POTENTIAL FACTORS INFLUENCING RESPONSES TO INTERVAL EXERCISE

POTENTIAL FACTORS INFLUENCING THE ACUTE AND CHRONIC RESPONSE OF HUMAN SKELETAL MUSCLE TO INTERVAL EXERCISE TRAINING

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LAY ABSTRACT

This thesis considered the role of biological sex, fibre type and contraction pattern on skeletal muscle responses to exercise. It focused on mitochondria, which are important components of the cell that affect health and performance. The responses of genes that regulate the formation of new mitochondria to a single session of brief, "sprint" interval exercise were similar between men and women. Acute sprint interval exercise also activated molecular pathways similar to traditional endurance exercise in the two main types of muscle fibres; however, 12 weeks of endurance training induced greater increases in mitochondrial content in type I ("slow-twitch") fibres, whereas the increases in type II ("fast-twitch") fibres were similar compared to interval training. Lastly, mitochondrial content increased after continuous training but not interval training when the intensity and total amount of exercise was matched. Our findings improve our understanding of the mechanisms by which exercise elicits mitochondrial remodelling in humans.

ABSTRACT

This thesis considered three potential factors that may influence skeletal muscle responses to interval exercise training in healthy adults, with a focus on mitochondrial remodelling: biological sex, fibre type and contraction pattern. Study 1 assessed the acute response to a sprint interval training (SIT) protocol that involved three, 20-second 'all-out' cycling efforts interspersed with 2 minutes of recovery. It found similar exercise-induced increases in the mRNA expression of genes linked to mitochondrial biogenesis in men and women. Study 2 showed that a single session of SIT and moderate-intensity continuous training (MICT) induced similar increases in the phosphorylation of signalling proteins linked to mitochondrial biogenesis in type I and IIa fibres when compared within the same individuals. It also assessed responses to 12 weeks of training in two different groups and found MICT elicited greater increases in markers of mitochondrial content, including cytochrome *c* oxidase subunit IV (COXIV) protein content, in type I fibres, whereas the increase in type IIa fibres was similar compared to SIT. Study 3 utilized single-leg cycling as a within-subject model to compare interval and continuous training protocols that were matched for exercise intensity and total training volume, but the pattern of contraction differed. Measurements of mitochondrial content, including subsarcolemmal mitochondrial area, the maximal activity of citrate synthase and COXIV protein content, increased after 4 weeks of continuous training but were unchanged after interval training. Overall, this thesis advances our understanding of the influence of biological sex, muscle fibre type and contraction pattern on skeletal muscle mitochondrial responses to exercise. Major findings include: the acute response to SIT was similar

between sexes, acute responses to interval and continuous exercise were similar but training elicited some divergent fibre type-specific responses, and mitochondrial content increased following continuous training but was unchanged following work- and intensity-matched interval training.

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LIST OF ABBREVIATIONS

36B4	Acidic ribosomal protein 36B4
ACC	Acetyl-CoA carboxylase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATP5A	ATP synthase α-subunit
b2M	β2 microglobulin
β-HAD	3-β-hydroxyacyl CoA dehydrogenase
BMI	Body mass index
BSA	Bovine serum albumin
CaMK	Ca^{2+}/calmodulin-dependent kinase
cDNA	Complementary DNA
CONT	Continuous
COX	Cytochrome *c* oxidase
COXIV	Cytochrome *c* oxidase subunit IV
CS	Citrate synthase
FFM	Fat free mass
FOXO3	Forkhead box O3
GLUT4	Glucose transporter 4
GS	Glycogen synthase
GSK3α	Glycogen synthase kinase-3α
HIIT	High-intensity interval training
HKII	Hexokinase II
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HR_{max}	Maximal heart rate
HSL	Hormone sensitive lipase
IGF1	Insulin-like growth factor 1
INT	Interval
LPL	Lipoprotein lipase
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MHC	Myosin heavy chain
MICT	Moderate-intensity continuous training
MitoPS	Mitochondrial protein synthesis
mRNA	Messenger ribonucleic acid
miRNA	Micro-ribonucleic acid
MURF-1	Muscle ring-finger protein 1
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation 1
NDUFA9	NADH:ubiquinone oxidoreductase subunit A9
OPA1	Optic atrophy 1

p38 MAPK	p38 mitogen-activated protein kinase
PBST	Phosphate buffered saline with Tween
PCr	Phosphocreatine
PDK4	Pyruvate dehydrogenase kinase isozyme 4
PDP1	Pyruvate dehydrogenase phosphatase catalytic subunit 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PPARD	Peroxisome proliferator-activated receptor delta
PRC	PGC-1α-related coactivator
RIP140	Receptor interacting protein 1
RIPA	Radioimmunoprecipitation assay
RPE	Ratings of perceived exertion
SDH	Succinate dehydrogenase
SDHA	Succinate dehydrogenase subunit A
SIRT1	Sirtuin 1
SIT	Sprint interval training
SL	Single-leg
TBP	TATA-box binding protein
TBST	Tris-buffered saline-tween
TEM	Transmission electron microscopy
TFAM	Mitochondrial transcription factor A
TFP-β	Trifunctional protein β-subunit
UQCRC2	Ubiquinol-cytochrome *c* reductase core protein 2
VEGFA	Vascular endothelial growth factor A
$\dot{V}O_{2peak}$	Peak aerobic capacity
W_{peak}	Peak power output

PREFACE:
DECLARATION OF ACADEMIC ACHIEVEMENT

FORMAT AND ORGANIZATION OF THESIS

This thesis is prepared in the "sandwich" format as outlined in the School of Graduate Studies Guide for the Preparation of Theses. It includes a general introduction, three independent studies prepared in journal article format, and a general discussion. The candidate is the first author on all of the manuscripts. At the time of the thesis preparation Chapter 2 was published in a peer-reviewed journal and Chapters 3 and 4 were in preparation for submission.

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L.E. Skelly, M.J. MacDonald, M.A. Tarnopolsky and M.J. Gibala designed the study. L.E. Skelly, J.B. Gillen, B.J. Martin, M.J. MacDonald, M.A. Tarnopolsky and M.J. Gibala were involved in data collection. L.E. Skelly, J.B. Gillen, A. Safdar and M. Akhtar completed data analyses. L.E. Skelly, J.B. Gillen, B.J. Martin, M.J. MacInnis and M.J. Gibala interpreted results of the experiments. L.E. Skelly drafted the manuscript, with input from M.J. Gibala and all authors.

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Physiological responses to interval training have been studied for at least a half century (Astrand *et al.*, 1960; Christensen *et al.*, 1960*b*, 1960*a*), but scientific interest in the topic has intensified over the last decade. This has been paralleled by a marked increase in attention by fitness enthusiasts and the media. Interval training can be simply defined as intermittent bouts of higher intensity exercise, interspersed with periods of lower intensity effort or complete rest for recovery. There are numerous variables that can be manipulated that make for almost infinite variety (Buchheit & Laursen, 2013*a*, 2013*b*) and many different types of interval training protocols have been studied. Recent efforts have been made to standardize the general terminology used to broadly categorize interval training protocols (Weston *et al.*, 2014; MacInnis & Gibala, 2017) and this thesis will adopt the approach based on exercise intensity. Sprint interval training (SIT) is characterized by brief, intermittent periods of 'all-out' or supramaximal efforts performed at an intensity that is equal to or exceeds the power output eliciting peak aerobic capacity ($\dot{V}O_{2peak}$). High-intensity interval training (HIIT) involves intervals that are performed at a relatively intense but submaximal intensity (i.e., below the power output eliciting $\dot{V}O_{2peak}$) and elicit ≥80% of maximal heart rate (HR_{max}). Interval training protocols also vary with respect to total exercise volume, which is particularly relevant when comparisons are made *versus* traditional moderate-intensity continuous training (MICT). "Low-volume" interval training generally refers to protocols involving ≤10 min of intense exercise in a total time commitment of ≤30 min (Gillen & Gibala, 2014).

Exercise training can robustly increase skeletal muscle oxidative capacity (Holloszy, 1967), which is associated with improved exercise capacity (Holloszy & Coyle, 1984; Jacobs *et al.*, 2011) and health (Hood *et al.*, 2019). Increases in mitochondrial content following low-volume SIT are remarkably similar to MICT despite the discrepancies in total work and time (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*; Gillen *et al.*, 2016). In work-matched comparisons, HIIT demonstrates superior (Daussin *et al.*, 2008; MacInnis *et al.*, 2017*c*) or similar (Henriksson & Reitman, 1976; Bækkerud *et al.*, 2016) mitochondrial adaptations compared to MICT, although one study found no change following both training interventions (Granata *et al.*, 2016). Mitochondrial adaptive responses following low-volume SIT and HIIT may be influenced by factors intrinsic to the exercise prescription of interval training (e.g., the intensity or pattern of exercise), extrinsic to components of an interval training protocol (e.g., fasted vs. fed training) and/or specific to the populations studied (e.g., sex). Investigating factors that may impact skeletal muscle adaptations to interval training will increase our understanding of the mechanisms by which interval training elicits responses despite a low total volume of exercise and may better inform the exercise prescription of interval training for improving health and fitness.

The present thesis sought to examine factors that may impact acute and chronic mitochondrial responses to interval training by addressing three major questions: 1) are there sex-based differences in the acute skeletal muscle response to low-volume SIT?; 2) are there fibre type-specific responses to a single session and 12 weeks of low-volume

SIT *versus* MICT?; and 3) does the inherent intermittent pattern of exercise during interval training influence mitochondrial adaptations? While various physiological measures were examined, this thesis focused on skeletal muscle mitochondrial responses to acute and chronic interval training. The introductory chapter of the thesis will provide an overview of low-volume SIT and higher volume HIIT-induced skeletal muscle mitochondrial responses in humans, including direct comparisons to MICT.

1.2 Effect on interval exercise on skeletal muscle

The molecular mechanisms that regulate skeletal muscle adaptations to exercise training are proposed to begin with disturbances in homeostasis within contracting skeletal muscle (Coffey & Hawley, 2007; Egan & Zierath, 2013). These exercise-induced perturbations in homeostasis activate signalling proteins to increase the transcription of target genes. The pulsatile and transient changes in mRNA expression following repeated exercise sessions precede the observed increases in protein content (Perry *et al.*, 2010; Egan *et al.*, 2013) and overall, the accumulation of these acute molecular responses are proposed to underpin phenotypic changes in skeletal muscle over a training period. This section of the chapter will discuss acute and chronic effects of interval exercise on skeletal muscle, with a focus on a few key components involved in the molecular cascade linked to training-induced increases in skeletal muscle mitochondrial content.

1.2.1. Substrate metabolism during interval training

The high intensity nature of SIT and HIIT imposes a large demand for ATP that requires a rapid breakdown of substrates to meet the energy requirements within skeletal muscle. Higher intensity exercise relies to a greater extent on phosphocreatine (PCr) hydrolysis and non-oxidative glycolysis compared to lower intensity exercise, as evidenced by greater reductions in PCr and increases in muscle lactate (Sahlin *et al.*, 1987, 1989; Howlett *et al.*, 1998). In addition, there is a greater reliance on carbohydrate oxidation as opposed to fat oxidation, with increasing exercise intensity (van Loon *et al.*, 2001).

Substrate utilization patterns change over the multiple intervals performed during SIT where over time the contribution from non-oxidative sources and oxidative metabolism to ATP decreases and increases, respectively (Spriet *et al.*, 1989; Gaitanos *et al.*, 1993; Bogdanis *et al.*, 1996; Parolin *et al.*, 1999). Parolin *et al.* (1999) conducted an elegant study to examine substrate metabolism during repeated 30-sec 'all-out' sprints, interspersed with 4 minutes of recovery. During the first half of the first sprint there was a predominant reliance on PCr hydrolysis and glycolysis for ATP production but the contribution from oxidative metabolism increased progressively and accounted for ~50% of ATP production in the latter half (Parolin *et al.*, 1999). Importantly, during the 4 minutes of recovery between sprints PCr was resynthesized to near resting levels and also contributed a large proportion of ATP during the initial 15-sec of the third sprint (Parolin *et al.*, 1999). In contrast, there was a limited contribution of glycolysis and a much greater reliance on oxidative metabolism in the third compared to the first sprint (Parolin *et al.*,

1999). These findings are consistent with other investigations demonstrating a diminishing reliance on glycolysis over repeated sprints (Spriet *et al.*, 1989; Gaitanos *et al.*, 1993; Bogdanis *et al.*, 1996) and provide evidence that SIT is an aerobic exercise stimulus.

Limited investigations have compared substrate metabolism during a single session of interval exercise and MICT using a within-subject design. A low-volume SIT protocol involving 6 x 20-sec sprints demonstrated greater changes in ATP, PCr and muscle lactate compared to MICT (Fiorenza *et al.*, 2018), whereas SIT-induced glycogen depletion was found to be both lower (Fiorenza *et al.*, 2018) and similar (Scribbans *et al.*, 2014*a*) compared to MICT. HIIT protocols have elicited similar glycogen breakdown and greater increases in muscle lactate compared to work-matched MICT (Bartlett *et al.*, 2012; Kristensen *et al.*, 2015). Given high power outputs produced and the multiple work to rest transitions during interval exercise, it is not surprising that rates of anaerobic metabolism are higher during interval training compared to MICT.

1.2.2. Cell signalling responses to acute interval exercise

1.2.2.1 Signalling kinases

Exercise-induced perturbations in metabolic stress are sensed by signalling proteins which initiate a molecular cascade to upregulate mitochondrial biogenesis. Three important and well-studied signalling kinases proposed to be involved in this response are adenosine monophosphate (AMP)-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38 MAPK) and Ca^{2+}/calmodulin-dependent kinase (CaMK)

(Egan & Zierath, 2013; Hood *et al.*, 2019). These signalling kinases are activated (phosphorylated) in response to signals within skeletal muscle that reflect increased contractile activity and metabolic demand. Specifically, AMPK is a sensor of cellular energy stress and is phosphorylated, in part, by increases in the AMP/ATP ratio (Gowans *et al.*, 2013). One metabolic signal that is proposed to activate p38 MAPK is reactive oxygen species, which are generated by NADPH oxidase, xanthine oxidase and mitochondria (Powers *et al.*, 2010). Lastly, the phosphorylation of CAMKII is thought to be related to fluctuations in intracellular calcium that occur during skeletal muscle contraction (Chin, 2010).

Given that high intensity exercise requires higher rates of ATP turnover and elicits greater disturbances in homeostasis compared to lower exercise intensity (Sahlin *et al.*, 1987, 1989; Constantin-Teodosiu *et al.*, 1991; Howlett *et al.*, 1998), it is not surprising that some researchers have found the activation of signalling kinases to be dependent on exercise intensity. Numerous investigations have demonstrated greater phosphorylation of AMPK at higher intensities of continuous exercise compared to lower intensities (Wojtaszewski *et al.*, 2000; Sriwijitkamol *et al.*, 2007; Rose *et al.*, 2008; Egan *et al.*, 2010; Popov *et al.*, 2015) . Acetyl-CoA carboxylase (ACC), a downstream target of AMPK that is often measured as an indicator of AMPK activity (Winder *et al.*, 1997; Birk & Wojtaszewski, 2006; Jäger *et al.*, 2007), has also demonstrated intensity-dependent responses (Chen *et al.*, 2003; Rose *et al.*, 2008; Egan *et al.*, 2010), although this is not a consistent finding (Popov *et al.*, 2015). Importantly, intensity-dependent phosphorylation of AMPK and ACC are observed in work-matched comparisons

(Wojtaszewski *et al.*, 2000; Egan *et al.*, 2010) suggesting that the activation of the AMPK pathway with higher intensity continuous exercise is independent of total exercise volume. In one of the work-matched comparisons (Egan et al. 2010), the phosphorylation of CAMKII was also greater following cycling at 80% of $\dot{V}O_{2peak}$ vs. 40%; however, the phosphorylation of p38 MAPK was increased to a similar extent.

In contrast to comparisons of continuous exercise at different intensities, work-matched HIIT and MICT have elicited similar increases in the phosphorylation of AMPK, ACC and p38 MAPK (Bartlett *et al.*, 2012; Kristensen *et al.*, 2015; Parker *et al.*, 2017; Trewin *et al.*, 2018). A single session of low-volume SIT increases the phosphorylation of AMPK, ACC, p38 MAPK and CAMKII immediately following exercise (Gibala *et al.*, 2009; Little *et al.*, 2011*b*; Serpiello *et al.*, 2012; Cochran *et al.*, 2014; Scribbans *et al.*, 2014*a*; Metcalfe *et al.*, 2015; Taylor *et al.*, 2016*b*, 2016*a*; Broatch *et al.*, 2017; Granata *et al.*, 2017; Parker *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018), although there are equivocal data in this regard (Gibala *et al.*, 2009; Serpiello *et al.*, 2012; Scribbans *et al.*, 2014*a*; Metcalfe *et al.*, 2015). In within-subject investigations, low-volume SIT consisting of 20-30 sec repeated sprints has induced similar phosphorylation of AMPK and ACC and greater phosphorylation of p38 MAPK and CAMKII compared to MICT (Granata *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018). Interestingly, Fiorenza *et al.* (2018) found that changes in metabolic variables during exercise (e.g., muscle lactate) predicted the changes in the phosphorylation of p38 MAPK and CAMKII following SIT but not MICT. This may suggest that one mechanism explaining the comparable or possibly greater activation of signalling kinases following SIT *versus* MICT relates to the

initial stress imposed in skeletal muscle as a result of a higher exercise intensity during SIT.

1.2.2.2. PGC-1α activation

AMPK, p38 MAPK and CAMKII activate peroxisome proliferator-activated receptor gamma coactivator (PGC-1*α*), frequently considered a "master regulator" of mitochondrial biogenesis (Fernandez-Marcos & Auwerx, 2011; Hood *et al.*, 2019). PGC-1α translocates to the nucleus where it can coactivate numerous transcription factors, including nuclear receptor factors 1 and 2 and the peroxisome proliferator-activated receptors, to upregulate the expression of mitochondrial genes. Due to the central role of PGC-1α in upregulating mitochondrial biogenesis and evidence that PGC-1α can coactivate its own transcription through the myocyte enhancer factor 2 transcription factor in cells (Handschin *et al.*, 2003), researchers have examined exercise-induced increases in *PGC-1α* mRNA expression to gain insight into post-exercise PGC-1α activation. Examining the subcellular location of PGC-α is also measured as an indicator of its activity based on evidence that increased nuclear PGC-1α protein content coincides with increased mitochondrial gene expression in rodents (Wright *et al.*, 2007) and more recently, humans (Little *et al.*, 2011*b*; Heesch *et al.*, 2016; Granata *et al.*, 2017). Exercise-induced changes in the transcription of genes have been shown to precede changes in protein content in humans (Perry *et al.*, 2010; Egan *et al.*, 2013), and it is generally accepted that mitochondrial biogenesis occurs in response to exercise training due to the cumulative effects of transient increases in gene expression that lead to the increased

translation of mitochondrial proteins (Coffey & Hawley, 2007; Egan & Zierath, 2013). Thus, examining acute gene expression responses to interval exercise may increase our understanding of the mechanisms by which interval training increases mitochondrial content.

