeated shortening contractions compared to isometric contractions, 2) how contraction-type affects muscle energetics from the perspective of ATP and ATPase activity, and 3) evidence related to metabolite accumulation affecting skeletal muscle function.

2.1: Evidence of greater fatigue in shortening vs. isometric contraction tasks

Various studies have identified that repeated shortening contractions produce greater fatigue than compared to isometric contractions, and the details of these studies are shown in Table 1. In human subjects, shortening contractions required less contractions to reach task failure when compared to isometric contractions (Cheng & Rice., 2009). Moreover, in an animal model using mouse diaphragm, muscles undergoing isotonic contractions experience greater fatigue when compared to isometric contractions (Seow et al., 1988), and canine diaphragm muscles also experience a greater loss of force in isovelocity compared to isometric contractions (Ameredes & Clanton., 1990). In addition, using a small bundle of intact rat flexor hallucis brevis at 20 °C, Roots and colleagues (2009) demonstrate that force decreases during shortening contractions to a greater extent when compared to isometric contractions. Moreover, another study using rodent muscle showed that isotonic contractions had a greater fatigue-induced decline in maximal rate of force development when compared to isometric fatiguing contractions (Vedsted et al., 2003). Overall, shortening contractions experience greater fatigue when compared to isometric contractions seen from the human in-vivo level down to the small muscle fibre bundle level in rodent skeletal muscle. However, there is some evidence that suggests that shortening contractions experience greater energy consumption when compared to isometric contractions and may in part explain the fatigue differences between both isometric and shortening contractions. Within the next section we will cover how increased energy consumption through increased ATP use may explain the greater fatigue seen in shortening contractions when compared to isometric contractions.

2.2: ATP and ATPase activity

ATP provides skeletal muscle free energy for contraction and ATP demand increases with exercise (Baker et al., 2010). In contracting muscles, most of the available ATP is

consumed by the actin-myosin cross bridge cycling, SR Ca^{2+} reuptake and preserving membrane propagation at the Na^{+}/K^{+} pumps (Cheng et al., 2018). Evidently, skeletal muscle can meet this high ATP demand by three energy systems including: phosphocreatine, glycolysis, and aerobic metabolism (Baker et al., 2010; Hargreaves & Spriet, 2020; Nogueira et al., 2013). Short bouts of high intense exercise predominantly rely on phosphocreatine and glycolysis for ATP production whereas low-moderate intensity, long-duration endurance exercise primarily relies on aerobic energy production via mitochondrial oxidative phosphorylation (Hargreaves & Spriet., 2020). These three different energy systems can use a variety of substrates and yield different values of ATP generation to meet their demand (Baker et al., 2010). In summary, ATP provides the muscle with energy and can be met by three different systems to fulfill a required task, and impairments with providing the muscle with energy can be related to fatigue (Cheng et al., 2018). Moreover, ATP utilization in different intensities is varied and may be due to different sources throughout skeletal muscle including sarcolemmal membrane excitability, SR Ca^{2+} reuptake, and crossbridge force generation which will be described in further detail below.

Skeletal muscle contractions use ATP hydrolysis to convert chemical energy into 1) mechanical energy through cross bridge cycling of actin and myosin, and 2) heat (Barclay et al., 1993; Hill AV., 1938). Therefore, ATP break down of chemical energy can also convert to heat, and which allowed for indirect methods to study ATP consumption in the pioneering studies that discovered the regulators of energy use during muscle contraction by A.V. Hill and others (Barclay et al., 1993). With regards to contractions and exercise, a study by Edwards and colleagues (1975) used isometric contractions performed in human quadricep muscles and found that muscle temperature generation is also positively proportional to the force produced by the muscle. Interestingly, heat generation is also proportional to the velocity of a muscle, and if the

load is high enough to slow down the velocity, the heat production is lower when compared to an unloaded muscle (Hill AV., 1938). Moreover, heat generation also appears to plateau when steady state exercise is met (González-Alonso J., 2012) whereas during dynamic high intensity exercise, heat generation fails to plateau, with the greatest rise in heat production seen at the start of exercise (González-Alonso J., 2000). This suggests that the constant shortening of the myofilaments during contraction could be responsible for the greater heat generation during shortening contractions compared with isometric contractions. In summary, heat production is proportional to the force, power, work, and shortening velocity that a muscle produces. Furthermore, shortening contractions have greater ATP usage (i.e., as measured by increased heat generation) compared to isometric contractions due to repeated myofibrillar contractile shortening as opposed to contractile force produced at a fixed muscle length that requires relatively less ATP usage.

ATPase are enzymes that utilize ATP hydrolysis (ATP → ADP + P_i) and are important in conducting cellular functions such as, ion pumping, muscle contractions and other process like replication and transcription (Rule et al., 2016). P-type ATPases are membrane transporters that can transport ions against a concentration gradient, and in skeletal muscle can include Na^+/K^+ ATPase, and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) (Kühlbrandt, w., 2004; Tadini-Buoninsegni et al., 2018). Na^+/K^+ ATPase pumps three Na^+ ions out of a cell in exchange for two K^+ ions and is essential for membrane potential preservation (Pirkmajer & Chibalin., 2016). Within skeletal muscle, the Na^+-K^+- ATPase plays a role in membrane potential preservation following each successive action potential and allows for action potentials to undergo sustained propagation across both sarcolemma and t-tubules during repeated tetanic contractions (Wyckelsma & McKenna., 2016). Under exercise condition Na^+/K^+ ATPase contributes for 5-

10% of ATP demand (Nielsen et al., 2022). However, Na^+-K^+- ATPase are unlikely to contribute to fatigue impairments during shortening contraction because M-wave amplitudes, which are an indirect surface electromyography measure of sarcolemmal excitability (Rodriguez-Falces & Place., 2021), do not show any fatigue-induced alteration during repeated shortening and isometric contractions (Cheng & Rice., 2009). Since sarcolemmal excitability is typically well-preserved during both dynamic and intermittent isometric fatiguing tasks, fatigue is unlikely explained by impairments Na^+/K^+ ATPase activity, nor is any difference in fatigue between contraction-types explained by sarcolemmal excitability.

Downstream from the sarcolemma, SERCA is the most abundant protein on the SR membrane and is critically involved with the SR Ca^{2+} reuptake post-contraction that allows for muscle relaxation to occur (Xu & Van Remmen., 2021). SERCA also plays a critical role in refilling the SR with Ca^{2+} to allow enough Ca^{2+} to be released during SR Ca^{2+} release, whereby the myoplasmic free Ca^{2+} concentration is a major regulator of the myofibrillar force being produced by skeletal muscle (Periasamy et al., 2008). SERCA has 3 different isoforms, SERCA 1, SERCA 2, and SERCA 3 with, SERCA 1 being the dominant isoform in fast twitch skeletal muscle, SERCA 2a as the primary isoform in slow twitch skeletal muscle and cardiac muscles, and SERCA 3 the primary isoform at low expression in epithelial and neuronal cells (Xu & Van Remmen., 2021; Chemaly et al., 2018; Lytton et al., 1992). The primary mechanism of the SERCA pump in skeletal muscle is utilizing ATP hydrolysis to pump ions (cytosolic Ca^{2+}) across a gradient back into the SR (Periasamy & Kalyanasundaram., 2007; Xu & Van Remmen., 2021) at a rate of two Ca^{2+} per one ATP molecule (Periasamy & Kalyanasundaram., 2007; Tadini-Buoninsegni et al., 2018; Kühlbrandt, w., 2004; Smith et al., 2013). Moreover, under exercise conditions, SERCA activity increases ATP consumption (40 to 50 percent of ATP

demand) (Nielsen et al., 2022; Nogueira et al., 2013). Additionally, SERCA Ca^{2+} reuptake in slow-type fibres occurs with lower [Ca^{2+}] and is greater in fast twitch fibres (Lamboley et al., 2014; Schiaffino & Reggiani., 2011). In summary, SERCA helps sustain the integrity of a muscle by controlling SR Ca^{2+} reuptake and muscle relaxation. Moreover, given the large ATP demand of SERCA under exercise conditions, greater fatigue during shortening contractions may be partially attributed to fatigue at this cellular site (Cheng et al., 2018; Nogueira et al., 2013; Tupling AR., 2004).

