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## **Mechanisms and Mitigation of Skeletal Muscle Fatigue in Single Fibers from Older Adults**

Aurora Foster

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Mechanisms and Mitigation of Skeletal Muscle Fatigue in Single Fibers from  
Older Adults

A Thesis Presented

By

AURORA DANAE FOSTER

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
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Kinesiology

Mechanisms and Mitigation of Skeletal Muscle Fatigue in Single Fibers from  
Older Adults

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AURORA DANAE FOSTER

Approved as to style and content by:

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Mark Miller, Chair

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Jane Kent, Department Head  
Department of Kinesiology

## DEDICATION

I dedicate this work to my family...

Joey – You made this possible.

Little Bear & Alex – May this inspire you to aim high and never settle.

Mom & Dad – Always my support.

## ACKNOWLEDGEMENTS

Thank you to my advisor, Mark S. Miller, for your guidance and support, both professionally and personally. You have helped me develop the skills I need to succeed and grow into the professional that I am today. Thank you to the rest of my committee, Jane Kent and Edward (Ned) Debold, for your valuable input and support.

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## ABSTRACT

### MECHANISMS AND MITIGATION OF SKELETAL MUSCLE FATIGUE IN SINGLE FIBERS FROM OLDER ADULTS

MAY 2019

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Directed by: Professor Mark S. Miller

Skeletal muscle fatigue is the contraction-induced decline in whole muscle force or power, and can be greater in older versus young adults. Fatigue primarily results from increased metabolism elevating phosphate ( $P_i$ ) and hydrogen ( $H^+$ ), which alters myosin-actin interactions; however, which steps of the myosin-actin cross-bridge cycle are changed and their reversibility are unclear. **PURPOSE:** This study sought to: 1) Examine the effects of elevated  $P_i$  and  $H^+$  on molecular and cellular function, and 2) Test the ability of deoxyadenosine triphosphate (dATP), an alternative energy to adenosine triphosphate (ATP), to reverse the contractile changes induced with high  $P_i$  and  $H^+$ . **METHODS:** Maximal tension (force/cross-sectional area), myofilament mechanics and myosin-actin cross-bridge kinetics were measured in 214 single fibers (104 type I) from the vastus lateralis of eight (4 men) healthy, sedentary older adults ( $71 \pm 1.3$  years) under normal (5 mM  $P_i$ , pH 7.0), simulated fatigue (30 mM  $P_i$ , pH 6.2) and simulated fatigue with dATP conditions. **RESULTS:** Tension declined with high  $P_i$  and  $H^+$  in slow- (type I, 23%) and fast-contracting (type II, 28%) fibers due to fewer strongly bound myosin heads

(28-48%) and slower cross-bridge kinetics (longer myosin attachment times ( $t_{on}$ ) (18-40%) and reduced rates of force production (18-30%)). Type I myofilaments became stiffer with high  $P_i$  and  $H^+$  (48%), which may have partially mitigated fatigue-induced tension reduction. Elevated  $P_i$  and  $H^+$  with dATP moderately improved force production similarly in both fiber types (8-11%) compared to high  $P_i$  and  $H^+$  with ATP. In type I fibers, high  $P_i$  and  $H^+$  with dATP returned the number of myosin heads strongly bound and  $t_{on}$  to normal, while the rate of force production became faster than normal (16%). In type II fibers, high  $P_i$  and  $H^+$  with dATP did not change the number of myosin heads bound, but cross-bridge kinetics were 16-23% faster than normal. **CONCLUSION:** These results identified novel fiber-type specific changes in myosin-actin cross-bridge kinetics and myofilament stiffness that help explain fatigue-related force reduction in human single skeletal muscle fibers as well as an alternative energy source that partially to fully reverses contractile changes of elevated  $P_i$  and  $H^+$  that occur with fatigue.

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# CHAPTER 1

## INTRODUCTION

### 1.1 Background

Skeletal muscle fatigue occurs when hard or continuous contractions reduce the ability of muscles to generate power due to reduced force and/or velocity (Power = Force  $\times$  Velocity) (Callahan & Kent-Braun, 2011; Katsiaras et al., 2005; Kent-Braun et al., 2012). Older adults are especially susceptible to fatigue-related decrements since they tend to fatigue quicker and to a greater extent than young adults during rapid dynamic contractions (Hicks et al., 2001; Katsiaras et al., 2005; Callahan & Kent-Braun, 2011; Christie et al., 2011; Broxterman et al., 2017; Sundberg, Kuplic et al., 2018), which may be due to altered cellular and molecular contractile properties with age (Trappe et al., 2003; Ochala et al., 2007; Callahan & Kent-Braun, 2011; Miller et al., 2013). Decreased muscle power that characterizes fatigue increases an older adult's risk for falls (Schwendner et al., 1997; Wolfson et al., 1995) and frailty (Theou et al., 2008), indicating reduced physical function. Older adults with reduced physical function are more likely to be hospitalized (Cawthon et al., 2009), which leads to greater personal and societal burden. These fatigue-related issues in older adults will be exacerbated by the fact that the older population (aged 65+ years) in the U.S. is increasing and is expected to constitute 22% of our population (82 million people) by the year 2040 (Ortman et al., 2014).

The build-up of metabolic by-products, primarily phosphate ( $P_i$ ) and hydrogen ( $H^+$ ) (low pH), are temporally associated with fatigue (Kent-Braun et al., 1993; Kent-

Braun et al., 2002; Broxterman et al., 2017) and are thought to inhibit muscle performance at the molecular and cellular levels (Debold et al., 2016), ultimately leading to decreased whole muscle and whole body function. Understanding the mechanisms and potential treatments for fatigue at the cellular and molecular levels are important to mitigate or reverse fatigue-related declines in whole muscle and whole body function, especially in older adults who are most affected by fatigue. Fatigue can be studied *in vitro* by elevating metabolites in the bathing solutions of single muscle fibers and isolated myosin-actin proteins in order to simulate the biochemical changes that occur during fatigue *in vivo*. Elevated  $P_i$  and  $H^+$  are presumed to be the primary metabolites that decrease muscle function during fatigue. Most other metabolites and substrates that change with fatigue are thought to play a minimal role in altering contractile properties (elevated adenosine diphosphate and reduced adenosine triphosphate, (Cooke, 2007)) or are lacking accurate measurement techniques (elevated reactive oxygen and nitrogen species, (Debold et al., 2016; Westerblad & Allen, 2011)), while others may not be as important for relatively short duration fatigue *in vivo* (reduced calcium concentration, (Allen et al., 1989; Lee et al., 1991; Westerblad et al., 1989)). Therefore, this study focused on simulated fatigue *in vitro* with elevated  $P_i$  and low pH.

Reduced power that characterizes fatigue can be caused by a reduction in force and/or velocity production, therefore single fiber and isolated myosin-actin studies have examined the effects of the putative agents of fatigue on power, force, velocity, and the molecular determinants of these parameters. Simulated fatigue at the molecular and cellular levels has mostly been studied using young animal tissue, with only one recent study in tissue from young and older men. Both slow- (myosin heavy chain I or MHC I)

and fast- (MHC II) contracting single skeletal muscle fibers from young animals and men exposed to elevated  $P_i$  and low pH at near-physiological temperatures (30°C) to simulate fatigue exhibited decreased maximal velocity and force production (Karatzaferi et al., 2008; Nelson et al., 2014; Sundberg, Hunter et al., 2018). The most recent fiber studies in rats (Nelson et al., 2014) as well as young and older men (Sundberg et al., 2018) showed a leftward shift in the force-velocity curve with fatigue, indicating depressed force and velocity across the entire range of both parameters, which decreased power production throughout the entire fiber force-generating range. Reduced fiber force and velocity with fatigue are largely due to alterations in the interactions between the two primary contractile proteins, myosin and actin, though the exact mechanisms remain uncertain (Debold et al., 2016).

Slowed single fiber velocity with high  $P_i$  and  $H^+$  may be due to longer myosin attachment times to actin ( $t_{on}$ ) (slower cross-bridge kinetics) at the molecular level. Results from unloaded isolated protein *in vitro* motility assays have suggested that elevating the putative agents of fatigue lengthened  $t_{on}$  in single myosin molecules, if velocity is assumed to be limited by myosin detachment (H. Huxley, 1990; Debold et al., 2008; Debold et al., 2012). Single fiber experiments under loaded, control conditions not simulating fatigue also suggest that slowed fiber velocity occurred because of longer  $t_{on}$  (Piazzesi et al., 2007; Miller et al., 2017), making longer  $t_{on}$  a probable cause for slowed velocity with fatigue assuming a detachment-limited model of the cross-bridge cycle. However,  $t_{on}$  has not yet been measured in single fibers under conditions that simulate fatigue.

Fiber force and therefore tension (force/cross-sectional area (CSA)) are largely determined by the number of strongly bound myosin-actin cross-bridges multiplied by the force exerted by each cross-bridge (A. F. Huxley, 1957; Brenner, 1988; Linari et al., 2004), so both or either of these parameters may decrease in order to reduce force with fatigue (Nocella et al., 2011; Nelson et al., 2014). The number of strongly bound cross-bridges is dictated by myosin-actin cross-bridge kinetics, myosin attachment time ( $t_{on}$ ) and rate of myosin force production, which influences myosin detachment time ( $t_{off}$ ). If we assume that, in fibers exposed to high  $P_i$  and  $H^+$ , slowed velocity occurs due to longer  $t_{on}$  in a detachment-limited model, the concomitant decrease in force could occur due to 1) an even greater increase in  $t_{off}$  than  $t_{on}$  that reduces the number of strongly bound heads at any given time and/or 2) a reduction in the amount of force generated by each myosin-actin cross-bridge. The few studies that have examined these mechanisms reached different conclusions. One study found that force initially declined due to reduced force generation per cross-bridge (cross-bridge stiffness), while greater force reductions occurred as the number of strongly bound myosin heads were reduced over time (Nocella et al., 2011). Another study showed that the rate of myosin weak to strong binding transition (measured by  $k_{tr}$ , or rate of force redevelopment) was unchanged in single fibers in elevated  $P_i$  and low pH conditions, but maximal fiber force was hypothesized to decrease due to reduced force per cross-bridge (Nelson et al., 2014). A new study in fibers from young and older men found that  $k_{tr}$  slowed with high  $P_i$  and  $H^+$  (Sundberg et al., 2018), suggesting that reduced force with simulated fatigue may be due to longer  $t_{off}$  and therefore fewer strongly bound cross-bridges. Finally, one recent isolated protein study using the laser trap assay found that high  $P_i$  and low pH reduced the force produced

by each myosin molecule and its energy utilization rate (ATPase), which indicates longer  $t_{on}$  and/or  $t_{off}$  (Woodward & Debold, 2018). Overall, more work needs to be done to determine the molecular and cellular determinants of reduced fiber force under fatiguing conditions.

Some promising data suggests that fatigue-related decrements in muscle function can be reversed by substituting the muscle's primary energy source, adenosine triphosphate (ATP), with a naturally occurring ATP analogue, deoxyadenosine triphosphate (dATP). One *in vitro* motility study using isolated proteins from young animal muscle showed that dATP reversed the decrease in regulated thin filament velocity as a result of elevated  $P_i$  and low pH back to control levels (Longyear et al., 2014). Non-fatigued young rabbit fibers activated with dATP produced similar tensions and faster contractile velocities compared to fibers activated with ATP (Regnier et al., 1998; Regnier & Homsher, 1998), demonstrating that dATP also improves cellular-level contractile function under non-fatigue conditions. These studies suggest that dATP improves single fiber function both with and without elevated  $P_i$  and  $H^+$  conditions, making it a potential target for a fatigue countermeasure.

## **1.2 Statement of the Problem**

The exact cellular and molecular mechanisms of fatigue are currently unclear since few single fiber or molecular studies have simulated fatigue *in vitro* by combining high  $P_i$  and  $H^+$ , the two major metabolites that alter muscle function and change concurrently with fatigue *in vivo*. The mechanisms of the reductions in the determinants of fiber power, maximal velocity and force, with elevated  $P_i$  and low pH are unclear. The



effects of high  $P_i$  and  $H^+$  on an important determinant of fiber velocity and force,  $t_{on}$ , have only been hypothesized using *in vitro* motility experiments with low pH only (Debold et al., 2008; Debold et al., 2012; Debold et al., 2016). These molecular data should be verified in single fibers exposed to high  $P_i$  and  $H^+$  conditions since single fiber experiments maintain the sarcomere's three-dimensional structure and the load that actin imposes on myosin (Miller et al., 2013). Other important determinants of fiber force at the cellular (myofilament stiffness) and molecular (number of strongly bound myosin heads, force per cross-bridge and rate of myosin force production) levels should be further examined since current studies have shown inconsistent results in how these parameters change with fatigue.

To my knowledge, most single fiber studies have examined the effects of high  $P_i$  and low pH in fibers from young animals with only one recent study using fibers from young and older men. Fibers from humans have inherently different contractile properties than those from animals, such as slower velocity due to slower cross-bridge kinetics (Pellegrino et al., 2003), which implies that different species may have distinct responses to high  $P_i$  and  $H^+$  conditions. In addition, simulated fatigue studies using fibers from older adults are needed since this population is more affected by fatigue during dynamic whole muscle contractions than their young counterparts (Hicks et al., 2001; Katsiaras et al., 2005; Callahan & Kent-Braun, 2011; Christie et al., 2011; Broxterman et al., 2017; Sundberg et al., 2018), which may be due to altered mechanical and kinetic properties with age (Trappe et al., 2003; Ochala et al., 2007; Miller et al., 2013). Importantly, men and women may respond differently to fatigue at the whole muscle level (Hicks et al.,

2001; Labarbera et al., 2013) and to aging at the cellular and molecular levels (Miller et al., 2013), indicating the importance of examining the response of both sexes to fatigue.

### **1.3 Study Approach**

The objectives of this study were to: 1) Determine the mechanisms of fatigue that decrease skeletal muscle performance at the cellular and molecular levels in single human skeletal muscle fibers from older men and women and 2) examine the efficacy of a potential countermeasure, dATP, to mitigate or reverse fatigue-related declines in skeletal muscle function at the cellular and molecular levels. Skeletal muscle cellular and molecular function was measured in 214 single fibers (104 type I, 110 type II) from the vastus lateralis of eight healthy, sedentary older adults (4 men). *In vivo* fatigue was simulated by activating fibers in solutions containing the highest recorded levels of  $P_i$  (30 mM vs. 5 mM) and  $H^+$  (pH 6.2 vs. 7) in fatigued human whole muscles (Hermansen & Osnes, 1972; Metzger & Fitts, 1987; Cady et al., 1989; Broxterman et al., 2017). Single fiber function was characterized by measuring tension, which is force normalized to cross-sectional area, to allow for comparison among fibers of different sizes. Small-amplitude sinusoidal length perturbation analysis was used to determine myofilament mechanical properties, including myosin-actin cross-bridge mechanics (cross-bridge number and stiffness) and kinetics (myosin attachment time and rate of myosin force production) as well as myofilament stiffness. Aims and hypotheses are outlined below and in Figure 1.

## **1.4 Aims and Hypotheses**

### **1.4.1 Aim 1**

Determine the effects of simulated fatigue on tension and myofilament mechanical properties, including myosin-actin cross-bridge kinetics, in single fibers from older men and women.

#### **1.4.1.1 Hypotheses for Aim 1**

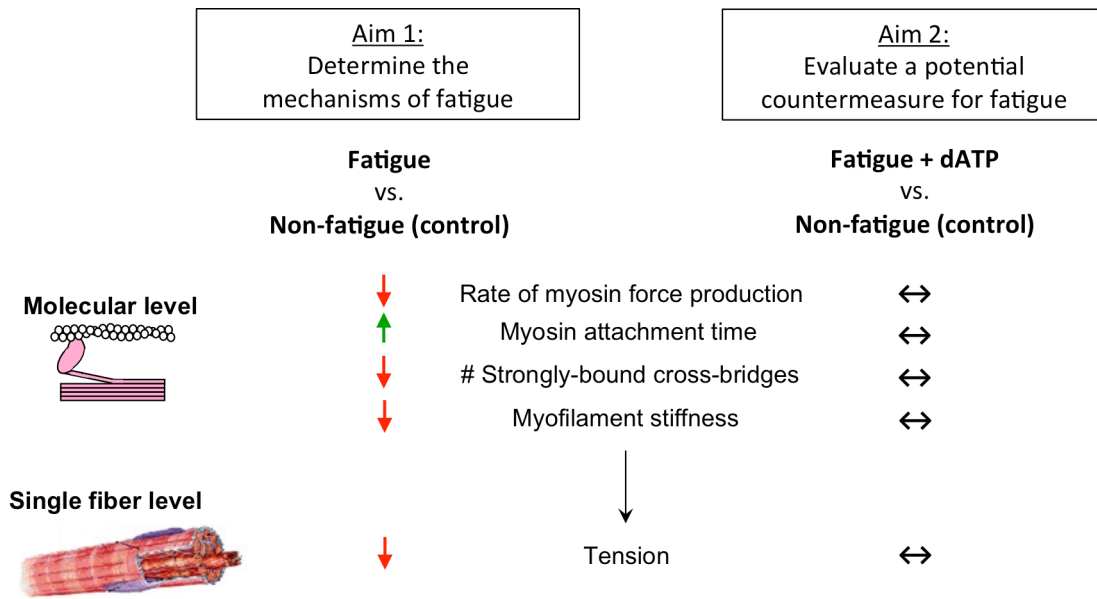
Simulated fatigue will 1A) decrease single fiber tension, due to 1B) a reduction in the number or stiffness of strongly bound myosin heads, and 1C) slower cross-bridge kinetics, including increasing myosin attachment time ( $t_{on}$ ), in type I and II fibers.

### **1.4.2 Aim 2**

Determine the ability of dATP to reverse simulated fatigue-induced changes in tension and myofilament mechanical properties in single fibers from older men and women.

#### **1.4.2.1 Hypotheses for Aim 2**

Simulated fatigue with dATP will 2A) recover tension back to control values, due to its ability to 2B) recover the number or stiffness of strongly bound myosin heads and 2C) cross-bridge kinetics, in type I and II fibers.