A recent review by Granata, Jamnick & Bishop (2018) demonstrated that higher exercise-induced increases in *PGC-1α* mRNA are associated with higher submaximal exercise intensities. Despite this potential positive relationship between post-exercise *PGC-1α* mRNA expression and exercise intensity, the data regarding work-matched HIIT and MICT comparisons are equivocal: one study has found greater interval HIIT-induced increases in *PGC-1α* mRNA expression (Popov *et al.*, 2014), whereas two other studies have found similar changes (Bartlett *et al.*, 2012; Trewin *et al.*, 2018). Interestingly, there may be an optimal intensity of approximately aerobic peak power output (W_{peak}) for increasing *PGC-1α* mRNA following interval exercise. Edgett *et al*. (2013) compared work-matched interval exercise at 73%, 100% or 133% of W_{peak} and found similar post-exercise increases in *PGC-1α* mRNA expression between the 73% and 133% W_{peak} efforts with greater responses following the 100% W_{peak} protocol. This finding aligns with the similar increases in *PGC-1α* mRNA expression observed following MICT and work-matched (Wang *et al.*, 2009; Wang & Sahlin, 2012), or lower volume SIT (Granata *et al.*, 2017; Fiorenza *et al.*, 2018), although one study found greater increases following low-volume SIT (Skovgaard *et al.*, 2016). There is mounting evidence that low-volume SIT is a very potent exercise stimulus for increasing the mRNA expression of *PGC-1α* (Gibala *et al.*, 2009; Little *et al.*, 2011*b*; Serpiello *et al.*, 2012; Edgett *et al.*, 2013, 2016; Cochran

et al., 2014; Metcalfe *et al.*, 2015; Taylor *et al.*, 2016*b*; Broatch *et al.*, 2017; Granata *et al.*, 2017; Fiorenza *et al.*, 2018). Similar to the activation of signalling kinases, it is likely that the enhanced energetic stress during SIT counteracts its reduced volume to induce at least comparable increases in *PGC-1α* mRNA expression as compared to MICT, as changes in PCr, [H+], glycogen and plasma adrenaline were shown to significantly predict increases in *PGC1α* mRNA following SIT, based on multiple linear regression analyses (Fiorenza *et al.*, 2018).

1.2.3. Skeletal muscle adaptations to interval exercise

Transmission electron microscopy (TEM) is considered the gold standard method for measuring mitochondrial content in human skeletal muscle (Medeiros, 2008; Larsen *et al.*, 2012); however, other biomarkers that are less technically demanding and expensive, namely the activity or protein content of key mitochondrial enzymes, are more widely utilized owing to their greater accessibility, lower cost and time efficiency. The maximal activity of citrate synthase (CS) and the protein content of cytochrome *c* oxidase (COX) in particular have been reported to show positive correlations with TEM-based mitochondrial content (Larsen *et al.*, 2012). Positive correlations between the maximal activity of CS and mitochondrial volume measured using TEM have also been found before and after 6 weeks of MICT; however, there was no relationship between the change in the maximal activity of CS and the change in mitochondrial volume over the 6 weeks (Meinild Lundby *et al.*, 2018).

As little as six sessions of SIT over 2 weeks increases the maximal activity and/or protein content of mitochondrial enzymes (Burgomaster *et al.*, 2005, 2006; Gibala *et al.*, 2006; Little *et al.*, 2010; Jacobs *et al.*, 2013). Training-induced increases in mitochondrial content are comparable following low-volume SIT and MICT, as measured by increases in the maximal activity of CS (Burgomaster *et al.*, 2008; Bækkerud *et al.*, 2016; Gillen *et al.*, 2016), activity or protein content of COX (Gibala *et al.*, 2006; Shepherd *et al.*, 2013), activity of succinate dehydrogenase (Scribbans *et al.*, 2014*a*), and mitochondrial volume using TEM (Shepherd *et al.*, 2017). With respect to work-matched comparisons, similar (Bækkerud *et al.*, 2016), or superior (MacInnis *et al.*, 2017*c*), increases in the maximal activity of CS are reported in response to short-term HIIT vs. MICT.

Interval training also has the potential to induce other skeletal muscle adaptations characteristic of traditional MICT in untrained and recreationally active individuals. Short-term SIT and HIIT increases mitochondrial respiration (Daussin *et al.*, 2008; Jacobs *et al.*, 2013; Vincent *et al.*, 2015; Granata *et al.*, 2016; MacInnis *et al.*, 2017*c*), with some investigations demonstrating superior improvements following interval training compared to MICT (Daussin *et al.*, 2008; Granata *et al.*, 2016; MacInnis *et al.*, 2017*c*). The protein content of mitofusin 1 (MFN1) or 2 (MFN2), important proteins involved the regulation of mitochondrial dynamics and the maintenance of a healthy mitochondrial network within skeletal muscle (Yan *et al.*, 2012), is also reported to increase following low-volume SIT and HIIT (Perry *et al.*, 2010; Little *et al.*, 2011*a*; Granata *et al.*, 2016; MacInnis *et al.*, 2017*c*). With respect to substrate metabolism, there is evidence that interval training increases resting muscle glycogen (Burgomaster *et al.*, 2005; Perry *et al.*,

2008; Little *et al.*, 2010; Scribbans *et al.*, 2014*b*, 2014*a*), and intramuscular triglyceride content (Shepherd *et al.*, 2013; Scribbans *et al.*, 2014*a*), carbohydrate and lipid transport proteins (Talanian *et al.*, 2007; Burgomaster *et al.*, 2007; Perry *et al.*, 2008; Little *et al.*, 2010, 2011*a*; Gillen *et al.*, 2014, 2016), the maximal activity of glycolytic and lipid enzymes (Jacobs *et al.*, 1987; Linossier *et al.*, 1993; MacDougall *et al.*, 1998; Rodas *et al.*, 2000; Perry *et al.*, 2007; Talanian *et al.*, 2007; Perry *et al.*, 2008; Raleigh *et al.*, 2018), and reduces the rate of glycogenolysis and lactate production during submaximal exercise (Burgomaster *et al.*, 2006; Talanian *et al.*, 2007). Lastly, low-volume SIT increases skeletal muscle capillarization density to a similar extent as MICT (Cocks *et al.*, 2013, 2016; Scribbans *et al.*, 2014*a*).

1.3. Potential factors influencing the skeletal muscle response to interval exercise

1.3.1. Sex-based differences

Women compared to men have been shown to oxidize a greater proportion of fat *versus* carbohydrate during a single session of MICT (Venables *et al.*, 2005; Tarnopolsky, 2008); however, sex-based comparisons of the acute response to interval exercise are limited. Using expired gas analyses, Hill *et al*. (1993) found that aerobic metabolism contributed to a greater proportion of total work during a single 30-sec sprint in women compared to men. In support of this potential sex-based difference in substrate metabolism during a sprint, women were reported to break down ~20% less muscle glycogen during SIT despite similar net ATP breakdown, suggesting that women produced more ATP through aerobic metabolism (Esbjörnsson-Liljedahl *et al.*, 2002).

The lower SIT-induced increases in muscle blood lactate found in women *versus* men also supports the reported lower rates of glycogenolysis in women (Esbjörnsson-Liljedahl *et al.*, 2002; Esbjörnsson *et al.*, 2009). Lower basal activities of phosphofructokinase (Jaworowski *et al.*, 2002), lactate dehydrogenase (Esbjörnsson Liljedahl *et al.*, 1996; Jaworowski *et al.*, 2002), and lower catecholamine responses to SIT (Esbjörnsson-Liljedahl *et al.*, 2002), in women compared to men may contribute to lower glycogen use during high intensity exercise. In contrast, the contribution of PCr in the production of ATP during SIT does not seem sex-specific as similar rates of PCr breakdown are reported in men and women (Esbjörnsson-Liljedahl *et al.*, 2002). With respect to acute responses in the recovery period following exercise, similar increases in the phosphorylation of AMPK and ACC following a single 30-sec sprint has been shown in in men and women (Fuentes *et al.*, 2012). Increases in mitochondrial gene expression are reported following SIT in mixed cohorts of men and women (Serpiello *et al.*, 2012; Metcalfe *et al.*, 2015); however, no study has been designed to specifically address sex-based differences in mitochondrial gene expression following interval exercise.

A few studies have suggested sex-specific skeletal muscle adaptations exist in response to short-term SIT. Compared to men, women are reported to experience blunted increases in GLUT4 protein content and the maximal activity of 3-β-hydroxyacyl CoA dehydrogenase (β-HAD) (Gillen *et al.*, 2014), greater increases in type II fibre-cross sectional area and total muscle glycogen (Esbjörnsson Liljedahl *et al.*, 1996), and similar increases in whole-body fat oxidation (Astorino *et al.*, 2011) following SIT. While increases in the activity and protein content of CS appear to be similar between sexes

following SIT (Gillen *et al.*, 2014; Scalzo *et al.*, 2014), rates of mitochondrial protein synthesis showed a tendency to be lower over 3 weeks of SIT in women *versus* men (Scalzo *et al.*, 2014). Other studies assessing mitochondrial adaptations to interval training in mixed cohorts of men and women have not reported sex-based differences, although these studies were not designed to make sex-specific comparisons (Perry *et al.*, 2008; Burgomaster *et al.*, 2008; Hood *et al.*, 2011; Scribbans *et al.*, 2014*a*).

Discrepancies regarding whether sex-based differences exist in response to interval training may relate to potential confounding factors, such as training status and fluctuations in sex-hormone levels. When matched for $\dot{V}O_{2peak}$ relative to kilogram of body mass, women typically complete more habitual exercise training compared to men (Tarnopolsky, 2008). Owing to a greater proportion of body fat in women *versus* men (Lundsgaard & Kiens, 2014), it is considered best practice to match sexes based on $\dot{V}O_{2peak}$ relative to kilogram of fat free mass (Tarnopolsky, 2008), and this method is associated with similar training backgrounds and lactate thresholds between groups (Phillips *et al.*, 1993; McKenzie *et al.*, 2000; Kiens *et al.*, 2004; Horton *et al.*, 2006). Concentrations of endogenous estradiol and progesterone fluctuate over a naturally occurring menstrual cycle and are suppressed in females using oral contraceptives (Sims & Heather, 2018). Although likely smaller than the influence of sex, menstrual cycle phase and oral contraceptive use can also alter substrate metabolism during MICT (Horton *et al.*, 2002; Suh *et al.*, 2003; Devries *et al.*, 2006; Tarnopolsky, 2008), but the impact of these factors on acute skeletal muscle responses to interval exercise is unknown. Studies that control for various factors, including training status, menstrual

cycle phase and oral contraceptive use, are needed to clarify whether sex-based differences, in fact, exist in response to interval training.

1.3.2. Fibre type-specific responses

Most research investigating responses to interval training has focused on mixed muscle analyses, which may mask fibre type-specific differences. One mechanism by which interval training may induce similar skeletal muscle adaptations as MICT is by greater recruitment and remodelling of type II fibres owing to a higher exercise intensity. In agreement with the motor unit size principle whereby with increasing force production small motor units containing type I fibres are recruited before larger motor units containing type II fibres (Henneman, 1957; Henneman & Olson, 1965), skeletal muscle fibre recruitment patterns, assessed using glycogen depletion measurements, correspond to exercise intensity (Vollestad & Blom, 1985). Both greater (Gollnick *et al.*, 1973; Essén, 1978; Thomson *et al.*, 1979; Vollestad *et al.*, 1992; Greenhaff *et al.*, 1994), and similar (Gollnick *et al.*, 1974; Essén, 1978), glycogen depletion in type II compared to type I fibres has been reported during supramaximal continuous and intermittent exercise. Higher depletion of ATP and PCr and greater accumulation of muscle lactate and inosine monophosphate has also been found in type II fibres compared to type I fibres following a single sprint (Esbjörnsson-Liljedahl *et al.*, 1999; Karatzaferi *et al.*, 2001).

There is a paucity of research investigating acute fibre type-specific responses to interval training. One study comparing glycogen depletion in work-matched HIIT and MICT showed greater depletion in type II *versus* I fibres following HIIT only (Kristensen

et al., 2015). In the same study, the phosphorylation of AMPK and ACC was higher in type II vs. I fibres following high volume HIIT, whereas MICT did not induce a differential response in the activation of the AMPK pathway between fibres types (Kristensen *et al.*, 2015). With respect to low-volume SIT, similar fibre type-specific glycogen depletion as MICT was measured but no changes in the phosphorylation of AMPK was found following either exercise type (Scribbans *et al.*, 2014*a*). The lack of change in post-exercise fibre type-specific activation of the AMPK pathway was surprising and requires further investigation given that other studies have shown that SIT and MICT increase the phosphorylation of AMPK in whole muscle homogenates (Bartlett *et al.*, 2012; Kristensen *et al.*, 2015; Fiorenza *et al.*, 2018). Lastly, SIT increased the mRNA expression of *PGC-1α* in type I and II fibres to a similar extent as work-matched MICT (Wang & Sahlin, 2012).

Short-term SIT interventions have demonstrated increases (Scribbans *et al.*, 2014*b*; Edgett *et al.*, 2016), or no change (Raleigh *et al.*, 2018), in succinate dehydrogenase (SDH) activity in type I and type II fibres. There are also reports of training-induced increases in COX and SDH activity within both fibre types following low-volume SIT, HIIT and MICT (Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*; Tan *et al.*, 2018). Two weeks of single-leg HIIT and MICT did not change protein content of COXIV and NDUFA9 in type I and type II fibres (MacInnis *et al.*, 2017*c*), but 12 weeks of double-leg HIIT in older adults increased COXIV in both fibre types and NDUFA9 in type II fibres only (Wyckelsma *et al.*, 2017). Longer training interventions may be required to detect small differences in fibre type-specific adaptations.

Investigations that examine fibre type-specific responses of other proteins shown to increase in whole muscle homogenates following SIT, such as GLUT4 (Burgomaster *et al.*, 2007; Little *et al.*, 2010, 2011*a*; Gillen *et al.*, 2014, 2016), are also warranted.

1.3.3. Role of contractile pattern

The potential for interval training to elicit comparable or superior skeletal muscle responses as MICT may be related to a higher exercise intensity per se and/or the inherent intermittent contractile pattern. A recent editorial by Jimenez-Pavon & Lavie (2017) highlighted that exercise intensity is often identified as the underlying factor behind the potency of interval training despite the stimulus pattern also differing compared to MICT (intermittent vs. continuous). The authors called for future research to examine moderate-intensity interval training in order to better understand the benefits of an intermittent pattern of exercise in the absence of high exercise intensity (Jiménez-Pavón & Lavie, 2017).

Studies examining high-intensity intermittent and continuous exercise have reported similar signalling responses (Cochran *et al.*, 2014; Taylor *et al.*, 2016*b*). The phosphorylation of AMPK, ACC and p38 MAPK and the exercise-induced increase in the mRNA expression of *PGC-1α* is similar following SIT and 2-4 minutes of 'all-out' continuous exercise (Cochran *et al.*, 2014; Taylor *et al.*, 2016*b*). These similar acute responses were reported following two protocols of equal work (Cochran *et al.*, 2014) or exercise duration (Taylor *et al.*, 2016*b*). Surprisingly, despite the similar acute responses of high-intensity continuous exercise and SIT, 6 weeks of training involving the same

continuous protocol did not increase the maximal activity of citrate synthase (Cochran *et al.*, 2014). The lack of change in a biomarker of mitochondrial content contrasts the increases in protein content and maximal activity of mitochondrial proteins observed following SIT protocols consisting of approximately the same volume of exercise per session in a similar population (Burgomaster *et al.*, 2006; Gibala *et al.*, 2006), and may suggest that an intermittent contractile pattern contributes to SIT-induced increases in mitochondrial content.

An investigation comparing interval and continuous exercise matched for intensity supports that an intermittent pattern of exercise influences the activation of signalling pathways involved in mitochondrial biogenesis. Combes *et al.* (2015) demonstrated superior phosphorylation of AMPK, p38 MAPK and CAMKII following interval exercise *versus* continuous exercise. The two work-matched protocols involved exercising at 70% W_{peak} for a total exercise duration of 30 minutes but the interval protocol consisted of 30 x 1 min intervals of exercise with 1 min of passive rest and thus, required double the total duration of the continuous protocol (Combes *et al.*, 2015). Whether these acute differences would lead to enhanced skeletal muscle adaptations following moderate interval *versus* continuous training is unknown. No study to date has directly assessed mitochondrial adaptations to intermittent and continuous training interventions that are matched for work, absolute intensity and duration.

1.4. Single-leg exercise as a model to study adaptations to training

1.4.1. Traditional single-leg exercise

Unilateral exercise has been used to investigate physiological responses to exercise for decades (Duner, 1959; Freyschuss & Strandell, 1968; Pernow & Saltin, 1971; Andersen & Saltin, 1985). Exercising only one leg has been a useful approach for assessing adaptations to a training program compared to a within-subject control (Saltin *et al.*, 1976; Henriksson, 1977), and the impact of nutritional strategies alone or in combination with exercise (Witard *et al.*, 2009; Churchward-Venne *et al.*, 2012; McGlory *et al.*, 2016). Investigations have assigned different conditions to each leg in order to assess the effect of different protocols (e.g., interval *versus* continuous training) (Saltin *et al.*, 1976; MacInnis *et al.*, 2017*c*), environments (e.g., normoxia *versus* hypoxia) (Melissa *et al.*, 1997; Bakkman *et al.*, 2007), or different physiological states (e.g., low *versus* high muscle glycogen) (Hansen *et al.*, 2005), on training adaptations. Single-leg exercise has also greatly contributed to our current understanding that central factors primarily limit whole-body $\dot{V}O_{2peak}$. Andersen & Saltin (1985) measured rates of relative peak muscle blood flow during single-leg kicking exercise that exceeded rates previously observed during whole-body exercise, indicating that the capacity for skeletal muscle to receive blood exceeds the capacity of the circulatory system to supply it.

Single-leg exercise models permit the comparison of two treatments within the same subject, which reduces the potential influence of confounding factors on outcomes of interest, increases statistical power and lowers the financial cost and time commitment compared to between-subject designs (MacInnis *et al.*, 2017*a*). The reported large

variability in responses to exercise training (Bouchard *et al.*, 1999; Churchward-Venne *et al.*, 2015; Ross *et al.*, 2015; Bonafiglia *et al.*, 2016), may occur as a result of differences in nutrition, sleep or baseline fitness (Hawley *et al.*, 2011; Halson, 2014). The use of a unilateral exercise model reduces the impact of these factors on the relative changes induced by different treatments by allowing participants to undergo the two treatments simultaneously or within a relatively short time frame. Within-subject designs also require a lower sample size compared to between-subject designs to obtain adequate statistical power (MacInnis *et al.*, 2017*a*). These advantages apply to crossover designs as well; however, when comparing the effects of two training interventions within the same participant washout periods lasting months are typically utilized to ensure adaptations from the first treatment are eliminated (Bonafiglia *et al.*, 2016). Unilateral exercise designs do not require a washout period, enabling the reduction of costs related to controls implemented in the study (e.g., providing standardized diets), the duration of subject burden and potential time required for the completion of data collection.

One of the major limitations of utilizing single-leg exercise models is that only two treatments can be compared within the same individual over the same time frame. In addition, the examination of some common training-induced physiological changes is not compatible with a unilateral design. The effect of two different exercise protocols on whole-body and central adaptations cannot be assessed since both interventions may contribute to systemic responses. Furthermore, measurements of strength following single-leg exercise must be interpreted with caution as approximately half of the increases in strength induced by unilateral strength training are observed on the contralateral side

(Carroll *et al.*, 2006). Critically, single-leg designs are appropriate for studies examining skeletal muscle adaptations because there appears to be no cross-over of biochemical adaptations between limbs. Unchanged maximal activity of CS, β-HAD and SDH (Saltin *et al.*, 1976; Bell *et al.*, 2001; Lindholm *et al.*, 2014*a*, 2016), capillary density (Jensen *et al.*, 2004), and cross-sectional area (Houston *et al.*, 1983; Wilkinson *et al.*, 2006), were found in untrained legs following unilateral exercise training. Moreover, the activity of mitochondrial enzymes (Saltin *et al.*, 1976; Henriksson, 1977; Essen-Gustavsson & Borges, 1986; Lindholm *et al.*, 2014*a*, 2016; MacInnis *et al.*, 2017*c*), capillary density (Jensen *et al.*, 2004), whole muscle and fibre type-specific cross sectional area (Saltin *et al.*, 1976; Essen-Gustavsson & Borges, 1986; Lexell & Taylor, 1991; Wilkinson *et al.*, 2006; Tarnopolsky *et al.*, 2007), and gene expression (Tarnopolsky *et al.*, 2007; Lindholm *et al.*, 2014*b*) at rest is similar between legs prior to a training intervention. Overall, unilateral exercise models offer unique advantages for assessing skeletal muscle adaptations to exercise training as compared to between-group designs.