Myofibrillar ATPase is the third ATP consuming site within skeletal muscle. Myosin are responsible for executing muscle contraction and have two heavy chain and four light chains. The head region of myosin has an ATPase and an actin-binding region whereby myosin is responsible for converting chemical energy into mechanical work (actin-myosin cross bridge cycling) via ATP hydrolysis (Fischer et al., 2005; Geeves & Holmes., 2005; Koppole et al., 2007; Schiaffino & Reggiani., 2011). With skeletal muscle, there are 4 main myosin heavy chain isoform types that correspond to fibre types of the same name which includes, type I, type IIA, type IIX, type IIB. Rodent muscle possesses all four fibre types whereas humans express three fibre types (type I, type IIA, type IIX) (Hyatt et al., 2016; Schiaffino & Reggiani., 2011). Each fibre type possesses unique profiles as type I skeletal muscle fibres have the lowest ATPase activity while type IIX fibres express the greatest ATPase activity (Vikne et al., 2020). The ATPase activity of a muscle fibre is proportional to the speed of contraction (Barany M., 1967). Fast twitch fibre possesses a faster ATPase hydrolysis rate when compared to slow twitch fibres (Taylor et al., 1974). Generally, ATP demand increases during exercise and can be partially explained by increased cross bridge cycling consuming 50 to 60 percent (Nielsen et al., 2022; Nogueira et al., 2013). In summary, each muscle fibre possesses a unique profile that

differentiates from one another. However, each skeletal muscle fibre utilizes myosin ATPase albeit at different consumption levels.

ATP usage may be dependent on type and intensity of exercise. Human muscle biopsies taken from individuals immediately after 30 knee extensions revealed small decreases in type I and II ATP values (10 % and 20% respectively) (Jansson et al., 1987). However, in another study using human muscle by Karatzaferi and colleagues (2001), individuals undergoing an all-out high intensity cycling exercise over 25 seconds saw a significantly large decrease (up to 80%) in type II ATP concentration immediately after exercise when compared to pre-exercise values. These results express that ATP in skeletal muscle fibres can deplete rapidly during very intense acute exercise. Moreover, it has been suggested that the greater fatigue-induced decline in muscle contractile function during shortening compared to isometric contractions could partially be attributed to the greater metabolic cost of muscle shortening. In a study by Newham and colleagues (1995), the metabolic cost of rapid shortening contractions (100ms stimulation every second, over 50 seconds) were roughly double the cost of a sustained isometric contraction (sustained over 5 seconds) while under the same stimulation (50 Hz). Evidently, this greater metabolic cost during shortening contraction could lead to an accelerated decline in skeletal muscle contractile performance due to the increased energy demand of working muscle especially in type II fibres. It also appears that ATP utilization rates vary among contraction types as using chemically skinned rabbit psoas muscle fibres or bundles, muscles undergoing shortening were found to have greater ATP consumption when compared to isometric contractions (Potma & Stienen., 1996). Moreover, assessing ATP hydrolysis rate through fluorescence labelled phosphate binding protein in human skeletal muscle, muscle fibres

undergoing shortening experienced greater ATP consumption in proportion to shortening velocity (He et al., 2000).

In summary, the three major sites that consume ATP in skeletal muscle are Na^+/K^+ ATPase, SERCA, and myosin ATPase. With respect to total ATP usage during exercise, myosin ATPases are presumed to consume the greatest amounts of ATP (50 – 60%) followed by SERCA (40 – 50%), with Na^+/K^+ ATPase consuming the least ATP (5 – 10%) (figure 1). Ca^{2+} reuptake into the SR via SERCA may be compromised under high intensity contractions. Evidently, the increased ATP consumption at two major sites may explain for the greater ATP usage in shortening contractions seen in Newham and colleagues (1995) results. Given the importance of ATP and cellular function, there are mechanisms present to preserve muscle integrity by impairing ATP depletion in muscle undergoing high intensity exercise which can be done by inhibiting SR Ca^{2+} release. A decrease in ATP would lead to skeletal muscle impairments (figure 2). Therefore, preserving ATP through the means of impairing SR Ca^{2+} would blunt ATP consumption at both the cross bridge and SERCA levels resulting in ATP preservation for muscle function. However, there are indirect mechanisms related to increased ATP consumption that can lead to fatigue and impair SR Ca^{2+} release, which includes increased accumulation of metabolic byproducts.

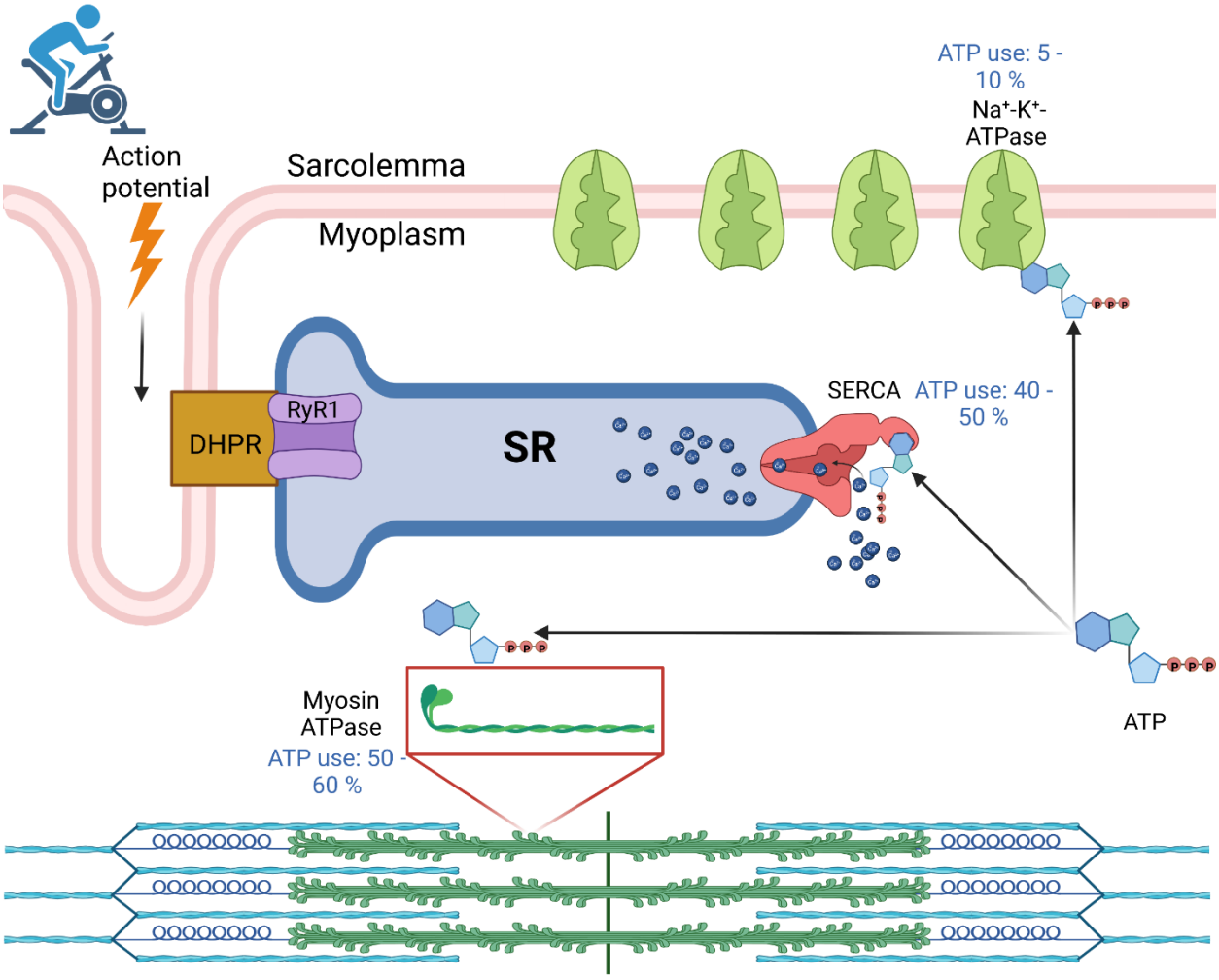

Figure 1**: ATP utilization and specific ATPases in skeletal muscle.** When a muscle undergoes contraction during exercise, the total ATP pool is divided and utilized in regions that help sustain contraction. Myosin ATPase consumes 50 – 60% of total ATP followed by SERCA at 40 – 50% and Na^+-K^+- ATPase consumes the least ATP during skeletal muscle contraction. ATPase consumption values were obtained from Nielsen and colleagues (2022). Dihydropyridine receptor = DHPR; ryanodine receptor = RyR1; sarcoplasmic reticulum = SR; sarcoplasmic reticulum calcium ATPase = SERCA; Adenosine triphosphate = ATP. Created with BioRender.com