**Figure 1. Proposal aims and hypotheses.** Aim 1 (left column) compares fatigue (elevated  $P_i$  and  $H^+$ ) to non-fatigue (control) conditions. Aim 2 (right column) compares fatigue with the addition of deoxyadenosine triphosphate (dATP) instead of adenosine triphosphate (ATP) to non-fatigue conditions. Various parameters at the single fiber and molecular levels (middle column) were examined. A red downward arrow indicates a relative decrease with fatigue or fatigue + dATP compared to control, a green upward arrow indicates a relative increase, and a black horizontal arrow indicates no difference.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Introduction

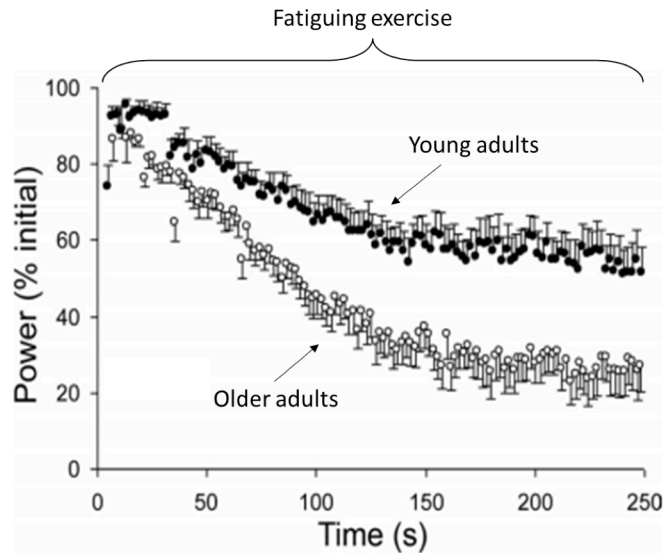
This literature review will first provide a general overview about the effects of skeletal muscle fatigue on older men and women at the whole body and whole muscle levels and why cellular and molecular alterations are important determinants of fatigue. Second, the *in vivo* biochemical changes that characterize fatigue in humans will be examined. Third, the currently known effects of simulated fatigue on cellular and molecular function will be examined from primarily animal studies as only one human study has been published to date. Finally, this review will examine the efficacy of deoxyadenosine triphosphate (dATP) as a potential treatment to counteract fatigue-induced decrements in skeletal muscle performance at the cellular and molecular levels.

#### 2.2 Skeletal Muscle Fatigue

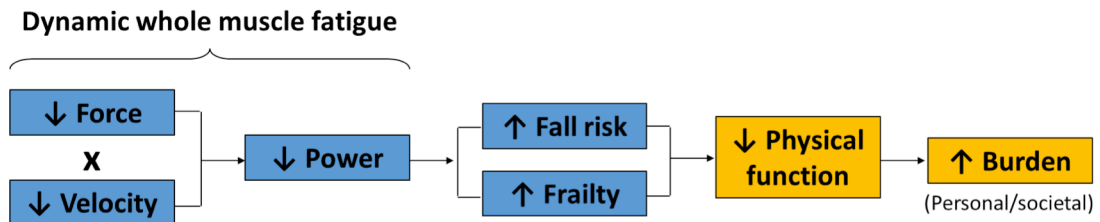
##### 2.2.1 Whole Body and Whole Muscle Alterations in Older Adults

Intense and/or continuous muscle contractions eventually cause whole skeletal muscles to fatigue, or decrease their ability to produce power, because of reduced velocity and/or force generation ( $\text{Power} = \text{Force} \times \text{Velocity}$ ) (Katsiaras et al., 2005; Callahan & Kent-Braun, 2011; Kent-Braun et al., 2012). Older men and women fatigue sooner and to a greater extent than their young counterparts during dynamic contractions (Figure 2) (Hicks et al., 2001; Katsiaras et al., 2005; Callahan & Kent-Braun, 2011;

Christie et al., 2011; Broxterman et al., 2017; Sundberg et al., 2018). Depressed whole muscle function that characterizes fatigue in older adults has been associated with a higher risk for falls (Wolfson et al., 1995; Schwendner et al., 1997) and increased frailty (Theou et al., 2008), indicating reduced physical function (Figure 3) in a population that is already experiencing whole body functional decline due to aging (Bassey et al., 1992; Cuoco et al., 2004; J. Holmes et al., 2009). Older adults with reduced physical function have a greater likelihood of being hospitalized (Cawthon et al., 2009), which increases their physical and financial burden upon themselves and society overall. The impact of these decrements with fatigue will be exacerbated by the growing population of people aged 65 years and older that is projected to nearly double from 48 million in 2015 to 82 million by 2040 (Ortman et al., 2014). Therefore, skeletal muscle fatigue in older adults is an important phenomenon to understand and counteract due to its detrimental functional implications and increasing prevalence in the future.



**Figure 2. Dynamic skeletal muscle fatigue in young versus older adults.** Changes in normalized power over time in the quadriceps muscles of 11 young (dark circles) and 10 older (open circles) activity-matched (sedentary) women in response to high velocity maximal voluntary dynamic knee extensions ( $270^\circ/\text{sec}$ ) ( $P < 0.01$  young vs. older). Reduced power characterizes fatigue. Adapted from (Callahan & Kent-Braun, 2011).



**Figure 3. Effects of dynamic skeletal muscle fatigue in older adults.** Blue boxes indicate associations observed in the literature. Yellow boxes indicate hypothesized relationships.

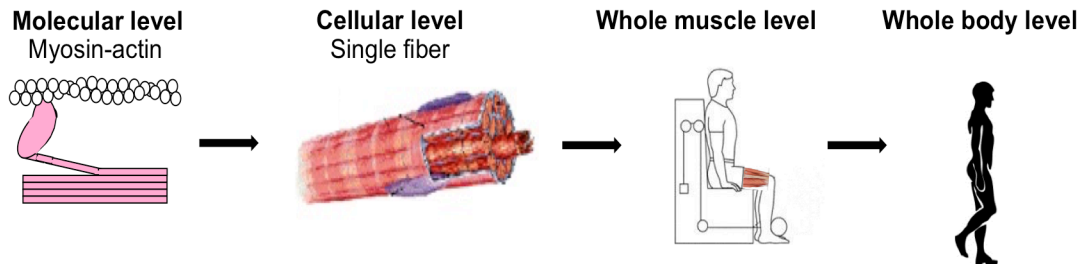
### 2.2.2 Rationale for Studying Cellular and Molecular Interactions

Although skeletal muscle contraction depends upon both neural and contractile factors, fatigue primarily results from altered contractile properties. Contractile factors account for up to 80% of fatigue in healthy young adults (Kent-Braun, 1999) and

potentially even more in older adults since neural activation has not been shown to play a significant role in muscle fatigue in this population (Kent-Braun et al., 2002). Thus, this study focused on the effects of fatigue on the contractile components of skeletal muscle.

Whole body, skeletal muscle tissue and cellular performance are dictated in large part by molecular-level myosin-actin cross-bridge interactions. The molecular level interactions between myosin and actin largely determine single muscle fiber force and velocity at the cellular level (Bottinelli, 2001; Miller et al., 2013). Single fiber type and function largely influence whole muscle force and velocity (Harridge et al., 1996; D'Antona et al., 2006; Miller et al., 2013), which have been shown to significantly dictate physical function at the whole body level (Figure 4) (Manini & Clark, 2012; Reid & Fielding, 2012). Therefore, studying the molecular and cellular mechanisms of altered skeletal muscle contraction is vital to understanding changes in performance at higher anatomical levels, such as fatigue. Studying the mechanisms of fatigue will deepen our understanding of this phenomenon and help provide the foundation to develop potential molecular-based treatments to counteract fatigue-related declines in muscle function, leading to improved physical function. These advancements are especially important for older adults who are most vulnerable to the detrimental physical effects of fatigue.





**Figure 4. Anatomical levels of skeletal muscle function.** Myosin-actin interactions at the molecular level dictate single fiber (cellular) function, which largely determines whole muscle function. Whole muscle performance is a major determinant of whole body function.

### 2.2.3 Biochemical Changes *In Vivo*

Under non-fatiguing conditions, when adenosine triphosphate (ATP) is hydrolyzed to provide energy for the muscle cell, adenosine diphosphate (ADP) and inorganic phosphate ( $P_i$ ) are produced. Our body's energy systems resynthesize ATP with ADP and  $P_i$  and, when energy production and energy usage are in balance, metabolite and substrate concentrations are maintained at concentrations of 5-8 mM ATP (Kushmerick et al., 1992), 8-11  $\mu$ M ADP (Kushmerick et al., 1992) and 4-5 mM  $P_i$  (Pathare et al., 2005; Kemp et al., 2007). When these metabolic processes are quickened during continuous and/or higher intensity fatiguing contractions, the build-up of products exceeds the rate that ATP is resynthesized, leading to increased levels of ADP ( $\leq$  200-300  $\mu$ M) (Chase & Kushmerick, 1995) and  $P_i$  (30 mM) (Dawson et al., 1978; Wilson et al., 1988; Cady et al., 1989) as well as reduced levels of ATP (3 mM) (Sahlin et al., 1989) (Table 1). Increased contractile activity also leads to a greater reliance on glycolytic metabolism and therefore a build-up of hydrogen/acid ( $H^+$ ), characterized by a reduction

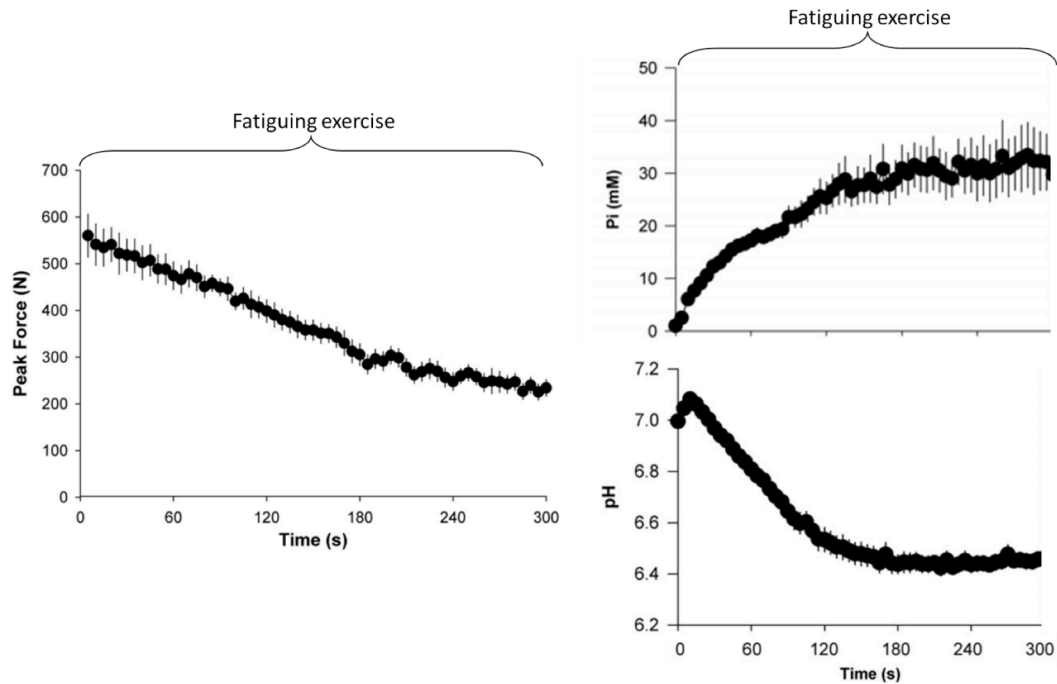
in intramuscular pH from 7 (rest) to as low as 6.2 (fatigue) (Table 1) (Hermansen & Osnes, 1972; Metzger & Fitts, 1987; Cady et al., 1989; Broxterman et al., 2017).

	<b>P<sub>i</sub></b>	<b>pH</b>	ADP	ATP	ROS, RNS	[Ca <sup>2+</sup> ]
Baseline	<b>4-5 mM</b>	<b>7</b>	8-11 μM	5-8 mM	-	pCa 4.5 or greater
Change w/ fatigue	↑	↓	↑	↓	↑	↑/↓
Max/min w/ fatigue	<b>30-40 mM</b>	<b>6.8-6.2</b>	≤ 200-300 μM	3 mM	-	pCa 5.3-5.9
Time to max/min	<b>2-3 min</b>	<b>2-3 min</b>	-	-	-	Fast: 2 min Slow: 8 min
Stim. Freq.	<b>10-26 Hz</b>	<b>10-26 Hz</b>	-	-	-	20-83 Hz

**Table 1. Changes in muscle intracellular milieu with fatigue.** Arrows illustrate direction of change with fatigue (↑ = increase, ↓ = decrease). Includes baseline (rest) concentrations, maximum/minimum metabolite concentrations with fatigue, time (in minutes) to reach maximal/minimal concentrations for each metabolite when fatigue is induced via voluntary whole muscle contraction (P<sub>i</sub>, pH, ADP, ATP) or electrical stimulation in fibers (ADP, ATP, ROS/RNS, [Ca<sup>2+</sup>]) at specified stimulation frequencies. **Bold text** indicates metabolites hypothesized to most affect contractile function. P<sub>i</sub> = inorganic phosphate, pH = potential of hydrogen (inversely related to H<sup>+</sup>), ADP = adenosine diphosphate, [Ca<sup>2+</sup>] = peak transient calcium concentration, ROS, RNS = reactive oxygen and nitrogen species. For [Ca<sup>2+</sup>], data is separated by fast and slow-contracting fibers. Data obtained from (Hermansen & Osnes, 1972; Dawson et al., 1978; Metzger & Fitts, 1987; Wilson et al., 1988; Cady et al., 1989; Sahlin et al., 1989; Kushmerick et al., 1992; Chase & Kushmerick, 1995; Pathare et al., 2005; Kemp et al., 2007; Broxterman et al., 2017).

Much of the cellular and molecular literature suggests that fatigue is mainly due to elevated P<sub>i</sub> and H<sup>+</sup>, while other substrate changes with fatigue *in vivo* play a more minimal role. Single fiber and isolated myosin and actin protein studies have shown that increased concentrations of P<sub>i</sub> and H<sup>+</sup> inhibit the myosin-actin cross-bridge and/or myofilament protein activation (Allen et al., 2008; Fitts, 2008; Debold et al., 2016). During fatiguing exercise in the quadriceps (Broxterman et al., 2017) and dorsiflexors (Kent-Braun et al., 2002) of young and older men and women, a rise in P<sub>i</sub> and lowering

of pH correlate temporally with reduced whole muscle force (Figure 5), which provides *in vivo* evidence that changes in  $P_i$  and pH are linked to fatigue. Other metabolites and substrates besides  $P_i$  and  $H^+$  change during fatigue (Table 1), though most are presumed to play a minimal role in decreased muscle performance and/or are not easily measured with current techniques. Single fiber evidence suggests that concentrations of ADP (8-11  $\mu\text{M}$  to  $\leq 200\text{-}300 \mu\text{M}$ ) (Chase & Kushmerick, 1995) and ATP (5-8 mM to  $\geq 3 \text{ mM}$ ) (Sahlin et al., 1989) do not change enough to significantly affect contractile function at the cellular and molecular levels (Cooke, 2007). Reactive oxygen and nitrogen species (ROS and RNS) may also increase and contribute to fatigue, but measurements of ROS and RNS concentrations are difficult to quantify, making their exact contributions to fatigue unknown (Cooke, 2007; Westerblad & Allen, 2011; Debold et al., 2016).



**Figure 5. Changes in force, inorganic phosphate ( $P_i$ ) and  $H^+$  (pH) during fatigue.** Changes in peak force,  $P_i$  and pH over time in the quadriceps muscles in 8 young men fatigued with 5 minutes of all-out, intermittent maximal isometric single leg knee extensions (3-s contraction, 2-s relaxation). Metabolite concentrations were measured using magnetic resonance spectroscopy. Adapted from (Broxterman et al., 2017).

Studies that have electrically stimulated single fibers from frogs have shown that peak transient calcium concentration ( $[Ca^{2+}]$ ) first increases from resting levels (pCa 8, where  $pCa = \log_{10}[Ca^{2+}]$ ) to maximal activation (pCa 4.5), but then falls to submaximal levels (pCa 5.3-5.9) during fatigue (Table 1) (Allen et al., 1989; Westerblad et al., 1989; Lee et al., 1991). Reduced  $[Ca^{2+}]$  can impair cross-bridge function and therefore decrease fiber force and velocity. For example, fibers exposed to high  $P_i$  and low pH conditions exhibited greater force reductions when activated at lower  $[Ca^{2+}]$  (pCa 5.3-5.9) than at maximally activated  $[Ca^{2+}]$  (Nelson & Fitts, 2014; Debold, 2016).

However, reduced  $[Ca^{2+}]$  with fatigue may not contribute to decrements in cross-bridge function as much as elevated  $P_i$  and low pH. During fatiguing contractions,  $P_i$  and

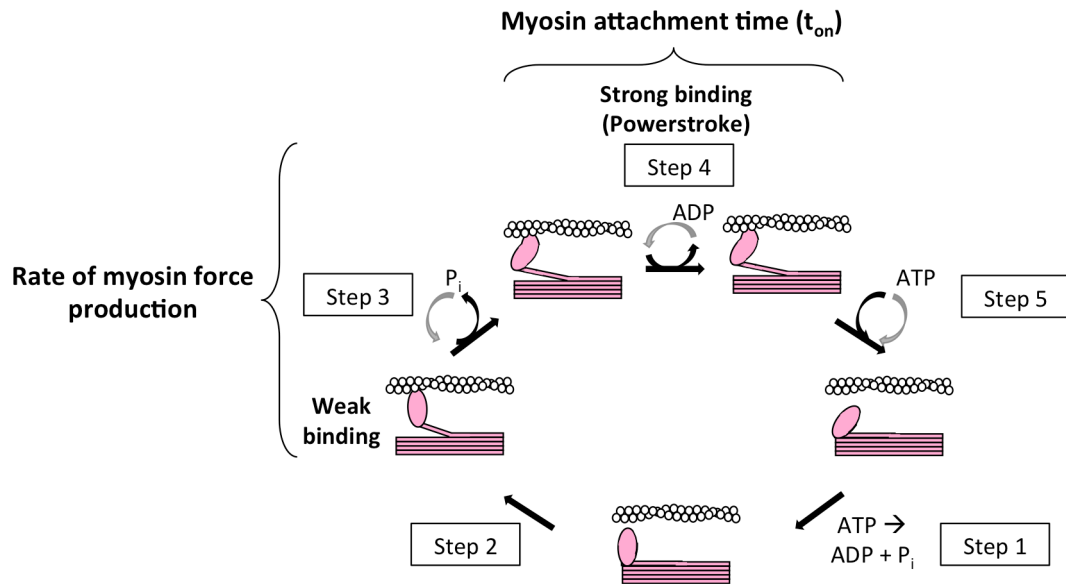
H<sup>+</sup> begin to rise first (within < 1 min of starting contractions) before reaching their maximum within 2-3 minutes (Kent-Braun et al., 2002; Broxterman et al., 2017), whereas single fiber data suggests that [Ca<sup>2+</sup>] increases during the first minute or so then steadily decreases mainly during long duration fatigue (Allen et al., 1989; Westerblad et al., 1989; Lee et al., 1991). In addition, stimulation frequency differs between single fiber and whole muscle fatigue studies and may significantly affect [Ca<sup>2+</sup>]. In single fibers fatigued with electrical stimulation, the stimulation frequency starts at 20 Hz and ramps up to a maximum of 83 Hz (Allen et al., 1989; Westerblad et al., 1989; Lee et al., 1991). In quadriceps muscles of young and older men, stimulation frequency measured with electromyogram (EMG) was 10-20 Hz when 75% of the motor units discharged and 26 Hz during maximum voluntary contraction (MVC) (Roos et al., 1999). These data suggest that [Ca<sup>2+</sup>] may not immediately decrease enough to account for the large decrements in cross-bridge function during the first few minutes of contractions. Furthermore, fatigued single fibers are stimulated at a greater intensity (up to 83 Hz) than what occurs *in vivo* (10-26 Hz), suggesting that [Ca<sup>2+</sup>] may decline less and at a slower rate *in vivo* compared to electrically stimulated fibers. These hypotheses still need to be confirmed, however, because no studies have measured *in vivo* [Ca<sup>2+</sup>] in human skeletal muscle. In conclusion, although reduced [Ca<sup>2+</sup>] may contribute to fatigue, the effects of elevated P<sub>i</sub> and H<sup>+</sup> on myosin-actin cross-bridge function may be the most important alterations to examine during fatigue and were therefore the focus of this study.