1.4.2. Counterweighted single-leg cycling

Single-leg cycling has been utilized to increase our understanding of physiological responses to endurance and interval training (Saltin *et al.*, 1976; Henriksson, 1977; Bell & Wenger, 1988; Rud *et al.*, 2012*b*; Vincent *et al.*, 2015; MacInnis *et al.*, 2017*c*). Single-leg cycling can be performed using unmodified cycle ergometers (Saltin *et al.*, 1976; Dolmage *et al.*, 2014), but the exercise may feel uncomfortable, in part, due to the increased reliance on the hip flexor muscles in the active leg during the upstroke phase

(Abbiss *et al.*, 2011). The addition of a counterweight on the opposing crank arm (Fig. 1) assists with the upstroke phase of the active limb and makes single-leg cycling easier to coordinate (Burns *et al.*, 2014). Muscle recruitment patterns during double-leg cycling are more similar to counterweighted single-leg cycling *versus* unassisted single-leg cycling (Bini *et al.*, 2015; Elmer *et al.*, 2016). In addition, while cycling at a constant power output, using a counterweight elicits the same oxygen consumption and heart rate as bilateral cycling and lower cardiovascular responses compared to unassisted single-leg cycling (Burns *et al.*, 2014). When power output is normalized to account for relative intensity (i.e., set as a percentage relative to either double-leg or single-leg peak power) counterweighted unilateral cycling elicits a lower oxygen uptake, heart rate and self-perceived exertion compared to bilateral cycling (MacInnis *et al.*, 2017*b*).

Figure 1. A cycle ergometer adapted for counterweighted single-leg cycling. An 11.4 kg counterweight is placed on the crank arm of the non-active limb.

1.4.3. Skeletal muscle adaptations to single-leg cycling

The lower central demands of single-leg cycling may allow for a greater relative intensity for a given cardiorespiratory cost and lead to augmented skeletal muscle adaptations *versus* traditional double-leg cycling (Abbiss *et al.*, 2011; MacInnis *et al.*, 2017*a*). Indeed, greater workloads per leg relative to double-leg cycling are reported during incremental tests to exhaustion (Bundle *et al.*, 2006; Rud *et al.*, 2012*a*; MacInnis *et al.*, 2017*b*), and 'all-out' interval exercise (Bundle *et al.*, 2006; Abbiss *et al.*, 2011). Cyclists who performed single-leg HIIT at the highest power output they could maintain had greater training-induced increases in COXIV and GLUT4 compared to cyclists who performed the same HIIT protocol using two legs (Abbiss *et al.*, 2011). Given the potential superior peripheral adaptations, single-leg cycling may be an appealing exercise strategy for athletes or individuals with central limitations as a result of chronic disease (e.g., chronic obstructive pulmonary disease). The greater relatively intensity per leg during single-leg cycling compared to double-leg cycling should also be taken into consideration when comparing exercise intensities across studies that employ either method.

Valuable insight into the effects of different exercise protocols on skeletal muscle adaptations can be gained from studies using single-leg exercise but comparisons of unilateral cycling protocols are relatively limited. Using the more traditional unassisted single-leg cycling model, Saltin *et al*. (1976) found similar increases in SDH activity following work-matched single-leg MICT and SIT. MacInnis *et al*., (2017c) utilized the more recently studied counterweighted single-leg cycling model and found greater

mitochondrial adaptations following HIIT compared to work-matched MICT. More unilateral investigations comparing various exercise protocols will increase our understanding of the influence of different exercise prescription factors on training-induced adaptations.

1.5. Purpose of thesis

The overall purpose of the present thesis was to advance our understanding of potential factors that may influence acute and chronic responses to interval training. Numerous studies have shown that interval training elicits mixed muscle adaptations traditionally characteristic of MICT. Identifying factors that impact or underpin adaptations to interval training may provide insight into the mechanisms by which interval training induces skeletal muscle adaptations. This thesis seeks to clarify the influence of three potential factors on skeletal muscle responses to interval training: sex, fibre type-specific responses and contractile pattern.

First, sex-based differences in exercise metabolism are evident during MICT (Tarnopolsky, 2008), but less is known regarding the influence of sex on responses to interval training. The purpose of Study 1 was to examine whether acute skeletal muscle responses to SIT differs between sexes, with a primary focus on mRNA expression for genes associated with skeletal muscle remodelling. Groups of sedentary men and women with similar relative cardiorespiratory fitness completed a single bout of low-volume SIT and women completed the trial in the mid-follicular phase of their menstrual cycles. Based on recent findings of greater skeletal muscle adaptations in men relative to women

(Gillen *et al.*, 2014; Scalzo *et al.*, 2014), we hypothesized that SIT-induced increases in the mRNA expression of genes linked to skeletal muscle remodelling would be greater in men compared to women.

Divergent fibre-type specific responses to low-volume SIT and MICT may form the basis for the similar responses observed in mixed muscle samples. The inherent higher exercise intensity of SIT may recruit and remodel type II fibres to a greater extent relative to MICT. Short-term studies that have directly compared fibre-type specific responses to low-volume SIT and MICT have demonstrated generally similar training responses (Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*), and the only acute comparison found no exercise-induced changes in the phosphorylation of AMPK (Scribbans *et al.*, 2014*a*). Study 2 sought to examine fibre type-specific changes in more acute and chronic markers of mitochondrial biogenesis following SIT and MICT utilizing Western blotting and following a 12-week training intervention. We hypothesized that type II responses to acute and chronic SIT would be greater *versus* MICT.

Finally, an intermittent contractile pattern may contribute to the potential superior mitochondrial adaptations following work-matched HIIT relative to MICT (MacInnis *et al.*, 2017*c*). Study 3 utilized counterweighted single-leg cycling as a model to examine mitochondrial adaptations to short-term interval and continuous training, where absolute intensity, duration and volume were matched but contractile pattern differed. In light of recent findings that the activation of signalling proteins is greater following work- and intensity-matched interval and continuous exercise (Combes *et al.*, 2015), we hypothesized that intermittent training would elicit greater mitochondrial adaptations

compared to continuous training. This study was the first to compare two protocols matched for work, absolute intensity, and duration using unilateral cycling.

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CHAPTER 2: Effect of sex on the acute skeletal muscle response to sprint interval exercise. Published in *Exp Physiol* 102: 354-365, 2017

Experimental Physiology

Research Paper

Effect of sex on the acute skeletal muscle response to sprint interval exercise

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New Findings

- **What is the central question of this study?**
  Are there sex-based differences in the acute skeletal muscle response to sprint interval training (SIT)?
- **What is the main finding and its importance?**
  In response to a SIT protocol that involved three 20 s bouts of 'all-out' cycling, the expression of multiple genes associated with mitochondrial biogenesis, metabolic control and structural remodelling was largely similar between men and women matched for fitness. Our findings cannot explain previous reports of sex-based differences in the adaptive response to SIT and suggest that the mechanistic basis for these differences remains to be elucidated.

A few studies have reported sex-based differences in response to several weeks of sprint interval training (SIT). These findings may relate to sex-specific responses to an acute session of SIT. We tested the hypothesis that the acute skeletal muscle response to SIT differs between sexes. Sedentary but healthy men (n = 10) and women (n = 9) were matched for age (22 ± 3 *versus* 22 ± 3 years old) and cardiorespiratory fitness [45 ± 7 *versus* 43 ± 10 ml O_2 (kg fat-free mass)$^{-1}$ min^{-1}], with women tested in the mid-follicular phase of their menstrual cycles. Subjects performed three 20 s 'all-out' cycling efforts against a resistance of 5% of body mass, interspersed with 2 min of recovery. Relative mean power outputs [7.6 ± 0.5 *versus* 7.5 ± 0.9 W (kg fat-free mass)$^{-1}$] were similar between men and women (P > 0.05). Furthermore, there were no differences in the exercise-induced changes in mRNA expression of *PGC-1α*, *PRC*, *PPARD*, *SIRT1*, *RIP140*, *HSL*, *HKII*, *PDK4*, *PDP1*, *FOXO3*, *MURF-1*, *Myf5*, *MyoD* and *VEGFA* at 3 h of recovery *versus* rest (P < 0.05, main effect of time). The only sex-specific responses to exercise were an increase in the mRNA expression of *GLUT4* and *LPL* in women only and *Atrogin-1* in men only (P < 0.05). Women also had higher expression of *HKII* and lower expression of *FOXO3* compared with men (P < 0.05, main effect of sex). We conclude that the acute skeletal muscle response to SIT is largely similar in young men and women. The mechanistic basis for sex-based differences in response to several weeks of SIT that has been previously reported remains to be elucidated.

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Introduction

Sprint interval training (SIT) is characterized by brief intermittent bursts of 'all-out', or supramaximal, exercise interspersed with short periods of recovery (Weston *et al.* 2014). This type of training is a potent stimulus to induce physiological remodelling, with responses to SIT and traditional endurance training being similar despite a large difference in total exercise volume (Gibala *et al.* 2006; Burgomaster *et al.* 2008). For example, 2–6 weeks of SIT has been demonstrated to increase skeletal muscle mitochondrial content, reduce carbohydrate utilization during matched-work exercise and enhance the maximal capacity for lipid oxidation (Burgomaster *et al.* 2005, 2008; Gibala *et al.* 2006). The most commonly studied SIT protocol involves repeated Wingate tests, typically four to six 'all-out' 30 s cycling efforts interspersed by several minutes of recovery (Gibala *et al.* 2014). More recently, SIT protocols involving two to three 20 s 'all-out' cycling efforts, within a time commitment of 10 min per session, have also demonstrated skeletal muscle remodelling similar to traditional endurance training despite a very small total volume (i.e. ≤1 min) of intense exercise (Gillen *et al.* 2014, 2016).

A few studies have suggested potential sex-based differences in the response to SIT (Metcalfe *et al.* 2012; Gillen *et al.* 2014; Scalzo *et al.* 2014). We (Gillen *et al.* 2014) and others (Metcalfe *et al.* 2012) have observed a sex-specific improvement in glycaemic control following 6 weeks of SIT, with improvements in 24 h blood glucose concentrations observed in men only and a higher SIT-induced increase in GLUT4 protein content in men compared with women. Men were also reported to experience a greater increase in mixed muscle protein synthesis compared with women after 3 weeks of SIT (Scalzo *et al.* 2014). In the same study, there was a tendency for greater rates of mitochondrial biogenesis in men, although the protein content of mitochondrial enzymes increased to a similar extent in both sexes (Scalzo *et al.* 2014). However, other common adaptations to SIT, such as improvements in peak oxygen uptake ($\dot{V}_{O_2peak}$; Astorino *et al.* 2011; Metcalfe *et al.* 2012, 2016; Gillen *et al.* 2014; Scalzo *et al.* 2014) and increases in fat oxidation during submaximal exercise (Astorino *et al.* 2011), are reportedly similar in men and women. Notably, none of the above-mentioned studies was specifically designed to make sex-based comparisons according to established practice, i.e. by matching men and women for baseline fitness relative to fat-free mass and standardizing the menstrual cycle testing phase (Tarnopolsky, 2008). Therefore, the mechanistic basis for purported sex-based differences in skeletal muscle remodelling in response to SIT is unclear.

It is generally accepted that exercise training induces skeletal muscle remodelling as a result of the cumulative effects of transient exercise-induced disturbances in skeletal muscle homeostasis (Coffey & Hawley, 2007; Egan & Zierath, 2013). These disturbances within skeletal muscle activate signalling pathways that co-ordinate an increase in the transcription of target genes and, ultimately, protein content (Coffey & Hawley, 2007; Perry *et al.* 2010; Egan & Zierath, 2013). Thus, sex-based differences in the acute metabolic response to SIT may lay the foundation for sex-specific adaptations following several weeks of SIT. The purpose of the present study was to examine the effect of sex on the acute skeletal muscle response to SIT, with a primary focus on mRNA expression for genes associated with skeletal muscle remodelling. Groups of men and women were matched for fitness relative to fat-free mass, and women were tested during a standardized phase (mid-follicular) of their menstrual cycles. Given that specific skeletal muscle adaptations have been reported to be greater in men relative to women (Gillen *et al.* 2014; Scalzo *et al.* 2014), we hypothesized that men compared with women would have a greater exercise-induced utilization of glycogen and increases in mRNA expression. We also hypothesized that SIT would induce an increase in the mRNA expression for genes linked to skeletal muscle remodelling.

Methods

Ethical approval

All experimental procedures were approved by the Hamilton Integrated Research Ethics Board and conformed in all respects with the *Declaration of Helsinki*. All subjects completed routine medical screening and provided written informed consent before study participation.

Subjects

A total of 20 sedentary but otherwise healthy adult men and women were recruited ($n = 10$ each). The two groups were matched based on cardiorespiratory fitness relative to fat-free mass. None of the women was using oral contraceptives. One female participant experienced an adverse reaction to a blood sampling procedure and did not complete the study. Descriptive characteristics for the 10 men and nine women who completed all experimental procedures are presented in Table 1.

Pre-experimental procedures

Participants reported to the laboratory for baseline testing and familiarization procedures a minimum of 4 days before the main experimental trial. The $\dot{V}_{O_2peak}$ was assessed during a ramp test on an electronically braked cycle ergometer (Lode Excalibur Sport V 2.0, Groningen,

Table 1. Descriptive characteristics of the subjects

Characteristic	Men	Women
Age (years)	22 ± 3	22 ± 3
Height (cm)	175 ± 9	164 ± 6*
Weight (kg)	82 ± 20	61 ± 17*
FFM (kg)	66 ± 12	45 ± 7*
Body fat (%)	17 ± 8	24 ± 9
$\dot{V}_{O_2peak}$ (l min^{-1})	3.0 ± 0.6	1.9 ± 0.5*
$\dot{V}_{O_2peak}$ (ml kg^{-1} min^{-1})	37 ± 5	33 ± 8
$\dot{V}_{O_2peak}$ [ml (kg FFM)$^{-1}$ min^{-1}]	45 ± 7	43 ± 10

Data are means ± SD; n = 10 men and n = 9 women. Abbreviations: FFM, fat-free mass; and $\dot{V}_{O_2peak}$, peak oxygen consumption. *Significantly different from men ($P < 0.05$).

The Netherlands) using an online gas collection system (Moxus modular oxygen uptake system; AEI Technologies, Pittsburgh, PA, USA). Briefly, following a 2 min warm-up at 50 W, the workload was increased by 1 W every 2 s until volitional exhaustion or the point at which pedal cadence fell below 60 r.p.m. The $\dot{V}_{O_2peak}$ was defined as the highest average oxygen consumption over a 30 s period. After an overnight fast, fat and fat-free masses were determined through air-displacement plethysmography (BodPod®; COSMED Inc., Concord, CA, USA). On a separate visit, participants completed a familiarization trial of the SIT protocol (explained below in '*Experimental protocol*') on a different electronically braked cycle ergometer (Velotron; RacerMate, Seattle, WA, USA).

Experimental protocol

Participants refrained from exercise, alcohol and caffeine and food for a minimum of 48, 24 and 10 h prior to testing, respectively. Women were tested in the mid-follicular phase of their menstrual cycles (day 9 ± 2 following the onset of menstruation). A fasting blood sample was collected from an antecubital vein. A resting muscle biopsy was obtained from the vastus lateralis under local anaesthesia (1% lidocaine) using a Bergström needle adapted with suction, as previously described (Tarnopolsky *et al.* 2011). Samples were cleaned of excess blood, sectioned into several pieces, immediately frozen in liquid nitrogen, and stored at −80°C for subsequent analyses. After the first muscle biopsy, participants completed the SIT protocol, which consisted of a 2 min warm-up at 50 W and three 20 s 'all-out' cycling efforts against a load corresponding to 0.05 kg (kg body mass)$^{-1}$, interspersed by 2 min of cycling at 25 W. Unlike previous studies from our laboratory using this protocol (Gillen *et al.* 2014, 2016), there was no cool-down period. Instead, immediately following exercise, a second needle muscle biopsy was obtained ∼5 cm distal to the first incision site. A final needle muscle biopsy was obtained from the contralateral leg 3 h after exercise. Heart rate was measured throughout the SIT protocol using telemetry (Polar A3, Lake Success, NY, USA).

Blood analyses

Serum samples were collected using the appropriate collection tubes (BD Vacutainer®; Becton, Dickinson and Company, Franklin Lakes, NJ, US), processed according to the manufacturer's instructions, aliquoted and stored at −20°C for subsequent analyses. The Hamilton Regional Medicine Program Core Laboratory measured serum estradiol and progesterone using chemiluminescent microparticle immunoassays (Abbott Architect; Abbott Laboratories, Chicago, IL, USA) and total testosterone using a chemiluminescent enzyme immunoassay (Immulite 2000; Siemens, Erlangen, Germany). The estradiol chemiluminescent microparticle immunoassay has a sensitivity of 37 pmol l^{-1} and a total standard deviation of 5 pg ml^{-1}. The progesterone chemiluminescent microparticle immunoassay and testosterone chemiluminescent enzyme assay have sensitivities of 0.3 and 0.5 nmol l^{-1}, respectively, and coefficients of variation of ≤10 and ≤24%, respectively.

Muscle analyses

Glycogen. One piece of each muscle sample was freeze-dried, powdered and dissected free of connective tissue for the determination of glycogen, as we have previously described (Cochran *et al.* 2010). Briefly, ∼2 mg of powdered muscle was incubated in 2 M HCl for 2 h at 98°C to hydrolyse glycogen to glucosyl units. This solution was neutralized with an equal volume of 2 M NaOH and analysed for glucose concentration via a commercially available hexokinase kit (Pointe Scientific, Canton, MI, USA).

