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Ryanodine Receptor Modifications Following Sprint Interval Exercise: Time Course and Fibre-Specific Responses in Human Skeletal Muscle

Tripp, Thomas R.

UNIVERSITY OF CALGARY

Ryanodine Receptor Modifications Following Sprint Interval Exercise: Time Course and Fibre-Specific Responses in Human Skeletal Muscle

by

Thomas Tripp

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Abstract

It was recently discovered that a single session of sprint interval training (SIT) resulted in fragmentation of the sarcoplasmic reticulum calcium (Ca^{2+})-release channel, ryanodine receptor 1 (RyR1), leading to an increase in intracellular [Ca^{2+}], a known signal for mitochondrial biogenesis; however, it was unclear whether fragmentation could initiate mitochondrial biogenesis, as RyR1 fragmentation had only been observed 24 h post-exercise in humans, and most signalling events occur much earlier. The primary objective of this thesis was to characterize the time course of RyR1 modifications following SIT in whole muscle and pooled type I and type IIa muscle fibres in humans. The secondary objective was to assess if SIT affected the content of other Ca^{2+}-handling proteins alongside RyR1. We collected muscle biopsy samples from recreationally-active males (n = 6) and females (n = 4) before and 3 h, 6 h, and ~24 h after they completed a single session of SIT (6 x 30-s 'all-out' with 4.5 min rest). Western blotting experiments showed that full-length RyR1 content was significantly lower at 6 h (-44 ± 31%; p = 0.01) and 24 h (-35 ± 42%; p = 0.02) compared to pre-exercise. RyR1 content was also lower than pre-exercise at 6 h in type IIa fibres (-21 ± 17%; p = 0.01) but not type I fibres (-2 ± 43%; p > 0.05). Aside from FKBP12, which was higher at 24 h compared to 3 h (26 ± 29%; p = 0.03) and 6 h (24 ± 23%; p = 0.03), Ca^{2+}-handling protein content was stable in whole muscle (all p > 0.05). Our results indicate that RyR1 fragmentation is fibre type-dependent and occurs on a similar time course to other relevant signalling events reported elsewhere in the literature. RyR1 fragmentation, in conjunction with stable content of other Ca^{2+}-handling proteins, supports previous work suggesting a post-SIT increase in intracellular [Ca^{2+}] is primarily due to RyR1-related Ca^{2+} leak. Overall, this thesis supports a role of RyR1 modifications in triggering mitochondrial adaptations in response to sprint interval training.

Preface

This thesis is original, unpublished work by the author, Thomas Tripp. The experiment reported in chapter 3 was covered by Ethics Certificate number 19-0423, issued by the University of Calgary Conjoint Health Ethics Board for the project “Ryanodine receptor fragmentation in response to exercise intensity” on May 28, 2019.

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Thank you to my excellent committee members, Dr. Juan Murias and Dr. Jane Shearer. Your feedback and support throughout this thesis—from the original planning, to the several times those plans had to change, to preparing to defend—is immensely appreciated. Thank you as well to Dr. Preston Wiley and Dr. Victor Lun for performing the biopsy procedures while working around busy clinic schedules to make this type of time course study possible.

Thank you to my lab mates for making time inside and outside of the lab so enjoyable. I am grateful for the breaks from lab work to go outside and play Spikeball and the countless walks over to Mac Hall for coffee; I apologize for any caffeine addictions I may have contributed to. Thank you especially to those who assisted with this project: Austin and Madi, your help with biopsy collection and running testing sessions made everything so much smoother; Barney and Graham, learning from and working alongside both of you in the wet lab contributed substantially to the success of this project.

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List of Symbols, Abbreviations, and Nomenclature

Abbreviation	Definition
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATF-2	Activating transcription factor-2
ATP	Adenosine triphosphate
BHAD	3-hydroxyacyl-CoA dehydrogenase
Ca^{2+}	Calcium ion
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CAT	Catalase
CO_2	Carbon dioxide
COX	Cytochrome c oxidase
CRE	Cyclic-AMP response element
CREB	Cyclic-AMP response element binding protein
CS	Citrate synthase
CSQ	Calsequestrin
DHPR	Dihydropyridine receptor
DSHB	Developmental Studies Hybridoma Bank
DXA	Dual-energy x-ray absorptiometry
E-C coupling	Excitation-contraction coupling
FKBP12	FK506-binding protein 12-kDa
FSR	Fractional synthetic rate
GPX	Glutathione peroxidase
H^+	Hydrogen ion
HCl	Hydrogen chloride
HDAC	Histone deacetylase
HIIT	High-intensity interval training
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMP	Inosine monophosphate
KCl	Potassium chloride
MAPK	Mitogen-activated protein kinase
Mg^{2+}	Magnesium ion
MHC	Myosin heavy chain
MICT	Moderate-intensity continuous training
mRNA	Messenger RNA
N_2	Molecular nitrogen
NaCl	Sodium chloride
NaN_3	Sodium azide
NRF1	Nuclear respiratory factor 1

O_2	Molecular oxygen
PBST	Phosphate-buffered saline with Tween-20
PCr	Phosphocreatine
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
P_i	Inorganic phosphate
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
RST	Repeated sprint training
RyR1	Ryanodine receptor 1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERCA	Sarco(endo)plasmic reticulum ATPase
SIT	Sprint interval training
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TBST	Tris-buffered saline with Tween-20
TFAM	Mitochondrial transcription factor
VL	Vastus lateralis muscle
$\dot{V}O_2max$	Maximal oxygen uptake

Chapter One: Introduction

Aerobic exercise training markedly changes skeletal muscle to improve fatigue resistance and attenuate homeostatic disruptions in response to future metabolic challenges. One of the most notable adaptations to aerobic exercise is an increase in mitochondrial content, which improves aerobic energy provision, glycogen sparing, and submaximal exercise performance (Holloszy & Coyle, 1984). While aerobic exercise is a potent stimulus to increase mitochondrial content in skeletal muscle through the production and incorporation of new proteins into the mitochondrial reticulum, the mechanisms through which this adaptation occurs are complex. Adaptive responses can be manipulated through the various parameters that comprise an aerobic exercise session or training program, such as structure (i.e., continuous vs. interval exercise), intensity (i.e., rate of work), duration (i.e., session length), frequency (i.e., sessions/week), or volume (i.e., intensity • duration • frequency). Understanding how alterations to exercise parameters can maximize training adaptations—and how seemingly disparate types of exercise can lead to similar adaptive responses—is of interest to researchers, exercise physiologists, coaches, high-level athletes, recreationally-active individuals, and clinicians.

In recent years, the effectiveness of sprint interval training (SIT), involving only a small amount of total work per session (e.g., 3 minutes divided into six "all-out" intervals), has been a specific research focus because it induces adaptations traditionally associated with prolonged, lower intensity exercise (i.e., traditional endurance training). Within this field of research, the mechanisms through which small volumes of high-intensity exercise augment increases in mitochondrial content have been studied intensely. Recently, Place and colleagues discovered that the sarcoplasmic reticulum (SR) calcium (Ca^{2+})-release channel, ryanodine receptor 1 (RyR1), was fragmented 24 h after a single session of sprint interval exercise (Place *et al.*, 2015),

a finding that has since been replicated by the same research group (Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020). Modifications to RyR1, including fragmentation, cause Ca^{2+} leak and may generate a signal leading to mitochondrial biogenesis (Wright *et al.*, 2007; Ivarsson *et al.*, 2019), but many aspects of the RyR1 fragmentation mechanism remain unknown. Specifically, the time course of this mechanism, the simultaneous response of related Ca^{2+}-handling proteins, and the mediating role of skeletal muscle fibre-type require further investigation. Studying these aspects of the RyR1 fragmentation mechanism will enhance our understanding of its relevance and role in sprint exercise-induced mitochondrial adaptations.

The following chapter contains a literature review examining our current understanding of the acute time course of exercise-induced mitochondrial biogenesis, the role of Ca^{2+} as a signal for adaptations, and the Ca^{2+}-handling modifications that may produce an adaptive signal. Throughout the literature review, the focus is on sprint interval exercise. Chapter Three contains our novel contribution to the research field, written as a research manuscript to be published at a later date. Finally, the concluding chapter includes the implications and limitations of the presented research, as well as future directions for the research area.

Chapter Two: Literature Review

2.1 Introduction

The purpose of this literature review is to explore past research in several areas surrounding the RyR1 modification mechanism. It will provide an overview of the acute adaptive time course of molecular events involved in expanding the mitochondrial reticulum in response to a single aerobic exercise session, followed by an examination of how exercise intensity affects signalling responses. Subsequently, a description of skeletal muscle Ca^{2+}-handling and the specific role of Ca^{2+} as an adaptive signal will lead into what is currently known about exercise-induced RyR1 modifications. Finally, reactive oxygen species production (ROS) and skeletal muscle fibre-type—two factors that may mediate RyR1 modifications—will be discussed. This review will highlight why studying this mechanism is important for improving our understanding of mitochondrial adaptations to aerobic exercise.

2.2 Mitochondrial Adaptation to Exercise: Molecular Events and their Time Course

Though the exact mechanisms remain unknown, the general molecular process through which aerobic exercise stimulates production of mitochondrial proteins in skeletal muscle can be described with reasonable accuracy. A critical component of the adaptive response is the time course of molecular events. Molecular responses are not dictated solely by the time at which they peak, as other aspects of the response, such as magnitude of the increase and duration of the response, also influence downstream effects. Measuring molecular events at key time points aids in evaluating the relevance of novel mechanisms and helps compare diverse exercise interventions (Granata *et al.*, 2018). Before RyR1's potential role in this molecular pathway can be addressed, an overview of our current understanding of the other steps along the pathway is necessary. This section will describe the steps in the signal transduction process (summarized in

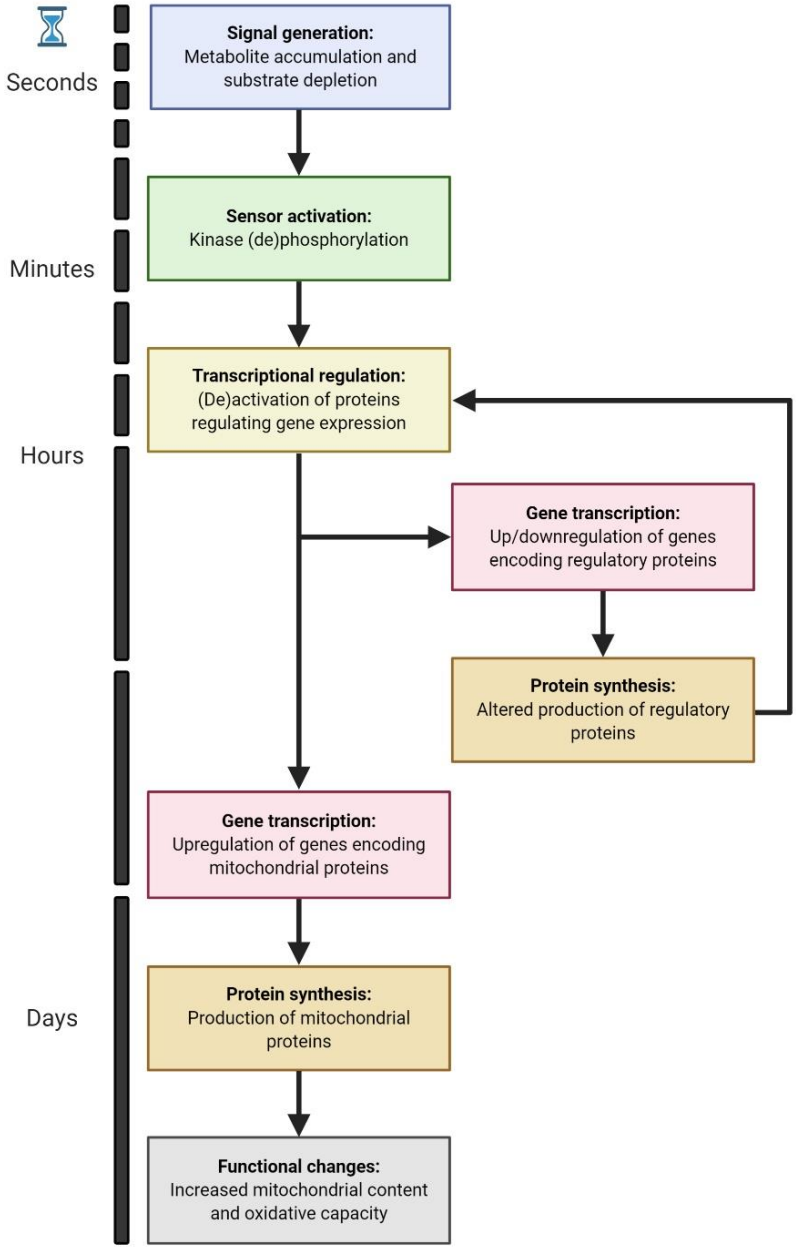

Figure 2.1: Molecular events leading to mitochondrial biogenesis. This schematic depicts the steps of exercise-induced increases in mitochondrial content in skeletal muscle. Muscle contraction generates signals (*blue box*; ADP, P_i, Ca^{2+}, etc.) that are sensed by signalling kinases (*green box*; AMP-activated protein kinase, p38 mitogen-activated protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, etc.). When activated, these signalling kinases alter the activity of proteins regulating gene expression (*yellow box*; peroxisome proliferator-activated gamma receptor coactivator 1-alpha, histone deacetylases, cyclic AMP response element binding proteins, activating transcription factor-2, etc.). These proteins interact with genes to affect gene expression *(red boxes*): They promote transcription of genes encoding mitochondrial proteins (*lower red box*) but also affect genes encoding transcriptional co-activators such as themselves (*higher red box*). Once these genes are translated into proteins (*higher gold box*), they create a loop to amplify the original signalling effect. Greater transcription leads to more mitochondrial gene transcription, followed by protein synthesis (*lower gold box*). Proteins are assembled and incorporated into the cellular structure, and functional adaptations are realized (*grey box*) For simplicity, the pathways contributing to other aspects of mitochondrial content regulation like mitophagy, fusion, and fission are not included. Created with BioRender.com.

Figure 2.1) and when each response occurs from the beginning of a single session of exercise through the recovery period.

From the onset of exercise, muscle contractions markedly increase the cellular energy requirement compared to rest and generate signals for adaptation. These signals include Ca^{2+} cycling, ROS production, oxygen (O_2) consumption, metabolite accumulation (i.e., ADP, AMP, inorganic phosphate (P_i), lactate, H^+, etc.) and substrate depletion (i.e., ATP, phosphocreatine (PCr), glycogen) (Hood, 2009). Homeostatic perturbations start at the beginning of exercise but may stabilize or continue to change throughout the session, depending on the intensity of exercise being performed. In the heavy-intensity domain, concentrations of metabolites such as ADP, AMP, and lactate increase from rest, but stabilize as exercise continues (Howlett *et al.*, 1998; Black *et al.*, 2017). In the severe-intensity domain (e.g., short, high-intensity bouts or prolonged exercise) decreases in [ATP], [PCr], and glycogen and increases in [lactate], [ADP], and [AMP] continue until exercise ends (Howlett *et al.*, 1998; Black *et al.*, 2017; Fiorenza *et al.*, 2018).

Metabolite and substrate concentration changes activate signalling cascades, including the phosphorylation or dephosphorylation of a network of kinases (Hoffman *et al.*, 2015) such as AMP-activated protein kinase (AMPK) (Marcinko & Steinberg, 2014), p38 mitogen-activated protein kinase (MAPK) (Menzies *et al.*, 2013), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Rose & Hargreaves, 2003). These kinases are signal sensors and trigger downstream events based on the status of the signal they detect by phosphorylating regulatory proteins. Kinase activation appears to occur immediately after the onset of exercise (Rose *et al.*, 2006): CaMKII phosphorylation increased ~12-fold from rest after 1 min of exercise before stabilizing at ~2-fold resting activity from minutes 10 to 90 of a continuous exercise session (Rose *et al.*,

2006). Unfortunately, few other studies provide the same temporal information during exercise as that of Rose and colleagues (2006). Most other research suggests that signalling kinase activity reaches its maximum level either during or by the end of exercise: kinase phosphorylation (AMPK, CaMKII, and p38 MAPK) peaks immediately after the cessation of the exercise session (Fiorenza *et al.*, 2018) then declines, returning to resting values by the next sample time point (most frequently 3 h into recovery) (Gibala *et al.*, 2009; Little *et al.*, 2011; Bartlett *et al.*, 2012; Granata *et al.*, 2017). Overall, it seems kinase activation closely mirrors the signals that activate them: kinases activate rapidly upon signal generation and remain active throughout exercise and for a short period following the cessation of exercise, when signals gradually subside.

Signalling kinases activate proteins that regulate gene expression, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, considered the 'master regulator' of mitochondrial biogenesis (Hood, 2009)) and the tumor suppressor protein p53 (Egan & Zierath, 2013; Granata *et al.*, 2018). AMPK and p38 MAPK phosphorylate PGC-1α, increasing its stability (Jäer *et al.*, 2007), and allow it to translocate from the cytosol to the nucleus. PGC-1α deacetylation, also required for increased PGC-1α activity, is further modulated by AMPK via sirtuin 1 (Cantó *et al.*, 2009). A session of continuous exercise increased nuclear PGC-1α without a change in total PGC-1α immediately post-exercise (Little *et al.*, 2010), and all-out interval exercise did the same 3 h post-exercise (Little *et al.*, 2011). Similar timing was observed for an increase in phosphorylated p53 in both the nucleus and cytosol (Granata *et al.*, 2017). Transcription factor DNA binding activity (e.g., histone deacetylase 4, 5, or 7 (HDAC 4/5/7); cyclic-AMP response element binding protein (CREB); or activating transcription factor-2 (ATF-2)) is also altered by upstream kinases: phosphorylation of HDAC 4/5/7 and ATF-2 is

elevated immediately following exercise, and CREB phosphorylation is elevated 3 h into recovery (Egan *et al.*, 2010). HDAC phosphorylation results in its exclusion from the nucleus, so it no longer inhibits the activity of myocyte enhancing factor 2 to promote gene transcription. CREB—phosphorylated by AMPK and CaMKII—and ATF-2—activated by p38 MAPK—act on the CRE site in the *PGC1A* gene promoter to increase its expression (Egan *et al.*, 2010).

Upregulation of multiple regulatory proteins coordinates mitochondrial gene expression. PGC-1α, in conjunction with other factors such as CREB and ATF-2, amplifies the initial signalling effect by promoting its own expression. *PGC1A* mRNA increases with the progression of recovery from exercise, with no changes observed immediately following an exercise session, marked increases apparent by 2 h and lasting for up to 6 h, and a return to resting levels at 24 h (Pilegaard *et al.*, 2003; Perry *et al.*, 2010). These findings from human skeletal muscle have since been supported by numerous studies of a variety of exercise sessions, with sampling most frequently performed at 3 h post-exercise (Egan *et al.*, 2010; Little *et al.*, 2011; Bartlett *et al.*, 2012; Serpiello *et al.*, 2012; Stepto *et al.*, 2012; Cochran *et al.*, 2014; Granata *et al.*, 2018). Upregulation of genes encoding other transcription factors, such as nuclear respiratory factor 1 (*NRF1*) and the mitochondrial transcription factor (*TFAM*), occur simultaneously (Psilander *et al.*, 2013; Fiorenza *et al.*, 2018) or on slightly longer time scales (i.e., up to 24 h) than that of *PGC1A* (Perry *et al.*, 2010; Psilander *et al.*, 2013).

Upregulation of genes encoding mitochondrial proteins—such as components of the electron transport chain and enzymes that facilitate substrate oxidation within the mitochondria (e.g., citrate synthase (*CS*) and 3-hydroxyacyl-CoA dehydrogenase (*BHAD*))—occurs later in recovery from exercise. Several studies have shown no changes in mitochondrial gene expression within the first 3-5 h of recovery (Perry *et al.*, 2010; Stepto *et al.*, 2012; Percival *et*

al., 2015), albeit with some conflicting evidence (Little *et al.*, 2011). In another study, gene expression significantly increased between 10 and 24 h post-exercise, although the patterns and timing of peak expression varied by gene (Leick *et al.*, 2010). Certain genes may require greater increases in transcriptional regulators to increase their expression, which may explain why only *BHAD* mRNA was elevated at 4-8 h post-exercise (Leick *et al.*, 2010; Perry *et al.*, 2010) although both *CS* and *BHAD* mRNA were increased at later timepoints (18-24 h).

