EFFECTS OF CARBOHYDRATE AVAILABILITY ON FATIGUE AND FATIGUE PRE-CONDITIONING IN MOUSE FDB MUSCLE

By

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ABSTRACT

To prevent damaging ATP depletion during periods of intense activity, intrinsic mechanisms within skeletal muscle are activated and lead to myoprotection; a process known as muscle fatigue. It has been proposed that the primary mechanism of fatigue is a submaximal sarcoplasmic reticulum Ca^{2+} release and decreased force generation, however, what triggers this mechanism remains controversial. It is possible that glycogen may act as a trigger as studies have repeatedly shown a direct correlation between glycogen content at the beginning of activity and time to fatigue. In previous studies, a fatigue bout and/or period of fasting to deplete glycogen was used. However, this leaves investigators to differentiate between the effects of glycogen depletion methodology causing a metabolic stress and effects of glycogen itself. One objective of this M.Sc. project was to produce a low glycogen model without a prior metabolic stress that could forgo these limitations. It was extended to differentiate between the role of glycogen and extracellular glucose during fatigue as well as fatigue pre-conditioning (FPC), a phenomenon in which fatigue resistance increases for about 2 hours after a first fatigue bout. During a single, first fatigue bout (one contraction every sec for 3 min) a mean decrease in glycogen from 95 to 20 μmol/g dry wt. had no effect on the decrease in tetanic $[Ca^{2+}]_i$, i.e. the $[Ca^{2+}]_i$ during tetanic contractions, whereas removing glucose from the physiological solution led to a 46% greater decrease in tetanic $[Ca^{2+}]_i$ than when glucose was present. During a subsequent fatigue bout (i.e. FPC) a greater amount of glycogen was used as glycogen content was 27% greater than prior to the first fatigue bout. When glycogen and/or glucose was limited, FPC was abolished. It is concluded that extracellular glucose is critical to prevent fatigue. Additionally, whereas glycogen is important for FPC, it appears to be much less important during a first fatigue bout initiated in absence of any prior metabolic stress.

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LIST OF ABBREVIATIONS

$[]_e$	Extracellular ion concentration
$[]_i$	Intracellular ion concentration
Ach	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	5’ adenosine monophosphate-activated protein kinase
AP	Action potential
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
CHO	Carbohydrate
CK	Creatine kinase
CNS	Central nervous system
DHPR	Dihydropyridine receptor
E_m	Membrane potential
FDB	Flexor digitorum brevis
FFA	Free fatty-acid
FPC	Fatigue pre-conditioning
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-1-phosphate
GLUT-4	Glucose transporter type 4

GP	Glycogen phosphorylase
GS	Glycogen synthase
IMF	Intermyofibrillar
IMP	Inositol monophosphate
Intra	Intramyofibrillar
K^+	Potassium ion
K_{ATP}	Adenosine triphosphate-sensitive potassium channel
L.S.D	Least square difference
MHC	Major histocompatibility complex
Na^+	Sodium ion
Nav	Voltage-gated sodium channel
NMJ	Neuromuscular junction
OXPHOS	Oxidative phosphorylation
PCr	Phosphocreatine
PCr	Phosphocreatine
P_i	Inorganic phosphate
ROS	Reactive oxygen species
RyR	Ryanodine receptor
S.E.	Standard error
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SR	Sarcoplasmic reticulum
SS	Subsarcolemmal
T_m	Tropomyosin

T_n	Troponin
T-tubules	Transverse tubules
UDP	Uridine diphosphate

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CHAPTER 1

INTRODUCTION

Skeletal muscles must adapt to different intensities of activity quickly and require equally adaptable metabolic capabilities to effectively regulate the energy production required to sustain different levels of muscle activity. During each muscle contraction, ATP is required at multiple sites within the individual muscle fiber. At rest, the requirement for ATP is minimal and metabolic rates are accordingly low. However, during repeated, intense muscle contraction, metabolic rate can increase up to 100-fold (Allen et al., 2008). If maintained, periods of high muscle activity will lead to an ATP demand exceeding that of ATP generation. If the subsequent ATP depletion becomes too large, then cell damage as well as cell death may occur. It is therefore critical that mechanisms be in place to prevent excessive depletion of ATP before it occurs.

It has been proposed that muscle fatigue, i.e. a recoverable decline in the ability of the muscle to produce force, is the primary mechanism responsible for the prevention of damaging ATP depletion (Cifelli et al., 2007, 2008). This is because less force equates to less ATP demand. There is evidence for a role of several intracellular signals acting as energy sensors that could initiate the fatigue response (Pedersen et al., 2009, Allen et al., 2008, Cifelli et al., 2007, Wan et al., 2017). It remains unclear, however, where these signals originate and what triggers an increase in their activity.

Numerous studies have reported strong correlations between the rate at which force declines during fatigue and muscle glycogen content (Hermansen et al., 1967, Chin & Allen, 1997, Allen et al., 2008, Ørtenblad et al., 2013, Green, 1991). It is also known that the role of glycogen is

not simply that of a glucose reservoir, as its content in muscle (or depletion thereof) can influence intracellular signaling, ultimately leading to changes in glucose availability for use in energy production as well as metabolic capabilities within a muscle fiber (Allen et al., 2008, Green, 1991, Ørtenblad et al., 2013). To study the correlation between glycogen and muscle fatigue, glycogen depletion has been achieved either by means of a fast or a previous bout of fatiguing stimulation. This leaves investigators to differentiate between the effects due to glycogen content and the effects of a previous metabolic stress associated with either fasting or a fatigue bout.

A first objective of this study was to develop a protocol to deplete glycogen in an acute manner, without metabolically stressing the fibers, to investigate the effect of glycogen on muscle fatigue. This was extended to include an investigation of the influence of extracellular glucose. A second objective was to investigate the role of carbohydrate availability in the etiology of fatigue pre-conditioning (FPC), a recently described phenomenon in which fatigue resistance activity increases after a first fatigue bout for about 2 hours.

MUSCLE CONTRACTION

Muscle force is generated upon stimulation by the nervous system. Impulses originating in the central nervous system (CNS) are eventually propagated as action potentials (AP) along α-motor neurons (the neurons that innervates skeletal muscle) until they reach a specialized synapse called the neuromuscular junction (NMJ). In the synaptic button of the motor neuron, APs induce the release of the neurotransmitter acetylcholine (Ach). Ach diffuses across the synaptic cleft of the NMJ to a nicotinic acetylcholine receptor rich region of the muscle fiber termed the motor end plate. Ach binds to these receptors causing a conformational change and an opening of the receptor pore which, once open, allows a large cation influx, particularly Na^+, into the muscle fiber.

Muscle fiber membrane potential at rest (resting E_m) is approximately -80 mV and is maintained primarily by Kir2.1 inward rectifying K^+ channels and ClC-1 chloride channels (Pedersen et al., 2009a, 2009b). The net effect of the Na^+ influx is a localized depolarization of the motor end plate, activating neighboring voltage-gated Na^+ (Nav) channels. As a consequence of these channels opening and the large and rapid Na^+ influx, an AP is triggered, and the cell membrane depolarizes to approximately +30 mV. The depolarization phase ends as Nav channels are inactivated and as voltage-gated K^+ channels (K_V) are activated. The net effect is a K^+ efflux, causing a repolarization of the membrane potential back to resting E_m.

From the NMJ, APs propagate along the muscle fiber membrane (sarcolemma) and down transverse tubules (t-tubules). The t-tubules are invaginations of the sarcolemma that allow APs to propagate deeply within muscle fibers and are closely associated with the sarcoplasmic reticulum (SR). Voltage sensitive Ca^{2+} ($Ca_{V1.1}$) channels, also known as L-type Ca^{2+} channels or dihydropyradine receptors (DHPR), are located along the t-tubules and are activated during APs when membrane potentials become less negative than -50 mV. Once activated, conformational changes to the channel allow for a small influx of Ca^{2+} into the fiber from the extracellular space. However, most of the increase in myoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) involves a release of Ca^{2+} from the SR via the ryanodine receptor (RyR). This SR Ca^{2+} release channel is activated as DHPR changes conformation which then, through a physical interaction with RyR, allows for a rapid and large release of Ca^{2+} into the myoplasm.

The sarcomere is the contractile unit that produces force or does work in skeletal muscle. The sarcomere is composed of thick and thin filaments. The major protein that makes up the thick filament is myosin, which interacts with the contractile protein in thin filaments, i.e. actin. Interaction

between actin and myosin is regulated at the level of the thin filament. In the relaxed state, myosin binding sites on actin are blocked by tropomyosin (Tm), another protein in thin filaments, and thus a cross-bridge, or myosin-actin link, cannot be formed. When $[Ca^{2+}]_i$ rises, it binds to troponin (Tn), also present in thin filaments, and causes a conformational change in Tn that moves Tm from the myosin binding site on actin. As myosin binds to actin the energy released by the hydrolysis of ATP by myosin-ATPase is used to shorten the sarcomere, allowing it to generate force or do work. This may continue until $[Ca^{2+}]_i$ is sufficiently lowered as it is transported back into the SR by the Ca^{2+} ATPase (SERCA), allowing the muscles to relax.

There are three major sites of energy consumption during muscle contraction: *i*) the myosin-ATPase accounts for approximately 70% of the ATP utilization; *ii*) the Ca^{2+}-ATPase, which transports Ca^{2+} back into the SR, accounts for 30% of ATP utilization; and *iii*) the Na^+/K^+-ATPase, which pumps 3 Na^+ out of the cell and 2 K^+ into the cell, playing a role in restoring Na^+ and K^+ electrochemical gradients necessary for AP generation, accounts for less than 1% of ATP utilization (Kushmerick et al., 1969, Rall et al., 1975).

Energy metabolism during exercise

The increased ATPase activity during exercise creates a need for a concomitant increase in metabolic production of ATP to constantly maintain cellular energy levels. Despite substantial ATP consumption during exercise, most studies report that overall $[ATP]_i$ after exercise is only reduced by approximately 20% (Hirvonen et al., 1992, Jansson et al., 1987, Taylor et al., 1986); a clear indication of the upregulation of ATP producing pathways. Several pathways are involved in the generation of ATP.

Phosphocreatine (PCr) is the first pathway that provides ATP according to the following reaction:

$$PCr + ADP + H^+ \leftrightarrow Cr + ATP$$

PCr is utilized and exhausted within 20 to 50 seconds of activity (Scott et al., 2016). As PCr is consumed, so too is H^+, elevating the pH which subsequently activates glycolysis through the relief of inhibition on the rate-limiting phosphofructokinase reaction. Although ATP generation from glycolysis is rapid, it only produces 2 and 3 ATP per glycosyl unit from extracellular glucose and glycogen stores respectively (Scott et al., 2016). Extracellular glucose enters muscle fibers via GLUT-1 and GLUT-4 glucose transporters, the latter being translocated in large amounts from intracellular vesicles to the sarcolemma during muscle contraction or in the presence of insulin (Richter & Hargreaves, 2013). Contraction and insulin induced GLUT-4 translocation to the sarcolemma and T-Tubules has been shown to occur through different signaling pathways using different GLUT-4 pools so when both are present their action are additive; thus, both of these factors can influence muscle energetics by increasing glucose availability within muscle fibers (Lund et al., 1995, Lauritzen, 2014, Tremblay et al., 2003). Once glucose has entered a fiber, it is then phosphorylated to glucose-6-phosphate (G-6-P) by the ATP-dependent hexokinase and can undergo glycolysis through the following reaction:

$$G\text{-}6\text{-}P + 2\ ADP + 2\ NAD^+ + 2P_i \rightarrow 2\ Pyruvate + 2\ ATP + 2\ NADH^+ + 2\ H^+$$

Glycosyl units originating from the breakdown of glycogen molecules are released as glucose-1-phosphate (G-1-P) and undergo glycolysis by the following reaction:

$$G\text{-}1\text{-}P + 3\ ADP + 2\ NAD^+ + 3\ P_i \rightarrow 2\ Pyruvate + 3\ ATP + 2\ NADH^+ + 2\ H^+$$

The major end-product of glycolysis, pyruvate, can have two fates.

When exercise intensity is high, or under anaerobic conditions, pyruvate is converted to lactate by lactate dehydrogenase. Once considered a waste product, recent evidence suggests that lactate, and it's release from skeletal muscle, is an important source of energy production (Brooks, 1998, Kemppainen et al., 2002). Briefly, lactate production works intracellularly to replenish NAD^+, required as a substrate to fuel glycolysis, but has been shown to be taken up by mitochondria where it is oxidized back to pyruvate, ultimately fueling ATP production (Brooks, 1998, Kane, 2014). Intercellularly, during high intensity exercise, lactate originating in skeletal muscle travels through the blood stream and has been shown to be taken up by cardiac muscle tissue as an important source of high energy compounds fueling increased cardiac activity (Kemppainen et al., 2002). Thus, lactate produced as a result of glycolysis is gaining acceptance as an important energy source during exercise.

Alternatively, pyruvate can be directly converted to acetyl coenzyme-A (Acetyl-CoA) by pyruvate dehydrogenase and enter the Krebs cycle to produce more ATP per glucose by oxidative phosphorylation:

$$\text{Glucose} + 36\ \text{ADP} + 36\ P_i + 6\ O_2 \leftrightarrow 36\ \text{ATP} + 6\ H_2O + 6\ CO_2$$

This theoretical yield of 36 ATP however does not typically occur in living cells due to the presence of a 'proton leak' which reduces the efficiency of oxidative phosphorylation (Jastroch et al., 2010).

Acetyl-CoA can also be derived from free fatty-acids (FFA) by a process termed β-oxidation in which 2 carbon at a time are cleaved from FFAs. The general reaction for β-oxidation is as follows:

$$\text{FFA}(n) + n\text{ATP} + n\text{CoA} + n\text{FAD} \leftrightarrow n\text{Acetyl-CoA} + 2nP_i + n\text{AMP} + n\text{NADH} + n\text{FADH2},$$

where n represent the number of 2 carbon cleavages of a given FFA molecule.

Lastly, ATP can be generated with the aid of adenylate kinase. As ATP is depleted and the ATP:ADP ratio is reduced, adenylate kinase catalyzes the transfer of one inorganic phosphate (P_i) from one ADP molecule to another by the following reaction:

$$2\ ADP \leftrightarrow ATP + AMP$$

This has the net effect of reducing the accumulation of ADP while replenishing ATP. Adenylate kinase is however a near-equilibrium enzyme and to ensure the reaction moves forward AMP must be removed. Thus, AMP deaminase catalyzes the removal of AMP by its conversion to IMP through the following reaction:

$$AMP + H_2O \rightarrow IMP + NH_3$$

Upregulation of these ATP producing pathways effectively enhances ATP producing capabilities of the muscle and, as mentioned, works to maintain ATP concentration within levels that will prevent fiber damage. However, it is known that ATP production during prolonged low intensity or shorter high intensity exercise eventually fails to match the ATP demand, creating an ATP deficit (Allen et al., 2008). The mechanism by which muscles protect against damaging ATP depletion resulting from the imbalance of energy consumption to energy utilization is termed muscle fatigue.

SKELETAL MUSCLE FIBERS TYPES

Although the mechanics of a muscle contraction do not change between individual fibers, muscles are subdivided into fiber types based on their myosin heavy chain (MHC) isoform expression. Fiber type grouping also extends to metabolic and functional properties.

Type I, or slow-twitch fibers, are highly resistant to fatigue. They also have slow contractile speeds and shortening velocity. As such, they are well suited for maintaining posture as opposed to, for example, sprinting. They are characterized by a high capacity to produce ATP through oxidative

phosphorylation (OXPHOS) and have concomitantly high levels of mitochondria, myoglobin, and blood vasculature (Schiafinno & Reggiani, 2011). Additionally, they express troponin isoforms which are less sensitive to Ca^{2+}, and SERCA channel isoforms that are associated with slower relaxation between contractions when compared to type II fibers.

Type II, or fast-twitch fibers, are divided into three subcategories: type IIA, IIX, and IIB. Type II fibers are characterized by faster shortening velocity, contractile speeds and greater force development than type I fibers. Furthermore, they express troponin isoforms that are more sensitive to Ca^{2+} and SERCA channels that allow for faster relaxation (Schiafinno & Reggiani, 2011). Except for shortening velocity, type IIA are similar to type I fibers in that they are highly fatigue resistant, because of a high oxidative capacity. They are well suited for endurance exercises such as jogging. Type IIB are the least resistant to fatigue and are characterized by a low oxidative capacity but high glycolytic rates and increased glycogen content when compared to type I and IIA fibers (Schiafinno & Reggiani, 2011). It is a result of this reliance on glycolytic energy that these fibers are not fatigue resistant. Type IIX fibers are intermediate to type IIA and IIB in terms of their oxidative capacity and shortening velocity. Due to their faster shortening velocity and greater force development, type IIX and IIB fibers become important during exercises such as sprinting and weight lifting. Interestingly, type IIB fiber are expressed in rodents, however not in humans, although humans do possess the myosin IIB gene.

Muscle fibers can also exist as hybrids, expressing two or more MHC isoforms (Schiafinno & Reggiani, 2011). As an example, in the mouse flexor digitorum brevis (FDB) muscle, fibers expressing exclusively type I represented only 2% of total fibers; type IIA, 19%; and type IIX, 21% (Banas et al., 2011). This leaves a total of 58% of total fibers being hybrids.

FATIGUE

Fatigue can be defined as a transient recoverable decline in muscle force or work when muscles are repeatedly activated. It has been proposed to prevent damaging ATP depletion (Cifelli et al., 2007, Selvin et al., 2015, Boudreault et al., 2010, Mckenna, 2008, Thabet et al., 2005). The decline in force generation during fatigue can be to 20 - 80% of pre-fatigue values and is highly dependent on fiber type as well as the intensity, length, and number of contractions. Despite numerous studies aimed at elucidating the mechanisms of fatigue, they are still not completely known. There is evidence for a central fatigue component, implicating mechanisms at the level of the CNS, for which there are decreases in motor drive. However, the exact role of central fatigue is controversial (reviewed in Batson, 2013). More importantly, to this study, there is strong evidence for peripheral fatigue, which implicates mechanisms intrinsic to muscle itself (Allen et al., 2008). The remainder of this discussion will focus on the intrinsic properties of muscles and peripheral mechanisms of fatigue.

Role of metabolites during fatigue

For many years fatigue was thought to be a result of changes in metabolite concentration and end-products of metabolic pathways acting at the level of the sarcomere; either changing its sensitivity to $[Ca^{2+}]_i$ or changing the amount of force generated by the myosin-actin link. With fatiguing exercise, major metabolic changes include decreases in intracellular the concentration of ATP and increases in ADP, AMP, IMP, P_i, adenosine and lactate (Allen et al., 2008, Godt & Nosek, 1989). The effects of various metabolites at the level of sarcomere have been extensively studied and are briefly summarized in the following section.

The role of purines during fatigue have been investigated by mimicking the changes that occur during fatigue using ‘skinned’ muscle fibers, which are obtained by removing the cell membrane to

give access to the intracellular milieu (Godt & Nosek, 1989, Myburgh et al., 1997). Godt and Nosek (1989) showed that decreasing ATP concentration from 6.18 to 4.7 mM, a value similar to that found during fatigue, has no effect on maximal force production while it increases Ca^{2+} sensitivity of the sarcomere. In fact, decreases in ATP to concentrations to < 1 mM have been showed to increase maximal force production (Best et al., 1977). Godt and Nosek also reported 6 and 7 % increases in maximal force production and no effect on Ca^{2+} sensitivity when AMP and ADP are increased to levels observed during fatigue, i.e. 0.06 and 0.7 mM respectively. Additionally, when IMP levels are increased to fatigue levels (from 0 to 5 mM) no effect on either maximal force production or Ca^{2+} sensitivity are observed (Myburgh et al., 1997). Taken together these results indicate that adenine nucleotides and IMP have a minimal role in the etiology of muscle fatigue at the level of the sarcomere.

Lactate build-up is the classical explanation for skeletal muscle fatigue, however closer examination of its role suggests otherwise. Higher intensity exercise favors glycolysis to readily produce ATP which results in an increase in intracellular lactate. It has been thought that lactate accumulation causes lactic acidosis and thus a drop in pH, ultimately impairing force production in muscle. Firstly, increasing lactate concentration from 0 to 30 mM has been reported to have no effect on force production (Andrews et al., 2006). Secondly, experiments conducted at 22˚C do report a decrease in force production at lowered pH levels; however, these effects are abolished at physiological temperature (Pate et al., 2005). Thirdly, low levels of acidosis in muscles reverse the K^+ induced force depression (a potential mechanism of fatigue; see section intitled 'Membrane excitability and SR Ca^{2+} release during fatigue') through a mechanism involving increased membrane excitability (Nielsen et al.,2001, Pedersen et al., 2005).

The increased hydrolysis of ATP and creatine phosphate during fatiguing exercise has been shown to lead to an accumulation of myoplasmic P_i. Studies using creatine kinase knockout ($CK^{-/-}$) mice to prevent increases in intracellular P_i show a decrease in muscle fatigue of up to 30% (Dahlstedt et al., 2001). Other studies have reported a correlation between high levels of intracellular P_i and impaired SR Ca^{2+} release (Duke & Steele, 2000, Westerblad et al., 2002). These studies and others (Allen et al., 2008) do provide strong evidence that P_i does play at least some role in the etiology of fatigue, however it does not fully account for the up 80% decline in force observed during fatiguing stimulation.

Overall, although evidence does suggest some role of metabolites in skeletal muscle fatigue, they alone cannot fully explain the decline in force and $[Ca^{2+}]_i$ observed during intense or sustained exercise.

Decreased Ca^{2+} released by the SR during fatigue

Allen & Westerblad groups were the first to demonstrate large decreases in $[Ca^{2+}]_i$ during contractions, here termed tetanic $[Ca^{2+}]_i$, as the primary cause for the decrease in force during fatigue (Westerblad et al., 1991, Allen et al., 1989). They elicited repeated 350 msec long tetanic contractions to measure tetanic $[Ca^{2+}]_i$ and force, first at an interval of 4 sec, then reducing the interval every 2 minutes thereby making the muscle activity increasingly demanding. A pattern in the changes of tetanic $[Ca^{2+}]_i$ and force over time, consisting of 3 phases (P1, P2, and P3), was observed (Fig 1-1A). During P1, force declines by roughly 10%, while $[Ca^{2+}]_i$ actually increases by approximately 10 – 40%. It has been hypothesized that this decline is due to a decrease in the sensitivity of the contractile apparatus to Ca^{2+} (Chin & Allen, 1997), as shown in Fig 1-1C as a shift in the force-Ca^{2+} curve towards higher $[Ca^{2+}]_i$ in fatigued muscle when compared to non-fatigue muscle. P2 is characterized by a

steady state during which tetanic force and $[Ca^{2+}]_i$ remain relatively constant. Lastly, during P3 a large decline in tetanic force and $[Ca^{2+}]_i$ is observed. Both the decline in tetanic force and $[Ca^{2+}]_i$ returned to near pre-fatigue levels when caffeine (a RYR opener) is added (Fig 1-1B), which suggests that insufficient Ca^{2+} remaining in the SR is not the reason for the decline in Ca^{2+}-activated force. Instead, large reductions in tetanic force are only observed when tetanic $[Ca^{2+}]_i$ is submaximal, as is observed during P3 as a decline to levels lower than 500 nM (Fig 1-1C). Although a large body of evidence now supports a major role for Ca^{2+} homeostasis in fatigue (Selvin & Renaud, 2015, Chin & Allen, 1997, Westerblad & Allen, 1993, Helander et al., 2002,Ørtenblad et al., 2013, Westerblad et al., 1991, Kabbara et al., 1999, Glass et al., 2018), specifically that a decrease in Ca^{2+} release from the SR plays a major role in the etiology of muscle fatigue, the mechanisms responsible for the decrease remain to be elucidated. At least five cellular components may be involved in the decrease in Ca^{2+}. The first two are the DHPR and RYR. Although regulation of these two channels has not been extensively studied there is evidence of an ATP dependence, where a decrease in ATP reduces their activity (Fill & Copello, 2002, Laver et al., 2001). P_i is another component that has been proposed to influence Ca^{2+} release. There is evidence for an anion channel in the SR membrane that is activated in response to a decrease in ATP, allowing an influx of P_i that precipitates SR Ca^{2+} reducing its availability for release (Dutka et al., 2005, Allen et al., 2008).

The last two components are the ATP-sensitive K^+ channel (K_{ATP}) and ClC-1 channels, which are also ATP dependent and for which a decrease in ATP activates the channels to eventually reduce AP amplitude and membrane excitability. The role of membrane excitability on Ca^{2+} release have been extensively studied and will be discussed in the following section.

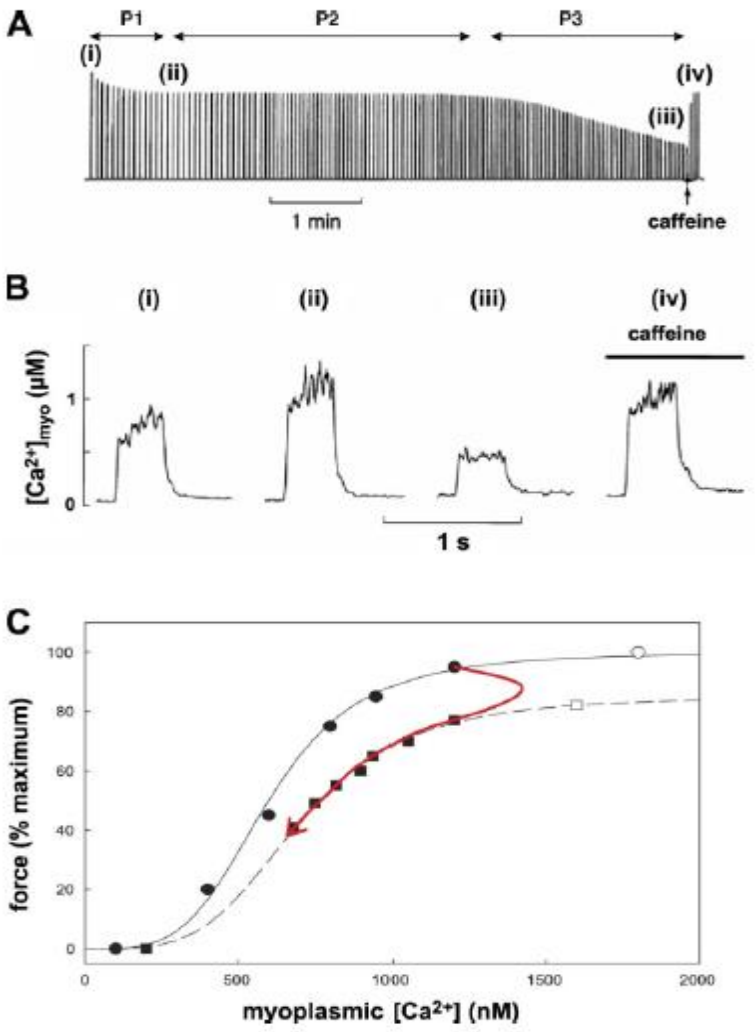

Figure 1-1. Relationship between force and intracellular Ca^{2+} concentration during muscle fatigue (Westerblad & Allen, 1993). Fatigue was induced by tetani of 350 msec duration elicited at 100 Hz at an initial interval of 4 sec, reduced every min by 1 sec until force reached 30% of original. A) Force recordings of the fatigue period, showing 3 phases of fatigue (P1, P2, and P3), and a caffeine contracture (10 mM) at the end of the fatigue period. B) Representative traces of $[Ca^{2+}]_i$ during the phase shown in A. C) Steady state Ca^{2+}-force relationship; (●) unfatigued muscle; (○)application of caffeine in unfatigued muscle. (■) Represents muscle in P3 fatigue. (□) represents final application of caffeine after fatigue.