Gene expression. RNA was extracted from a third piece of muscle (∼30 mg) in 1 ml of Isol-RNA Lysis Reagent (5 Prime, Gaithersburg, MD, USA). Samples were homogenized using the TissueLyser LT (Qiagen Inc., Venlo, The Netherlands), and 0.2 ml of chloroform was added to each sample. After vigorous mixing for 15 s by hand, samples were centrifuged at 12,000*g* for 15 min at 4°C. RNA was isolated from the aqueous phase using a commercially available kit (RNeasy Mini Kit; Qiagen Inc.). All samples were treated with DNase (Qiagen Inc.) to minimize potential contamination with genomic DNA. The RNA purity and quantity were determined using the Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Rockville, MD, USA). RNA was reverse transcribed to cDNA using a commercially available High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster

Table 2. Primer sequences for RT-PCR

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
36B4	GCAATGTTGCCAGTGTCTGT	TCGTTTGTACCCGTTGATGA
Atrogin-1	GTGAGCGACCTCAGCAGTTA	TCTTCTTGGCTGCAACATCA
b2M	TGTCTTTCAGCAAGGACTGG	CGGCAGGCATACTCATCTTT
COXIV	TCGCTCCCAGCTTATATGGA	CAGACAGGTGCTTGACATGG
CS	CCCTTACCTGTCCTTTGCAG	GCACTTCCTGATTTGCCAGT
FOXO3	GGTGTTCGGACCTTCATCTC	GCTGGCTTGTTCTCTTGGAT
GLUT4	TTGGCATGGGTTTCCAGTAT	CAGGAGGACCGCAAATAGAA
GSK3α	TGGCTTACACGGACATCAAA	GCGACTAGTTCCCTGGTCTCT
HKII	GTCCACTCCAGATGGGACAG	CTTTCACCCAAAGCACACG
HPRT	TGGCGTCGTGATTAGTGATG	ACCCTTTCCAAATCCTCAGC
HSL	TCCAAGCAGGGATCTTTGAC	TTCCACTCTAGGGCTGATCG
IGF1	CTGAGCTGGTGGATGCTCTT	ATACCCTGTGGGCTTGTTGA
LPL	ATCAACTGGATGGAGGAGGA	CTCCAAGGCTGTATCCCAAG
MURF-1	CTGCCAAGCAACTCATCAAA	CAAAGCCCTGCTCTGTCTTC
Myf5	CACCTCCTCAGAGCAACCTG	AGTTGCAGGCTGTGAATCG
MyoD	CGCCTGAGCAAAGTAAATGA	AACCGCTGGTTTGGATTG
p53	CCTCACCATCATCACACTGG	CACAAACACGCACCTCAAAG
PDP1	CCTCACACCTCCACAAGTCA	CCGTCAAATTCTGGCACTTT
PDK4	GACAGAGGAGGTGGTGTTCC	CGTTGGTGCAGTGGAGTATG
PGC-1α	TTAGGGCTTCTCCAAAGCTG	TACCTGGGCTTCTTTGATGG
PPARD	CCCTTTGTGATCCACGACAT	TTCACCAACTGCTTCCACAC
PRC	ACCAACCCATCTCTCATTGG	CAGTGGGATCAGCTTCAACA
RIP140	TCCTGCCACCTCACCTAAAC	TACTGCTGCAAATGGGCTTC
SIRT1	GGGTGTCTGTTTCATGTGGA	TCTACAGCAAGGCGAGCATA
TBP	CACGAACCACGGCACTGAT	TCCAAGAACTTAGCTGGA
TFAM	AAATATGGTGCTGAGGAGTGTT	CTGGTTTCCTGTGCCTATCC
TFP-β	TGTTGGCTTGATTGCTTCTG	CGAATAGGGACATCGGACAT
VEGFA	AGGGCAGAATCATCACGAAG	CTCGATTGGATGGCAGTAGC

Abbreviations: *36B4*, acidic ribosomal protein 36B4; *b2M*, β2 microglobulin; *COXIV*, cytochrome *c* oxidase subunit IV; *CS*, citrate synthase; *FOXO3*, forkhead box O3; *GLUT4*, glucose transporter 4; *GSK3α*, glycogen synthase kinase-3α; *HKII*, hexokinase II; *HPRT*, hypoxanthine-guanine phosphoribosyltransferase; *HSL*, hormone sensitive lipase; *IGF1*, insulin-like growth factor 1; *LPL*, lipoprotein lipase; *MURF-1*, muscle ring-finger protein 1; *Myf5*, myogenic factor 5; *MyoD*, myogenic differentiation 1; *PDK4*, pyruvate dehydrogenase kinase isozyme 4; *PDP1*, pyruvate dehydrogenase phosphatase catalytic subunit 1; *PGC-1α*, peroxisome proliferator-activated receptor γ coactivator 1α; *PPARD*, peroxisome proliferator-activated receptor delta; *PRC*, PGC-1α-related coactivator; *RIP140*, receptor interacting protein 1; *SIRT1*, Sirtuin 1; *TBP*, TATAA-box binding protein; *TFAM*, mitochondrial transcription factor A; *TFP-β*, trifunctional protein β-subunit; and *VEGFA*, vascular endothelial growth factor A.

City, CA, USA) and a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Complementary DNA samples were stored at −20°C until further analysis. Quantitative real-time PCR was performed using SYBR Green Master Mix (Quanta Biosciences, Gaithersburg, MD, USA) and gene-specific primers on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Primers used for quantitative real-time PCR analysis are listed in Table 2. Genes linked to skeletal muscle remodelling through a range of molecular pathways were selected to provide a comprehensive comparison of mRNA expression between men and women. Priority was given to genes that have been previously shown to increase following SIT or have shown a sex-specific response to exercise. Changes in gene expression were analysed using the $2^{-\Delta C_T}$ method, as previously described (Schmittgen & Livak, 2008). For each gene, the HUGO Gene Nomenclature Committee standard symbol is provided in addition to the more commonly used abbreviation, when different. Gene expression was normalized to the geometric mean expression of four housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase (*HPRT*, *HPRT1*), TATAA-box binding protein (*TPB*), acidic ribosomal protein 36B4 (*36B4*, *RPLPO*) and β2 microglobulin (*B2M*), which did not change as a result of the exercise bout (data not shown). Data are also presented in graphical or tabular form as the fold change at 3 h of recovery normalized to their respective pre-exercise values, using $2^{-\Delta\Delta C_T}$.

Statistical analyses

Descriptive characteristics and serum hormone data were analysed using Student's independent-samples *t* tests. All

Table 3. Serum hormone concentrations in men and women

Hormone	Men	Women
Estradiol (pmol l^{-1})	127 ± 38	213 ± 100*
Progesterone (nmol l^{-1})	0.8 ± 0.2	0.8 ± 0.2
Testosterone (nmol l^{-1})	16.9 ± 6.2	1.0 ± 0.4*

Data are means ± SD; $n = 9$ men and $n = 9$ women. Values below the detection limit were included as the detection limit value ($n = 2$ for estradiol; $n = 4$ for total testosterone). *Significantly different from men ($P < 0.05$).

other data were analysed using a two-factor (time × sex) ANOVA. Mauchly's sphericity test was used to validate data sets before interpreting ANOVA results, and the Greenhouse–Geisser correction was applied to data sets that violated the assumption of sphericity. Statistical significance was set at $P \leq 0.05$. *Post hoc* tests were performed using Tukey's honestly significant difference test. All data are presented as mean values ± SD. Serum hormone values reflect $n = 9$ for each sex, because the estradiol value for one male subject was 2.7 SD above the mean and removed from the analyses. Messenger RNA expression data are based on $n = 8$ per group owing to low RNA quality for samples from three subjects.

Results

Descriptive exercise data

The SIT protocol elicited an average intensity of 92 ± 3 and 94 ± 4% of maximal heart rate during intervals in men and women, respectively, with no difference between sexes ($P = 0.10$). Men produced higher absolute mean power than women (men *versus* women, 509 ± 115 *versus* 339 ± 82 W, $P = 0.002$), but values normalized to fat-free mass were not different [men *versus* women, 7.6 ± 0.5 *versus* 7.5 ± 0.9 W (kg fat-free mass)$^{-1}$, $P = 0.69$].

Serum hormone concentrations

Relative to men, serum estradiol concentration was higher ($P = 0.04$; Table 3) and total testosterone concentration lower ($P < 0.001$; Table 3) in women. Serum progesterone concentration was not different between men and women ($P = 0.75$; Table 3).

Muscle data

The exercise-induced decrease ($P < 0.001$) in muscle glycogen was not different between men and women ($P = 0.14$; Fig 1).

Exercise increased the mRNA expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator 1α (*PGC-1α*, *PPARGC1A*), PGC-1α-related coactivator (*PRC*, *PPRC1*), PPARδ (*PPARD*), Sirtuin 1 (*SIRT1*) and receptor interacting protein 1 (*RIP140*, *NRIP1*) at 3 h of recovery relative to rest and immediately post-exercise ($P < 0.01$), but there were no differences between men and women (Fig. 2*A–D* and Table 4). There was no effect of exercise or sex on the expression of cytochrome *c* oxidase subunit IV (*COXIV*, *COX4I1*), citrate synthase (*CS*), tumor protein 53 (p53, *TP53*) or mitochondrial transcription factor A (*TFAM*; $P > 0.05$, Table 4).

The mRNA expression of hexokinase II (*HKII*, *HK2*), pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) and hormone sensitive lipase (*HSL*, *LIPE*) increased following exercise at 3 h of recovery *versus* rest and immediately postexercise ($P < 0.001$), with no differences between men and women ($P > 0.05$; Fig. 3*A* and *B* and Table 4). The mRNA expression of pyruvate dehydrogenase phosphatase catalytic subunit 1 (*PDP1*) was decreased following exercise to a similar extent in men and women ($P < 0.001$; Table 4). Exercise increased the mRNA expression of glucose transporter 4 (*GLUT4*, *SLC2A4*) and lipoprotein lipase (*LPL*) in women only ($P < 0.05$; Fig. 3*C* and *D*). Women had higher expression of *HKII* compared with men across all time points ($P < 0.01$; Fig. 3*A*) and higher expression of *LPL* immediately and 3 h following exercise ($P < 0.05$, Fig. 3*D*). Women also had lower resting expression of *GLUT4* ($P < 0.05$; Fig. 3*C*). There was a significant interaction for the mRNA expression of trifunctional protein β-subunit (*TFP-β*, *HADHB*; $P = 0.03$; Table 4), but the *post hoc* test revealed no significant differences between time points or sex. There was no effect of exercise or sex on the expression of glycogen synthase kinase-3α (*GSK3A*; $P > 0.05$; Table 4).

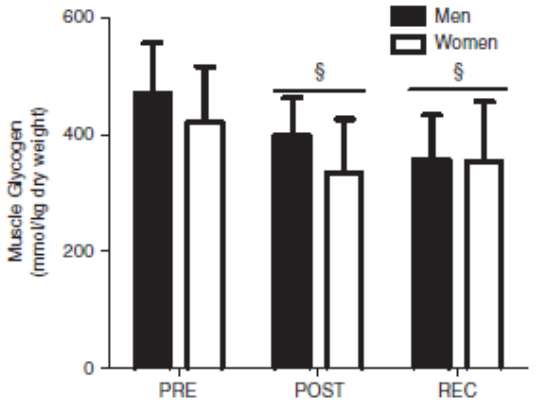

Figure 1. Muscle glycogen content before (PRE), immediately after (POST) and 3 h into recovery (REC) following an acute bout of sprint interval training (SIT) in men ($n = 10$, filled bars) and women ($n = 9$, open bars)
§Significantly different *versus* PRE ($P < 0.001$).

Exercise increased the mRNA expression of myogenic differentiation 1 (*MyoD*, *MYOD1*), vascular endothelial growth factor A (*VEGFA*), forkhead box O3 (*FOXO3*, *FOXO3A*) and muscle ring-finger protein 1 (*MURF-1*, *TRIM63*) at 3 h of recovery *versus* rest and immediately postexercise to a similar extent in men and women ($P < 0.001$; Fig. 4*A–C* and Table 4). The mRNA expression of myogenic factor 5 (*MYF5*) was decreased after exercise in both men and women ($P < 0.01$; Table 4). Exercise increased the mRNA expression of *Atrogin-1* (*FBXO32*) at 3 h of recovery in men only, and the expression of *Atrogin-1* was higher in men compared with women at 3 h of recovery ($P < 0.05$; Fig. 4*D*). Men had higher expression of *FOXO3* compared with women ($P = 0.01$; Fig. 4*C*). There was no effect of exercise or sex on the expression of insulin-like growth factor 1 (*IGF1*; $P > 0.05$; Table 4).

Discussion

The major new finding from the present study was that the acute skeletal muscle response to a single bout of sprint interval exercise was generally similar in men and women. Subjects were matched for age and relative fitness, and women were tested in the mid-follicular phases of their menstrual cycles. Contrary to our hypothesis, glycogen use and the acute gene expression response to exercise, including *PGC-1α* and *PPARD*, was similar in men and women, apart from the sex-specific responses of *GLUT4*, *LPL* and *Atrogin-1*.

Effect of sex on the gene expression response to SIT

The present study is the first to examine sex-based differences in the acute response of genes associated with skeletal muscle remodelling to SIT. The mRNA expression of genes involved in mitochondrial biogenesis (i.e. *PGC1α*, *PRC*, *PPARD* and *SIRT1*) increased following exercise without any differences between men and women. In the mid-follicular phase of their menstrual cycles, women have a blunted response in the expression of *PGC-1α* following endurance exercise performed at 65% of $\dot{V}_{O_2peak}$ (Fu *et al.* 2009), but the present data suggest similar expression in men and women at a higher intensity of exercise. The expression of *RIP140*, a transcriptional corepressor proposed to inhibit mitochondrial biogenesis, was also elevated following SIT in both men and women, and we are not aware of previous sex-based comparisons of

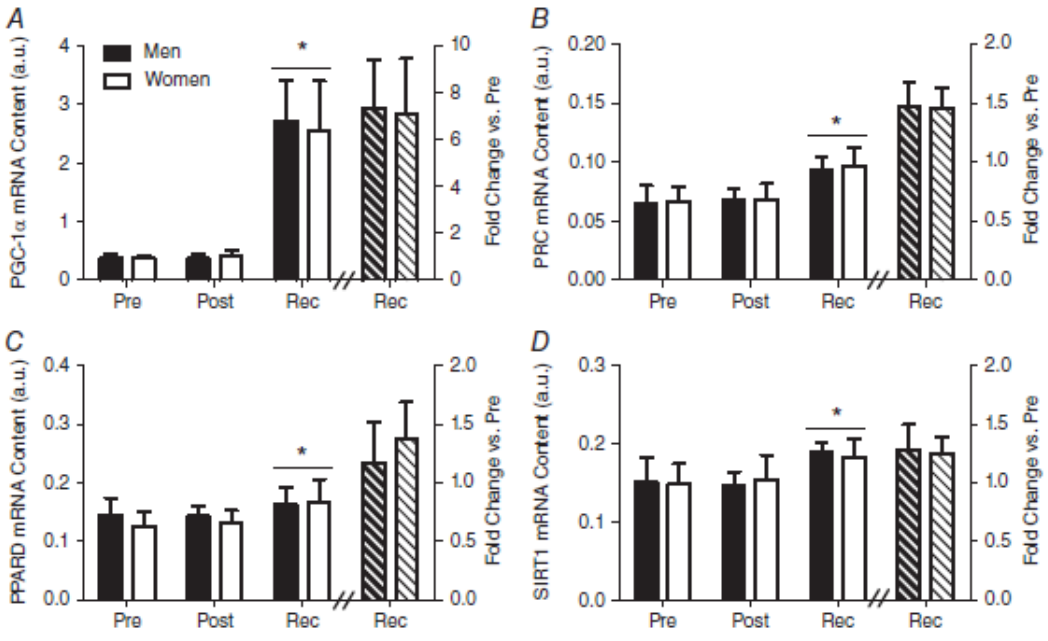

Figure 2. Messenger RNA content of mitochondrial genes in men ($n = 8$, filled bars) and women ($n = 8$, open bars) PRE, POST and REC following an acute about of SIT
Data are also presented as the fold change at 3 h of recovery normalized to their respective pre-exercise values. Peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*; *A*), PGC-1α-related coactivator (*PRC*; *B*), peroxisome proliferator-activated receptor delta (*PPARD*; *C*) and Sirtuin 1 (*SIRT1*; *D*) are shown separately. *Significantly different *versus* PRE and POST ($P < 0.05$).

Table 4. Messenger RNA expression in men and women

Gene	Sex	Messenger RNA content (a.u.) PRE	POST	REC	PRE *versus* REC (fold change)	*P* Values
COXIV	M	4.8 ± 1.0	4.6 ± 0.6	4.5 ± 0.8	1.0 ± 0.2	0.49, 0.88,
	W	4.7 ± 1.0	4.9 ± 0.7	4.7 ± 1.1	1.0 ± 0.1	0.22
CS	M	3.0 ± 0.4	2.9 ± 0.4	2.8 ± 0.3	0.9 ± 0.1	0.55, 0.67,
	W	2.7 ± 0.5	2.9 ± 0.4	2.8 ± 0.5	1.0 ± 0.1	0.09
p53	M	6.0 ± 1.2 ($\times10^{-2}$)	5.9 ± 1.5 ($\times10^{-2}$)	5.8 ± 1.3 ($\times10^{-2}$)	1.0 ± 0.2	0.58, 0.18
	W	5.3 ± 1.1 ($\times10^{-2}$)	5.0 ± 1.1 ($\times10^{-2}$)	5.1 ± 0.7 ($\times10^{-2}$)	1.0 ± 0.2	0.83
RIP140	M	5.1 ± 1.8 ($\times10^{-1}$)	5.3 ± 1.7 ($\times10^{-1}$)	6.5 ± 2.2 ($\times10^{-1}$)*	1.3 ± 0.5	<0.001, 0.44
	W	4.6 ± 0.4 ($\times10^{-1}$)	4.7 ± 1.0 ($\times10^{-1}$)	6.0 ± 0.8 ($\times10^{-1}$)*	1.3 ± 0.2	0.97
TFAM	M	2.3 ± 0.3 ($\times10^{-1}$)	2.2 ± 0.4 ($\times10^{-1}$)	2.4 ± 0.5 ($\times10^{-1}$)	1.1 ± 0.2	0.06, 0.51,
	W	2.3 ± 0.3 ($\times10^{-1}$)	2.5 ± 0.4 ($\times10^{-1}$)	2.5 ± 0.4 ($\times10^{-1}$)	1.1 ± 0.2	0.12
GSK3α	M	4.5 ± 0.5 ($\times10^{-1}$)	4.1 ± 0.7 ($\times10^{-1}$)	4.3 ± 0.8 ($\times10^{-1}$)	1.0 ± 0.2	0.17, 0.72
	W	4.3 ± 0.7 ($\times10^{-1}$)	4.2 ± 0.5 ($\times10^{-1}$)	4.2 ± 0.6 ($\times10^{-1}$)	1.0 ± 0.1	0.61
HSL	M	9.2 ± 6.1 ($\times10^{-3}$)	9.5 ± 6.3 ($\times10^{-3}$)	1.2 ± 0.8 ($\times10^{-2}$)*	1.5 ± 1.0	<0.001, 0.30
	W	6.5 ± 2.0 ($\times10^{-3}$)	7.0 ± 1.7 ($\times10^{-3}$)	1.0 ± 0.2 ($\times10^{-2}$)*	1.7 ± 0.6	0.97
PDP1	M	1.2 ± 0.3	1.2 ± 0.3	0.9 ± 0.3*	0.7 ± 0.2	<0.001, 0.15
	W	0.9 ± 0.3	1.0 ± 0.4	0.7 ± 0.3*	0.8 ± 0.1	0.27
TFP-β	M	2.0 ± 0.8	1.8 ± 0.5	1.7 ± 0.5	0.9 ± 0.2	0.34, 0.95
	W	1.7 ± 0.4	1.9 ± 0.6	1.8 ± 0.5	1.0 ± 0.1	0.03
IGF1	M	2.5 ± 0.7 ($\times10^{-2}$)	2.2 ± 0.6 ($\times10^{-2}$)	2.2 ± 0.7 ($\times10^{-2}$)	0.9 ± 0.3	0.43, 0.85
	W	2.2 ± 0.6 ($\times10^{-2}$)	2.3 ± 0.9 ($\times10^{-2}$)	2.1 ± 0.5 ($\times10^{-2}$)	1.0 ± 0.3	0.15
MURF-1	M	3.1 ± 0.7	3.3 ± 0.8	11.4 ± 1.9*	3.7 ± 0.6	<0.001, 0.13
	W	3.2 ± 0.7	3.2 ± 0.9	9.2 ± 3.4*	3.0 ± 1.3	0.15
MYF5	M	2.0 ± 0.3 ($\times10^{-2}$)	1.9 ± 0.4 ($\times10^{-2}$)	1.6 ± 0.2 ($\times10^{-2}$)*	0.8 ± 0.1	0.001, 0.19
	W	2.3 ± 0.6 ($\times10^{-2}$)	2.1 ± 0.5 ($\times10^{-2}$)	1.9 ± 0.6 ($\times10^{-2}$)*	0.8 ± 0.2	0.85

Data are means ± SD before (PRE), immediately after (POST) and 3 h into recovery (REC); $n = 8$ men (M) and 8 women (W). *P* Values are listed in order for main effect of time, main effect of sex and interaction. Abbreviations: *COXIV*, cytochrome *c* oxidase subunit IV; *CS*, citrate synthase; *GSK3α*, glycogen synthase kinase-3α; *HSL*, hormone sensitive lipase; *IGF1*, insulin-like growth factor 1; *MURF-1*, muscle ring-finger protein 1; *Myf5*, myogenic factor 5; *PDP1*, pyruvate dehydrogenase phosphatase catalytic subunit 1; *RIP140*, receptor interacting protein 1; *TFAM*, mitochondrial transcription factor A; and *TFP-β*, trifunctional protein β-subunit. *Significantly different *versus* PRE and POST ($P < 0.05$).