After mitochondrial genes are transcribed, proteins are produced from the mRNA. Protein synthesis comprises the production of new proteins via translation of mRNA and occurs over a longer duration than the other steps of the signal transduction pathway. As measures of protein synthesis are integrative, they capture rates over time rather than a value at a single point. An endurance exercise session increased mitochondrial protein fractional synthetic rate (FSR; the amount of newly synthesized protein as a percentage of total protein per unit time) compared to rest during the 4 h post-exercise (Wilkinson *et al.*, 2008; Donges *et al.*, 2012). Higher intensity exercise also leads to greater sarcoplasmic FSR over 24 h following exercise (Bell *et al.*, 2015); however, sarcoplasmic FSR encompasses all non-myofibrillar proteins, not only those in the mitochondria. These results are supported by the findings of Di Donato and colleagues, which showed greater mitochondrial FSR at 24-28 h following a high-intensity session compared to a work-matched low-intensity session but with only a "trend" to be greater than resting rates (Di Donato *et al.*, 2014).

Insights from the response to a single session of exercise are useful in uncovering mechanistic information, but lasting protein expression and functional changes rarely occur with just one exercise session (Leick *et al.*, 2010; Perry *et al.*, 2010; Egan *et al.*, 2013). Rather, periods of regular exercise and recovery trigger repeated increases in mitochondrial gene

expression, which eventually lead to increased mitochondrial protein content (Perry *et al.*, 2010; Egan *et al.*, 2013). Exercise-induced changes in mitochondrial content are commonly assessed using measures of mitochondrial DNA abundance, content or activity of specific proteins (e.g., CS and cytochrome c oxidase [COX]), respiration rates of isolated mitochondria or permeabilized muscle fibres, or mitochondrial density via electron microscopy (Larsen *et al.*, 2012; Miller & Hamilton, 2012). Frequently, exercise protocols are compared on their ability to acutely trigger molecular events related to mitochondrial biogenesis or on the adaptations produced when the protocol is performed regularly as a training program. While acute signalling and chronic adaptations are linked, they are not necessarily interchangeable with respect to the information they provide. Different exercise sessions can trigger similar acute responses, like increased p38 MAPK phosphorylation and *PGC1A* mRNA content, without driving similar chronic increases in mitochondrial protein content (Cochran *et al.*, 2014; Gunnarsson *et al.*, 2019).

Acutely, any exercise expected to stimulate mitochondrial biogenesis must generate contractile and metabolic demands sufficient to activate the appropriate signalling cascades. As adaptations develop, the same absolute exercise demands produce diminishing responses: the adaptations themselves blunt the homeostatic disruptions that initiate molecular responses (Neufer & Booth, 2005; Williams & Neufer, 2011; Serpiello *et al.*, 2012; Stepto *et al.*, 2012; Granata *et al.*, 2019; Gunnarsson *et al.*, 2019). In this context, effective aerobic exercise training protocols are expected to produce detectably stable increases in the abundance of mitochondrial proteins in skeletal muscle cells (Granata *et al.*, 2020).

2.3 The Importance of Exercise Intensity

2.3.1 Common Aerobic Exercise Training Protocols

Intensity, the rate of energy transfer in the body, is a key determinant of muscular adaptations to exercise training (MacInnis & Gibala, 2017; MacInnis *et al.*, 2019). Interval exercise, comprised of intermittent high-intensity bouts interspersed with periods of active or passive recovery, has been used to interrogate questions related to the role of exercise intensity in promoting endurance adaptations in skeletal muscle. Variations in session structure, interval duration, number of intervals, rest duration, and type of rest are found throughout the interval training literature and allow for the creation of a virtually limitless number of unique interval exercise sessions. Researchers have defined general categories of interval exercise to facilitate comparisons within and across studies. For consistency, the terminology used here will incorporate the definitions from Buchheit and Laursen (2013) and those used by MacInnis and Gibala (2017), as proposed by Weston et al. (2014). These definitions divide interval training into three main categories, moving from the longest to the shortest interval durations: high-intensity interval training (HIIT), sprint interval training (SIT), and repeated sprint training (RST). HIIT is most often prescribed as intervals eliciting ≥ 80-90% of maximum heart rate or > 90% of maximal oxygen uptake ($\dot{V}O_2max$) but should occur in the severe exercise intensity domain (i.e., above the maximal lactate steady state or critical power). These intervals typically last 1.5-6 minutes and are separated by recovery periods of equal or slightly lesser duration. SIT is comprised of short near-maximal or 'all-out' efforts (i.e., 20-90 s at power outputs ranging from the peak power output from an incremental test (PPO), up to 400-800 W (Gibala *et al.*, 2009; Schlittler *et al.*, 2019)), interspersed with proportionally longer rest periods of around 2-4 minutes (Buchheit & Laursen, 2013). RST consists of extremely brief 'all-out' sprints (3-10 s)

broken up by relatively short rest periods and performed as repeated sets of sprints (e.g., 3 sets of 5 x 4-s sprints with 20 s rest between sprints and 4.5 minutes between sets; Serpiello et al., 2012).

Researchers often compare interval exercise methods to 'traditional' endurance exercise: prolonged, constant-intensity efforts at workloads sustainable for at least 20 minutes and up to several hours. This type of training is referred to as moderate-intensity continuous training (MICT) (Weston *et al.*, 2014; MacInnis & Gibala, 2017), though depending on the prescribed intensity these sessions most likely fall in the heavy-, rather than the moderate-intensity, domain (Iannetta *et al.*, 2020). Comparing different intensities of constant-workload exercise has also been used to understand the importance of exercise intensity.

2.3.2 Acute and Chronic Adaptive Responses to Different Aerobic Exercise Intensities

When performing continuous exercise, higher intensities stimulate a greater acute adaptive response compared to exercise at a lower intensity (Chen *et al.*, 2003; Rose *et al.*, 2007; Egan *et al.*, 2010). Exercising at a greater intensity for a given amount of time—even as brief as 10 (Chen *et al.*, 2003) or 20 minutes (Rose *et al.*, 2006)—leads to more signalling kinase activity, indicated by greater AMPK phosphorylation (Chen *et al.*, 2003) and phospholamban phosphorylation (a downstream target of CaMKII) (Rose *et al.*, 2006). Matching total work across individual exercise protocols eliminates the possibility that a greater response is simply driven by a greater exercise volume (i.e., intensity • duration), which is a limitation of the studies by Chen et al. and Rose et al. When work-matched continuous exercise at 40% and 80% of $\dot{V}O_2$peak were compared, the higher intensity session elicited a greater increase in *PGC1A* mRNA and significant increases in AMPK and CaMKII phosphorylation, whereas the low-intensity session did not activate either kinase (Egan *et al.*, 2010). Similarly, 60 min of cycling at

75% of $\dot{V}O_2$peak markedly increased AMPK phosphorylation immediately after the session, while 90 min of cycling at 50% of $\dot{V}O_2$peak did not result in any changes to AMPK phosphorylation (Wojtaszewski *et al.*, 2000).

Work-matched comparisons are also useful in understanding the acute effects of interval training relative to continuous exercise, with most studies showing that HIIT and MICT similarly activate signalling pathways. Increases in AMPK and p38 MAPK phosphorylation, as well as *PGC1A* gene expression, were comparable 3 h post-exercise in both HIIT and MICT (Bartlett *et al.*, 2012; Trewin *et al.*, 2018). Protocols that match total work between very brief intervals (i.e., SIT or RST) and continuous exercise have shown similar acute responses (Wang *et al.*, 2009; Cochran *et al.*, 2014; Gunnarsson *et al.*, 2019). Work-matched SIT (4 x 30-s) and continuous exercise (~4 min) both increased post-exercise acetyl-CoA carboxylase (ACC, a downstream target of AMPK) and p38 MAPK phosphorylation and *PGC1A* mRNA (Cochran *et al.*, 2014). Substantially longer exercise sessions also similarly stimulated signalling pathways: 90 min of cycling at 60% of PPO and 90 minutes broken into 12 s at 120% of PPO and 18 s at 20% of PPO similarly increased *PGC1A* mRNA post-exercise (Wang *et al.*, 2009).

Work-matched exercise protocols performed as training programs provide additional evidence for the importance of exercise intensity in eliciting stable increases in muscle mitochondrial density. Indeed, in studies with work-matched HIIT and MICT interventions, HIIT induced superior (Daussin *et al.*, 2008; MacInnis *et al.*, 2017) or similar (Henriksson & Reitman, 1976; Baekkerud *et al.*, 2016) mitochondrial adaptations compared to MICT. Fewer studies have examined adaptations following extended periods of work-matched SIT and MICT. Looking at enzyme markers of mitochondrial content, MICT produces similar increases in succinate dehydrogenase (SDH) activity (Saltin *et al.*, 1976) and greater increases in citrate synthase

activity (Gorostiaga *et al.*, 1991) compared to equal (i.e., atypically large) volumes of SIT. Interestingly, inclusion of an all-out sprint every 10 minutes during an hour-long MICT session (adjusted to keep total work and session duration equal across conditions) induced increases in mitochondrial content with training (measured via COX subunit IV and CS protein content) that were not found with MICT alone (Gunnarsson *et al.*, 2019).

The best evidence for the importance of exercise intensity mediating mitochondrial adaptations to exercise comes from studies of low-volume SIT, involving a fraction of the total work of HIIT or MICT. Low-volume SIT triggers signalling cascades that promote mitochondrial biogenesis following a single session: various studies showed similar (Scribbans *et al.*, 2014; Parker *et al.*, 2017) or greater p38 MAPK phosphorylation (Fiorenza *et al.*, 2018), similar (Scribbans *et al.*, 2014; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018) or greater AMPK/ACC phosphorylation (Granata *et al.*, 2017), and significantly increased CaMKII phosphorylation (compared to no change in MICT) (Fiorenza *et al.*, 2018) after a SIT session. Common sensor activation responses likely explain similar responses further down the mitochondrial biogenesis pathway too: SIT induced similar increases in post-exercise *PGC1A* mRNA content compared with MICT (Granata *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018) or HIIT (Psilander *et al.*, 2010; Trewin *et al.*, 2018). The similar acute signalling response, occurring despite a large discrepancy in exercise volume between MICT and SIT protocols, further stresses the importance of exercise intensity in mediating mitochondrial adaptations to exercise training. When performed as a regular training program, low-volume SIT also improved muscle oxidative capacity and endurance performance (Burgomaster *et al.*, 2006; Gibala *et al.*, 2006; Gillen *et al.*, 2016), indicating that high intensities can compensate for low total volumes of exercise. Given

that work-matched SIT and MICT protocols generate similar responses, these results indicate that there are diminishing returns with high-volume SIT protocols.

While there may be divergent upstream mechanism that trigger the acute responses (Fiorenza *et al.*, 2018), it seems that a greater exercise intensity leads to greater acute signals when protocols are work-matched (Wojtaszewski *et al.*, 2000; Egan *et al.*, 2010). Further, high intensities of exercise can compensate for a much lower total volume of exercise, generating comparable signalling activation to that of MICT protocols (Psilander *et al.*, 2010; Scribbans *et al.*, 2014; Granata *et al.*, 2017; Parker *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018). One potential explanation for the compensatory effect of intense exercise in situations where exercise volume is low is alterations of muscle Ca^{2+} cycling, namely the ability to regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence of modifications to Ca^{2+}-handling proteins (Bellinger *et al.*, 2008; Place *et al.*, 2015; Ivarsson *et al.*, 2019).

2.4 Skeletal Muscle Ca^{2+}-Handling

Ca^{2+} movement from the SR to the cytosol in the muscle cell is tightly regulated as part of excitation-contraction (E-C) coupling, the process whereby electrical signals from the nervous system initiate biochemical processes that lead to muscle contraction. A brief description of the Ca^{2+} cycle during E-C coupling (summarized in Figure 2.2) and the roles of key Ca^{2+}-handling proteins follows.

When the muscle is not actively contracting, the vast majority of skeletal muscle Ca^{2+} is contained within the SR bound to the Ca^{2+}-sequestering protein, calsequestrin (CSQ), the amount of which determines both endogenous Ca^{2+} storage and maximal Ca^{2+} storage capacity (Lamboley *et al.*, 2013). After a motor neuron delivers an action potential, the electrical signal

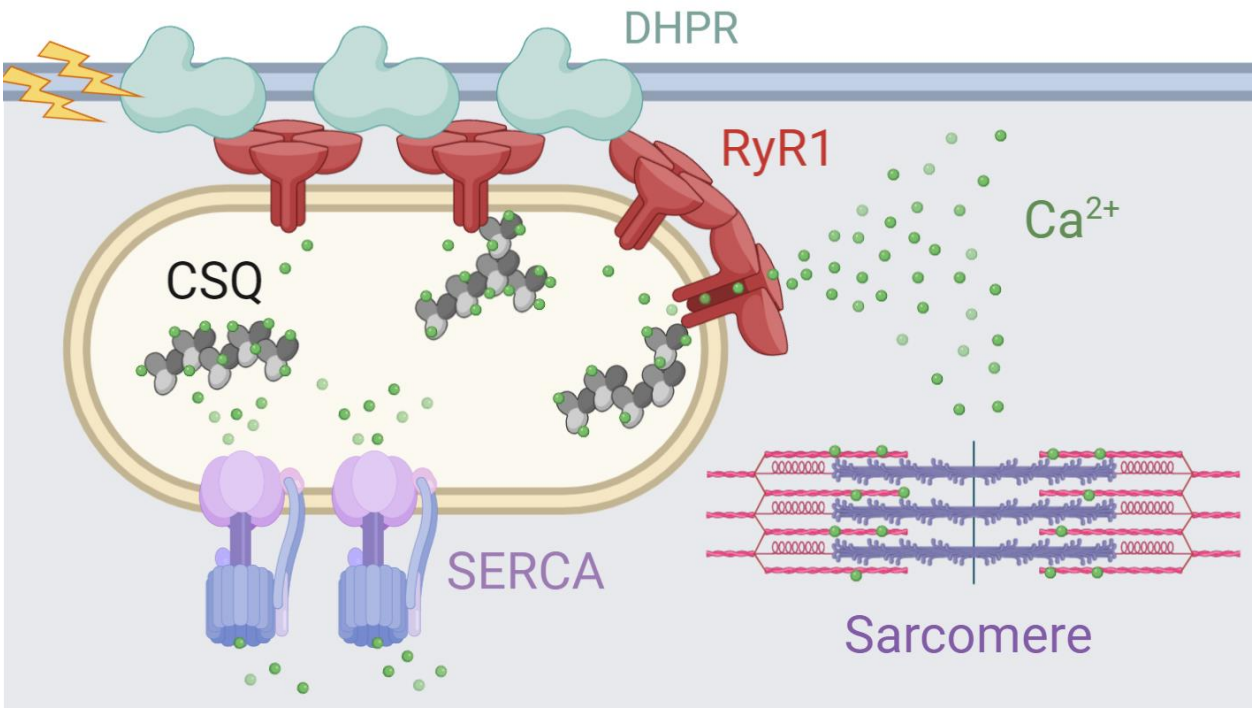

Figure 2.2: Calcium handling in skeletal muscle. Movement of calcium ions (Ca^{2+}) is tightly regulated in skeletal muscles. At rest, Ca^{2+} is stored in the sarcoplasmic reticulum, where most is bound to the buffer calsequestrin (CSQ). To initiate muscle contraction, an action potential (lightning bolts) propagates into the cell along membrane invaginations called t-tubules. The action potential triggers voltage-sensing ion channels (dihydropyridine receptors, DHPR), which open the Ca^{2+} release channels (ryanodine receptor 1, RyR1) to which they are mechanically coupled. Ca^{2+} floods into the cytosol, where it travels to the sarcomere to initiate muscle contraction. Sarco(endo)plasmic reticulum calcium ATPases (SERCA) hydrolyse ATP to pump Ca^{2+} back into the sarcoplasmic reticulum, and Ca^{2+} re-uptake occurs constantly to ensure adequate Ca^{2+} is available for release the next time the muscle is stimulated. Created with BioRender.com.

travels into the muscle cell via sarcolemma invaginations where it activates voltage-gated Ca^{2+} channels (dihydropyridine receptors; DHPR) (Schneider & Chandler, 1973; Rios & Brum, 1987). Subsequently, mechanical coupling between DHPR and RyR1 opens the RyR1 channel (Paolini *et al.*, 2004), releasing Ca^{2+} from the SR to diffuse into the sarcomere. After flooding into the cytosol, Ca^{2+} binds to troponin to facilitate contraction, interacts with various buffers, and immediately begins re-entering the SR via sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) pumps, which bring two Ca^{2+} into the SR per ATP molecule hydrolyzed (Toyoshima, 2009; Lamboley *et al.*, 2014). Once Ca^{2+} release has ended, the RyR1 channel closes, and this conformation is stabilized by FK506-binding protein 12-kDa (FKBP12). SERCA activity allows

for rapid muscle relaxation and ensures the muscle cell is prepared for the next action potential. More details for each of these proteins is provided in the following subsections.

2.4.1 Sarco(endo)plasmic Reticulum Calcium ATPase (SERCA)

SERCA Ca^{2+} pumping is immensely important to maintaining the SR:cytosol concentration gradient, which is a 10,000-fold difference between the two cellular compartments under normal conditions (Toyoshima, 2009). The SERCA pump possesses a large transmembrane region (M), with binding sites for two Ca^{2+} and a cytoplasmic headpiece separated into three domains (A, N, and P) (Toyoshima *et al.*, 2000). Of the cytoplasmic portion, the central P region houses the ATP hydrolysis site, though the actual binding site for nucleotides is further away in the N domain (Toyoshima *et al.*, 2000). This spatial separation means the SERCA pump bends during ATP hydrolysis, which is accomplished by rotation of the A domain (Toyoshima *et al.*, 2000; Toyoshima, 2009). This rotation deactivates the Ca^{2+}-binding sites in the M region, allowing the two Ca^{2+} ions to be released into the SR (Toyoshima, 2009). SERCA activity is regulated through several mechanisms, including total protein content (Stammers *et al.*, 2015), interactions with the regulatory proteins, phospholamban and sarcolipin (both of which act to inhibit SERCA activity by lowering the affinity of SERCA for Ca^{2+}) (Fajardo *et al.*, 2013), and post-translational modifications such as glutathionation or glycosylation (Stammers *et al.*, 2015).

Several SERCA isoforms exist within different cells in the human body. Two isoforms are expressed in skeletal muscle, SERCA1 and SERCA2a. Type I skeletal muscle fibres exclusively express SERCA2a and type II fibres predominantly express SERCA1 (Lamboley *et al.*, 2014), with only a very small amount of SERCA2a (Fajardo *et al.*, 2013). Each SERCA isoform possesses distinct functional properties that match the demands of the fibres in which

they are found. In experiments on intact, mechanically skinned muscle fibres, Lamboley and colleagues found that type I fibres had a significantly higher sensitivity of Ca^{2+} uptake than type II fibres (Lamboley *et al.*, 2014). The authors proposed that expressing the more sensitive SERCA2a isoform allowed type I fibres—whose contractile apparatus has greater Ca^{2+} sensitivity—to ensure sufficient Ca^{2+} re-uptake for relaxation (Lamboley *et al.*, 2014). The SERCA1 isoform is faster (Lamboley *et al.*, 2014), facilitating rapid relaxation in type IIa fibres despite the fact that more Ca^{2+} must be re-sequestered to the SR to fully relax the fibre (Lamboley *et al.*, 2013).