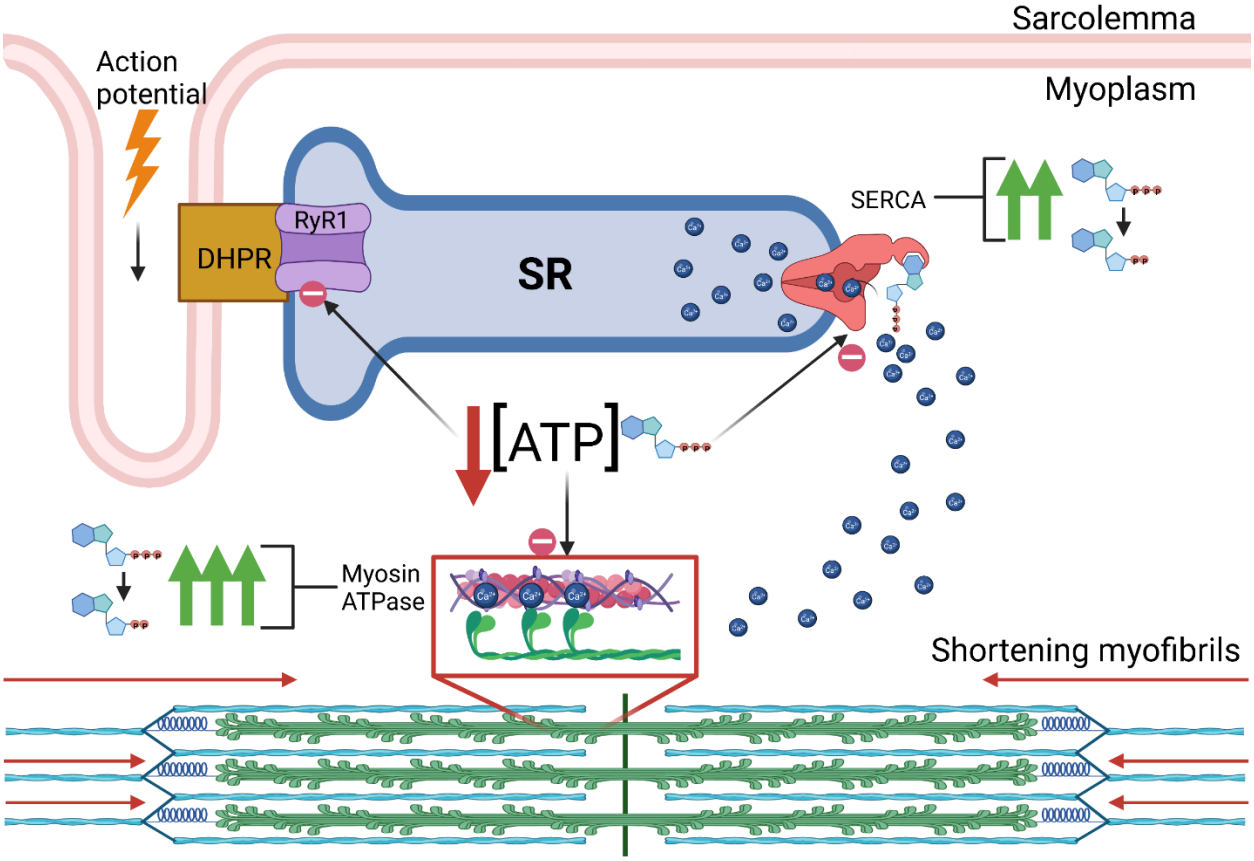

Figure 2: Decreased myoplasmic [ATP] leads to impaired skeletal muscle function. Myosin ATPase and SERCA collectively consume the most amount of ATP in contracting muscle. To preserve ATP for cellular function, when [ATP] decreases muscle function is impaired. This decrease in [ATP] would impair SR Ca^{2+} release and potentially blunts cross bridge cycling and SERCA function. Dihydropyridine receptor = DHPR; ryanodine receptor = RyR1; sarcoplasmic reticulum = SR; sarcoplasmic reticulum calcium ATPase = SERCA; Adenosine triphosphate = ATP; Created with BioRender.com

2.3: Metabolites and muscle function

A byproduct of ATP hydrolysis and anaerobic glycolysis during exercise are the accumulation of metabolites, which consequently contribute to fatigue (Cheng et al., 2018; Debold et al., 2016). For instance, the accumulation of metabolites or ions such as inorganic phosphate (P_i) hydrogen ion (H^+) and magnesium (Mg^{2+}) can hinder contractions through impaired force and velocity and thus promote fatigue-induced power loss at the level of ECC (Allen et al., 2008; Cheng et al., 2018; Debold et al., 2016). The following sections will cover how the effects of metabolite impair 1) SR Ca^{2+} release, and 2) force and velocity generation at the crossbridge level.

SR Ca^{2+} release can diminish in the presence of greater P_i, Mg^{2+} and decreases in ATP concentration. P_i is generated when phosphocreatine is broken down into P_i and creatine (Allen et al., 2008). In a study using mechanically skinned rat muscle fibres by Duke & Steele (2001), the presence of P_i impaired the release of SR Ca^{2+}. Moreover, ATP is associated with RyR1 binding and Mg^{2+} serves as an inhibitor (Allen et al., 2008; Cheng et al., 2018) which can impair the release of Ca^{2+} from the SR (Allen et al., 2008b). Generally, a rise in [Mg^{2+}] during contraction is attributed to a lower affinity in ADP, AMP and IMP (Allen et al., 2008). To see impairments in SR Ca^{2+} release, cytoplasmic [ATP] levels and [Mg^{2+}] need to significantly decrease and increase respectively (Cheng et al., 2018). To illustrate SR Ca^{2+} release, a study by Dutka and Lamb (2004) showed that a decrease in [ATP] independent of additional metabolites exerted only small impairments to SR Ca^{2+} release (10-20%) when [ATP] was lowered to 1mM and 0.5mM respectively. Additionally, when looking at Mg^{2+} in the same study, 3 mM of [Mg^{2+}] exerted a 40% decrease in SR Ca^{2+} release (Dutka & Lamb., 2004). In summary, metabolites can impair the release of SR Ca^{2+} release conditions with decreased ATP paired with increased P_i and Mg^{2+} (Duke & Steele., 2001). Furthermore, SR Ca^{2+} release can also be blunted due to an

increase in [P_i] which would impair SR Ca^{2+} release via P_i entering the SR through chloride channels in the SR membrane and then binding to Ca^{2+} to form Ca^{2+}-P_i precipitate that reduces the releasable Ca^{2+} from the SR (Allen & Trajanovska 2012; Ferreira et al., 2021). Given the nature of skeletal muscle contractions, P_i, H^+, and Mg^{2+} would be present which would collectively blunt SR Ca^{2+} release. Moreover, metabolites like H^+ and P_i can play multiple roles in fatigue such as blunting both force generation and velocity.

The decrease in intracellular pH is due to augmented H^+ generated from high rates of ATP hydrolysis paired with increased glycolytic flux (Robergs et al., 2004; Debold et al., 2016). Generally, human skeletal muscle exhibits pH levels of 7.05 to 7 and under intense exercise, can drop to roughly 6.5 – 6.2pH (Allen et al., 2008; Debold et al., 2016). This decrease in pH by H^+ impairs the strong binding of cross bridges, and crossbridge detachment rates, the latter of which impairs maximal shortening velocity (Debold et al., 2016; Woodward & Debold., 2018). Evidently, Knuth and colleagues (2006) have demonstrated that using the skinned muscle fibre method of fast (lateral head of gastrocnemius) and slow twitch rat muscles (soleus), acidosis can impair muscle shortening velocity. In this study, both fast and slow twitch muscles were placed in pH of 6.2 or 7 pH at 15 °C and 30 °C and when stimulated, the 6.2 pH group experienced greater reductions in unloaded velocity and smaller decreases of force when compared to the 7 pH group at both temperatures. Moreover, in another study utilizing single molecule laser trap assays of chicken pectoralis muscle at pH level of 6.4, velocity was blunted in comparison to a pH of 7.4 (Debold et al., 2008). Additionally, force generation declines in the presence of P_i (Debold et al., 2016). Furthermore, one speculated idea of P_i and muscle fatigue revolves around a concept that P_i impairs peak isometric force through a reduction of high force generating cross bridges (Debold et al., 2016) and P_i may play a role in premature myosin detachment from actin

during the high force state (Debold et al., 2013) therefore reducing force (Debold et al., 2016). Moreover, H^+ could impair myofibrillar Ca^{2+} sensitivity as H^+ outcompetes Ca^{2+} from binding to troponin C (Debold, 2016) reducing contractile force. Moreover, SERCA function appears to be blunted with increases in H^+ (Stienen et al., 1999) or [ADP] (Macdonald & Stephenson., 2001). In summary, H^+ and P_i both play a role at the crossbridge by impairing velocity and force.