RIP140 expression following exercise. Additionally, we assessed the acute response to a number of substrate metabolism-related genes. SIT increased the mRNA expression of *HKII* and *HSL* to a similar extent in men and women. Following 90 min of endurance exercise, men and women are reported to have a similar increase in *HKII* (Fu *et al.* 2009), but neither men nor women (Roepstorff *et al.* 2006) or only men (Fu *et al.* 2009) have demonstrated an upregulation of *HSL* expression. A single bout of SIT also increased the expression of *PDK4* and decreased the expression of *PDP1* in both men and women following SIT. This is the first investigation into the expression of these genes following acute exercise in men *versus* women, with our results revealing no difference among sexes. Lastly, despite a reported higher *TFP-β* mRNA expression in women compared with men (Maher *et al.* 2009), this difference was not observed in the present study, which is in agreement with evidence of similar TFP-β protein content among men and women (Maher *et al.* 2009). Discrepancies between the findings of the present study and previous reports (Roepstorff *et al.* 2006; Fu *et al.* 2009; Maher *et al.* 2009) may relate to the type and duration of exercise [sprint interval exercise (7 min) *versus* endurance exercise (90 min)], the time course of biopsies and the training status of the participants (sedentary *versus* moderately active).

In the present study, we observed a lower baseline expression of *GLUT4* mRNA in women, as well as a sex-specific increase in the expression of *GLUT4* and *LPL*. The lower baseline expression and exercise-induced increase in women only for *GLUT4* is in contrast to previous reports of higher baseline expression and unchanged *GLUT4* expression following endurance exercise in women also in the mid-follicular phase of the menstrual cycle (Fu *et al.* 2009). Despite this gene expression result, men and women have similar GLUT4 protein content (Høeg *et al.* 2009). More work is needed to elucidate whether GLUT4 translocation in response to SIT is similar between sexes. Interestingly, higher *LPL* mRNA expression in women compared with men has been shown at rest (Kiens *et al.* 2004), but our data are the first to show a greater acute response of *LPL* mRNA expression

following exercise, possibly indicating greater potential for training-induced increases in the capacity for hydrolysis of triglycerides carried by lipoproteins in women following SIT. Furthermore, we observed higher mRNA expression of *HKII* across all time points in women compared with men, which is consistent with previous findings of both higher *HKII* mRNA (Fu *et al.* 2009) and protein expression (Høeg *et al.* 2011) in women.

We also report new data on the response of genes involved in structural remodelling. FOXO transcription factors co-ordinate the upregulation of the ubiquitin ligases, MURF-1 and Atrogin-1, which are involved in the regulation of muscle protein breakdown (Egan & Zierath, 2013). SIT induced an increase in the expression of *FOXO3* and *MURF-1* at 3 h of recovery in both men and women, whereas the expression of *Atrogin-1* increased in men only. No measurements of protein breakdown-related genes have been made previously following SIT, but men have displayed a greater response of *MURF-1* expression following protein ingestion 24 h after resistance exercise (West *et al.* 2012). We also observed similar exercise-induced increases across sexes for the expression of *MyoD*, a myogenic regulatory factor involved in satellite cell proliferation. This finding is in line with reported increases in the number of active and differentiating satellite cells following 6 weeks of SIT in both men and women (Joanisse *et al.* 2015) and a similar increase in the expression of *MyoD* mRNA following endurance exercise (Vissing *et al.* 2008). Finally, SIT increased the mRNA expression of the pro-angiogenic factor *VEGFA* in both men and women. A recent study reported a 3.5-fold increase in the expression of *VEGF* following Wingate-based SIT in trained men (Taylor *et al.* 2016), and increases in skeletal muscle capillarization have been shown in men and a small sample of women ($n = 3$) following 6 weeks of SIT (Cocks *et al.* 2013; Scribbans *et al.* 2014); however, whether the extent of the increase in capillarization is similar in men and women has not been adequately tested.

The potency of very low-volume SIT

The robust skeletal muscle remodelling observed in the present study occurred in response to only 1 min of 'all-out' intermittent exercise. Very brief SIT protocols involving two to three 20 s 'all-out' cycling efforts, within a 10 min training session, have been shown to improve glycaemic control and increase skeletal muscle

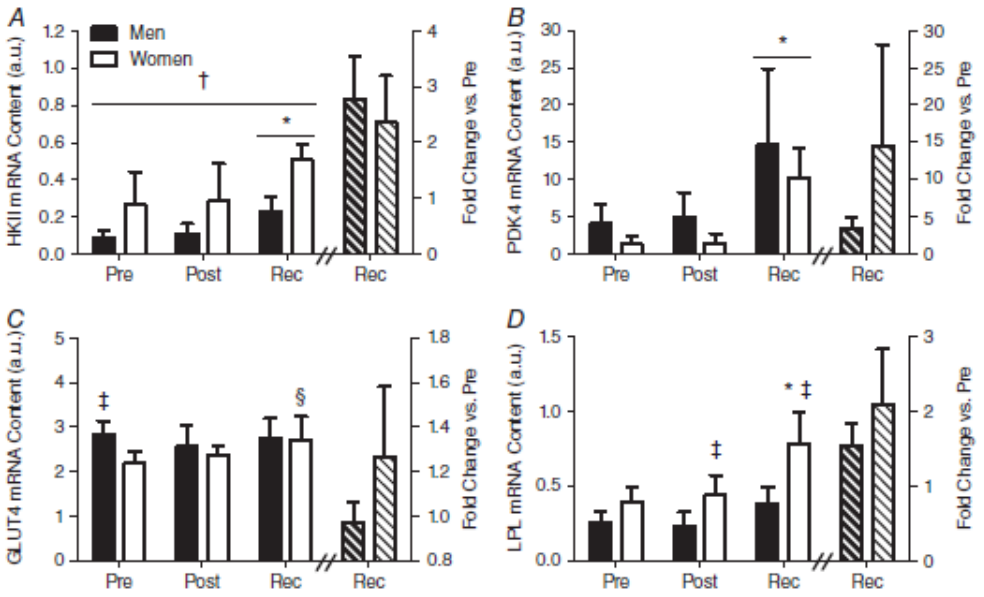

Figure 3. Messenger RNA content of substrate metabolism-related genes in men ($n = 8$, filled bars) and women ($n = 8$, open bars) PRE, POST and REC following an acute about of SIT
Data are also presented as the fold change at 3 h of recovery normalized to their respective pre-exercise values. Hexokinase II (*HKII*; *A*), pyruvate dehydrogenase kinase isozyme 4 (*PDK4*; *B*), glucose transporter 4 (*GLUT4*; *C*) and lipoprotein lipase (*LPL*; *D*) are shown separately. The symbols indicate a significant difference *versus* PRE and POST ($^{*}P < 0.05$), a significant difference *versus* PRE only ($^{\S}P < 0.05$), a main effect of sex ($^{\dagger}P < 0.01$) and a significant difference *versus* opposite sex at same time point ($^{\ddagger}P < 0.05$).

mitochondrial protein content following 6 weeks of training (Metcalfe *et al.* 2012; Gillen *et al.* 2014). Recently, our laboratory demonstrated that 12 weeks of the same SIT protocol as that described in the present manuscript increased insulin sensitivity, cardiorespiratory fitness and skeletal muscle mitochondrial content to a similar extent as a 50 min moderate-intensity continuous exercise protocol in untrained men (Gillen *et al.* 2016). The present study is the most comprehensive assessment to date of the acute skeletal muscle response to very low-volume SIT and further supports the suggestion that brief, intense exercise is a potent stimulus to induce a molecular cascade that ultimately increases mitochondrial content (MacInnis & Gibala, 2016). One previous study reported a large increase in the mRNA expression of *PGC-1α* (~17 fold) following a 10 min SIT protocol (Metcalfe *et al.* 2015), and our work expands on this finding, highlighting that 1 min of intense intermittent exercise is an effective stimulus to increase the expression of numerous genes involved in skeletal muscle remodelling. The relative changes of expression for mitochondrial genes (i.e. *PGC1α*, *SIRT1* and *RIP140*) following very low-volume SIT are consistent with previous acute SIT studies involving higher total exercise volumes (Little *et al.* 2011; Edgett *et al.* 2013).

Similar acute responses cannot explain sex-specific training adaptations

The similar responses of genes involved in mitochondrial biogenesis to acute SIT in men and women are in agreement with previous reports of comparable increases in the maximal activity of CS (Gillen *et al.* 2014) and the protein content of CS, COXIV and PGC-1α following 3–6 weeks of SIT (Gillen *et al.* 2014; Scalzo *et al.* 2014). In contrast, Scalzo *et al.* (2014) reported a tendency for higher mitochondrial protein synthetic rates, measured by the incorporation of deuterium oxide (D_2O), in men compared with women, following 3 weeks of SIT. Interestingly, the authors also reported that women had greater COXIV protein content compared with men, indicative of greater mitochondrial content (Larsen *et al.* 2012), but that neither group increased COXIV protein content in response to training (Scalzo *et al.* 2014). It is possible that this discrepancy relates to differences in the sensitivity of the analytical methods to detect a training-induced change, or that the net mitochondrial protein turnover response to SIT is similar in men and women (i.e. women might have lower synthetic rates in conjunction with lower degradation rates relative to men).

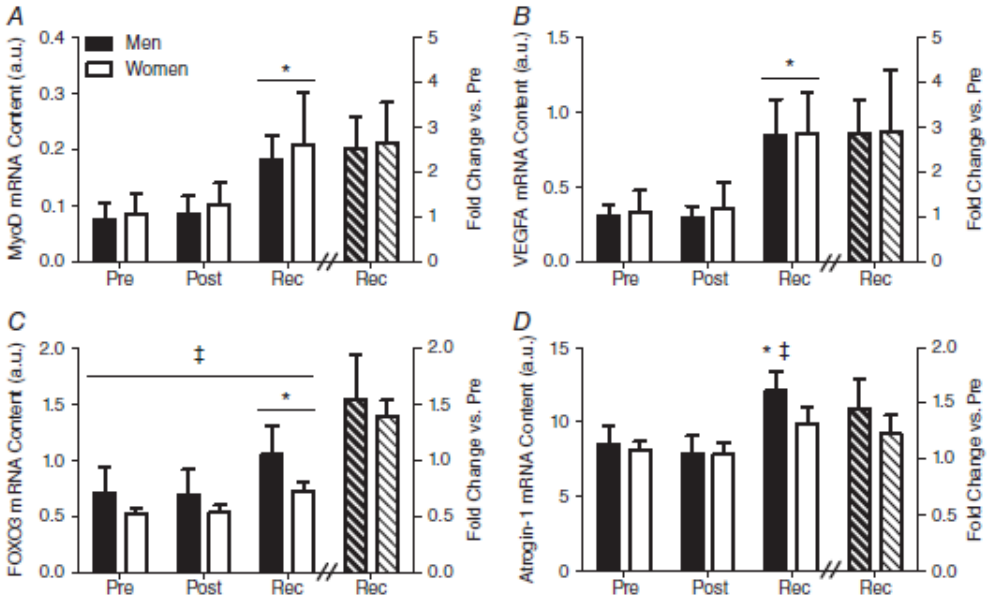

Figure 4. Messenger RNA content of genes involved in skeletal muscle structural remodelling in men ($n = 8$, filled bars) and women ($n = 8$, open bars) PRE, POST and REC following an acute about of SIT
Data are also presented as the fold change at 3 h of recovery normalized to their respective pre-exercise values. Myogenic differentiation 1 (*MyoD*; *A*), vascular endothelial growth factor A (*VEGFA*; *B*), forkhead box O3 (*FOXO3*; *C*) and *Atrogin-1* (*D*) are shown separately. The symbols indicate a significant difference *versus* PRE and POST ($^*P < 0.05$), a main effect of sex ($^\dagger P < 0.001$) and a significant difference *versus* opposite sex at same time point ($^\ddagger P < 0.05$).

Future studies should consider integrating measures of mitochondrial protein synthetic rates with more direct measures of mitochondrial volume (i.e. transmission electron microscopy) or stronger biomarkers, such as CS maximal activity (Larsen *et al.* 2012).

Glycogen depletion is recognized as an important molecular signal linked to the regulation of gene expression (Philp *et al.* 2012), and the high rates of glycogen breakdown and resynthesis following high-intensity exercise may contribute to the improvements in insulin sensitivity following 6 weeks of SIT (Metcalfe *et al.* 2012). The SIT-induced reduction in muscle glycogen of ∼17% in the present study is consistent with previous data (Metcalfe *et al.* 2015), and we show that whole-muscle glycogen depletion during low-volume SIT is similar between men and women. In contrast, Esbjornsson-Liljedahl *et al.* (1999, 2002) reported similar glycogen use in type II fibres but lower glycogen use in type I fibres in women compared with men following single and repeated Wingate sprints. This effect might be masked in whole muscle, or our sprint protocol might not elicit the same effect, as our participants completed three 20 s 'all-out' sprints (*versus* one to three 30 s 'all-out' sprints), at a lower resistance (5.0 *versus* 7.5% of body weight) and had a shorter recovery time between sprints (2 *versus* 20 min). Alternatively, the differences in glycogen use might be related to disparities in power output between sexes. The relative mean power output (per kilogram fat-free mass) was similar between men and women with our protocol, whereas during the Wingate tests, women appear to produce lower relative mean power outputs than men (Esbjörnsson-Liljedahl *et al.* 1999, 2002). Our data suggest that glycogen use during SIT does not account for the sex-based differences in the increase in GLUT4 protein content and improvements in glycaemic control following 6 weeks of SIT (Metcalfe *et al.* 2012; Gillen *et al.* 2014). The metabolic basis for the improvements in glycaemic control in men but not women remains unknown but may relate to a higher baseline insulin sensitivity in women compared with men (Lundsgaard & Kiens, 2014). A recent study by Metcalfe *et al.* (2016), in a large mixed cohort of men ($n = 17$) and women ($n = 18$), reported a significant negative correlation between baseline insulin sensitivity and the percentage change following SIT. Interestingly, no sex-based differences were found; however, the authors reported large variability in the response to SIT and did not detect an improvement in insulin sensitivity based on an oral glucose tolerance test.

Limitations

Although our research provides new insight into the acute response to SIT in men and women, we recognize that acute skeletal muscle responses may not accurately predict training-induced adaptations (Cochran *et al.* 2014). Changes in mRNA expression do not necessarily result in similar changes in protein content or enzyme activity, and future research should confirm whether sex-based differences exist in increases in protein content and enzyme activity following longer periods of SIT. It is also worth noting that women were tested only in the mid-follicular phase of their menstrual cycles. Menstrual cycle phase has a small influence on gene expression at rest and following endurance exercise (Fu *et al.* 2009); therefore, although it would be insightful to compare our measures in the luteal phase, the effect of menstrual cycle phase is likely to be minor compared with the influence of sex (Tarnopolsky, 2008).

We used a 10 h overnight fast and advised participants to maintain their habitual diet leading up to the experimental trial, in addition to controlling for physical activity. Diet was not specifically controlled given the between-subject design, and it is possible that differences in food intake between individuals during the day before the experimental trials might have influenced the gene expression response. It is also possible that our relatively small sample size might have limited our ability to detect potential sex-based differences in the acute response to SIT.

Conclusion

In summary, the major finding from the present study was that the gene expression response to an acute session of SIT was largely similar in men and women matched for fitness. Therefore, the mechanistic basis for reported sex-based differences in response to several weeks of SIT remains to be elucidated. Additional studies using rigorous controls (e.g. for menstrual phase and relative fitness) are required to confirm whether sex-based differences exist in the adaptive response to SIT and to identify potential mechanisms if necessary. We also demonstrate the potency of 1 min of intense intermittent exercise to increase the expression of genes involved in skeletal muscle metabolism and structural remodelling and provide insight into the mechanisms of very low-volume SIT-induced increases in skeletal muscle oxidative capacity.

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Additional information

Competing interests

None declared.

Author contributions

Conception and design of the experiments: L.E.S., M.J.M.D., M.A.T. and M.J.D. Collection, analysis and interpretation of the data, and drafting the article or revising it critically for important intellectual content: L.E.S., J.B.G., M.J.M.I., B.J.M., A.S., M.A., M.J.M.D., M.A.T. and M.J.G. All authors approved the final version for publication and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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CHAPTER 3: Human skeletal muscle fibre type-specific responses to sprint interval and moderate-intensity continuous exercise: acute and training-induced changes. *In preparation.*

Title: Human skeletal muscle fibre type-specific responses to sprint interval and moderate-intensity continuous exercise: acute and training-induced changes

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Running Title: Muscle fibre type-specific responses to acute exercise and training

Key words: exercise, fibre type, mitochondria, Western blotting

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KEY POINTS SUMMARY

- Sprint interval training (SIT) can elicit responses similar to moderate-intensity continuous training (MICT) when assessed in biopsies of mixed human skeletal muscle, but there are limited and equivocal data regarding potential fibre type-specific responses.
- We assessed fibre type-specific responses of proteins involved in mitochondrial biogenesis to a single session of SIT and MICT in men and women, and markers of mitochondrial content after 12 weeks of training in men, using Western blotting.
- An acute session of SIT and MICT elicited similar increases in the phosphorylation of signalling proteins in type I and IIa fibres.
- MICT elicited greater increases in markers of mitochondrial content in type I fibres but similar increases in type IIa fibres, compared to SIT.
- These data reveal evidence of potential differences in the response of type I but not type IIa human skeletal muscle fibres to 12 weeks of SIT and MICT, which may be related to differences in recruitment patterns.

ABSTRACT

Low-volume sprint interval training (SIT) can elicit responses similar to moderate-intensity continuous training (MICT) when assessed in biopsies of mixed human skeletal muscle, but there are limited and equivocal data regarding potential fibre type-specific differences. We assessed mixed skeletal muscle, and type I and type IIa fibre-specific responses to a single session (study 1) and 12 weeks (study 2) of MICT and SIT in young healthy adults using Western blotting. MICT consisted of 45 min of cycling at ~70% of maximal heart rate and SIT involved 3 x 20-s 'all-out' sprints interspersed with 2 min of recovery. The exercise-induced increase ($p<0.04$) in the ratios of phosphorylated to total acetyl CoA carboxylase and p38 mitogen activated protein kinase protein content in mixed muscle and pooled fibre samples were similar after SIT and MICT. With respect to training responses, MICT, compared to SIT, increased the protein content of cytochrome *c* oxidase subunit IV and NADH:ubiquinone oxidoreductase subunit A9 to a greater extent ($p<0.04$) in type I fibres, but there were similar increases in type IIa fibres. Training also increased optic atrophy 1 (OPA1) protein content in pooled fibre samples ($p<0.01$) and both OPA1 and mitofusin 2 mixed muscle protein content ($p<0.02$). In summary, while responses to acute exercise were similar, we found evidence of potential differences in the response of type I but not type IIa human skeletal muscle fibres to 12 weeks of SIT and MICT, which may be related to differences in recruitment patterns during training.