Membrane excitability and SR Ca^{2+} release during fatigue

Membrane excitability is determined by the intra- and extracellular concentrations of K^+, Na^+, and Cl^- as well as their conductance across the sarcolemma. Due to the large Na^+ influx during multiple APs, intracellular concentration of Na^+ can increase from 8 - 14 mM at rest to 10 - 58 mM during fatigue (Mckenna et al., 1985). This change in intracellular Na^+ concentration has little effect on resting E_m as Na^+ conductance is very low at rest. On the other hand, the decrease in the Na^+ concentration gradient results in lower AP amplitude because of a reduced Na^+ influx during the depolarization (Pedersen et al., 2005, McKenna et al., 1985). Mimicking the change in the Na^+ gradient by reducing extracellular Na^+ concentration demonstrated that only extreme decreases in the Na^+ gradient depresses force. It has thus been suggested that changes in intra- and extracellular Na^+ concentration alone have a minor role in the decrease in membrane excitability and thus force.

K^+ efflux, largely responsible for the repolarization phase during an AP, results in an increase in $[K^+]_e$ from ≈ 4.5 to 10 mM during sustained muscle contraction, with a concomitant decrease in $[K^+]_i$ from 160 to 100-140 mM (McKenna et al., 2008). As K^+ is permeable at rest, the resulting change in the K^+ gradient leads to depolarization of the resting E_m. When tested *in vitro* a depolarization of approximately 10 mV is observed with an increase in $[K^+]_e$ to 6 mM and up to 30 mV when $[K^+]_e$ increases past 10 mM (Cairns & Lindinger, 2008). As a consequence of the depolarization, a large number of Nav channels become inactivated, resulting in lower Na^+ influx during AP depolarization and thus lower AP amplitude and force. However, changes in K^+ at 37°C are not large enough to suppress force as $[K^+]_e$ must reach 12 – 13 mM (Cairns & Lindinger, 2008).

Maintenance of Na^+ and K^+ gradients during muscle activity is largely accomplished through the activity of the electrogenic Na^+/K^+-ATPase. Increases in pump activity play a major role in resetting K^+ gradients across the sarcolemma, which helps prevent suppression of force resulting from high

$[K^+]_e$. In fact, increases in pump activity have been shown to induce hyperpolarization and increase M-wave area, effectively increasing contractility (Kuiack & McComas, 1992, Hicks & McComas, 1989, Clausen, 2003). However, it is known that Na^+/K^+-ATPase activity decreases during intense exercise and as such could severely affect muscle performance (Nielsen & Overgaard, 1996, Sandiford et al., 1985). Both glucose supplements and the presence of insulin have been shown to increase human muscle in vitro Na^+/K^+-ATPase activity via a PKC dependent mechanism (Green et al., 2007, Chibalin et al., 2001, Sampson, 1995). Additionally, AMP-activated protein kinase (AMPK), central in cellular energy homeostasis, has been shown to upregulate Na^+/K^+-ATPase activity. These factors could play a role in fatigue resistance by countering the decrease in pump activity during prolonged exercise, and maintaining Na^+ and K^+ gradients across the sarcolemma. The K^+-force relationship can be affected by other factors as well, specifically, ClC-1 and K_{ATP} channel activity.

The channel primarily responsible for Cl^- conductance in muscle is the ClC-1 channel. The channel is voltage-sensitive, but its kinetics are too slow for any change in the number of open channels during an AP (Pedersen et al., 2016). ClC-1 channel activity is, however, regulated in a complex manner during exercise. At the onset of exercise, the channel is inhibited and Cl^- conductance decreases by 70% via a PKC dependent mechanism (Pedersen et al., 2009). The major reason is to counteract the K^+ induced force depression by means of a resulting shift in the K^+-force relationship to higher $[K^+]_e$. The shift results in increased fiber excitability and increase AP amplitude as leak conductance due to Cl^- influx during depolarization is less. Thus, the decrease in open ClC-1 channels at the onset of exercise prevents changes in Na^+ and K^+ gradients from decreasing AP amplitude.

When fast-twitch muscle is continuously stimulated, there comes a point when membrane conductance suddenly increases as ClC-1 channels are activated (Pedersen et al., 2009). The increase seems to be related to metabolic stress because the occurrence *i)* is accelerated under glucose-free conditions, *ii)* occurs sooner as the frequency of contractions increases, *iii)* is not observed in more oxidative, fatigue resistant, soleus muscles and, *iv)* ClC-1 channels have been shown to be activated under metabolic stress and to be ATP sensitive (Pedersen et al., 2016, 2009, Bennetts et al., 2007, Gong et al., 2003). The increase in Cl^- influx resulting from ClC-1 channel opening counters depolarization during action potentials leading to a decrease in membrane excitability.

The ATP-sensitive potassium channel (K_{ATP} channel) is a second channel that is ATP-dependent for which a decrease in ATP causes channel opening. Once open the channel allows K^+ efflux which acts as a leak conductance, counteracting Na^+ influx during the depolarization phase of an AP, decreasing AP amplitude and force generation (Gong et al., 2003, Cairns & Lindinger, 2008). In addition, in the absence of K_{ATP} activity, either via pharmaceutical intervention or in KO models, continuous stimulation leads to fiber damage (Thabet et al., 2005, Gong et al., 2003, Cifelli et al., 2007) and metabolic dysfunction (Scott et al., 2016), suggesting that they are involved in myoprotection, a hallmark of muscle fatigue.

Although the evidence is quickly accumulating to support a central role for membrane excitability, decreased AP amplitude, and decreased Ca^{2+} release from the SR in the etiology of muscle fatigue, what initiates this process remains largely unknown. The relationship between ClC-1 and K_{ATP} channel activation and the energy status of a given muscle fiber does however appear to be integral to the biological process and offers a parsimonious explanation for how declining energy reserves lead to decreases in muscle force and myoprotection. As mentioned, ATP levels themselves do not

decrease to such a point where they effect force production. It is however possible that the intracellular storage of ATP producing substrate, glycogen, may be central in decoding the energy status of muscle and translating it to muscle fatigue.

GLYCOGEN: MORE THAN JUST GLUCOSE STORAGE

The association between skeletal muscle glycogen content and fatigue is well established and has been known for decades (Bergström et al., 1967, Hermansen et al., 1967, Chin & Allen, 1997, Ørtenblad et al., 2013). However, only recently has the focus been shifted toward the molecular mechanisms involved in this association. Emerging concepts combining both early studies and more recent work implicate glycogen as a central component to muscle fatigue.

<u>The glycogen molecule</u>

Glycogen is an optimized and efficient polysaccharide granule composed of glucose subunits. Its synthesis begins with glucose which is converted to G-6-P by hexokinase once it enters a muscle fiber. G-6-P is converted to G-1-P by phosphoglucomutase and G-1-P to a glycosyl unit, UDP-glucose, by UDP-glucose pyrophosphorylase. UDP-glucose is then directed towards the autoglycosylating, homodimeric protein, glycogenin. The enzyme acts as both a substrate and enzyme, catalyzing the addition of UDP-glucose monomers to itself at a specific tyrosine residue creating a chain connected by α-1,4-glycosidic linkages. Once the nascent chain begins to grow, glycogen synthase (GS) takes over from the autoglycosylating enzyme, catalyzing the addition of further UDP-glucose subunits. Every 10 – 14 residues, glycogen branching enzyme catalyzes the transfer of 7 glycosyl units to a deeper residue, creating a branch via an α-1,6-glycosidic linkage. This branching is done in a highly ordered manner, creating 'tiers' consisting of what are termed A- and B- branches. Because one B-branch always gives rise to two A-branches, glucose storage increases exponentially with each new

tier. Maximum size is predicted to be 12 tiers (or 42 nm) due to steric hindrance, however granules typically reach 6 – 9 tiers under physiological conditions (Meléndez-Hevia et al., 1993). Due to the ordered and progressive branching, a 12 tier glycogen molecule can be predicted to have approximately 55 000 glycosyl units, with 34.6% (or 19 000) being in the outermost tier, 2100 of which can be estimated to have non-reducing ends (i.e. the site at which glycogen breakdown is initiated) (Meléndez-Hevia et al., 1993). The branched configuration and high quantity of non-reducing ends are crucial in allowing for very rapid mobilization of glucose where and when it is needed.

Glycogen granules are not uniformly distributed throughout a given muscle fiber but are localized primarily in 3 distinct subcellular locations (Nielsen et al., 2011). Most glycogen granules have been found to be stored between myofibrils (repeated sarcomere units), located in close proximity to the t-tubules and the SR and are termed intermyofibrillar (IMF). The remainder are stored in the subsarcolemmal region (SS) and, to a lesser extent, within the myofibrils themselves (Intra). Interestingly, these regions share the microenvironments with sites of high ATP consumption during contraction mentioned above; IMF and SS with the Ca^{2}-ATPase and Na^{+}/K^{+}-ATPase; and Intra with the myosin-ATPase.

Glycogen breakdown occurs when an activated glycogen phosphorylase enzyme (GP), acting on a non-reducing end, cleaves the α-1,4-glycosidic bond by substitution of a phosphoryl group, releasing G-1-P. GP acts repeatedly until it reaches a glycosyl unit 4 residues before a branching point. Glycogen debranching enzyme then catalyzes the transfer of three glycosyl units to a neighboring branch, exposing the branching point. The residue at the branching point is cleaved by α-1,6-glucosidase, releasing glucose as opposed to G-1-P (Jensen & Richter, 2012).

Regulation of this process of storing and liberating glucose is multifactorial and will be discussed only briefly. Reactions catalyzed by both GS and GP are the rate-limiting step in their respective role in glycogen metabolism and are thus crucial points for regulation of glycogen granules (Jensen & Richter, 2012). Situations favoring synthesis are during low metabolic demand and high availability of glucose. As such, whereas insulin and G-6-P activate GS, AMP inhibits it. GS is also regulated by various kinases, particularly GS kinase 3. GP is regulated in a reciprocal manner. Situations favoring breakdown are during or post exercise where metabolic demand is high and glucose availability is low. G-6-P and insulin inhibit GP, whereas AMP activates it. In addition, both enzymes are regulated by glycogen content itself, with GS being down-regulated with high content and GP being down-regulated with low content (Jensen & Richter, 2012). This prevents too great of granule size causing steric hindrance and over-depletion respectively.

Glycogen and skeletal muscle fatigue

The correlation between muscle glycogen content and muscle fatigue was first described over forty years ago in a set of experiments by Hermansen et al., (1967) and Bergström et al., (1967). Bergström and colleague used the biopsy technique along with dietary manipulation and found a strong correlation between the amount of glycogen at the onset of fatiguing exercise and time to fatigue. In the early study conducted by Hermansen and colleagues, trained and untrained individuals biked to complete exhaustion at 75% VO_2 max, either intermittently or continuously. Biopsies taken after the exercise revealed that glycogen content was not different between all conditions at the time of exhaustion. It was suggested that this could explain the phenomenon of 'hitting the wall' experienced by some marathon runners and suggests a critical limit of glycogen content. In a more recent study (Chin & Allen, 1997), mouse flexor digitorum brevis (FDB) muscle bundles and single

fibers were subjected to a fatigue protocol in which a first bout of fatigue was followed by a recovery period in the absence of glucose, and a second fatigue bout. Muscle glycogen content at the prior to the second fatigue bout was significantly less in muscle recovering in the absence of glucose. The lowered glycogen content coincided with both a more rapid decline in force generation and tetanic $[Ca^{2+}]_i$ during the second fatigue bout when compared to fibers that had recovered in the presence of glucose. Similar studies using cane toad muscle fibers (Stephenson et al., 1999) and mouse EDL muscle bundles (Pederson et al., 2009) have yielded similar results. These results have also been confirmed in humans, using a 24 hour fast to lower glycogen content and have been extended to include subcellular localization of glycogen (Ørtenblad et al., 2011). Biopsies taken before and after a 20 km cross-country ski time trial were analyzed using electron microscopy and it was determined that Intra glycogen store were preferentially depleted and more closely correlated with fatigue when compared to IMF and SS stores (Nielsen et al., 2010). The mechanism by which glycogen effects muscle fatigue, however, remains elusive.

One hypothesis is that the lack of glycogen leads to an energy deficiency. As discussed, ATP levels do not correlate with muscle fatigue, however due to difficulties with technique, these measurements represent only whole fiber ATP levels and do not consider intracellular microenvironments. During exercise, as glycogen stores are depleted in these microenvironments, it is possible that readily available ATP therein is insufficient to power the ATPases responsible for maintaining muscle contraction. In addition, evidence shows that membrane channels rely on glycolysis for energy (Dhar-Chowdhury et al., 2007, Hong et al., 2011, Hüser et al., 2000). This, and evidence of glycogen subcellular localization, could provide a concrete link between glycogen levels and membrane excitability, as both K_{ATP} and ClC-1 channels, as well as Na^+/K^+ ATP-ases, are known

to be reliant on ATP for their regulation. Thus, as glycogen is depleted, energy reserves within these IMF, SS, and Intra compartments are depleted, and membrane excitability is decreased via the activation of K_{ATP} and ClC-1 channels and a decrease in Na^+/K^+-ATPase activity. There is also evidence that K_{ATP} channels are positively regulated by AMPK, which itself is regulated by the presence of glycogen as its binding to branching points within glycogen granules inhibits the kinase (McBride et al., 2009). Thus, in addition to providing ATP to regulate membrane channels involved in membrane excitability, glycogen appears to also regulate kinases involved with channel regulation.

One way to test an energy deficiency hypothesis is to investigate the effects of carbohydrate supplementation during exercise. Keralis et al. (2010) conducted a meta-analysis of studies investigating carbohydrate supplementation in 1117 subjects and reported an average 40% increase in performance during exercise when compared to controls. Although the underlying mechanisms are certainly multifactorial, this could be interpreted as a compensatory effect of extracellular glucose and support an energy deficiency hypothesis. However, it does not fully account for decreases in performance resulting from glycogen depletion (which can reach up to 80% declines in muscle force (Chin & Allen, 1997, Allen et al., 2008)), suggesting either *a)* diffusion of glucose into the fiber is not sufficiently efficient to penetrate deeply into muscle fibers or *b)* the role of glucose and glycogen are, at least in part, distinct during fatigue. Interestingly, it has been reported that the presence of glycogen itself is a determining factor in the amount a glucose that is transported into the fiber (Hargreaves, 2010). In studies investigating glucose uptake in muscles with low glycogen content an increased glucose uptake was observed which the authors attributed to a decreased intracellular G-6-P (effectively increasing the concentration gradient and driving force on glucose) (Lai et al., 1985).

Therefore, regardless, any role of extracellular glucose in muscle fatigue is, at least in part, dependent on glycogen content.

Another important consideration is that glycogen may influence $[Ca^{2+}]_i$ homeostasis. Evidence has shown that glycolytic complexes associate with isolated SR membranes, and link glycolysis to SERCA activity (Kockskämper et al., 2005, Xu & Becker, 2008). Furthermore, glycogen itself has been shown to associate with SR membrane fractions (Lees et al., 2001). Having both glycogen and glycolytic complexes co-localize directly with the SR suggest a coupling of energy provided by glycogen and SR function. In addition to this effect on Ca^{2+} reuptake, low glycogen content has been shown to correlate with decreased SR Ca^{2+} release.

In a study conducted by Chin & Allen (1997), rat FDB muscle were subjected to a fatigue bout, followed by a 60 min recovery period in either the presence or absence of glucose. After recovery In the presence of glucose, both glycogen and tetanic $[Ca^{2+}]_i$ recovered to 85 and 87% of their values prior to the fatigue bout, respectively. Conversely, after the 60 min recovery in the absence of glucose, a significant decrease in glycogen replenishment was observed which correlated with a decrease in tetanic $[Ca^{2+}]_i$, being only 25 and 57% of their values prior to fatigue. Thus, it appears that when glycogen does not recover fully, neither does SR Ca^{2+} release. A link is supported by a study on humans subjected to fatiguing exercise followed by a recovery in the presence or absence of CHO (Ørtenblad et al., 2011). Biopsies of muscles taken immediate after, and 4 hours after exercise show that SR vesicle Ca^{2+} release is impaired after fatiguing exercise and remains impaired throughout a 4-hour recovery period in the absence of carbohydrate (CHO). Free access to CHO during the recovery resulted in glycogen replenishment as well as recovery of SR Ca^{2+} release. The RyR channel, responsible for the large majority of the increase in $[Ca^{2+}]_i$ during contraction, has been shown to be

negatively regulated by active GP (Hirata et al, 2003, Quinlan et al., 2010, Lees et al., 2001) and activated by high ATP/AMP ratios (Laver et al., 2001). Evidence also exists of a SR-glycogen complex which contains proteins involved with glycogenolysis and resynthesis, glycolysis, and regulation of glycolysis (Ørtenblad et al., 2011). Additionally, glycolytic sugar phosphate intermediates have been shown to increase open probability of RyR channels (Hüser et al., 2000, Kermode et al., 1998, Zima et al., 2006).

Taken together, these studies strongly suggest a close relationship between glycogen, glycolysis, and RyR activity, in which a decrease in glycogen would decrease glycolytic intermediates, the ATP/AMP ratio, and release GP from glycogen in the microenvironment of the triadic gap, leading to RyR inhibition. Further studies have also reported a decline in SR Ca^{2+} release closely associated with Intra glycogen content when compared to IMF and SS glycogen (Nielsen et al., 2014, 2009).

Although evidence supports that the role of glycogen content in the etiology of muscle fatigue can at least in part be explained by an energy deficiency hypothesis, it does not fully account for effects observed within muscles during fatigue. A link between membrane excitability, SR Ca^{2+} release and glycogen are supported by current literature and suggests a more complex mechanism through which glycogen effects muscle performance.

Current methods in the study of glycogen and fatigue

Previous studies on the importance of glycogen in muscle fatigue have been carried out by depleting glycogen either via extended fasting protocols or by fatiguing a muscle followed by a recovery period in the absence of extracellular glucose. Although effective at lowering glycogen content, extenuating factors resulting from the methodology may influence muscle performance.

Using fasting protocols can lead to whole body changes that can affect the onset of fatigue. In a study conducted by Williams et al., (2013), it was shown that although fasted rats exhibit significant decreases in voluntary treadmill exercise, direct stimulation of the motor nerves did not affect force production when compared to non-fasted rats. In another study, although glycogen associated with the SR was depleted in fasted rats, no change in SR cycling was observed (Batts et al., 2009); these changes are typically observed when glycogen is depleted using a previous bout of fatigue (Chin & Allen, 1997, Allen et al., 2008). Other studies even suggest a 'glycogen sparing' effect resulting from an increase in fatty acid utilization during exercise in fasted rats (Dohm et al., 1983). In humans, fasting before fatiguing exercise led to an increased rate of muscle fatigue with no glycogen sparing effect (Ørtenblad et al., 2011). The effects however could not be separated from extenuating factors resulting from a central fatigue component because force production during direct nerve stimulation was not tested. Taken together, it is apparent that evidence from studies using fasting protocols is conflicting.

A depletion of glycogen using a first fatigue bout to determine the effect of a lack of glycogen during a second fatigue bout is complicated by the fact that prior to the second fatigue bout, muscle have not fully recovered from the first bout; i.e. it is still in a metabolic stress mode. Briefly, increases in free radicals, changes in metabolites, effects of P_i, any damage incurred, and kinase activation are just some examples of extenuating factors that could be involved (Allen et al., 2008, Gong et al., 2003, Yoshida et al., 2012). In addition, many experiments have been conducted at 25°C, and at this temperature some proteins, like KATP channels, are not fully active during fatigue as discussed by Selvin & Renaud (2015).

Lastly, the usage of whole muscle bundles vs. single muscle fibers is of relevance to the research of glycogen and fatigue. Individuals fibers that make up muscle bundles are divided by fiber type based on their individual metabolic profiles as well as contractile speeds, as discussed above. Briefly, some fiber types are highly oxidative while others, glycolytic. Evidence from whole muscle is an average of the individual fiber types and thus may obscure some valuable insight at the level of the single fiber. Furthermore, whole muscle bundles *in vitro* have been shown to possess an anoxic core and concomitant restrictions on glucose diffusion (Sogaard et al., 2008, Henriksen & Holloszy, 1991, Barclay et al., 2005). In the study of the relationship between glycogen content and fatigue, fiber type and diffusional restriction are certainly important factors to keep in mind.

The first aim of this study was therefore to establish the effect of glycogen during fatigue at a physiological temperature while using a method that foregoes the confounding effects of fasting and previous fatigue bouts, lowering glycogen content in a more acute manner in single muscle fibers. This first aim was extended to distinguishing the effect of glycogen from that of extracellular glucose to address the possibility that the influence of glycogen on fatigue, in both single fibers and whole muscle bundles, is merely as a source of glucose.

FATIGUE PRE-CONDITIONING

The decline in force observed during fatigue can be attenuated with training over weeks. However, it can also be acutely attenuated by a previous bout of fatigue, a recently observed phenomenon known as 'fatigue pre-conditioning' (FPC); that is, the decrease in tetanic force and $[Ca^{2+}]_i$ during a second fatigue bout (FAT2) is slower than during a first fatigue bout (FAT1). FPC is observed if FAT2 is elicited 30 min after FAT1 and lasts about 2 hours (Boudreault et al., 2010).

Furthermore, FPC leads to a change in the response of muscle to a lack of K_{ATP} activity during fatigue (Boudreault et al., 2010).

As discussed above, K_{ATP} channels are activated during metabolic insult and prevent 'contractile dysfunction', defined as any event from the generation of action potentials to the actin–myosin interaction that is depressed in a manner not associated with the normal process of fatigue or any metabolic stress, and that eventually incapacitate muscle from generating force (Gong et al., 2003, Cifelli et al., 2007, 2008, Thabet et al., 2005). These studies outline the importance of K_{ATP} channels in myoprotection during a metabolic stress such as fatigue. During FPC the lack of K_{ATP} channel activity does not cause any contractile dysfunction during FAT2. The mechanism underlying FPC is unknown, however it has been suggested that the attenuation of the loss of force can be attributed to an increased capacity to generate ATP, reducing the need for K_{ATP} activation. Perhaps an increase in glucose availability and oxidative capacity of mitochondria are part of the mechanism.

Glycogen depletion, along with its subsequent effects on metabolism and glucose uptake, occurring during an initial fatigue bout may have direct bearing on the increase in fatigue resistance observed during a subsequent fatigue bout. It is well documented that there is an increase in glucose transport as well as glycogen synthesis after glycogen depleting exercise (Hingst et al., 2018, Mamedova et al., 2003, Jensen & Richter, 2012, Garcia-Roves et al., 2003). In fact, studies have shown that glycogen replenishment following exercise can increase muscle glycogen content to greater levels than before exercise (Helander et al., 2002, Jensen & Richter, 2012, Hingst et al., 2018). The mechanism that allows for this supercompensation has been proposed to involve increased activation of GS and AMPK (Hingst et al., 2018). AMPK serves as a cellular energy regulator by monitoring nucleotide status within muscle; becoming activated with high AMP/ATP ratios. Its

activation is also inversely related to glycogen content, as binding of the kinase to glycogen inhibits its activity (McBride et al., 2009). Thus, as glycogen is depleted, and AMP/ATP ratios increase, AMPK is activated. Once activated AMPK increases GLUT-4 translocation, and, coupled with an increase in GS activity, glycogen is rapidly replenished. AMPK also induces a switch to fatty acid metabolism which spares carbohydrate usage for energy, creating an intracellular environment strongly favoring glycogen production. This effect has been reported to persist for more than 2 hours post-exercise, meaning glucose transport as well as glycogen content is expected to be higher prior to FAT2 (Hingst et al., 2018, Jensen & Richter, 2012, Sano et al., 2012). In consideration of these factors implicit to glycogen, it is an intriguing possibility that glycogen may play a central role to FPC.

It is therefore the second aim of this study to examine the effect of glycogen content on FPC. Similarly to the first aim, the second aim was extended to examine the distinction of the effects glycogen and extracellular glucose on FPC. Ultimately this aim and the preceding aim will provide baseline results necessarily to elaborate on the potential role of glycogen as a central component in the etiology of muscle fatigue.

CHAPTER 2

METHODS AND MATERIALS

ANIMALS AND MUSCLES

Experiments were carried out using the flexor digitorum brevis muscle from 2 -3-month-old, CD-1 mice (Charles River, Canada). All mice weighed 20–25 g, were fed ad libitum, and housed according to the guidelines of the Canadian Council for Animal Care (CCAC). The Animal Care Committee of the University of Ottawa approved all experimental procedures used in this study. Mice were anaesthetized with a single intraperitoneal injection of 2.2 mg ketamine, 0.44 mg xylazine, and 0.22 mg acepromazine per 10 g of body mass. They were then sacrificed by cervical dislocation. Flexor digitorum brevis muscles were excised from the hind paw. Bundles controlling the 4^{th} digit were isolated for force measurements as described by Cifelli et al. (2008) or the whole FDB was used to isolate single fibers to measure $[Ca^{2+}]_i$.

SINGLE FIBER PREPARATION

FDB muscles bundles were isolated by a method involving a collagenase digestion as optimized by Selvin et al. (2015). Briefly, bundles were incubated for 3h at 37°C in a minimal essential media with Earle's salts and L-glutamine (MEM, Gibco, Canada) containing 0.2% (w/v) type I collagenase (Worthington, USA), 10% (v/v) heat inactivated fetal bovine serum (FBS, Gibco, Canada), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Gibco, Canada). Following incubation, fibers were dispersed by gentle trituration in 3 ml of collagenase-free MEM.

100 µl aliquots from the 3 ml concentrated fiber-containing solution were placed on matrigel (VWR, Canada) pre-coated 12 mm circular glass coverslips (VWR, Canada). Fibers were allowed to settle onto the coverslips for 30 min at 37˚C. MEM was then added to completely cover each coverslip. Fibers were incubated for at least 30 min before loading them with Fura-2 as needed for $[Ca^{2+}]_i$ measurements.

PHYSIOLOGICAL SOLUTION

For force and $[Ca^{2+}]_i$ measurements FDB muscle bundles or single fibers were continuously immersed in a physiological saline solution. Control physiological solution contained (in mM): 118.5 NaCl, 4.7 KCl, 2.4 $CaCl_2$, 3.1 $MgCl_2$, 25 $NaHCO_3$, 2 NaH_2PO_4, and 5.5 D-glucose as well as 0.2% v/v fetal bovine serum (FBS). Insulin-containing solutions were prepared by adding the appropriate concentration of insulin (Humulin-R, USA) to a final activity of 300 µU/ml. All solutions used to superperfuse single fibers for Ca^{2+} measurements (including control) contained 0.1% (v/v) DMSO (Sigma, USA) This is because 0.1% DMSO (giving rise to 14 mM) is necessary during Fura-2 loading in MEM (see next section); and, if DMSO is not present in the physiological solutions then an osmotic shock occurs separating t-tubules from the surface membrane resulting in no contraction (Albadrani and Renaud, personnel communication). All solutions were continuously gassed with 95% O_2/5% CO_2 to keep pH at 7.4. Experimental temperature was 37°C for all experiments.