Conversely, results surrounding metabolite production and different contractions appear to be conflicted. In one study using rat muscle, Vedsted and colleagues (2003) showed that lactate levels are not different between isometric and shortening contractions post fatiguing contractions. However, lactate is not an effective measure in assessing fatigue as lactate has minimal impact on contractile activity or SR Ca^{2+} reuptake (Allen et al., 2008; Posterino et al., 2001). Moreover ATP, PCr and glycogen use also appear to be non-significant when comparing isometric and shortening contractions (Vedsted et al., 2003). These results would contradict the finding that isometric contractions utilize less ATP when compared to shortening contractions seen in Newham and colleagues (1995) results. However, a weakness in measuring metabolites in the Vedsted and colleagues (2003) study is that there was a delayed period after the muscles were fatigued, and before the muscles were frozen for analysis, which allowed for muscle recovery. This would be seen in the time window in which unmounting the muscle from a chamber and flash freezing the muscle with liquid nitrogen would not fully reflect the metabolite changes that occurred at task failure. During this process, the fatigued muscle would regenerate ATP and decrease P_i due to the ATP resynthesis pathways reverting the levels of ATP present in the muscle. Overall, within the current literature, there is difficulties in assessing metabolites between both isometric and shortening contractions.

In summary, metabolites can play a role in impairing ECC processes through decreased power, force, and velocity generation by impairing SR Ca^{2+} release, cross bridge force as well as crossbridge cycling rates and potentially impairing SERCA function (Figure 3). Fatigue would blunt the ECC function and would preserve ATP to help sustain the muscle integrity. Furthermore, the potentially greater accumulation of these metabolites in skeletal muscles during fatiguing shortening contractions may play a major role in explaining why shortening contractions experience greater fatigue when compared to isometric contractions.

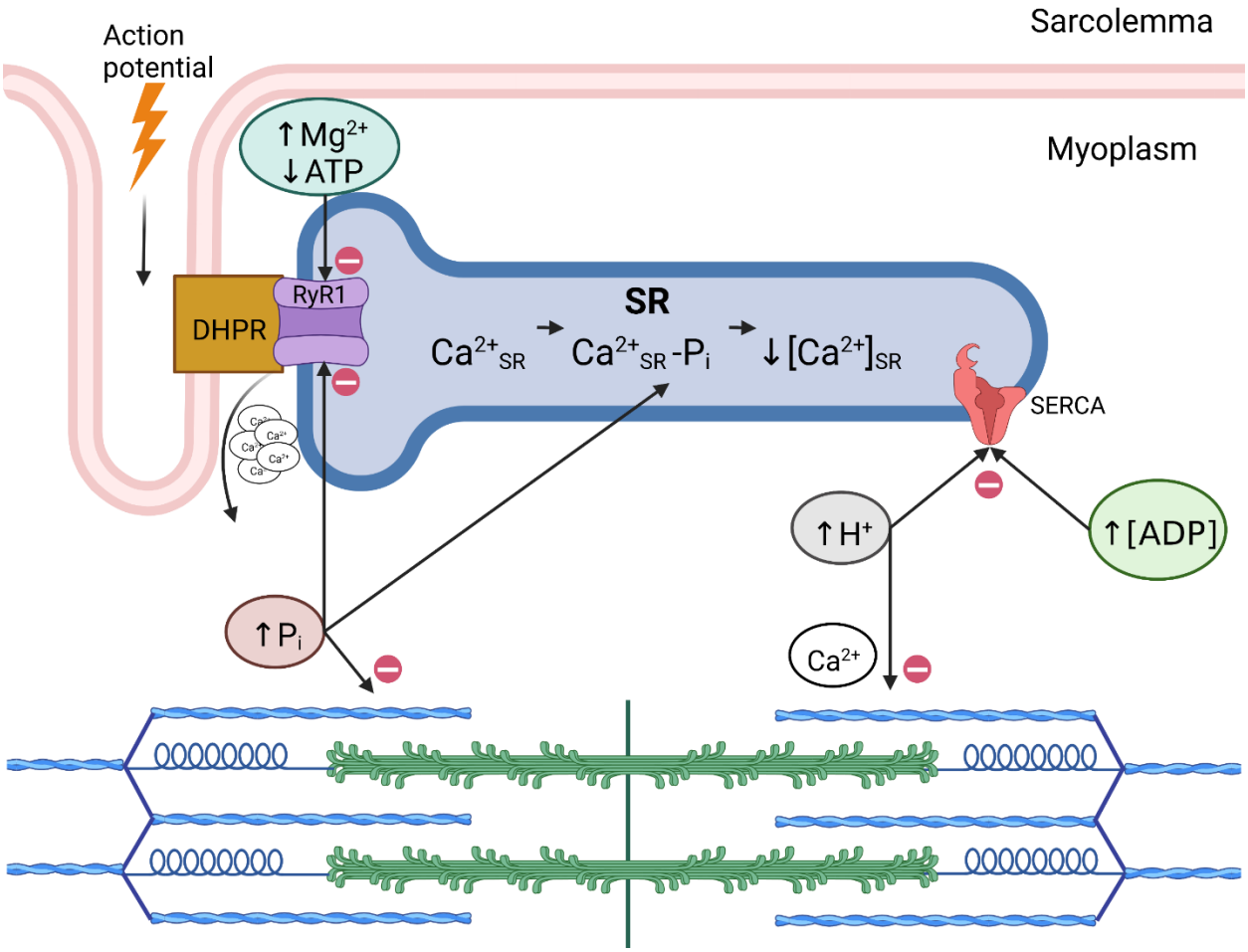

Figure 3. Metabolites and their effect on the ECC. Skeletal muscle produces metabolites and can impact various sites along the excitation contraction coupling process which can include impairments in cross bridge cycling, impairments in Ca^{2+} release via RyR1 and inefficient SERCA Ca^{2+} reuptake. Dihydropyridine receptor = DHPR; ryanodine receptor = RyR1; sarcoplasmic reticulum = SR; sarcoplasmic reticulum calcium ATPase = SERCA; adenosine triphosphate = ATP; adenosine diphosphate = ADP; Inorganic Phosphate = Pi; Calcium = Ca^{2+}; Hydrogen = H^{+}. Created with BioRender.com

2.4: Summary mechanisms - Suspected mechanisms related to greater fatigue during shortening vs. isometric contractions

To summarize, greater muscle fatigue induced by repeated shortening contractions compared with repeated isometric contractions may be attributed to direct mechanisms related to increased crossbridge cycling causing greater ATP utilization or indirectly through increased metabolite accumulation. Given the potential effects of increased ATP cost in shortening contractions compared to isometric contractions (Newham et al., 1995), a combination of greater ATP use and the simultaneous increase in metabolite accumulation may play a role in explaining increased impairments in SR Ca^{2+} release following shortening compared to isometric fatiguing contractions (Cheng et al., 2018).

Chapter 3: Objective and hypothesis:

Objective

The aim of this study is to investigate fatigue in shortening contractions when compared to isometric contractions and to observe if greater impairments in SR Ca^{2+} release are responsible for the greater fatigue-induced force loss following repeated shortening contractions.

Hypothesis

We hypothesize that under the same intensity and contraction duration, intense shortening contractions would result in greater force declines when compared to isometric contractions. This force decline in intense repeated shortening contractions would be attributed to reduced SR Ca^{2+} release when compared to repeated isometric contractions under the same stimulus conditions.

Chapter 4: Methods

4.1: Ethics and muscle dissection procedure

This study was approved by the York University Animal Care Committee (certificate # 2020-08). Female adult C57BL/6 mice (age 11-14 weeks) were housed and provided with chow and water ad libitum. Mice were anesthetized with 5% isoflurane (Fresenius Kabi, Canada) and then sacrificed via cervical dislocation. Flexor digitorum brevis (FDB) muscles were removed from the hindlimb via dissection and then submerged in an experimental tyrode solution consisting of 121 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH2PO_4, 24 mM $NaHCO_3$, 0.1 mM EDTA, and 5.5 mM glucose which is then bubbled with 95% O_2 and 5% CO_2 to get a pH of 7.4 (Cheng & Westerblad., 2017) at room temperature (23 °C). The FDB muscle was then mechanically dissected to extract an intact single muscle fibre (Cheng & Westerblad, 2017). Type 1 fibers are generally slow twitch fibers and are more fatigue resistant and type 2 fibres (type IIa and type IIx) can generate higher power but are more fatigable (Lievens et al., 2020). The FDB consists of predominantly (90%) fatigable type IIA and IIX muscle fibres (Tarpey et al., 2018) which would be ideal for studying fatigue mechanisms due to its predominant fatigable type II fibre-type composition. To check the viability of the fibres during the dissection, the FDB fibres were stimulated periodically with a custom-built pen-type stimulation electrode connected to an electrical stimulator (Model 701C, Aurora Scientific, Aurora, Canada) until a viable contracting single fibre was finally isolated (Cheng & Westerblad., 2017).