INTRODUCTION

Sprint interval training (SIT) involves brief intermittent efforts performed in an 'all-out' manner or at an intensity equal to or greater than the power output eliciting peak aerobic capacity ($\dot{V}O_{2peak}$) (Weston *et al.*, 2014; MacInnis & Gibala, 2017). SIT can elicit skeletal muscle responses including increased mitochondrial content similar to moderate-intensity continuous training (MICT) despite a lower total exercise volume and time commitment, when assessed in biopsies of mixed human skeletal muscle (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2017). For example, a SIT protocol that consisted of 3 x 20-sec 'all-out' sprints, within a 10-min session of otherwise low-intensity cycling, elicited similar increases in the maximal activity of citrate synthase compared to 50 min of MICT, when each protocol was performed three times per week for 12 weeks by independent groups of inactive men (Gillen *et al.*, 2016).

The mechanism(s) by which SIT can elicit skeletal muscle responses similar to MICT despite lower exercise volume is unclear, but may involve a greater rate of metabolic disturbance owing to the higher absolute intensity or the intrinsic "hard-easy" pattern of contraction. Exercise training-induced skeletal muscle remodelling is largely mediated through the repeated, transient activation of signalling proteins that increase the transcription of target genes and augment protein content (Coffey & Hawley, 2007; Perry *et al.*, 2010; Egan & Zierath, 2013). There is evidence from acute studies that, despite involving a lower exercise volume, SIT can elicit disturbances to cellular energy state that are comparable to or greater than MICT, as reflected by changes in metabolites such as phosphocreatine, the phosphorylation state of AMP-activated protein kinase (AMPK) and

p38 mitogen-activated protein kinase (p38 MAPK), and mRNA expression of the key transcriptional co-activator, peroxisome proliferator activated receptor gamma coactivator 1α (PGC-1α) (Skovgaard *et al.*, 2016; Granata *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018).

There is also limited evidence that acute interval exercise may elicit fibre type-specific responses that differ from continuous exercise, including greater phosphorylation of AMPK in type II fibres that was attributed to a higher rate of glycogen degradation (Kristensen *et al.*, 2015). In contrast, another group reported similar type I and II glycogen depletion and no difference in fibre-type specific AMPK phosphorylation state immediately following an acute bout of MICT and SIT (Scribbans *et al.*, 2014*a*). With respect to short-term training responses examining changes in cytochrome *c* oxidase (COX) or succinate dehydrogenase (SDH) activities, Henriksson & Reitman (1976) reported divergent fibre type-specific responses following 7-8 wks of continuous and interval training, whereas more recent studies found similar increases in both fibre types in response to 4-6 wks of SIT and MICT (Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*). The discrepancies among investigations may relate to the technique utilized to assess fibre type-specific responses, where Western blotting (e.g., Kristensen *et al.*, 2015) may be more sensitive to detect small differences in protein content as compared to immunofluorescence (Murphy, 2011; Murphy & Lamb, 2013). It is also possible that longer training interventions are required to detect divergent fibre type-specific responses to SIT and MICT.

The purpose of the present investigation was to examine fibre type-specific responses of proteins involved in mitochondrial biogenesis to a single session of SIT and MICT and changes in mitochondrial protein content after 12 weeks of training in young healthy adults using Western blotting. In study 1, we assessed the acute response of signalling proteins, including the phosphorylation of acetyl-CoA carboxylase (ACC) and p38 MAPK, to a single session of SIT and MICT using a repeated measures design in men and women. In study 2, we examined changes in markers of mitochondrial protein content, including cytochrome *c* oxidase IV (COXIV), in two groups of men matched for cardiorespiratory fitness after 12 weeks of either SIT and MICT. We hypothesized that SIT, compared to MICT, would induce greater acute increases in the phosphorylation of signalling proteins and larger training-induced changes in markers of mitochondrial content in type II but not type I fibres.

METHODS

Ethical approval

All experimental procedures were approved by the Hamilton Research Ethics Board and conformed to the *Declaration of Helsinki*. All subjects completed routine medical screening and, after being advised of the purpose and potential risks of the study, provided written informed consent.

Subjects

Descriptive characteristics for the total of 29 subjects who volunteered to participate in the two studies are presented in Table 1. Ten recreationally active men (n=6) and women (n=4) took part in the acute investigation (study 1), which involved a repeated-measures design to investigate skeletal muscle responses to acute bouts of MICT and SIT. Nineteen inactive men took part in the training study (study 2), which examined skeletal muscle responses to 12 weeks of MICT or SIT. Descriptive characteristics for the men in study 2 have been published previously (Gillen *et al.*, 2016), but the focus of the present analyses was distinct and has not been published previously.

Pre-experimental procedures

For both study 1 and study 2, participants initially performed a ramp test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur Sport V 2.0, Groningen, The Netherlands) to determine whole-body peak oxygen uptake ($\dot{V}O_{2peak}$) and peak power output (W_{peak}). Following a ~2 min warm-up at 50 W, the workload was increased 1 W every 2 s until volitional exhaustion or the point at which pedal cadence fell below 60 revolutions per minute. The $\dot{V}O_{2peak}$ was defined as the highest average oxygen consumption over a 30-s period. On a separate visit, participants were familiarized with the exercise protocols. The MICT protocol consisted of 45 min of continuous cycling at ~70% of maximal heart rate (HR_{max}) on a cycle ergometer (Kettler, Ergo Race I, Germany). The SIT protocol consisted of 3 x 20-sec 'all-out' sprint efforts performed against 7.5% of body mass, interspersed with 2 min of recovery at 50 W

performed on an electronically-braked cycle ergometer (Velotron; RacerMate, Seattle, WA, USA).

Experimental trials

For study 1, the main experiment consisted of two trial days separated by at least 6 days. Trials were conducted in a randomized, counterbalanced manner. Participants were instructed to refrain from exercise, alcohol and caffeine for a minimum of 48, 24, and 10 h prior to each experimental trial, respectively. Participants recorded a 24 h diet log, were provided a standardized dinner before the first trial and were instructed to repeat this diet and consume the same standardized dinner prior to the second trial. On the day of each trial, participants arrived at the laboratory in the morning following an overnight fast. A resting muscle biopsy was obtained from the vastus lateralis under local anaesthesia (1% xylocaine) using a Bergström needle modified for suction (Tarnopolsky *et al.*, 2011). Samples were cleaned of excess blood, sectioned into several pieces, immediately frozen in liquid nitrogen, and stored at -80°C until analysis. After the first muscle biopsy, participants were fitted with a heart rate (HR) monitor (A300, Polar Electro Oy, Kempele, Finland) and completed a warm-up consisting of 2 min of cycling at 50 W. Participants then performed the designated exercise protocol. Immediately following exercise, a second needle muscle biopsy was obtained ~5 cm distal to the first incision site. During the exercise protocol heart rate was measured continuously using a HR monitor (Polar). Ratings of perceived exertion (RPE) were measured every 15 minutes during MICT and following the completion of each 20-sec sprint during SIT.

For study 2, the experimental procedures have been reported previously (Gillen *et al.*, 2016) and are briefly summarized below. Participants were matched for age, BMI and $\dot{V}O_{2peak}$ and assigned to SIT (n=9) or MICT (n=10). Participants completed 12 weeks of exercise training, which included a 2-wk lead-in phase consisting of one and two exercise sessions in the first and second week, respectively. Three sessions per week were completed thereafter, apart from week 7 where only one session was completed to allow for mid-training assessments not reported in the present manuscript. The same MICT and SIT protocols as study 1 were performed throughout the training intervention, with the exception that the sprints in SIT were initially performed against 5% of body mass, instead of 7.5%, and were progressively increased to maintain the desired relative exercise intensity. Heart rate was recorded every 5 seconds during each training session. RPE were recorded at 15, 30 and 45 minutes of exercise (MICT) or at the end of each sprint (SIT) on the 1st, 15th, and 30th sessions. Resting muscle biopsies were obtained ~1 week prior to training and 96 h after the final exercise session, as described for study 1.

Muscle analyses

Mixed muscle homogenate preparation

Muscle samples (~30 mg) were freeze dried for 48 h. A portion of each muscle sample (~2 mg dw) was weighed and homogenized on ice (1:200 w/v) in Na-EGTA solution (165 mM Na+, 50 mM EGTA, 90 mM Hepes, 1mM free Mg^{2+} (10.3 mM total Mg^{2+}), 8 mM total ATP, 10 mM creatine phosphate, pH 7.10). Samples were diluted to 33 ug wet weight/uL using 3x SDS loading buffer (0.125 M Tris-HCl, 10% glycerol, 4%

SDS, 4 M urea, 10% 2-mercaptoethanol and 0.001% Bromophenol Blue, pH 6.8) and then further diluted to 2.5 ug wet weight/uL with 1x SDS loading buffer (diluted 2:1 with 1x Tris-HCl (pH 6.8)) . A small amount of mixed muscle homogenate from each sample was mixed together to generate a pooled sample for calibration curves.

Single fibre collection, fibre-typing and pooling

Segments of single fibres were collected from freeze-dried muscle, as previously described (Murphy, 2011). Briefly, segments of individual fibres were removed using jeweller's forceps under a microscope and each segment was added to a tube containing 10 uL of 1x SDS denaturing solution. Each tube was vortexed, incubated at room temperature for 1 h and frozen at -80°C until analysis.

The dot blotting method was used to fibre-type each fibre segment as previously described (MacInnis *et al.*, 2017; Wyckelsma *et al.*, 2017; Christiansen *et al.*, 2019). Briefly, a PVDF membrane was activated in 96% ethanol, equilibrated in transfer buffer, and 1 uL of each fibre segment sample was spotted to the membrane. The membrane was dried, re-activated in 96% ethanol, equilibrated in 1x transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol, pH 8.3), washed in 1x Tris-buffered saline-Tween (TBST) for 5 min and then blocked in 5% skim milk powder in 1x TBST (blocking buffer) for 5 min. Membranes were incubated in primary antibody overnight (see details below) at 4°C and 2 h at room temperature the next day, all with constant rocking. After washing in blocking buffer, the membrane was incubated in secondary antibody for 1 h at room temperature, exposed to chemiluminescent substrate (Super

signal West Femto, Pierce, Rockford, IL, USA), imaged using a Chemidoc MP (Bio-Rad) and analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA). The presence of myosin heavy chain type IIa (MHC IIa, mouse, monoclonal IgG, A4.74, Developmental Studies Hybridoma Bank (DSHB), 1:200 in 1% bovine serum albumin (BSA) in phosphate-buffed saline with 0.025% Tween and 0.02% NaN_3 (PBST)) was determined first in each sample. After imaging, stripping buffer was used to remove MHC IIa antibodies and membranes were incubated in primary antibody to determine the presence of myosin heavy chain I (MHC I, mouse, monoclonal IgM, A4.840, DSHB, 1:200 in 1% BSA in PBST). Fibre segments expressing only MHC I or MHC IIa were identified and pooled separately to create a type I sample (study 1: 10 ± 2; study 2: 8 ± 3 fibres) and a type IIa sample (study 1: 14 ± 2; study 2: 14 ± 2 fibres) for each muscle biopsy. Hybrid fibres expressing both MHC I and MHC IIa were not analyzed further.

Western blotting

Western blotting was conducted on mixed muscle and pooled fibre samples as previously described (Murphy, 2011; MacInnis *et al.*, 2017; Wyckelsma *et al.*, 2017). Samples were separated on 26-well, 4-15%, pre-cast Criterion TGX Stain-Free gels (Bio-Rad) and run for 45 min at 200 V. A protein ladder (Fermentas PageRuler Prestained Ladder, ThermoFisher Scientific, Waltham, MA, USA) and a calibration curve (e.g., 2, 4, 8, 16 uL) of pooled mixed muscle homogenates were run on every gel. The total protein loaded was visualized using ultraviolet (UV) activation of the gel and analysed with Image Lab 6.0 (Bio-Rad). Protein was wet-transferred to a nitrocellulose membrane at

100 V for 30 min in transfer buffer at 4°C. Membranes were incubated in Pierce Miser solution (Pierce, Rockford, IL, USA) and blocked in blocking buffer. Sections of membranes were incubated with antibodies diluted in 1% BSA in PBST overnight at 4°C and 2 h at room temperature, all with constant rocking. After washing in blocking buffer, membranes were incubated in secondary antibody for 1 h at room temperature, washed in 1x TBST, exposed to chemiluminescent substrate (West Femto, ThermoScientific, IL, USA), and imaged using a Chemidoc MP and analyzed Image Lab software (Bio-Rad).

The primary antibodies used for Western blotting were COXIV (rabbit, polyclonal IgG, Cell Signaling Technology, 1:1000), NADH:ubiquinone oxidoreductase subunit A9 (complex I) (NDUFA9; rabbit, polyclonal, 1:1000), mitofusin 2 (MFN2; 1:1000, rabbit), optic atrophy 1 (OPA1; 1:1000, mouse, BD Biosciences, 1:1000), glucose transporter 4 (GLUT4; rabbit, polyclonal, Thermo Fisher Scientific, 1:1000), phosphorylated acetyl-CoA carboxylase (ACC^{Ser79}; rabbit, polyclonal, Cell Signaling Technology, 1:1000), total ACC (rabbit, polyclonal, Cell Signaling Technology, 1:1000), phosphorylated p38 mitogen-activated protein kinase (p38 $MAPK^{Thr180/Tyr182}$; rabbit, polyclonal, Cell Signaling Technology, 1:1000), total p38 MAPK (rabbit, Cell Signaling Technology, 1:1000), phosphorylated glycogen synthase (GS^{Ser641} rabbit, monoclonal, Epitomics, 1:1000) and total GS (rabbit, monoclonal, Epitomics, 1:1000). The horseradish peroxidase-conjugated secondary antibodies used were goat anti-mouse IgG and IgM (Pierce ThermoScientific) and goat-anti rabbit (Pierce Thermoscientific). Each secondary antibody was diluted 1:20,000 in blocking buffer. Professor Michael Ryan (Monash University, Australia) kindly provided the NDUFA9 and MFN2 antibodies.

A calibration curve containing pooled mixed muscle homogenates was run on every gel. The abundance of a given protein for each muscle sample was expressed relative to the calibration curve and then normalized to total protein, which was also expressed relative to the calibration curve. If a gel contained a sample with protein content approaching or below zero on the calibration curve, then all samples on the gel were normalized to total protein but not calibrated to the calibration curve. In order to reduce variability across gels, mixed muscle samples were expressed relative to the average MICT pre-exercise (study 1) or pre-training (study 2) value and pooled fibre samples were expressed relative to the average MICT pre-exercise (study 1) or pre-training (study 2) type I values on each gel. Samples with no detectable protein content for a given protein of interest or a very small amount close to detection limits were not included in the final analyses. This reduced the sample size for type I phosphorylated to total ACC (n=9), type I phosphorylated to total p38 MAPK (n=9), type I phosphorylated to total GS (n=8), type IIa phosphorylated to total GS (n=9), type I NDUFA9 (MICT: n=9, SIT: n=8), type IIa NDUFA9 (MICT: n=9, SIT: n=7) and type I and IIa MFN2 (MICT: n=10, SIT: n=8) protein content. Mixed muscle data for COXIV, NDUFA9 and GLUT4 following 12 weeks of SIT and MICT in the biopsy samples has been reported previously (Gillen *et al.*, 2016); however, the present study used a separate piece of biopsy material to analyze these markers using different primary antibodies and a different protocol.

Statistical analyses

Data were assessed for normality using the Shapiro-Wilk test and the majority of data were normally distributed. Muscle data from study 1 were analyzed using a two-factor repeated measures ANOVA with the within factors of time (pre-exercise, post-exercise) and group (MICT, SIT). Muscle data from study 2 were analyzed using a two-factor mixed ANOVA with the within factor, time (pre-training, post-training), and between factor, group (MICT, SIT). All fibre-type specific analyses were performed within a fibre type. Post hoc testing, involving Tukey's honestly significant difference tests, was performed after identification of significant interactions. Analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Data are reported relative to the average MICT Pre or MICT Pre Type I samples for presentation purposes, but statistical analyses were performed on the data prior to the group-level normalization. Statistical significance was set at $p<0.05$ and all data are presented as mean ± SD.

RESULTS

Study 1

Exercise data

Mean power output during the acute bout of MICT and SIT was 106 ± 30 and 133 ± 20 W, respectively, which elicited 73 ± 4% and 83 ± 5% of HR_{max} (n=9) and corresponded to RPE of 12 ± 1 and 17 ± 1. Mean total work was 300 ± 85 kJ for MICT and 56 ± 8 kJ for SIT.

Mixed muscle protein content

The protein content ratios of phosphorylated ACC to total ACC and phosphorylated p38 MAPK to total p38 MAPK increased in mixed muscle homogenates following acute sessions of MICT and SIT, with no differences between groups (main effects for time $p<0.03$; Fig 1C and 1D). In both groups, the ratio of mixed muscle phosphorylated GS to total GS abundance decreased to a similar extent following MICT and SIT (main effect for time $p<0.001$; Fig 2B).

Single-fibre protein content

The ratio of phosphorylated ACC to total ACC increased following MICT and SIT in both type I and type IIa fibres (main effects for time $p<0.03$; Fig. 1E and 1G), with no differences between groups. Phosphorylated p38 MAPK relative to total p38 MAPK also increased following MICT and SIT in both type I and type IIa fibres to a similar extent (main effects for time $p<0.04$; Fig. 1F and 1H). There was no change in phosphorylated GS to total GS in type I or type IIa fibres ($p>0.05$, Fig. 2C and 2D).

Study 2

Exercise data

Participants completed a total of 31 ± 1 and 32 ± 2 sessions in the MICT and SIT groups, respectively. Over the 12 weeks of training, mean HR during MICT was 71 ± 5% of HR_{max} and mean HR during SIT was 79 ± 4% of HR_{max}. Mean RPE, measured during

the 1st, 15th and 30th session was 13 ± 1 and 16 ± 1 for MICT and SIT, respectively. Mean total work per session was ~310 kJ for MICT and ~60 kJ for SIT.

Mixed muscle protein content

The protein contents of COXIV (Fig. 3C), NDUFA9 (Fig. 3D), MFN2 (Fig. 4C), OPA1 (Fig. 4D) and GLUT4 (Fig. 5B) increased following MICT and SIT (main effects for time $p<0.02$).

Single-fibre protein content

In type I fibres, only MICT increased the protein content of COXIV (Fig. 3E) and NDUFA9 (Fig. 3F) and the abundances of COXIV and NDUFA9 were greater in type I fibres post-training in MICT compared to SIT (group x time interactions $p<0.04$). In type IIa fibres, training increased COXIV (Fig. 3G) and NDUFA9 (Fig. 3H) protein contents to a similar extent following MICT and SIT (main effects for time $p<0.03$). There were no training-induced changes in MFN2 content in type I or type IIa fibres following MICT and SIT (main effects for time $p>0.05$; Fig. 4E and Fig. 4G). OPA1 content increased after training in both type I and type IIa fibres (main effects for time $p<0.01$; Fig. 4F and 4H). There was also a main effect for group for OPA1 content in type I fibres, where MICT had greater OPA1 abundance compared to SIT (main effect for group $p=0.003$). Both MICT and SIT increased GLUT4 protein content in type I and type IIa fibres (main effects for time $p<0.03$; Fig 5C and 5D).

DISCUSSION

The overarching goal of the present study was to examine human skeletal muscle fibre type-specific responses to an acute bout of low-volume SIT and MICT and changes induced by 12 weeks of training, using Western blotting. Contrary to our hypothesis, increases in the phosphorylation state of signalling proteins related to mitochondrial biogenesis were similar following an acute bout of MICT and SIT. With respect to training responses, MICT, compared to SIT, elicited greater increases in markers of mitochondrial content in type I fibres but similar changes in type IIa fibres. Finally, training-induced changes in mitochondrial fusion proteins were similar after MICT and SIT in both mixed muscle and pooled fibre samples.