$[Ca^{2+}]_i$ MEASUREMENTS

Intracellular Ca^{2+} was measured using the fluorescent Ca^{2+} indicator Fura-2. Fibers were loaded with Fura-2 by incubating them 30 min at 37°C in culture medium containing Fura-2 AM (Molecular Probes, Canada) dissolved in DMSO, giving a final concentration of 5 µM Fura-2 AM and 0.1% (v/v) DMSO. After loading, the coverslip containing single FDB fibers was mounted into a 370 µl

chamber (model 128 RC-25, Warner Instruments, USA). Fibers were continuously superperfused with physiological solution at a rate of 5 ml/min. Experimental temperature of 37°C was controlled for by simultaneously heating the plate in which the chamber was mounted, and pre-heating the physiological solution, using a dual channel heater controller (model TC-344B, Warner Instruments, USA). As discussed by Selvin et al. (2015), the temperature was increased from 22°C to 37°C at a rate of 2°C every 100 sec to prevent loss of fiber contractility.

Fura-2 was alternatively excited at wavelengths of 340 and 380 nm using the IonOptix Dual Fluorescence system (U.S.A), and light emission was measured at 505 nm with a Hamamatsu photomultiplier tube (Japan). Filters were respectively as follows: 340 ± 12 nm, 380 ± 6 nm and 505 ± 6 nm. Fibers were viewed using a Zeiss Axo Observer A1 (Canada) at a magnification of 200x. Data acquisition was set at 200 Hz. $[Ca^{2+}]_i$ was calculated as previously described (Selvin et al., 2015) using the following equation:

$$[Ca^{2+}]_i = K_d \bullet \beta \bullet (R - R_{MIN})/(R_{MAX} - R)$$

where, R, the ratio of the fluorescence from 340 nm excitation over the fluorescence from 380 nm excitation; K_d, the dissociation constant of Fura-2 for Ca^{2+} (37°C: 224 nM); R_{MIN}, the minimum ratio measured at low $[Ca^{2+}]_i$, being 89 ± 0.7% (n =7 fibers) of R measured in resting fibers; R_{MAX}, the maximum ratio at saturating $[Ca^{2+}]_i$, being 126.1 ± 7.5% of R during a tetanic contraction; β, the fluorescence at 380 nm excitation of Ca^{2+}-free divided by Ca^{2+}-bound Fura-2, being 3.17 ± 0.72. Values for R_{MIN}, R_{MAX} and β were obtained as described by Selvin et al. (2015). Two parameters are reported in this study. The first parameter, unstimulated $[Ca^{2+}]_i$, is defined as the $[Ca^{2+}]_i$ when fibers are not stimulated and was determined by averaging the $[Ca^{2+}]_i$ during the 100 ms period preceding a

contraction. The second parameter, tetanic $[Ca^{2+}]_i$, is defined as the maximum $[Ca^{2+}]_i$ observed during a tetanic contraction and determined by averaging the $[Ca^{2+}]_i$ during the tetanic plateau phase.

FORCE MEASUREMENT

Muscles bundles were mounted horizontally into a chamber and attached to a force transducer at one end (Model #400A, Aurora Scientific, Canada), and to a stationary hook at the other end. Physiological solution entered the chamber just below and above the muscle at a constant rate of 15 ml/min. Force transducers were connected to a KCP13104 data acquisition system (Keithley, USA) and data sampling was set at 5 kHz. Parameters of the contraction were later obtained with a computer analysis program. Unstimulated force was termed to be consistent with the term for $[Ca^{2+}]_i$ measurement, and is defined as the tension when muscles were not stimulated. Peak force was defined as the force generated as a result of electrical stimulation and was calculated as the difference between maximum force during stimulation and baseline force measured 5 msec before stimulation.

STIMULATION AND FATIGUE PROTOCOL

Muscle bundle and single fibers were stimulated by field stimulation generated by two platinum electrodes. For force measurements the platinum electrodes were positioned 6 mm apart, located on opposite sides of the muscle bundle. For Ca^{2+} measurements platinum electrodes were running along each side of the chamber containing the fibers. The electrodes were connected to a Grass S88 stimulator with isolation unit (Grass Technologies, West Warwich, RI, USA) which were used to generate 200 ms trains of 0.4 ms pulses at 10 V; unless indicated, the stimulation frequency was 140 Hz for $[Ca^{2+}]_i$ measurements and 200 Hz for force measurements.

Muscle bundles and single fibers were allowed to equilibrate for a period of 30 min prior to any fatiguing stimulation, during which they were stimulated once every 100 sec to ensure muscle stability. Following the equilibrium period, muscles were subjected to a 180 sec fatigue bout during which they were stimulated once every sec. In experiments investigating FPC, the first fatigue bout (FAT1) was followed by a recovery period during which muscles were stimulated once every 100 sec. The recovery period lasted 1 h for force measurements for muscle bundles and 30 min for Ca^{2+} measurements for single fibers. According to Boudreault et al., (2010) FPC was fully activated after a 30 min recovery, lasting at least 2 hrs. So, for Ca^{2+} measurements from single fibers the recovery period was 30 min to minimize Fura-2 loss which is significant at 37°C (Ciffeli et al., 2007). Muscle were then subjected to a subsequent second fatigue bout (FAT2) similar to FAT1.

GLYCOGEN DETERMINATION

Glycogen content was measured in both muscle bundles and single fibers. Muscle bundles were freeze clamped using metal clamps pre-cooled in liquid nitrogen prior to or after a fatigue bout. For single fibers, glycogen was determined after various times of incubation following trituration. One ml of fiber-rich collagenase-free suspension was centrifuged 10 sec at 600 g. Fibers from the pellet were washed in an ice-cold control physiological solution and centrifuged a 2^{nd} time. The pellet was flash frozen directly in the centrifuge tube by pressing it into a metal casing pre-cooled in liquid nitrogen with a metal rod pushed on the pellet, also pre-cooled in liquid nitrogen. Muscle bundles and fibers were stored at -80°C until glycogen determination. Muscle bundles and single fibers were freeze-dried overnight using a lyophilizer (Freezemobile 6, Virtis, USA). Lyopholized fibers from bundles were separated from tendons where single fibers were separated from crystalized ions before being weighed on a 6 decimal analytical balance (Mettler Toledo, XS105, USA).

Lyophilized muscle tissue was added to 66 µl of 1 N NaOH and incubated at 80°C for 15 min to degrade endogenous glucose. The NaOH solution was then diluted by adding 198 µl of double distilled H_2O. Sixty-six µl of the diluted solution was added to 132 µl of 0.15 M sodium acetate buffer. Glycogen was hydrolyzed to β-D-glucose by adding 6 µl of 260 U/ml amyloglucosidase (Sigma, USA) and incubated at 30°C for 2 hours. β-D-glucose was specifically oxidized to D-glucono-δ-lactone by the addition of glucose oxidase forming hydrogen peroxide in the process. Hydrogen peroxide, in the presence of horseradish peroxidase, reacts with 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) in a 1:1 stoichiometry to generate the highly fluorescent product resorufin which was measured with a plate reader (Perkin Elmer, Model LS50B, USA) using an excitation wavelength of 535 nm and measuring fluorescence at 590 nm. The enzymatic equations are as follows:

Glucose oxidase:

$$C_6H_{12}O_6 + O_2 \rightarrow C_6H_{10}O_6 + H_2O_2$$

Horseradish peroxidase:

$$H_2O_2 + C_{14}H_{11}NO_4 + 2O_2 \rightarrow C_{12}H_7NO_3 + 3H_2O + 2CO_2$$

STATISTICAL ANALYSIS

Data are presented as mean ± standard error (S.E.). ANOVA was used to determine significant differences. For glycogen results, 2-way ANOVAs were used. For force and Ca^{2+}, split plot designs were used because fibers were tested at all time levels. ANOVA calculations were made using the Version 9.3 GLM (General Linear Model) procedures of the Statistical Analysis Software (SAS Institute Inc., Cary, NC USA). When a main effect or an interaction was significant, the least square difference (L.S.D.) was used to locate the significant differences. The word “significant” refers only to a statistical difference ($P<0.05$).

CHAPTER 3

RESULTS

SINGLE MUSLE FIBER GLYCOGEN CONTENT

One goal of this study was to create a low glycogen model that neither relied on fasting or a previous bout of fatigue (here termed an 'acute' low glycogen model) using single FDB muscle fibers. The mean glycogen content of FDB muscle bundles flash frozen immediately following dissection was 95 µmole/g dry wt and is considered to be representative of normal physiological levels in resting muscle (Fig 3-1). After collagenase digestion and fiber separation by trituration, glycogen content in single fibers was significantly lowered, being 48 µmole/g dry wt. When single fibers were incubated for 1 hr, glycogen was replenished, reaching a near physiological level of 86 µmole/g dry wt. Interestingly, further incubation to 2 and 3 hrs resulted in glycogen content that significantly decreased to 47 and 60 µmole/g dry wt, respectively.

To circumvent the decrease in glycogen content after 2 hrs of incubation, insulin was included in the culture media post-trituration at an activity of 300 µU/ml; higher insulin activity did not result in more glycogen (data not shown). In the presence of insulin, glycogen content after a 1 hr incubation was not significantly different from the content measured under control conditions, i.e. in the absence of insulin. However, after 2 hrs of incubation, the decrease in glycogen content was less in the presence of insulin than under control conditions, being 69 vs. 47 µmole/g dry wt respectively.

For an acute low glycogen model, fibers were incubated in 0 mM glucose DMEM culture medium following trituration to prevent glycogen replenishment. After a 1, 2, and 3 hr incubation in

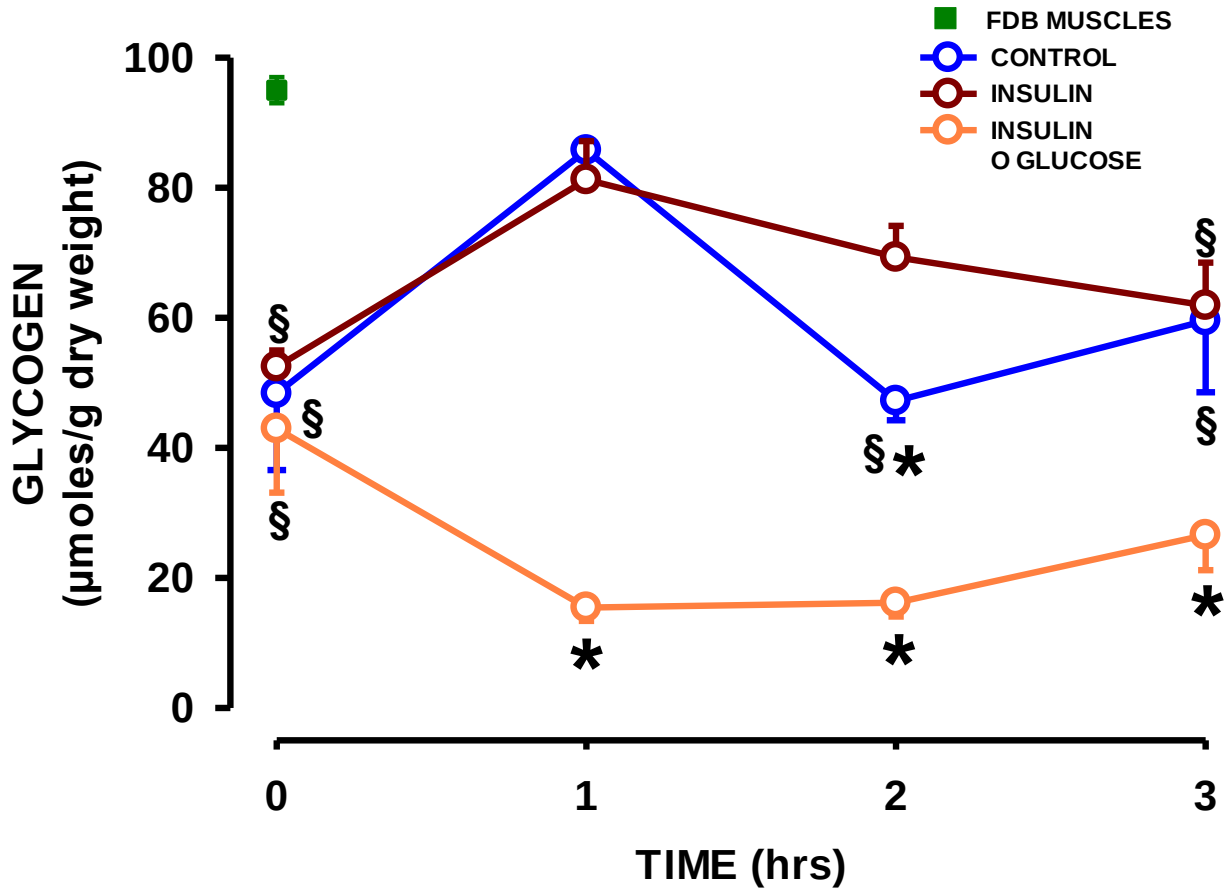

Figure 3-1. Insulin is necessary to maintain normal glycogen content while a lack of extracellular glucose depleted glycogen reserves in single FDB fibers. Glycogen was measured in FDB whole muscles immediately after dissection (■) and in single fibers at various time after incubation at 37°C in MEM supplemented with 10% FBS in the absence (○, CONTROL) or presence of 300 µU/ml insulin (○, 300 µU/ml insulin) or in glucose-free DMEM supplemented with 10% FBS and 300 µU/ml insulin (○, 300 µU/ml insulin – 0 glucose). Vertical bars represent the S.E. of 3-9 FDB preparations.

§ Mean glycogen content significantly different from the mean content at 1 hr;

* Mean glycogen content significantly different from the mean content for INSULIN;

ANOVA and L.S.D. $P < 0.05$.

0 mM glucose culture media, glycogen content was significantly decreased to 15, 16, and 27 μmole/g dry wt, respectively. So, for the experiments described below, FDB fibers were incubated in normal MEM or in 0 glucose DMEM media, both media containing 300 μU/ml insulin.

EFFECT OF CARBOHYDRATE AVAILABILITY ON $[Ca^{2+}]_i$ DURING FATIGUE

Carbohydrate availability during fatigue was manipulated as follows. Fibers incubated in MEM and insulin, when transferred to the Ca^{2+} measuring apparatus, were initially exposed to a physiological saline solution containing 5.5 mM glucose. Then, fibers were fatigued either in the presence of 5.5 mM glucose to represent condition #1, '5.5 glucose/high glycogen', or in the absence of glucose (removed 10 min prior to fatigue) to represent condition #2, '0 glucose/high glycogen'. Fibers incubated in 0 mM glucose DMEM and insulin were initially exposed to a 0 mM glucose physiological solution for Ca^{2+} measurements. Fibers were then fatigued in the presence of 5.5 mM glucose (added 10 min prior to fatigue) to represent condition #3, '5.5 glucose/low glycogen' or in the absence of glucose to represent condition #4, '0 glucose/low glycogen'. To verify whether insulin affects fatigue kinetics, fibers were tested in the absence or in the presence of 300 μU/ml insulin and all fatigue bouts were elicited 30 min after being transferred to the to the fiber chamber to measure Ca^{2+}. An original Ca^{2+} trace is presented in Fig. 3-2, showing changes in Fura-2 AM fluorescent ratio over the 180 sec fatigue period. Changes in the fluorescent ratio for individual tetanic contractions are also shown for the 1^{st}, 31^{st}, and 180^{th} contraction (Fig 3-2B, C, D, respectively). These individual traces show an initial increase in the plateau fluorescence ratio during contraction followed by a net decrease by the end of the fatigue period. A slight, but continuous increase in unstimulated fluorescence ratio was also observed.

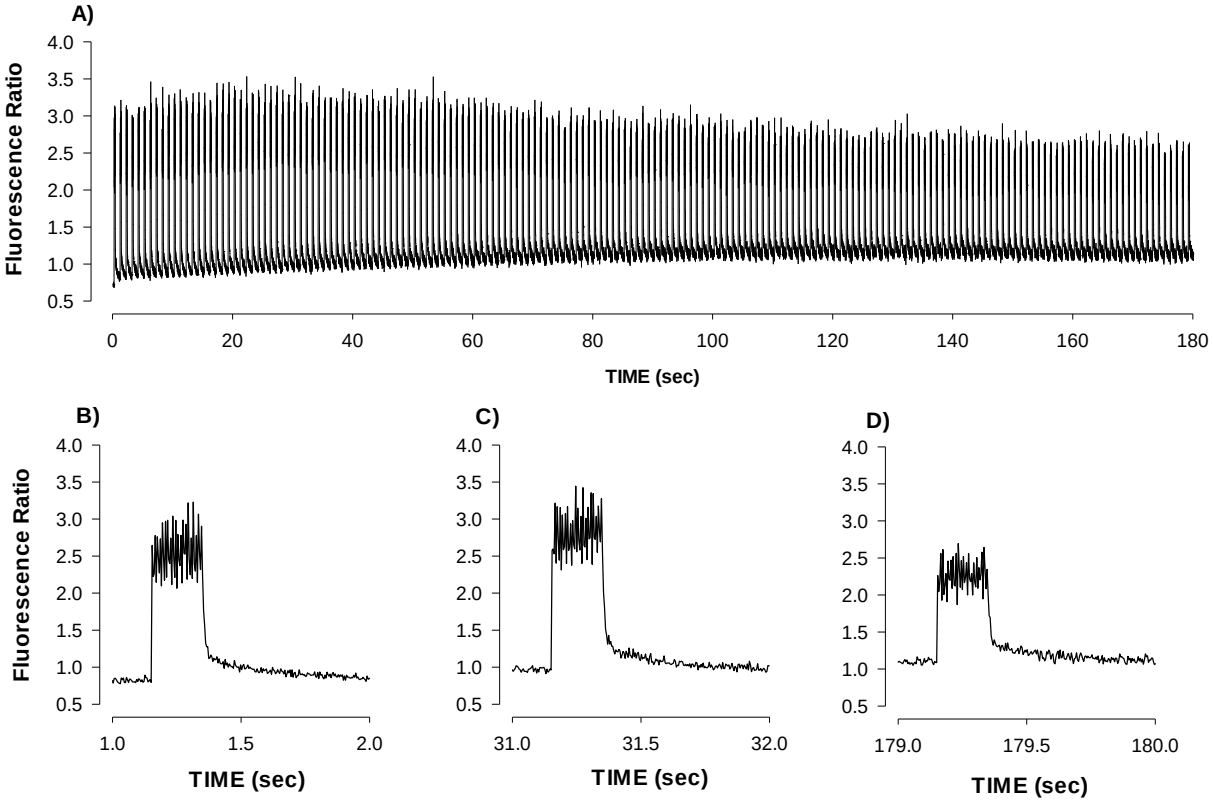

Figure 3-2. Example of an original record of 360/380 ratio signals obtained from a fatigue bout of a fiber loaded with fura-2 AM. Fibers were under 5.5 glucose/high glycogen conditions and in the absence of insulin. Fatigue was elicited with one contraction every sec for 3 min. (A) Fluorescence ratio signals from the entire fatigue bout, (B-D) Fluorescence ratio signals of individual traces from times indicated by the time scale.

For 5.5 glucose/high glycogen fibers, in the absence of insulin, mean tetanic $[Ca^{2+}]_i$ was 0.96 μM prior to fatigue and reached a maximum of 1.62 μM after 30 sec of fatiguing stimulation (Fig 3-3A). Mean tetanic $[Ca^{2+}]_i$ subsequently decreased, returning to the pre-fatigue levels by 120 sec, at which point it remained stable for the remainder of the fatigue bout. The addition of 300 μU/ml insulin did not significantly affect fatigue kinetics for fibers having high glycogen and exposed to 5.5 mM glucose. When glucose was removed from the physiological solution 10 min prior to fatigue (0 glucose/high glycogen), pre-fatigue tetanic $[Ca^{2+}]_i$ was 1.08 μM (Fig 3-3B), not significantly different from 5.5 glucose/high glycogen fibers (Fig 3-3A). It also did not affect the initial increase in tetanic $[Ca^{2+}]_i$ observed during the first 30 sec of stimulation. The subsequent decrease in tetanic $[Ca^{2+}]_i$ however was significantly altered as it steadily declined until the end of fatigue becoming significantly less than pre-fatigue levels after 150 sec.

In the presence of insulin, a depletion of glycogen while glucose was present did not affect fatigue kinetics. That is, the mean pre-fatigue, initial increase and subsequent decrease in tetanic $[Ca^{2+}]_i$ (Fig 3-3C) were similar to that observed with normal glycogen content (Fig 3-3A). Under the same conditions but in the absence of insulin, tetanic $[Ca^{2+}]_i$ was slightly higher throughout the majority of the fatigue bout when compared to normal glycogen conditions (Fig 3-3C).

When glycogen was depleted and fibers exposed to 0 mM glucose (Fig 3-3D), the pre-fatigue tetanic $[Ca^{2+}]_i$ was similar to 5.5 glucose/high glycogen conditions (Fig 3-3A). During the fatiguing stimulation in the absence of insulin, the initial increase and subsequent decrease in tetanic $[Ca^{2+}]_i$ (Fig 3-3D) was similar to that from fibers having high glycogen but no glucose (Fig 3-3B). In the presence of insulin, the decrease in tetanic $[Ca^{2+}]_i$ was actually delayed and slower than in the absence of insulin for and 0 glucose/low glycogen (Fig 3-3D).

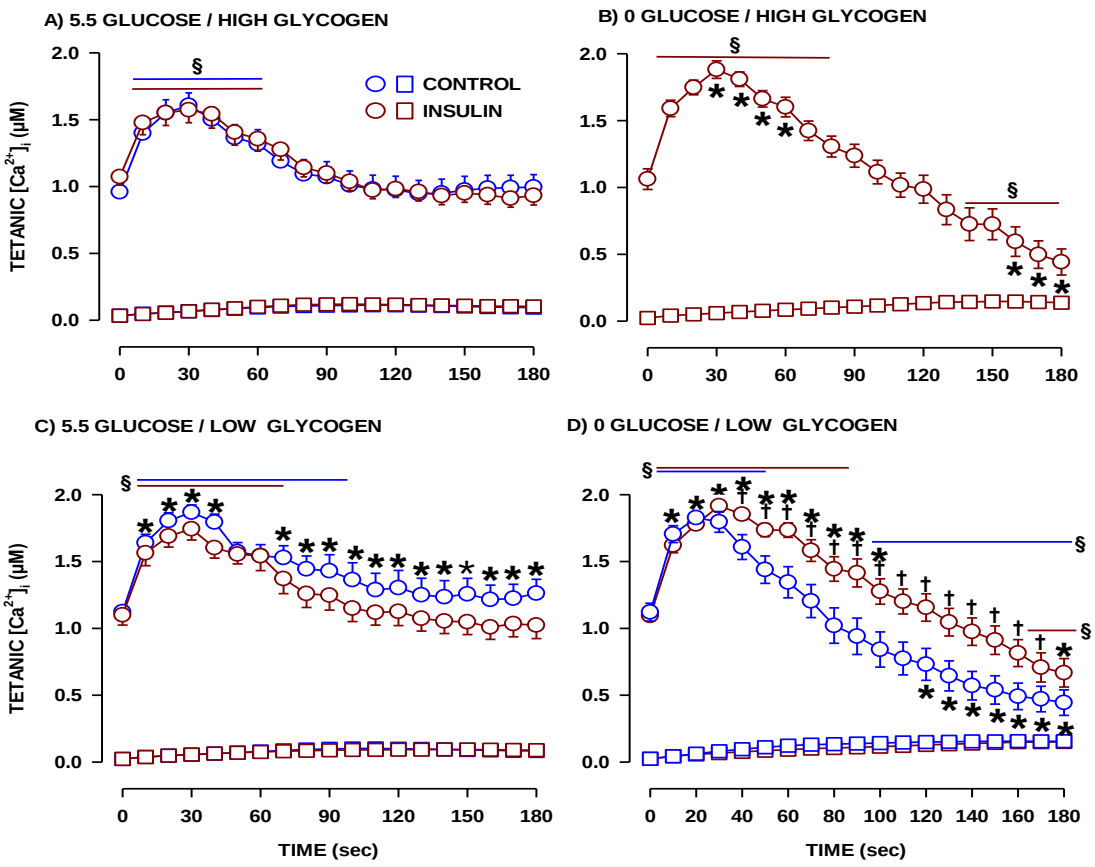

Figure 3-3. The lack of extracellular glucose (0 glucose) but not low glycogen content increased the rate of fatigue. Fatigue was elicited with one tetanic contraction every sec for 3 min and mean unstimulated (squares) and tetanic (circles) $[Ca^{2+}]_i$ are shown every 10 sec. Here, the unstimulated $[Ca^{2+}]_i$ is shown to clearly show how much $[Ca^{2+}]_i$ increased during contractions, being the difference between tetanic and unstimulated $[Ca^{2+}]_i$. The effects of carbohydrate availability on unstimulated $[Ca^{2+}]_i$ are shown in Fig 3-4 using a different scale. Vertical bars represent the S.E. of 12-15 fibers.

§ Mean tetanic $[Ca^{2+}]_i$ was significantly different from that of time 0 sec;

* Mean tetanic $[Ca^{2+}]_i$ was significantly different from mean tetanic $[Ca^{2+}]_i$ in Control or insulin conditions;

† Mean tetanic $[Ca^{2+}]_i$ significantly different from the mean in the absence of insulin;

ANOVA and L.S.D., $P < 0.05$.

Unstimulated $[Ca^{2+}]_i$ during fatigue

The unstimulated $[Ca^{2+}]_i$ represented the $[Ca^{2+}]_i$ while fibers were not being stimulated, being measured 100 msec prior to each contraction. Unstimulated $[Ca^{2+}]_i$ significantly increased as fatigue occurred in all conditions tested (Fig 3-4). In 5.5 glucose/high glycogen fibers, unstimulated $[Ca^{2+}]_i$ increased during the first 40 sec , changing very little thereafter; the increases were affected only minimally by insulin (Fig 3-4A). The same was observed with 5.5 glucose/low glycogen, except for a slightly lower unstimulated $[Ca^{2+}]_i$ between 70 and 120 sec in the presence of insulin (Fig 3-4C). When glucose was removed and with high glycogen, unstimulated $[Ca^{2+}]_i$ continuously increased until the 120^{th} sec, becoming significantly higher than in the presence of glucose (high glycogen) (Fig 3-4B). A similar situation was observed for 0 glucose/low glycogen (Fig 3-4D) when compared to 0 glucose/high glycogen (Fig3-4B). Interestingly, the presence of insulin caused slower increases in unstimulated $[Ca^{2+}]_i$ without an effect on the final level at 180 sec (Fig 3-4D).