## 2.3 Overview of Skeletal Muscle Contraction

### 2.3.1 The Cross-bridge Cycle

According to the sliding filament theory, contraction occurs when the head of the myosin filament binds to and causes the actin filament to slide past myosin and shorten (contract) the sarcomere (H. E. Huxley, 1969). This model has been the predominant theory for over 50 years due to strong structural, physiological and biochemical supporting data (K. C. Holmes & Geeves, 2000). Myosin hydrolyzes ATP into ADP and  $P_i$  to re-cock its head (the cross-bridge) (Figure 6, step 1) to the starting position in preparation for binding to actin (Lymn & Taylor, 1971; Steffen et al., 2003). Once  $Ca^{2+}$  makes the binding sites on actin available, myosin can weakly bind to actin (Figure 6, step 2). Either prior to or immediately after myosin strongly binds to actin, myosin releases inorganic phosphate ( $P_i$ ) (Figure 6, step 3), generates force and goes through the powerstroke (Lymn & Taylor, 1971; Takagi et al., 2004). During the powerstroke, the myosin head undergoes a conformational change and pulls the actin filament, causing the actin filament to slide past the myosin filament. Next, the myosin-actin cross-bridge releases adenosine diphosphate (ADP) (Figure 6, step 4) and completes the second, smaller conformational change of the powerstroke (Takagi et al., 2004). After the powerstroke is complete, a new ATP molecule binds to the myosin head (Figure 6, step 5), causing it to dissociate from actin (Lymn & Taylor, 1971). These steps are collectively known as the cross-bridge cycle and ultimately result in muscle contraction when performed ensemble by millions of myosin heads across many fibers throughout each whole muscle. Importantly, specific steps of the cross-bridge cycle can be altered

and/or reversed under certain conditions, such as when the putative agents of fatigue are elevated (Figure 6, gray arrows at each step).



**Figure 6. Schematic representation of the cross-bridge cycle.** The myosin head (pink oval) interacts with the actin filament (string of white circles). The myosin head is connected to its tail (pink cylindrical shape), which eventually is incorporated into the thick filament (pink cylindrical shapes together). Steps 1-5 indicate one full cross-bridge cycle. Black arrows represent a normal cross-bridge cycle; gray arrows indicate steps in the cross-bridge cycle where metabolites/substrates can rebind to or dissociate from the myosin head under certain conditions (e.g. high  $P_i$  and  $H^+$ ). Myosin-actin cross-bridge kinetics measured in this study include the rate of myosin force production (step 3, transition from weak to strong binding states) and myosin attachment time ( $t_{on}$ ) (steps 3-5, strong binding state)).

### 2.3.2 The Cross-bridge Cycle Dictates Single Fiber Velocity and Force

The cross-bridge cycle largely determines single fiber velocity and force or tension (force/CSA). Fiber velocity is mainly dictated by the amount of time that myosin is strongly bound to actin, or myosin attachment time ( $t_{on}$ ) (Figure 6, steps 3-5), with longer attachment times lengthening the cross-bridge cycle and therefore slowing fiber velocity (Piazzesi et al., 2007; Miller et al., 2017). Single fiber force production occurs

when myosin is strongly bound to actin (Figure 6, steps 3-5) and undergoes the powerstroke (Figure 6, step 3). Fiber force is determined by the number of myosin heads strongly bound to actin multiplied by the force exerted by each head (A. F. Huxley, 1957; Brenner, 1988; Linari et al., 2004), which is generated during the powerstroke. Hence, increasing the number of strongly bound heads and/or the force exerted per myosin head will increase fiber force. The number of myosin heads strongly bound to actin is influenced by the two components of total cross-bridge cycle time, myosin attachment time ( $t_{on}$ ; Figure 6, steps 3-5) and myosin detachment time ( $t_{off}$ ; Figure 6, steps 5-2). When  $t_{on}$  alone is lengthened, the overall amount of time myosin remains strongly bound compared to the total cycle time, or its duty ratio  $[ t_{on} / (t_{on} + t_{off}) ]$ , is increased. In a population of myosin, an increase in the duty ratio leads to more strongly bound myosin heads at any given time and an increase in fiber force (Linari et al., 2004). When  $t_{off}$  alone is lengthened, the duty ratio is decreased, fewer heads are strongly bound at any given time, and fiber force is decreased. The rate of myosin force production (Figure 6, step 3), or the sum of the forward and reverse rate constants of this step, and  $t_{on}$  can be measured using our small amplitude sinusoidal oscillations. A decrease in the myosin rate of force production typically occurs due to a decrease in the forward rate constant, causing  $t_{off}$  to become longer, fewer myosin heads to be strongly bound, and fiber force production to decrease.

Overall, slower cross-bridge kinetics, meaning longer  $t_{on}$  and  $t_{off}$ , will decrease single fiber velocity due to the inverse relationship between velocity and  $t_{on}$ . However, alterations in force depend upon the relative changes in  $t_{on}$  and  $t_{off}$ . If  $t_{on}$  increases to a greater extent relative to the total cycle time, or an increased duty ratio, a greater number



of myosin heads will be strongly bound at a given time and fiber force will increase. In addition, an increase in the force exerted by each myosin cross-bridge could also increase fiber force.

## **2.4 Skeletal Muscle Fatigue: Cellular and Molecular Contractile Alterations**

Studies in single fibers (chemically skinned or mechanically peeled) and isolated myosin and actin molecules (laser trap and *in vitro* motility experiments) using young animal models and one human model have enhanced our understanding about how the putative agents of fatigue ( $P_i$  and  $H^+$ ) may compromise the ability of myosin and actin to generate velocity, force and power (Debold et al., 2012; Debold et al., 2013; Nelson et al., 2014; Debold et al., 2016; Sundberg et al., 2018; Woodward & Debold, 2018).

Because concomitant changes in  $P_i$  and pH have been linked to fatigue *in vivo* and both metabolites are known to inhibit myosin-actin cross-bridge function, this literature review will primarily focus on studies that have examined the combined effects of elevated  $P_i$  and  $H^+$ . This review will also focus on fatigue research conducted at near-physiological temperatures (25°C or higher) since this is most applicable to *in vivo* conditions (37°C) as studies have shown that single fiber contractile properties and alterations with elevated  $P_i$  and  $H^+$  are temperature dependent. Older studies traditionally examined fibers at colder temperatures (10-15°C) to maintain sarcomere integrity (Cooke et al., 1988; Metzger & Moss, 1990), leading to artificial differences in fiber function due to temperature because certain aspects of the cross-bridge cycle become slower as temperature decreases (Pate et al., 1995). Relatively recent advancements now provide the ability to quickly change solution temperatures, which helps maintain sarcomere integrity and enables single fiber

experiments to be performed at near-physiological temperatures (25-30°C *in vitro* vs. 37°C *in vivo*) (Pate et al., 1995; Miller et al., 2010; Nelson et al., 2014). Single fiber studies that simulated fatigue with isolated metabolites and at colder temperatures will also be briefly discussed in order to provide a complete picture of the cellular and molecular fatigue literature.

Few cellular and molecular studies have used conditions that closely mimic fatigue *in vivo* with elevated  $P_i$  (5 to 30 mM) and low pH (7 to 6.2) at near-physiological temperatures (25-30°C *in vitro* vs. 37°C *in vivo*). Furthermore, most of these studies have used young animal tissue and only one study has been performed in single fibers from young and older men. This is important since human fibers have different underlying properties than animal fibers such as slower cross-bridge kinetics because cross-bridge kinetics tend to slow as the species become larger (Pellegrino et al., 2003). In addition, women are more fatigue-resistant than men at submaximal, whole muscle contractions (Hicks et al., 2001; Labarbera et al., 2013) and older women tend to have slower cross-bridge kinetics than older men when matched for physical activity (Miller et al., 2013), suggesting that women have different underlying contractile properties than men with age. Thus, although the animal studies presented in this review provide valuable insights, performing experiments in human skeletal muscle tissue from men and women may be necessary to understand human and sex-specific responses to fatigue. The following sections will review current literature about how skeletal muscle function at lower anatomical levels is altered with elevated  $P_i$  and/or low pH to simulate fatigue.

### **2.4.1 Effects of Elevated $P_i$ and $H^+$ (low pH) on Contractile Function**

Consistent with whole muscles, fatigue simulated with elevated  $P_i$  and low pH in single fibers and isolated myosin and actin proteins depressed power production due to declines in maximal velocity and force, which were largely due to inhibited myosin-actin cross-bridge function (Table 2). Elevated  $P_i$  and  $H^+$  have synergistic effects on cross-bridge function when examined in combination, indicating the importance of studying the effects of these molecules together. The following sections will review the effects of elevated  $P_i$  and low pH on single fiber force and the potential molecular mechanisms for reduced force with high  $P_i$  and  $H^+$ , since these were the focus of the present study. This study focused on examining the mechanisms of reduced fiber force with high  $P_i$  and  $H^+$  because of discrepancies and uncertainty in the current literature.

	Fiber type	Low pH (pH 6.2-6.5)	High P <sub>i</sub> (10-30 mM)	Low pH + high P <sub>i</sub>	Low pH + high P <sub>i</sub>
<b>Single fibers: Animal</b>					Human
<b>Velocity</b>	MHC I	↓,↓↓	↔	↓	↓↓
	MHC II	↓,↓↓	↔	↓,↓↓	↓↓↓
<b>Force</b>	MHC I	↓	↓	↓↓	↓
	MHC II	↔	↔,↓↓	↓↓↓	↓↓
<b>Power</b>	MHC I	↓↓	↓↓	↓↓↓↓	↓↓↓
	MHC II	↓	↓	↓↓↓↓	↓↓↓
<b>Isolated proteins: Animal</b>					Human
<b>Velocity</b>	MHC I	-	-	-	-
	MHC II <sup>+</sup>	↓↓↓,↓↓↓	↔	↓↓↓,↓↓↓	-
<b>Force</b>	MHC I	-	-	-	-
	MHC II <sup>+</sup>	↔, ↓	↓↓↓↓	↓	-
<b>Power</b>	MHC I	-	-	-	-
	MHC II <sup>+</sup>	-	-	-	-

**Table 2. Effects of high P<sub>i</sub> and/or low pH on single fiber and isolated protein function.** Most animal experiments examined tissue from young rat, rabbit (single fibers) or chicken pectoralis<sup>+</sup> (isolated proteins; mainly type II muscle (Verdiglione & Cassandro, 2013)) studied at 30°C with low pH (6.2-6.5) and/or high P<sub>i</sub> (10-30 mM). Arrows indicate direction of change (↓ = decrease, ↔ = no significant difference) and percent change (↓ = 10-20%; ↓↓ = 21-40%; ↓↓↓ = 41-60%; ↓↓↓↓ ≥ 60%) compared to control, non-fatigue conditions (P<sub>i</sub> 0-5 mM; pH 7-7.4) with results from studies separated by a comma (.). Data are separated by fiber type into slow- (myosin heavy chain (MHC) I) and fast- (MHC II) contracting fibers. Animal single fiber data was obtained from ((Debold et al., 2004; Debold et al., 2006; Karatzaferi et al., 2008; Knuth et al., 2006; Nelson et al., 2014). Human single fiber data was obtained from (Sundberg et al., 2018), where all MHC II data was obtained at 15°C. Isolated protein data was obtained from (Debold et al., 2008; Debold et al., 2011; Debold et al., 2012; Debold et al., 2013; Woodward & Debold, 2018).

#### 2.4.1.1 Single Fiber Force

Most recent studies have shown that simulating fatigue with both elevated P<sub>i</sub> (30 mM) and low pH (6.2) at temperatures close to *in vivo* (30°C *in vitro* vs. 37°C *in vivo*) depressed single fiber peak force and therefore tension (force/CSA). The most recent

study in young and older men showed that high  $P_i$  and  $H^+$  reduced peak force in MHC I (21-22%) and II (40-43% at 15°C) fibers (Sundberg et al., 2018), which was less than what was observed in rat fibers (MHC I: 36%; MHC II: 46%) (Nelson et al., 2014) and rabbit psoas fibers (52% for MHC I and II combined) under similar conditions (Table 2) (Karatzaferi et al., 2008). Elevated  $P_i$  and  $H^+$  also depressed the fiber force-velocity relationship (Nelson et al., 2014; Sundberg et al., 2018), indicating that submaximal force declined across the entire range of velocities. Other studies in young animal fibers that examined elevated  $P_i$  (S. Palmer & Kentish, 1994; Coupland et al., 2001; Debold et al., 2004; Debold et al., 2006; Nelson et al., 2014) or low pH (S. Palmer & Kentish, 1994; Pate et al., 1995; Pedersen et al., 2004; Kristensen et al., 2005; Knuth et al., 2006; Nelson et al., 2014) individually at high temperatures observed decreased peak fiber force compared to control conditions, but to a lesser extent than studies simulating fatigue with high  $P_i$  and low pH combined. These data suggest that both  $P_i$  and pH decrease maximal fiber force, therefore  $P_i$  and pH must individually inhibit myosin-actin cross-bridge function. Some evidence suggests that the combination of elevated  $P_i$  and  $H^+$  may reduce force in a synergistic manner (Chase & Kushmerick, 1988; Cooke et al., 1988; Potma et al., 1995), reinforcing the importance of simulating fatigue with both metabolites combined because both  $P_i$  and pH change concurrently with fatigue *in vivo*.

Single fiber fatigue studies in young animal tissue conducted at relatively low temperatures (10-15°C) with high  $P_i$  and low pH combined (Cooke et al., 1988; Karatzaferi et al., 2008; Nelson et al., 2014), high  $P_i$  only (Cooke et al., 1988; Martyn & Gordon, 1992; Wahr et al., 1997; Coupland et al., 2001; Debold et al., 2004; Debold et al., 2006; Caremani et al., 2008; Nelson et al., 2014; Caremani et al., 2015) and low pH

only (Chase & Kushmerick, 1988; Cooke et al., 1988; Metzger & Moss, 1990; Pate et al., 1995; Westerblad et al., 1997; Knuth et al., 2006; Nelson et al., 2014) showed greater declines in peak force compared to fibers exposed to similar conditions at high temperatures (25-30°C). Low temperatures introduced artificial differences in force due to a greater slowing of the rate constant of myosin force generation, leading to fewer strongly bound heads and reduced fiber force compared to high temperatures (Zhao & Kawai, 1994; Galler & Hilber, 1998; Fitts, 2008). Therefore, although the decline in force is less pronounced at high temperatures, studying fiber function at temperatures closer to *in vivo* (37°C) is more physiologically relevant.

#### **2.4.1.2 Myosin-actin Cross-bridge Function**

Reductions in single fiber force may be the result of elevated  $P_i$  and  $H^+$  decreasing the number of strongly bound cross-bridges and/or reducing the force exerted per cross-bridge (Force =  $N \cdot F_{CB}$ , where  $N$  = number of strongly bound cross-bridges,  $F_{CB}$  = force per cross-bridge). Many studies have measured fiber stiffness in order to examine the number and/or stiffness of bound myosin heads. However, measurement methods and experimental results vary regarding the effects of high  $P_i$  and low pH on fiber stiffness and there is currently no consensus. Most studies on fiber stiffness have only examined the effects of elevated  $P_i$  or  $H^+$  in isolation. At low temperatures (10-12°C), low pH (6.2) reduced stiffness in fast (MHC II) but not slow (MHC I) contracting fibers (Metzger & Moss, 1990), while elevated  $P_i$  decreased stiffness in MHC II fibers (Caremani et al., 2008), both of which indicate fewer strongly bound cross-bridges in MHC I but not MHC II fibers. One recent study (Nelson et al., 2014) that examined the effect of high  $P_i$  and

low pH on fiber stiffness (assumed to represent the total number of bound myosin heads) at 30°C in young rodent fibers found that stiffness was not altered in MHC I or II fibers, indicating no change in the number of bound heads. Since force was reduced with high  $P_i$  and low pH but stiffness was unchanged, this implied that there was a reduction in the force generated per cross-bridge. Another study that simulated fatigue in young mouse fibers (primarily MHC II) at 22-24°C suggested that force was initially reduced due to each cross-bridge generating less force, while greater force reductions over time occurred due to a reduction in the number of strongly bound myosin heads (Nocella et al., 2011). At the molecular level in isolated myosin-actin proteins, elevated  $P_i$  and low pH reduced the number of strong binding events (fewer strongly bound myosin heads) (Woodward & Debold, 2018), which provides direct evidence of the changes in myosin-actin cross-bridges that can only be inferred about at the single fiber level. Overall, the effects of high  $P_i$  and low pH on fiber stiffness and its components at high temperatures are understudied and results are somewhat inconsistent. Stiffness measurements under high  $P_i$  and  $H^+$  conditions have also not been performed in fibers from humans or older populations, both of which are important for determining the mechanisms of fatigue and the applicability of current simulated fatigue data to older adults.