We measured the phosphorylation of ACC and p38 MAPK to gain insight into the activation of signalling pathways known to be involved in upregulating mitochondrial biogenesis following exercise (Egan & Zierath, 2013). The expression of *PGC-1α* mRNA is proposed to be regulated by signalling proteins, including AMPK and p38 MAPK, which respond to indices of metabolic stress during exercise. To our knowledge, the present study is the first to compare the fibre type-specific phosphorylation states of ACC, a downstream target of AMPK (Winder *et al.*, 1997), and p38 MAPK in response to low-volume SIT and MICT. Consistent with previous investigations in mixed muscle samples, we observed similar phosphorylation of ACC immediately post-exercise (Scribbans *et al.*, 2014*a*; Granata *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018) and we build on these findings by also demonstrating similar responses of ACC in type I and IIa fibres. We also found similar phosphorylation of p38 MAPK in type I and type IIa

fibres as well as mixed muscle homogenates. In contrast, other laboratories have reported greater phosphorylation of p38 MAPK following SIT compared to MICT (Granata *et al.*, 2017; Fiorenza *et al.*, 2018) or no change following either exercise protocol (Scribbans *et al.*, 2014*a*). The SIT protocols demonstrating greater phosphorylation of mixed muscle p38 MAPK involved 2-3x longer durations of total intense exercise *versus* the present study, and it is possible that a greater duration of intense exercise, and thus greater metabolic stress during SIT, elicits superior activation of p38 MAPK compared to MICT. The work by Kristensen *et al.* (2015) assessed responses to a total of 9 minutes of intense intermittent exercise performed at close to peak aerobic power in comparison to work-matched MICT. These authors reported greater phosphorylation of AMPK and ACC in type II *versus* I fibres after the acute interval protocol only, despite similar mixed muscle changes. Thus, the potential for acute fibre-type specific responses to SIT *versus* MICT may depend in part on the total volume of intense intermittent exercise performed.

Many studies have shown comparable increases in markers of mitochondrial content in mixed muscle samples following low-volume SIT and MICT (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*; Gillen *et al.*, 2016). With respect to potential fibre type-specific responses, a few studies using immunofluorescence reported similar increases in SDH and COX activities in type I and II fibres after 4-6 weeks of low-volume SIT and MICT (Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*), although such studies cannot show how great the increases were because the system cannot be calibrated (Murphy & Lamb, 2013). Using Western blotting to assess potential changes over a longer (12-week) training period, we report larger increases in

COXIV and NDUFA9 protein content in type I fibres after MICT *versus* SIT, but similar changes in type IIa fibres across training groups. These data provide insight into potential fibre-specific differences in the response to SIT, although the observed changes were incongruent with our original hypothesis. The increases in mitochondrial content within both type I and IIa fibres following MICT was consistent with previous investigations (Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*a*), suggesting that both fibre types are recruited during MICT and undergo metabolic stress, despite the lower relative intensity of exercise performed. Progressively more and higher threshold motor units may have been recruited over the course of the longer duration bout of MICT. Furthermore, while type I fibres are recruited during high-intensity exercise in accordance with the motor unit size principle (Henneman, 1957; Henneman *et al.*, 1965), the relatively brief total duration of hard effort (i.e., one minute) was seemingly insufficient to induce increases in mitochondrial content in this fibre type. A greater volume of intense exercise may be required to elicit type I fibre-specific increases in markers of mitochondrial content. In support of this interpretation, low-volume SIT protocols involving longer durations of intense exercise (~2-3 min) seemingly elicit increases in type I fibre SDH and COX activity (Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014*b*, 2014*a*; Edgett *et al.*, 2016). The incongruent findings between the previous studies and the present investigation may also relate to the methodology utilized to assess fibre type-specific mitochondrial content (immunofluorescence *versus* Western blotting, where calibration is possible in the latter only) or the length of intervention (4-6 weeks *versus* 12 weeks). Western blotting may be a superior method for detecting small differences in protein content compared to

immunofluorescence (Murphy, 2011; Murphy & Lamb, 2013) and longer training periods may be needed to detect small differences between MICT and SIT.

We did not observe any differences in type IIa responses to the two diverse training approaches, which was in contrast to our hypothesis. A potential explanation for the similar mixed muscle changes following training despite a blunted type I response following SIT may be that type IIX fibres experienced an augmented response in SIT *versus* MICT. However, the present study did not examine responses in type IIX fibres due to the low relative proportion of type IIX only fibres in skeletal muscle obtained from young, healthy individuals (Russell *et al.*, 2003; Daugaard & Richter, 2004), which makes it difficult to obtain a sufficient number within each skeletal muscle biopsy. It was also not possible to examine hybrid IIa/IIx fibres separately within our samples because the MHC IIx antibody cross-reacts with other MHC isoforms when assessed by dot blotting (Christiansen *et al.*, 2019).

A novel aspect of the present study was the assessment of fibre type-specific mitochondrial fusion proteins following low-volume SIT and MICT. The process of mitochondrial fusion creates a larger connected network within skeletal muscle by joining healthy mitochondria, and the proteins MFN2 and OPA1 are involved in the regulation of outer and inner mitochondrial membrane fusion, respectively (Yan *et al.*, 2012). The present study and Granata *et al.* (2016) show similar increases in mixed muscle MFN2 protein content following SIT and MICT. The unchanged fibre type-specific MFN2 protein content following 12 weeks of training concurs with two previous studies in our laboratories finding inconsistent MFN2 single fibre and mixed muscle training responses

(MacInnis *et al.*, 2017; Wyckelsma *et al.*, 2017). The lack of fibre type-specific changes in MFN2 found in the present investigation may be influenced by the relatively smaller increase in mixed muscle content of MFN2 relative to other proteins examined. In contrast, the MICT and SIT-induced mixed muscle changes in OPA1 were relatively larger compared to MFN2, and there were increases in both type I and type IIa OPA1 content following both training interventions. Together, our findings highlight that SIT elicits similar changes in mitochondrial fusion proteins as MICT despite a reduced exercise volume. More research examining mitochondrial fission proteins, in particular the mitochondrial specific protein, MiD49, following SIT is needed to provide a more comprehensive understanding of how SIT impacts mitochondrial dynamics proteins.

In addition to our primary focus on mitochondrial responses, we assessed fibre-specific changes in proteins involved in carbohydrate metabolism. Skeletal muscle glycogen resynthesis following an acute exercise has been suggested to contribute to the improvement in glycemic control for ~24 hr post-exercise (Jensen *et al.*, 2011). Glycogen synthase is a key regulator of glycogen synthesis (Danforth, 1965) and lower phosphorylation of GS is associated with higher GS activity and lower muscle glycogen content (Prats *et al.*, 2009). While there was a decrease in mixed muscle phosphorylation of GS^{Ser641} following both SIT and MICT, we did not detect exercise-induced changes in type I and IIa fibres. Similar exercise-induced glycogen depletion in type I and II fibres was found following low-volume SIT and MICT (Scribbans *et al.*, 2014*a*). In contrast, Kristensen *et al.* (2015) reported a greater decrease in the phosphorylation of GS^{3a+3b} and glycogen content in type IIa fibres compared to type I fibres after an interval training

protocol and similar changes within fibre types following MICT. The mixed muscle changes in the phosphorylation of GS^{3a+3b} by Kristensen *et al.* (2015) were relatively larger than the present study and Scribbans *et al*. (2014) and it is possible that only higher volumes of high intensity exercise elicit fibre type-specific responses in changes in the phosphorylation of GS and glycogen content.

Short-term, low-volume SIT is also a potent stimulus for increasing GLUT4 protein content in mixed muscle homogenates (Burgomaster *et al.*, 2007; Little *et al.*, 2010; Gillen *et al.*, 2014). Similar fibre type-specific increases in GLUT4 intensity, measured using immunofluorescence, were reported after 6 weeks of SIT and MICT (Bradley *et al.*, 2014). We confirm these findings in another cohort of inactive men following a longer (2x) training intervention and using semi-quantitative Western blotting to assess protein content. Longer training interventions may have greater potential to detect changes in GLUT4 content within fibre types, as two weeks of single-leg MICT elicited increases in GLUT4 content in type I fibres but not type IIa fibres (Daugaard *et al.*, 2000).

A strength of the present investigation is the examination of both acute and chronic fibre type-specific responses to SIT and MICT. Our findings demonstrate a discrepancy between acute and chronic responses, where the blunted training responses found in type I fibres following SIT were not evident in our acute measures. While our data suggest that acute responses of mitochondrial signalling pathways do not necessarily predict training responses, there are a few caveats to acknowledge. Firstly, the acute and chronic analyses were conducted in two separate groups of participants, which limits the

comparison of responses across experiments. Secondly, while we measured the phosphorylation of two proteins implicated in upregulating mitochondrial biogenesis, many other proteins with a role in skeletal muscle adaptation (Egan & Zierath, 2013) were not assessed. We also may have missed potential fibre type-specific differences in the acute response of ACC and p38 MAPK by only examining exercise-induced changes immediately post-exercise. Furthermore, there are numerous processes downstream of the activation of signalling pathways that may be responsible for the blunted increases in mitochondrial content within type I fibres, including protein transcription and translation and mitophagy (Hood *et al.*, 2016).

In summary, the present study shows that an acute bout of MICT and low-volume SIT induce similar changes in the phosphorylation of ACC and p38 MAPK in mixed muscle homogenates and pooled type I and IIa fibres. We also measured skeletal muscle training adaptations following 12 weeks of MICT and SIT and observed similar mixed muscle and type IIa responses but superior increases in COXIV and NDUFA9 protein content within type I fibres in MICT compared to SIT, indicating greater increases in mitochondrial content. The protein content of MFN2 and OPA1 increased after MICT and SIT in mixed muscle samples, and OPA1 increased in pooled fibre samples. Overall, these findings suggest that the mechanism(s) by which SIT elicits comparable skeletal muscle responses as MICT is not related to enhanced type IIa responses immediately after acute exercise or in response to a 12-week training intervention.

ADDITIONAL INFORMATION

Competing interests

The authors have no competing interest to declare.

Author contributions

All human experimental trials and exercise training were completed at McMaster University. All muscle analyses were conducted at La Trobe University. LES, JBG, MJM, MAT, RMM and MJG contributed to the conception and design of the work. LES, JBG, BPF, MJM, FEG, MAT, RMM and MJG collected samples, performed analyses, and/or interpreted data. LES, JBG, BPF, MJM, FEG, MAT, RMM and MJG drafted or revised the manuscript critically for important intellectual content.

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Table 1. Descriptive characteristics of the subjects completing an acute bout (study 1) or 12 weeks (study 2) of MICT vs. SIT.

	Acute study (MICT & SIT)	**Chronic study (MICT)**	**Chronic study (SIT)**
Participants	6 men; 4 women	10 men; 0 women	9 men; 0 women
Age (yr)	22 ± 3	28 ± 9	27 ± 7
Height (cm)	177 ± 12	176 ± 10	177 ± 11
Weight (kg)	76 ± 12	84 ± 20	84 ± 23
$\dot{V}O_{2peak}$ (L/min)	3.3 ± 0.8	2.7 ± 0.5	2.6 ± 0.8
$\dot{V}O_{2peak}$ (mL/kg/min)	44 ± 7	33 ± 6	32 ± 7
Peak Power Output (W)	305 ± 65	248 ± 30	243 ± 68

$\dot{V}O_{2peak}$, peak oxygen consumption. Data are means ± SD.

FIGURES

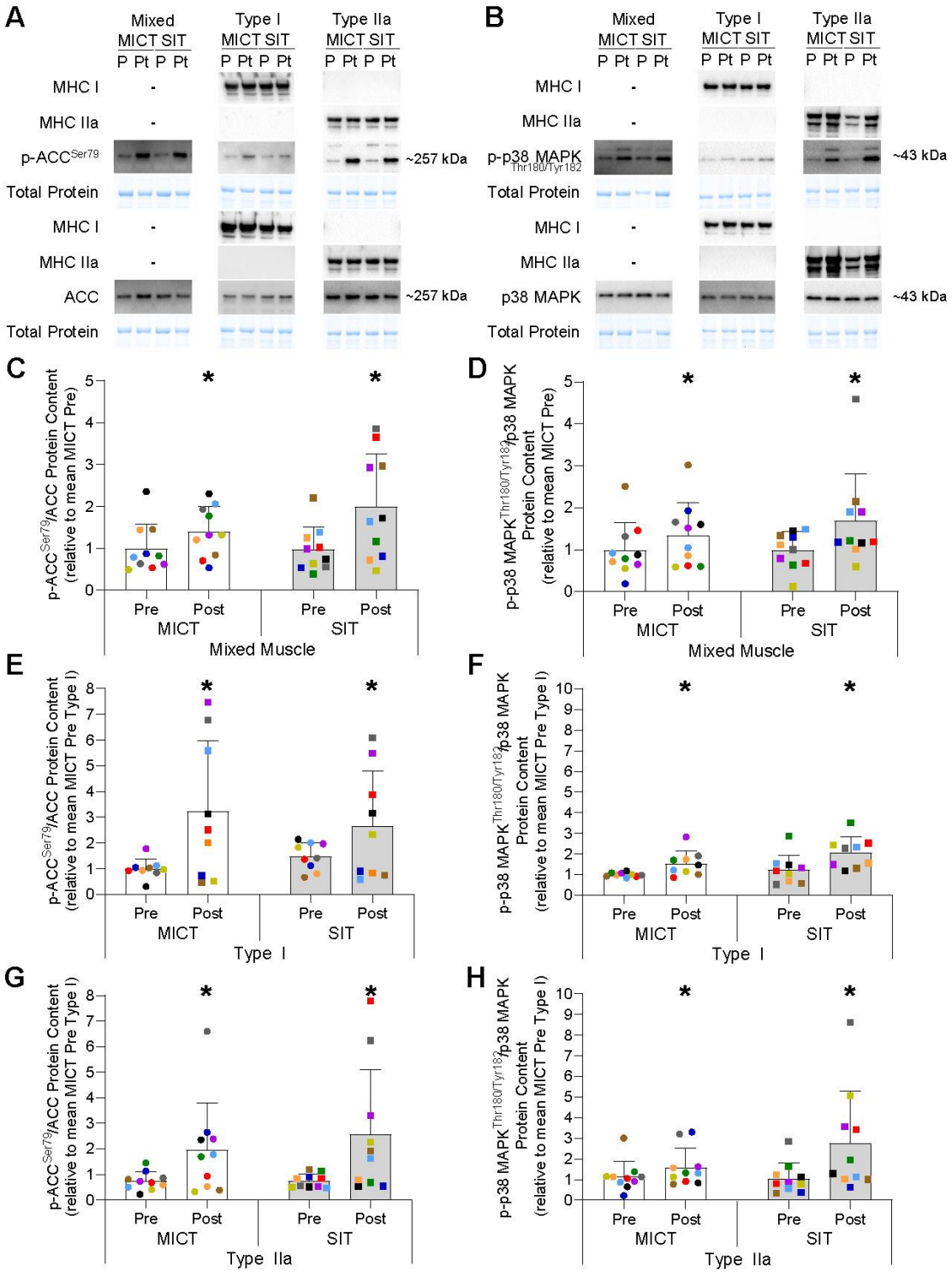
A
Mixed
Type I
Type IIa
MICT SIT
P Pt P Pt
MHC I
MHC IIa
p-ACCSer79
~257 kDa
Total Protein
ACC
B
p-p38 MAPKThr180/Tyr182
~43 kDa
p38 MAPK
C
p-ACCSer79/ACC Protein Content (relative to mean MICT Pre)
Pre
Post
MICT
SIT
Mixed Muscle
D
p-p38 MAPKThr180/Tyr182/p38 MAPK Protein Content (relative to mean MICT Pre)
E
p-ACCSer79/ACC Protein Content (relative to mean MICT Pre Type I)
Type I
F
p-p38 MAPKThr180/Tyr182/p38 MAPK Protein Content (relative to mean MICT Pre Type I)
G
Type IIa
H

Figure 1. Phosphorylated acetyl-CoA carboxylase (ACC) to total ACC and phosphorylated p38 mitogen activated protein kinase (p38 MAPK) to total p38 MAPK protein content before and following a single session of MICT and SIT. Representative blots (A, B), mixed muscle samples (C, D) pooled type I muscle fibres (E, F), and pooled type IIa muscle fibres (G, H) are displayed separately. Mixed muscle samples were normalized to the average pre-training MICT value and pooled muscle fibre samples were normalized to the average pre-training type I value. Myosin heavy chain (MHC) I and IIa were not measured in mixed muscle samples. The symbol indicates a significant difference vs. pre (*; $p < 0.05$). Data are means ± SD. Each colour represents one individual and is consistent across panels and figures within the same experiment. Pre (P); post (Pt).

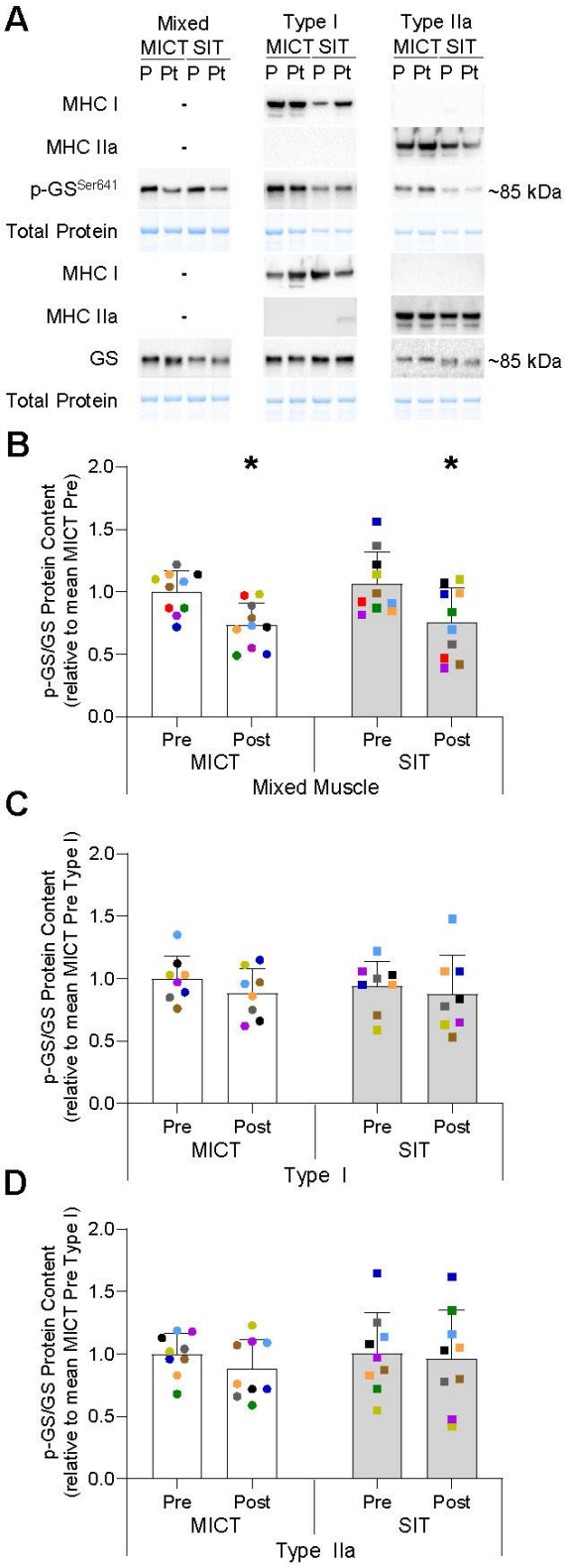
A
Mixed
MICT SIT
P Pt P Pt
Type I
MICT SIT
P Pt P Pt
Type IIa
MICT SIT
P Pt P Pt
MHC I
MHC IIa
p-GSSer641
~85 kDa
Total Protein
MHC I
MHC IIa
GS
~85 kDa
Total Protein
B
p-GS/GS Protein Content
(relative to mean MICT Pre)
2.0
1.5
1.0
0.5
0.0
*
*
Pre
Post
Pre
Post
MICT
SIT
Mixed Muscle
C
p-GS/GS Protein Content
(relative to mean MICT Pre Type I)
2.0
1.5
1.0
0.5
0.0
Pre
Post
Pre
Post
MICT
SIT
Type I
D
p-GS/GS Protein Content
(relative to mean MICT Pre Type I)
2.0
1.5
1.0
0.5
0.0
Pre
Post
Pre
Post
MICT
SIT
Type IIa

Figure 2. Phosphorylated glycogen synthase (GS) to total GS protein content before and following a single session of MICT and SIT. Representative blots (A), mixed muscle samples (B) pooled type I muscle fibres (C), and pooled type IIa muscle fibres (D) are displayed separately. Mixed muscle samples were normalized to the average pre-training MICT value and pooled muscle fibre samples were normalized to the average pre-training type I value. Myosin heavy chain (MHC) I and IIa were not measured in mixed muscle samples. The symbol indicates a significant difference vs. pre (*; $p < 0.05$). Data are means ± SD. Each colour represents one individual and is consistent across panels and figures within the same experiment. Pre (P); post (Pt).