Variability Among Individual Fibers

FDB fiber type composition includes the fatigue resistant type I fibers; intermediate fatigue resistant type IIA fibers; fatiguable type IIX fibers; as well as hybrid fibers containing 2 or 3 of the myosin type I, IIA, IIX. So, as expected, there is great variability in the fatigue kinetics among FDB fibers. This variability is best studied by measuring the extent of the change in tetanic $[Ca^{2+}]_i$ over the 180 sec of fatigue stimulation for individual fibers, as described by Selvin et al. (2015). Under 5.5 glucose/high glycogen conditions, some fibers had greater tetanic $[Ca^{2+}]_i$ at 180 sec compared to the pre-fatigue levels; that is, 8 fibers out of 15 (53%) in the absence of insulin and 1 out of 13 (8%) in the presence of insulin (Fig. 3-5A, B). Other fibers had a net decrease in tetanic $[Ca^{2+}]_i$, the decreases being less than 0.25 μM for 4 fibers (27%) in the absence of insulin and 9 fibers (69%) in the presence

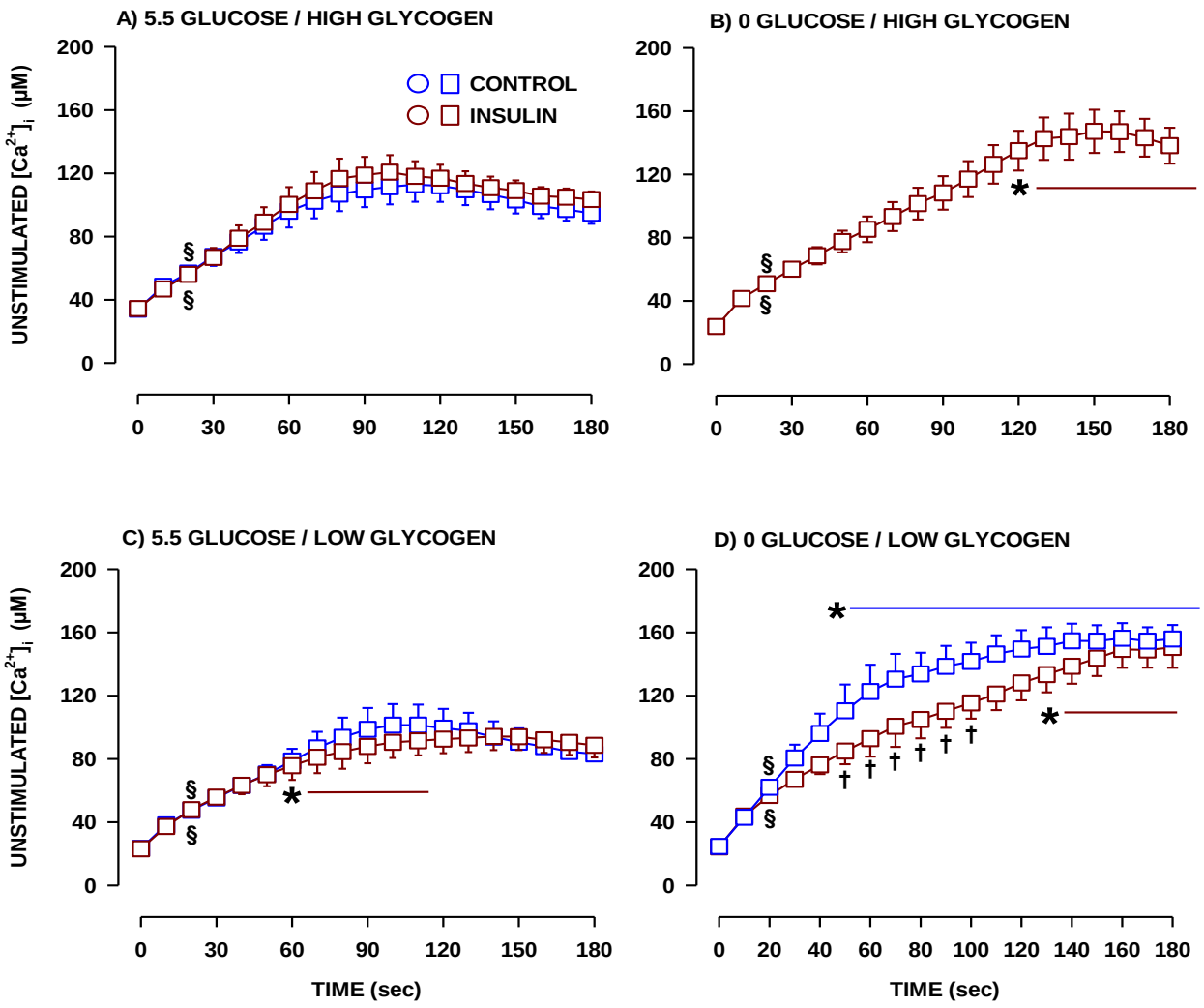

Figure 3-4. Removal of extracellular glucose but not low glycogen resulted in greater increases in unstimulated $[Ca^{2+}]_i$, an effect attenuated by insulin in 0 glucose/low glycogen fibers. Unstimulated $[Ca^{2+}]_i$ was obtained by averaging the $[Ca^{2+}]_i$ 100 msec prior to an elicited contraction, as defined by Selvin et al., (2015). Vertical bars represent the S.E. of 12-15 fibers.

§ Unstimulated $[Ca^{2+}]_i$ became significantly greater than the mean at time 0;

* Mean unstimulated $[Ca^{2+}]_i$ that were significantly different from the mean under 5.5 glucose and high glycogen;

† Mean unstimulated $[Ca^{2+}]_i$ significantly different from the mean in the absence of insulin;

ANOVA and L.S.D., $P < 0.05$.

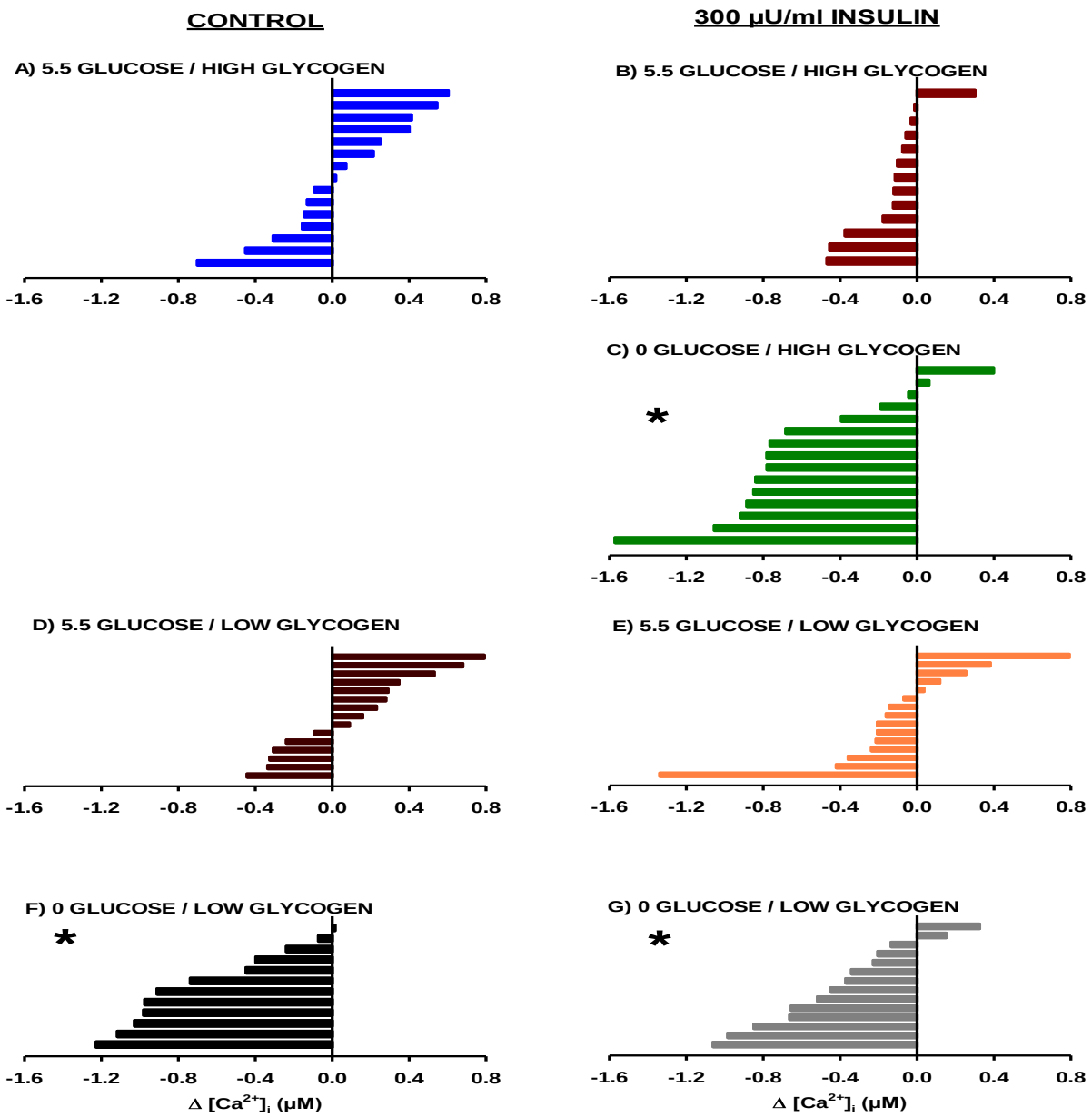

Figure 3-5. The absence of extracellular glucose but not low glycogen significantly increased the frequency of FDB fibers that had large decreases in tetanic $[Ca^{2+}]_i$ during fatigue. Changes in tetanic $[Ca^{2+}]_i$ were calculated as the difference in $[Ca^{2+}]_i$ between the first and the last contraction of the fatigue bout; a negative value signifies a net decrease in $[Ca^{2+}]_i$ during fatigue.

* Number of fibers that had decreases in tetanic $[Ca^{2+}]_i$ greater than 0.25 µM significantly different from 5.5 glucose/high glycogen with the same insulin concentration;

Fisher's exact t-test, 2 tailed. $P < 0.05$.

Only 3 fibers had decreases in tetanic $[Ca^{2+}]_i$ exceeding 0.25 μM, regardless of the presence of insulin. So, while on an average basis insulin appeared to have no effect on tetanic $[Ca^{2+}]_i$, major differences were observed among individual fibers.

The removal of glucose for fibers with high glycogen, in the presence of insulin, resulted in a drastic increase in the proportion of fibers with decreases in tetanic $[Ca^{2+}]_i$ exceeding 0.25 μM, increasing from 23% in the presence of glucose to 73% (11 of 15 fibers) in the absence of glucose (Fig. 3-5B, D). However, when glycogen was depleted, and glucose was present, the variability among fibers was not significantly different from 5.5 glucose/high glycogen fibers (Fig. 3-5D, E vs. A, B). When glycogen was depleted and 0 glucose was present, the number of fibers with decreases in tetanic $[Ca^{2+}]_i$ exceeding 0.2.5 μM were 64 and 75% of fibers tested in the presence and absence insulin respectively (Fig 3-5F, G), values that were, again, greater than in the presence of glucose.

EFFECT OF GLUCOSE AVAILABILITY ON FORCE DURING FATIGUE

Tetanic force cannot be measured in single FDB fibers as there is no tendon onto which a force transducer can be attached; thus here, small FDB muscle bundles were used. However, lowering glycogen content in these bundles could not be done without a previous fatigue bout or fasting, so, only the effect of extracellular glucose availability on tetanic force during fatigue was studied.

Decreases in tetanic force became significant within only 10 sec of fatiguing stimulation in all conditions tested (Fig. 3-6). The decrease in force continued until approximately 90 sec at which point tetanic force had dropped on average by 78%. Thereafter tetanic force stabilized until the end of the fatigue period. There were no significant differences between the presence and absence of insulin conditions, while a slight but not significantly greater decrease in tetanic force was observed between

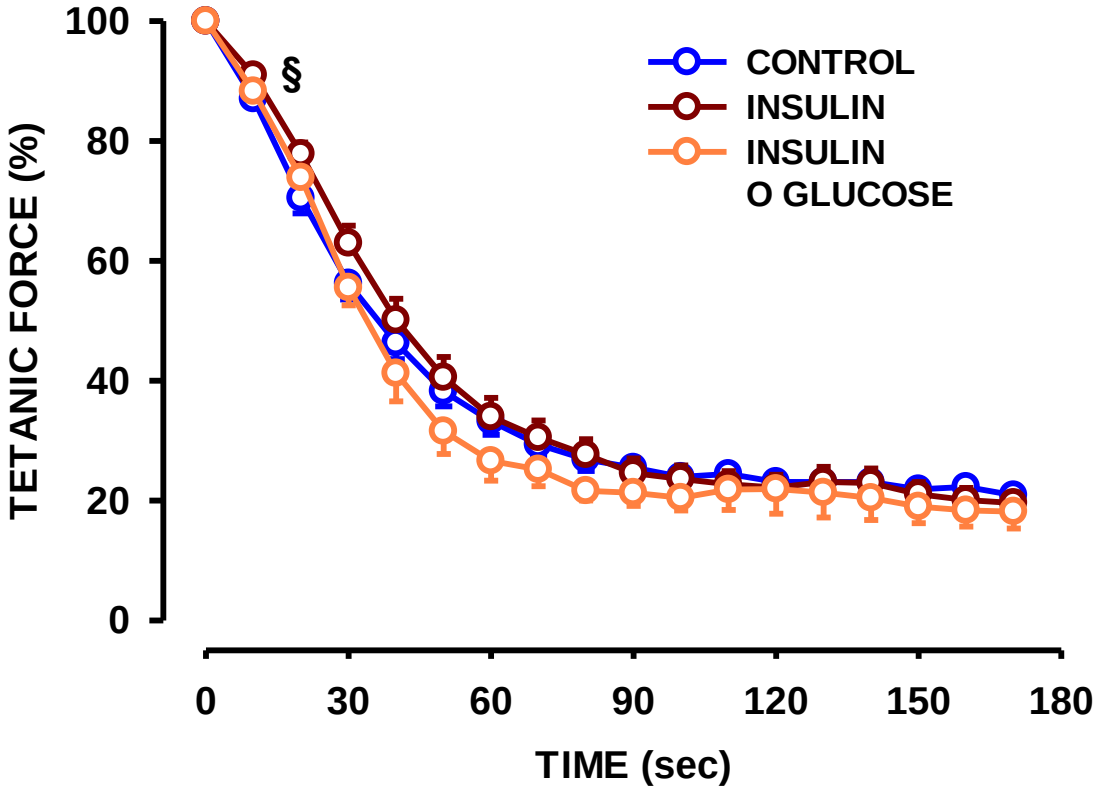

Figure 3-6. Removing glucose from the physiological solution did not significantly affect the decrease in tetanic force during fatigue when small FDB bundles were used. FDB bundles were exposed to physiological solutions containing 5.5 mM glucose in the absence (CONTROL) or in the presence of 300 µU/ml insulin or in glucose-free physiological (0 GLUCOSE) solution containing 300 µU/ml insulin. Tetanic force is expressed as a percent of the force prior to fatigue, which was elicited with one 200 msec contraction every sec for 3 min (data are shown at every 10 sec). Vertical bars represent the S.E. of 6 FDB bundles.

§ Tetanic force became significantly different from 100%. There was no significant differences between the three groups of FDB bundles;

ANOVA and L.S.D., $P < 0.05$.

30 & 90 sec in the absence of extracellular glucose. No differences in unstimulated force were observed between conditions.

EFFECTS OF CARBOHYDRATE AVAILABILITY ON FPC

As described in the introduction, FPC is a phenomenon in which a first fatigue bout (FAT1) increases resistance to fatigue using a second fatigue bout (FAT2), elicited 30 – 60 min after FAT1 (Boudreault et al., 2010). So, the aim here was to determine the role of carbohydrate availability on FPC, first with FDB bundles, then in single FDB fibers. Regardless of the condition tested during FAT2, all FAT1 were elicited, in the presence of 5.5 mM glucose with bundles or fibers having a high glycogen content. FAT1 and FAT2 were also elicited either in the absence or presence of 300 µU/ml insulin.

Glycogen content during FPC

Glycogen content was measured before and after both FAT1 and FAT2 to determine the extent of glycogen depletion during fatigue and replenishment during the recovery periods. In the absence of insulin and under 5.5 glucose/high glycogen conditions, glycogen content dropped from 95 µM/g dry wt prior to FAT1 to 32 µmoles/g dry wt at the end of FAT1 (Fig 3-7A), for a difference of 63 µmoles/g dry wt (Fig 3-7B). A similar decrease was observed when insulin was present, having 112 µmoles/g dry wt. at the beginning and 48 µmoles/g dry wt at the end of FAT1, for a difference of 64 µmoles/g dry wt. After a 60 min recovery in the presence of 5.5 glucose and immediately prior to FAT2, glycogen levels were significantly greater than the content prior to FAT1, without an effect from insulin. In the absence of insulin, it coincided with a significant increase in glycogen consumption from 63 µmoles/g dry wt during FAT1 to 109 µmoles/g dry wt during FAT2 (Fig 3-7B). Similar to the situation during FAT1, glycogen depletion was not affected by insulin during FAT2.

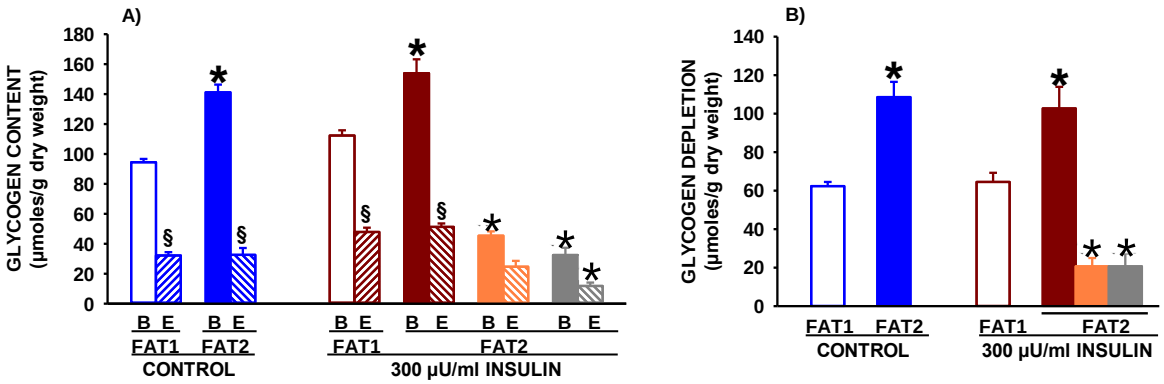

Figure 3-7. FDB bundles used more glycogen during FAT2 than during FAT1, except when the glycogen stores were not allowed to recover after FAT1. A) Glycogen content at the beginning (B) and end (E) of FAT1 or FAT2 in the absence (CONTROL) or in the presence of 300 µU/ml insulin. 5.5 glucose/low glycogen (orange bars) content during FAT2 was obtained by removing glucose immediately after FAT1 and reintroducing glucose 10 min prior to FAT2; 0 mM glucose/low glycogen (gray bars): glucose was not reintroduced prior to FAT2. Blue and red bars represent 5.5 glucose/high glycogen conditions. Vertical bars represent the S.E. of 4-7 FDB bundles.

§ Mean glycogen content at the end of fatigue significantly different from the mean content before fatigue.

ANOVA, $P < 0.05$.

Glycogen levels were not replenished after FAT1 when muscle bundles recovered in the absence of glucose; mean glycogen levels before FAT2 ranged between 33 and 45 µmoles/g dry wt (Fig. 3-7A). For one group of FDB bundles, glucose was reintroduced 10 min prior to FAT2, while for a second group FAT2 was elicited with no glucose. Under both conditions, glycogen breakdown during FAT2 was 20 µM/g dry wt. (Fig 3.7B). Although this accounted for approximately 40% of their pre-FAT2 glycogen content, it did not represent any significant breakdown of glycogen.

Tetanic Force during FPC

During recovery after FAT1, and prior to FAT2, tetanic force increased to 87 – 89% of it's values prior to FAT1 in the presence of 5.5 mM glucose. This incomplete recovery was observed regardless of the presence of insulin. When glucose was not present during recovery after FAT1, tetanic force recovered to, on average, 85% of its value prior to FAT1.

FDB muscle bundles under 5.5 glucose/high glycogen conditions had a significantly slower decline in tetanic force during the early phase of FAT2 in both the absence and presence of insulin (Fig. 3-8A, B). However, the final extent of the force decline was no longer significant after 80 sec in the absence of insulin or 50 sec in its presence.

When glucose was present during recovery but removed from the physiological solution 10 min prior to FAT2, the slower decline in tetanic force during FAT2 was significant only between 10 and 20 sec (Fig. 3-8C). Thereafter there was no difference in force between FAT1 and FAT2. Preventing replenishment of glycogen stores with 0 mM glucose during recovery but reintroduced 10 min prior to FAT2 completely abolished the difference in fatigue kinetics between FAT1 and FAT2 (Fig. 3-8D). Lastly, under low glycogen condition and in the total absence of glucose (recovery and

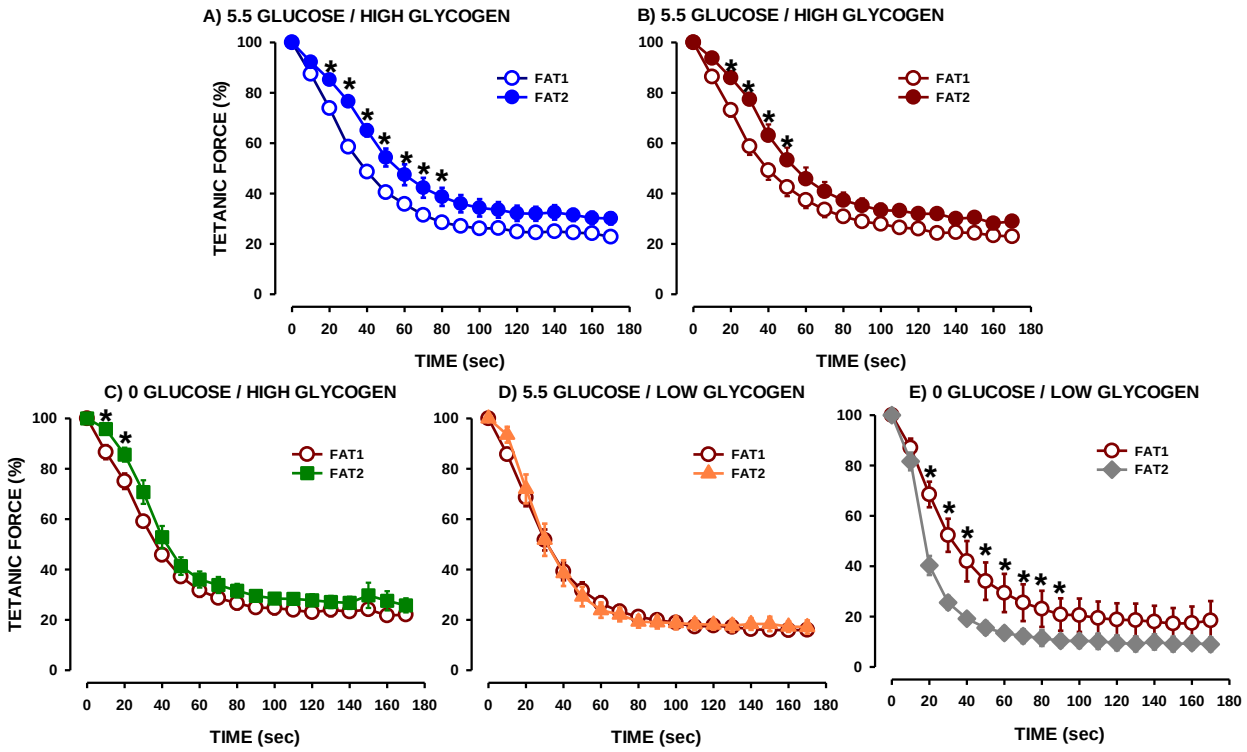

Figure 3-8. Compared to FAT1, the decrease in tetanic force was significantly slower during FAT2 for 5.5 glucose/high glycogen bundles in the absence (A) and presence of 300 µU/ml insulin (B), an effect diminished in 0 glucose/high glycogen bundles (C) or abolished in 5.5 glucose/low glycogen (D) and 0 glucose/low glycogen bundles (E). Except in (A), FDB were exposed to 300 µU/ml throughout the experiment (B-E). All fatigue bouts consisted of one tetanic contraction every sec for 3 min and data are shown at every 10 sec. For all FAT1, FDB were fatigued under the 5.5 glucose/high glycogen conditions. Recovery period (60 min) following FAT1 occurred while 5.5 mM glucose was present to replenish glycogen (high glycogen) (A-C) or in 0 mM glucose to prevent any increase in glycogen (low glycogen) (D, E). FAT2 was then elicited in the absence (C, E) or presence of glucose (A, B, D). Changes in glucose were made 10 min prior to FAT2 (i.e. its removal in C or reintroduction in D). Vertical bars represent the S.E. of 5 muscles (not shown when smaller than symbol).

* Mean tetanic force during FAT2 was significantly different from mean tetanic force during FAT1;

ANOVA L.S.D., $P < 0.05$.

FAT2), the decrease in tetanic force during FAT2 was significantly faster when compared to FAT1 (Fig 3.8E).

Unstimulated force during FPC

Although under 5.5 glucose/high glycogen conditions unstimulated forces were not significantly different between FAT1 and FAT2, it consistently increased during FAT2 but not FAT1 (Fig. 3-9A, B). The situation was the same for about 100 sec in 0 mM glucose/high glycogen before unstimulated force clearly increased after 145 sec (Fig 3-9C). There were significant increases in unstimulated force with low glycogen, when glucose was reintroduced 10 min prior to FAT2 (5.5 glucose/low glycogen bundles), becoming significant after 145 sec of fatiguing stimulation (Fig 3-9D). Lastly, 0 glucose/low glycogen bundles had the largest increases in unstimulated force during FAT2, beginning at 30 sec and continuing until the end of FAT2 (Fig 3-9E). The increases reached 11% of the pre-fatigue force in FAT2, compared to 2% in FAT1.