The number of strongly bound myosin heads is largely dictated by myosin-actin cross-bridge kinetics ( $t_{on}$  and rate of myosin force production, which determines  $t_{off}$ ), which can be altered by elevated  $P_i$  and  $H^+$ .  $H^+$  is thought to slow the release of ADP from myosin (Figure 6, step 4), which prolongs the strong binding state and therefore  $t_{on}$  as hypothesized by molecular studies using *in vitro* motility (Debold et al., 2008; Debold et al., 2012). Longer  $t_{on}$  decreases the rate at which myosin uses energy (ATPase) (Potma

et al., 1995), which lengthens the total cross-bridge cycle time ( $t_{on} + t_{off}$ ). In support of this, a recent isolated protein study using the laser trap assay (Woodward & Debold, 2018) as well as earlier single fiber work (Cooke et al., 1988) have shown that ATPase declines with high  $P_i$  and  $H^+$  combined, indicating longer  $t_{on}$  and/or  $t_{off}$ . However, earlier work that separated myosin and actin proteins speculated that  $P_i$  is more likely to rebind to the myosin head as  $H^+$  prolongs the strongly bound state (Figure 6, step 3), causing myosin to dissociate from actin faster and shorten  $t_{on}$  (Debold et al., 2011; Debold et al., 2012; Longyear et al., 2014). This contradicts single fiber work showing that only high  $H^+$  slows contractile velocity, whereas elevated  $P_i$  has no effect (Knuth et al., 2006; Karatzaferi et al., 2008; Nelson et al., 2014) even when in combination with high  $H^+$  (Karatzaferi et al., 2008; Nelson et al., 2014; Sundberg et al., 2018), indicating that  $t_{on}$  either does not change or slows with high levels of  $P_i$  and/or  $H^+$ . The effects of high  $P_i$  and low pH on  $t_{on}$  has only been hypothesized using results from *in vitro* motility and laser trap assays in isolated myosin and actin proteins but not measured in single fibers until the present study, which is important because of discrepancies between single fiber and isolated protein results.

If  $t_{on}$  slows with high  $P_i$  and low pH like single fiber and isolated protein studies suggest, then the strongly bound state would be prolonged and increase the number of strongly bound myosin heads, which would increase fiber force. Because previous literature has consistently shown that fiber force declines with high  $P_i$  and  $H^+$  conditions, myosin detachment time ( $t_{off}$ ) (the time when myosin heads are not strongly bound and exerting force) must lengthen to a greater degree than  $t_{on}$  to prolong the non-force generating state. As the rate of myosin force production (Figure 6, step 3) slows,  $t_{off}$



becomes longer, which leads to a reduction in the number of strongly bound, force-producing myosin heads and a reduction in fiber force. A recent human single fiber study showed that elevating both  $P_i$  and  $H^+$  reduced the rate that myosin transitions from weak to strong binding measured by  $k_{tr}$  (rate of force redevelopment) in both MHC I and II fibers (Sundberg et al., 2018), which would prolong  $t_{off}$ . An relatively older study in rat fibers under the same conditions showed only a trend ( $P=0.07$ ) towards a significant decrease in  $k_{tr}$  in MHC I fibers, but ultimately no significant change in either fiber type with high  $P_i$  and  $H^+$  (Nelson et al., 2014). Lack of significance could have been due to low fiber numbers (23 fibers total, averaging three MHC I and two MHC II fibers per rat vs. 254 fibers total, averaging 4-10 fibers per person in the study by Sundberg et al.). One isolated protein study (Debold et al., 2013) supported single fiber data by suggesting that elevated  $P_i$  speeds up the reverse rate constant of the myosin head (Figure 6, step 3), causing myosin to dissociate from actin shortly after initiating the strong binding state, which shortens  $t_{on}$  and may therefore increase  $t_{off}$  (Chock, 1979). Molecular work also suggests that high levels of  $H^+$  may inhibit the myosin forward rate constant (Figure 6, step 3) (Debold et al., 2008), which would also lengthen  $t_{off}$  (Figure 6, weak binding state). Finally, a more recent isolated protein study found a reduction in strongly bound cross-bridges with elevated  $P_i$  and  $H^+$ , which they hypothesized was due to slower myosin weak-to-strong binding and therefore longer  $t_{off}$  (Woodward & Debold, 2018).

Overall, more work is needed to determine how cross-bridge kinetics are altered with elevated  $P_i$  and  $H^+$  in human single fibers and how this reduces fiber force, which has important implications for whole muscle and ultimately whole body function with

fatigue. This study sought to address these gaps by examining the effects of elevated  $P_i$  and  $H^+$  on molecular and cellular function in single fibers from older men and women.

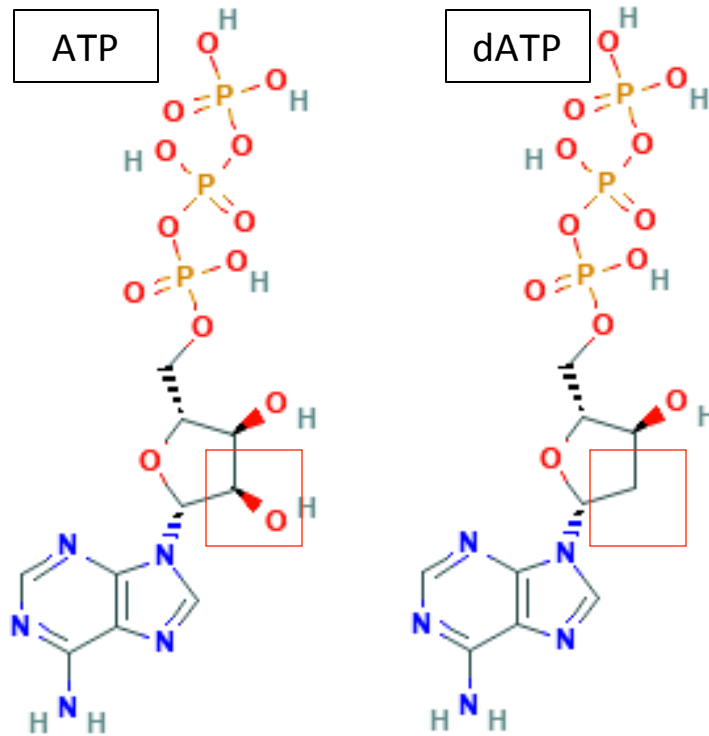
## **2.5 dATP as a Potential Countermeasure for Skeletal Muscle Fatigue**

This section will review basic evidence about using deoxyadenosine triphosphate (dATP) as a potential countermeasure for fatigue simulated with high  $P_i$  and low pH and future implications for treatment development to ultimately reverse fatigue-related decrements in physical function.

### **2.5.1 What is dATP?**

dATP is a nucleotide triphosphate (NTP) molecule, meaning that dATP is an analog of ATP that serves as another form of energy because dATP is biologically similar enough to ATP to be recognized as such (Figure 7) (Regnier et al., 1998; Regnier & Homsher, 1998; Cid=15993.accessed Jan. 8, 2019; Cid=5957.accessed Jan. 8, 2019). NTPs are valuable experimental tools since they are biologically recognized by the target tissue but have slightly altered properties that make them useful for establishing mechanistic and/or causative effects. For example, the mechanical behavior in NTPs can be correlated with fluctuations in rate constants throughout different steps of NTP hydrolysis to provide insights into mechanical and energetic aspects of muscle contraction (Regnier et al., 1998; Regnier & Homsher, 1998). There are several NTPs that occur naturally, including dATP, cytosine triphosphate (CTP), deoxycytosine triphosphate (dCTP), uridine diphosphate (UTP), deoxyuridine diphosphate (dUTP),

inosine 5'-triphosphate (ITP) and guanosine triphosphate (GTP). This review will focus on dATP because evidence has revealed positive effects of dATP on single skeletal muscle fiber function, which may help counter contractile declines such as those observed with fatiguing conditions.



**Figure 7. ATP and dATP structures.** Chemical structures of adenosine triphosphate (ATP) (left) and deoxyadenosine triphosphate (dATP) (right). The only structural difference is that dATP has one less oxygen molecule than ATP, as indicated by the red boxes. Adapted from PubChem Compound Database (Cid=15993.accessed Jan. 8, 2019; Cid=5957.accessed Jan. 8, 2019).

### 2.5.2 dATP Improves Single Fiber Function and Possibly Simulated Fatigue

Few studies have examined the effects of dATP on skeletal muscle function, but the results are promising. Regnier et al. demonstrated that, compared to single psoas

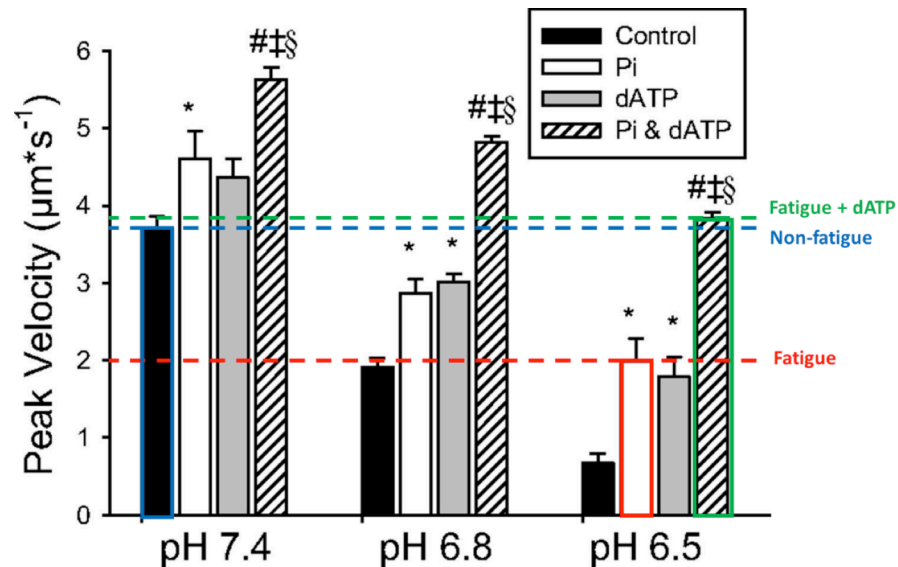
fibers from young rabbits (primarily MHC II) (Hamalainen & Pette, 1993) using ATP as their substrate, dATP was the only NTP that resulted in faster contractile velocity and similar maximal force production due to similar fiber stiffness (Regnier et al., 1998; Regnier & Homsher, 1998). All other naturally occurring analogs studied (CTP, dCTP, UTP, dUTP, ITP and GTP) led to slower contractile velocity and/or less maximal force than with ATP (Table 3). Fibers with dATP also exhibited a faster rate of energy utilization (measured with NTPase, or the rate at which myosin uses energy to complete the cross-bridge cycle) and force redevelopment ( $k_{tr}$ ) compared to ATP, indicating faster kinetics. In cardiac tissue of various mammals, dATP enhanced both fiber velocity and force compared to ATP (Regnier et al., 2000; Cheng et al., 2016; Thomson et al., 2016).

dATP leads to faster fiber velocity since dATP speeds up myosin detachment rate (Figure 6, step 5) due to quicker dADP release from myosin (Regnier et al., 1998; Regnier et al., 2000), indicating faster cross-bridge kinetics. dADP is able to release quickly since it has a weaker affinity for myosin because of subtle structural differences from ADP (Regnier et al., 1998; Regnier et al., 2000). The release of ADP from myosin (Figure 6, step 4) is the step that limits unloaded velocity (Siemankowski et al., 1985; Weiss et al., 2001; Nyitrai et al., 2006), most likely by altering myosin attachment time ( $t_{on}$ ). Therefore, quicker release of dADP versus ADP could explain the faster fiber velocity observed with dATP relative to ATP. In addition, faster hydrolysis of a nucleotide triphosphate (NTP) may reduce  $t_{off}$  (Koretz & Taylor, 1975; Chock, 1979) and speed up cross-bridge kinetics. This hypothesis suggests that dATP speeds up the cross-bridge cycle compared to ATP, resulting in faster cross-bridge kinetics and therefore quicker single fiber contractile velocity.

	dATP vs. ATP	Other NTPs vs. ATP (n=6)
Maximal force	↔	↓
Unloaded shortening velocity	↑	↓
Stiffness	↔	↓
NTPase	↑	↓
$k_{tr}$	↑	↑, ↓

**Table 3. Effects of NTPs on single fiber and isolated protein function.** Adenosine triphosphate (ATP) was substituted as an energy substrate with different nucleotide triphosphates (NTPs) (dATP, CTP, dCTP, UTP, dUTP, ITP and GTP) in skinned, single muscle fibers and isolated proteins from young rabbit psoas muscle. Arrows indicate change with a particular NTP versus ATP (↔ = no significant difference, ↑ = increase, ↓ = decrease) with different results separated by commas (.). NTPase is an enzymatic assay that measures energy utilization rate (rate of completing one cross-bridge cycle) and  $k_{tr}$  is rate of force redevelopment. (Regnier et al., 1998; Regnier & Homsher, 1998).

The benefits of dATP on improving single skeletal fiber function may be applicable to fatiguing conditions. To date, Longyear et al. (2014) is the only study to have examined the efficacy of dATP as a countermeasure for skeletal muscle contractile decline with elevated  $P_i$  and low pH. Substituting ATP with dATP in isolated myosin and actin proteins from young chicken pectoralis muscle placed in an *in vitro* motility assay fatigued with high  $P_i$  (15 and 30 mM) and low pH (6.5) showed that peak velocity (measured via actin filament velocity) was restored to control levels after being slowed with high  $P_i$  and low pH (Figure 8) (Longyear et al., 2014). As described above, an increased rate of dATP release from myosin (Regnier et al., 1998; Regnier et al., 2000) most likely shortens  $t_{on}$  and may speed up the rate of myosin force production, both of which would quicken cross-bridge kinetics and therefore fiber velocity and possibly increase fiber force under fatiguing conditions.



**Figure 8. Effect of elevated  $P_i$  and low pH with dATP in isolated proteins.** *In vitro* motility assay performed in isolated myosin and actin proteins from young chicken pectoralis muscle. Peak actin filament velocity in non-fatigue (0 mM  $P_i$ , pH 7) (blue) fatigue (15 & 30 mM  $P_i$  and pH 6.5) (red), and fatigue with dATP vs. ATP (green) conditions. Adapted from (Longyear et al., 2014).

These data indicate that, in the future, dATP may be a viable treatment option to reverse declines in skeletal muscle function observed with fatigue, though more evidence is needed. Additionally, experiments should be conducted in older humans at higher anatomical levels to determine the applicability of these molecular results to larger scale function in older adults who are most burdened by fatigue. Further studies should measure additional functional parameters at all anatomical levels to obtain a better understanding about the molecular mechanisms and effects of dATP on mitigating and/or reversing fatigue-induced decrements in cross-bridge kinetics and fiber force, velocity and power, which are important determinants of whole muscle and physical function. This study began to explore answers to these questions by examining changes in cellular

and molecular function in response to dATP in single fibers from older men and women under conditions simulating fatigue with high  $P_i$  and low pH.

### **2.5.3 Implementing dATP as a Treatment for Fatigue**

In the future, dATP could be implemented as a treatment for fatigue-related declines in skeletal muscle function. dATP is primarily involved with deoxyribose nucleic acid (DNA) synthesis and tends to quickly convert to ATP (Thomson et al., 2016), but a translational medicine technique can reverse this process and subsequently increase levels of dATP in the body. One method of gene therapy, called BB-R<sub>12</sub>, directly targets myosin and can up-regulate ribonucleotide reductase (R<sub>1</sub>R<sub>2</sub>), which is the rate-limiting enzyme in dATP synthesis in cardiomyocytes (Thomson et al., 2016). While this technique targets cardiac contractility, similar techniques can theoretically be developed and employed to up-regulate these same types of enzymes in skeletal muscle, thus improving contractility and potentially reversing fatigue. Although these advancements would take years to accomplish, a therapy for fatigue may be possible and worth investigating in the future.

## **2.6 Summary**

Skeletal muscle fatigue is characterized by functional decline at all anatomical levels and is especially evident in older adults who fatigue sooner and to a greater extent than young adults during dynamic muscle contractions. Fatigue decreases physical function in older adults by increasing their risk for falls and becoming frail, thus increasing likelihood of hospitalization and therefore personal and societal burden.

Fatigue is a decrease in whole muscle velocity and/or force and therefore power (Power = Force x Velocity) that occurs as a result of hard and/or continuous contractions. Fatigue is temporally associated with elevated  $P_i$  and reduced pH in the muscle, indicating that changes in these metabolites are linked to reduced muscle function with fatigue. Whole muscle function is largely determined by single muscle fiber and molecular function, which can be used to study the contractile mechanisms of fatigue *in vitro* by mimicking *in vivo* conditions. Elevated  $P_i$  and low pH conditions lead to reductions in single fiber velocity, force and power, though the molecular mechanisms for these declines are not clear and sometimes inconsistent among single fiber and isolated myosin-actin protein studies. All but one study to date have been performed in young animal tissue and may not be applicable to older adults due to humans and older adults both having inherently different contractile properties than animals and young adults, respectively. Furthermore, the one human single fiber study was done in young and older men and studies have shown that women may fatigue differently and have different underlying contractile properties than men, especially with age. Finally, there are no treatments for fatigue despite promising evidence that dATP can enhance single fiber velocity and maintain force due to improved cross-bridge kinetics in normal conditions. Improved velocity also occurred in high  $P_i$  and  $H^+$  conditions in isolated proteins. Understanding the mechanisms of fatigue by studying single fibers from older men and women is important to ultimately develop countermeasures for fatigue-related decrements in skeletal muscle function, which will improve physical function and reduce personal and societal burden.



## **CHAPTER 3**

### **METHODS**

#### **3.1 Introduction**

This study aimed to deepen our understanding about the mechanisms of skeletal muscle fatigue in older adults and determine the efficacy of deoxyadenosine triphosphate (dATP) as a potential treatment for the fatigue-induced decline in muscle function at the cellular and molecular levels. Individual skinned skeletal muscle fibers from older men and women were exposed to control (5 mM  $P_i$  (inorganic phosphate); pH 7), fatigue (30 mM  $P_i$ ; pH 6.2) or fatigue with dATP instead of adenosine triphosphate (ATP) solutions. In each condition, fiber tension (force/cross-sectional area) and myofilament mechanical properties, including myosin-actin cross-bridge kinetics, were measured using small amplitude sinusoidal analysis.