2.4.2 Calsequestrin (CSQ)

The ability of the SR to store such an immense amount of Ca^{2+}, although partly a function of SERCA pumps re-sequestering Ca^{2+} from the cytosol, is largely due to CSQ, a monomer consisting of three similar domains that dimerizes and polymerizes as $[Ca^{2+}]$ increases (Wang *et al.*, 1998). CSQ is a high-capacity, low-affinity Ca^{2+} buffer capable of storing 40-50 Ca^{2+} per CSQ molecule (Wang *et al.*, 1998). CSQ Ca^{2+} storage keeps SR free $[Ca^{2+}]$ low enough that it does not limit further Ca^{2+} uptake. Like SERCA, human skeletal muscle has two CSQ isoforms (CSQ1 and CSQ2) that are expressed differently across fibre types. A large amount of CSQ2 is found in type I muscle fibres along with relatively lower CSQ1 expression (Lamboley *et al.*, 2013). Type II fibres show the opposite expression pattern, with low CSQ2 and high CSQ1 expression (Lamboley *et al.*, 2013). Type II fibres have slightly higher absolute SR Ca^{2+} content, though because of their much greater maximal Ca^{2+} capacity, are endogenously loaded to a relatively lower percentage of maximum compared to type I fibres (Lamboley *et al.*, 2013, 2014).

2.4.3 Dihydropyridine Receptor (DHPR)

Ca^{2+} is released from the SR in response to an action potential, and DHPR is responsible for sensing and transferring this electrical signal to the SR (Schneider & Chandler, 1973; Rios & Brum, 1987). DHPR is comprised of five subunits ranging from 32-175 kDa (Leung *et al.*, 1987) that form a relatively flat disk shape with an "arm" extending into the t-tubule, while the protein is embedded in the cell membrane (Samsó, 2015). A portion of DHPR extends into the cytoplasm, where it mechanically interacts with RyR1 to open the channel upon sensing the voltage change (Paolini *et al.*, 2004). DHPR expression has been found to be higher in fast- compared to slow-twitch fibres (Mänttäri & Järvilehto, 2005; Schiaffino & Reggiani, 2011), which may improve excitation-contraction coupling via greater direct interaction between DHPR and RyR1.

2.4.4 Ryanodine Receptor 1 (RyR1)

Ryanodine receptors (RyRs) release Ca^{2+} from the SR. Three RyR isoforms (RyR1-3) exist in the human genome, but the predominant isoform in skeletal muscle is RyR1 (Lanner *et al.*, 2010). The RyR1 protein is a tetrameric ion channel, made up of a transmembrane region for ion conductance and a large cytoplasmic region that interacts with modulators to regulate Ca^{2+} release (Takeshima *et al.*, 1989; Yan *et al.*, 2015). Association with FKBP12 (also known as calstabin1) is necessary to stabilize RyR1 in its closed conformation and ensure normal channel function (Gaburjakova *et al.*, 2001; Yan *et al.*, 2015). Though RyR1 opening is thought to be mechanically coupled to DHPR, other factors modulate its function, including $[Ca^{2+}]$ itself. RyR1 is capable of binding Ca^{2+} directly, which appears to increase RyR1 affinity to another regulator, calmodulin (CaM) (Rodney *et al.*, 2000). CaM can either promote or inhibit RyR1 activity in response to Ca^{2+}, depending on $[Ca^{2+}]_i$, and the binding status of CaM to Ca^{2+}

(Rodney *et al.*, 2000). Specifically, at low $[Ca^{2+}]_i$, and with CaM unbound to Ca^{2+}, RyR1 is sensitized to Ca^{2+}, promoting Ca^{2+}-induced Ca^{2+} release, as occurs in EC coupling; however, to prevent runaway Ca^{2+} release, at higher $[Ca^{2+}]_i$, Ca^{2+}-bound CaM inhibits RyR1 activity (Rodney *et al.*, 2000).

2.5 Ca^{2+}-Handling and Fatigue

Significant decreases in muscle [ATP] have destructive effects within the cell, so skeletal muscle possesses mechanisms to limit ATP demand when supply cannot keep up. These mechanisms present as muscle fatigue and reduce muscle performance to preserve muscle integrity. During exercise, Ca^{2+} initiates the largest energy consumption activities: its presence in the cytosol permits cross-bridge ATP hydrolysis and also requires ATP hydrolysis to be pumped back into the SR by SERCA. As such, many fatigue-related mechanisms seemingly target Ca^{2+} as an efficient method to limit muscular ATP demand when necessary (Cheng *et al.*, 2018). Ca^{2+}-based fatigue mechanisms can be divided into two primary categories: impaired myofibrillar function and reduced SR Ca^{2+} release (Cheng *et al.*, 2018). Though impairments in myofibrillar function are important in the early development of muscular fatigue, the focus here will be on reductions in SR Ca^{2+} release, particularly those directly affecting the Ca^{2+}-handling apparatus.

As discussed previously, Ca^{2+} release capacity is related directly to RyR1 function. Metabolite concentration changes, occurring as part of fatigue development, affect proper RyR1 channel opening. Increases in $[P_i]$ (Duke & Steele, 2001; Steele & Duke, 2003) and reductions in [ATP] (associated with increases in [ADP], [AMP], and $[Mg^{2+}]$) (Dahlstedt *et al.*, 2000) limit Ca^{2+} release during fatiguing contractions in single muscle fibres through an apparent decrease in RyR1 activity (Dahlstedt *et al.*, 2000; Duke & Steele, 2001; Steele & Duke, 2003). The amount of Ca^{2+} available for rapid release from the SR also contributes to Ca^{2+} release capacity and may

be altered as part of fatigue development. It appears this loss of releasable Ca^{2+} is primarily attributable to precipitation of free Ca^{2+} by P_i in the SR (Fryer *et al.*, 1995; Westerblad & Allen, 1996), rather than Ca^{2+} loss from the cell. Net loss of cellular Ca^{2+} is prevented by store-operated Ca^{2+} entry, whereby sensors monitoring SR [Ca^{2+}] trigger an influx of extracellular Ca^{2+} to maintain total Ca^{2+} content (Pan *et al.*, 2014).

2.6 Ca^{2+} as a Signal for Mitochondrial Adaptation

Ca^{2+} is crucial for muscular contraction as part of E-C coupling and is a key target of fatigue mechanisms. Given these major roles in typical muscle function, it is unsurprising that changes in muscular Ca^{2+} concentration (specifically $[Ca^{2+}]_i$) can activate signalling cascades and stimulate mitochondrial biogenesis (Ojuka *et al.*, 2003; Wright, 2007; Wright *et al.*, 2007). Work in cell lines showed that although chronically elevated $[Ca^{2+}]_i$ decreases cell viability (Ojuka *et al.*, 2002), intermittent increases stimulate signalling pathways and increase mitochondrial content (Ojuka *et al.*, 2002, 2003; Wright *et al.*, 2007). CaMKII senses increases in $[Ca^{2+}]_i$ (Fluck *et al.*, 2000; Wright *et al.*, 2007) and increases *PGC1A* expression through a p38 MAPK-mediated pathway (Wright *et al.*, 2007). CaMKII activity increases during (Rose *et al.*, 2006) and following a single session of MICT (Rose & Hargreaves, 2003; Combes *et al.*, 2015), and CaMKII content and maximal activity are also increased by regular exercise training (Rose *et al.*, 2007).

Taken together, the duration and magnitude of $[Ca^{2+}]_i$ fluctuations accompanying muscle contraction during exercise appear sufficient to stimulate the CaMKII pathway in a long, continuous exercise session like MICT. Even greater increases in CaMKII activity were seen when the same intensity and duration of exercise was split into intervals (i.e., repeating one minute at the MICT intensity and one minute of rest until total working time equalled the

continuous session) (Combes *et al.*, 2015). Higher intensity exercise appears to activate CaMKII to a greater extent (Rose & Hargreaves, 2003; Rose *et al.*, 2006; Egan *et al.*, 2010), possibly due to activation of a greater number of active muscle fibres. With much shorter exercise durations, such as repeated sprints, results are somewhat mixed. Both RST (~4-5 s, repeated 18-20 times) (Serpiello *et al.*, 2012; Fiorenza *et al.*, 2018) and SIT (20-30s, repeated four to six times) (Gibala *et al.*, 2009; Fiorenza *et al.*, 2018) have produced conflicting findings with regards to activating CaMKII; however, provided the exercise poses a sufficient metabolic challenge, it appears likely that even very brief exercise bouts increase CaMKII phosphorylation, with multiple studies showing increases in CaMKII phosphorylation 3 h post-exercise (Serpiello *et al.*, 2012; Fiorenza *et al.*, 2018) or an increase in the activity of p38 MAPK, a downstream target of CaMKII, immediately post-exercise (Gibala *et al.*, 2009).

$[Ca^{2+}]_i$ fluctuations triggering muscle contraction are of sufficient concentration to stimulate signalling pathways; however, lower magnitude, more prolonged $[Ca^{2+}]_i$ changes may also occur, particularly when the function of key Ca^{2+}-handling proteins is altered. The rates of Ca^{2+} leak from the SR (through either SERCA or RyR1) and pump activity, as well as their relative contributions to resting $[Ca^{2+}]_i$ can be assessed by examining the recovery of $[Ca^{2+}]_i$ following a tetanus contraction (Klein *et al.*, 1991; Westerblad & Allen, 1994; Bruton *et al.*, 2010; Ivarsson *et al.*, 2019). Decreased pump activity, or increased leak without a compensatory increase in pump activity, can increase $[Ca^{2+}]_i$ (Bruton *et al.*, 2010; Ivarsson *et al.*, 2019). Increased $[Ca^{2+}]_i$, primarily due to greater Ca^{2+} leak, increased mitochondrial biogenesis in muscles of cold-acclimated (Bruton *et al.*, 2010) and exercising (Ivarsson *et al.*, 2019) mice, improving fatigue resistance in both situations. Treatment with RyR1-destabilizing drugs also increased CS protein content and endurance performance in sedentary mice compared to controls

(Ivarsson *et al.*, 2019). Ca^{2+} leak due to RyR1 modifications leading to an increase in resting $[Ca^{2+}]_i$ is a promising explanation for high-intensity, low-duration exercise promoting endurance adaptations (Place *et al.*, 2015; Ivarsson *et al.*, 2019).

2.6.1 Modifications to Ryanodine Receptor 1

Alterations to RyR1 affect its ability to regulate Ca^{2+} release from the SR. There are multiple ways RyR1 function may be altered to increase Ca^{2+} leak, including fragmentation (Place *et al.*, 2015), phosphorylation (Bellinger *et al.*, 2008; Gehlert *et al.*, 2012), oxidation (Andersson *et al.*, 2011), and dissociation from FKBP12 (Bellinger *et al.*, 2008; Place *et al.*, 2015; Ivarsson *et al.*, 2019). Various interventions and conditions modify RyR1. For example, intense resistance exercise and cold-acclimation increased RyR1 phosphorylation (Aydin *et al.*, 2008; Gehlert *et al.*, 2012), and aging increased RyR1 oxidation (Andersson *et al.*, 2011).While evidence exists for endurance exercise-induced RyR1 phosphorylation and nitrosylation (at least in swimming mice) (Bellinger *et al.*, 2008), fragmentation and dissociation from FKBP12 appear the most likely candidates for modifications related to endurance/aerobic exercise.

In human muscle, a single SIT session triggered drastic RyR1 fragmentation (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020)—in one study, only ~15% of the full-length RyR1 protein was present 24 h post-exercise (Place *et al.*, 2015). Significant FKBP12 dissociation occurred within 1 h following long-duration exercise, including completion of a marathon (Place *et al.*, 2015) and 3 h of cycling at 70% $\dot{V}O_2$max (Bellinger *et al.*, 2008). Rodent models demonstrate a similar acute RyR1 response to SIT, including significant fragmentation 3 h post-exercise, a time point not measured in the human participants (Place *et al.*, 2015). FKBP12 dissociation was also observed at rest in mouse muscle following 3 weeks of daily wheel running (Ivarsson *et al.*, 2019), and both of these modifications resulted in increased

resting $[Ca^{2+}]_i$ (Place *et al.*, 2015; Ivarsson *et al.*, 2019). Though not reaching sufficient concentrations to cause muscle contraction, Ca^{2+} leak through RyR1 may raise resting $[Ca^{2+}]_i$ enough to activate signalling pathways related to mitochondrial biogenesis (Ojuka *et al.*, 2002, 2003; Wright *et al.*, 2007; Bruton *et al.*, 2010), and may also link exercise-induced ROS production to subsequent adaptations.

2.7 Reactive Oxygen Species and Antioxidant Defenses

ROS are a class of molecules with one or more unpaired electrons, characterized by instability and reactivity (Powers *et al.*, 2011). During exercise, skeletal muscle is a major site of ROS production (Powers *et al.*, 2011; Jackson *et al.*, 2016), including superoxide ($O_2^{\cdot -}$), which can be dismutated to hydrogen peroxide (H_2O_2). Both $O_2^{\cdot -}$ and H_2O_2 can generate highly reactive hydroxyl radicals ($OH^{\cdot}$) (Powers *et al.*, 2011). $O_2^{\cdot -}$ is produced in the mitochondria by the electron transport chain and by nicotinamide adenine dinucleotide phosphatase oxidases (NOX) (Kayani *et al.*, 2012) found in multiple skeletal muscle organelles, including the SR and transverse tubule (Powers *et al.*, 2011; Kayani *et al.*, 2012; Abramson *et al.*, 2013); however, which of these sites produces the majority of ROS during exercise remains an area of active debate (Powers *et al.*, 2011; Cheng *et al.*, 2016).

Single bouts of SIT, HIIT, and MICT all similarly affect post-exercise mitochondrial H_2O_2 production (Trewin *et al.*, 2018), and although large amounts of oxidative stress may be harmful to muscle function (Reid *et al.*, 1993), certain levels of ROS are critical for muscle force production (Reid *et al.*, 1993) and cellular signalling (Gomez-Cabrera *et al.*, 2005). With sprint interval exercise in particular, ROS are implicated in multiple signalling pathways. Experiments using antioxidant administration to blunt ROS formation during sprints in humans show that both AMPK and CaMKII activation following exercise depend on ROS production (Morales-Alamo

et al., 2013, 2017). Differences in kinase activation occurred despite similar sprint performance and metabolic conditions for the placebo and antioxidant trials (Morales-Alamo *et al.*, 2013), pointing to a primary signalling mechanism centered around ROS signalling.

To keep ROS from quickly becoming detrimental to muscle function—or even cell survival during times of increased production—skeletal muscles possess a robust system of enzymatic and non-enzymatic antioxidant compounds that scavenge ROS (Powers *et al.*, 2011). The primary antioxidant enzymes in skeletal muscle are superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). These enzymes target different oxidants, but all serve to convert ROS (e.g., $O_2^{\cdot -}$, H_2O_2) to less active molecules (i.e., H_2O, O_2) (Powers *et al.*, 2011). Among non-enzymatic antioxidant compounds, glutathione (GSH) is particularly important (Meister & Anderson, 1983) and performs its antioxidant role by directly scavenging free radicals (Yu, 1994) or as a substrate for reactions catalyzed by GPX (Björnstedt *et al.*, 1994).

Given the highly reactive nature of ROS, the proteins most likely to be affected by ROS-induced modifications, like oxidation or carbonylation, are those located nearest the sites of ROS production. RyR1 is a likely target because of its position on the SR in the triad (Xia *et al.*, 2003): SR and transverse tubule NOX are thought to be a main source of ROS production during intense exercise (Xia *et al.*, 2003; Morales-Alamo & Calbet, 2014). In terms of RyR1 modifications, exposure to a general antioxidant before and during simulated HIIT prevented RyR1 fragmentation in mouse muscle (Place *et al.*, 2015). Further, fragmentation was observed in recreationally-active participants but not in endurance-trained humans, who had relatively greater antioxidant enzyme content (i.e., SOD2 and CAT) (Place *et al.*, 2015). This finding is in accordance with other studies showing increased antioxidant gene and protein expression

following sufficiently intense exercise training protocols (Hellsten *et al.*, 1996; Ristow *et al.*, 2009; Yfanti *et al.*, 2010; Gliemann *et al.*, 2013; Mohr *et al.*, 2017). Interestingly, research has shown that young women have greater levels of skeletal muscle antioxidants than young men (Pansarasa *et al.*, 2000; Fanò *et al.*, 2001), suggesting the potential for a blunted RyR1 fragmentation response; however, no experiment has tested if RyR1 fragmentation is altered in women.

2.8 Skeletal Muscle Fibre Types

One factor that underlies all explorations of muscle adaptations to exercise is the potential for different responses across skeletal muscle fibre types. Human skeletal muscle consists of two main fibre types, type I and type II, the latter of which can be further sub-divided into type IIa and type IIx (Saltin & Gollnick, 2011; Egan & Zierath, 2013). The vastus lateralis muscle (VL) is frequently sampled in human studies and displays a mixed fibre type distribution. In most individuals, type I (~50%, ranging from 30-70%) (Lexell *et al.*, 1983; Schantz *et al.*, 1983; Lexell & Downham, 1991) and type IIa (~35%, ranging from 20-60%) (Schantz *et al.*, 1983) make up the vast majority of VL fibres, though the wide ranges show the drastic interindividual differences present in fibre type distribution. A proportion of fibres are hybrid fibres (~7-10% Type I/IIa, ~15-25% Type IIa/IIx), possessing properties of multiple fibre types (Biral *et al.*, 1988; Smerdu *et al.*, 1994; Gallagher *et al.*, 2005).

Distinguishing muscle fibre types has evolved from simply noting that white fibres contract quickly (type II) and red fibres contract slowly (type I) (Schiaffino & Reggiani, 2011) to today's understanding that a vast number of properties relating to contractile function, metabolism, and morphology differ between fibre types (Schiaffino & Reggiani, 2011; Egan & Zierath, 2013; Tobias & Galpin, 2020). Each of these properties specialize fibres for specific

types of activity/recruitment and are produced by differential gene and protein expression, including production of specific protein isoforms that allow for fibre type identification. Type I fibres are characterized by their slow-twitch, fatigue-resistant, highly oxidative phenotype (Egan & Zierath, 2013). Type I fibres express myosin heavy chain (MHC) type I, SERCA2a (Lamboley *et al.*, 2014), and a greater proportion of CSQ2 compared to CSQ1 (Lamboley *et al.*, 2013). Conversely, type IIa fibres display a faster-twitch phenotype with a heavier reliance on glycolytic metabolism, though they are still considered fatigue resistant (Egan & Zierath, 2013). Type IIa fibres express MHC type IIa, SERCA1 (Lamboley *et al.*, 2014), and a greater abundance of CSQ2 than CSQ1 (Lamboley *et al.*, 2013). Recruitment during muscular contraction also varies across fibre types according to the size principle (Henneman *et al.*, 1965). Smaller motor neurons innervating type I fibres are activated to produce lower forces, with activation of larger motor neurons, and therefore type II fibres, occurring when larger forces are required (Henneman *et al.*, 1965). Further, smaller slow-twitch units may deactivate early (i.e., they do not actively contract for as long) at high contraction velocities (Blake & Wakeling, 2014), such as those required during sprint interval exercise, due to their relatively longer relaxation times.

Clearly, the distinct phenotypic and activation differences of type I and type IIa fibres may lead to different levels of metabolic stress during exercise. This varied metabolic stress can differentially activate adaptive signalling pathways after a session of exercise (Kristensen *et al.*, 2015) and can lead to fibre type-specific adaptations with training (Henriksson & Reitman, 1976). Adaptations do not always display fibre type differences (Scribbans *et al.*, 2014; Tan *et al.*, 2018), and differences may be a function of the type of exercise performed (Kristensen *et al.*,

2015). For example, in a 6-week training study, Tan et al. (2018) observed similar training-induced changes in capillarization and mitochondrial content between type I and type II fibres.

Recent advances in Western blotting have made it easy and reliable to quantify protein content from very small quantities of protein (Tobias & Galpin, 2020), including from single muscle fibre segments (Mollica *et al.*, 2009; Murphy, 2011). The development of a fast, reliable method of fibre type identification using dot blotting (Christiansen *et al.*, 2019) makes preparing pooled samples of each fibre type more feasible. Despite the knowledge of skeletal muscle fibre type differences, most studies examining skeletal muscle exercise responses perform analyses in whole muscle. When a mixed fibre muscle is studied, this approach accepts the aggregate response of both major fibre types, as well as type IIx and hybrid fibres. As Murphy notes, the vastus lateralis is ~50% type II and ~50% type I in healthy, recreationally active people, but the distribution can range widely based on population, from endurance athletes with 70-80% type I fibres to sedentary individuals with a greater proportion of type II fibres (Murphy, 2011). Analysis of whole muscle may obscure divergent responses between populations based on fibre-type differences or between fibre-types within a population or intervention (Murphy, 2011; Tobias & Galpin, 2020).