Dissected fibres were then clipped with aluminum T clips attached to each tendon, and thereafter mounted onto the metal hooks in the chamber of an Aurora Scientific 1500A Isolated Muscle System Microscope Mountable (Aurora, Canada) placed on an inverted microscope

(Nikon Eclipse Ts2R-FL, Tokyo, Japan). To determine the optimal length of force production, fibre length was adjusted, and the fibre was stimulated with 70 Hz tetani (300ms duration, 0.5 ms pulse duration) until peak force was achieved. Using a digital camera (DMK 42BUC03, The Imaging Source, Charlotte, NC, USA) and PC, fibre cross-sectional area was calculated at the optimal length by measuring fibre diameter at two cross-sections and taking the average of these two diameters. To determine fibre viability at optimal length, the fibre was stimulated at 120 Hz (300ms duration, 0.5ms pulse duration) and the specific force (i.e., force per cross-sectional area) was assessed with a minimum requirement of 300 kN/m^2 and a maximum of 450 kN/m^2, which is within the normal range for the specific force for a healthy intact single FDB fibre (Cheng & Westerblad., 2017). Mechanically dissected single fibres that failed to meet the minimum 300 kN/m^2 requirement at optimal length with a 120 Hz (300ms duration, 0.5ms pulse duration) stimulation were discarded.

4.2: Calcium and force measurements

Myoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured using a fluorescent dye, indo-1 AM (Cat. No. I1223, Invitrogen, Eugene, OR, USA), which is excited at 346 ± 5nm wavelength via xenon bulb and then dual emissions wavelengths were recorded using two photomultipliers with band pass filters at 405±5 nm and 495±5 nm (Horiba Ratiomaster, London, ON, Canada) (Cheng et al., 2019; Cheng & Westerblad., 2017). The advantage of using indo-1 is that it is a high-affinity Ca^{2+} indicator that can accurately detect changes in $[Ca^{2+}]_i$ at both rest and during maximal contractions (Cheng & Westerblad., 2017). Dissected fibres were then loaded with a concentration of 37 µM indo-1 AM for 30 minutes. After loading, the fibres were washed with tyrode for 30 min to remove any remaining indo-1 dye and to allow for AM ester hydrolysis (Cheng & Westerblad., 2017; Bannwarth et al., 2009). During the wash period and throughout

the remainder of the experiment, the fibres were continuously superfused with room temperature (23°C) experimental tyrode and newborn calf serum (Cat. No. 16010159, Gibco, New Zealand) bubbled with 95% O_2 – 5% CO_2.

4.3: Force-frequency relationship assessment

A force-frequency relationship describes how much isometric force a desired muscle can exert with a given frequency of activation, and typically provides a baseline phenotype assessment of single fibre contractile function (MacDougall et al., 2020). Using a similar protocol as Cheng and colleagues (2015), the force-frequency relationship was assessed in the current study by stimulating the fibre with individual 300 ms tetani (0.5ms pulse duration) at 15-150Hz (15, 20, 30, 40, 50, 70, 100, 120, 150Hz) with 2-minute intervals of rest between each stimulation. The resting and tetanic $[Ca^{2+}]_i$ values will be recorded as a ratio of the Indo-1 emissions at 405 and 495 nm wavelengths, along with baseline and peak force values at 300 ms using Felix32 (Horiba Scientific, Kyoto, Japan) and asi600a software (Aurora Scientific, Aurora, Canada), respectively. Fibres that displayed a leftward shift in the force frequency relationship were omitted from the study as these would represent fibres with a fatigue resistant slow-twitch phenotype.

4.4: Shortening and isometric contraction fatigue protocol

Fatigue was induced during the shortening contraction protocol by stimulating the fibre with repeated 70 Hz tetani (of 600 ms duration,0.5 ms pulse width) at 4 s intervals (Dahlstedt & Westerblad., 2001; Olsson et al., 2020) for a total of 50 contractions. The FDB muscle is preferred for mechanical dissection of intact single fibres due to short fibre lengths (0.5-1mm) and long tendons (Cheng & Westerblad., 2017). As performed previously (Roots et al., 2009), fibres undergoing the shortening protocol were stimulated to produce an initial isometric tetanus

at 300ms, and then the fibres were shortened to 10-20% of their optimal length while still undergoing a stimulation for an additional 300ms. Similar to the shortening fatigue protocol, the isometric fatigue protocol was induced with an identical stimulation parameter (70 Hz, 600 ms duration, 0.5ms pulse width, 4 s rest between contractions for 50 repetitions). Force development during the initial 300ms isometric phase of the isometric and shortening contractions were used to compare the fatigue-induced changes in contractile force between the fatigue protocols. Furthermore, resting and tetanic $[Ca^{2+}]_i$ along with force values were recorded during the initial 300 ms isometric phase of each contraction at 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 contractions. Fibres that failed to lose 50% of force production by repetition 50 (i.e., indicative of fatigue resistant fibres), or that were considered outliers (i.e., > 2 standard deviation above or below mean force values during the fatigue protocol) were also excluded from the study.

4.5: Statistics

Values are presented as means (± S.E.M) unless stated otherwise. Unpaired t-tests were used to determine statistically significant differences in fibre cross-sectional area, force and [Ca2+]i for baseline pre-fatigue measures. Two-way repeated measures ANOVA were used to determine statistically significant differences in force frequency, force $[Ca^{2+}]$ relationships, force and $[Ca^{2+}]_i$ between groups over time. Tukey post hoc test would be utilized provided a significant difference is identified using repeated measures ANOVA. A $p < 0.05$ will be considered as a statistically significant difference. Graph Prism 10 software (Graphpad, San Diego, CA, USA) will be used for all statistical analysis.

Chapter 5: Results

5.1: Baseline measurements

Fibre cross sectional area was not significantly different between both isometric (25 μm ± 0.86) and shortening groups (24.75μm ± 0.83) (Fig. 4A, $p = 0.81$). With regards to the specific force generated by the single fibres between groups (i.e., maximal force per cross sectional area), no significant difference was observed in 150 Hz specific force between isometric (411 kN/m^2 ± 18.3) and shortening (413 kN/m^2 ± 12.2) contractions (Fig. 4B, $p = 0.93$). Regarding the tetanic Ca^{2+} at the 150 Hz stimulation, the indo-1 ratio was not significantly different when comparing isometric (1.49 ± 0.08) and shortening (1.29 ± 0.09) contractions (Fig. 4C, $p = 0.11$). Furthermore, there were no difference in the indo-1 ratio representative of resting Ca^{2+} between the isometric (1.05 ± 0.08) and shortening (0.91 ± 0.07) contractions (Fig. 4D, $p = 0.22$). Each experiment started with the assessment of the force-$[Ca^{2+}]_I$ relationship for each fibre which found no differences in force frequency ($p = 0.71$) and $[Ca^{2+}]$ frequency ($p = 0.14$).

5.2: Fatigue measurements

5.2.1 Force

There was no significant difference in the 70 Hz force at the start of the fatiguing protocol for both isometric (355 kN/m^2 ± 18) and shortening groups (347 kN/m^2 ± 15.15) ($p = 0.73$). When examining the percentage force decline from the initial contraction between both isometric and shortening conditions, there was no significant difference in the percent reduction in 70 Hz force at the end of the fatigue protocol for both isometric (20 % ± 6 of initial value) and shortening (14 %± 5 of initial value) (Fig. 5A, $p = 0.20$).

5.2.2 Tetanic Ca^{2+}

There was no significant difference in the tetanic Ca^{2+} ratio at the start of the fatigue protocol between both isometric (1.40 ± 0.08) and shortening (1.23 ± 0.08) contractions ($p =$

0.16). Additionally, the percentage decline in tetanic Ca^{2+} from the initial contraction to the end of the fatigue protocol was not significantly different between the isometric (94% ± 2 of initial value) and shortening (89% ± 4 of initial value) fatigue protocol (Fig. 5B, $p = 0.39$).

5.2.3 Resting Ca^{2+}

No significant differences were reported with the initial resting Ca^{2+} ratio at the start of the fatigue protocol for the isometric (1.06 ± 0.09) and shortening (0.92 ± 0.08) contractions ($p = 0.23$). Additionally, no significant differences were seen in the percentage change in resting Ca^{2+} from the initial contraction through to the end of the fatigue protocol (Fig. 5C, $p = 0.64$).