Tetanic and unstimulated $[Ca^{2+}]_i$ during FPC

FPC experiments described above were repeated using single FDB muscle fibers to measure changes in $[Ca^{2+}]_i$ during FAT1 and FAT2. The experimental protocol was the same except the recovery period was reduced to 30 min in order to decrease the loss of Fura-2 AM from fibers while still allowing enough time for FPC to occur. In 5.5 glucose/high glycogen fibers, tetanic $[Ca^{2+}]_i$ prior to FAT1 and prior to FAT2 was not significantly different. However, while an initial increase in tetanic $[Ca^{2+}]_i$ was similar in FAT1 and FAT2, the subsequent decrease was delayed during FAT2, beginning at 40 sec during FAT1 and 80 sec during FAT2. As a consequence, tetanic $[Ca^{2+}]_i$ between 50 and 140 sec was significantly higher during FAT2 when compared to FAT1 (Fig. 3.10A, B). Thus, on an average basis, fibers showed improved fatigue resistance during FAT2 when compared to FAT1 when they

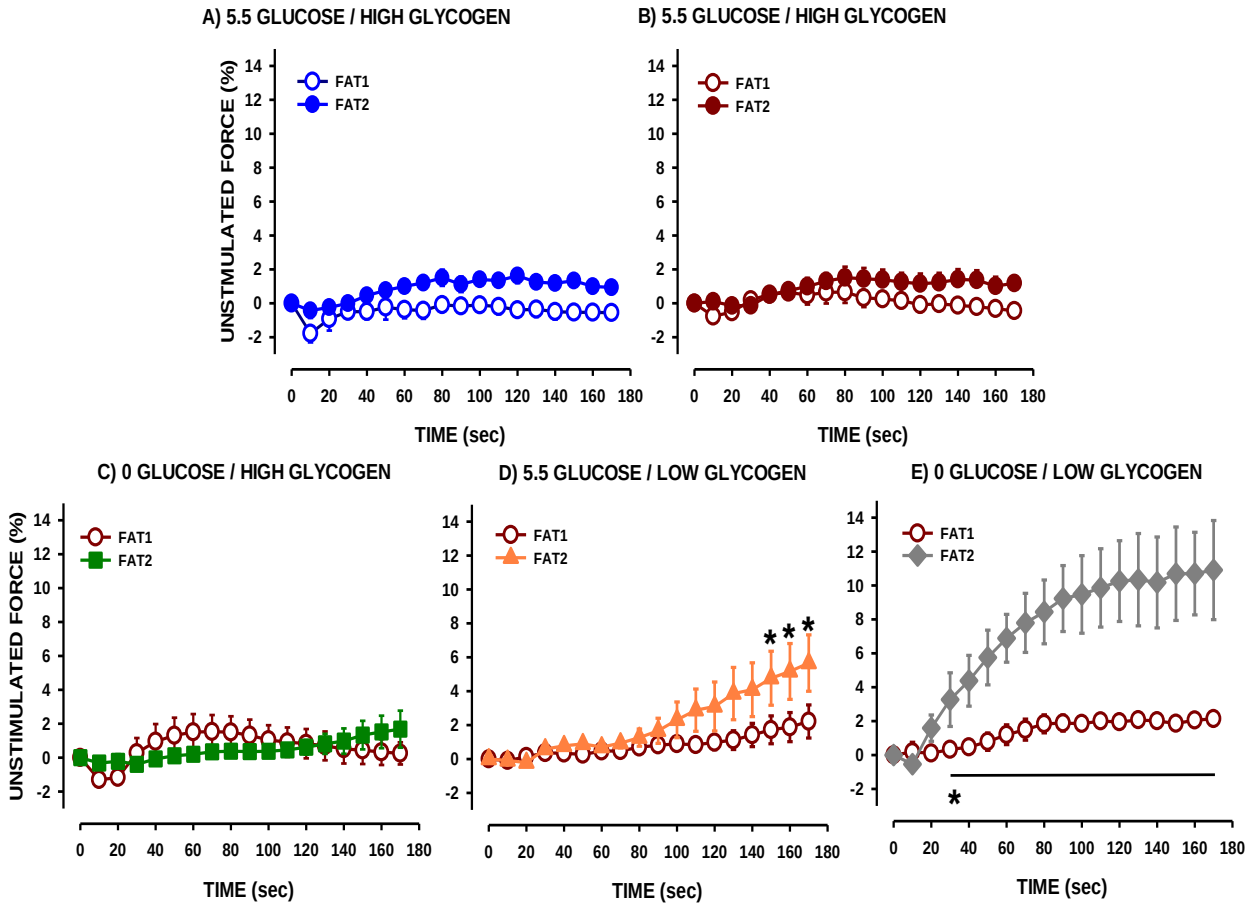

Figure 3-9. When compared to FAT1, the increase in unstimulated force during FAT2 was significantly greater only under low glycogen conditions. All conditions are all as described in Fig. 3-8. Vertical bars represent the S.E. of 5 muscles (not shown when smaller than symbol).

* Mean unstimulated force during FAT2 was significantly different from mean unstimulated force during FAT1.

ANOVA L.S.D., $P < 0.05$.

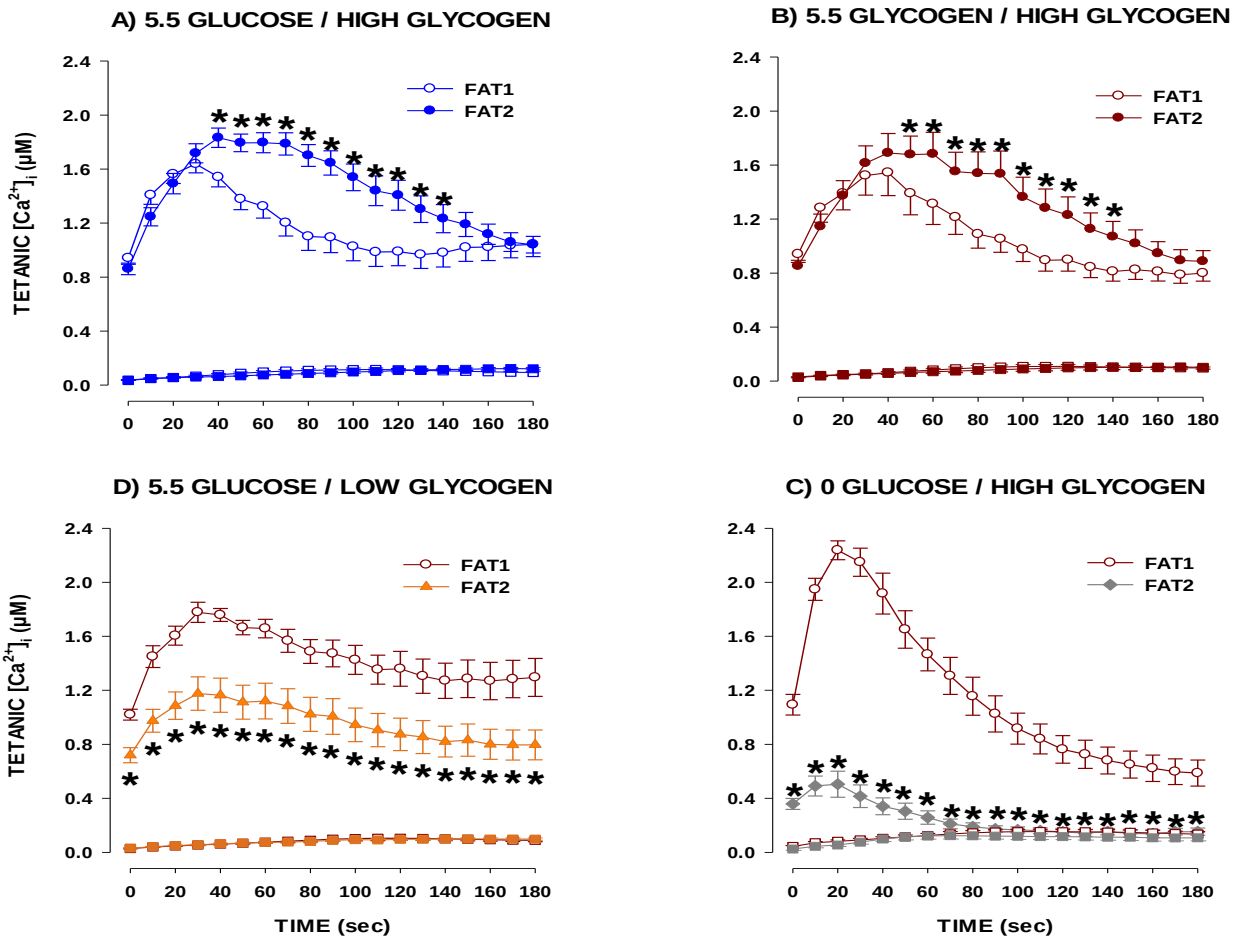

Figure 3-10. The decrease in tetanic $[Ca^{2+}]_i$ was significantly slower during FAT2 than during FAT1 for 5.5 glucose/high glycogen fibers in the absence (A) and presence (B) of 300 μU/ml insulin, whereas tetanic $[Ca^{2+}]_i$ was lower throughout FAT2 than in FAT1 in 5.5 glucose/low glycogen (C) and 0 mM glucose/low glycogen (D) fibers. Except in (A), FDB were exposed to 300 μU/ml throughout the experiment (B-D). All fatigue bouts consisted of one tetanic contraction every sec for 3 min and data are shown at every 10 sec. For all FAT1, FDB were fatigued under the 5.5 glucose/high glycogen conditions. Recovery period (30 min) following FAT1 occurred while 5.5 mM glucose was present to replenish glycogen (high glycogen) (A, B) or in 0 mM glucose to prevent any increase in glycogen (low glycogen) (C, D). FAT2 was then elicited in the absence (D) or presence of glucose (A-C). Glucose was reintroduced 10 min prior to FAT2 in (C). Square symbols (□, ■) represent unstimulated $[Ca^{2+}]_i$ and circles (○, ●) represent tetanic $[Ca^{2+}]_i$ with open symbol for FAT1 and closed symbols for FAT2. Both unstimulated and tetanic $[Ca^{2+}]_i$ are presented to clearly show how much

$[Ca^{2+}]_i$ increased during a tetanus. Vertical bars represent the S.E. of 7-13 fibers from 4-5 mice (not shown when smaller than symbol).

* Mean tetanic force during FAT2 was significantly different from mean tetanic force during FAT1;

ANOVA L.S.D., $P < 0.05$.

had 5.5 mM glucose and high glycogen. The effects were the same whether insulin was present or not (Fig 3-10A, B).

When fibers recovered after FAT1 in the absence of glucose to prevent glycogen replenishment, and 5.5 mM glucose was reintroduced 10 min before FAT2, tetanic $[Ca^{2+}]_i$ was significantly less prior to FAT1, being 0.72 μM, compared to prior to FAT1, being 1.02 μM (Fig 3-10C). Tetanic $[Ca^{2+}]_i$ remained significantly lower throughout all of FAT2 in glycogen depleted fibers (Fig 3-10C, D). The lack of tetanic $[Ca^{2+}]_i$ recovery was worse when under 0 glucose/low glycogen conditions during FAT2 as tetanic $[Ca^{2+}]_i$ prior to FAT2, was only 0.36 μM compared to 1.09 μM prior to FAT1 (Fig 3-10D). Furthermore, the initial increase in tetanic $[Ca^{2+}]_i$ was barely observable and by 80 sec (and for the remainder of the fatigue period) it had decreased to such an extent that it was not different from unstimulated $[Ca^{2+}]_i$ upon stimulation.

Unstimulated $[Ca^{2+}]_i$ became significantly greater than prior to FAT1 or FAT2 after 30 sec in all conditions tested except with 0 glucose/low glycogen during FAT2, which became significantly greater after 20 sec (Fig 3-11). Under 5.5 glucose/high glycogen conditions, unstimulated $[Ca^{2+}]_i$ was significantly lower starting at 50 and 70 sec in the absence and presence of insulin respectively, and continued until 120 sec (Fig 3-11A, B). This was not the case under low glycogen conditions as there were no differences in unstimulated $[Ca^{2+}]_i$ between FAT1 and FAT2 with low glycogen in the presence of 5.5 mM glucose (Fig 3-11C) and a significant increase in unstimulated $[Ca^{2+}]_i$ was present between 40 – 90 sec with low glycogen in the absence of glucose (Fig 3-11D).

Variability in fatigue kinetics between single muscle fibers

A total of 29 FDB fibers were used under the 5.5 glucose/high glycogen condition and, as shown in Fig. 3-12, there was a large variability in the difference between tetanic $[Ca^{2+}]_i$ at the

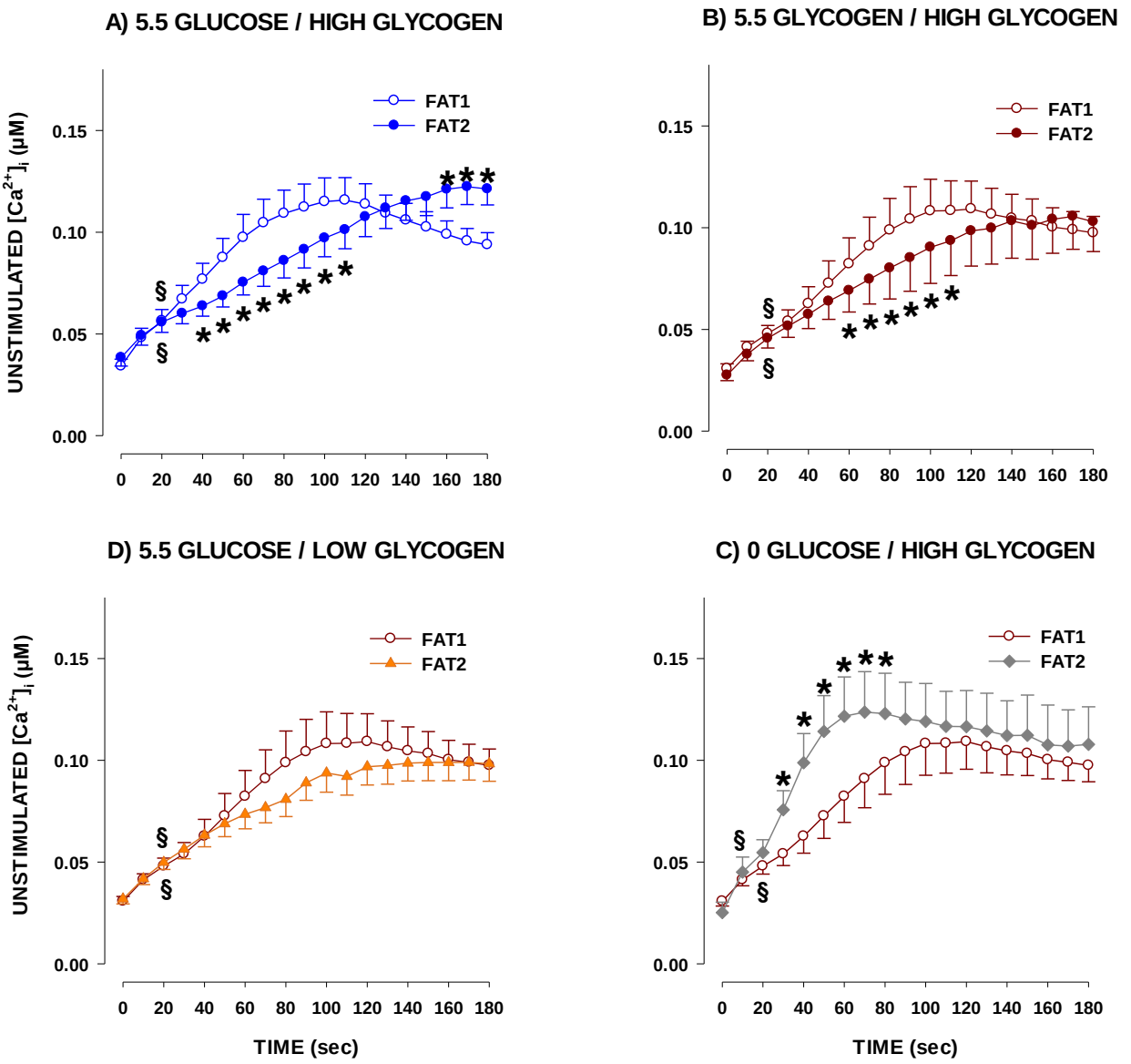

Figure 3-11. The increase in unstimulated $[Ca^{2+}]_i$ was slower during FAT2 than during FAT1 except under low glycogen conditions. All conditions are all as described in Fig. 3-10. Vertical bars represent the S.E. of 7-13 fibers from 4-5 mice (not shown when smaller than symbol).

§ Unstimulated $[Ca^{2+}]_i$ became significantly greater than the mean at time 0;

* Mean unstimulated $[Ca^{2+}]_i$ that were significantly different from the mean during FAT1;

ANOVA L.S.D., $P < 0.05$.

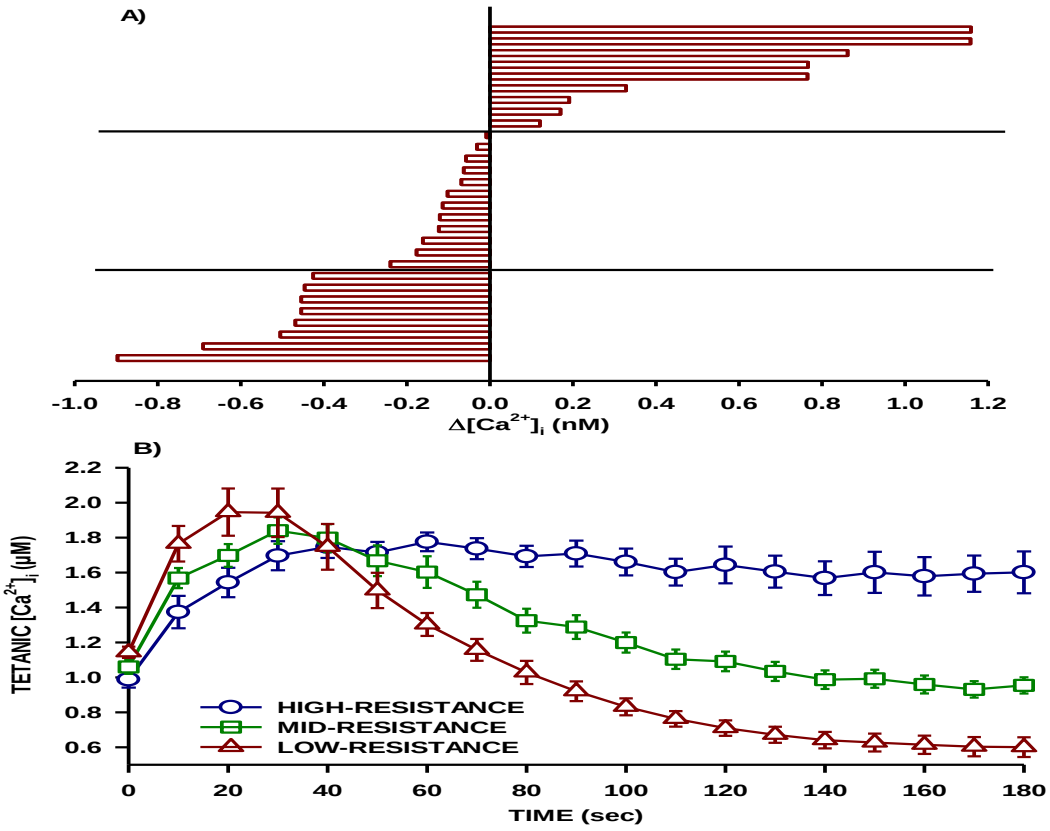

Figure 3-12. Variability in fatigue kinetics between single FDB fibers. A) The extent of the change in tetanic $[Ca^{2+}]_i$ for each individual fiber during FAT1 in the presence of 300 μU/ml insulin (taken from figure 3-10A-B). The change in $[Ca^{2+}]_i$ was calculated as the difference in $[Ca^{2+}]_i$ at time 0 and 180 sec with a negative value indicating a net decrease in tetanic $[Ca^{2+}]_i$. B) Mean tetanic $[Ca^{2+}]_i$ traces from three groupings of fibers: i) high-resistance: fatigue resistant fibers for which the tetanic $[Ca^{2+}]_i$ at 180 sec was still above the pre-fatigue level, *n* = 9; ii) Mid-resistance: fibers with mid-fatigue resistance were those for which the decrease in $[Ca^{2+}]_i$ did not exceed 0.25 μM, *n* = 12; and, iii) Low-resistance: fatigable or least fatigue resistant fibers for which the decreases exceeded 0.25 μM, *n* = 8. High-resistance fiber tetanic $[Ca^{2+}]_i$ was significantly different from mid-resistance and low-resistance tetanic $[Ca^{2+}]_i$ after 50 and 70 sec (respectively) until the end of the fatigue bout. Mid-resistance fiber tetanic $[Ca^{2+}]_i$ was significantly different from low-resistance from 60 to 180 sec. Tetanic $[Ca^{2+}]_i$ was significantly different from at 0 sec from 10 – 180 sec in high-resistance fibers; 5 – 90 sec in mid-resistance fibers; and 5 – 55 sec and 90 – 180 sec in low-resistance fibers. Vertical bars represent the S.E., ANOVA L.S.D., $P < 0.05$.

beginning and at the end of fatigue. For some fibers, tetanic $[Ca^{2+}]_i$ was greater at the end of FAT1 than at the beginning, the increases reaching up to 1.12 μM, while for other fibers there was a net decrease in tetanic $[Ca^{2+}]_i$, being up to 0.93 μM. Fibers were divided into three groups according to the extent to which tetanic $[Ca^{2+}]_i$ changed during fatigue, marked by the horizontal black lines in Fig. 3-12. High-resistance fibers were considered as those that had a net increase in tetanic $[Ca^{2+}]_i$ at 180 sec compared to pre-fatigue tetanic $[Ca^{2+}]_i$; mid-resistance fibers were those that had small decreases in tetanic $[Ca^{2+}]_i$ not exceeding 0.25 μM at 180 sec; and low-resistance fibers were those that had decreases in tetanic $[Ca^{2+}]_i$ exceeding 0.25 μM at 180 sec (Fig 3-12). On an average basis, there was no significant differences in tetanic $[Ca^{2+}]_i$ prior to fatigue between the groupings, as mean values ranged between 0.96 and 1.16 μM. After 180 sec of fatigue, high-resistance fibers had an average tetanic $[Ca^{2+}]_i$ of 1.7 μM, compared to 1.1 μM in mid-resistance fibers and 0.6 μM in low-resistance fibers. In addition, those fibers that had the greatest decreases in tetanic $[Ca^{2+}]_i$ during a given fatigue bout, had the most rapid declines in tetanic $[Ca^{2+}]_i$. While there was a great variability in how tetanic $[Ca^{2+}]_i$ changed during fatigue among individual fibers, there were a lot less differences in the kinetics of the tetanic $[Ca^{2+}]_i$ during fatigue within each individual fiber group (Appendix 1).

The same grouping criteria was used to divide fibers as either high-resistance, mid-resistance, or low-resistance from the kinetics for each of the glucose/glycogen conditions during FAT1. Fatigue kinetics between FAT1 and FAT2 were then compared within each of the three groups. In 5.5 glucose/high glycogen fibers, in the absence of insulin, the changes in tetanic $[Ca^{2+}]_i$ were the same between FAT1 and FAT2 in the high-resistance group (Fig 3-13A). In fibers that qualified as having mid-resistance on the other hand, there was a delayed decrease in tetanic $[Ca^{2+}]_i$ during FAT2 so that tetanic $[Ca^{2+}]_i$ during FAT2 became significantly greater between 50 and 130 sec when compared to

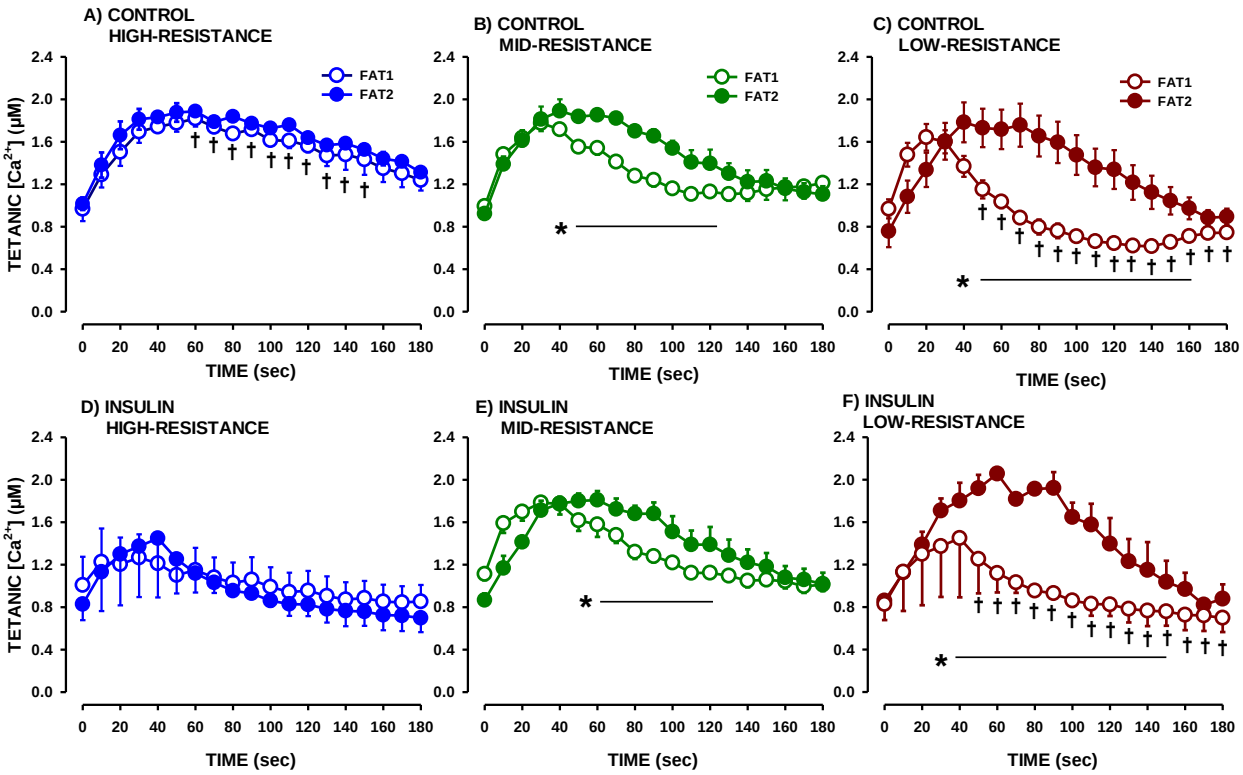

Figure 3-13. The extent of FPC was in the order of low-resistance > mid-resistance > high-resistant fibers in both in the absence (Control, A-C) or presence of 300 µU/ml insulin (D-F). FPC results subdivided based on FAT1 fiber groupings as described in Fig. 3-12. FAT1 and FAT2 were elicited while fibers had high glycogen and were exposed to 5.5 mM glucose throughout the experiment. Vertical bars represent the S.E. of 2 to 6 fibers.

* Mean tetanic $[Ca^{2+}]_i$ during FAT2 was significantly different from the mean value during FAT1.

† Mean tetanic $[Ca^{2+}]_i$ was significantly different from the mean value from mid-resistant fibers. ANOVA and L.S.D., $P < 0.05$.