#### **3.2 Participant Characteristics**

##### **3.2.1 Ethical Approval**

Written, informed consent was obtained from each participant prior to being enrolled in the study. Protocol approval was obtained from the Institutional Review Board (IRB) at the University of Massachusetts, Amherst.

### **3.2.2 Overview**

This study used tissue taken under baseline/control conditions from another study in our laboratory, the Cultivating Healthy Aging and Muscle Performance (CHAMP) study. From this study, we obtained a sample of convenience by attaining tissue from the first 8 sedentary, healthy older adults (4 men) who were recruited and functionally tested 214 fibers (104 slow- (type I) and 110 fast-contracting (type II); 26 per person on average) single skeletal muscle fibers obtained via biopsy of the vastus lateralis for each participant. Participant characteristics are outlined in further detail in Chapter 4: Results (Table 4).

The following inclusion and exclusion criteria for our participants were obtained directly from the CHAMP study:

### **3.2.3 Inclusion Criteria**

- Aged 65-75 years
- Generally healthy, by self-report
- Ambulatory without the use of walking aids
- Living independently in the community
- Non-smokers; for at least the preceding year
- Relatively sedentary (no more than two 30-minute exercise sessions per week, by self-report) verified by multilateral accelerometry using Actigraph
- Women were postmenopausal, defined as cessation of menses for at least 12 months prior to study
- Must pass the Physical Activity Readiness Questionnaire for Everyone (PAR-Q+)

### **3.2.4 Exclusion Criteria**

- History of major neurological or neuromuscular condition that may impact physical function, including cerebrovascular disease, peripheral neuropathy,

neurodegenerative disease, demyelinating disease, cerebellar or extrapyramidal disease, etc.

- History of myocardial infarction, angina, peripheral vascular disease, surgical or percutaneous coronary artery revascularization
- History of severe pulmonary disease (i.e., dyspnea that limits activities of daily living such as household ambulation and self-care)
- History of rheumatoid arthritis
- History of diabetes or other metabolic disease that may impact neuromuscular function
- Uncontrolled hypertension (blood pressure > 140/90)
- History of smoking in the past 1 year
- Moderate to severe lower extremity arthritis or pain (i.e., pain on level walking or that limits activities of daily living such as household ambulation and self-care)
- Pain, muscle cramps, joint stiffness, dyspnea, angina, light-headedness or other symptoms upon exertion
- The use of beta-blockers, sedatives, tranquilizers, or other medication that may impair physical function
- Individuals taking statin medications who report symptoms of muscle pain or myopathy
- Body-mass index  $>30 \text{ kg}\cdot\text{m}^{-2}$ , as increased fat mass may alter single muscle fiber performance
- Body-mass index  $<18 \text{ kg}\cdot\text{m}^{-2}$ , as this may be an early sign of frailty
- Any persons taking anti-coagulant medication or with known coagulopathies will be excluded, due to increased bleeding risk from biopsy procedure
- Participants with a contraindication for magnetic resonance testing, including a pace-maker or other implant
- Men and women undergoing hormone replacement therapy, because this treatment may circumvent normal age-related declines in sex hormone levels (if taken, hormone therapy must have been > 5 years ago)
- Unintentional weight loss of greater than 2.5 kg during the last 3 months
- Currently participating in or have participated in a weight loss or exercise training program in the last year
- An inability to understand written and spoken English
- An inability to follow instructions, as determined by the investigators during the consenting process

### **3.3 Experimental Preparation**

#### **3.3.1 Location**

All fiber preparation and functional analysis measures were completed in the Muscle Biology Laboratory at University of Massachusetts, Amherst in Totman Physical Education Building, room 140. The muscle biopsies were performed in an examination room in the Human Testing Center at the Institute of Applied Life Sciences (IALS) at University of Massachusetts, Amherst.

#### **3.3.2 Muscle Tissue Processing**

Muscle tissue processing has been described previously (Miller et al., 2010). Biopsy tissue was placed immediately into cold (4°C) dissecting solution (in mM: 20 N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 5 EGTA, 5 MgATP, 1 free Mg<sup>2+</sup>, 1 dithiothreitol and 0.25 phosphate (P<sub>i</sub>) with an ionic strength of 175 mEq, pH 7.0, and at pCa 8 (pCa=-log<sub>10</sub> [Ca<sup>2+</sup>]) for isolation of single fibers for mechanical measurements. Muscle fibers were processed for mechanical measurements by dissecting the tissue sample into bundles of approximately 50+ fibers and tying each bundle to a glass rod at a slightly stretched length at 4°C. Each tied bundle was then placed in skinning solution (in mM: 170 potassium propionate, 10 imidazole, 5 EGTA, 2.5 MgCl<sub>2</sub>, 2.5 Na<sub>2</sub>H<sub>2</sub>ATP, 0.05 leupeptin and 0.05 antipain at pH 7.0) for 24 h at 4°C. After skinning, fibers were placed in storage solution (identical to skinning solution, but without leupeptin and antipain) with increasing concentration of glycerol (10% v/v glycerol for 2 h, 25% v/v glycerol for 2 h) until reaching the final storage solution (50%

v/v glycerol), in which they were incubated at 4°C for 18–20 h. Thereafter, bundles were stored at –20°C until isolation of single fibers for mechanical measurements, which occurred within 4 weeks of the biopsy to ensure that there were no functional declines due to prolonged storage.

### **3.3.3 Preparation of Single Fibers for Mechanical Analysis**

Single fiber preparation has been described previously (Miller et al., 2010). Muscle bundles were incubated in dissection solution containing 1% Triton X-100 (v/v) for 30-40 min at 4°C. Segments (~2.0-2.5 mm) of single fibers were manually isolated from muscle bundles, aluminum T-clips placed at both ends of the fiber, and the fiber was mounted onto hooks in dissecting solution at room temperature on the cross-sectional area/fixation rig. Top and side diameter measurements were made at three positions along the length of the middle 1-2 mm of the fiber using a filar eyepiece micrometer (Lasico, Los Angeles, CA, USA) and a right-angled, mirrored prism to calculate average cross-sectional area. Fibers were then fixed at two points approximately 1-2 mm apart with glutaraldehyde, as described elsewhere (Chase & Kushmerick, 1988; Hilber & Galler, 1998), with some modifications. Fixation was necessary to ensure that the fiber did not tear during activation since this tends to occur in human fibers at elevated temperatures (Miller et al., unpublished observations). Briefly, fibers were placed in rigor solution (in mM: 134 potassium propionate, 10 imidazole, 7.5 EDTA and 2.5 EGTA; 5 2,3-butanedione monoxime at pH 6.8) and glutaraldehyde fixative (~0.01% bromophenol blue (v/v), 30% glycerol (v/v), 2% glutaraldehyde (v/v)) was applied (20 s per end) using the gravity feed method (Hilber & Galler, 1998). The fiber was placed in dissecting

solution with 1% bovine serum albumin to absorb any remaining glutaraldehyde and stop it from spreading further along the fiber (Chase & Kushmerick, 1988). New T-clips were placed on the fixed regions, which were evident because of bromophenol blue indicator dye. Any fiber remaining outside of the fixed regions was cut off along with the original T-clips. The fiber was incubated in dissecting solution containing 1% Triton X-100 (v/v) for 30 min at 4°C to ensure removal of the sarcolemma and sarcoplasmic reticulum, which allowed for control over the intracellular environment (e.g. contraction was initiated with high  $[Ca^{2+}]$  in order to measure fiber function).

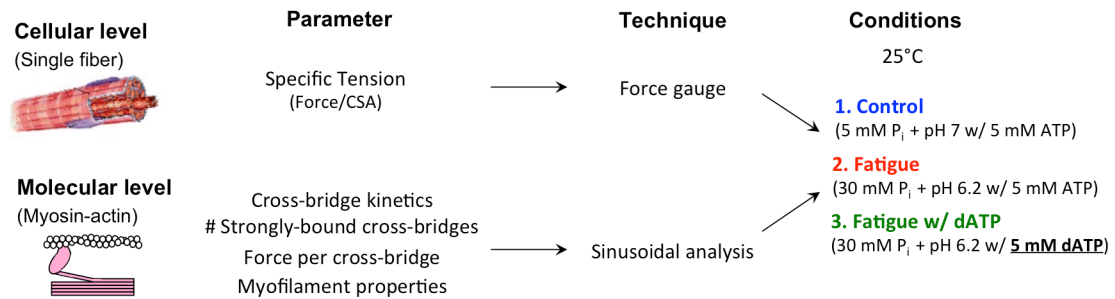
### 3.3.4 Experimental Solutions

Constituents of all solutions used during mechanical measurements were calculated using the equations and stability constants according to Godt & Lindley (Godt & Lindley, 1982), as described previously (Miller et al., 2010). Relaxing solution was dissecting solution with 15 mM creatine phosphate and 300 units/ml<sup>-1</sup> of creatine phosphokinase. Pre-activating solution was the same as relaxing solution, except at an EGTA concentration of 0.5 mM. Control activating solution was the same as relaxing solution, except at pCa 4.5, and P<sub>i</sub> levels were 5 mM to correspond with resting P<sub>i</sub> levels in healthy human gastrocnemius (5 mM; (Pathare et al., 2005)) and quadriceps (4.5 mM; (Kemp et al., 2007)) muscles, each comprised of both slow and fast fiber types. Single fiber work in mouse and rat tissue demonstrated that [P<sub>i</sub>] tends to be higher in type I and IIX fibers (6 mM) compared to IIA fibers (0.8 mM) (Kushmerick et al., 1992), indicating that the [P<sub>i</sub>] used in this study may not be representative of exact *in vivo* values for each specific fiber type, but instead were a good estimate. Furthermore, fiber type was

determined after fiber measurements were obtained, so resting  $[P_i]$  could not have been varied by fiber type. All solutions had an ionic strength of 175 mEq using sodium methane sulfate. Fatigue activating solution was the same as control activating solution except that  $P_i$  levels were increased to 30 mM and pH was lowered to 6.2 to simulate the highest *in vivo* metabolite levels in fatigued whole muscle (Hermansen & Osnes, 1972; Metzger & Fitts, 1987; Cady et al., 1989; Broxterman et al., 2017). Fatigue with dATP activating solution was the same as fatigue solution except that 5 mM ATP was replaced with 5 mM dATP.

### **3.4 Experimental Protocol**

The experimental protocol is outlined below and in Figure 9. First, each fiber was tested for maximal tension production in each of the three experimental conditions in a random order with minimal time (< 5 seconds) between each trial: Control (5 mM  $P_i$ , pH 7, 5 mM ATP), fatigue (30 mM  $P_i$ , pH 6.2, 5 mM ATP), and fatigue with dATP (30 mM  $P_i$ , pH 6.2, 5 mM dATP) (Figure 9). Second, myofilament mechanical properties, including myosin-actin cross-bridge kinetics, were measured using small amplitude sinusoidal analysis in all three conditions. Each sinusoidal analysis trial took ~5 minutes with ~2 minutes between each trial. Each fiber went through all three experimental conditions for tension measures, then was tested in two of the three experimental conditions for sinusoidal analysis. It was not possible for each fiber to undergo sinusoidal analysis testing in three conditions because there is generally considerable rundown by the third trial, meaning that fiber function would be artificially reduced (Muscle Biology Laboratory, unpublished observations).



**Figure 9. Overview of experimental protocol.** Parameters measured with corresponding techniques at single fiber and molecular levels under three experimental conditions. Cross-bridge kinetics include myosin attachment time ( $t_{on}$ ) and rate of myosin force production (derived from  $2\pi b$ ).  $P_i$  = inorganic phosphate, ATP = adenosine triphosphate, dATP = deoxyadenosine triphosphate.

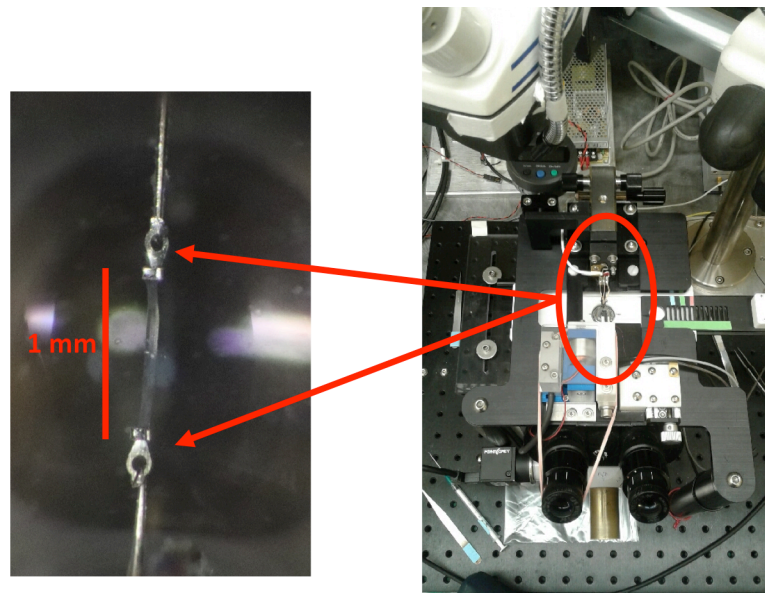
### 3.4.1 Single Fiber Mechanical Analysis

Single muscle fiber mechanical analysis was adapted from (Miller et al., 2010).

The T-clipped ends of the fiber were attached to a piezoelectric motor (Physik Instrumente, Auburn, MA, USA) and a strain gauge (SensorNor, Horten, Norway) on the sinusoidal analysis rig (Figure 10) in relaxing solution at 15°C to maintain fiber integrity, and the fiber was manually stretched until the sarcomere length was set to 2.65  $\mu\text{m}$  (IonOptix, Milton, MA, USA) since this resembles *in vivo* sarcomere length in human skeletal muscle (Chen et al., 2016). A camera (Point Grey, FLIR Integrated Imaging Solutions, Inc., Richmond, BC, Canada) was used to image the fiber and a computer program (ImageJ) performed a fast-fourier transformation (FFT) of the visible light and dark sarcomere patterns to determine sarcomere length. The length of the fiber was measured using a micrometer to determine the distance between the inside edges of the T-clips on each end of the fiber. Fiber top width was measured in the middle of the fiber



once it was stretched and side width was estimated using the ratio of the side/top widths obtained on the cross-sectional area/fixation rig. Fiber top and estimated side widths were used to normalize fiber force to cross-sectional area (CSA) and obtain tension (force/CSA).



**Figure 10. Sinusoidal analysis rig.** Right: Top view of sinusoidal analysis rig. Left: Magnified view of single muscle fiber with T clipped ends mounted on hooks connected to the force gauge (top) and length motor (bottom).

Sinusoidal length perturbations and isometric tension measurements were performed under maximal  $\text{Ca}^{2+}$ -activation (pCa 4.5) at 25°C for control and fatigue conditions as this approaches *in vivo* temperature (37°C) without compromising sarcomere integrity; for example, researchers have reported difficulty obtaining data for human MHC II fibers at 30°C (Sundberg et al., 2018). Furthermore, studies suggest that 25°C is a reasonable representation of *in vivo* temperature regarding force and rate of force development since these parameters are much less temperature dependent between 25 and 37°C compared to colder temperatures (Ranatunga & Wylie, 1983; Bottinelli et

al., 1996; Davis & Epstein, 2007). On the other hand, fiber velocity was shown to be very temperature dependent between 12-22°C and above with a Q10 of 5.88 (Bottinelli et al., 1996), suggesting that our cross-bridge kinetics measurements, which ultimately determine fiber velocity, are also temperature dependent and would quicken at temperatures higher than 25°C. Ultimately, 25°C was chosen as the experimental temperature to provide a reasonable representation of *in vivo* conditions without compromising fiber integrity.

Starting in relaxing solution at 15°C, the fiber was slackened completely, the force gauge zeroed, the fiber pulled back to its original position, allowed to equilibrate for 1 min and relaxed isometric tension measured. This process was known as setting the tension baseline. The fiber was transferred to pre-activating solution for 30 s and then to control activating solution, with tension recorded at its plateau. After tension plateau, the fiber was returned to relaxing solution, given time to relax, and then stretched as necessary so that sarcomere length was 2.65  $\mu\text{m}$ . Starting again in relaxing solution, temperature was increased to 25°C over the course of 1-2 min, tension baseline was set, the fiber was transferred from pre-activating to control activating solution in the same manner as before, and tension was recorded at its plateau. Tension plateau was obtained and recorded in control (non-fatigue), fatigue, and fatigue with dATP conditions in a randomized order. The fiber was then transferred back to relaxing solution to set sarcomere length to 2.65  $\mu\text{m}$  and tension baseline determined. Once force equilibrated, the fiber was transferred from pre-activating to activating solutions using the same protocol as described above. After recording the fiber's tension at plateau, small-amplitude, sinusoidal length changes (0.05% of L) were applied to the fiber at 48

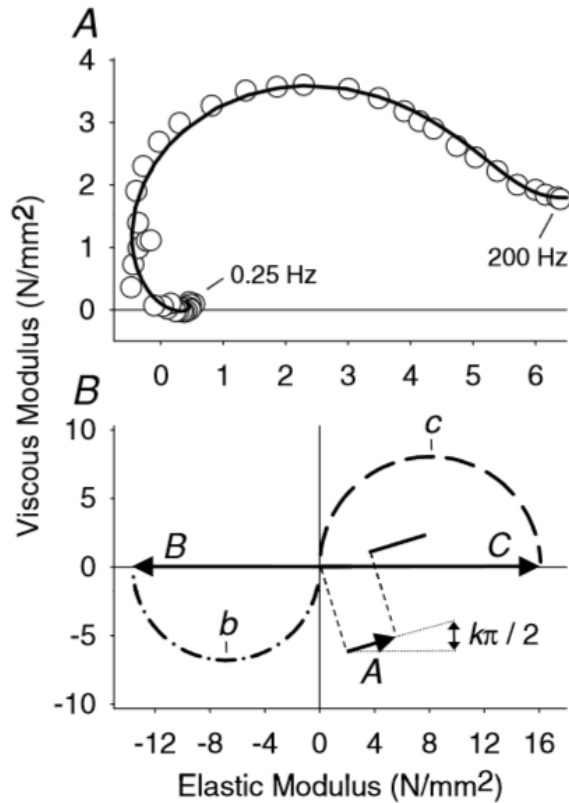
frequencies (0.25 to 200 Hz) in control and fatigue conditions or in control and fatigue with dATP conditions. Between each condition, sarcomere length was reset and tension baseline determined. Length and force were normalized to determine fiber strain ( $\Delta L/L$ ) and tension (force/CSA) by dividing the length change ( $\Delta L$ ) by L and by dividing the force (F) by the fiber average cross-sectional area (CSA). Elastic ( $E_e$ ) and viscous ( $E_v$ ) moduli ( $\text{kNm}^{-2}$ ) were calculated from the tension transient by determining the magnitudes of the in-phase and out-of-phase components (0 deg and 90 deg with respect to strain, respectively). The elastic and viscous moduli are the real and imaginary parts, respectively, of the complex modulus, the ratio of the tension response to the strain.