2.9 Research Objectives and Hypotheses

2.9.1 Objectives

Modification of the SR Ca^{2+} release channel, RyR1, following a single session of SIT is a promising mechanism that may link the roles of Ca^{2+} in skeletal muscle function, fatigue, and mitochondrial adaptations; however, important aspects of the RyR1 modification response and factors regulating the response remain unknown. The **primary objective** of this thesis is to

examine the time course of RyR1 fragmentation following a single session of SIT in young, healthy, recreationally-active males and females in whole muscle and pooled type I and type IIa muscle fibres. Additionally, the **secondary objective** is to determine the acute effects of SIT on the abundance of other components of the skeletal muscle Ca^{2+}-handling apparatus (i.e., DHPR, CSQ1, CSQ2, SERCA1, SERCA2a, and FKBP12) in whole muscle and pooled type I and type IIa muscle fibres.

2.9.2 Hypotheses

Based on the timing of signalling events proposed to be downstream of RyR1 fragmentation and results from simulated SIT experiments in rodents (Place *et al.*, 2015), I hypothesized that there would be a significant reduction in full-sized RyR1 (i.e., an increase in RyR1 fragmentation) by 3 h post-SIT that would persist until 24 h post-SIT, in agreement with previous human studies (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020). I expected that content of other Ca^{2+}-handling proteins would remain unchanged: they would not be acutely increased to compensate for greater Ca^{2+} leak (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020) nor would they be significantly reduced and contributing to greater $[Ca^{2+}]_i$, as leak appears to be the biggest factor for increased resting $[Ca^{2+}]_i$ (Bruton *et al.*, 2010; Ivarsson *et al.*, 2019). Accordingly, I hypothesized that the content of other Ca^{2+}-handling proteins (i.e., SERCA1, SERCA2a, CSQ1, CSQ2, and DHPR) would not be affected by a single session of SIT. Finally, based on the fibre type-dependent responses observed following intense interval exercise (6 x 90s; Kristensen et al., 2015), I hypothesized that RyR1 fragmentation would occur in a fibre type-dependent manner. Specifically, I hypothesized that while significant reductions in full-length RyR1 would occur in both type I and type IIa fibres, the level of fragmentation would be greater in type IIa fibres. Greater fragmentation in type IIa fibres is

expected due to potential type I fibre dropout at high contraction velocities (Blake & Wakeling, 2014) as well as greater antioxidant capacity in type I fibres (Criswell *et al.*, 1993; Leeuwenburgh *et al.*, 1997).

Chapter Three: Research Manuscript

3.1 Introduction

Exercise training increases skeletal muscle mitochondrial content through the production of new mitochondrial proteins and expansion of the mitochondrial reticulum (Morgan *et al.*, 1971; Holloszy & Coyle, 1984). This process, known as mitochondrial biogenesis, is regulated through a complex molecular signalling network (Egan & Zierath, 2013) that appears sensitive to exercise intensity, the rate at which work is performed (MacInnis & Gibala, 2017; MacInnis *et al.*, 2019). A notable example demonstrating the importance of exercise intensity in mitochondrial biogenesis is sprint interval training (SIT), which involves several brief intense efforts interspersed with rest periods (e.g., 3-6 intervals of 20-30s separated by 2-4 minutes of rest). Despite involving a much smaller volume of work, a single bout of SIT increases mitochondrial biogenesis-related signalling kinase activation and gene expression to a similar extent as prolonged endurance exercise at a moderate or heavy intensity (Granata *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018). SIT also leads to similar increases in mitochondrial content compared with traditional endurance exercise when performed for 2-12 weeks (Burgomaster *et al.*, 2006; Gibala *et al.*, 2006; Gillen *et al.*, 2016). Recently, multiple studies suggested that SIT triggers modifications to ryanodine receptor 1 (RyR1)—the skeletal muscle sarcoplasmic reticulum (SR) calcium (Ca^{2+})-release channel—that increase intracellular [Ca^{2+}] (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020), a signal for mitochondrial biogenesis (Ojuka *et al.*, 2002, 2003; Wright *et al.*, 2007).

A variety of RyR1 modifications—including fragmentation (Kohl *et al.*, 2015; Neyroud, 2017), dissociation from FK506-binding protein 12 kDa (FKBP12) (Gaburjakova *et al.*, 2001; Zalk *et al.*, 2007), and oxidative damage (Bellinger *et al.*, 2008; Bruton *et al.*, 2010)—reduce the

stability of the RyR1 channel's closed state and result in elevated Ca^{2+} leak from the SR (Bruton *et al.*, 2010; Ivarsson *et al.*, 2019). While Ca^{2+} fluctuations in and out of the SR during an exercise session are known to stimulate downstream signalling (Rose & Hargreaves, 2003; Rose *et al.*, 2006), smaller, stable increases in intracellular [Ca^{2+}] post-exercise may activate similar signalling pathways (Bruton *et al.*, 2010; Ivarsson *et al.*, 2019). RyR1 modifications in skeletal muscle have been observed in response to individual exercise sessions. From human data, there is evidence that RyR1 fragmentation occurs 24 h post-SIT (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020) and FKBP12 dissociation occurs immediately post-3h of cycling (Bellinger *et al.*, 2008) and 1 h post-marathon (Place *et al.*, 2015). Similarly, RyR1 modifications occurred immediately following SIT-simulating electrical stimulation procedures in rodents (Place *et al.*, 2015). These studies have mostly focused on a potential link to muscle fatigue and have not examined earlier time points (i.e., between 1 and 24 h post-exercise) when other key signalling events are known to occur (Hood, 2009; Hood *et al.*, 2015; Islam *et al.*, 2020). Without time course data, the role of RyR1 modifications in mitochondrial biogenesis is unclear: if RyR1 is fragmented only after key signalling events have happened, it is doubtful that this mechanism initiates the adaptive response. Furthermore, to our knowledge, no studies have examined RyR1 modifications at the fibre type-level, which may be a mediating factor due to fibre recruitment patterns (Henneman *et al.*, 1965; Blake & Wakeling, 2014), fibre type-specific signaling responses to interval exercise (Kristensen *et al.*, 2015), and fibre type-specific antioxidant defence systems (Criswell *et al.*, 1993; Leeuwenburgh *et al.*, 1997).

In addition to RyR1 modifications, numerous proteins have specific Ca^{2+}-handling functions and could be involved in the molecular response to SIT. The dihydropyridine receptor (DHPR) senses action potentials to trigger Ca^{2+} release (Rios & Brum, 1987); the

sarco(endo)plasmic reticulum Ca^{2+} ATPases (SERCA) pump Ca^{2+} back in to the SR (Toyoshima *et al.*, 2000); and calsequestrins (CSQ) buffer SR Ca^{2+} to increase the Ca^{2+} storage capacity of the SR (Lamboley *et al.*, 2013). While some studies have examined one or two of these Ca^{2+}-handling proteins in human skeletal muscle after a session of SIT (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020), SERCAs and CSQs have fibre type-specific isoforms (Lamboley *et al.*, 2013, 2014), so analysis based on only one isoform in whole muscle samples offers limited insight.

In order to understand the relevance of RyR1 modifications with respect to initiating mitochondrial adaptations to SIT, we sought to characterize the time course of RyR1 modifications (i.e., fragmentation) and the abundance of Ca^{2+}-handling proteins in whole muscle and type I and IIa muscle fibres following a single SIT session. We hypothesized that (i) RyR1 fragmentation (i.e., reductions in full-length RyR1) would be detectable 3 h and 6 h post-SIT and would persist until 24 h post-SIT in whole muscle, (ii) RyR1 fragmentation would be evident in pooled fibre samples and greater in type IIa compared to type I fibres; and (iii) the content of other Ca^{2+}-handling proteins would not be affected at any time point post-SIT in whole muscle or type I and IIa fibres.

3.2 Methods

3.2.1 Participants

10 healthy, young females (n = 4) and males (n = 6) were recruited to take part in the study (age: 24 ± 4 yrs; height: 175 ± 8 cm; weight: 72 ± 9 kg; lean body mass: 56 ± 10 kg; body fat percentage: 18 ± 7 %; $\dot{V}O_2$max: 48.4 ± 7.7 ml/kg/min). All participants were recreationally active (i.e., performing endurance exercise fewer than three times per week). Prior to the

commencement of testing, an investigator explained the testing procedures to each participant. All participants completed the Get Active Questionnaire (Canadian Society of Exercise Physiology) and provided written, informed consent (see Appendix D for letter of consent). The study was approved by the University of Calgary Conjoint Health Research Ethics Board (19-0423).

3.2.2 Experimental Protocol

Participants arrived at the laboratory fasted (overnight) and underwent a dual-energy x-ray absorptiometry (DXA) scan to quantify lean mass, before completing a ramp incremental exercise test and a brief familiarization to the sprint interval exercise session. At least 72 h after the initial visit, participants returned to the lab for the experimental visits. Participants underwent a skeletal muscle biopsy prior to performing a session of sprint interval exercise and then returned to the lab for follow-up biopsies 3, 6, and ~24 h after completing the exercise session.

Incremental Exercise Test

Participants completed a 30 W/min (i.e., 1 W/ 2s) ramp incremental exercise test on an electromagnetically braked cycle ergometer (Velotron; Racermate Inc., Seattle, WA, USA) for determination of maximal oxygen uptake ($\dot{V}O_2max$). Expired gases were collected using a mixing chamber and analyzed using a metabolic cart in 10 s intervals (Quark CPET; Cosmed, Rome, Italy). Gas analyzers were calibrated using a gas mixture of known concentrations (15.90% O_2, 4.95% CO_2, balance N_2), and the turbine flowmeter was calibrated with a 3 L syringe. A 4-min warm-up at 50 W was performed prior to the commencement of the ramp portion of the test. Participants were instructed to cycle at a consistent, self-selected cadence

between 80-100 rpm for the duration of the test. Verbal encouragement was provided throughout the test, and each test was stopped when the participant's cadence dropped below 60 rpm.

Gas exchange data were analyzed using Origin graphing software (OriginLab Corporation, Northampton, MA, USA). Data were interpolated to second-by-second values and $\dot{V}O_2$max was calculated as the highest 30-s average during the test. $\dot{V}O_2$max was expressed relative to total body mass as well as lean mass derived from the DXA scan.

Sprint Interval Exercise Protocol

The sprint interval exercise protocol, which was selected to closely match Place et al. (2015), consisted of 6 x 30 s all-out sprints (i.e., Wingate tests) performed on an electromagnetically-braked cycle ergometer (Velotron; Racermate Inc.) with loads of 7.5% of body weight, separated by 4.5 min of rest. Participants completed a set of two sprints approximately 15 min following their incremental exercise test to become familiar with performing repeated all-out efforts and the associated discomfort. Participants were instructed to increase their cadences leading up to each sprint (i.e., 5 s before the load was applied) so that peak cadence was achieved within 1 s of the load being applied. Verbal encouragement was provided throughout exercise and particularly during intervals, and ratings of perceived exertion (1-10 Borg Scale) and ratings of fatigue (1-10) were collected following each interval. Power data from the sprint interval exercise sessions were collected at 10 Hz and exported to Microsoft Excel. The highest single power value and the average of all power values from each interval were recorded as peak power and mean power, respectively.

Muscle Biopsy Procedure

Muscle biopsy samples were collected via needle biopsy from the vastus lateralis muscle using a microbiopsy needle (Temno Evolution; Merit Medical Systems Inc., South Jordan, UT, USA) (Hayot *et al.*, 2005; Perry *et al.*, 2015; Bonafiglia *et al.*, 2020). Muscle biopsies were performed under local anesthetic (1% lidocaine with epinephrine). Incisions for each biopsy were made 2 cm apart, and a new incision was made for each biopsy. Samples were dissected free of visible fat and connective tissue, blotted free of blood, and immediately snap frozen in liquid nitrogen. Samples were stored at -80 °C until further analyses. For nine participants, all four biopsies were collected from one randomly-selected leg; however, due to local swelling, the final biopsy from one participant was collected from the contralateral leg.

Exercise and Dietary Controls

To limit the effects of previous exercise, participants were instructed to abstain from exercise for 24 h prior to each testing session. Participants were also not permitted to exercise—aside from activities of daily living—in the 24 h after the sprint interval exercise session. To limit the influence of dietary factors, participants were instructed to abstain from alcohol or caffeine for 12 h prior to each exercise session and were asked to fast overnight prior to the first lab visit and experimental session. Participants were also asked to avoid taking any anti-inflammatory drugs between the first and last muscle biopsies.

3.2.3 Muscle Analysis

Whole Muscle Homogenization for Western Blotting

A small piece of muscle (10-20 mg wet weight) was freeze-dried for 48 h (FreeZone Tray Drier and FreeZone 8L; Labconco, Kansas City, MO), reducing the sample weight by an average

of 73% (standard deviation: 5.8%). A small piece of freeze-dried muscle (0.2-1.0 mg) was homogenized and solubilized in 1x solubilizing buffer (0.125 M Tris-HCI, 10% glycerol, 4% SDS, 4 M urea, 10% 2-mercaptoethanol and 0.001% bromophenol blue [pH 6.8] diluted 2:1 with 1 M Tris-HCl [pH 6.8]) to a final concentration of 0.2 µg dry weight muscle/µL buffer. The muscle was powdered in the bottom of the tube using fine-tipped forceps, and half of the required solubilizing buffer was added. A plastic pestle was used to further immerse and homogenize the powdered muscle in the buffer. The remaining solubilizing buffer was added, the tubes were vortexed, and finally allowed to solubilize at room temperature for 1 h. Samples were stored at -80 °C until Western blotting experiments were performed.

Single Fibre Collection and Fibre Pooling

Single fibre segments were collected from selected freeze-dried muscle samples (pre-exercise and 6 h and 24 h post-exercise) to perform Western blotting on pooled type I and IIa fibres. With the goal of identifying at least 10 type I and 10 type IIa fibres, 40 fibre segments were collected from each sample initially; an additional 10-30 fibres were collected if needed (see Appendix B for total fibre counts).

Single fibre collection was performed according to the method of Christiansen et al. (2019). The freeze-dried sample was transferred to a petri dish under the dissecting microscope for fibre collection. Fibres were separated using jeweller's forceps and placed in individual microcentrifuge tubes containing 10 µL of 1x solubilizing buffer (Christiansen *et al.*, 2019). The fibre was deposited on the side of the tube and visually verified to be inside the tube prior to vigorous vortexing and brief centrifugation to ensure fibres were submersed in buffer. Samples were left to solubilize at room temperature for at least 1 h, after which they were stored at -80 °C until dot blotting.

Dot Blotting

Dot blotting was used to determine the fibre type of each fibre segment. The dot blotting protocol used was similar to that described by Christiansen et al. (2019) but modified to allow for faster experiment completion and optimized for our laboratory (see Appendix A for more information). This method allowed a greater number of samples to feasibly be tested per day (up to 160 fibre segments) without sacrificing fibre-typing accuracy. Polyvinylidene fluoride (PVDF) membranes (0.45 µm pore size) were activated in 95% ethanol for 2 min, then equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 2 min. Membranes were placed on transfer buffer-soaked filter paper for sample spotting: 1.0 µL of each sample and 2.0 µL of a pooled human whole muscle sample (positive control for both myosin heavy chain (MHC) antibodies) were spotted on the membrane in a pre-determined grid. Membranes were fully dried on a piece of filter paper (~15 min) before being re-activated in 95% ethanol (2 min) and equilibrated in transfer buffer (2 min). Membranes were blocked for 15 min in blocking buffer (5% skim milk powder in 1x tris-buffered saline with Tween-20 [1x TBST; 20 mM Tris Base, 150 mM NaCl, 0.1% Tween-20 (w/v), pH 7.6]), rinsed in 1x TBST, and incubated in mouse anti-MHC type IIa primary antibody (MHCIIa) (see Appendix C for antibody details) while rocking gently for 1 h at room temperature. Membranes were washed in blocking buffer and then incubated in the secondary antibody (1:20 000; goat anti-mouse IgG-horseradish peroxidase (HRP), Invitrogen; Carlsbad, CA, USA) for 30 min at room temperature. Membranes were imaged using chemiluminescent reagent (Clarity ECL Substrate, BioRad Laboratories Inc.; Hercules, CA, USA) in an Amersham Imager 600 (GE Healthcare; Chicago, IL, USA), and then stripped for 30 min at room temperature (Restore Western Blot Stripping Buffer, ThermoFisher Scientific; Waltham, MA, USA). Subsequently, the same procedure was

repeated for the MHC type I probe, using mouse anti-MHCI primary antibody and goat anti-mouse IgM-HRP secondary antibody (1:20 000; Invitrogen). All primary antibodies were diluted in 1% BSA in 1x phosphate-buffered saline with Tween-20 (1x PBST; 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20 (w/v), pH 7.4) and 0.02% NaN_3. Secondary antibodies were diluted in blocking buffer.

Membrane images were analyzed to assign a fibre type to each fibre segment. Any segment that produced a signal for both fibre types was considered a 'hybrid' fibre segment and discarded. Some spots did not produce a signal for either MHCIIa or MHCI. It is possible that these samples were type IIx fibres—which represent a small percentage of total fibres in human vastus lateralis (Biral *et al.*, 1988; Smerdu *et al.*, 1994; Gallagher *et al.*, 2005) and were not examined in this study—or that the fibre was not solubilized. Samples containing type I or type IIa fibres were combined into separate pooled samples, mixed, and stored at -80 °C until further analysis. An average of 16 fibres were pooled for each sample (range: 10-20 fibres).

SDS-PAGE and Western Blotting

All samples were run in triplicate on 4-12% Criterion XT Bis-Tris gels (BioRad Laboratories Inc.). All gels included a 3-6-point calibration curve comprised of whole muscle protein aliquots from every sample (Mollica *et al.*, 2009) as well as a protein ladder (PageRuler Plus; ThermoFisher Scientific). Proteins were separated via SDS-PAGE at 200 V (65 min for RyR1, 30 min for other proteins) and transferred to nitrocellulose membranes via wet transfer for 60 min at 100 V (Criterion Blotter; BioRad Laboratories Inc.). After transfer, membranes were stained with 1x Ponceau S staining solution (VWR Life Sciences; Radnor, PA, USA) to quantify total lane protein for normalizing sample loading. Membranes were de-stained using 1x TBST, rocked in Miser antibody extender (ThermoFisher Scientific) for 10 min, washed in double

distilled water 5 times, and blocked for 2 h at room temperature. Membranes were then were cut at specific molecular weights to allow for multiple probes to be performed simultaneously. Membranes were incubated in the respective primary antibodies for 2 h at room temperature and overnight at 4 °C. The following primary antibodies were used (see Appendix C for more details): mouse anti-RyR1, mouse anti-DHPR, mouse anti-SERCA1, rabbit anti-SERCA2a, mouse anti-CSQ1, rabbit anti-CSQ2 and rabbit anti-FKBP12. Pooled fibre samples were additionally probed using mouse anti-MHCIIa and mouse anti-MHCI to verify fibre pooling accuracy. Subsequently, membranes were washed in blocking buffer and rocked in the appropriate secondary antibody (1:20 000; goat anti-mouse IgM-HRP for MHCI primary antibody, goat anti-mouse IgG-HRP for all other mouse primary antibodies, goat-anti rabbit IgG-HRP for all rabbit primary antibodies) for 1 h at room temperature. Membranes were imaged using an Amersham Imager 600 (GE Healthcare) after rocking in chemiluminescent substrate for 5 min. To avoid the loss of sample protein, membranes were not stripped at any point except between MHC probes. Between subsequent probes of similarly sized proteins (e.g., SERCA1 and SERCA2a), membranes were washed several times in 1x TBST prior to incubation in the next primary antibody.

Western Blotting Image Analysis

Western blotting images were analyzed using Image Lab software (BioRad Laboratories Inc.). Total protein (i.e., Ponceau S) and protein band (i.e., Western blotting) densities were adjusted using calibration curves, after which the abundance of proteins of interest were normalized to the total protein and expressed relative to the average of pre-exercise samples for each protein on the same gel (whole muscle) or the average of type I pre-exercise samples on the

same gel (pooled fibre samples). SERCA1 was expressed relative to the average of type IIa pre-exercise samples on the same gel because it was not present in type I fibres.