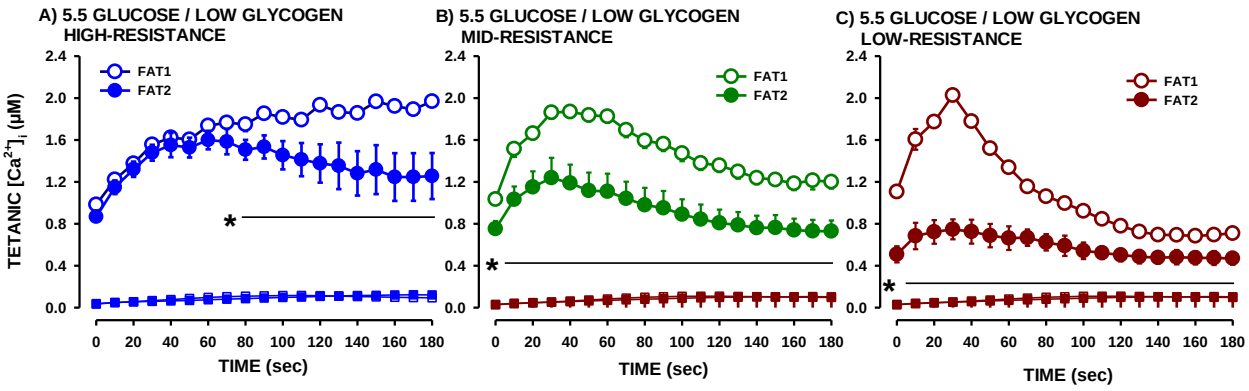

Figure 3-14. The extent of which low glycogen content reduced $[Ca^{2+}]_i$ during FAT2 was in the order of low-resistance > mid-resistance > high-resistant fibers. As described n Fig. 3-13 except after FAT1 fibers recovered for 30 min in the absence of extracellular glucose to prevent recovery of glycogen stores. Extracellular glucose was reintroduced 10 min prior to FAT2. Vertical bars represent the S.E. of 3 to 6 fibers.

* Mean tetanic $[Ca^{2+}]_i$ during FAT2 was significantly different from the mean value during FAT1. ANOVA and L.S.D., $P < 0.05$.

FAT1 (Fig 3-13B). For high-resistance fibers, that difference between FAT1 and FAT2 was even greater (Fig 3-13C). In the presence of insulin differences in fatigue kinetics between FAT1 and FAT2 in all three groups were the similar to those in the absence of insulin (Fig 3-13D-F).

Interestingly, unlike FAT1, there were no significant differences in the fatigue kinetics between the three groups for FAT2.In the 5.5 glucose/low glycogen condition, high-resistance fibers had similar increases in tetanic $[Ca^{2+}]_i$ during the first 60 sec between FAT1 and FAT2 (Fig 3-14A). Thereafter, tetanic $[Ca^{2+}]_i$ decreased during FAT2, but not during FAT1. Mid-resistance fibers did not fully recover tetanic $[Ca^{2+}]_i$ prior to FAT2, had a smaller initial increase in tetanic $[Ca^{2+}]_i$, and tetanic $[Ca^{2+}]_i$ remained lower throughout FAT2 when compared to high-resistance fibers (Fig 3-14B). In low-resistance fibers the recovery was the lowest of the groups, and initial as well as tetanic $[Ca^{2+}]_i$ throughout FAT2 was also decreased (Fig 3-14C). In the absence of glucose (0 glucose/low glycogen), a similar pattern was observed in that there was a decreased tetanic $[Ca^{2+}]_i$ at the onset of FAT2 when compared to FAT1 in mid-resistance and low-resistance fibers (no high-resistance fibers were found). The decrease persistent throughout the entire fatigue period, albeit to a greater extent in the respective groups in 0 glucose/low glycogen fibers when compared 5.5 glucose/low glycogen conditions (Fig. 3.15A-C).

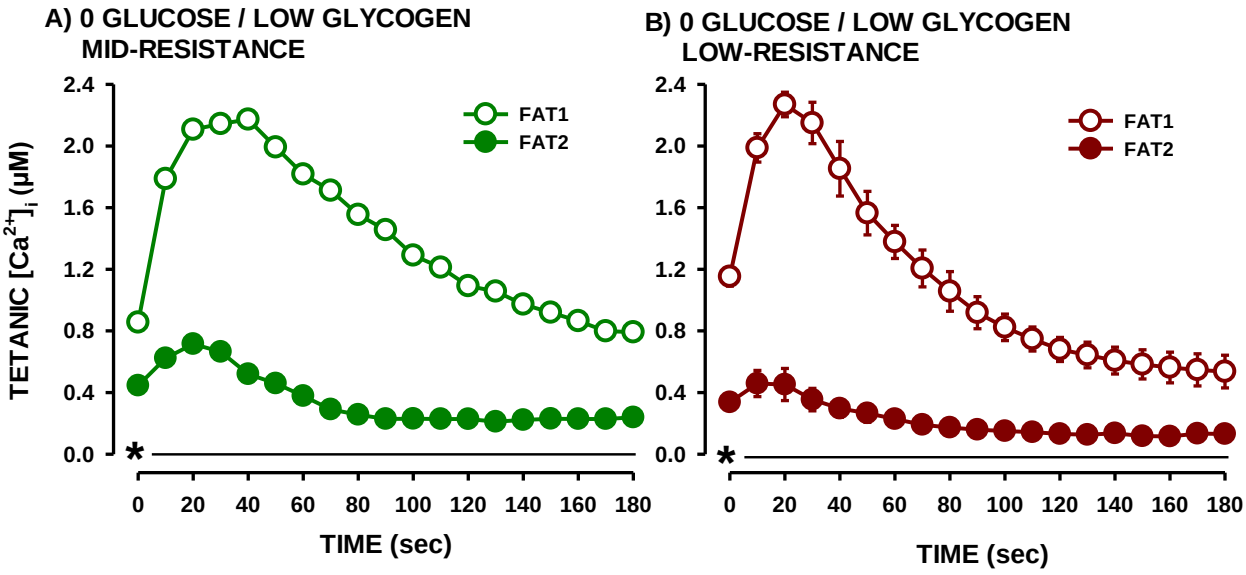

Figure 3-15. The extent of which low glycogen content reduced tetanic $[Ca^{2+}]_i$ during FAT2 was in the order of low-resistance > mid-resistant. As described in figure 3-14 except extracellular glucose remained absent during FAT2. Vertical bars represent the S.E. of 1 – 4 fibers.

* Mean tetanic $[Ca^{2+}]_i$ during FAT2 was significantly different from the mean value during FAT1. ANOVA and L.S.D., $P < 0.05$.

CHAPTER 4

DISCUSSION

The current study investigated the effects of carbohydrate availability on a single bout of fatigue (FAT1) and fatigue pre-conditioning (FAT1 vs. FAT2). These effects have been well studied in the past (Allen et al., 2008, Chin & Allen, 1997, Ørtenblad et al., 2011, Stephensen et al., 1999, Boudreault et al., 2010), however the current study has produced a novel method for depleting glycogen content within single muscle fibers prior to FAT1. This new method forgoes the need for fasting protocols and/or a prior bout of fatigue as well as addresses diffusional restrictions in whole muscle bundles. The addition of insulin was needed to optimize the methodology and thus the hormone's role in conjunction with that of glycogen and extracellular glucose during muscle fatigue and FPC was investigated.

The major findings can be summarized as follows: 1) glycogen content in single muscle fibers following a collagenase digestion and trituration was low compared to normal levels, and can be further lowered, and maintained at low levels, by incubating dispersed fibers in a 0 mM glucose culture medium. 2) There was no effect of low glycogen content on tetanic $[Ca^{2+}]_i$ during a single fatigue bout when using the current glycogen depletion methodology. 3) Whereas the absence of extracellular glucose had little effect tetanic force during a single bout of fatigue in whole muscle bundles, it resulted in a marked decrease in tetanic $[Ca^{2+}]_i$ in single fibers. 4) FPC was observed as slower decreases in tetanic force and $[Ca^{2+}]_i$ as well as increased glycogen usage. 5) FPC was greatest in fibers with a low resistance to fatigue during FAT1. 6) During FAT1, fibers could be divided into 3 groups based on the changes in tetanic $[Ca^{2+}]_i$; high-resistance, mid-resistance, and low-resistance.

Whereas during FAT2 all fibers appeared as high-resistance under 5.5 glucose/high glycogen conditions, this effect was abolished when carbohydrate availability was decreased. 7) Insulin had a minimal effect on muscle fatigue during an initial fatigue bout, FPC, and under low glycogen conditions in the presence of glucose. However, under low glycogen conditions in the absence of glucose, the presence of insulin increased tetanic $[Ca^{2+}]_i$ during fatigue.

GLYCOGEN CONTENT IN SINGLE MUSCLE FIBERS

The current study represents the first time a low glycogen model in collagenase-dispersed, single muscle fibers has been used to investigate the effect of low glycogen content during a single fatigue bout. However, the first major hurdle in producing a low glycogen model in single muscle fibers was to establish if, under normal conditions, glycogen content was similar to what is observed *in vivo*. FDB glycogen content when muscles are rapidly excised and, within 1 min, freeze-clamped, was 95 µmol/g dry wt, which agrees with other studies measuring glycogen in isolated muscle that report a content anywhere between 62 to 146 µmol/g dry wt (Scott et al., 2016, Helander et al., 2002, Lai et al., 2009, Spriet, 1990). Immediately following the trituration process, centrifugation, and freeze-clamping, single fiber glycogen content fell to half that measured following dissection. The reason for the decline in glycogen content cannot be determined from the present study. However, unpublished results (Cairns & Renaud) show that muscles contain large amounts of lactate and lower glycogen content when they are carefully excised for *in vitro* force measurements (a 25 – 30 min procedure). Glycogen content may have further decrease when FBD muscles were incubated at 37˚ with collagenase (prior to fiber dispersion). The most important factor was the capacity to replenish the glycogen levels within one hour of incubation in MEM once fibers had been dispersed, which was achieved, with levels replenished to 86 µmol/g dry wt.

Glycogen content of single fibers, did however decrease after the first hour of incubation. The decrease in glycogen content was attenuated with the inclusion of insulin in the culture media, which allowed for the maintenance of higher glycogen levels, most likely by increasing glucose transport, as insulin stimulates GLUT-4 translocation to the sarcolemma (Richter, 2013). So, normal and stable glycogen content was maintained in single fibers in the presence of insulin providing a condition of high glycogen content.

Glycogen content in single fibers fell to, on average, 20% of physiological levels when glucose was removed post-trituration. Interestingly this value closely approximates values reported at time of exhaustion during exhausting exercise (Hermansen et al., 1967, Rauch et al., 2005). The primary source of energy in resting muscle is from fatty acids, through β-oxidation (Hultman, 1995). However, a lack of free fatty acids in the culture media was most likely compensated by an increased glycogen usage resulting in further decreases in glycogen content overtime. So, the present protocol allowed for a unique approach to study the effect of glycogen content approximating that at the time of exhaustion (or a critical lower limit) without any metabolic stress, such as fasting or a previous fatigue bout.

SINGLE FIBERS VS. MUSCLE BUNDLES IN THE STUDY OF MUSCLE FATIGUE

As discussed in the introduction, the primary cause for a decrease in force is a reduction in Ca^{2+} release from the SR, which becomes submaximal during fatigue. In the present study, with 5.5 glucose/high glycogen FDB muscle bundles, tetanic force declined by 80% during the first 90 sec of stimulation and stabilized at that point thereafter until the end of fatigue, as previously reported (Selvin et al., 2015, Boudreault et al., 2010, Helander et al., 2002). This suggests that tetanic $[Ca^{2+}]_i$ decreased at the onset of the fatigue stimulation. Contrary to the situation with FDB bundles, FDB

single fibers showed a rise in tetanic $[Ca^{2+}]_i$ during the first 30 sec of a similar fatiguing stimulation, followed by a decrease (the extent of which appears to be fiber type dependent). These differences have been well documented (Allen et al., 2008, Chin & Allen, 1997, Westerbad & Allen, 1991) and suggest that fatigue is not immediately triggered in single fibers as it is in muscle bundles.

The difference in results between the muscle preparations was also particularly prominent in 0 glucose/high glycogen conditions. In FDB bundles decreases in tetanic force during a single bout of fatigue were barely faster in 0 mM glucose compared to in 5.5 mM glucose. This in agreement with previous studies showing that extracellular glucose has little significant effect on tetanic force in whole muscles throughout a fatiguing stimulation (Chin & Allen, 1997, Kabbara et al., 2000). Again, contrary to the FDB bundle, and for the first time, a significant and robust effect of 0 mM extracellular glucose on tetanic $[Ca^{2+}]_i$ in single fibers was observed. That is, in the presence of glucose, mean tetanic $[Ca^{2+}]_i$ did not drop below values prior to fatigue, whereas it decreased by 48% in the absence of glucose. These results suggest that extracellular glucose plays a major role during fatigue that has not been observed to-date in whole muscle models.

Finally, results the from FPC experiments reveal a significantly more robust effect in single fibers when compared to muscle bundles. In this case, a significantly slower decrease in force during FAT2 compared to FAT1 was observed with FDB bundles, but for single fibers the difference was more prominent, especially in low-resistance fibers.

Studies have shown that fibers at the center of a muscle suffer from diffusional limitations and display symptoms of metabolic stress more readily than surface fibers (Barclay et al., 2005, Han et al., 2011, Sogaard et al., 2009). According to the Barclay model of 0_2 diffusion (2005), the size of the FDB muscle bundle does not cause an 0_2 diffusional limitation at 35˚C when stimulated once every

100 sec. However, when stimulation frequency is increased to once every second, the maximum radius for proper 0_2 diffusion decreases within seconds, leading to the development of an anoxic core, even in an FDB bundle of 1 – 2 mg. The development of an anoxic core has also been linked to a glycogen poor region in the center of muscle bundles after fatigue (Han et al., 2011). Therefore, it is more than likely that in the FDB bundles a glucose diffusion limitation was indeed present and, as a consequence, the lack of a major effect of 0 mM glucose was not observed because even at 5.5 mM glucose many FDB fibers were still glucose-deprived. This situation does not occur with single fibers because the entire perimeter of the fiber is exposed to the physiological solution and diffusion of oxygen and glucose was no longer hindered, leading to the effect of removing glucose becoming more prominent. Thus, from this point on the availability of CHO during fatigue and FPC will be discussed only using data from single fibers.

INSULIN ONLY EFFECTS MUSCLE FATIGUE UNDER EXTREME CARBOHYDRATE LIMITATION

As discussed previously, the presence of insulin was needed to maintain glycogen content in single fibers in culture. In order to be able to compare with previous studies (which are conducted in the absence of insulin) the current study was repeated in both the presence and absence of insulin.

Results from the current study show no significant effect of 300 µU/ml insulin on the progression of fatigue during an initial (FAT1) and subsequent (FAT2) bout of fatigue. In fact, results showed that tetanic $[Ca^{2+}]_i$ actually increased slightly under low glycogen conditions, during which insulin sensitivity has been reported to be increased (Kawanaka et al., 1999). This was of interest given that evidence shows that contraction and insulin have an additive effect on GLUT-4 translocation and glucose uptake in skeletal muscle, acting through separate signalling pathways (Lund et al., 1995). Studies have shown that glucose transport resulting from increases in GLUT-4

content has a significant effect under basal conditions, however not under hyperinsulinaemia or during exercise (Fueger et al., 2004, 2005). Under these conditions it has been proposed that glucose phosphorylation by hexokinase is the rate-limiting step for glucose influx. Furthermore, it has been shown that an increase in extracellular glucose concentration from 5.5 to 11 mM has no significant effect on fatigue progression, supporting this proposition (Helander et al., 2002). This would suggest that glucose uptake is already maximal at a concentration of 5.5 mM during exercise due to the maximal activity of hexokinase and could explain the lack of any insulin effect during a short, intense fatigue bout.

One intriguing finding from this study was that the presence of insulin in 0 mM glucose/low glycogen fibers had the effect of increasing tetanic $[Ca^{2+}]_i$ and fatigue resistance when compared to the absence of insulin. The reason for a difference cannot be determined from the present results, although insulin has been reported to increase Na^+/K^+ -ATPase activity (Novel-Chaté et al., 2001, Chibalin et al., 2001) and decrease GP activation (Hartmann et al., 1985). This could lead to better maintenance of Na^+ and K^+ gradients and membranes excitability as well as a glycogen sparing effect (Lees et al., 2001, Quinlan et al., 2010). Additionally, it is possible that an increased mitochondrial activity, known to occur in the presence of insulin and under low glycogen content conditions (Craig et al., 2003, O'Neill et al., 2011) may have allowed for greater use of intracellular lipid stores. Overall, it appears that insulin can contribute to fatigue resistance in a manner that is independent of its effect on glucose transport, a mechanism that has not been addressed in current literature.

In the absence of any major insulin effects in the fatigue kinetics, the following discussion on CHO availability will no longer take into consideration whether insulin was absent or present.

LOW GLUCOSE NOT LOW GLYCOGEN LOWERS FATIGUE RESISTANCE DURING A SINGLE BOUT OF FATIGUE

Under control conditions, i.e., 5.5 glucose/high glycogen, mean pre-fatigue tetanic $[Ca^{2+}]_i$ was in the range of 0.94 – 1.12 μM compared to concentrations anywhere between 0.89 – 1.2 μM reported by previous studies (Selvin & Renaud, 2015, Chin & Allen, 1997, Westerblad & Allen, 1993, Helander et al., 2002, Kabbara et al., 1999). The same studies also reported an initial increase in tetanic $[Ca^{2+}]_i$ reaching up to 162%, followed by a decline, as was observed in the current study. It has been suggested that an initial increase in tetanic $[Ca^{2+}]_i$ is likely due to a reduced $[Ca^{2+}]_i$ buffering capability of the fiber (Westerblad & Allen, 1991). As previously reported (Selvin & Renaud, 2015), the decline in tetanic $[Ca^{2+}]_i$ varies tremendously among fibers, from small to large declines, likely from highly fatigue resistant type I and IIA fibers or low fatigue resistant type IIX fibers, respectively. Unstimulated $[Ca^{2+}]_i$ steadily increased until reaching a peak after 90 sec, at which point it reached a steady-state. These unstimulated $[Ca^{2+}]_i$ values are in agreement with previous reports (Selvin & Renaud, 2015, Boudreault et al., 2010, Westerblad et al., 1997, Cifelli et al., 2008, 2009). As the fatigue kinetics under control conditions agree with previous studies, they will not be discussed further.

One of the primary objectives of this study was to determine the role of two sources of carbohydrate, extracellular glucose and glycogen, during skeletal muscle fatigue.

<u>Extracellular glucose availability is critical in maintaining fatigue resistance</u>

When eliciting fatigue in 0 glucose/high glycogen fibers, mean initial tetanic $[Ca^{2+}]_i$ and initial increase thereof was the same as was observed in 5.5 glucose/high glycogen fibers, which indicates that these aspects of fatigue kinetics are not based on a metabolic affect but rather a decreased

buffering capacity as has been suggested (Westerblad & Allen, 1991). The major effect of removing extracellular glucose was observed in the subsequent decrease in tetanic $[Ca^{2+}]_i$. In control, mean tetanic $[Ca^{2+}]_i$ was 0.95 μM at the end of the fatigue period, while in the absence of glucose, mean tetanic $[Ca^{2+}]_i$ fell to 0.44 μM. Removing glucose from the physiological solution also resulted in a significant increase in unstimulated $[Ca^{2+}]_i$ when compared to 5.5 mM glucose/high glycogen fibers. Lastly, individual fiber variability showed that the majority of fibers (12 of 15) had a decrease in tetanic $[Ca^{2+}]_i$ exceeding 0.25 μM in 0 glucose/high glycogen fibers when compared to only 3 of 15 in 5.5 glucose/high glycogen fibers. Thus, in 0 mM glucose very few fibers retained a high resistance to fatigue. These changes in tetanic and unstimulated $[Ca^{2+}]_i$ strongly suggest that glucose plays an essential role in maintaining normal $[Ca^{2+}]_i$ dynamics within muscle fibers as the lack of extracellular glucose reduces the fiber's ability to release $[Ca^{2+}]_i$ during contraction as well as sequester it into the SR during relaxation.

To our knowledge, there are no studies investigating a complete lack of glucose during a single fatigue bout *in vitro*. Many have, however, investigated the effects of glucose supplementation and may provide insight into the role of glucose during fatigue. A meta-analysis of 72 studies focused on the effect of carbohydrate supplementation (oral ingestion and/or intravenous) during different modes of exercise, reports an, on average, 40% increase in performance (Karelis et al., 2010). An intriguing mechanism through which glucose supplementation may increase fatigue resistance is through better maintenance of fiber electrical properties. The attenuation of muscle fatigue with glucose supplementation has been associated with increased M-wave peak-to-peak amplitude, duration, and area (which has been shown to coincide with improved AP generation and muscle excitability (Harrison & Flatman, 1999)) in rats *in situ* (Karelis et al., 2002), as well as humans *in vivo*

(Stewart et al., 2007). Three membrane components may be involved in improving membrane excitability. An increase in glucose can improve ATP generation to maintain the activity of electrogenic Na^+/K^+ -ATPase pumps, which than maintain [K^+] and [Na^+] gradients necessary for AP generation as well as countering any depolarization of the resting membrane potential as observed during fatigue, ultimately maintaining membrane excitability (Green et al., 2007, Karelis et al., 2005). Slower decreases in ATP is also expected to delay activation of K_{ATP} and ClC-1 channels, both known to reduce AP amplitude (and thus SR Ca^{2+} release and force) and decrease the number of excitable fibers when activated (Pedersen et al., 2009, 2016, Zhang et al., 2008, Zhu et al., 2014).

It stands to reason that with 0 mM glucose, glycolytically derived ATP would be severely limited as glycogen would become the only availability source and its reserves are finite. As the three membrane components are known to be the preferential dependent on glycolytically derived ATP (Okamoto et al., 2001, Sepp et al., 2014, Dhar-Chowdhury et al., 2005, Bennetts et al.,2012), their activity would be increasingly altered in the absence of extracellular glucose and as glycogen becomes depleted. The overall result being a decreased membrane excitability and an increase in AP failure, ultimately impairing depolarization induced SR Ca^{2+} release and tetanic $[Ca^{2+}]_i$ during fatigue. Results for individual fibers support this idea in that not all fibers are affected by 0 mM extracellular glucose, but likely type IIA and IIX fibers, which are more reliant on glycolysis for energy production than type I fibers.

The increase in unstimlated $[Ca^{2+}]_i$ in the absence of glucose was also potentially mediated by a decrease in local glycolysis and ATP production, leading to decrease SERCA channel activity. It is now known that glycogenolytic and glycolytic complexes are bound to the SR membrane and that ATP generated by SR-associated glycolytic enzymes provide energy for the nearby SR Ca^{2+} -ATPases

(Xu et al., 1995, 1998). 0 glucose/low glycogen fiber results support this probability as the increase in unstimulated $[Ca^{2+}]_i$ occurred earlier (at approximately 60 rather than 120 sec) when glycolysis was severely impaired due to a near-complete lack of substrate.

Based on the present results from single fibers stimulated to fatigue in the absence of extracellular glucose it is concluded that glucose is critical in maintaining SR Ca^{2+} release as well as SR Ca^{2+} sequestration and thus fatigue resistance. This effect is likely mediated through the maintenance of membrane components that rely heavily on glycolytically derived ATP.

Without a prior metabolic insult glycogen does not affect muscle fatigue

A glycogen depletion in single FDB fibers prior to a single fatigue bout did not affect fatigue resistance; i.e. there were only minor differences in mean tetanic $[Ca^{2+}]_i$ fatigue kinetics between low glycogen and high glycogen conditions (both with 5.5 mM glucose). When examining individual fibers, a similar distribution of fatigue kinetics between low glycogen and high glycogen fibers was also observed. In addition, unstimulated $[Ca^{2+}]_i$ was actually slightly decreased in low glycogen compared to high glycogen conditions. This was not expected considering the large number of studies correlating increased fatigue resistance with increased glycogen content (Chin & Allen, 1997, Helander et al., 2002, Kabbara & Allen, 1999, Nielsen et al., 2011, Duhamel et al., 2006, Bergström et al., 1967, Ørtenblad et al., 2011).

Considering the importance of glucose, discussed in the previous section, one possibility for the lack of any substantial effect of glycogen could be the reintroduction of glucose to the physiological solution 10 min prior to a fatigue bout. Fibers were incubated in the presence of insulin and under conditions that favor AMPK activation (i.e. low glycogen and glucose availability) leading

to an expected increase in GLUT-4 translocation (Jessen et al., 2002). Upon glucose reintroduction, it is possible that rapid glucose transport may have sufficiently replenished glycogen stores to above a critical level prior to a fatigue bout. However, this is an unlikely explanation in that results from 5.5 glucose/low glycogen in the presence of insulin had a reduce fatigue resistance when compared to in the absence of insulin. It is also unlikely because if the addition of glucose 10 min prior to fatigue allowed for a replenishment, even partially, of glycogen, then the decrease in tetanic $[Ca^{2+}]_i$ should have been much greater in 0 glucose/low glycogen compared to 0 glucose/high glycogen. Instead the decrease in tetanic $[Ca^{2+}]_i$ was the same, suggesting that in mouse FDB muscle a lack of glucose is more important to fatigue resistance, without a previous bout of fatigue, than a lack of glycogen.

In low glycogen FDB fibers, regardless of the presence of glucose, tetanic $[Ca^{2+}]_i$ prior to a fatigue bout was similar to control values. Some studies show a decrease in tetanic $[Ca^{2+}]_i$ during the first contraction of a fatigue bout under low glycogen conditions (present results from FPC, Chin & Allen, 1997, Ørtenblad et al., 2011) while some show no difference from controls (Helander et al., 2002, Duhamel et al., 2006, Kabbara & Allen, 2009). Interestingly, when comparing studies that show a decreased initial tetanic $[Ca^{2+}]_i$ prior to fatigue and those that do not, the extent of glycogen depletion highlights the possibility of a 'glycogen limit' at which point glycogen becomes sufficiently low to significantly effect SR Ca^{2+} release. In the studies where glycogen was depleted by greater than 60% an effect was observed whereas those in which depletion was only to 40 - 50%, the effect was not observed. Additionally when comparing the progression of tetanic $[Ca^{2+}]_i$ decline it is evident that fatigue resistance is more severely impaired when fatigue begins with glycogen content that has been depleted by more than 60%. In the present study, despite the approximate 80% decrease in glycogen in fibers, tetanic $[Ca^{2+}]_i$ was similar between high and low glycogen conditions at the onset of a single

fatigue bout. It is however important to note that many of the aforementioned studies used a prior fatigue bout to deplete glycogen and that any decrease in tetanic $[Ca^{2+}]_i$ prior to fatigue may be dependent on prior metabolic stress or a lack of recovery thereof.

In vitro studies involve a glycogen depletion using a strong metabolic stress, i.e. a fatigue bout followed by recovery in 0 mM glucose to prevent glycogen storage to be replenished (Chin & Allen, 1997, Duhamel et al., 2006, Kabbara & Allen, 2009). The problem with this approach is that muscles have not fully recovered both force and $[Ca^{2+}]_i$, and thus are still in a metabolic stress mode. It is possible that glycogen depletion in the absence of metabolic stress does not lower fatigue resistance. If this is the case, than studies using low glycogen models produced by fasting should show no effect on fatigue resistance. Two studies, one on rats (Williams et al., 2013) and one on humans (Duhamel et al., 2006), used a protocol that involved a glycogen depleting bout of exercise followed by a 24-hour fast and low carbohydrate diet respectively. These studies showed a decreased time to fatigue in low glycogen rats and humans, however, the criteria for fatigue was based on volition. Volition implicates a potential central fatigue component, which may have been involved as blood glucose levels were significantly decreased. Interestingly, when the low glycogen rats, that displayed decrease time to volitional fatigue, were tested *in situ* (with direct muscle stimulation) no difference in force production was observed between fed and fasted rats. Studies using only a fast and no prior glycogen depleting exercise show no difference (Koubi et al., 1985) and even increases in fatigue resistance (Dohm et al., 1983) (which the authors attribute to a 'glycogen sparing' effect of increased β – oxidation). These studies support the current findings and a hypothesis that lowering glycogen alone is insufficient to reduce fatigue resistance during a short period of intense muscle activity, and

suggests the reductions in fatigue resistance commonly observed require an additional event to occur.