To relate sinusoidal analysis with specific steps in the cross-bridge cycle, the complex modulus at peak calcium activation was characterized by the following mathematical expression (A):

$$\text{Expression A: } Y(\omega) = A(i\omega/\alpha)^k - Bi\omega/(2\pi b+i\omega) + Ci\omega/(2\pi c+i\omega),$$

where  $\omega=2\pi f$  in  $\text{s}^{-1}$ , A, B and C are magnitudes expressed in  $\text{kNm}^{-2}$ ,  $2\pi b$  and  $2\pi c$  are characteristic rates expressed in  $\text{s}^{-1}$ ,  $i=-1^{1/2}$ ,  $\alpha=1\text{s}^{-1}$ , and k is a unitless exponent. For example, a Nyquist plot, or a plot of the viscous versus elastic modulus, for a human MHC I skeletal muscle fiber from a heart failure patient was fitted using the above equation (Figure 11A) and broken down into its three processes (A, B and C). The A-process (described by parameters A and k) is a linear relationship between the viscous and elastic moduli, while the B-process (described by parameters B and b) and C-process (described by parameters C and c) are semi-circles (Figure 11B). These six parameters have been related to various aspects of muscle mechanics through experimentation and

modeling (Kawai et al., 1993; Zhao & Kawai, 1993; Mulieri et al., 2002; B. M. Palmer et al., 2007). Although different models vary in their precise interpretation, the following summarizes our laboratory's current thoughts on the meaning of these parameters. The A-process has no kinetic or enzymatic dependence ( $Q_{10}$  of  $\sim 0.9$ ; (Mulieri et al., 2002)) and reflects the viscoelastic properties of the structural elements of the fiber across the oscillation frequency range. Under fully relaxed conditions, where no myosin heads are attached, the A-process represents the viscoelastic properties of the underlying fiber structure. Under  $\text{Ca}^{2+}$ -activated conditions where myosin heads are attached, the A-process represents the underlying lattice structure, as well as a portion that increases with  $\text{Ca}^{2+}$  concentration that is ascribed to the attached myosin heads (Mulieri et al., 2002; B. M. Palmer et al., 2004). The parameter  $A$  indicates the magnitude of a viscoelastic modulus and  $k$  represents the angle at which the A-process lies relative to the x-axis. Thus,  $k$  reflects the viscous-to-elastic modulus relationship of the A-process. The magnitude part of the B-process ( $B$ ) is proportional to the number of myosin heads strongly bound to actin and the cross-bridge stiffness (Kawai et al., 1993). The characteristic rate of the B-process ( $2\pi b$ ) is hypothesized to represent the apparent (observed) rate of myosin force production or, in other words, the rate of myosin transition between the weakly- and strongly bound states (Zhao & Kawai, 1993). For the C-process,  $C$  is equivalent to the number of myosin heads strongly bound to actin multiplied by the cross-bridge stiffness, and is therefore proportional to  $B$ , and  $(2\pi c)^{-1}$  represents the average myosin attachment time to actin,  $t_{\text{on}}$  (B. M. Palmer et al., 2007).



**Figure 11. Six-parameter model used to fit Nyquist plots from sinusoidal analysis.** A, the Nyquist plot of a  $\text{Ca}^{2+}$ -activated (pCa 4.5) MHC I fiber, where each open circle represents 1 of 48 oscillation frequencies (0.25 to 200 Hz) performed during sinusoidal analysis, provides information on mechanical properties (elastic and viscous moduli) of a muscle fiber and its components. Sinusoidal analysis results (open circles) are well-characterized by the continuous line, calculated using a six-parameter model (expression A). The six model parameters can be related to cross-bridge function and myofilament structural properties and are paired into three different processes: the linear A-process (continuous line, described by parameters A and  $k$ ), the semicircular B-process (dash-dot line, described by parameters B and  $b$ ) and the C-process (dashed line, described by parameters C and  $c$ ). As a negative viscous modulus indicates positive work production, the B-process is work producing and the A- and C-processes are work absorbing. The B and C parameters, displayed as arrows the length of the diameter of the semicircular B- and C-processes, are magnitudes ( $\text{N mm}^{-2}$ ) proportional to the number of strongly bound cross-bridges and cross-bridge stiffness. The parameters  $b$  and  $c$  indicate the frequencies (Hz) at which the B- and C-processes exhibit viscous modulus values that are the most negative (or largest oscillatory work production) and positive (or largest oscillatory work absorption), respectively. Importantly,  $2\pi b$  and  $2\pi c$  are characteristic rates ( $\text{s}^{-1}$ ) related to cross-bridge kinetics, with  $(2\pi c)^{-1}$  being equivalent to myosin attachment time ( $t_{\text{on}}$ ). The A-process represents the viscoelastic properties of the underlying lattice structure as well as strongly bound cross-bridges. Parameter A, represented by an arrow, indicates a magnitude ( $\text{N mm}^{-2}$ ) and  $k$  (a unitless exponent) is the angle at which the A-process lies relative to the x-axis (Adapted from (Miller et al., 2010)).

### 3.4.2 Fiber Typing

During all functional measurements, single fibers were typed as either type I or II based on the speed of their kinetics; specifically, the rate of myosin force production ( $2\pi b$ ). Fibers were determined as type I if the viscous modulus dip, which is related to  $2\pi b$ , occurred at  $< 2$  Hz and as type II if the dip occurred at  $> 2$  Hz; this method has been fairly accurate for predicting fiber type relative to gel electrophoresis (Miller lab, unpublished observations). This method cannot discern between hybrid fiber types, but this analysis will be done with these data using gel electrophoresis in the near future to determine the myosin heavy chain isoform of each fiber. Therefore, all fibers for these analyses were categorized as either type I or II with no distinction of hybrids. Fibers that had extremely fast (type II: dip  $> 5$  Hz) cross-bridge kinetics were considered extreme outliers and were excluded from this analysis (n=5 fibers) since these fibers are likely hybrids or MHC IIX isoforms, potentially skewing the data. These outliers will be reintegrated into the data after gel electrophoresis determines their isoforms. Other fibers were also excluded for tension values that were abnormally low ( $< 60$  mN/mm<sup>2</sup>) (n=4 fibers) or high ( $> 400$  mN/mm<sup>2</sup>) (n=1 fiber), which often indicates a bad fiber or mix up of top and side width for cross-sectional area calculations, respectively. 1 fiber was also removed due to poor sinusoidal analysis data output that was unable to be fitted by expression (A).

### 3.5 Statistics

All data were reported as mean  $\pm$  standard error of mean (S.E.M.) and differences were considered significant at  $P \leq 0.05$ . All variables were assessed for outliers within each participant and by fiber type using boxplots of means and variability. Fibers that appeared as outliers were reassessed to make sure that the fiber was functioning well during testing per experimental notes as well as to make sure that the data output was reasonable and fit properly with expression (A).

To determine the mechanisms of fatigue (Aim 1) and the ability of dATP to reverse fatigue (Aim 2), differences in fiber tension as well as mechanics and kinetics between control (5 mM  $P_i$ , pH 7, 5 mM ATP), fatigue (30 mM  $P_i$ , pH 6.2, 5 mM ATP) and fatigue with dATP (30 mM  $P_i$ , pH 6.2, 5 mM dATP) conditions in type I and II fibers were evaluated using a one-way analysis of variance (ANOVA) for each fiber type. A linear mixed model was used with group assignment as the between-subject factor (i.e. control group vs. fatigue group vs. fatigue with dATP group) as previously described (Callahan et al., 2014). A linear mixed model was used because the general linear model assumes that each measurement is independent, which is not the case for multiple fibers evaluated within each participant (i.e. fibers from the same participant are related). Accordingly, we have included a repeated effect in the model to account for variations in fiber characteristics within each individual. By accounting for this within-subject variance, this approach can be considered more conservative than the simple process of taking an average value for each fiber characteristic for each subject, which yields a single value for each individual with no estimate of variance. If a main effect was noted, least significant difference post-hoc tests were performed to determine pairwise

differences between the three experimental conditions. The additional between-subject factor of sex was included in the same model in a second, separate analysis to evaluate whether high  $P_i$  and  $H^+$  with ATP or dATP conditions had different effects on fiber function in men and women. Least significant difference post-hoc tests were performed for this secondary analysis as stated above. All analyses were conducted using IBM SPSS Statistics for Windows version 25.0 (IBM, Armonk, NY) and SAS software version 9.4 (SAS Institute, Cary, NC).



## CHAPTER 4

### RESULTS

#### 4.1 Introduction

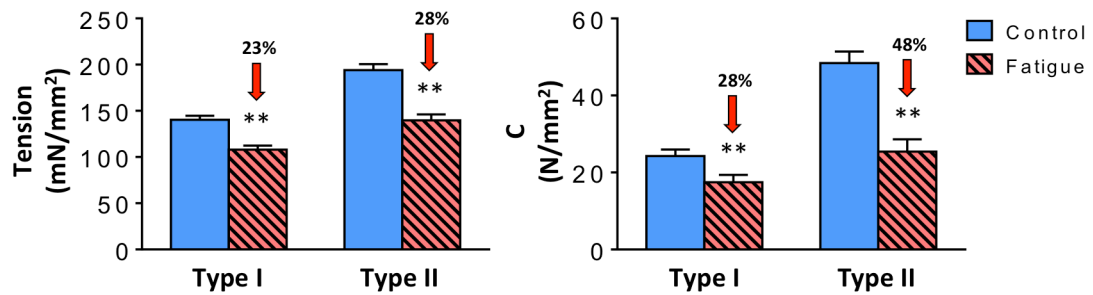
This study examined the effects of elevating two putative agents of fatigue,  $P_i$  and  $H^+$  (low pH), and a potential countermeasure for fatigue, deoxyadenosine triphosphate (dATP), on cellular and molecular function in single human skeletal muscle fibers from older adults. We functionally tested 214 fibers (104 type I and 110 type II; 26 fibers per person on average) from the vastus lateralis of 8 sedentary, healthy older adults (Table 4). Overall, elevated  $P_i$  and  $H^+$  (simulated fatigue) reduced contractile function at the cellular and molecular levels and activating the fiber with dATP versus adenosine triphosphate (ATP) under the same conditions partially reversed these contractile deficits. There were no experimental condition by sex effects in any of the parameters of cellular or molecular function with high  $P_i$  and  $H^+$  for type I or II fibers ( $P > 0.05$  for all parameters, both fiber types), indicating that tension, number of heads, cross-bridge kinetics and stiffness were altered similarly in fibers from older men and women. Therefore, single fiber data from men and women were analyzed together but separated by fiber type.

	<b>Men</b>	<b>Women</b>	<b>Total</b>
<b>N</b>	4	4	8
<b>Age (years)</b>	70 ± 2.2	71 ± 1.7	71 ± 1.3
<b>Height (cm)</b>	175 ± 3.0	153 ± 2.1*	164 ± 4.5
<b>Weight (kg)</b>	77.5 ± 1.7	58.8 ± 5.7*	68.1 ± 4.5
<b>BMI (kg/m<sup>2</sup>)</b>	25.5 ± 1.1	25.2 ± 2.5	25.4 ± 1.3
<b>MVPA (min/day)</b>	18.7 ± 1.65	24.8 ± 3.6	21.8 ± 5.0

**Table 4. Participant characteristics.** Values represent variable means ± S.E.M.  
\*P<0.05 men vs. women.

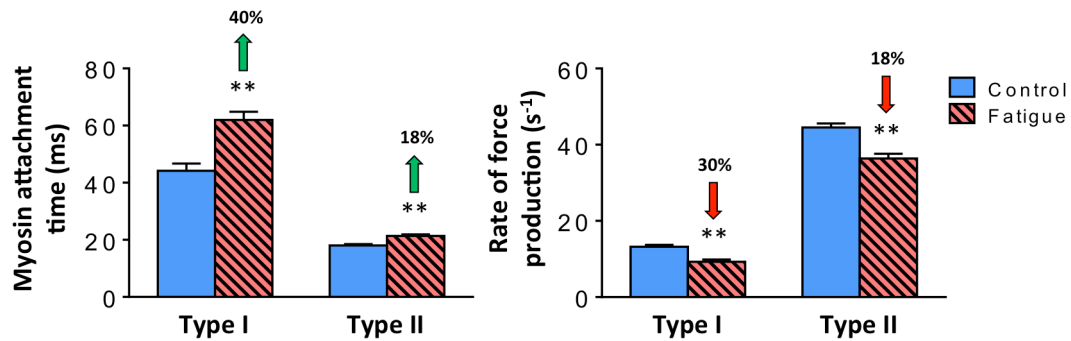
#### **4.2 Cellular and Molecular Changes in Response to Elevated P<sub>i</sub> and H<sup>+</sup> (low pH)**

In single skeletal muscle fibers of older adults, maximal fiber tension (force/cross-sectional area (CSA)) declined in both type I and II (23 and 28%, P<0.01 for both) fibers under fatiguing conditions (elevated P<sub>i</sub> (30 mM) and H<sup>+</sup> (pH 6.2)) compared to control conditions (5 mM P<sub>i</sub>, pH 7) (Figure 12, left). High P<sub>i</sub> and H<sup>+</sup> reduced the number or stiffness of strongly bound myosin heads (parameter C) in type I and II fibers (28% and 48%, P<0.01 for both) (Figure 12, right), leading to lower fiber tension.



**Figure 12. Tension and strongly bound myosin heads response to elevated  $P_i$  and  $H^+$ .** Single fiber tension (force/CSA) and parameter C, the number of strongly bound myosin heads, in type I and II fibers under control (5 mM  $P_i$ ; pH 7) and fatigue (30 mM  $P_i$ ; pH 6.2) conditions with maximal  $Ca^{2+}$  activation (pCa 4.5) at 25°C. Number of fibers analyzed for Tension: Type I = 90 and II = 91; Parameter C: Type I = 93 and II = 97. Significant pairwise differences indicated by \*\* $P < 0.01$  vs. control. Percent change relative to control indicated by arrows above fatigue bars.

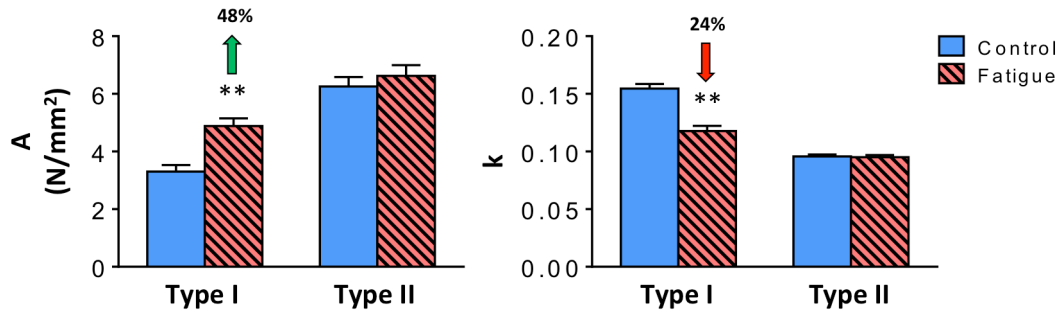
Fibers exposed high levels of  $P_i$  and low pH exhibited a slowing of cross-bridge kinetics compared to control;  $t_{on}$  (derived from  $2\pi\tau_c$ ) became longer in type I and II fibers (40 and 18%,  $P < 0.01$  for both) (Figure 13, left) and the rate of myosin force production (derived from  $2\pi b$ ) slowed in type I and II fibers (30 and 18%,  $P < 0.01$  for both) (Figure 13, right).



**Figure 13. Cross-bridge kinetics response to elevated  $P_i$  and  $H^+$ .** Myosin attachment time ( $t_{on}$ ) (derived from  $2\pi c$ ) and rate of myosin force production (derived from  $2\pi b$ ) in type I and II fibers under control (5 mM  $P_i$ ; pH 7) and fatigue (30 mM  $P_i$ ; pH 6.2) conditions with maximal  $Ca^{2+}$  activation (pCa 4.5) at 25°C. Number of fibers analyzed: Type I = 93 and II = 97. Significant pairwise differences indicated by \*\* $P < 0.01$  vs. control. Percent change relative to control indicated by arrows above fatigue bars.

Parameter A, or myofilament stiffness, represents the magnitude of the viscoelasticity of the myofilament structure, including the number and force of strongly bound cross-bridges. Elevated  $P_i$  and  $H^+$  caused type I fibers to become stiffer, as shown by an increase in parameter A (48%,  $P < 0.01$ ), while type II fibers experienced no significant change in stiffness ( $P = 0.47$ ) (Figure 14, left). Parameter k characterizes the viscous-to-elastic relationship of parameter A, and represents the degree to which the fiber is purely elastic ( $k=0$ ) (behaves like a spring and does not absorb work) or viscous ( $k=1$ ) (behaves like a dashpot and absorbs work). Parameter k decreased with high  $P_i$  and  $H^+$  in type I (24%,  $P < 0.01$ ) but not II ( $P = 0.27$ ) fibers (Figure 14, right), indicating that type I fibers were becoming more elastic with high  $P_i$  and  $H^+$  and increases in parameter A represented greater elasticity in both fiber types. The fact that fiber stiffness was increased (type I) or unchanged (type II) with high  $P_i$  and  $H^+$  suggests that there was no decrease in the stiffness of the strongly bound myosin heads (force per cross-bridge).

Thus, the decrease in parameter C is interpreted as a decrease in the number of strongly bound myosin heads, not a change in their stiffness.