3.2.4 Statistical Analysis

All statistical tests were performed in Prism 8 (GraphPad Software; San Diego, CA, USA). Data were checked for normality using the Shapiro-Wilk test and for sphericity using Mauchley's W. The RPE and fatigue rating values were not normally distributed, so the effect of interval on these variables was assessed using non-parametric Friedman's tests. The effect of interval number on peak power output and mean power output during the SIT session was assessed using repeated-measures ANOVAs. Aside from two time points for FKBP12 (type I, 6 h and 24 h), all groups (i.e., each protein at each time point) were normally distributed, so all protein analyses used parametric tests for consistency (note that the choice of test did not affect the outcomes). The effect of time on protein content in whole muscle and pooled fibres was also assessed using repeated-measures ANOVAs. Where an ANOVA detected a significant effect, Tukey's honestly significant difference tests were used to compare individual intervals or time points. Differences in basal protein content between fibre types were assessed using paired t-tests comparing the pre-exercise time points (MacInnis *et al.*, 2017). Relationships between percent changes in RyR1 expression from pre-exercise to 6 h and 24 h post-exercise between whole muscle and each fibre type were examined using simple linear regression analysis. Statistical tests were considered significant at $p \leq 0.05$. Data are presented as mean ± standard deviation.

3.3 Results

3.3.1 Sprint Interval Exercise Session

Participants performed a total of 97 ± 20 kJ of work across the six 30-s intervals. The decline in power throughout each interval, characteristic of 'all-out' exercise, is illustrated in Figure 3.1A. Along with power decreasing within the intervals, peak power (Figure 3.1B; effect of interval: $p < 0.001$) and mean power (Figure 3.1C; effect of interval: $p < 0.001$) also decreased across intervals, though participants were able to maintain peak power (-11 ± 8% from interval 1 to 6) slightly better than mean power (-21 ± 5%). Rating of perceived exertion (effect of interval: $p < 0.001$) and rating of fatigue (effect of interval: $p < 0.001$) each rose throughout the session (Figure 3.1D and Figure 3.1E, respectively).

3.3.2 Whole Muscle Protein Content

SIT significantly reduced full-length RyR1 protein content (Figure 3.3A; effect of time, $p = 0.01$). At 3 h, RyR1 content had decreased by 26 ± 44% but was not significantly different from pre-exercise ($p = 0.07$). Significant reductions in RyR1 were apparent at 6 h (- 44 ± 31% compared to pre-exercise; $p = 0.01$) and persisted until 24 h (-35 ± 42% compared to pre-exercise; $p = 0.02$). In some participants, reductions in full-length RyR1 were accompanied by the appearance of several smaller bands in the range of ~200-400 kDa (Figure 3.2). We also observed notable interindividual variability in the recovery of RyR1 fragmentation: among participants who experienced a large decline in RyR1 content at 6 h some showed a full recovery by 24 h, while others had their RyR1 content continue to decrease. When looking at the peak fragmentation response, regardless of time point, there was a 62 ± 21% reduction in full-length RyR1 protein content compared to pre-exercise (Figure 3.3A; $p = 0.001$).

We did not find any differences post-SIT (i.e., no effect of time) in the protein content of DHPR (Figure 3.3G; $p = 0.17$), SERCA1 (Figure 3.5A; $p = 0.57$), SERCA2a (Figure 3.5D; $p = 0.49$), CSQ1 (Figure 3.5G; $p = 0.11$), or CSQ2 (Figure 3.5J; $p = 0.48$) in whole muscle samples. There was a significant effect of time on FKBP12 protein content (Figure 3.3D; $p = 0.02$), such that FKBP12 was significantly higher at 24 h compared to 3 (26 ± 29%; $p = 0.03$) and 6 h (24 ± 23%; $p = 0.03$).

3.3.3 Pooled Fibre Protein Content

MHC probes confirmed that fibre pooling was successful, shown in Figure 3.2 (i.e., no MHCIIa present in type I samples and no MHCI in type IIa samples). There were several significant fibre-type differences in protein expression at baseline. SERCA1 was expressed exclusively in type IIa fibres (Figure 3.5B-C; $p < 0.001$) and SERCA2a was expressed exclusively in type I fibres (Figure 3.5E-F; $p < 0.001$). Type I fibres also had greater CSQ2 protein content (728 ± 692%; $p < 0.001$), while pooled type IIa fibres had significantly greater concentrations of DHPR (71 ± 23%; $p < 0.001$), CSQ1 (51 ± 40%; $p = 0.003$), and FKBP12 (30 ± 38%; $p = 0.01$). Resting RyR1 content was not significantly different between fibre types ($p = 0.72$).

We found several fibre type-specific responses, including several responses that were not detected in the whole muscle analysis. Pooled fibre protein content following SIT is shown in Figure 3.3 (Type I in panels B, E, and H, type IIa in panels C, F, and I) and Figure 3.5 (Type I in panels B, E, H, and K, type IIa in panels C, F, I, and L). Though both fibre types showed a similar pattern in the response of RyR1 expression—lower at 6 h and recovering towards pre-exercise values at 24 h—the effect of time was only significant in type IIa fibres. In type IIa, but not type I fibres, there was a small decrease in DHPR from pre-exercise to 6 h post-exercise (-12

± 13%; p = 0.008). In type I fibres, we found a significant effect of time on protein content of both CSQ1 and CSQ2, with CSQ1 elevated by 15 ± 18% and CSQ2 elevated by 16 ± 26% at 6 h post-exercise. CSQ1 was significantly greater at 6 h compared to pre-exercise (p = 0.02), while CSQ2 only showed a significant difference between 6 h and 24 h post-exercise (p = 0.03). The effect of time was not significant for CSQ1 (p = 0.92) and CSQ2 (p = 0.65) in type IIa fibres. FKBP12 content increased from pre-exercise to 6 h in type IIa fibres (21 ± 19%; p = 0.01), with no changes in type I fibres. Finally, neither SERCA isoform was altered by exercise in type I (effect of time: SERCA1, p = 0.526; SERCA2a, p = 0.090) or type IIa fibres (effect of time: SERCA1, p = 0.209; SERCA2a, p = 0.613).

3.3.4 Relationship Between Changes in RyR1 in Whole Muscle and Pooled Fibres

There was not a significant relationship between the amount of fragmentation at 6 h or 24 h in whole muscle and either fibre type (Figure 3.6A-D); however, there was a significant relationship between the amount of fragmentation present in each fibre type at both 6 h (Figure 3.6E; $r^2 = 0.81$, $p < 0.001$) and 24 h (Figure 3.6F; $r^2 = 0.72$, $p = 0.002$).

3.4 Discussion

In this study, we demonstrated that a single session of SIT (6 x 30 s ‘all-out’ with 4.5 min rest between intervals) led to significant RyR1 fragmentation in whole skeletal muscle 6 and 24 h post-exercise and that SIT-induced RyR1 fragmentation is fibre type-dependent, occurring in type IIa but not type I muscle fibres. These results generally agreed with our hypotheses, though we expected fragmentation to be detectable by 3 h as well as present in type I fibres, which it was not. As we hypothesized, the content of other Ca^{2+}-handling proteins was stable in whole muscle (DHPR, SERCA1/2a, CSQ1/2), aside from FKBP12, which was increased at 24 h post-

exercise. Contrary to our hypothesis, we observed small, fibre-specific differences in the abundance of several of these proteins, highlighting the importance of fibre-specific analyses and the potential of whole muscle analysis to obscure relevant changes in protein content. While several studies have reported RyR1 fragmentation in response to SIT, these studies were all performed by one research group, and to our knowledge, this is the first study to independently replicate these findings.

Our data supports previous findings of RyR1 fragmentation following SIT but provides important details on the time course and fibre type-specific nature of these modifications. The reduction reported herein is notably less than Place et al. found in the original RyR1 fragmentation study, where only ~15% of full-length RyR1 remained 24 h post-exercise (Place *et al.*, 2015) but is similar to recent studies showing ~50% (Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020). Modifications to RyR1, including fragmentation, cause the channel to become 'leaky' (Bellinger *et al.*, 2008; Neyroud, 2017) and have been shown alongside increases in basal intracellular [Ca^{2+}] in skeletal muscle (Place *et al.*, 2015; Ivarsson *et al.*, 2019). Prolonged increases in intracellular [Ca^{2+}]—such as increases induced via destabilization of RyR1 (Ivarsson *et al.*, 2019)—are a known signal for mitochondrial biogenesis and lead to increased peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1A*) gene expression (Ojuka *et al.*, 2002, 2003; Wright *et al.*, 2007). With our finding that RyR1 is significantly fragmentated from 6 h until 24 h post-SIT, the time course for RyR1 initiating a Ca^{2+} signal, or possibly prolonging the Ca^{2+} signal generated during an exercise session, appears to occur earlier and for a longer time than previously thought. Furthermore, although fragmentation was not statistically significant at the 3 h time point, some individuals demonstrated marked fragmentation at this time, suggesting the time course is heterogeneous and that in some individuals RyR1 may

fragment even earlier than 3 h. The interindividual variability in the timing of peak RyR1 fragmentation is highlighted by Figure 3.3A; the greatest reduction in RyR1 content, regardless of time point, was -62 ± 21% relative to pre-exercise, notably larger than at any single time point we measured.

Though this study was not powered to detect sex-based differences in our outcomes, this is the first time that female subjects have been included in a study measuring RyR1 fragmentation. While the reduction in full-length RyR1 was similar between our male and female participants 6 h post-exercise (males: -45 ± 34%; females: -48 ± 15%), the pattern of fragmentation was different: male participants seem to have a more sustained fragmentation response (-45 ± 34% at 3h, -59 ± 23% at 24h) than females (2 ± 49% at 3h, 2 ± 38% at 24 h). This may simply be a product of our low group sizes (males, n = 6; females, n = 4), so future work should aim to assess if there are sex-based differences in RyR1 fragmentation.

The results of this study place the RyR1 fragmentation mechanism closer temporally to other important signalling events in the mitochondrial adaptive pathway, though not without some discrepancies. For signalling kinases sensitive to [Ca^{2+}], namely Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and p38 mitogen-activated protein kinase (MAPK) (Wright *et al.*, 2007), phosphorylation appears to peak during or immediately following exercise and return to baseline by 3 h (Gibala *et al.*, 2009; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018). Phosphorylation of these proteins is not usually measured late in recovery (i.e., > 3 h post-exercise), so it is unclear if the Ca^{2+} leak associated with RyR1 fragmentation produces a second, late increase in kinase phosphorylation. Beyond signalling kinases, gene expression of proteins co-ordinating the mitochondrial adaptive response, such as *PGC1A*, has been shown to increase as the recovery period progresses, peaking between 2-6 h post-exercise (Pilegaard *et al.*, 2003;

Perry *et al.*, 2010; Trewin *et al.*, 2018); however, spikes in *PGC1A* mRNA have been reported as late as 18 h post-exercise as well (Leick *et al.*, 2010). Gene expression for mitochondrial proteins is upregulated more slowly, and increases are typically observed in the range of 10-24 h depending on the gene (Leick *et al.*, 2010; Perry *et al.*, 2010) rather than within 3-5 h post-exercise (Stepto *et al.*, 2012; Percival *et al.*, 2015). Given these longer times before notable increases in downstream gene expression, we speculate that the RyR1 fragmentation (and the related increase in intracellular [Ca^{2+}]) potentially acts to sustain the adaptive signal generated by the exercise session, prolonging increased expression of transcriptional regulators and supporting the upregulation of mitochondrial genes.

To investigate any fibre-specific effects of SIT on RyR1 fragmentation, we collected single fibre segments from selected time points (pre-, 6 h post-, and 24 h post-exercise) and studied pooled type I and type IIa fibres. We did not find a baseline difference in RyR1 protein content between fibre types, and although the expression pattern between the fibre types appeared to be similar, the decline in full-length RyR1 was significant only in type IIa fibres. A possible explanation for this fibre-type difference is differential recruitment and metabolic stress during sprint exercise. Type I fibres may experience earlier deactivation at high contraction frequencies (Blake & Wakeling, 2014), such as those required during 'all-out' sprint exercise. Furthermore, following a 30 s Wingate, type II fibres had a markedly greater increase in lactate and IMP than type I fibres (Esbjörnsson-Liljedahl *et al.*, 1999). It appears that a certain combination of intensity and duration are required for fibre-specific stresses to be produced: 6 intervals of 90 s at a workload eliciting ~100% of $V\dot{O}_2$peak interspersed with 2.5 min rest lead to greater glycogen depletion in type II fibres than type I fibres (Kristensen *et al.*, 2015), but 8 intervals of 20 s with 10 s of rest lead to similar glycogen depletion (Scribbans *et al.*, 2014).

Previous studies have also shown fibre-specific adaptive responses to exercise, such as greater phosphorylated AMP-activated protein kinase and acetyl-CoA carboxylase after a single high-intensity interval training session (Kristensen *et al.*, 2015) and increased succinate dehydrogenase activity after interval training (Henriksson & Reitman, 1976) in type II fibres; however, other studies employing low-volume high-intensity interval exercise interventions have found similar adaptations between fibre types (Scribbans *et al.*, 2014; Tan *et al.*, 2018). Based on the present data, RyR1 appears to fragment in a fibre type-dependent manner.

The fibre-specific changes in RyR1 protein content, and specifically type IIa fibres, are not sufficient to explain the extent of fragmentation we saw in whole muscle homogenate. Furthermore, there was not a significant relationship between fragmentation in either fibre type and fragmentation in whole muscle at either 6 or 24 h (Figure 3.6A-D). One potential explanation for the discrepancy in the whole muscle response is that greater RyR1 fragmentation occurs in pure and hybrid type IIx fibres and these fibres drive the whole muscle response, despite their lower proportion of total fibres at around 15-25% (Biral *et al.*, 1988; Smerdu *et al.*, 1994; Gallagher *et al.*, 2005). Type IIx fibres have an extremely fast-contracting/fast-fatiguing phenotype and are recruited for contractions requiring the highest force production (Egan & Zierath, 2013; Tobias & Galpin, 2020). Assuming that the factors differentiating type I and type IIa fibres—including lower oxidative capacity, a faster myosin heavy chain isoform, and greater recruitment due to the exercise intensity—underly the different RyR1 responses we report here, type IIx fibres could be prone to even greater fragmentation than type IIa fibres. Assuming typical percentages of type IIx fibres (Biral *et al.*, 1988; Smerdu *et al.*, 1994; Gallagher *et al.*, 2005) and that baseline RyR1 protein content in type IIx fibres is similar to type I and type IIa fibres, RyR1 fragmentation would have to be near 100% in type IIx fibres to account for the

remaining portion of the whole muscle response. Our finding that RyR1 fragmentation was significantly correlated between type I and type IIa fibres (Figure 3.6E-F) despite no relationship with whole muscle, supports the idea that another portion of the muscle experiences a disproportionately greater amount of fragmentation. Future work should explore if there are baseline differences in RyR1 content between type IIx fibres and other fibre types, as well as examine the type IIx fibre response to SIT.

We also assessed protein content of other Ca^{2+}-handling proteins in whole muscle and found no changes in the protein content of DHPR, SERCA1, SERCA2a, CSQ1, or CSQ2, in agreement with previous work (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020). Unlike Place et al. (2015), who found no change in whole muscle FKBP12 content, we saw an increase in FKBP12 24 h post-SIT (significantly different from 3 h and 6 h). FKBP12 associates with RyR1 to stabilize the channel's closed conformation (MacMillan, 2013), and dissociation from RyR1 results in the channel becoming 'leaky', similar to fragmentation (Bellinger *et al.*, 2008). Though not assessed in the present study, FKBP12 dissociation has been shown to occur following exercise, including immediately post-3 h of cycling and 1 h post-marathon in human muscle (Place *et al.*, 2015), and during regular wheel running in mice (Ivarsson *et al.*, 2019). Thus, FKBP12 protein content may be acutely upregulated in an attempt to accommodate for RyR1 instability due to dissociation/fragmentation. In agreement with this idea, FKBP12 increased post-SIT in type IIa fibres, where fragmentation was present, but not in type I fibres.

Fibre-specific analyses of other Ca^{2+}-handling proteins showed significant fibre-type variation in protein expression, as well as small time course differences not detected in whole muscle. Our results for SERCA and CSQ expression are in agreement with previous studies

(Lamboley *et al.*, 2013, 2014), while type IIa fibres additionally had greater DHPR (Mänttäri & Järvilehto, 2005) and FKBP12 content. Several time course differences in protein expression were found in fibre-specific measures, despite only RyR1 and FKBP12 significantly changing in whole muscle samples. In type I fibres, both CSQ isoforms were elevated at 6 h post-SIT. CSQ content determines endogenous SR Ca^{2+} loading (Lamboley *et al.*, 2013), so this increase may allow type I fibres to store a greater amount of Ca^{2+}; however, it is unclear if this would alter intracellular [Ca^{2+}] in any way. Our fibre-type analysis also revealed a small but significant decline in DHPR in type IIa fibres. As DHPR is directly associated with a portion of RyR1 channels (Paolini *et al.*, 2004), a decrease in DHPR could indicate that there are fewer RyR1 channels opened directly by DHPR when it senses an action potential; however, given that full-length RyR1 content was reduced to a similar extent, the ratio of DHPR to RyR1 was likely unchanged. Overall, our results indicate that, aside from the SERCA pumps, other parts of the Ca^{2+}-handling system in skeletal muscle are affected by single session of SIT, though these effects are fibre type-specific and relatively small compared to the effect on RyR1.

The present study did not measure changes in signalling kinase activity, gene expression, or mitochondrial protein content; to further improve our understanding of this mechanism, future studies should aim to characterize the relationships between each of these downstream events and RyR1 fragmentation. Several other questions relating to RyR1 modifications post-exercise remain to be answered, including potential sex differences, responses in type IIx fibres, and whether or not ‘all-out’ sprint exercise is required to elicit RyR1 fragmentation, and comprise promising areas for future research.

3.5 Conclusions

In the present study, we showed that RyR1 fragmentation occurs earlier than previously reported, with a significant reduction in full-length RyR1 protein by 6 h post-SIT. This study provides additional evidence for the relevance of RyR1 fragmentation as a mechanism for low-volume, high-intensity exercise to elicit increases in mitochondrial content by positioning RyR1 fragmentation within the same time window as other important signalling events. We also show that RyR1 fragmentation is fibre type-specific, indicating that SIT may be a more effective stimulus for mitochondrial biogenesis in type IIa rather than type I muscle fibres. Aside from small changes to CSQs and DHPR in specific fibre-types, we did not find large effects of SIT on other Ca^{2+}-handling proteins. Our results support previous research proposing that the basal $[Ca^{2+}]$ increase is primarily generated by Ca^{2+} leak through RyR1, rather than decrements in Ca^{2+} uptake or storage capacity.