However, one cannot eliminate the possibility that glycogen was not depleted to less than critical values in all fibers, as glycogen measurements were taken as a mean of several fibers. The most viable fibers in terms of appearance and contractility may have been chosen for tetanic $[Ca^{2+}]_i$ measurements and therefore may have had a glycogen level sufficiently high enough above critical levels to have no effect on tetanic $[Ca^{2+}]_i$. On an individual fiber basis, tetanic $[Ca^{2+}]_i$ kinetics of high-, mid-, and low-fatigue resistant fibers was the same regardless of glycogen content. One would expect the more fatigue resistant fibers to be type I or IIA and the less fatigue resistant fibers to be type IIX. It appears unlikely that over 15 fibers that a difference in pattern was not observed because one would expect type IIX fibers to become less resistant under low glycogen conditions. Future studies will need to determine variability in glycogen content in various fibers. If a high degree of variability exists, differences in Ca^{2+} release prior to fatigue might vary between fibers with different glycogen contents. It is possible that a decreased Ca^{2+} release could be observed only in fibers with the lowest glycogen content and that those fibers were not chosen for experiments. However, a small decrease in fatigue resistance was observed in 0 glucose/low glycogen compared to 0 glucose/high glycogen, indicating that there was in fact a difference in glycogen content between low glycogen and high glycogen conditions as well as indicating some effect of glycogen independent of glucose.

With respect to the aim of determining the effect of carbohydrate (CHO) availability during a single bout of fatigue, the current study provides evidence to support the novel insight that without a prior metabolic stress, extracellular glucose is more important to fatigue resistance than glycogen. Moreover, whereas a lack of glucose leads individual fiber tetanic $[Ca^{2+}]_i$ kinetics to more closely

resembles those from type IIX fibers, individual low glycogen fiber tetanic $[Ca^{2+}]_i$ kinetics are no different then high glycogen fibers. It is proposed that extracellular glucose is essential in buffering glycogen depletion as an alternative source of CHO, leading to better maintenance of membrane excitability and improving Ca^{2+} handling within fibers.

FATIGUE PRE-CONDITIONING (FPC)

FPC is a phenomenon in which a one fatigue bout (FAT1) triggers an acute (lasting 30 to 120 min after FAT1) physiological process during a second fatigue bout (FAT2). It involves *i)* increased fatigue resistance (as seen in the present study as well as in the study by Boudreault et al., 2010) and, *ii)* prevention of contractile dysfunctions normally induced in the absence of K_{ATP} channel activity during FAT1. The fact that K_{ATP} are activated under conditions of metabolic stress and that they have no role during FPC have led Boudreault et al. (2010) to suggest that FPC allows muscle to better cope with a metabolic insult during FAT2.

<u>The least fatigue resistant single fibers experience the greatest effect of FPC</u>

In the Boudreault et al. (2010) study, only the mean tetanic $[Ca^{2+}]_i$ had been shown. In this study, we are now showing that not all fibers are capable of FPC. Fibers were first divided into high-, mid-, and low-resistance to fatigue groups, based on FAT1 tetanic $[Ca^{2+}]_i$ kinetics. High-resistance fibers were those for which tetanic $[Ca^{2+}]_i$ was greater at the end of FAT1 than at the beginning; mid-resistance fibers, those for which tetanic $[Ca^{2+}]_i$ decreased by no greater than 0.25 µM by the end of FAT1; and low-resistance fibers, those for which tetanic $[Ca^{2+}]_i$ decrease by greater than 0.25 µM by the end of FAT1. Such an approach indicated that the least fatigue resistant fibers during FAT1 (low-resistance) had the largest increases in fatigue resistance during FAT2. Moreover, the differences

observed during FAT1 among all three groups completely disappeared during FAT2; i.e., all fibers behave like high-resistance fibers during FAT2. Although, fiber type was not determined in this study, it can be suggested that high-resistance fibers are type I fibers, while mid-resistance and low-resistance fibers are type IIA and IIX respectively (type IIB are not present in the FDB muscle; Banas et al., 2011). These results provide a novel contribution to our understanding of FPC and suggest that FPC is a property of fast twitch fibers, occurring to a greater extent in type IIX than IIA fibers.

Here, the aim was to document how CHO availability could play a role in this phenomenon.

Glycogen supercompensation and FPC

When glucose was present during recovery, glycogen content in FDB bundles was 37 - 49% greater prior to FAT2 compared to the content prior to FAT1. These values agree with previous studies which report glycogen supercompensation between 35 – 55% following recovery from fatigue (Helander et al., 2002, Sano et al., 2012, Chin & Allen, 1997, Ivy et al., 1985). It is possible that the mechanism is mediated by an activation of AMPK, commonly observed during a fatigue bout (in this case FAT1; Morales-Alamo & Calbet, 2016, Chen et al., 2003). Glycogen depletion and Ca^{2+} release increases activation of AMPK and glycogen synthase (GS) activity (Jørgensen et al., 2007). Once activated AMPK increases glucose transport (2-fold), and, coupled with an increase in GS activity (up to 3-fold), glycogen is rapidly replenished (Hingst et al., 2018, Merill et al., 1997).

Although glycogen content could not be measured in single fibers, a similar situation is expected. When FDB bundles recovered in the absence of glucose, tetanic force failed to return to pre-FAT1 levels. Similarly, when single fibers recovered in the absence of glucose, tetanic $[Ca^{2+}]_i$ failed to return to pre-FAT1 levels. This suggests that glycogen was consumed during FAT1 in both bundles

and single fibers, and not replenished prior to FAT2 when glucose was absent during recovery. A supercompensation in single fibers is also expected as there are no indications that any metabolite effects in muscle bundles are different from those in single fibers. So, for the following discussion, it is assumed that the changes in glycogen content observed during FAT1 and FAT2 in FDB bundles reflect the changes in single fibers.

The glycogen supercompensation prior to FAT2 observed in the current study corresponded with an increased glycogen usage and slower decreases in tetanic $[Ca^{2+}]_i$ during FAT2. It is likely that an increased glucose availability via glycolysis contributed to the slower decrease in tetanic $[Ca^{2+}]_i$ as membrane excitability is expected to be better maintained (as discussed above). In terms of oxidative metabolism, no significant activation has been reported during a 3 min fatigue (Scott et al., 2016). However, as AMPK becomes more active it induces an increased activity of oxidative metabolism (Rabinovitch et al., 2017, Cantó et al., 2009). AMPK also induces a switch to fatty-acid metabolism, increasing fatty-acid oxidation 2.8-fold (Merill et al.,1997). Thus, it is also likely that an increase in glucose availability from glycogen and a greater oxidative capacity significantly increase ATP production. Lastly, AMPK activation has been reported to persist for at least 30 minutes following a fatigue bout (Musi et al., 2001), which suggests that it's effect at increasing the energy turnover, whether from glycogen usage, increased glucose uptake, or increased fatty-acid oxidation (or, more likely, increasing all three) persisted into FAT2 and contributed to FPC.

Although glycogen content was greater at the beginning of FAT2, it's content (as well as final tetanic $[Ca^{2+}]_i$ levels) at the end of both FAT1 and FAT2 was the same. This is in agreement with the hypothesis that, when glycogen reaches a critical limit during a metabolic stress, intracellular

mechanisms are activated that decrease SR Ca^{2+} release (Hermansen et al., 1965, Helander et al., 2002, Chin & Allen, 1997).

Considering the strong possibility of a role of glycogen in FPC and the critical limit causing large decreases in tetanic $[Ca^{2+}]_i$, the question is now what role CHO availability in general plays in FPC.

Carbohydrate availability is a central component of FPC

One marked observation was a lack of tetanic $[Ca^{2+}]_i$ recovery when glycogen was not replenished, leading to lowered tetanic $[Ca^{2+}]_i$ prior to FAT2. Tetanic $[Ca^{2+}]_i$ then remained depressed throughout FAT2, more so when glucose was omitted. Although there are no reports, using a similar protocol, on the effects of 0 mM glucose during FPC, previous studies on low glycogen support the current study in that preventing replenishment of glycogen consistently correlates with decreased fatigue resistance, an effect that has been extended to highlight coinciding decreases in glycogen and SR Ca^{2+} release (Ørtenblad et al., 2011, Chin & Allen, 1997, Kabbara et al., 2000, Kabbara & Allen, 1999).

Unstimulated force, in the absence of glucose (high glycogen), was only slightly decreased during the first 100 sec of fatigue compared to in its presence, indicating that glycogen supercompensation provided enough endogenous ATP production via glycolysis to fuel Ca^{2+} reuptake into the SR. Results from low glycogen conditions support this possibility in that unstimulated force and $[Ca^{2+}]_i$ increased at a more rapid rate than with high glycogen. In the absence of glucose and low glycogen unstimulated force and $[Ca^{2+}]_i$ was significantly elevated shortly after the beginning of FAT2. Thus, as glycogen was depleted, the unstimulated force and $[Ca^{2+}]_i$ rose steadily. It is possible that

glycogen is the primary source of energy for SERCA channels but is heavily buffered by ATP generated from exogenous glucose. This possibility is well supported by relatively large increases in unstimulated force and $[Ca^{2+}]_i$ under 0 glucose/low glycogen conditions.

Taken together, low glycogen and 0 mM glucose results from FAT2 suggest that they play a critical role in maintaining Ca^{2+} handling during FAT2, suggesting that they are a central to the mechanisms underlying FPC. They also suggest that the source of substrate for glycolysis does not matter *per se*. However, it can also be argued that a decrease CHO availability is not the reason for preventing FPC but rather it prevented the normal recovery of force and Ca^{2+} handling, which resulted in FAT2 being elicited in a fatigue state resulting in an apparent decrease in fatigue resistance and thus no FPC.

Role of glycogen: FAT1 vs. FAT2

While it is clear that the presence of extracellular glucose is critical during FAT1, the subsequent recovery of tetanic force and $[Ca^{2+}]_i$, and in FPC (at least in bundles as equipment problems prevented measurements in single fibers under 0 glucose/high glycogen in FPC), the role of glycogen seems to be more complex.

The effect of low glycogen during FAT2 was as previously reported, even at the presently used experimental temperature of 37˚C when compared to the more commonly used room temperature (Chin & Allen, 1997, Helander et al., 2002, Westerblad & Allen, 1993). However, no major effect of low glycogen was observed during a short, but intense fatigue bout (FAT1). The prevailing difference between FAT1 and FAT2 results was the metabolic state of the muscle.

AMPK is known to be a master regulator of metabolism and is maximally activated during periods of metabolic stress (Hardie et al., 2007). It's activation has been shown to increase ATP production but has also been implicated in depressing membrane excitability (Ikematsu et al., 2011, Yoshida et al., 2012). Additionally, AMPK has been shown to bind to glycogen, inhibiting the activity of the former (McBride et al., 2009). Thus, when glycogen is present, AMPK is sequestered and inhibited, and any decrease of membrane excitability due to AMPK activation, and subsequent decreasing tetanic force and $[Ca^{2+}]_i$, are also inhibited. However, in low glycogen conditions, AMPK is liberated.

Prior to FAT1, the liberation of AMPK may not effect membrane excitability due to the lack of a metabolic stress that would otherwise cause AMPK activation. However, after a fatigue bout (FAT1) that stress is surely present, leading to AMPK activation, contributing to a fatigue state. If glycogen replenishment is prevented, this fatigue state would be expected to persist, as sequestering of AMPK does not occur and energy stores remain depleted. This could explain why low glycogen decreased fatigue resistance during FAT2 when compared to FAT1 in that AMPK availability and activation are likely higher prior to FAT2, priming muscle to be in a fatigue state at the onset of the second fatigue bout.

CONCLUSIONS

In conclusion, a novel method in which a metabolic stress was not used to deplete glycogen in single muscle fibers was produced. Surprisingly, lowered glycogen content, using this approach and measuring tetanic $[Ca^{2+}]_i$, did not affect fatigue resistance. A decrease in fatigue resistance associated

with low glycogen was observed only when glycogen stores were depleted during a first fatigue bout and not allowed to recover before a second fatigue bout. It is therefore suggested that, in the absence of a metabolic stress, low glycogen content does not affect fatigue resistance when fatigue is triggered with intense stimulation over a short period of time, i.e. 1 contraction every sec for 3 min.

A significant decrease in fatigue resistance was, however, observed when fibers were fatigued in the absence of glucose. This was observed for a single fatigue bout as well as FPC. Thus, for an intense stimulation and for a short period, glucose but not glycogen is important in maintaining force. Finally, contrary to a single fatigue bout, both glucose and glycogen play a major role in FPC, which is an acute increase in fatigue resistance after a first fatigue bout being in the order of type IIX > IIA > I fibers. It is proposed that the phenomenon of FPC is mediated by an increase in glycolytic capacity and perhaps an increase in oxidative metabolism. Lastly, insulin does not appear to play a major role in muscle fatigue unless there are extreme CHO limitations, indicating that insulin may contribute to fatigue resistance be mechanisms that do not involve glucose transport.

APPENDIX I

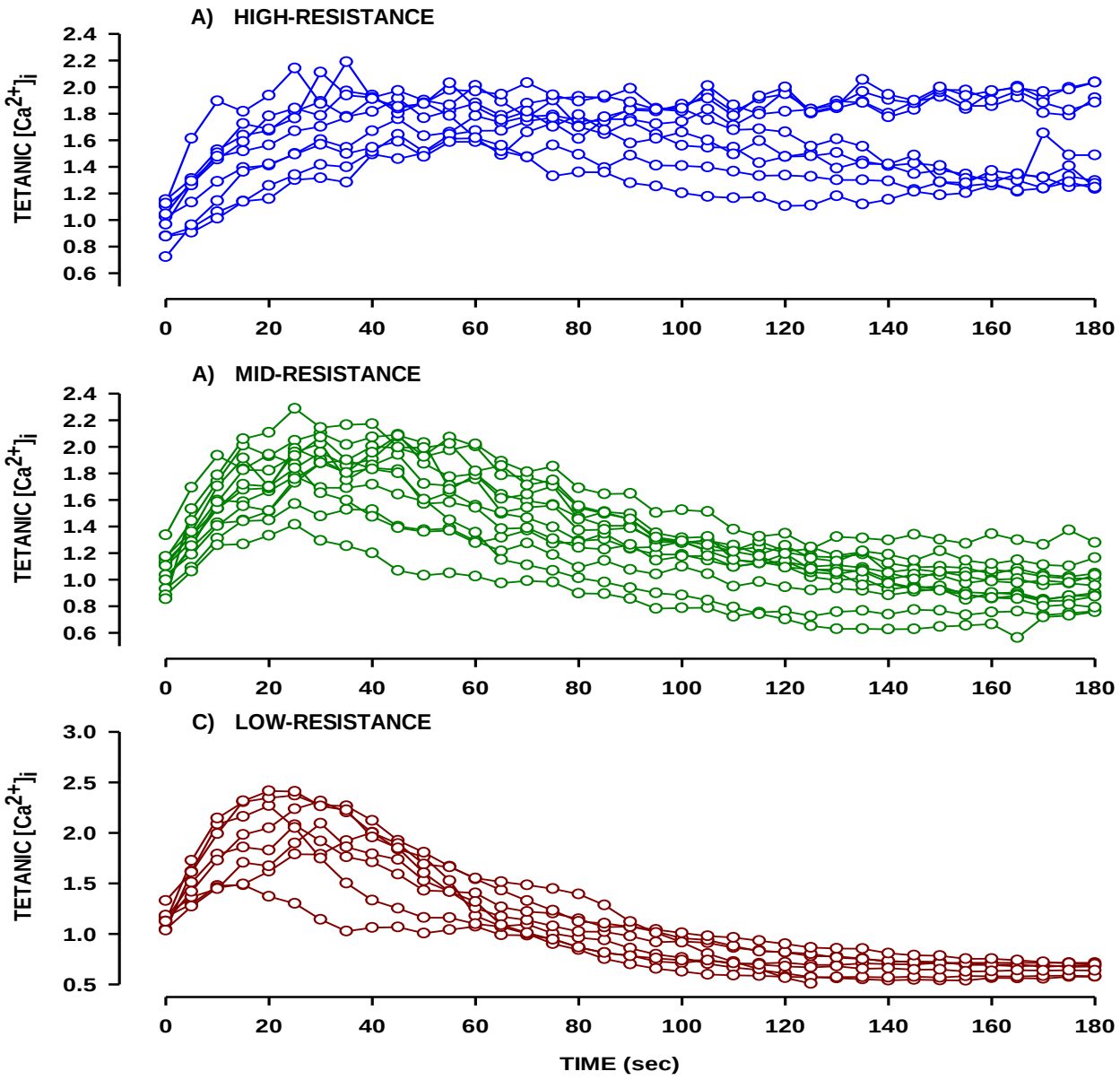

Figure A-1. Individual tetanic $[Ca^{2+}]_i$ traces for fibers shown in Fig 3-12. Fibers were grouped as described in Fig. 3-12**.** Notice the similarity in the kinetics of the changes in tetanic $[Ca^{2+}]_i$ traces for each of the three groups.

REFERENCES

Allen DG, Lamb GD, Westerblad H (2008). Skeletal muscle fatigue: cellular mechanisms. *Physiol Rev.* 88(1):287-332.

Andrews MA, Godt RE, Nosek TM (1985). Influence of physiological L(+)-lactate concentrations on contractility of skinned striated muscle fibers of rabbit. *J Appl Physiol*. 80(6):2060-5.

Bagby GJ, Green HJ, Katsuta S, Gollnick PD (1978). Glycogen depletion in exercising rats infused with glucose, lactate, or pyruvate. *J Appl Physiol Respir Environ Exerc Physiol*. 45(3):425-9.

Banas K, Clow C, Jasmin BJ, Renaud JM (2011). The KATP channel Kir6.2 subunit content is higher in glycolytic than oxidative skeletal muscle fibers. *Am J Physiol Regul Integr Comp Physiol.* 301(4):R916-25.

Bangsbo J, Graham TE, Kiens B, Saltin B (1992). Elevated muscle glycogen and anaerobic energy production during exhaustive exercise in man. *J Physiol*. 451:205-27.

Barclay CJ (2005). Modelling diffusive O_2 supply to isolated preparations of mammalian skeletal and cardiac muscle. *J Muscle Res Cell Motil.* 26(4-5):225-35.

Barnes M, Gibson LM, Stephenson DG (2001). Increased muscle glycogen content is associated with increased capacity to respond to T-system depolarisation in mechanically skinned skeletal muscle fibres from the rat. *Pflugers Arch*. 442(1):101-6.

Barnes WS, Worrell GA (1985). Glycogen utilization in isolated frog muscle: an effect of passive mechanical stretch. *Comp Biochem Physiol A Comp Physiol*. 81(2):243-6.

Batson G (2013). Exercise-induced central fatigue: a review of the literature with implications for dance science research. *J Dance Med Sci*. 17(2):53-62.

Bennetts B, Parker MW, Cromer BA (2007). Inhibition of skeletal muscle ClC-1 chloride channels by low intracellular pH and ATP. *J Biol Chem.* 282(45):32780-91.

Bennetts B, Rychkov GY, Ng HL, Morton CJ, Stapleton D, Parker MW, Cromer BA (2005). Cytoplasmic ATP-sensing domains regulate gating of skeletal muscle ClC-1 chloride channels. *J Biol Chem*. 280(37):32452-8.

Bennetts B, Yu Y, Chen TY, Parker MW (2012). Intracellular β-nicotinamide adenine dinucleotide inhibits the skeletal muscle ClC-1 chloride channel. *J Biol Chem*. 287(31):25808-20.

Benziane B, Björnholm M, Pirkmajer S, Austin RL, Kotova O, Viollet B, Zierath JR, Chibalin AV (2012). Activation of AMP-activated protein kinase stimulates Na+,K+-ATPase activity in skeletal muscle cells. *J Biol Chem.* 287(28):23451-63.

Bergström J, Hermansen L, Hultman E, Saltin B (1967). Diet, muscle glycogen and physical performance. *Acta Physiol Scand*. 71(2):140-50.

Best PM, Donaldson SK, Kerrick WG (1977). Tension in mechanically disrupted mammalian cardiac cells: effects of magnesium adenosine triphosphate. *J Physiol*. 265(1):1-17.

Blazev R, Lamb GD (1999). Low [ATP] and elevated [Mg^{2+}] reduce depolarization-induced Ca2+ release in rat skinned skeletal muscle fibres. *J Physiol*. 520 Pt 1:203-15.

Boudreault L, Cifelli C, Bourassa F, Scott K, Renaud JM (2010). Fatigue preconditioning increases fatigue resistance in mouse flexor digitorum brevis muscles with non-functioning K(ATP) channels. *J Physiol*. 588(Pt 22):4549-62.

Brooks GA (1998). Mammalian fuel utilization during sustained exercise. *Comp Biochem Physiol B Biochem Mol Biol*. 120(1):89-107.

Cairns SP, Buller SJ, Loiselle DS, Renaud JM (2003). Changes of action potentials and force at lowered [Na+]o in mouse skeletal muscle: implications for fatigue. *Am J Physiol Cell Physiol.* 285(5):C1131-41.

Cairns SP, Hing WA, Slack JR, Mills RG, Loiselle DS (1997). Different effects of raised $[K^+]_o$ on membrane potential and contraction in mouse fast- and slow-twitch muscle. *Am J Physiol.* 273(2 Pt 1):C598-611.

Cairns SP, Lindinger MI (2008). Do multiple ionic interactions contribute to skeletal muscle fatigue? *J Physiol*. 586(17):4039-54.

Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature*. 458(7241):1056-60.

Chen ZP, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, Kemp BE, McConell GK (2003). Effect of exercise intensity on skeletal muscle AMPK signaling in humans. *Diabetes.* 52(9):2205-12.

Chibalin AV, Kovalenko MV, Ryder JW, Féraille E, Wallberg-Henriksson H, Zierath JR (2001). Insulin- and glucose-induced phosphorylation of the Na(+),K(+)-adenosine triphosphatase alpha-subunits in rat skeletal muscle. *Endocrinology*. 142(8):3474-82.

Chin ER, Allen DG (1997). Effects of reduced muscle glycogen concentration on force, Ca2+ release and contractile protein function in intact mouse skeletal muscle. *J Physiol*. 498 (Pt 1):17-29.

Cifelli C, Boudreault L, Gong B, Bercier JP, Renaud JM (2008). Contractile dysfunctions in ATP-dependent K+ channel-deficient mouse muscle during fatigue involve excessive depolarization and Ca2+ influx through L-type Ca2+ channels. *Exp Physiol*. 93(10):1126-38.

Cifelli C, Bourassa F, Gariépy L, Banas K, Benkhalti M, Renaud JM (2007). KATP channel deficiency in mouse flexor digitorum brevis causes fibre damage and impairs Ca2+ release and force development during fatigue in vitro. *J Physiol*. 582(Pt 2):843-57.

Clausen T (2003). Na+-K+ pump regulation and skeletal muscle contractility. *Physiol Rev*. 83(4):1269-324.

Clausen T (2013). Quantification of Na+,K+ pumps and their transport rate in skeletal muscle: functional significance. *J Gen Physiol*. 142(4):327-45.

Coyle EF, Coggan AR, Hemmert MK, Ivy JL (1985). Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *J Appl Physiol*. 61(1):165-72.

Cuenda A, Nogues M, Henao F, Gutiérrez-Merino C (1995). Interaction between glycogen phosphorylase and sarcoplasmic reticulum membranes and its functional implications. *J Biol Chem*. 270(20):11998-2004.

Dahlstedt AJ, Katz A, Westerblad H (2001). Role of myoplasmic phosphate in contractile function of skeletal muscle: studies on creatine kinase-deficient mice. *J Physiol*. 533(Pt 2):379-88.

De Ruiter CJ, De Haan A (2000). Temperature effect on the force/velocity relationship of the fresh and fatigued human adductor pollicis muscle. *Pflugers Arch*. 440(1):163-70.

Debold EP, Dave H, Fitts RH (2004). Fiber type and temperature dependence of inorganic phosphate: implications for fatigue. *Am J Physiol Cell Physiol*. 287(3):C673-81.

Dhar-Chowdhury P, Malester B, Rajacic P, Coetzee WA (2007). The regulation of ion channels and transporters by glycolytically derived ATP. *Cell Mol Life Sci*. 64(23):3069-83.

Dohm GL, Tapscott EB, Barakat HA, Kasperek GJ (1983). Influence of fasting on glycogen depletion in rats during exercise. *J Appl Physiol Respir Environ Exerc Physiol*. 55(3):830-3.

Duhamel TA, Perco JG, Green HJ (2006). Manipulation of dietary carbohydrates after prolonged effort modifies muscle sarcoplasmic reticulum responses in exercising males. *Am J Physiol Regul Integr Comp Physiol.* 291(4):R1100-10.

Duke AM, Steele DS (2000). Characteristics of phosphate-induced Ca(2+) efflux from the SR in mechanically skinned rat skeletal muscle fibers. *Am J Physiol Cell Physiol*. 278(1):C126-35.

Dutka TL, Cole L, Lamb GD (2005). Calcium phosphate precipitation in the sarcoplasmic reticulum reduces action potential-mediated Ca2+ release in mammalian skeletal muscle. *Am J Physiol Cell Physiol.* 289(6):C1502-12.

Fairchild TJ, Armstrong AA, Rao A, Liu H, Lawrence S, Fournier PA (2003). Glycogen synthesis in muscle fibers during active recovery from intense exercise. *Med Sci Sports Exerc.* 35(4):595-602.

Farah CS, Reinach FC (1995). The troponin complex and regulation of muscle contraction. *FASEB J*. 9(9):755-67.

Fill M, Copello JA. Ryanodine receptor calcium release channels (2002). *Physiol Rev*. 82(4):893-922.

Flagg TP, Enkvetchakul D, Koster JC, Nichols CG (2010). Muscle KATP channels: recent insights to energy sensing and myoprotection. *Physiol Rev*. 90(3):799-829.

Fueger PT, Hess HS, Posey KA, Bracy DP, Pencek RR, Charron MJ, Wasserman DH (2004). Control of exercise-stimulated muscle glucose uptake by GLUT4 is dependent on glucose phosphorylation capacity in the conscious mouse. *J Biol Chem*. 279(49):50956-61.

Fueger PT, Shearer J, Bracy DP, Posey KA, Pencek RR, McGuinness OP, Wasserman DH (2005). Control of muscle glucose uptake: test of the rate-limiting step paradigm in conscious, unrestrained mice. *J Physiol*. 562(Pt 3):925-35.

Garcia-Roves PM, Han DH, Song Z, Jones TE, Hucker KA, Holloszy JO (2003). Prevention of glycogen supercompensation prolongs the increase in muscle GLUT4 after exercise. *Am J Physiol Endocrinol Metab*. 285(4):E729-36.

Gejl KD, Hvid LG, Frandsen U, Jensen K, Sahlin K, Ørtenblad N (2014). Muscle glycogen content modifies SR Ca2+ release rate in elite endurance athletes. *Med Sci Sports Exerc*. 46(3):496-505.