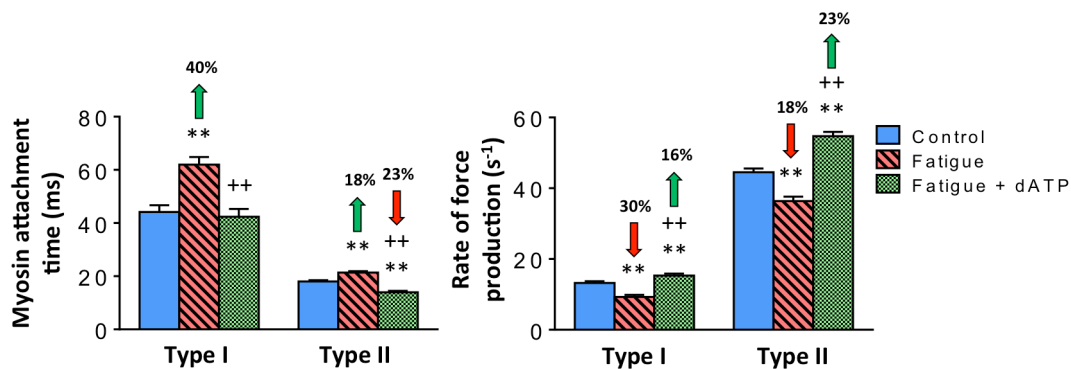


**Figure 14. Myofilament stiffness response to elevated  $P_i$  and  $H^+$ .** Parameter A, the viscoelastic magnitude of the myofilament lattice with strongly bound cross-bridges (greater values indicate greater stiffness) and parameter k, the degree to which the fiber is purely elastic ( $k=0$ ) or viscous ( $k=1$ ), in type I and II fibers under control (5 mM  $P_i$ ; pH 7) and fatigue (30 mM  $P_i$ ; pH 6.2) conditions with maximal  $Ca^{2+}$  activation (pCa 4.5) at 25°C. Number of fibers analyzed: Type I = 93 and II = 97. Significant pairwise differences indicated by  $**P < 0.01$  vs. control. Percent change relative to control indicated by arrows above fatigue bars.

Preliminary experiments using single fibers from 1 woman were performed to try and determine the mechanisms of increased stiffness with elevated  $P_i$  and low pH in type I fibers. When all cross-bridges were put into the weak-binding state using 80 mM 2,3-butanedione monoxime (BDM) (10 fibers), fiber stiffness did not differ between high  $P_i$  and  $H^+$  versus control conditions, suggesting that the myofilament lattice itself, independent of strongly bound cross-bridges, did not stiffen. When all cross-bridges were placed into the strong-binding state (rigor) in a 0 mM ATP solution with high  $P_i$  and  $H^+$  versus a 5 mM ATP control solution (11 fibers), there was still no increased stiffness. Given these results, the reason for the increase in myofilament stiffness remains unclear.

### 4.3 Cellular and Molecular Changes in Response to Elevated $P_i$ and $H^+$ (low pH) with dATP

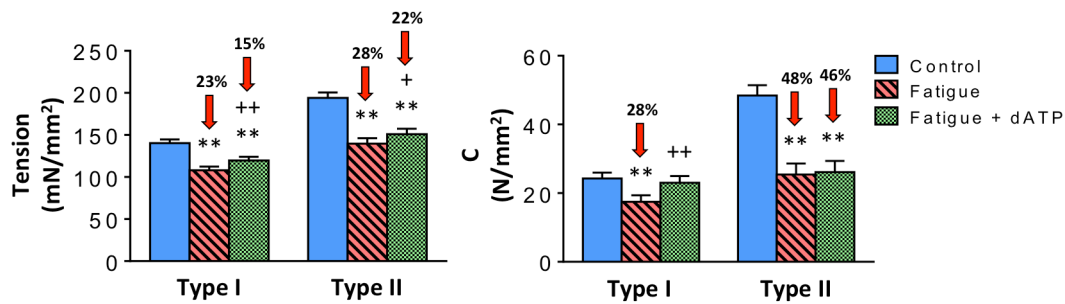
When muscle contracted with dATP instead of ATP, myosin-actin cross-bridge kinetics recovered back to control levels or beyond in conditions with elevated  $P_i$  and low pH (Figure 15). Myosin attachment time ( $t_{on}$ ) in high  $P_i$  and  $H^+$  conditions with dATP recovered back to control levels in type I fibers ( $P=0.47$  vs. control; 32%,  $P<0.01$  vs. fatigue) and exceeded control values for type II fibers (23%,  $P<0.01$  vs. control; 35%,  $P<0.01$  vs. fatigue) (Figure 15, left). The rate of myosin force production in high  $P_i$  and  $H^+$  conditions with dATP became faster than control values in type I (16%,  $P<0.01$  vs. control; 65%,  $P<0.01$  vs. fatigue) and II (23%,  $P<0.01$  vs. control; 50%,  $P<0.01$  vs. fatigue) fibers (Figure 15, right).



**Figure 15. Cross-bridge kinetics response to elevated  $P_i$  and  $H^+$  with dATP.**

Myosin attachment time ( $t_{on}$ ) (derived from  $2\pi\tau_c$ ) and rate of myosin force production (derived from  $2\pi\tau_b$ ) in type I and II fibers under control (5 mM  $P_i$ ; pH 7), fatigue (30 mM  $P_i$ ; pH 6.2) and fatigue with dATP (30 mM  $P_i$ ; pH 6.2; 5 mM dATP vs. ATP) conditions with maximal  $Ca^{2+}$  activation (pCa 4.5) at 25°C. Number of fibers analyzed: Type I = 93 and II = 97. Significant pairwise differences indicated by \*\* $P<0.01$  vs. control; ++ $P<0.01$  vs. fatigue. Percent change relative to control indicated by arrows above fatigue and fatigue with dATP bars.

Although cross-bridge kinetics completely recovered back to or exceeded control values in elevated  $P_i$  and low pH conditions with dATP versus ATP in both fiber types, the number of strongly bound myosin heads and tension did not always recover to the same degree (Figure 16). When ATP was replaced with dATP in high  $P_i$  and  $H^+$  conditions, the number of strongly bound heads fully recovered back to control values in type I fibers ( $P=0.44$  vs. control; 32%,  $P<0.01$  vs. fatigue), while the number of heads remained low and were not significantly different from values observed in high  $P_i$  and  $H^+$  conditions with ATP in type II fibers (46%,  $P<0.01$  vs. control;  $P=0.79$  vs. fatigue) (Figure 16, right), suggesting a fiber-type-specific recovery. Although the number of heads fully recovered in type I fibers, this only translated to a partial recovery of tension relative to high  $P_i$  and  $H^+$  conditions with ATP (11%,  $P<0.01$ ) as it was still lower than control (15%,  $P<0.01$ ) (Figure 16, left). Although the number of heads remained low in high  $P_i$  and  $H^+$  conditions with dATP in type II fibers (Figure 16, right), tension was also partially recovered relative to high  $P_i$  and  $H^+$  conditions with ATP (8%,  $P<0.05$ ) but was still lower than control (22%,  $P<0.01$ ) (Figure 16, left), most likely due to large improvements in cross-bridge kinetics with dATP versus ATP.

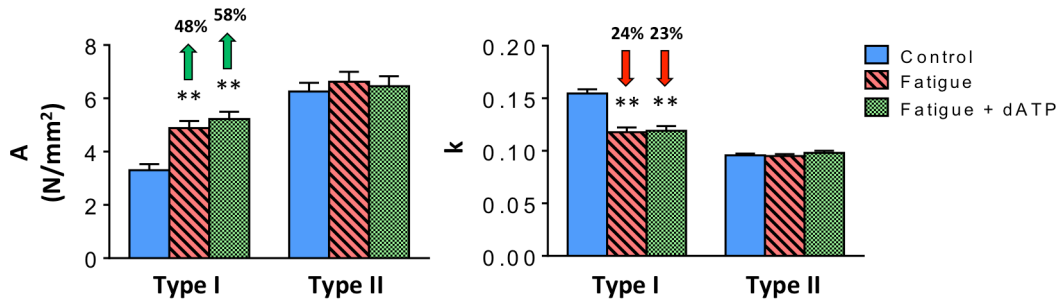


**Figure 16 Tension and strongly bound myosin heads response to elevated  $P_i$  and  $H^+$  with dATP.** Fiber tension and number of strongly bound myosin heads (parameter C) in type I and II fibers under control (5 mM  $P_i$ ; pH 7), fatigue (30 mM  $P_i$ ; pH 6.2) and fatigue with dATP (30 mM  $P_i$ ; pH 6.2; 5 mM dATP vs. ATP) conditions with maximal  $Ca^{2+}$  activation (pCa 4.5) at 25°C. Number of fibers analyzed for Tension: Type I = 90 and II = 91; Parameter C: Type I = 93 and II = 97. Significant pairwise differences indicated by \*\* $P < 0.01$  vs. control; + $P < 0.05$  and ++ $P < 0.01$  vs. fatigue. Percent change relative to control indicated by arrows above fatigue and fatigue with dATP bars.

Compared to the control condition, the myofilament lattice of type I fibers remained stiffer (larger parameter A) in elevated  $P_i$  and low pH conditions with dATP (58%,  $P < 0.01$  vs. control) and was not significantly different than high  $P_i$  and  $H^+$  with ATP ( $P = 0.22$ ) (Figure 17, left). On the other hand, the stiffness (parameter A) of type II fibers remained unchanged in high  $P_i$  and  $H^+$  conditions with dATP compared to both high  $P_i$  and  $H^+$  with ATP and control values ( $P = 0.47$ ) (Figure 17, left). In accordance with parameter A, parameter k remained low relative to control values in type I fibers exposed to high  $P_i$  and  $H^+$  conditions with dATP (23%,  $P < 0.01$ ) and was not significantly different than high  $P_i$  and  $H^+$  with ATP ( $P = 0.76$ ), meaning that fibers became more elastic in high  $P_i$  and  $H^+$  conditions and dATP did not further alter this parameter. In type II fibers, k remained unchanged across all three experimental conditions ( $P = 0.27$ ) (Figure 17, right). Since k was closer to 0 (elastic) than 1 (viscous) for all experimental



conditions in both fiber types, this indicated that parameter A represented the magnitude of the fiber's elasticity/stiffness. Overall, these data show that type I fibers from older adults became stiffer and more elastic with high  $P_i$  and low pH while type II fibers remained unchanged. Furthermore, substituting dATP for ATP in high  $P_i$  and  $H^+$  conditions had no effect on myofilament stiffness or elasticity in either fiber type.



**Figure 17. Myofilament stiffness response to elevated  $P_i$  and  $H^+$  with dATP.** Parameter A, the viscoelastic magnitude of the myofilament lattice with strongly bound cross-bridges (greater values indicate greater stiffness) and parameter k, the degree to which the fiber is purely elastic ( $k=0$ ) or viscous ( $k=1$ ), in type I and II fibers under control (5 mM  $P_i$ ; pH 7), fatigue (30 mM  $P_i$ ; pH 6.2) and fatigue with dATP (30 mM  $P_i$ ; pH 6.2; 5 mM dATP vs. ATP) conditions with maximal  $Ca^{2+}$  activation (pCa 4.5) at 25°C. Number of fibers analyzed: Type I = 93 and II = 97. Significant pairwise differences indicated by \*\* $P < 0.01$  vs. control. Percent change relative to control indicated by arrows above fatigue and fatigue with dATP bars.

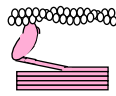

## CHAPTER 5

### DISCUSSION

#### 5.1 Introduction

This study aimed to 1) determine the cellular and molecular mechanisms of skeletal muscle fatigue by exposing single fibers from older adults to solutions with elevated levels of the putative agents of fatigue and 2) evaluate the efficacy of deoxyadenosine triphosphate (dATP) to reverse the negative effects caused by the putative agents of fatigue. Fatigue was simulated in single skeletal muscle fibers *in vitro* to closely mimic *in vivo* conditions by elevating inorganic phosphate ( $P_i$ ) (from 5 to 30 mM) and  $H^+$  (lowering pH from 7 to 6.2). The results from this study are summarized below in Figure 18. Elevated  $P_i$  and  $H^+$  decreased fiber tension (force/cross-sectional area (CSA)) in type I and II fibers due to fewer strongly bound myosin heads caused by slower cross-bridge kinetics (Figure 18). Elevated  $P_i$  and  $H^+$  with dATP instead of adenosine triphosphate (ATP) recovered cross-bridge kinetics and the number of strongly bound myosin heads in a fiber-type-specific manner, which led to partial recovery of tension in both fiber types. Interestingly, myofilament stiffness increased in high  $P_i$  and  $H^+$  conditions with ATP and dATP in type I fibers, but was unchanged in type II fibers in all three experimental conditions. This study deepened our knowledge about the mechanisms of skeletal muscle fatigue in human single fibers from older adults by illustrating how high  $P_i$  and low pH conditions slowed cross-bridge kinetics, which partially explained changes in maximal fiber tension. The present study also showed that

the contractile deficits in cross-bridge kinetics and tension induced by high  $P_i$  and low pH can be partially to fully reversed in these fibers.

		Aim 1		Aim 2	
		Fatigue vs. Control		Fatigue w/ dATP vs. Control	
		Type I	Type II	Type I	Type II
<b>Molecular level</b> (Myosin-actin) 	<b>Cross-bridge kinetics</b>	↓↓↓	↓	↔, ↑	↑↑
	<b># of strongly bound myosin heads</b>	↓↓	↓↓↓	↔	↓↓↓
<b>Cellular level</b> (Single fiber) 	<b>Tension</b> (Force/CSA)	↓↓	↓↓	↓	↓↓

**Figure 18. Results summary.** Summary of study results for Aims 1 & 2 by fiber type (type I or II) at the molecular and cellular levels. Cross-bridge kinetics include myosin attachment time ( $t_{on}$ ) (derived from  $2\pi c$ ) and rate of force production (derived from  $2\pi b$ ). Parameter C is assumed to represent the # of strongly bound myosin heads. Experimental conditions were the following: Control: 5 mM inorganic phosphate ( $P_i$ ), pH 7, 5 mM adenosine triphosphate (ATP); fatigue: 30 mM  $P_i$ , pH 6.2, 5 mM ATP; and fatigue with deoxyadenosine triphosphate (dATP): 30 mM  $P_i$  pH 6.2, 5 mM dATP. Arrows indicate direction of and percent change relative to control (increase: ↑ = 15-20%, ↑↑ = 20-30%; decrease: ↓ = 15-20%, ↓↓ = 20-30%, ↓↓↓ = ≥ 30%; no significant difference = ↔). This figure does not include parameters A and k (myofilament lattice stiffness) because these were not primary measurements for the study aims.

## 5.2 Aim 1: Cellular and Molecular Mechanisms in Response to High $P_i$ and $H^+$ (low pH)

**Aim 1:** Determine the effects of simulated fatigue on tension and myofilament mechanical properties, including myosin-actin cross-bridge kinetics, in single fibers from older men and women.

**Hypotheses for Aim 1:** Simulated fatigue will 1A) decrease single fiber tension, due to 1B) a reduction in the number or stiffness of strongly bound myosin heads, and 1C) slower cross-bridge kinetics, including increasing myosin attachment time ( $t_{on}$ ), in type I and II fibers.

### **5.2.1 Mechanisms for Reduced Fiber Tension with High $P_i$ and $H^+$ (low pH)**

Elevated  $P_i$  and low pH decreased maximal fiber tension (force/CSA) in type I and II fibers, similar to what has been observed in previous studies that mimicked fatigue with both high  $P_i$  (30 mM) and low pH (6.2) at high temperature ( $\geq 22^\circ\text{C}$ ) in fibers from animals (Fitts, 2008; Karatzaferi et al., 2008; Nocella et al., 2011; Nelson et al., 2014) and humans (Sundberg et al., 2018). High  $P_i$  and  $H^+$  decreased parameter C in type I and II fibers, which represents a reduction in either the number or stiffness of strongly bound myosin heads. If parameter C represented stiffness per cross-bridge, myofilament stiffness (parameter A), or the stiffness of the myofilament lattice with strongly bound cross-bridges, should have also decreased with high  $P_i$  and  $H^+$ . Because parameter A either increased (type I) or remained unchanged (type II) with elevated  $P_i$  and  $H^+$ , this suggested that the force exerted per cross-bridge (cross-bridge stiffness) was not reduced, thus the decrease in parameter C most likely represented fewer strongly bound myosin heads. Fiber force (and therefore tension) is the product of the number of strongly bound myosin heads and force per head, so fewer strongly bound heads could account for the decreased tension with elevated  $P_i$  and  $H^+$  observed in the present study. These data agree with previous work suggesting that elevated  $P_i$  and  $H^+$  reduced the number of strongly bound cross-bridges in fibers from animals (Nocella et al., 2011; Nelson et al., 2014) and

humans (Sundberg et al., 2018) at high temperatures ( $\geq 22^{\circ}\text{C}$ ;  $15^{\circ}\text{C}$  for myosin heavy chain (MHC) II human fibers), which was hypothesized to have been due to more weakly bound cross-bridges (Nelson et al., 2014; Sundberg et al., 2018).

The number of strongly bound myosin heads and force per cross-bridge cannot be directly determined or discerned in fiber experiments, so experiments in isolated myosin-actin proteins using the laser trap assay provide useful information about individual cross-bridge behavior. Using this technique at  $30^{\circ}\text{C}$ , elevating  $\text{P}_i$  only (30 mM) reduced maximal force production of a myosin mini-ensemble (multiple myosin heads in series) obtained from chicken pectoralis muscle (primary MHC II (Verdiglione & Cassandro, 2013)) due to fewer high force-generating myosin heads binding (Debold et al., 2013), while acidosis only (high  $\text{H}^+$ ) reduced peak force by decreasing both the number of heads strongly bound and the force generated per head (Woodward & Debold, 2018).

However, single fiber studies suggest that  $\text{P}_i$  and  $\text{H}^+$  synergistically depress force (Chase & Kushmerick, 1988; Cooke et al., 1988; Potma et al., 1995; Nelson et al., 2014); therefore these metabolites should be studied concurrently. One recent single molecule and myosin mini-ensemble laser trap study at  $30^{\circ}\text{C}$  also using chicken pectoralis muscle observed that elevated  $\text{P}_i$  (10 mM) and low pH (pH 6.5) combined also reduced peak force (20%) to a similar degree as observed in the present study (23-28%) due to elimination of all high-force binding events, which indicated that there were fewer strongly bound myosin heads at any given time (Woodward & Debold, 2018). Overall, isolated protein work using single molecules and myosin ensembles supports our hypothesis that the reduction observed in parameter C with high  $\text{P}_i$  and low pH represented fewer strongly bound myosin heads, which provides a mechanism for the

reduction in fiber tension. Future isolated protein work could examine the effects of more extreme metabolite concentrations ( $P_i$  30 vs. 10 mM; pH 6.2 vs. 6.5) in both MHC I and II muscle to clarify the applicability of these mechanisms to fatigue *in vivo* in different fiber types.