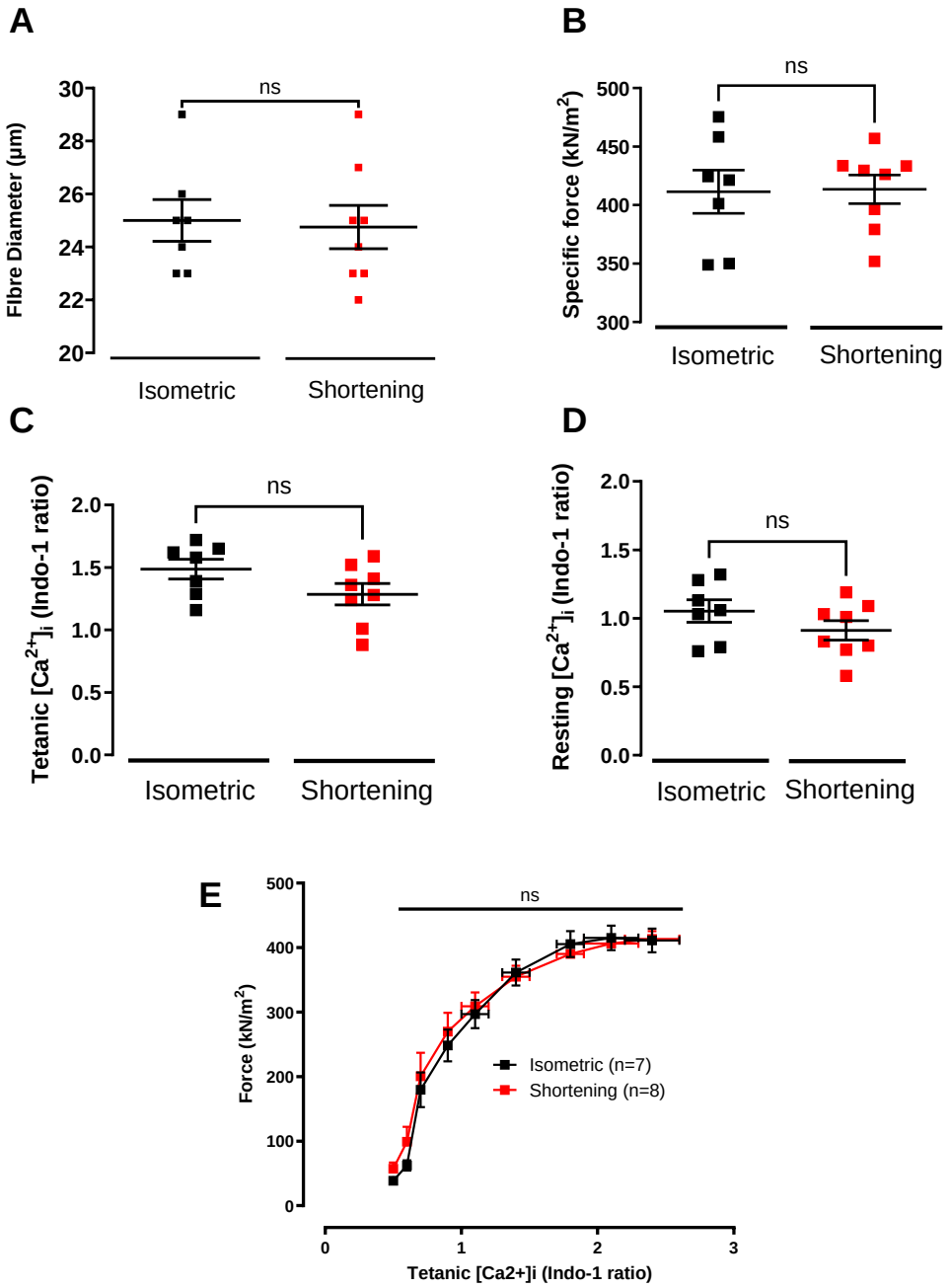

Figure 4: Baseline similarities between fibres in isometric and shortening contractions groups. A) Fibre diameter (µm) between isometric (Black boxes) and shortening (Red boxes). B) Specific force (kN/m^2) at 150 Hz stimulation. C) Tetanic [Ca2+]i (Indo-1 ratio) at 150 Hz stimulation. D) Resting [Ca2+]i (Indo-1 ratio) at 150 Hz stimulation. E) Force - [Ca2+] relationship for isometric and shortening groups.

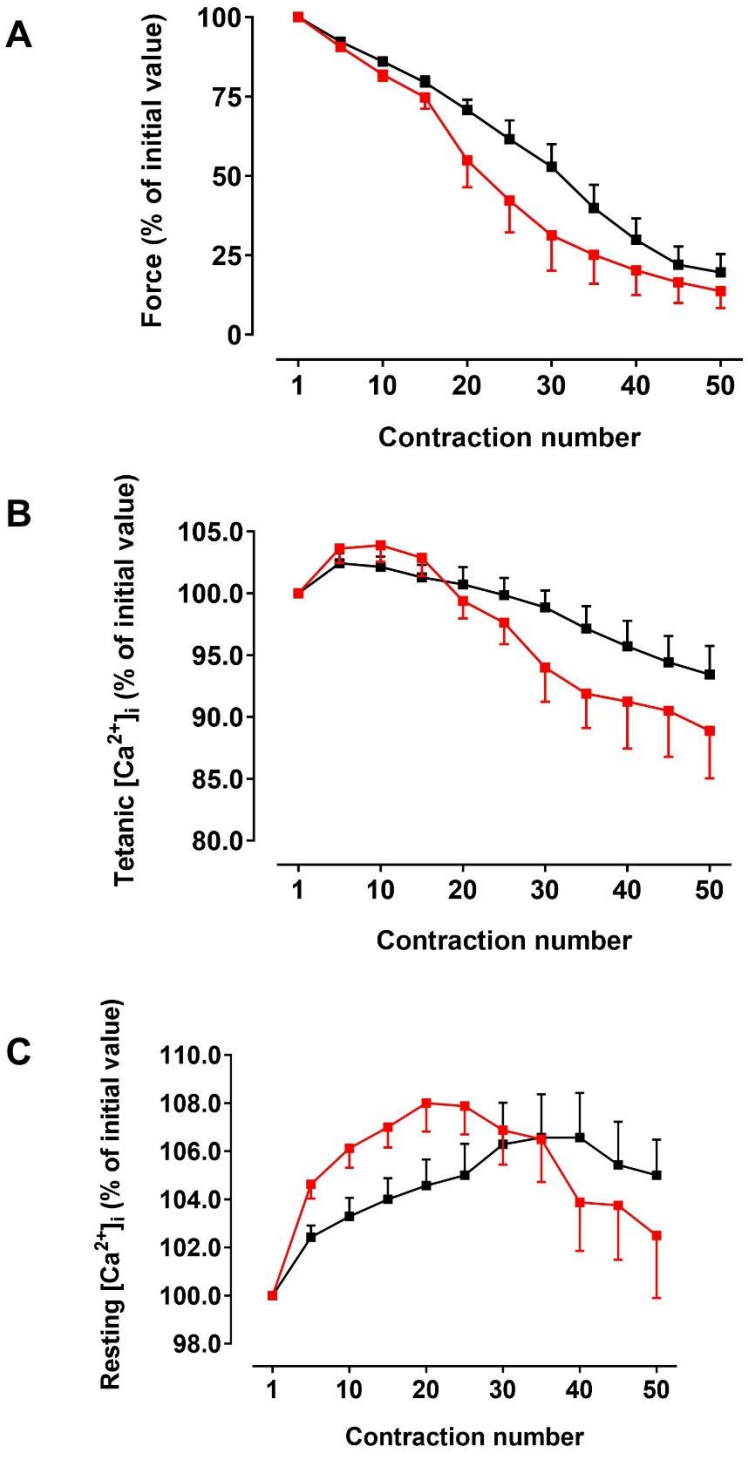

Figure 5: Effects of repeated isometric and shortening contractions on force and $[Ca^{2+}]$ **handling.** Fig. A) Percentage of force decline (kN/m^2) values over 50 contractions for both isometric (Black boxes) and shortening contractions (Red boxes). B) Percentage change in $[Ca^{2+}]$ ratio. C) Percentage change in resting $[Ca^{2+}]$ ratio values.

Chapter 6: Discussion

The primary purpose of this thesis was to investigate and compare fatigue induced by repeated shortening contractions compared to repeated isometric contractions. Consistent with previous literature, I hypothesized that repeated shortening contractions would cause greater fatigue-induced force loss when compared to repeated isometric contractions, and additionally, that greater impairments in SR Ca^{2+} release following repeated shortening contractions would explain their greater force loss when compared to repeated isometric contractions. Instead, the results of my study showed that repeated shortening contractions did not significantly result in greater reductions in force or SR Ca^{2+} release when compared to isometric contractions.

6.1: Phenotypic similarity between single fibres in each group

My baseline data examining fibre characteristics prior to the fatiguing protocol suggested that single fibres belonging to both isometric and shortening groups were not significant. No difference in fibre cross-sectional area or in specific force (i.e., force per cross-sectional area) eliminated the possibility of one group of fibres producing greater or weaker force (kN/m^2). A force-Ca^{2+} relationship was also utilized, and I saw no phenotypic differences between both groups, suggesting that fibres of both groups showed similar initial contractile properties. Moreover, my data for baseline and tetanic $[Ca^{2+}]_i$ at maximal 150 Hz stimulation also expressed no significant difference between fibres in the isometric compared to those in the shortening protocol.