Gellerich FN, Kapischke M, Kunz W, Neumann W, Kuznetsov A, Brdiczka D, Nicolay K (1994). The influence of the cytosolic oncotic pressure on the permeability of the mitochondrial outer membrane for ADP: implications for the kinetic properties of mitochondrial creatine kinase and for ADP channelling into the intermembrane space. *Mol Cell Biochem*. 133-134:85-104.

Glass, L. D., Cheng, A. J., & MacIntosh, B. R. (2018). Role of Ca^{2+} in changing active force during intermittent submaximal stimulation in intact, single mouse muscle fibers. *Pflugers Archiv : European journal of physiology*, *470*(8), 1243-1254.

Godt RE, Nosek TM (1989). Changes of intracellular milieu with fatigue or hypoxia depress contraction of skinned rabbit skeletal and cardiac muscle. *J Physiol*. 412:155-80.

Gong B, Legault D, Miki T, Seino S, Renaud JM (2003). KATP channels depress force by reducing action potential amplitude in mouse EDL and soleus muscle. *Am J Physiol Cell Physiol*. 285(6):C1464-74.

Goodman C, Blazev R, Stephenson G (2005). Glycogen content and contractile responsiveness to T-system depolarization in skinned muscle fibres of the rat. *Clin Exp Pharmacol Physiol*. 32(9):749-56.

Goodwin ML, Harris JE, Hernández A, Gladden LB (2017). Blood lactate measurements and analysis during exercise: a guide for clinicians. *J Diabetes Sci Technol*. 1(4):558-69.

Green HJ, Duhamel TA, Foley KP, Ouyang J, Smith IC, Stewart RD (2007). Glucose supplements increase human muscle in vitro Na+-K+-ATPase activity during prolonged exercise. *Am J Physiol Regul Integr Comp Physiol*. 293(1):R354-62.

Green HJ (1991). How important is endogenous muscle glycogen to fatigue in prolonged exercise? *Can J Physiol Pharmacol*. 69(2):290-7.

Han JC, Taberner AJ, Kirton RS, Nielsen PM, Archer R, Kim N, Loiselle DS (2011). Radius-dependent decline of performance in isolated cardiac muscle does not reflect inadequacy of diffusive oxygen supply. *Am J Physiol Heart Circ Physiol*. 300(4):H1222-36.

Hardie DG (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol*. 8(10):774-85.

Hargreaves M (2004). Muscle glycogen and metabolic regulation. *Proc Nutr Soc*. 63(2):217-20.

Harrison AP, Flatman JA (199). Measurement of force and both surface and deep M wave properties in isolated rat soleus muscles. *Am J Physiol*. 277(6):R1646-53.

Hartmann H, Probst I, Jungermann K, Creutzfeldt W (1987). Inhibition of glycogenolysis and glycogen phosphorylase by insulin and proinsulin in rat hepatocyte cultures. *Diabetes*. 36(5):551-5.

Helander I, Westerblad H, Katz A (2002). Effects of glucose on contractile function, [Ca2+]i, and glycogen in isolated mouse skeletal muscle. *Am J Physiol Cell Physiol*. 282(6):C1306-12.

Henriksen EJ, Holloszy JO (1991). Effect of diffusion distance on measurement of rat skeletal muscle glucose transport in vitro. *Acta Physiol Scand*. 143(4):381-6.

Hermansen L, Hultman E, Saltin B (1967). Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand*. 71(2):129-39.

Hicks A, McComas AJ (1989). Increased sodium pump activity following repetitive stimulation of rat soleus muscles. *J Physiol*. 414:337-49.

Hingst JR, Bruhn L, Hansen MB, Rosschou MF, Birk JB, Fentz J, Foretz M, Viollet B, Sakamoto K, Færgeman NJ, Havelund JF, Parker BL, James DE, Kiens B, Richter EA, Jensen J, Wojtaszewski JFP (2018). Exercise-induced molecular mechanisms promoting glycogen supercompensation in human skeletal muscle. *Mol Metab.* 16:24-34.

Hirvonen J, Nummela A, Rusko H, Rehunen S, Härkönen M (1992). Fatigue and changes of ATP, creatine phosphate, and lactate during the 400-m sprint. *Can J Sport Sci*. 17(2):141-4.

Ho K (2011). A critically swift response: insulin-stimulated potassium and glucose transport in skeletal muscle. *Clin J Am Soc Nephrol*. 6(7):1513-6.

Hong M, Kefaloyianni E, Bao L, Malester B, Delaroche D, Neubert TA, Coetzee WA (2011). Cardiac ATP-sensitive K+ channel associates with the glycolytic enzyme complex. *FASEB J*. 25(7):2456-67.

Hultman E (1995). Fuel selection, muscle fibre. *Proc Nutr Soc*. 54(1):107-21.

Hunter RW, Treebak JT, Wojtaszewski JF, Sakamoto K (2011). Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes.* 60(3):766-74.

Hüser J, Wang YG, Sheehan KA, Cifuentes F, Lipsius SL, Blatter LA (2000). Functional coupling between glycolysis and excitation-contraction coupling underlies alternans in cat heart cells. *J Physiol*. 524 Pt 3:795-806.

Ikematsu N, Dallas ML, Ross FA, Lewis RW, Rafferty JN, David JA, Suman R, Peers C, Hardie DG, Evans AM (2011). Phosphorylation of the voltage-gated potassium channel Kv2.1 by AMP-activated protein kinase regulates membrane excitability. *Proc Natl Acad Sci U S A.* 108(44):18132-7.

Inagaki N, Gonoi T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J, Seino S (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K+ channels. *Neuron*. 16(5):1011-7.

Ivy JL, Katz AL, Cutler CL, Sherman WM, Coyle EF (1985). Muscle glycogen synthesis after exercise: effect of time of carbohydrate ingestion. *J Appl Physiol*. 64(4):1480-5.

Jansson E, Dudley GA, Norman B, Tesch PA (1987). ATP and IMP in single human muscle fibres after high intensity exercise. *Clin Physiol*. 7(4):337-45.

Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR, Brand MD (2010). Mitochondrial proton and electron leaks. *Essays in biochemistry*, *47*, 53–67.

Jensen TE, Richter EA (2012). Regulation of glucose and glycogen metabolism during and after exercise. *J Physiol*. 590(5):1069-76.

Jentjens RL, Wagenmakers AJ, Jeukendrup AE (1985). Heat stress increases muscle glycogen use but reduces the oxidation of ingested carbohydrates during exercise. *J Appl Physiol*. 92(4):1562-72.

Jeppesen J, Kiens B (2012). Regulation and limitations to fatty acid oxidation during exercise. *J Physiol*. 590(5):1059-68.

Jørgensen SB, Jensen TE, Richter EA. Role of AMPK in skeletal muscle gene adaptation in relation to exercise. *Appl Physiol Nutr Metab*. 32(5):904-11.

Kabbara AA, Allen DG (1999). The role of calcium stores in fatigue of isolated single muscle fibres from the cane toad. *J Physiol*. 519 Pt 1:169-76.

Kabbara AA, Nguyen LT, Stephenson GM, Allen DG (2000). Intracellular calcium during fatigue of cane toad skeletal muscle in the absence of glucose. *J Muscle Res Cell Motil*. 21(5):481-9.

Karelis AD, Péronnet F, Gardiner PF (2002). Glucose infusion attenuates muscle fatigue in rat plantaris muscle during prolonged indirect stimulation in situ. *Exp Physiol*. 87(5):585-92.

Karelis AD, Péronnet F, Gardiner PF (2005). Resting membrane potential of rat plantaris muscle fibers after prolonged indirect stimulation in situ: effect of glucose infusion. *Can J Appl Physiol*. 30(1):105-12.

Karelis AD, Smith JW, Passe DH, Péronnet F (2010). Carbohydrate administration and exercise performance: what are the potential mechanisms involved? *Sports Med*. 40(9):747-63.

Kawanaka K, Han DH, Nolte LA, Hansen PA, Nakatani A, Holloszy JO (1999). Decreased insulin-stimulated GLUT-4 translocation in glycogen-supercompensated muscles of exercised rats. *Am J Physiol.* 276(5 Pt 1):E907-12.

Kemppainen J, Fujimoto T, Kalliokoski KK, Viljanen T, Nuutila P, Knuuti J (2002). Myocardial and skeletal muscle glucose uptake during exercise in humans. *J Physiol*. 15;542(Pt 2):403-12.

Kermode H, Chan WM, Williams AJ, Sitsapesan R (1998). Glycolytic pathway intermediates activate cardiac ryanodine receptors. *FEBS Lett*. 431(1):59-62.

Kockskämper J, Zima AV, Blatter LA (2005). Modulation of sarcoplasmic reticulum Ca2+ release by glycolysis in cat atrial myocytes. *J Physiol*. 564(Pt 3):697-714.

Kuiack S, McComas A (1992). Transient hyperpolarization of non-contracting muscle fibres in anaesthetized rats. *J Physiol.* 454:609-18.

Kushmerick MJ, Larson RE, Davies RE (1969). The chemical energetics of muscle contraction. I. Activation heat, heat of shortening and ATP utilization for activation-relaxation processes. *Proc R Soc Lond B Biol Sci.* 174(1036):293-313.

Lai YC, Lin FC, Jensen J (2009). Glycogen content regulates insulin- but not contraction-mediated glycogen synthase activation in the rat slow-twitch soleus muscles. *Acta Physiol (Oxf).* 197(2):139-50.

Lang F, Föller M (2014). Regulation of ion channels and transporters by AMP-activated kinase (AMPK). *Channels (Austin)*. 8(1):20-8.

Lauritzen HP (2013). Insulin- and contraction-induced glucose transporter 4 traffic in muscle: insights from a novel imaging approach. *Exercise and sport sciences reviews*, *41*(2), 77–86.

Laver DR, Lenz GK, Lamb GD (2001). Regulation of the calcium release channel from rabbit skeletal muscle by the nucleotides ATP, AMP, IMP and adenosine. *J Physiol.* 537(Pt 3):763-78.

Lee JA, Westerblad H, Allen DG (1991). Changes in tetanic and resting [Ca2+]i during fatigue and recovery of single muscle fibres from Xenopus laevis. *J Physiol*. 433:307-26.

Lees SJ, Franks PD, Spangenburg EE, Williams JH (1985). Glycogen and glycogen phosphorylase associated with sarcoplasmic reticulum: effects of fatiguing activity. *J Appl Physiol*. 91(4):1638-44.

Light PE (1999). Cardiac KATP channels and ischemic preconditioning: current perspectives. *Can J Cardiol.* 15(10):1123-30.

Lund S, Holman GD, Schmitz O, & Pedersen O. (1995). Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proceedings of the National Academy of Sciences of the United States of America*. 92(13), 5817–5821.

Mamedova LK, Shneyvays V, Katz A, Shainberg A (2003). Mechanism of glycogen supercompensation in rat skeletal muscle cultures. *Mol Cell Biochem*. 250(1-2):11-9.

Marcil M, Karelis AD, Péronnet F, Gardiner PF (2004). Glucose infusion attenuates fatigue without sparing glycogen in rat soleus muscle during prolonged electrical stimulation in situ. Eur *J Appl Physiol*. 93(5-6):569-74.

McBride A, Ghilagaber S, Nikolaev A, Hardie DG (2009). The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab*. 9(1):23-34.

McKenna MJ, Bangsbo J, Renaud JM (1985). Muscle K+, Na+, and Cl disturbances and Na+-K+ pump inactivation: implications for fatigue. *J Appl Physiol*. 104(1):288-95.

Meléndez-Hevia E, Waddell TG, Shelton ED (1993). Optimization of molecular design in the evolution of metabolism: the glycogen molecule. *Biochem J*. 295 (Pt 2):477-83.

Merrill GF, Kurth EJ, Hardie DG, Winder WW (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol*. 273(6 Pt 1):E1107-12.

Mookerjee SA, Gerencser AA, Nicholls DG, Brand MD (2017). Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. *J Biol Chem*. 292(17):7189-7207.

Moopanar TR, Allen DG (2005). Reactive oxygen species reduce myofibrillar Ca2+ sensitivity in fatiguing mouse skeletal muscle at 37 degrees C. *J Physiol*. 564(Pt 1):189-99.

Morales-Alamo D, Calbet JAL (2016). AMPK signaling in skeletal muscle during exercise: Role of reactive oxygen and nitrogen species. *Free Radic Biol Med*. 98:68-77.

Jessen N, Pold R, Buhl ES, Jensen LS, Schmitz O, Lund S (2002). Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscles. *J Appl Physiol*. 94(4):1373-9.

Musi N, Fujii N, Hirshman MF, Ekberg I, Fröberg S, Ljungqvist O, Thorell A, Goodyear LJ (2001). AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes*. 50(5):921-7.

Myburgh KH, Cooke R (1985). Response of compressed skinned skeletal muscle fibers to conditions that simulate fatigue. *J Appl Physiol*. 82(4):1297-304.

Nielsen J, Cheng AJ, Ørtenblad N, Westerblad H (2014). Subcellular distribution of glycogen and decreased tetanic Ca2+ in fatigued single intact mouse muscle fibres. *J Physiol*. 592(9):2003-12.

Nielsen J, Holmberg HC, Schrøder HD, Saltin B, Ortenblad N (2011). Human skeletal muscle glycogen utilization in exhaustive exercise: role of subcellular localization and fibre type. *J Physiol*. 589(Pt 11):2871-85.

Nielsen J, Suetta C, Hvid LG, Schrøder HD, Aagaard P, Ortenblad N (2010). Subcellular localization-dependent decrements in skeletal muscle glycogen and mitochondria content following short-term disuse in young and old men. *Am J Physiol Endocrinol Metab.* 299(6):E1053-60.

Nielsen OB, de Paoli F, Overgaard K (2001). Protective effects of lactic acid on force production in rat skeletal muscle. *J Physiol.* 536(Pt 1):161-6.

Nielsen OB, Overgaard K (1996). Ion gradients and contractility in skeletal muscle: the role of active Na+, K+ transport. *Acta Physiol Scand*. 156(3):247-56.

Novel-Chaté V, Rey V, Chioléro R, Schneiter P, Leverve X, Jéquier E, Tappy L (2001). Role of Na+-K+-ATPase in insulin-induced lactate release by skeletal muscle. *Am J Physiol Endocrinol Metab*. 280(2):E296-300.

Okamoto K, Wang W, Rounds J, Chambers EA, Jacobs DO (2001). ATP from glycolysis is required for normal sodium homeostasis in resting fast-twitch rodent skeletal muscle. *Am J Physiol Endocrinol Metab.* 281(3):E479-88.

O'Neill HM, Maarbjerg SJ, Crane JD, Jeppesen J, Jørgensen SB, Schertzer JD, Shyroka O, Kiens B, van Denderen BJ, Tarnopolsky MA, Kemp BE, Richter EA, Steinberg GR (2011). AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc Natl Acad Sci U S A.* 108(38):16092-7.

O'Neill HM, Lally JS, Galic S, Thomas M, Azizi PD, Fullerton MD, Smith BK, Pulinilkunnil T, Chen Z, Samaan MC, Jorgensen SB, Dyck JR, Holloway GP, Hawke TJ, van Denderen BJ, Kemp BE, Steinberg GR (2014). AMPK phosphorylation of ACC2 is required for skeletal muscle fatty acid oxidation and insulin sensitivity in mice. *Diabetologia*. 57(8):1693-702.

Ørtenblad N, Nielsen J, Saltin B, Holmberg HC (2011). Role of glycogen availability in sarcoplasmic reticulum Ca2+ kinetics in human skeletal muscle. *J Physiol*. 589(Pt 3):711-25.

Ørtenblad N, Westerblad H, Nielsen J. Muscle glycogen stores and fatigue (2013). *J Physiol.* 591(18):4405-13.

Owen VJ, Lamb GD, Stephenson DG (1996). Effect of low [ATP] on depolarization-induced Ca2+ release in skeletal muscle fibres of the toad. *J Physiol*. 493 (Pt 2):309-15.

Pate E, Bhimani M, Franks-Skiba K, Cooke R (1995). Reduced effect of pH on skinned rabbit psoas muscle mechanics at high temperatures: implications for fatigue. *J Physiol.* 486 (Pt 3):689-94.

Pedersen TH, de Paoli F, Nielsen OB (2005). Increased excitability of acidified skeletal muscle: role of chloride conductance. *J Gen Physiol*. 125(2):237-46.

Pedersen TH, de Paoli FV, Flatman JA, Nielsen OB (2009a). Regulation of ClC-1 and KATP channels in action potential-firing fast-twitch muscle fibers. *J Gen Physiol*. 134(4):309-22.

Pedersen TH, Macdonald WA, de Paoli FV, Gurung IS, Nielsen OB (2009b). Comparison of regulated passive membrane conductance in action potential-firing fast- and slow-twitch muscle. *J Gen Physiol*. 134(4):323-37.

Pedersen TH, Riisager A, de Paoli FV, Chen TY, Nielsen OB. Role of physiological ClC-1 Cl- ion channel regulation for the excitability and function of working skeletal muscle. *J Gen Physiol*. 147(4):291-308.

Quinlan KG, Seto JT, Turner N, Vandebrouck A, Floetenmeyer M, Macarthur DG, Raftery JM, Lek M, Yang N, Parton RG, Cooney GJ, North KN (2010). Alpha-actinin-3 deficiency results in reduced glycogen phosphorylase activity and altered calcium handling in skeletal muscle. *Hum Mol Genet*. 19(7):1335-46.

Rabinovitch RC, Samborska B, Faubert B, Ma EH, Gravel SP, Andrzejewski S, Raissi TC, Pause A, St-Pierre J, Jones RG (2017). AMPK Maintains Cellular Metabolic Homeostasis through Regulation of Mitochondrial Reactive Oxygen Species. *Cell Rep*. 21(1):1-9.

Rall JA (1985). Energetic aspects of skeletal muscle contraction: implications of fiber types. *Exerc Sport Sci Rev*. 1985;13:33-74.

Rauch HG, St Clair Gibson A, Lambert EV, Noakes TD (2005). A signalling role for muscle glycogen in the regulation of pace during prolonged exercise. *Br J Sports Med*. 39(1):34-8.

Richter EA, Hargreaves M (2013). Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev.* 93(3):993-1017.

Robergs RA, Ghiasvand F, Parker D (2004). Biochemistry of exercise-induced metabolic acidosis. *Am J Physiol Regul Integr Comp Physiol*. 287(3):R502-16.

Sampson SR (1995). Activation of protein kinase C mediates insulin regulation of the Na-K pump in cultured skeletal muscle. *Adv Exp Med Biol*. 381:47-56.

Sandiford SD, Green HJ, Duhamel TA, Perco JG, Schertzer JD, Ouyang J (1985). Inactivation of human muscle Na+-K+-ATPase in vitro during prolonged exercise is increased with hypoxia. *J Appl Physiol.* 96(5):1767-75.

Sano A, Koshinaka K, Abe N, Morifuji M, Koga J, Kawasaki E, Kawanaka K (2012). The effect of high-intensity intermittent swimming on post-exercise glycogen supercompensation in rat skeletal muscle. *J Physiol Sci.* 62(1):1-9.

Schiaffino S, Reggiani C (2011). Fiber types in mammalian skeletal muscles. *Physiol Rev*. 91(4):1447-531.

Scott K, Benkhalti M, Calvert ND, Paquette M, Zhen L, Harper ME, Al-Dirbashi OY, Renaud JM (2016). KATP channel deficiency in mouse FDB causes an impairment of energy metabolism during fatigue. *Am J Physiol Cell Physiol*. 311(4):C559-C571.

Selvin D, Hesse E, Renaud JM (2015). Properties of single FDB fibers following a collagenase digestion for studying contractility, fatigue, and pCa-sarcomere shortening relationship. *Am J Physiol Regul Integr Comp Physiol.* 308(6):R467-79.

Selvin D, Renaud JM (2015). Changes in myoplasmic Ca2+ during fatigue differ between FDB fibers, between glibenclamide-exposed and Kir6.2-/- fibers and are further modulated by verapamil. *Physiol Rep*. 3(3). pii: e12303.

Sepp M, Sokolova N, Jugai S, Mandel M, Peterson P, Vendelin M (2014). Tight coupling of Na+/K+-ATPase with glycolysis demonstrated in permeabilized rat cardiomyocytes. *PLoS One*. 9(6):e99413.

Serysheva II, Schatz M, van Heel M, Chiu W, Hamilton SL (1999). Structure of the skeletal muscle calcium release channel activated with Ca2+ and AMP-PCP. *Biophys J*. 77(4):1936-44.

Shulman RG, Rothman DL (2001). The "glycogen shunt" in exercising muscle: A role for glycogen in muscle energetics and fatigue. *Proc Natl Acad Sci U S A*. 98(2):457-61.

Shulman RG (2005). Glycogen turnover forms lactate during exercise. *Exerc Sport Sci Rev*. 33(4):157-62.

Söderlund K, Hultman E (1990). ATP content in single fibres from human skeletal muscle after electrical stimulation and during recovery. *Acta Physiol Scand*. 139(3):459-66.

Sogaard P, Szekeres F, Holmström M, Larsson D, Harlén M, Garcia-Roves P, Chibalin AV (2009). Effects of fibre type and diffusion distance on mouse skeletal muscle glycogen content in vitro. *J Cell Biochem*. 107(6):1189-97.

Spriet LL (1990). Anaerobic ATP provision, glycogenolysis and glycolysis in rat slow-twitch muscle during tetanic contractions. *Pflugers Arch*. 417(3):278-84.

Stephenson DG, Nguyen LT, Stephenson GM (1999). Glycogen content and excitation-contraction coupling in mechanically skinned muscle fibres of the cane toad. *J Physiol*. 519 Pt 1:177-87.

Stewart RD, Duhamel TA, Foley KP, Ouyang J, Smith IC, Green HJ (2007). Protection of muscle membrane excitability during prolonged cycle exercise with glucose supplementation. *J Appl Physiol.* 103(1):331-9.

Stump CS, Short KR, Bigelow ML, Schimke JM, Nair KS (2003). Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. *Proc Natl Acad Sci U S A.* 100(13):7996-8001.

Sukhodub A, Jovanović S, Du Q, Budas G, Clelland AK, Shen M, Sakamoto K, Tian R, Jovanović A (2007). AMP-activated protein kinase mediates preconditioning in cardiomyocytes by regulating activity and trafficking of sarcolemmal ATP-sensitive K(+) channels. *J Cell Physiol*. 210(1):224-36.

Sweeney G, Klip A (1998). Regulation of the Na+/K+-ATPase by insulin: why and how? *Mol Cell Biochem*. 182(1-2):121-33.

Tanaka T, Hidaka S, Masuzaki H, Yasue S, Minokoshi Y, Ebihara K, Chusho H, Ogawa Y, Toyoda T, Sato K, Miyanaga F, Fujimoto M, Tomita T, Kusakabe T, Kobayashi N, Tanioka H, Hayashi T, Hosoda K, Yoshimatsu H, Sakata T, Nakao K (2005). Skeletal muscle AMP-activated protein kinase phosphorylation parallels metabolic phenotype in leptin transgenic mice under dietary modification. *Diabetes.* 54(8):2365-74.

Taylor DJ, Styles P, Matthews PM, Arnold DA, Gadian DG, Bore P, Radda GK (1986). Energetics of human muscle: exercise-induced ATP depletion. *Magn Reson Med*. 3(1):44-54.

Thabet M, Miki T, Seino S, Renaud JM (2005). Treadmill running causes significant fiber damage in skeletal muscle of KATP channel-deficient mice. *Physiol Genomics*. 22(2):204-12.

Thomson DM, Winder WW (2009). AMP-activated protein kinase control of fat metabolism in skeletal muscle. *Acta Physiol (Oxf).* 196(1):147-54.

Tremblay F, Dubois MJ, Marette A (2003). Regulation of GLUT4 traffic and function by insulin and contraction in skeletal muscle. *Front Biosci*. Sep 1;8:d1072-84.

Vøllestad NK, Blom PC (1985). Effect of varying exercise intensity on glycogen depletion in human muscle fibres. *Acta Physiol Scand*. 125(3):395-405.

Vøllestad NK, Tabata I, Medbø JI (1992). Glycogen breakdown in different human muscle fibre types during exhaustive exercise of short duration. *Acta Physiol Scand*. 144(2):135-41.

Wan JJ, Qin Z, Wang PY, Sun Y, Liu X (2017). Muscle fatigue: general understanding and treatment. *Exp Mol Med.* 49(10):e384.

Westerblad H, Allen DG, Lännergren J (2002). Muscle fatigue: lactic acid or inorganic phosphate the major cause? *News Physiol Sci.* 17:17-21.

Westerblad H, Allen DG (1991). Changes of myoplasmic calcium concentration during fatigue in single mouse muscle fibers. *J Gen Physiol*. 98(3):615-35.

Westerblad H, Allen DG (1993). The contribution of [Ca2+]i to the slowing of relaxation in fatigued single fibres from mouse skeletal muscle. *J Physiol.* 468:729-40.

Westerblad H, Lännergren J, Allen DG (1997). Slowed relaxation in fatigued skeletal muscle fibers of Xenopus and Mouse. Contribution of [Ca2+]i and cross-bridges. *J Gen Physiol*. 109(3):385-99.

Widrick JJ, Romatowski JG, Norenberg KM, Knuth ST, Bain JL, Riley DA, Trappe SW, Trappe TA, Costill DL, Fitts RH (1985). Functional properties of slow and fast gastrocnemius muscle fibers after a 17-day spaceflight. *J Appl Physiol*. 90(6):2203-11.

Xu KY, Becker LC (1998). Ultrastructural localization of glycolytic enzymes on sarcoplasmic reticulum vesticles. *J Histochem Cytochem*. 46(4):419-27.

Xu KY, Zweier JL, Becker LC (1995). Functional coupling between glycolysis and sarcoplasmic reticulum Ca2+ transport. *Circ Res*. 77(1):88-97.

Yoshida H, Bao L, Kefaloyianni E, Taskin E, Okorie U, Hong M, Dhar-Chowdhury P, Kaneko M, Coetzee WA (2012). AMP-activated protein kinase connects cellular energy metabolism to KATP channel function. *J Mol Cell Cardiol*. 52(2):410-8.

Zając A, Chalimoniuk M, Maszczyk A, Gołaś A, Lngfort J (2015). Central and Peripheral Fatigue During Resistance Exercise - A Critical Review. *J Hum Kinet.* 49:159-69.

Zhang XD, Tseng PY, Chen TY (2008). ATP inhibition of CLC-1 is controlled by oxidation and reduction. *J Gen Physiol*. 132(4):421-8.

Zhu Z, Sierra A, Burnett CM, Chen B, Subbotina E, Koganti SR, Gao Z, Wu Y, Anderson ME, Song LS, Goldhamer DJ, Coetzee WA, Hodgson-Zingman DM, Zingman LV (2014). Sarcolemmal ATP-sensitive potassium channels modulate skeletal muscle function under low-intensity workloads. *J Gen Physiol*. 143(1):119-34.

Zima AV, Kockskämper J, Blatter LA (2006). Cytosolic energy reserves determine the effect of glycolytic sugar phosphates on sarcoplasmic reticulum Ca2+ release in cat ventricular myocytes. *J Physiol.* 577(Pt 1):281-93.