### **5.2.2 High $P_i$ and $H^+$ (low pH) Slowed Myosin-actin Cross-bridge Kinetics**

The number of strongly bound myosin heads is largely dictated by myosin-actin cross-bridge kinetics, specifically the duty ratio [ $t_{on}/(t_{on} + t_{off})$ , where  $t_{on}$  = myosin attachment time and  $t_{off}$  = myosin detachment time]. Elevating two putative agents of fatigue ( $P_i$  and  $H^+$ ) in human skeletal muscle fibers from older adults slowed cross-bridge kinetics by increasing  $t_{on}$ , which contradicts research in myosin mini-ensembles with elevated high  $P_i$  only (Debold et al., 2013) but agrees with work in single myosin molecules in low pH only (Debold et al., 2008). However, recent isolated protein work suggested that the combination of elevated  $P_i$  and low pH may still lengthen  $t_{on}$ , but this is most likely due to acidosis alone and is more pronounced at low pH only like in previous studies (Woodward & Debold, 2018).

In the present study, high  $P_i$  and  $H^+$  conditions also slowed the other component of cross-bridge kinetics, the rate of myosin force production. This agrees with previous single fiber work where elevated  $P_i$  and  $H^+$  conditions slowed the rate of force redevelopment measured with  $k_{tr}$  (Fitts, 2008; Nelson et al., 2014; Sundberg et al., 2018), which is a technique where the fiber is rapidly slacked and the rate of force recovery is fit with a line. This technique is slightly different from the one used in the present study, which measured the rate of force production (the sum of the forward and reverse rates of

the myosin head) derived from parameter  $2\pi b$  obtained during small amplitude sinusoidal analysis. Therefore, the kinetics parameters obtained from these different techniques are somewhat, but not completely similar. Isolated myosin-actin proteins exposed to elevated  $P_i$  and low pH showed a reduction in the number of high force-generating events, indicating fewer strongly bound myosin heads at a given moment due to a slower myosin weak-to-strong binding transition (Woodward & Debold, 2018). The rate of force development measured in the present study is thought to represent the rate of myosin's weak-to-strong binding transition, demonstrating that the isolated protein work supports the current results.

Although this study did not quantify all components of the cross-bridge cycle, the increase in  $t_{on}$  observed with high  $P_i$  and  $H^+$  is hypothesized to have been countered by a relatively larger increase in  $t_{off}$ , which would increase the duty ratio and explain the observed reduction in the number of strongly bound myosin heads. According to early work in rabbit psoas fibers (Cooke et al., 1988), the time that myosin takes to complete the cross-bridge cycle ( $t_{on} + t_{off}$ ) measured with ATPase was slowed with high  $P_i$  and  $H^+$  compared to control conditions at low temperature ( $10^\circ C$ ) due to longer  $t_{on}$  and/or  $t_{off}$ . Single molecule and myosin mini-ensemble data also showed reduced ATPase in solution with both high  $P_i$  and low pH at  $30^\circ C$ , hypothesized to be largely the result of a slower myosin weak-to-strong binding rate and thus longer  $t_{off}$  (Woodward & Debold, 2018), which supports the present hypothesis. The present study advanced our knowledge about the molecular mechanisms of skeletal muscle fatigue, as it was the first to examine the effects of high  $P_i$  and low pH on  $t_{on}$  in single skeletal muscle fibers. This study also quantified a new measurement of cross-bridge kinetics, myosin rate of force production,

to further characterize changes in the cross-bridge cycle with high  $P_i$  and low pH. Future work in our laboratory seeks to build upon the present study and quantify the entire cross-bridge cycle in response to high  $P_i$  and  $H^+$  at high temperature ( $\geq 25^\circ\text{C}$ ) by measuring the time to complete the total cross-bridge cycle ( $t_{\text{on}} + t_{\text{off}}$ ) using an ATPase assay in conjunction with measuring  $t_{\text{on}}$  obtained from sinusoidal analysis. This will allow for direct quantification of  $t_{\text{off}}$  [ $(t_{\text{on}} + t_{\text{off}}) - t_{\text{on}}$ ] with high  $P_i$  and  $H^+$  to test the hypothesis that  $t_{\text{off}}$  increases under these conditions relative to control.

### **5.2.3 High $P_i$ and $H^+$ (low pH) Increased Myofilament Lattice Stiffness in Type I Fibers**

This was the first study to observe that myofilament lattice stiffness increased with elevated  $P_i$  and low pH in type I fibers and did not change in type II fibers relative to control despite decreased tension in both fiber types. Under normal conditions, increases in parameter A have been associated with increased fiber tension (force/CSA) as a stiffer lattice allows for greater force transmission (Miller et al., 2015). Prior research has shown that high  $P_i$  and  $H^+$  reduced fiber stiffness (Nocella et al., 2011; Nelson et al., 2014), contrary to the present study. However, these prior studies measured fiber stiffness by oscillating the fiber at extremely high frequencies (2-4 kHz) in order to place all cross-bridges in the strongly bound state, whereas stiffness measurements were obtained from our fibers over a range of lower frequencies (0.25 to 200 Hz) where not all cross-bridges were strongly bound at any given time. Perhaps the increased stiffness observed in the present study highlights a compensatory mechanism to mitigate the decline in tension and



fatigability that is characteristic of type I fibers, but the mechanisms of this phenomenon are unknown.

Additional, preliminary experiments suggested that the myofilament lattice did not become stiffer with or without strongly bound cross-bridges, thus the reason for increased myofilament stiffness with high  $P_i$  and low pH in type I fibers remains unclear. However, only 10 and 11 fibers of unknown types from 1 female participant each were tested without any and with all strongly bound cross-bridges, respectively. Future experiments that expose a greater number of type I and II human fibers to high  $P_i$  and  $H^+$  conditions with low ATP (rigor) or adenosine diphosphate (ADP) concentrations could be used to verify whether parameter A increases due to greater force per cross-bridge, as decreased [ATP] or [ADP] prolongs the strongly bound state (Debold et al., 2012; Debold, 2012), which allows for manipulation of the number of cross-bridges in strong binding. In addition to these future experiments, further analysis of the current data should elucidate the possible mechanisms for increased parameter A with high  $P_i$  and  $H^+$  in type I fibers by parsing out how the elasticity and viscosity of this parameter are altered under these conditions.

### **5.3 Aim 2: Efficacy of dATP to Reverse Contractile Decrements with High $P_i$ and $H^+$ (low pH)**

**Aim 2:** Determine the ability of dATP to reverse simulated fatigue-induced changes in tension and myofilament mechanical properties in single fibers from older men and women.

**Hypotheses for Aim 2:** Simulated fatigue with dATP will 2A) recover tension back to control values, due to its ability to 2B) recover the number or stiffness of strongly bound myosin heads and 2C) cross-bridge kinetics, in type I and II fibers.

### **5.3.1 High $P_i$ and $H^+$ (low pH) with dATP Recovered Myosin-actin Cross-bridge Kinetics**

Myosin-actin cross-bridge kinetics recovered when fibers were subjected to elevated  $P_i$  and low pH conditions with dATP instead of ATP as an energy source. In high  $P_i$  and  $H^+$  with dATP, cross-bridge kinetics became faster compared to high  $P_i$  and  $H^+$  with ATP; specifically,  $t_{on}$  recovered back to (type I) or become faster than control (type II) and rate of myosin force production ( $2\pi b$ ) exceeded control levels in both fiber types. These results agree with previous non-fatigue studies in rabbit psoas fibers showing that, compared to ATP, dATP resulted in a faster rate of tension redevelopment ( $k_{tr}$ ), which indicates longer  $t_{off}$ , as well as increased unloaded fiber velocity by 30% (Regnier & Homsher, 1998), which indicates shorter  $t_{on}$ . Fibers that contracted with dATP versus ATP in normal conditions exhibited quicker N- (nucleotide) TPase (Regnier et al., 1998), which represents a slower cross-bridge cycle rate due to shorter  $t_{on}$  and/or  $t_{off}$ . Because rabbit psoas fibers are primarily fast-contracting, myosin-heavy chain (MHC) II isoforms (Hamalainen & Pette, 1993), this could explain why Regnier et al. observed quicker velocity (indicating shorter  $t_{on}$ ) with dATP versus ATP that was similar to changes in type II fibers in the present study; specifically, type II fibers exhibited shorter  $t_{on}$  and type I fibers had similar  $t_{on}$  relative to control conditions, even with high  $P_i$  and  $H^+$ . The current study also agrees with the only other study to examine high  $P_i$  and

low pH with dATP but in isolated proteins, which showed that actin filament velocity, and theoretically  $t_{on}$ , returned to control levels in high  $P_i$  and  $H^+$  conditions with dATP instead of ATP (Longyear et al., 2014). dATP most likely quickens cross-bridge kinetics because it has a lesser affinity for myosin than ATP (Regnier et al., 2000).

Compared to ATP and ADP, myosin is thought to hydrolyze dATP faster and therefore shorten  $t_{off}$ , while dADP may be released faster from myosin than dATP, thus shortening  $t_{on}$  (Koretz & Taylor, 1975; Chock, 1979). These hypotheses were supported by quicker fiber NTPase observed with dATP versus ATP as an energy source under the same conditions (Regnier et al., 1998). Future work could examine the effect of dATP on  $t_{off}$  using the ATPase assay ( $t_{on} + t_{off}$ ) and sinusoidal analysis ( $t_{on}$ ) in order to determine whether changes in  $t_{off}$  with dATP help explain the fiber-type-specific recoveries of the number of bound heads.

### **5.3.2 High $P_i$ and $H^+$ (low pH) with dATP Partially Recovered Fiber Tension**

Theoretically, because cross-bridge kinetics recovered back to control levels or beyond in elevated  $P_i$  and low pH conditions with dATP versus ATP, the number of strongly bound myosin heads and fiber tension (force/CSA) was also expected to recover. However, in high  $P_i$  and  $H^+$  conditions with dATP, parameter C (which likely represents the number of strongly bound myosin heads in this study) recovered back to control values in type I fibers but remained low in type II fibers. Even with this fiber-type-specific recovery of the number of strongly bound heads, fiber tension only partially recovered in high  $P_i$  and  $H^+$  conditions with dATP in both fiber types to levels higher than high  $P_i$  and  $H^+$  with ATP but significantly lower than control. Notably, non-fatigue

studies in animal fibers showed no difference in maximal force between ATP and dATP at 10°C (Regnier et al., 1998; Regnier & Homsher, 1998), which may partially explain why dATP did not fully recover tension back to control levels in human single fibers.

Overall, the exact mechanisms for this partial recovery of tension in high  $P_i$  and  $H^+$  conditions with dATP are unknown, but this work provides the foundation for future studies by characterizing the molecular basis for these single fiber alterations, as mentioned in the previous section. Our laboratory plans to test human skeletal muscle fibers from older adults for maximal tension in a control condition with dATP compared to control with ATP to further characterize the mechanisms of fiber improvements with dATP in human tissue and determine if elevated  $P_i$  and  $H^+$  metabolites are inhibiting full tension recovery.

### **5.3.3 dATP did not Alter Myofilament Lattice Stiffness with High $P_i$ and $H^+$ (low pH)**

Myofilament lattice stiffness (parameter A) was the same in elevated  $P_i$  and low pH conditions with dATP and ATP in type I and II fibers, indicating that stiffness was unaffected by dATP. Therefore, type I fibers remained stiffer than control in solution that contained high  $P_i$  and  $H^+$  with dATP, and stiffness of type II fibers remained unchanged in all three experimental conditions. These results suggest that elevated  $P_i$  and/or low pH are responsible for increased myofilament stiffness in type I fibers, whereas type II fibers are unaffected by increased levels of these metabolites. Previous studies also found similar fiber stiffness (determined with 0.5 kHz sinusoidal oscillations) in control conditions with ATP and dATP, which was hypothesized to be due to similar force per

cross-bridge in each condition (Regnier et al., 1998; Regnier & Homsher, 1998) because active fiber stiffness is partially dependent on cross-bridge stiffness. This could indicate that the increased stiffness observed in the present study was the result of increased force per cross-bridge despite the proposed reduction in the number of strongly bound myosin heads with high  $P_i$  and low pH.

Further analyses of the characteristics of parameter A may help elucidate why the number of strongly bound heads and tension are altered with dATP while myofilament stiffness is unaffected. Once again, future studies should examine ATPase to quantify both  $t_{on}$  and  $t_{off}$  in order to clarify whether the number of strongly bound myosin heads changes with dATP. This would help us clarify whether changes in parameter C represent changes in the number or stiffness of myosin heads, which would also help explain changes in myofilament stiffness.

#### **5.4 Limitations**

The present study observed a 23-28% drop in maximal fiber tension (force/CSA), which is similar to previous single fiber studies that similarly simulated fatigue by elevating  $P_i$  and  $H^+$  at near-physiological temperatures (Fitts, 2008; Nelson et al., 2014; Sundberg et al., 2018; Nocella et al., 2011) but is much less than the 70-80% decline in power observed in whole muscles of older adults (Hicks et al., 2001; Katsiaras et al., 2005; Callahan & Kent-Braun, 2011; Christie et al., 2011; Broxterman et al., 2017; Sundberg et al., 2018). Neural drive is thought to play a minimal role in fatigue (Kent-Braun, 1999; Kent-Braun et al., 2002) and therefore does not likely account for this difference. Another possibility is that there was a diffusion limitation during the *in vitro*

experiments, as early research showed that the diffusion rate of  $P_i$  varied by fiber size, which would be important because  $P_i$  affects myosin-actin interactions and therefore fiber force (Cooke & Pate, 1985). However, a diffusion limitation was most likely not present because maximal tension was measured at plateau and tension always leveled off before starting sinusoidal analysis for fiber mechanics and kinetics. If diffusion of substrates and metabolites were limited, tension would not be expected to plateau. Notably, previous work in single fibers shows that myosin-actin cross-bridge kinetics remain constant across fiber size, indicating that diffusion of small molecules, such as ADP or  $P_i$ , are not limited in the myofilament lattice (Miller et al., 2015). Finally, single fiber tension may not have declined as much as in whole muscles due to other metabolites and substrates that change with fatigue *in vivo* that can alter contractile function. Although we cannot measure calcium concentration  $[Ca^{2+}]$  *in vivo* and some evidence suggests that  $[Ca^{2+}]$  may not drop *in vivo* as much as *in vitro* (Roos et al., 1999), transient  $[Ca^{2+}]$  may be reduced with fatiguing contractions in whole muscle or electrical stimulation in single fibers (Allen et al., 1989; Westerblad et al., 1989; Lee et al., 1991), which can reduce sensitivity and ultimately inhibit activation and myosin-actin interactions. There has been extensive research suggesting that reduced calcium sensitivity accounts for most of the decline in force observed with fatigue (Allen et al., 2008; Fitts, 2008; Nelson & Fitts, 2014; Debold, 2016; Debold et al., 2016), so reduced transient  $[Ca^{2+}]$  may help explain the differences in force or tension reduction between single fibers and whole muscles in human skeletal muscle.

Another possible limitation is that the type II fibers in the present were not being oscillated fast enough to adequately observe the behavior of strongly bound cross-bridges

in active conditions. If this were the case, the myofilament stiffness (parameter A) values observed in type II fibers in the present study would represent the maximum measurable values and the fact that stiffness was unchanged for all three experimental conditions would only be a methodological artifact. However, any shifts in the elasticity of the fiber from control to high  $P_i$  and  $H^+$  conditions were observed across all oscillation frequencies and did not appear to converge at higher frequencies, which provides evidence that stiffness was not at its maximum for type II fibers.

Control solutions contained 5 mM  $P_i$  in order to correspond with resting  $P_i$  levels in human calf (Pathare et al., 2005) and quadriceps (Kemp et al., 2007) muscles. Nuclear magnetic resonance techniques have revealed that  $[P_i]$  varies by fiber type in rodent muscle, ranging from 0.8 mM in type IIA muscle to 6 mM in type I and IIX muscle (Kushmerick et al., 1992). Varying  $[P_i]$  leads to altered fiber force, especially at concentrations lower than 10 mM (Millar & Homsher, 1990). Due to the nature of our experiments, fiber type cannot be discerned before testing fibers so this is an inherent, but relatively minor, limitation of the study.

Finally, this study did not examine a control condition with dATP to determine the effects of dATP on contractile function independent of elevated  $P_i$  and  $H^+$ . This made it difficult to determine the exact mechanisms of dATP. For example, without a control with dATP condition, this study cannot determine whether the improvements observed with dATP in high  $P_i$  and  $H^+$  conditions were because replacing ATP with dATP improved fiber function and resulted in fibers starting at a higher baseline. In this case, fiber function would have remained better than control despite high  $P_i$  and  $H^+$  conditions instead of recovering the negative effects of elevated  $P_i$  and  $H^+$ .

## 5.5 Summary

This study provided novel mechanistic insight into skeletal muscle fatigue and its reversal in single fibers from older men and women. Fatigue simulated with elevated  $P_i$  (from 5 to 30 mM) and low pH (from 7 to 6.2) at 25°C reduced fiber tension (force/CSA) compared to control conditions due to fewer strongly bound myosin heads resulting from slower cross-bridge kinetics, including longer  $t_{on}$ , which had not been previously measured in single fibers with high  $P_i$  and  $H^+$  conditions. Elevated  $P_i$  and low pH with dATP instead of ATP as an energy source recovered cross-bridge kinetics back to control values, which resulted in full recovery of strongly bound myosin heads in type I but not type II fibers. High  $P_i$  and  $H^+$  with dATP only partially recovered tension in both fiber types. Interestingly, myofilament lattice stiffness increased with high  $P_i$  and  $H^+$  in type I fibers only and was unaffected by dATP in both fiber types. These results improve our understanding about the mechanisms of skeletal muscle fatigue in human single muscle fibers by revealing that slower cross-bridge kinetics (longer myosin attachment times ( $t_{on}$ ) and slower rates of myosin force production) partially explained reduced maximal tension in response to high  $P_i$  and low pH conditions. This study also found novel evidence that replacing ATP with dATP as an energy source for muscle fibers partially to fully reversed the contractile deficits induced with high  $P_i$  and  $H^+$ , which provides a basis for future research about dATP as a potential countermeasure for skeletal muscle fatigue. By understanding the mechanisms of how two putative agents of fatigue, high  $P_i$  and  $H^+$ , depressed tension in single fibers and how these decrements can be mitigated, we now further understand the cellular and molecular causes and a potential countermeasure of reduced force, velocity and therefore power that characterize fatigue at the whole muscle



level. Future mechanistic work should focus on characterizing the full cross-bridge cycle, especially  $t_{\text{off}}$ , under similar experimental conditions to further elucidate the mechanisms of fatigue and its reversal.

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