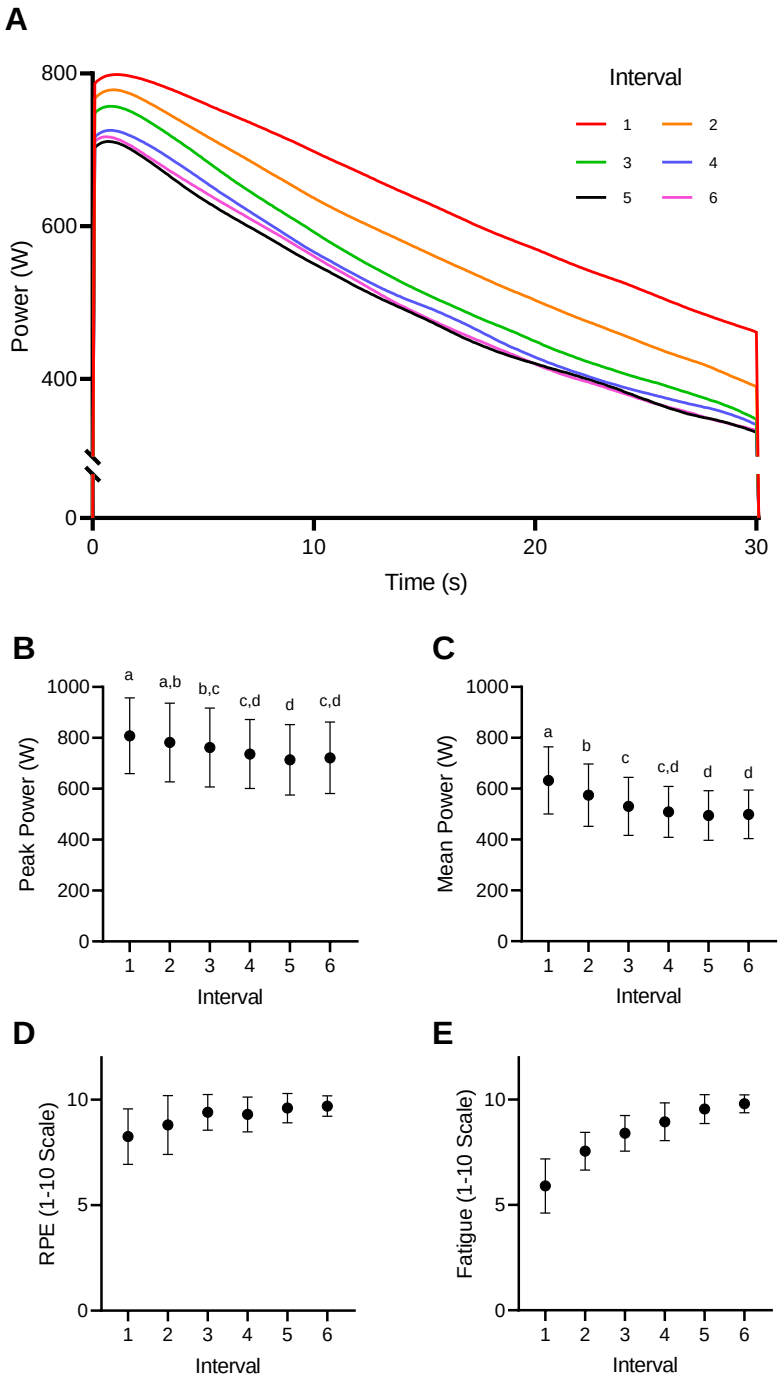

Figure 3.1: Descriptive information for the sprint interval training session. Participants performed six 'all-out' sprints on an electromagnetically-braked cycle ergometer against a resistance of 0.075kg/kg body mass. Power output traces showing the mean of each participant's trace (collected at 10 Hz) throughout each interval (**A**). In panels B-E, circles represent mean values and error bars represent standard deviations. Repeated measures ANOVAs revealed a significant effect of time for peak power and mean power, while non-parametric Friedman's tests revealed a significant effect of time for rating of perceived exertion (RPE) and fatigue rating (all $p < 0.001$). Tukey's multiple comparisons tests were used to examine significant differences between intervals for peak power and mean power. Points that do not share a common letter are significantly different at $p < 0.05$.

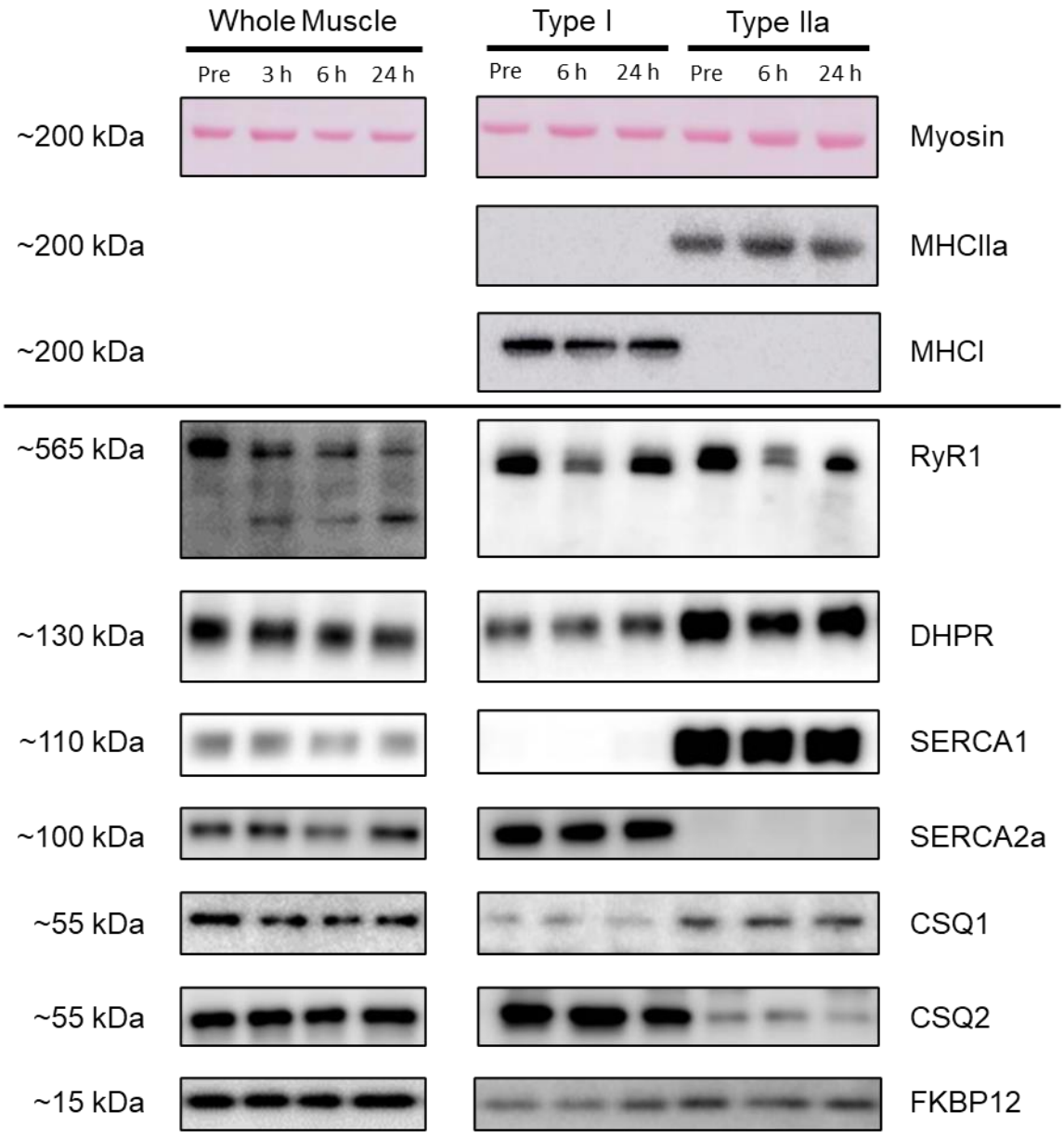

Figure 3.2: Representative Western blots for whole muscle and pooled type I and type IIa fibres. Whole muscle and pooled fibre samples were run on separate gels and transferred to nitrocellulose membranes for Western blotting. Total protein staining with Ponceau S solution (myosin band shown) was used to normalize loading. All gels included a 4-6 point calibration curve of pooled whole muscle samples (not shown). Total protein (Ponceau S) and protein band (Western blotting) densities were first adjusted to their respective calibration curves. Band densities were then normalized to total protein and expressed relative to the average pre-exercise value on each gel (whole muscle), to the average type IIa pre-exercise value of on each gel (pooled fibres, SERCA1 only), or the average type I pre-exercise value on each gel (pooled fibres, all other proteins). The presence of MHCIIa and MHCI were used to confirm fibre pooling was accurate.

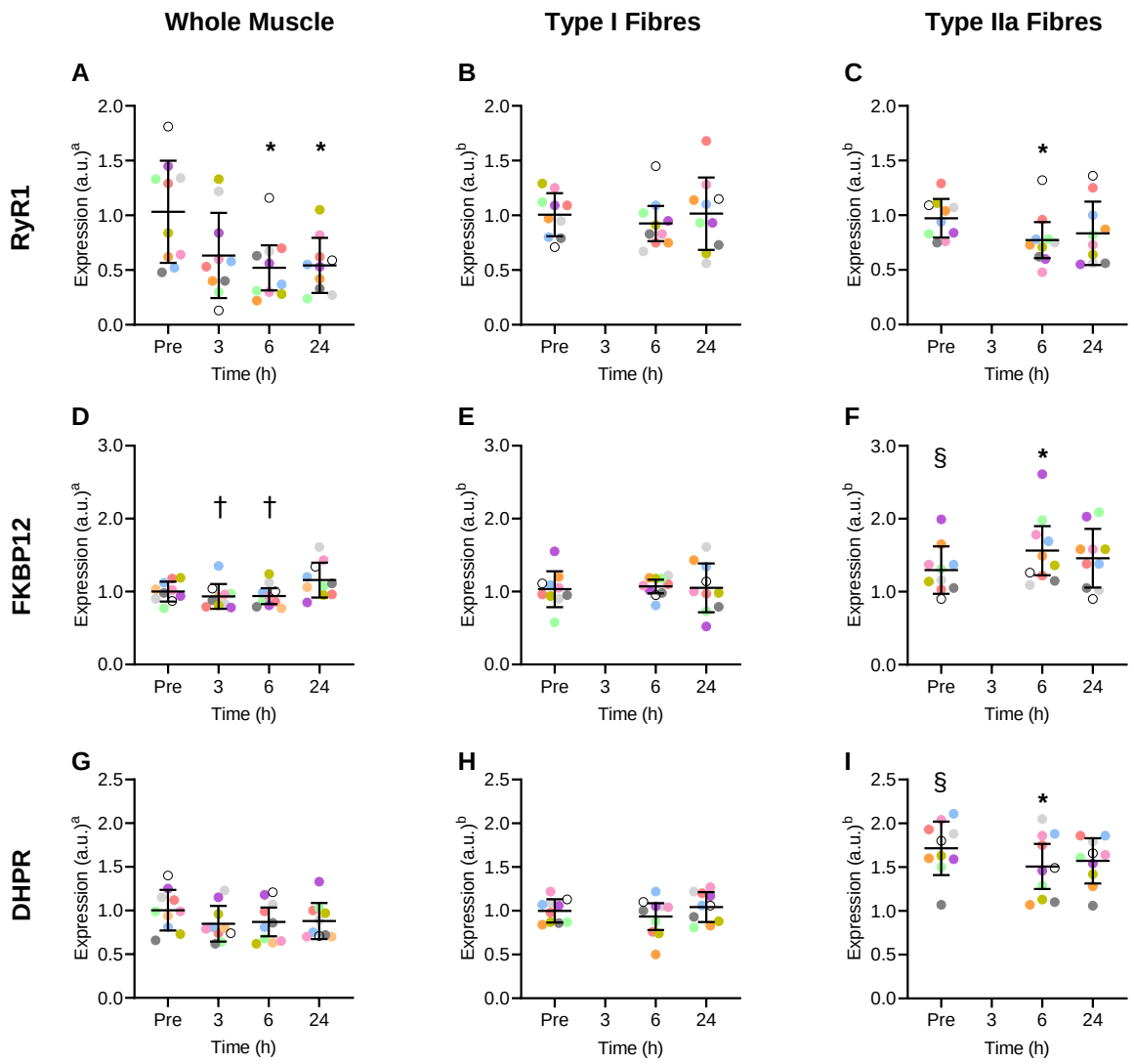

Figure 3.3: Relative expression of RyR1, FKBP12, and DHPR following acute SIT in whole muscle and pooled type I and type IIa fibres. Mean (centre line) and SD (error bars) with individual data (circles, n = 10) showing the effects of SIT on protein expression. Symbols indicate a significant difference from pre-exercise (*) or 24 h post-exercise (†) within the same sample type or a significant difference from pre-exercise type I fibres (§) at $p < 0.05$.

[a] Values expressed relative to pre-exercise in whole muscle from the same gel.

[b] Values expressed relative to pre-exercise in type I fibres from the same gel.

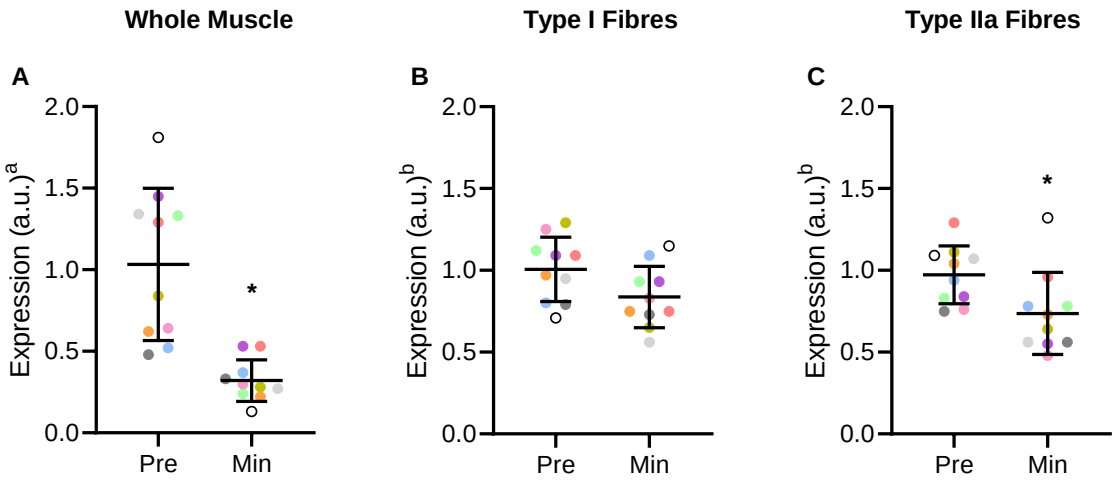

Figure 3.4: Comparison of pre-exercise and minimum post-exercise RyR1 content. Mean (centre line) and SD (error bars) along with individual data (circles, n = 10) showing pre-exercise (Pre) and the lowest post-exercise (Min) RyR1 protein content, regardless of the time point at which it occurred. This peak fragmentation response shows the interindividual variability in the timing of peak fragmentation, as there was a greater reduction in RyR1 in whole muscle (-62 ± 21%; p = 0.001), type I fibres (-12 ± 35%; p = 0.136), and type IIa fibres (-24 ± 20%; p = 0.007). * denotes statistical significance at $p < 0.05$.

[a] Values expressed relative to pre-exercise in whole muscle from the same gel.

[b] Values expressed relative to pre-exercise in type I fibres from the same gel.

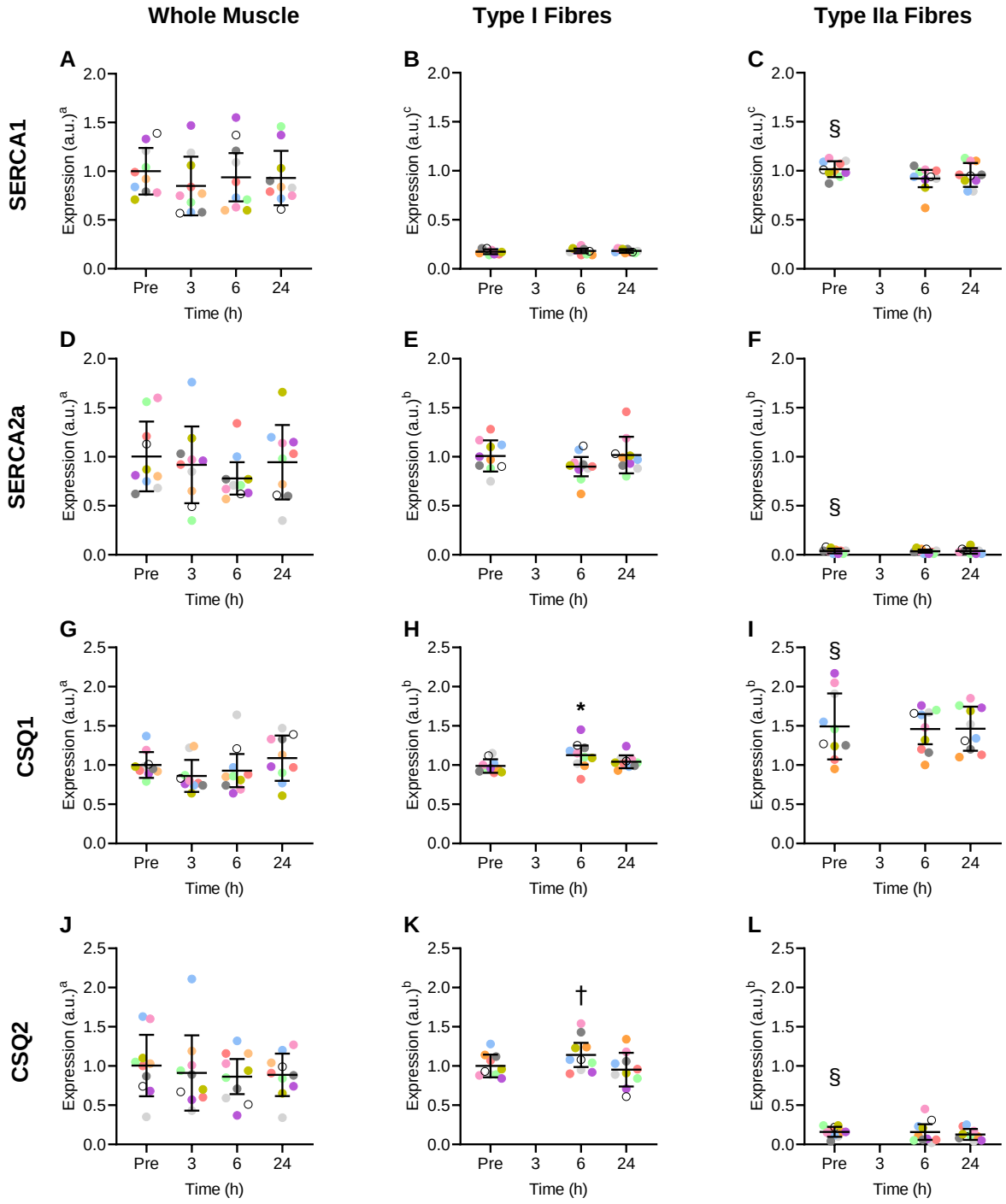

Figure 3.5: Relative expression of SERCA and CSQ isoforms following acute SIT in whole muscle and pooled type I and type IIa fibres. Mean (centre line) and SD (error bars) with individual data (circles, n = 10) showing the effects of SIT on protein expression. Symbols indicate a significant difference from pre-exercise (*), or 24 h post-exercise (†) within the same sample type, or a significant difference from pre-exercise type I fibres (§) at $p < 0.05$.

[a] Values expressed relative to pre-exercise in whole muscle from the same gel.

[b] Values expressed relative to pre-exercise in type I fibres from the same gel.

[c] Values expressed relative to pre-exercise in type IIa fibres from the same gel.