6.2: Isometric vs. shortening fatigue assessment

Muscle fatigue is defined as an exercise-induced reduction in muscle contractile force, which can be seen through impairments in force, velocity and power generation (Gandevia, SC., 2001; Allen et al., 2008). Failure in muscle contractile function can be caused by processes

anywhere in the neuromuscular system in-vivo, from the nervous system to the muscular intracellular level (Gandevia, SC., 2001). The focus of this thesis is rooted in the intramuscular contributions to fatigue such as the Ca^{2+} handling processes in the ECC. Moreover, a reduction in SR Ca^{2+} release is often related to fatigue considering that Ca^{2+} is the major regulator of muscle force generation (Allen et al., 2008b). My repeated stimulation protocol for both isometric and shortening contractions included the fibres being stimulated identically (ie, fifty 70 Hz tetani 600ms duration tetanus given every 4s) with the only difference in the shortening contractions pertaining to the contractile shortening during the final 300 ms of each tetanus, similar to that performed previously by Roots and colleagues (2009). To my knowledge, my study is one of the first attempts in this field of research to measure SR Ca^{2+} release in a mechanically dissected single fibre muscle undergoing repeated shortening contractions, as measuring $[Ca^{2+}]_i$ and force simultaneously during repeated shortening contractions require specialized equipment and technical expertise.

In my current study, the recorded changes in force and $[Ca^{2+}]_i$ for the isometric and shortening fatigue protocols are similar to other well previously established studies investigating fatigue-induced changes in contractile function during isometric fatigue protocols, whereby a reduction in force during repeated isometric contractions is largely caused by reduced tetanic $[Ca^{2+}]_i$ levels (Westerblad et al., 1997; Westerblad & Allen 1991; Westerblad & Allen., 1993; Cheng et al., 2019; Olsson et al., 2020). Generally, repeated stimulations in a single fibre such as those seen in an isometric fatigue protocol often follow 3 phases (Place et al., 2009). These phases are observed as, Phase 1, a quick decline in force (10-20 % reductions) followed by an increase in tetanic $[Ca^{2+}]_i$ which can be seen in the first 5 to 10 contractions (Place et al., 2009). Both the quick decline in force as well as the increase in tetanic $[Ca^{2+}]_i$ have been proposed to be

caused by increased [P_i], which is a metabolic by-product of muscle fatigue (Leijding et al., 2023). Phase 2 is then seen as a constant force output, where force and [Ca^{2+}]$_i$ are generally well-maintained during repeated contractions, and phase 3 is then characterized by a steep decline in tetanic [Ca^{2+}]$_i$ and force production. The decline in [Ca^{2+}]$_i$ seen in phase 3 is mainly attributed to a reduction in SR Ca^{2+} release (Allen et al 2008; Place et al., 2009). However, while not significant, in the current study there is a visual trend in which the initial tetanic [Ca^{2+}]$_i$ values seen in phase 1 during the shortening fatigue protocol appears higher than that of the isometric contractions, potentially suggesting greater metabolite accumulation of [P_i] being responsible for the heightened SR Ca^{2+} release in phase 1. Additionally, my tetanic [Ca^{2+}]$_i$ data for shortening contractions also follows a trend in which the reduction in force and tetanic [Ca^{2+}]$_i$ appear greater in phase 3, which may elude to shortening contractions potentially causing greater fatigue-induced force loss than isometric contractions, with a pattern toward the greater force loss during shortening contractions being caused by greater reductions in tetanic [Ca^{2+}]$_i$. Furthermore, greater metabolic fatigue induced by the repeated shortening contractions could be eluded to by the greater increase in resting [Ca^{2+}]$_i$ seen during repeated shortening contractions when compared to the isometric contraction protocol. An elevated resting [Ca^{2+}]$_i$ would indicate impairments in the SERCA function contraction (Cheng et al 2019), whereby Ca^{2+} reuptake back into the SR is an ATP-dependent process and greater elevations in resting [Ca^{2+}] could suggest an increased energy demand of repeated shortening contractions when compared to repeated isometric contractions (Potma & Stienen., 1996; Newham et al 2005). Nonetheless, given the lack of statistically significant differences, we can only speculate on potential visual trends eluding to repeated shortening contractions inducing greater fatigue than repeated isometric contractions, suggesting that further experiments may be required to strengthen any conclusions.

There are a limited number of studies that have compared isometric and shortening contractions in muscle fatigue (See table 1). A study by Roots and colleagues (2009) is the study that closely resembles my methods by using dissected rat muscle bundles. I therefore expected to see comparable results in my study. For example, they identified that using predominantly fast-twitch rat flexor hallucis brevis muscle bundles, repeated shortening contractions had a larger force decline (35%) when compared to isometric contractions (25%) at the end of 20 repetitions. However, I observed during the shortening and isometric fatigue protocols in my study, a 45% decrease and 29% decrease in force loss, respectively at 20 repetitions, indicating greater fatigue induced in my experimental protocol. Furthermore, in the study by Roots and colleagues (2009), although a strength of their study design was the paired experiment protocol whereby all muscles performed both the isometric and shortening fatigue protocols, a major weakness of their methods was that the isometric fatigue protocol always proceeded the shortening fatigue protocol. This could be a concern because it can take hours or even days to recover from fatigue induced by exhaustive exercise, and repeating another exercise on the same muscle after a brief rest period could predispose the second exercise session to greater fatigue susceptibility.

One limitation of my study could be the design of the shortening contraction protocol. Similar to the contraction protocol by Roots and colleagues (2009), I designed the study to allow for a shortening contraction to first undergo an isometric phase at the initial to 300ms stimulation, followed immediately by the muscle then undergoing a 300ms shortening step. While having an initial isometric phase in the shortening group was done to help us compare the real-time fatigue-induced decline in force in both groups, the fatigue-induced by the shortening contraction fatigue protocol is not purely caused to contractile shortening given that 50% of each 600ms contraction begins with a 300ms isometric contraction. Thus, perhaps the lack of any

difference in the fatigue-induced force loss during the isometric vs. shortening fatigue protocols in the current study can be attributed to the contractions in the shortening fatigue protocol not being purely shortening contractions. This is important because contractile shortening per se is assumed to cause greater myofibrillar ATPase consumption than isometric contractions, resulting in greater energy consumption within the muscle fibre as well as potentially leading to greater metabolite accumulation that would accelerate fatigue processes (Newham et al., 1995; Potma & Stienen., 1996).

Another limitation of this study that may have explained the lack of a difference in fatigability between the isometric and shortening fatigue task could be an under powered sample size. An n value was based off the study by Roots and colleagues (2009), which is a paired study design that utilized a total n=12 fibres in which the same fibre was used for both isometric and shortening fatigue protocols. In comparison, my experiment involves an unpaired study design with n=7-8 fibres per group involved in the isometric and shortening fatigue protocols. This underpowered sample size could be an additional explanation as for why my results suggest both isometric and shortening contractions are not significantly different in fatigability.

6.3: Potential advantage of mechanically dissected single fibre technique in assessing muscle fatigue

Traditionally, fatigue at the single fibre is assessed during isometric contractions as opposed to shortening contractions since isometric contractions are technically easier to study than shortening contractions where the latter requires precise equipment and associated software to control shortening speed and distance, and the imaging of $[Ca^{2+}]_i$ is more complex with the larger movement occurring with contractile shortening. While a few studies have compared shortening and isometric contraction, this is one of the first studies to my knowledge to use the

mechanically dissected technique to investigate fatigue when comparing both contraction types. I decided to use the mechanically dissected technique due to the various advantages. One advantage of the mechanically dissected single fibre technique is, unlike, enzymatically dissociated fibres, the user can simultaneously assess both global Ca^{2+} and force with the same fibre (Cheng & Westerblad, 2017). Possessing the ability to pair both Ca^{2+} and force measurements allows the user to observe when fatigue is developing, and when Ca^{2+} impairments begin to develop in relationship to the change in contractile force. Additionally, the mechanically dissected fibre technique requires a living intact fibre which allows for testing a muscle fibre during a fatigue protocol unlike the mechanically and chemically skinned fibre techniques. Additionally, Ca^{2+} measurements can be assessed with the use of a dual emission system paired with indo-1 dye (either injected of AM loaded) allows for a user to investigate the effects of Ca^{2+} handling within the skeletal muscle at rest and during contraction (Cheng & Westerblad., 2017). Moreover, while not utilized in this study, this technique is not limited to Indo-1 dye as additional dyes such as Mag-indo and Mag-Fluo-4 can be loaded into the fibre before experiments. For example, Mag-indo allows for the observation of ATP utilization during contractions and the Mag-fluo-4 can be used to assess rapid calcium reuptake or release kinetics by the SR (Cheng & Westerblad., 2017). Moreover, unlike mechanically or chemically skinned fibres, the sarcolemmna remains intact with this method, allowing for the presence of additional physiological relevant factors such as intact membrane and extracellular matrix functions. Finally, mechanically dissected single fibres can also be paired with other techniques. For example, this technique can be used for transmission electron imaging and assessing metabolic heat production (Cheng & Westerblad., 2017). Ultimately, I suspected this technique would be optimal to test both fatigue in contractions due to the ability to assess both force values and Ca^{2+}

handling to help elucidate if shortening contractions have greater Ca^{2+} impairments and if those impairments are related to the suspected greater fatigue.