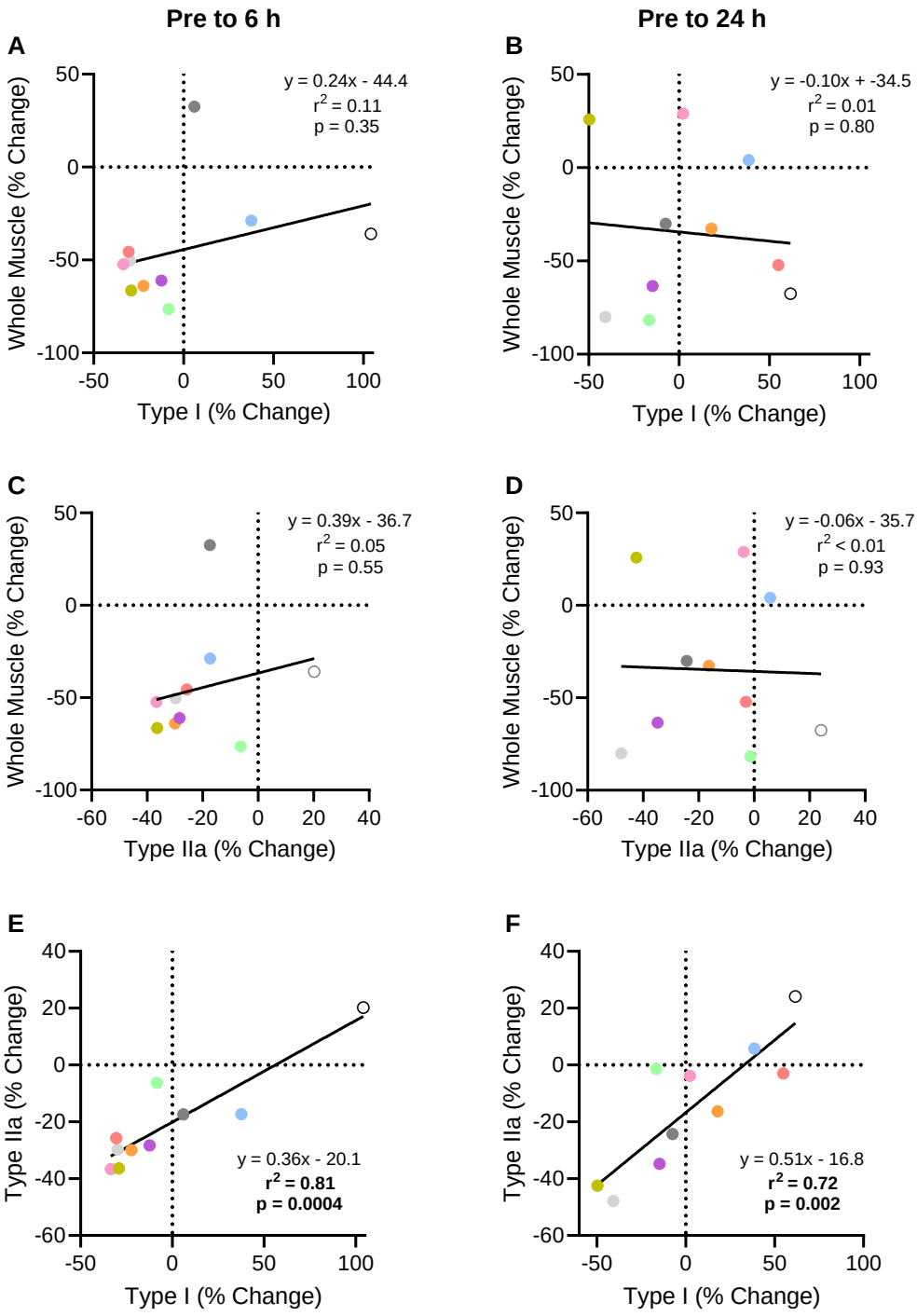

Figure 3.6: Relationships between RyR1 fragmentation responses in whole muscle, type I, and type IIa fibres from pre-exercise to 6 and 24 h post-exercise. Percent changes in RyR1 expression from pre-exercise to 6 and 24 h post-exercise compared between whole muscle and type I fibres (**A, B**), whole muscle and type IIa fibres (**C, D**), and between fibre types (**E, F**). Panels include equation of the linear regression line, r^2, and p-value. Significant relationships are denoted as bolding of the r^2 and p-value.

Chapter Four: Conclusion

Sprint interval exercise is capable of inducing mitochondrial adaptations similar to continuous endurance exercise despite requiring only a fraction of the total exercise volume (Gibala *et al.*, 2006; Gillen *et al.*, 2016). Significant research effort has centered on understanding the mechanisms and pathways through which SIT triggers mitochondrial biogenesis, including the time course of steps in the adaptive pathway (Little *et al.*, 2011; Granata *et al.*, 2017; Fiorenza *et al.*, 2018). Recent work suggested that modifications to RyR1 and altered Ca^{2+}-handling were a potential mechanism in SIT-induced mitochondrial biogenesis (Place *et al.*, 2015; Schlittler *et al.*, 2019; Wyckelsma *et al.*, 2020). Specifically, RyR1 fragmentation had been shown to be present 24 h post-exercise; however, considering that most acute signalling events (i.e., kinase activation, transcriptional regulator translocation, upregulation of gene expression) have been shown to occur within the 24 h post-exercise, it was unclear if RyR1 fragmentation could be involved in mitochondrial biogenesis. Our novel finding that RyR1 fragmentation is present markedly earlier than previously understood places RyR1 fragmentation within the time frame of other important signalling events. We also showed that RyR1 fragmentation is fibre-type dependent, supporting other research that has found fibre-specific effects of intense interval exercise both acutely and chronically. Furthermore, no study showing RyR1 fragmentation had comprehensively examined if the expression of other Ca^{2+}-handling proteins was similarly affected. Our results also support previous mechanistic studies showing that Ca^{2+} leak via RyR1, rather than impairments in other Ca^{2+}-handling proteins, is primarily responsible for an increase in $[Ca^{2+}]_i$ (Ivarsson *et al.*, 2019), as we did not observe changes in the content of calcium uptake (SERCA) or storage (CSQ) proteins in whole muscle post-SIT.

4.1 Limitations and Methodological Considerations

There are some limitations that must be noted when considering the results of this thesis. First, total protein content is not the only regulatory mechanism controlling protein function. Other factors, such as post-translational modifications (e.g., RyR1 phosphorylation (Bellinger *et al.*, 2008)) and interactions with other proteins (e.g., regulation of SERCA by phospholamban and sarcolipin (Fajardo *et al.*, 2013; Stammers *et al.*, 2015)), also regulate protein function. Though it was beyond the scope of this thesis to assess these other factors, it is important to acknowledge the potential for other mechanisms to interact with changes (or the lack thereof) in protein content when considering alterations to protein function.

The single fibre segment collection for pooled fibre samples presents another limitation of the findings reported here. Due to the necessity of physically separating a single fibre from fibre bundles while keeping it intact, fibres with a larger cross-sectional area may be easier to collect. This apparent limitation makes collecting type I fibres slightly more difficult and time-consuming in general, but it may mean that the type I fibres collected represent the larger end of the type I fibre size spectrum. Furthermore, the number of fibres pooled (10-20) may not be fully representative of that fibre type. Though setting a minimum number of fibres is necessary for experimental feasibility (Christiansen *et al.*, 2019)—for this study approximately 1500 fibre segments were collected and identified—there is a trade-off with how generalizable the findings are to the entire fibre population. Similarly, due to the nature of fibre segment collection and identification, we could not investigate pooled fibre responses at every time point in this thesis; we therefore chose to prioritize the time points where the whole muscle RyR1 fragmentation response was statistically significant. It is unlikely that investigating the 3 h timepoint would have altered the results of this thesis. Finally, as type IIx fibres are relatively rare, we chose to

study only type I and IIa fibres, which means that the relative magnitude of RyR1 fragmentation in type IIx human fibres remains unknown.

Finally, the lack of measured downstream effects of RyR1 fragmentation is a limitation of the present work. While we sought to characterize the RyR1 response alongside related proteins, we were not able to measure the downstream events potentially related to RyR1 fragmentation. Though we showed when RyR1 fragmentation is present, how the level of $[Ca^{2+}]_i$, phosphorylation of kinases such as CaMKII, and expression of genes like *PGC1A* relate to this specific time course remains unknown.

4.2 Future Directions

The findings presented in this thesis provide the rationale for further research interrogating the RyR1 modification mechanism as it pertains to exercise-induced mitochondrial adaptations. Overall, our research suggests that 6 h may be a more consistent time point to measure for detecting changes in full-length RyR1 in future studies, as the heterogeneity of the response is an important factor to consider. Though we were not powered to detect a sex-specific effect of SIT on RyR1 fragmentation, this was the first time that RyR1 fragmentation was shown to occur in females. Future work should include female participants and, considering the differences in relative fibre area between females and males (Glenmark *et al.*, 1992; Staron *et al.*, 2000) and our fibre type-specific results, should also aim to examine if there is an effect of sex on RyR1 fragmentation. The present work also presents solid reasoning for investigating type IIx fibres post-SIT: these extremely fast-twitch fibres are likely heavily recruited for ‘all-out’ exercise and the whole muscle RyR1 response is not explained by the fragmentation seen in type I and type IIa fibres.

Beyond sex- and fibre-specific analyses, other research should aim to expand upon the relationship between RyR1 fragmentation and purported downstream events in human participants. Though there is mechanistic evidence for RyR1 destabilization causing endurance adaptations even without exercise in mice (Bruton *et al.*, 2010; Ivarsson *et al.*, 2019), further investigation of signalling kinase and gene expression responses and their relationship to RyR1 fragmentation in humans is required.

Finally, whether RyR1 modifications are involved in the adaptive pathway following other types of exercise—both those using shorter bouts than SIT, like RST, and longer bouts like HIIT or MICT—is a question that remains to be answered. Studying the response of RyR1 after different exercise sessions would help shed light on the relevance of the mechanism to mitochondrial adaptations in general, as well as the role of exercise intensity, if it is in fact only present after 'all-out' efforts.

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Appendix A: Optimization of the Dot Blotting Technique for Identification of Single Fibre Segments

Background

Single fibre isolation, where individual muscle fibre segments are separated out from a muscle biopsy sample, is a valuable technique for investigating fibre type differences and fibre-specific responses to an intervention (Tobias & Galpin, 2020). Fibres are collected, identified as type I, type IIa, type IIx, or hybrids, and probed for differences in protein abundance, using techniques such as Western blotting. Individual fibres may also be pooled to reduce the number of samples that need to be probed (Christiansen *et al.*, 2019). Previously, the methods to identify single fibre segment fibre types, such as SDS-PAGE and Western blotting, have been time-consuming and use a larger portion of the fibre sample, leaving less material for subsequent experiments (Murphy, 2011). Recently, Christiansen et al. (2019) described a dot blotting method to determine fibre type in single fibre segments that is faster (single day vs. multiple days for regular Western blotting) and uses less sample (1/10 of a fibre vs. 1/5 of a fibre). Despite this advance in the methodology, the protocol still required almost 9 h to identify type I and IIa fibres on one membrane (i.e., 30-40 fibre segments). We aimed to reduce the time needed to complete the dot blotting procedure, making it feasible to complete multiple membranes within one day. The final result was a scalable protocol that required < 6 h to complete one membrane and ~7 h to complete four membranes simultaneously.

Procedural Differences

A workflow representation of the two protocols is shown in Figure A-1. The specific procedure we followed is described in section 3.2.3. The two main steps where we saved time

over the previous protocol were the antibody incubations. For regular Western blotting, we incubate membranes in the primary antibody for 2 h at room temperature and overnight at 4 ºC, and in the secondary antibody for 1 h at room temperature. While the Christiansen et al. protocol used a shorter primary antibody incubation time (2 h at room temperature) and the same secondary antibody incubation time, we further reduced these times to 1 h for the primary antibody and 30 min for the secondary antibody, both at room temperature. Additionally, we reduced the time of the stripping step from 45 min to 30 min. To account for these shorter incubation times, we increased the duration of the blocking step from 5 min to 15 min, aiming to limit non-specific binding.

Another consideration of our protocol is handling multiple membranes simultaneously. The protocol was designed so that four membranes could be processed together by a single researcher. There are two main steps where extra time is added while subsequent membranes are processed: during the initial dot blotting step and during the final imaging process. Briefly, the first membranes that are loaded are set aside for drying until all membranes are dry (up to ~45 min) even though drying only requires 2-5 min. Similarly, during imaging, membranes are allowed to rock in wash buffer while the others are being imaged. Creating these steps of variable length was necessary to process multiple membranes simultaneously. Using the Christiansen et al. (2019) protocol for four membranes, we would expect the same additional time (~45 min to 1 h) to be necessary. Although not validated here, there is no reason to expect that longer drying or washing times would influence the qualitative analysis needed for fibre typing. As evidence of this point, all of the pooled fibre type samples—which were created based on the results of dot blotting experiments—were pure type I or type IIa, as expected.

Validation

To validate our faster approach, we compared used pre-exercise and 24 h post-exercise pooled type I and type IIa fibre samples using three fibre type-identification methods: 1) our optimized protocol, 2) a dot blotting protocol using incubations of equal time to those frequently used in gel electrophoresis and Western blotting (i.e., primary incubation: overnight at 4 °C, secondary incubation: 1 h at room temperature), and 3) gel electrophoresis and Western blotting (Figure A-2). Our validation experiments showed that using shorter antibody incubation times did not affect our ability to identify fibres as type I or type IIa when dot blotting. Comparison to gel electrophoresis showed the same fidelity. These results support our protocol as an accurate and faster improvement to the previously described protocol.

Hour	Christiansen et al. 1		1	2	3	4
0	Membrane Prep		Membrane Prep	Membrane Prep	Membrane Prep	Membrane Prep
	Blot		Blot	Blot		
	Drying + Reactivation		Drying	Drying	Blot	Blot
	Blocking				Drying	Drying
1	MHCIIa		Reactivation	Reactivation	Reactivation	Reactivation
	Antibody Incubation		Blocking	Blocking	Blocking	Blocking
			MHCIIa	MHCIIa	MHCIIa	MHCIIa
			Antibody Incubation	Antibody Incubation	Antibody Incubation	Antibody Incubation
2						
			Wash	Wash	Wash	Wash
			Secondary	Secondary	Secondary	Secondary
3	Wash		Antibody Incubation	Antibody Incubation	Antibody Incubation	Antibody Incubation
	Secondary		Wash + Image	Wash+ Image	Wash	Wash
	Antibody Incubation				Image	Image
			Strip Antibodies	Strip Antibodies	Strip Antibodies	Strip Antibodies
4						
	Wash + Image		MHCI	MHCI	MHCI	MHCI
	Strip Antibodies		Antibody Incubation	Antibody Incubation	Antibody Incubation	Antibody Incubation
5						
	MHCI		Wash	Wash	Wash	Wash
	Antibody Incubation		Secondary	Secondary	Secondary	Secondary
			Antibody Incubation	Antibody Incubation	Antibody Incubation	Antibody Incubation
6			Wash + Image	Wash+ Image	Wash	Wash
					Image	Image
7						
	Wash					
	Secondary					
	Antibody Incubation					
8						
	Wash + Image					

Figure A-1: Daily workflow comparison of dot blotting protocols. Each step of the dot blotting process was assigned an approximate time to complete. The Christiansen et al. (2019) protocol takes almost 9 h to complete, from initial membrane preparation to capturing the final image. With our protocol, one researcher can process four membranes in ~6.5 h, and if extra time for the other membranes is removed, can complete a single membrane in under 6 h. MHC, myosin heavy chain. Note that if one wanted to complete four membranes with the Christiansen et al. (2019) method, the additional membranes could likely be completed with an additional hour of time (i.e., ~10 h).

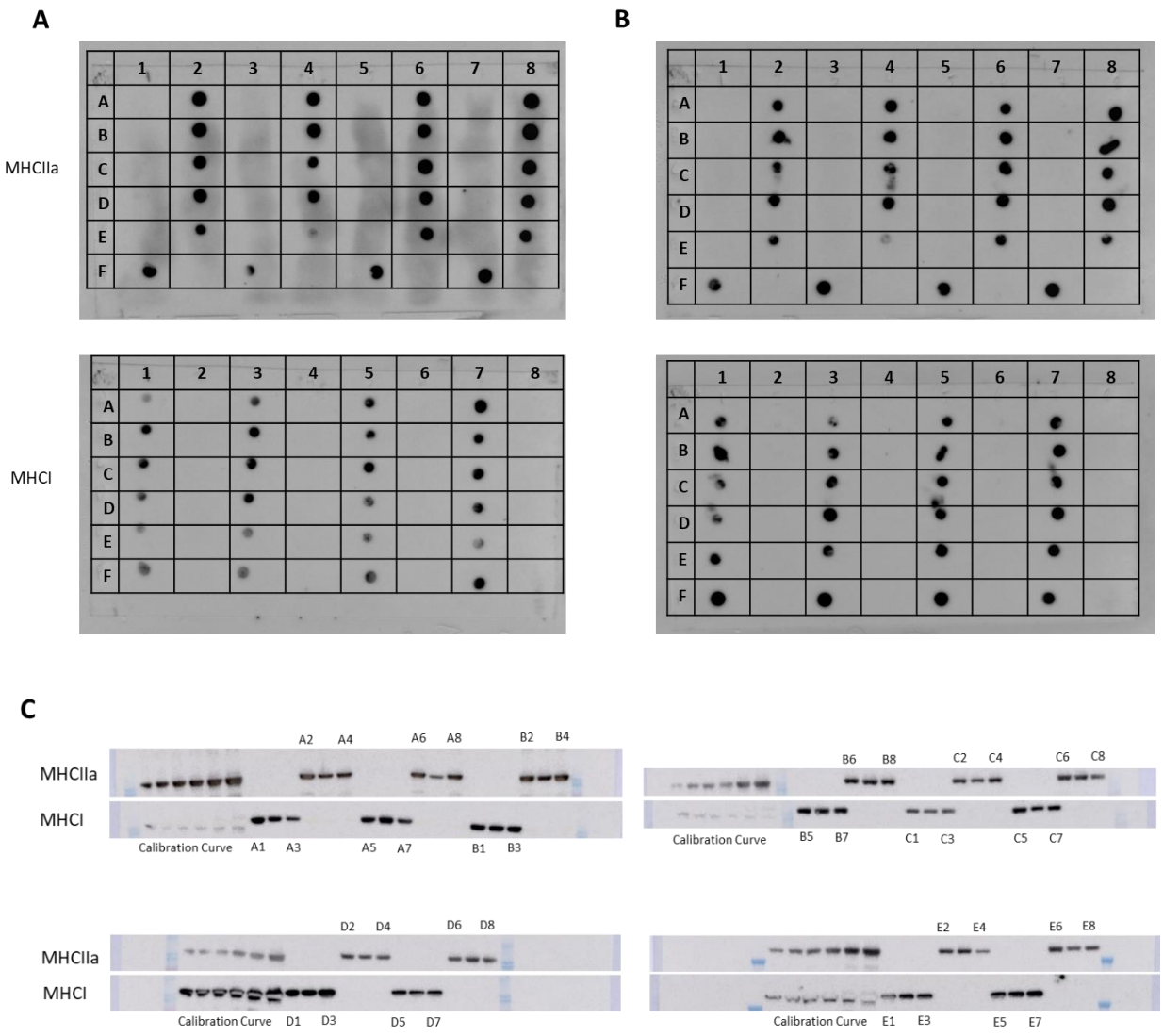

Figure A-2: Validation of the faster dot blotting protocol. Pre- and 24 h post-exercise pooled type I and type IIa muscle fibres were used to validate our faster dot blotting protocol. Samples from 10 participants were spotted on PVDF membranes in a predetermined grid (From left to right: Pre type I, Pre type IIa, 24 h type I, 24 h type IIa, then repeated) along with four whole muscle controls (row F of each grid) and processed using either our faster dot blot protocol (**A**) or a slower dot blot protocol where incubation times matched those used in our regular Western blotting protocol (**B**). Dot blotting was validated against samples run on 4-12% Bis-Tris gels, transferred to nitrocellulose membranes, and probed using standard Western blotting techniques (**C**). In all instances, membranes were stripped between antibody probes. Lanes in panel **C** are labelled with their corresponding grid identifier from panels **A/B**. Due to subsequent fibre collection after the initial dot blotting experiments, pooled fibre samples from the 6 h time point are also included on the membranes in panel **C** (unlabeled lanes), but these fibres are not shown on the images in panels A and B. MHC, myosin heavy chain.

Appendix B: Fibre Identification Counts

Table B-1: Number of each fibre type identified during dot blotting

	Type I (#)	Type IIa (#)	Hybrid (I/IIa; #)	Blank (#)	Total (#)
Pre	157	227	18	77	479
6 h	149	184	92	113	538
24 h	149	212	85	54	500
Total	455	623	195	244	1517

Table B-2: Percentage of each fibre type identified at each time point via dot blotting

	Type I (%)	Type IIa (%)	Hybrid (I/IIa; %)	Blank (%)	Total (%)
Pre	35.4	46.3	3.9	14.4	100
6 h	28.4	34.7	17.9	19.0	100
24 h	32.3	43.3	15.2	9.3	100
Average	32.0	41.4	12.3	14.2	100

Appendix C: Antibody Details

Table C-1: Primary antibody details

Protein	Concentration	Species	Mono/polyclonal?	Identifier	Supplier
RyR1	1:500	Mouse	Mono	34C	DSHB
DHPR	1:400	Mouse	Mono	III5ED1	DSHB
FKBP12	1:1000	Rabbit	Mono	ab2918	abcam
SERCA1	1:1000	Mouse	Mono	CaF2	DSHB
SERCA2a	1:1000	Rabbit	Poly	A010-20	Badrilla
CSQ1	1:500	Mouse	Mono	sc-53012	Santa Cruz
CSQ2	1:1000	Rabbit	Poly	ab3516	abcam
MHCIIa	1:200	Mouse	Mono	A4.74	DSHB
MHCI	1:200	Mouse	Mono	A4.840	DSHB

Appendix D: Letter of Consent

STANDARD CONSENT FORM

TITLE: The effects of exercise intensity on ryanodine receptor fragmentation in human skeletal muscle

SPONSOR: Natural Science and Engineering Research Council (NSERC)

INVESTIGATORS:

Principal Investigator

[redacted]

Co-Investigators

[redacted]

This consent form is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, please ask. Take the time to read this carefully and to understand any accompanying information. You will receive a copy of this form for your records.

BACKGROUND

You are being invited to participate in a study that examines how exercise intensity affects the signals to improve fatigue resistance within muscle, and to better understand the factors that influence this process. It is well known that regular endurance exercise can increase the body's aerobic fitness and endurance capacity. Interval exercise has emerged as a popular, time-efficient alternative for improving these same areas typically associated with long, low-intensity exercise. It appears that one key factor in interval exercise's ability to cause these improvements is the very high intensities that are achieved during the intervals. However, the mechanisms within the muscle by which exercise intensity is turned into beneficial adaptations remain unknown. One such potential mechanism was recently discovered, when a skeletal muscle protein (the

ryanodine receptor, RyR1) was shown to break following extremely intense exercise, generating a signal for the muscle to increase fatigue resistance. This study will attempt to replicate this finding, and will be the first to examine if the same response occurs at lower exercise intensities.

WHAT IS THE PURPOSE OF THE STUDY?

The primary purpose of this study is to characterize a potentially critical mechanism by which intensity influences muscular adaptations to exercise. This study will also examine how factors such as biological sex and muscle fibre type regulate the response to each exercise intensity.

WHAT WOULD I HAVE TO DO?

To be part of the study, the following inclusion criteria must be met:

- Between the ages of 18 and 40,
- Pass the physical activity readiness screening tool (CSEP Get Active Questionnaire; GAQ)
- Recreationally-active (performing moderate-vigorous intensity endurance exercise < 3 times per week, VO_2max < 50 ml/kg/min), and
- Women must be eumenorrheic (i.e., having regular periods).

Further, participants will be excluded if they meet any one of the following exclusion criteria:

- Being obese (BMI > 30 kg/m^2);
- Being pregnant
- Taking medications that are known to affect cardiovascular or metabolic responses to exercise (e.g., β-blockers, anti-inflammatories, anti-coagulants, insulin, etc.);
- Dieting for weight loss or following a low-carbohydrate diet;
- Smoking or using tobacco products (within the past year);
- Consuming excessive amounts of alcohol (>21 units/week);
- Self-identifying with any of the following conditions: renal or gastrointestinal disorders, metabolic disease, heart disease, vascular disease, rheumatoid arthritis, diabetes, poor lung function, uncontrolled blood pressure, dizziness, thyroid problems, or any other health conditions that are being treated and deemed likely to confound the results;
- Having orthopedic issues that limit exercise capacity;
- Using an investigational drug product within the last 30 days;
- Not understanding English.

This study involves two experiments. You have volunteered to participate in experiment ____.

Experiment 1

Time Commitment: Once accepted into the study, you will be required to come to the laboratory for three visits. These visits will be separated by 1 week (visits 1 and 2) or 24 hours (visits 2 and 3). The first two visits require approximately 2 hours, and the third visit will require approximately 30 minutes, for a total time commitment of 4.5 hours.

Visit 1: On the first visit, you will undergo a body scan to measure body composition, perform an aerobic fitness test, and be familiarized to the sprint interval exercise bout. The whole-body

body composition testing is performed using dual-energy x-ray absorptiometry (DXA). For this test, you will lay still on a bed that is open to the room while the scanning arm of the device passes over your body. This test uses x-rays to differentiate between bones, fat, and other tissues. The test requires approximately 7 minutes to complete. For the aerobic fitness test, you will pedal a stationary bicycle while wearing a facemask connected to a device that measures the volume and composition of gases you inspire and expire. The intensity of the test will increase progressively (1 W every 2 s) until you reach a point where it becomes too difficult and you decide to stop (i.e., exhaustion). Based on the amount of oxygen you consume, we will calculate your maximal oxygen uptake (VO2max), which is a commonly used indicator of aerobic fitness. This test will require approximately 30 min to complete. The sprint interval exercise familiarization will also be performed on the stationary bike. We will select a resistance based on your body weight, and you will be asked to bike as hard as possible for 30 seconds. You will then be given four and a half minutes to rest, before repeating the sprint once more. This familiarization will require approximately 30 min to complete. In total, this visit will require approximately 2 hours of your time.

Visit 2: On your second visit, you will undergo a muscle biopsy procedure and perform the full sprint interval exercise bout. A trained physician will perform the muscle biopsy, which involves collection of a small piece (~50-100mg, about the size of a pencil eraser) of muscle from your thigh. This procedure begins with the biopsy site being carefully cleaned using an alcohol swab. A small amount of local freezing with be injected into the skin and slightly below. After waiting for the freezing to begin acting, a small hollow needle will be used to pierce the skin and guide the biopsy needle to the muscle tissue. Four to five small cuts will be made using the needle to collect tissue (~10-20mg/cut), with the needle removed from the leg and the tissue removed from the needle following each cut. After the procedure is complete, pressure will be applied to the biopsy site for 2-5 minutes to minimize any bleeding. The site surrounding the biopsy will be cleaned once more, before a sterile bandage is applied. You will be provided with alcohol swabs, sterile bandages, and instructions on how to care for their biopsy site while it heals. This procedure will require approximately 30 minutes to complete. You will then perform the full sprint interval exercise bout on a stationary bike, completing six sprints identical to those you will be familiarized to during visit 1. This exercise will require approximately 30 minutes to complete. 3 and 6 hours after exercise completion, you will be asked to return to the lab for 30 minutes for collection of follow-up muscle biopsy samples. The procedure will be identical to the pre-exercise muscle biopsy. A total time commitment of approximately 2 hours of your time is required for visit 2.

Visit 3: For your third visit, you will return to the lab 24 hours after the second visit to undergo a second muscle biopsy. The procedure will be identical to those during the second visit. This visit will require approximately 30 minutes of your time.

Experiment 1 Biopsy Timing:

#1 – Immediately prior to exercise

#2 – Three hours after exercise

#3 – Six hours after exercise

#4 – 24 hours after exercise

Experiment 2

Time Commitment: Once accepted into the study, you will be required to come to the laboratory for nine visits. These visits will be separated by 1 week (visits 1 and 2, and between

each exercise intensity trial) or 24-48 hours (all other visits). Each visit will require between 30 minutes and 2 hours, for a total time commitment of approximately 11.5 hours.

Visit 1: On the first visit, you will undergo a body scan to measure body composition, perform an aerobic fitness test, and be familiarized to the sprint interval exercise bout. The whole-body body composition testing is performed using dual-energy x-ray absorptiometry (DXA). For this test, you will lay still on a bed that is open to the room while the scanning arm of the device passes over your body. This test uses x-rays to differentiate between bones, fat, and other tissues. The test requires approximately 7 minutes to complete. For the aerobic fitness test, you will pedal a stationary bicycle while wearing a facemask connected to a device that measures the volume and composition of gases you inspire and expire. The intensity of the test will increase progressively (1 W every 2 s) until you reach a point where it becomes too difficult and you decide to stop (i.e., exhaustion). Based on the amount of oxygen you consume, we will calculate your maximal oxygen uptake (VO2max), which is a commonly used indicator of aerobic fitness. This test will require approximately 30 min to complete. The sprint interval exercise familiarization will also be performed on the stationary bike. We will select a resistance based on your body weight, and you will be asked to bike as hard as possible for 30 seconds. You will then be given four and a half minutes to rest, before repeating the sprint once more. This familiarization will require approximately 30 min to complete. In total, this visit will require approximately 2 hours of your time.

Visits 2-3: On your second and third visits, you will perform a 30-minute cycling trial at a predetermined intensity. During the trial, your breathing parameters and muscle oxygenation will be continuously monitored, as well as blood lactate and your perceived exertion measures taken every 5 minutes. Each subsequent visit you will perform the same protocol, but at a slightly higher or lower intensity (+/- 10W), depending on whether steady-state (no significant change in blood lactate and breathing parameters) was observed. These visits will last approximately 1 hour each, and will allow us to identify the highest intensity that your aerobic system can support, which will be used to prescribe intensity for a subsequent visit.

Visits 4-9: The remaining six visits involve collection of a pre-exercise muscle biopsy and muscle fatigue assessment, completion of one of three exercise bouts in a random order, and a 24-hour follow-up visit. A trained physician will perform the muscle biopsy, which involves collection of a small piece (~50-100mg, the size of a pencil eraser) of muscle from your thigh. This procedure begins with the biopsy site being carefully cleaned using an alcohol swab. A small amount of local freezing with be injected into the skin and slightly below. After waiting for the freezing to begin acting, a small hollow needle will be used to pierce the skin and guide the biopsy needle to the muscle tissue. Four to five small cuts will be made using the needle to collect tissue (~10-20mg/cut), with the needle removed from the leg and the tissue removed from the needle following each cut. After the procedure is complete, pressure will be applied to the biopsy site for 2-5 minutes to minimize any bleeding. The site surrounding the biopsy will be cleaned once more, before a sterile bandage is applied. You will be provided with alcohol swabs, sterile bandages, and instructions on how to care for their biopsy site while it heals. This procedure will require approximately 30 minutes to complete. You will then perform one of the three exercise protocols on a stationary bike: sprint intervals, high-intensity intervals, or moderate-intensity continuous exercise. The full sprint interval exercise bout involves completing six sprints identical to those you will be familiarized to during visit 1. This exercise will require approximately 30 minutes to complete. The high-intensity interval exercise bout

involves completing five four-minute intervals at 110% of the power output determined by visits 2-3, with two-minute rest periods of light cycling in between each. This exercise will require approximately 45 minutes to complete. The moderate-intensity continuous exercise bout will be performed at a constant intensity determined from your aerobic fitness test, and its duration will be selected so that the total work completed is equal to that in the high-intensity interval bout. This protocol will require approximately 1 hour to complete. In total, the exercise trial visits will require between 1.5 and 2.5 hours of your time, depending on the exercise bout being performed.

24 hours following the exercise intensity trial, you will return to the laboratory for a follow-up muscle biopsy, identical to that described above. This visit will require approximately 30 minutes of your time.

In order to collect a data from each condition, you will perform the tasks described above on three separate occasions with 1 week between each exercise intensity trial. An example is shown below; however, the actual order of the exercise bouts will be randomized:

Visit 4: Perform moderate-intensity continuous bout and associated procedures

Visit 5: 24-hour follow-up for moderate-intensity continuous bout

Visit 6: 1 week after visit 4, perform sprint interval bout and associated procedures

Visit 7: 24-hour follow-up for sprint interval bout

Visit 8: 1 week after visit 6, perform high-intensity interval bout and associated procedures

Visit 9: 24-hour follow-up high-intensity interval bout

Experiment 2 Biopsy Timing:

#1 – Immediately prior to first exercise bout

#2 – 24 hours after first exercise bout

#3 – Immediately prior to second exercise bout

#4 – 24 hours after second exercise bout

#5 – Immediately prior to third exercise bout

#6 – 24 hours after third exercise bout

Measurements: All measurement techniques are used as routine measures in our laboratory. Trained personnel will administer each test, and all equipment will be properly cleaned prior to each test. All blood sampling will be collected from finger pricks made using single-use lancets, and all medical waste will be disposed of properly. In total, approximately 8-12 finger pricks will be performed, and less than 2 mL of blood will be collected for this study. All blood samples will be used immediately for the determination of blood lactate concentrations. All biopsy sampling will be done by a trained physician with single-use microbiopsy needles, and as with blood collection, all medical waste produced by the biopsy procedure will be disposed of properly. The results of the DXA scan will be available to participants, but they are not to be used for medical purposes (i.e., they are not suitable for interpretation by medical personnel).

Dietary Restrictions: For the exercise aerobic fitness tests (visit 1 in each experiment), you will be asked to fast overnight (at least 10 hours without food). You will be permitted to drink water during the fast but not food or other drinks (e.g., coffee, tea). You will be provided with a simple snack (e.g., juice and granola bar) after the exercise tests. For the exercise intensity trials (visits 4, 6, and 8 in experiment 2), we will provide two standardized meals to all participants to consume the evening before, and the morning of these visits. You will then arrive to the

laboratory for testing 2-3 hours following consumption of the provided breakfast. Meals will be chosen based on participants' preferences, but each participant will have the same meals the evening and morning before each visit.

Exercise Restrictions: Participants will be asked not to exercise in the 24 hours preceding each visit to the laboratory for this study, and not to exercise for the 24 hours following each exercise intensity trial.

WHAT ARE THE RISKS?

All exercise carries a minimal risk and may be uncomfortable if you are unfit or not used to doing exercise. In healthy adults who have no signs or symptoms, which may contraindicate exercise, there is no reason to expect any harmful effects of exercise protocols in the moderate intensity exercise domain. To minimize any potential risks during maximal testing, heart rate will be monitored throughout the test and study personnel are trained to handle adverse events. At least one member of the study team will have training in cardiopulmonary resuscitation (CPR). There might be some minor discomfort during the exercise testing. You may experience increased awareness of breathing, muscle pain and/or fatigue, increased sweating, or a general feeling of fatigue or nausea, all of which are not unexpected consequences of exercise. You may experience some minor discomfort from wearing the breathing mask necessary for VO_2 measurements. These sensations often become less noticeable with time during the tests. Additionally, mild discomfort from finger prick blood samples might also be experienced, and there is a risk of infection with all blood sampling; however, blood will be collected using a single-use lancet by trained personnel. The equipment used to measure VO_2 will be disinfected after each use, following manufacturers' instructions.

You may experience mild pain and discomfort during the muscle biopsy procedure. The local freezing will likely result in a burning feeling in the thigh at the time of the injection. This will last only 5 – 10 seconds. There is an extremely low risk of allergic reaction to the local injection (1 in 1 million). The chance of a local skin infection is less than 1 in 619. Carefully cleaning the skin and keeping the area clean and dry until the skin heals, and sterile procedures will minimize this risk. Most participants experience local soreness and stiffness in the leg for two or three days after the biopsy, similar to a deep bruise or Charlie Horse. There is a very low risk of internal bleeding at the biopsy site which can result in more prolonged pain and stiffness in the leg. On occasion, a small lump of scar tissue may form under the site of the incision, but this normally disappears within 2-3 months. A small visible scar often remains from the biopsy incision. Subsequent biopsies will be immediately adjacent to the initial biopsy area to minimize any scarring. There is a possibility of a small area of numbness (about the size of a one-dollar coin) around the biopsy site. This usually resolves over 5 – 6 months. There is a very low, theoretical risk (zero cases in 18 000 biopsies) of damage to a small nerve branch to the muscle. This would result in partial weakness of the vastus lateralis muscle (one of four muscles that straightens the knee) and would have no impact on day-to-day activities. Nerve injuries like this usually resolve in 8 – 12 months, but there is a theoretical risk of some muscle fibres being denervated which would not affect the functioning of the muscle.

Your participation in this study will require you to undergo multiple muscle biopsies in the same muscle within a short amount of time (2-4 biopsies in 3-24 hours). Repeated biopsy sampling is a common practice in research looking at the short-term responses of skeletal muscle to exercise

and does not compound the risk of soreness, pain, or complication beyond that of each individual biopsy itself. In other words, the risk of complication associated with collection of an individual biopsy sample is not affected by other recent biopsy samples.

In this study you will be asked to perform intense exercise immediately following biopsy collection. Intense exercise (including sprinting and heavy resistance training) is frequently performed in the minutes/hours after a muscle biopsy procedure without any adverse effects. It is possible that you may experience some discomfort at the biopsy site during exercise as the local anesthetic wears off, and the exercise may increase the soreness felt during recovery from the biopsy procedure. During the recovery, we suggest that you return to your typical physical activity levels based on your perceived recovery/soreness, and suggest that you limit additional intense exercise until you feel fully recovered to avoid the risk of increased muscle soreness or injury.

The DXA scanning procedure using x-rays to measure body composition. The radiation dose from one DXA scan is approximately 10 micro Sieverts, which is approximately the amount of radiation that an average person is exposed to in 24 hours from the environment. This procedure is painless and non-invasive.

All testing procedures will only be conducted when personnel that is certified in CPR is present. In the case of an emergency, 911 and campus security will be called using a telephone available in the testing laboratory. An automatic external defibrillator is also available within the testing building.

WILL I BENEFIT IF I TAKE PART?

This is a basic physiology study and, as such, there will be no direct benefits received because of participating in the study. If you are interested, the rationale for conducting the research and theory and significance of each of the tests will be explained, as will your individual results from each of the tests. You will also have the opportunity to learn about and better understand your physiological responses to an exercise situation. You are encouraged to ask questions regarding the purpose of the study, specific measures or outcomes of your exercise test, or overall findings and conclusions from this research study.

DO I HAVE TO PARTICIPATE?

Your participation in this research project is entirely voluntary. You can withdraw anytime by emailing [redacted] or by expressing this desire verbally to any of the investigators. All biological specimens will be stored indefinitely in a secure location at the University of Calgary. If you withdraw from the study, your data can be removed from the data set prior to the commencement of biological sample analysis (typically weeks to months following completion of data collection), and any biological samples you provided will be destroyed. After this point, data and biological samples cannot be withdrawn.

In addition, you might be withdrawn from the study for the following reasons:

- Changes in your status so you do not fit within the admission criteria for this study
- You cannot complete all testing sessions within the proposed period of the study

- You are not able to comply with the instructions prior to each testing session

If new information becomes available that might affect your willingness to participate in the study, you will be informed as soon as possible.

WHAT ELSE DOES MY PARTICIPATION INVOLVE?

Your participation in this study does not involve anything else beyond what is specified in this informed consent.

WILL I BE PAID FOR PARTICIPATING, OR DO I HAVE TO PAY FOR ANYTHING?

You will be paid $200 for participating in experiment 1 or $300 for participating in experiment 2. If you are coming to the University of Calgary campus specifically for testing purposes in this study, you may request to be reimbursed for your parking. Should you withdraw prior to completing the study, your payment will be pro-rated based on the percentage of experimental lab visits that you had completed. In experiment 1, visits 2-3 are considered experimental lab visits. In experiment 2, visits 4-9 are considered experimental lab visits.

WILL MY RECORDS BE KEPT PRIVATE?

Information obtained during this research project is confidential. Authorized representatives from the University of Calgary and the Conjoint Health Research Ethics Board may look at your identifiable study records held at the Faculty of Kinesiology for quality assurance purposes. Aside from these quality assurance checks, only the researchers will have access to your personal information. Further, your records are associated with an identification number rather than with your name. Published reports resulting from this study will not identify you by name. Thus, your right to privacy will be retained. If you require it, you will be given a summary of your results and the average results for all participants in this study.

IF I SUFFER A RESEARCH-RELATED INJURY, WILL I BE COMPENSATED?

In the event that you suffer injury as a result of participating in this research, no compensation will be provided to you by the University of Calgary or the Researchers. You still have all your legal rights. Nothing said in this consent form alters your right to seek damages.

SIGNATURES

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to participate as a participant. In no way does this waive your legal rights nor release the investigators or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time without any negative consequences. If you have further questions concerning matters related to this research, please contact:

[redacted]

Or

[redacted]

If you have any questions concerning your rights as a possible participant in this research, please contact the Chair, Conjoint Health Research Ethics Board, University of Calgary at 403-220-7990.

Participant's Name	Signature and Date
Investigator/Delegate's Name	Signature and Date
Witness' Name	Signature and Date

The University of Calgary Conjoint Health Research Ethics Board has approved this research study.

A signed copy of this consent form has been given to you to keep for your records and reference.