Some disadvantages of the mechanically dissected single fibre technique are the technical difficulties and the time-consuming process to acquire a single intact contracting fibre (Allen et al., 2008; Place et al., 2010). Evidently, this limits data output to a rate of one to two fibres per day (Cheng et al., 2017). Additionally, this technique requires an individual to mount a clean fibre to acquire Ca^{2+} signals, which can be difficult depending on dissection preparations which may result in disrupted Ca^{2+} signals. For example, using a fibre that has not been properly cleaned could result in an accumulation of debris around a region of interest. Once this fibre and localized debris is loaded with dye, Ca^{2+} readings can become obscured or difficult to interpret due to an increase in movement artifacts. Moreover, an additional criticism of the mechanically dissected single fibre technique is the lack of nerve innervation and blood flow to the muscle which can challenge the physiological relevance of the technique.

Chapter 7: Future directions

One future direction would be to revise my shortening and isometric protocol. I could revert to an isometric protocol which involves an isometric contraction at 70 Hz for 350ms duration and 2 seconds of rest (Dahlstedt & Westerblad., 2001). Additionally, my shortening contraction protocol would involve a purely shortening contraction as opposed to my current design which includes an initial 300 ms isometric contraction followed immediately by a 300ms shortening contraction. The shortening contraction first undergoing 300ms of isometric contractions results in my shortening contractions only comprising of 50% of muscle shortening which does not reflect physiological shortening contractions during locomotion. In order to compare the fatigue-induced force loss between the shortening vs. isometric fatigue protocols, I can instead periodically include a 70Hz isometric tetanus elicited every 5 or 10 repetitions during the repeated shortening contraction protocol. This alteration would allow me to fully investigate if repeated contractions that are purely consist of shortening contractions undergo greater fatigue when compared to repeated isometric contractions.

Skeletal muscle utilizes ATP hydrolysis to carry out muscle contraction function (Barclay et al., 1993). Additionally, ATP cost differs with contraction type, for example, Newham and colleagues (1995) demonstrated that the cost of shortening contractions were almost double that of sustained isometric contractions undergoing the same stimulation intensity (50 Hz). Therefore, I could investigate the effects of ATP cost between isometric and shortening contractions over 50 repeated bouts of contractions. I can investigate intracellular ATP breakdown measurements by using a Mag-Indo-1 dye. Generally, Mg^{2+} is typically bound to ATP and when ATP hydrolysis occurs, for example during cross bridge cycling, free myoplasmic [Mg^{2+}] would increase (Westerblad & Allen., 1992; Cheng et al., 2019b). I would expect that over the duration of the 50

contractions, mag-indo-1 ratios would increase, denoting an increase in ATP utilization. Given the three major locations of ATP consumption during a contraction (Na^+/K^+ ATPase, SERCA, and myosin ATPase), this could be considered beneficial to some degree because I can then observe if ATP costs are greater in repeated shortening contractions when compared to isometric contractions. However, while I can potentially determine overall energy cost of contractions between both the isometric and shortening contraction fatigue protocols, one of the limitations would reside in the uncertainty of which location uses more ATP compared to the others, such as, the uncertainty of SERCA function impairments during fatiguing bouts of stimulation when compared to ATP consumption by Na^+/K^+ ATPase as well as myosin ATPase.

Various contraction types influence functional independence and quality of life as all three, isometric, shortening and eccentric contractions allow us to interact with our environment. However, there appears to be a difference in which repeated shortening contractions create greater fatigue in an aging population when compared to the young. For example, in a study by Dalton and colleagues (2010), young (24 year old) and old (78 year old) individuals underwent 50 max velocity plantar flexion contractions at a 20% maximal voluntary isometric contraction expressed older individuals saw reductions in peak power generation when compared to younger individuals. Moreover, voluntary activation values were not significant suggesting central fatigue is not responsible for peak power loss and the fatigue may be rooted in mechanisms related to peripheral mechanisms. One potential mechanism that may explain the greater fatigue-induced reduction in power generation during dynamic than compared to isometric fatigue tasks could be the effects of metabolites (Sundberg et al., 2018). Evidently, elderly females undergoing dynamic knee extensions generate greater increase in $[P_i]$ and $[H^+]$ when compared to younger females at the end of exercise when doing the same fatiguing task (Sundberg et al., 2019). Given

that metabolites play a role in cross bridge function, both increased [Pi] and [H^+] would impair cross bridge kinetics through reduced force generation and velocity respectively (Debold et al., 2016). However, an additional mechanism that may be attributed to this may potentially be an impairment in Ca^{2+} function, which could be a result of metabolite accumulation and their impact on SR function. For example, an increase in [Pi] and [Mg^{2+}] can impair the release of Ca^{2+} from the SR (Duke & Steele., 2001). However, Sundberg and colleagues (2019) suggest that the mechanisms that contribute to the greater metabolite accumulation in elderly individuals during exercise remain unknown as [PCr] and [ATP] levels remain non-significant between both groups at the end of exercise.

Chapter 8: Conclusion

In the present study I investigated the comparison of fatigue induced by repeated isometric and shortening contractions. A novelty of my study is using the mechanically dissected intact single muscle fibre method to investigate the contribution of fatigue-induced changes in SR Ca^{2+} release to explore potential differences in the reductions in contractile force when comparing fatigue induced by repeated isometric vs. shortening contractions. Despite previous literature showing that repeated shortening contractions induce greater fatigue when compared to repeated isometric contractions, there was no significant differences in the fatigue-induced change in force, tetanic $[Ca^{2+}]_i$ and resting $[Ca^{2+}]_i$ over 50 contractions. Further investigation is potentially needed to elucidate whether there is a contraction-type dependency of muscle fatigue, with considerations including potential methodological modifications and an increased sample size. Therefore, further experiments are required to investigate the full extent of comparing force reduction and Ca^{2+} handling (resting and tetanic values) when comparing repeated isometric contractions to repeated shortening contractions undergoing the same stimulation protocol.

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Tables

Author	Animal/Human model - muscle	Assessment	Fatigue protocol	Fatigue Outcomes
Cheng, AJ & Rice, CL (2009)	Human – dorsiflexors	1s contraction at 2s intervals at 50% MVC	Contractions until task failure described below: Shortening: When 2 consecutive contractions declined to <50% velocity. 5 s after contraction the subject performed 1 isometric contraction Isometric: when maximum voluntary contraction torque decreased to values seen in the shortening contraction	Shortening contractions > Isometric contractions
Vedsted et al (2003)	Rat – soleus and extensor digitorum longus	Soleus - 60Hz, 400 ms for 100s Extensor digitorum longus – 60Hz 400 ms for 60s	F_{max} = Maximal force development dF/dtmax = maximal rate of force development	Isotonic contractions > Isometric contractions
Roots et al (2009)	Rat – Flexor hallucis brevis	Control was set by stimulating contraction every 1/60s at 70Hz. Fatigue was induced by stimulating contractions 1/5s	Decreases in tension	Shortening > Isometric

		at 70Hz for both isometric and shortening contractions.		
Ameredes, BT & Clanton, TL (1990)	Canine – Diaphragm	Diaphragm strips stimulated at 30 or 40Hz from optimal length	Decreases in peak tension	Greater loss of force in isovelocity compared to isometric
Seow, CY & Stephens, NL (1988)	Mouse - Diaphragm	100 Hz with 0.5 pulses every 2s for 0.5s. This was repeated over the duration of 160s	Percentage drop in isometric tension from start to end of fatigue process	Isotonic Contractions > Isometric contractions
Devrome, AN & MacIntosh BR (2018)	Rat – Medial gastrocnemius	1s stimulations at 170Hz over 100s	Greater decrease in Vmax	Isotonic Contractions > Isometric contractions

Table 1: Summary of studies which have compared fatigability during repeated shortening contractions compared to isometric contractions.