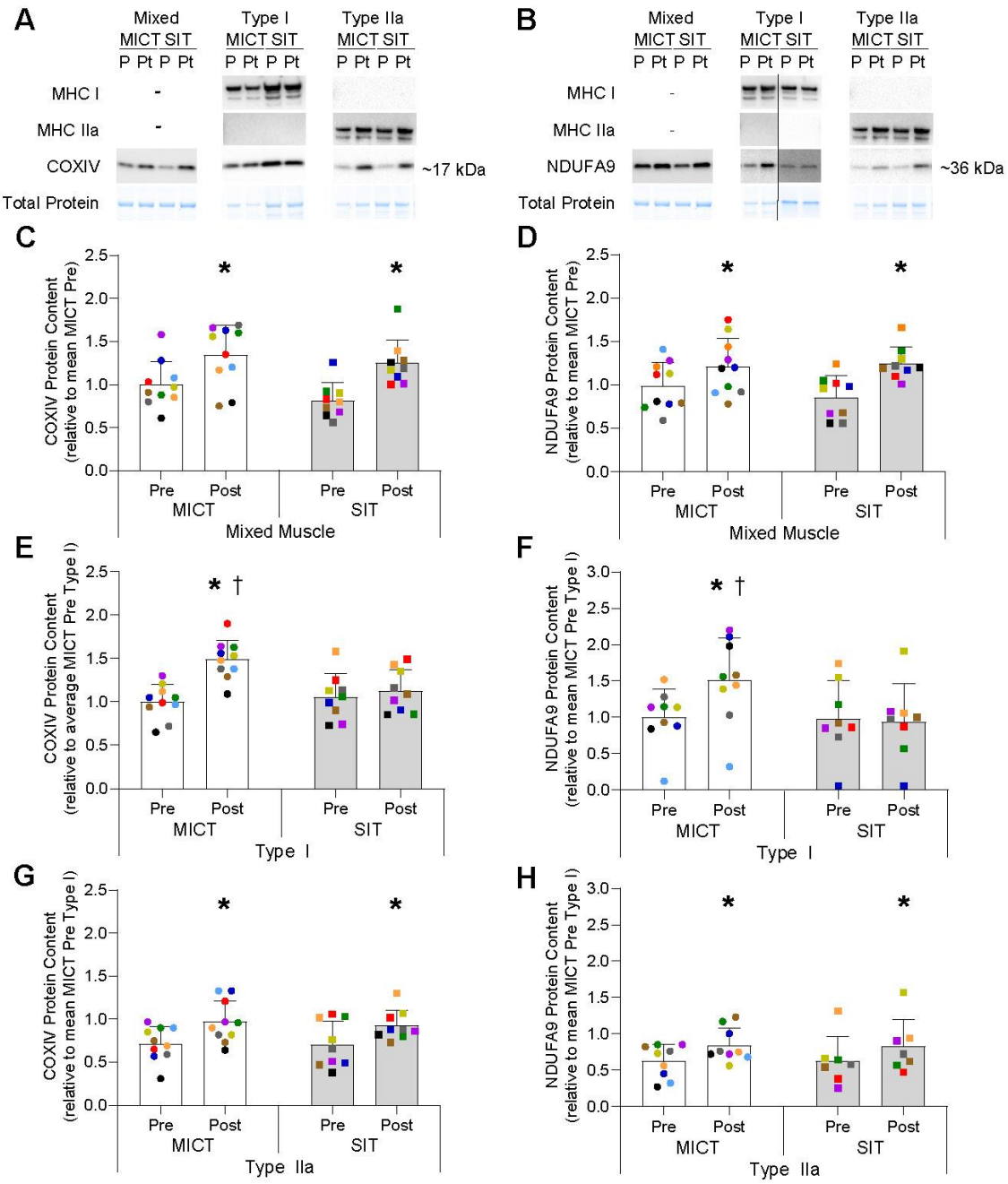
A
Mixed
Type I
Type IIa
MICT SIT
P Pt P Pt
MHC I
MHC IIa
COXIV
~17 kDa
Total Protein
B
NDUFA9
~36 kDa
C
COXIV Protein Content (relative to mean MICT Pre)
Pre
Post
MICT
SIT
Mixed Muscle
D
NDUFA9 Protein Content (relative to mean MICT Pre)
E
COXIV Protein Content (relative to average MICT Pre Type I)
Type I
F
NDUFA9 Protein Content (relative to mean MICT Pre Type I)
G
COXIV Protein Content (relative to mean MICT Pre Type I)
Type IIa
H
NDUFA9 Protein Content (relative to mean MICT Pre Type I)

Figure 3. Cytochrome *c* oxidase subunit IV (COXIV) and NADH:ubiquinone oxidoreductase subunit A9 (NDUFA) protein content before and following 12 weeks of MICT and SIT. Representative blots (A, B), mixed muscle samples (C, D), pooled type I muscle fibres (E, F), and pooled type IIa muscle fibres (G, H) are displayed separately. Mixed muscle samples were normalized to the average pre-training MICT value and pooled muscle fibre samples were normalized to the average pre-training type I value. Myosin heavy chain (MHC) I and IIa were not measured in mixed muscle samples. The symbols indicate a significant difference vs. pre (*; $p < 0.05$), and a significant difference vs. SIT (†; $p < 0.05$). Data are means ± SD. Each colour within a group represents one individual and is consistent across panels and figures within the same experiment. Pre (P); post (Pt).

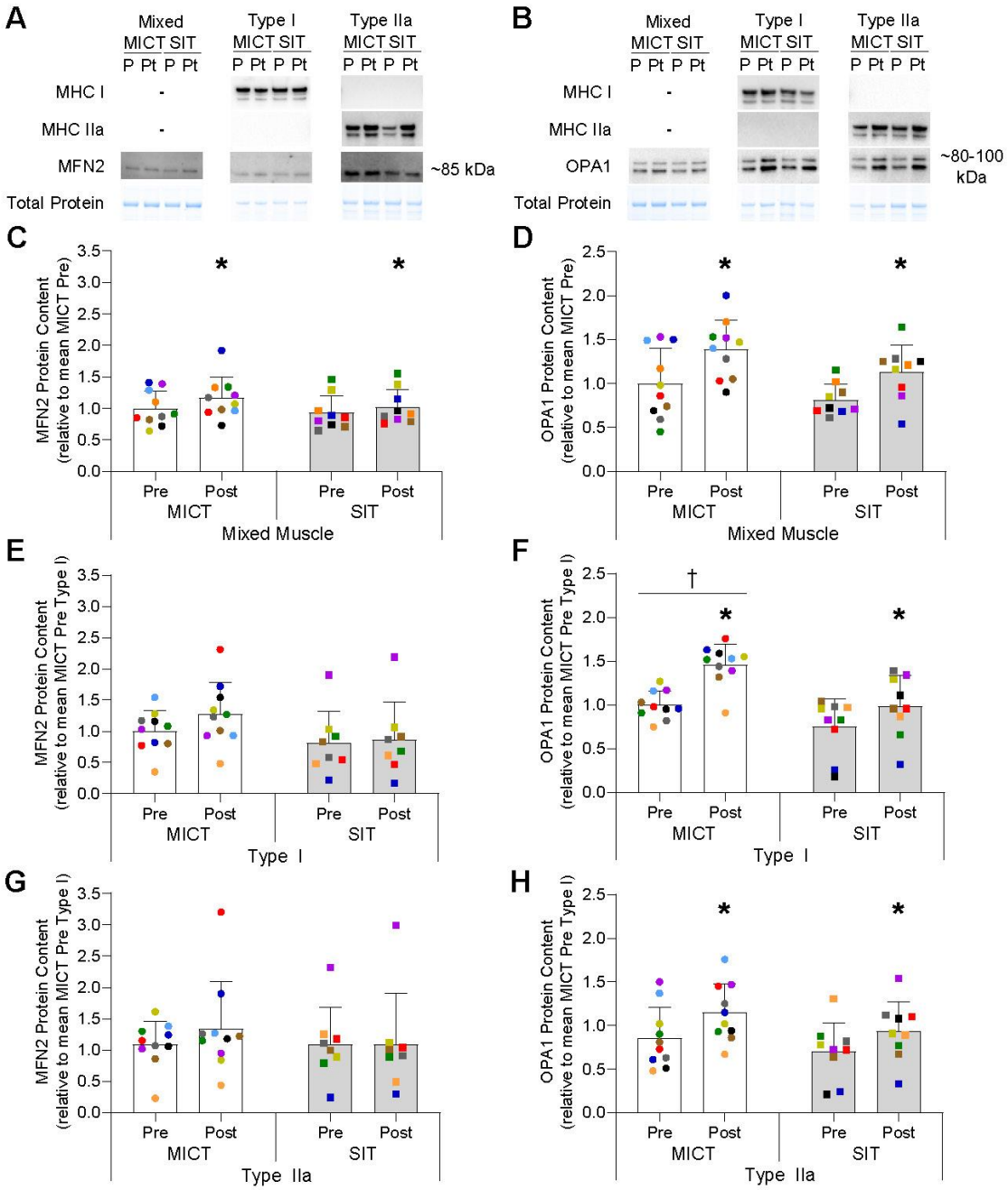
A
Mixed
MICT SIT
P Pt P Pt
Type I
MICT SIT
P Pt P Pt
Type IIa
MICT SIT
P Pt P Pt
MHC I
MHC IIa
MFN2
~85 kDa
Total Protein
B
Mixed
MICT SIT
P Pt P Pt
Type I
MICT SIT
P Pt P Pt
Type IIa
MICT SIT
P Pt P Pt
MHC I
MHC IIa
OPA1
~80-100 kDa
Total Protein
C
MFN2 Protein Content (relative to mean MICT Pre)
Pre Post Pre Post
MICT SIT
Mixed Muscle
D
OPA1 Protein Content (relative to mean MICT Pre)
Pre Post Pre Post
MICT SIT
Mixed Muscle
E
MFN2 Protein Content (relative to mean MICT Pre Type I)
Pre Post Pre Post
MICT SIT
Type I
F
OPA1 Protein Content (relative to mean MICT Pre Type I)
†
Pre Post Pre Post
MICT SIT
Type I
G
MFN2 Protein Content (relative to mean MICT Pre Type I)
Pre Post Pre Post
MICT SIT
Type IIa
H
OPA1 Protein Content (relative to mean MICT Pre Type I)
Pre Post Pre Post
MICT SIT
Type IIa

Figure 4. Mitofusin 2 (MFN2) and optic atrophy 1 (OPA1) protein content before and following 12 weeks of MICT and SIT. Representative blots (A, B), mixed muscle samples (C, D), pooled type I muscle fibres (E, F), and pooled type IIa muscle fibres (G, H) are displayed separately. Mixed muscle samples were normalized to the average pre-training MICT value and pooled muscle fibre samples were normalized to the average pre-training type I value. Myosin heavy chain (MHC) I and IIa were not measured in mixed muscle samples. The symbols indicate a significant difference vs. pre (*; $p < 0.05$) and a significant difference vs. SIT (†; $p < 0.05$). Data are means ± SD. Each colour within a group represents one individual and is consistent across panels and figures within the same experiment. Pre (P); post (Pt).

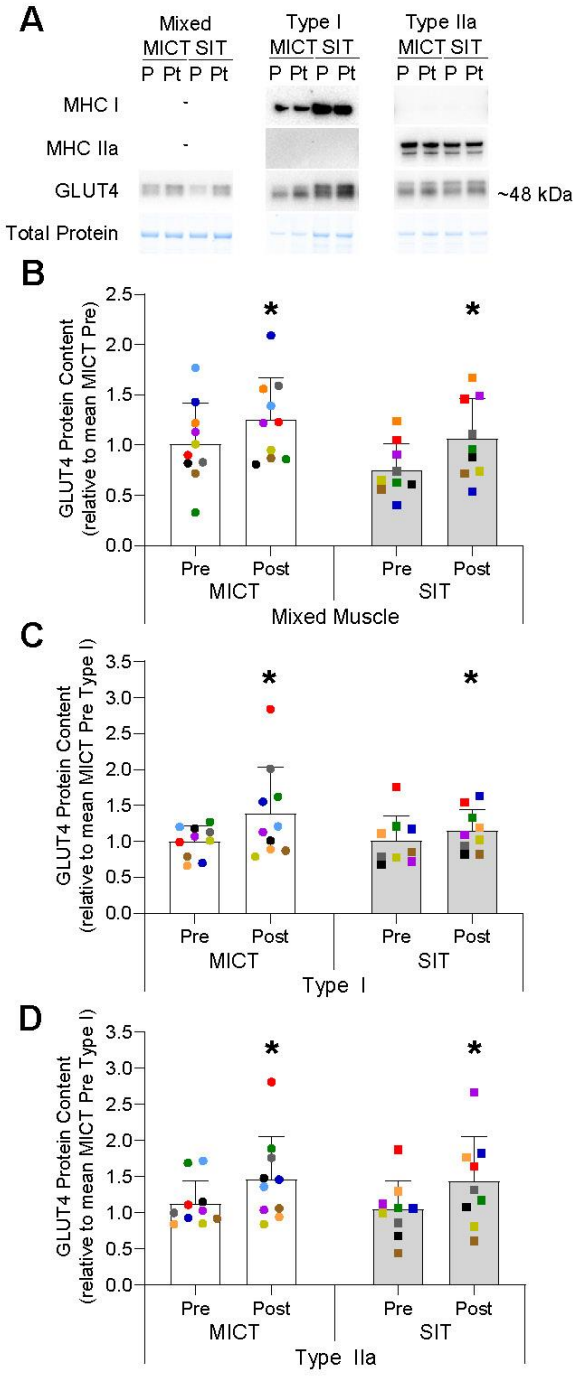
A
Mixed
MICT SIT
P Pt P Pt
Type I
MICT SIT
P Pt P Pt
Type IIa
MICT SIT
P Pt P Pt
MHC I
MHC IIa
GLUT4
~48 kDa
Total Protein
B
GLUT4 Protein Content
(relative to mean MICT Pre)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pre
Post
Pre
Post
MICT
SIT
Mixed Muscle
C
GLUT4 Protein Content
(relative to mean MICT Pre Type I)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pre
Post
Pre
Post
MICT
SIT
Type I
D
GLUT4 Protein Content
(relative to mean MICT Pre Type I)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pre
Post
Pre
Post
MICT
SIT
Type IIa

Figure 5. Glucose transporter 4 (GLUT4) protein content before and following 12 weeks of MICT and SIT. Representative blots (A), mixed muscle samples (B), pooled type I muscle fibres (C), and pooled type IIa muscle fibres (D) are displayed separately. Mixed muscle samples were normalized to the average pre-training MICT value and pooled muscle fibre samples were normalized to the average pre-training type I value. Myosin heavy chain (MHC) I and IIa were not measured in mixed muscle samples. The symbol indicates a significant difference vs. pre (*; $p < 0.05$). Data are means ± SD. Each colour within a group represents one individual and is consistent across panels and figures within the same experiment. Pre (P); post (Pt).

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CHAPTER 4: Training adaptations to single-leg moderate-intensity interval and continuous cycling: the role of contraction pattern. *In preparation.*

Title: Training adaptations to single-leg moderate-intensity interval and continuous cycling: the role of contraction pattern

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KEY POINTS

- Greater increases in the phosphorylation of signalling proteins involved in mitochondrial biogenesis have been shown following interval (INT) compared to work and intensity-matched continuous (CONT) exercise; however, whether these acute differences translate to divergent training responses is unknown.
- We tested the hypothesis that short-term INT training would elicit a greater increase in mitochondrial content as compared to work- and intensity-matched CONT training.
- Each leg from 10 healthy young men and women performed 12 sessions of either INT or CONT training over 4 weeks, using counterweighted single-leg cycling to facilitate a within-subject comparison.
- Contrary to our hypothesis, subsarcolemmal mitochondrial area, the maximal activity of citrate synthase and the protein content of cytochrome *c* oxidase subunit IV increased after CONT but not INT training.
- Skeletal muscle responses were not enhanced following INT training compared to CONT training, suggesting that an intermittent pattern of exercise is not a primary stimulus for training-induced changes in mitochondrial content.

ABSTRACT

Interval (INT) exercise has been reported to elicit greater phosphorylation of signalling proteins involved in skeletal muscle mitochondrial biogenesis compared to work and intensity-matched continuous (CONT) exercise; however, whether these acute differences translate into different training responses is unclear. We employed counterweighted, single-leg (SL) cycling to test the hypothesis that short-term INT training would elicit a greater increase in mitochondrial content as compared to work- and intensity-matched CONT training in young healthy adults. Each leg was randomly assigned to complete 12 sessions over 4 weeks of either INT [10 x 3 min at 50% SL peak power output (W_{peak}) interspersed with 1 min at 10% W_{peak}] or CONT (30 min at 50% W_{peak} followed by 10 min at 10% W_{peak}), such that the total time at each exercise intensity and overall training volume were matched, but the pattern of contraction differed. Contrary to our hypothesis, subsarcolemmal mitochondrial area, the maximal activity of citrate synthase and the protein content of cytochrome *c* oxidase subunit IV (COXIV) increased following CONT ($p<0.05$) but not INT training ($p>0.05$). Total mitochondrial area was positively correlated with COXIV protein content, when compared based on absolute values ($p<0.01$) and training-induced changes ($p=0.01$). INT and CONT training similarly improved SL W_{peak} ($p<0.01$) and time to exhaustion at 70% of SL W_{peak} ($p<0.01$). Skeletal muscle responses were not enhanced following INT training compared to intensity- and work-matched CONT training. These findings suggest that an intermittent pattern of exercise is not a primary stimulus for training-induced changes in mitochondrial content.

INTRODUCTION

Exercise training of sufficient intensity, duration and volume—whether performed in a continuous (CONT) or interval (INT) manner—increases skeletal muscle mitochondrial content (Holloszy, 1967; Saltin *et al.*, 1976; MacDougall *et al.*, 1998). INT training, particularly involving brief 'all out' or very intense exercise bouts, can elicit increases in mitochondrial content similar to CONT training despite lower exercise volume (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014; Gillen *et al.*, 2016). Limited evidence also suggests INT training can induce superior increases in mitochondrial content compared to CONT training, when total work is matched (MacInnis *et al.*, 2017*c*). The study by MacInnis *et al.* (2017c) employed single-leg (SL) cycling as a unilateral exercise model to compare training interventions within the same group of individuals. This approach affords greater statistical power to detect differences between treatments and eliminates the potential impact of confounding factors that affect between-subject designs (e.g., diet, baseline fitness) (MacInnis *et al.*, 2017*a*).

The potential for INT training to elicit superior increases in mitochondrial content for a given volume of exercise compared to CONT training may be related to a higher exercise intensity *per se* and/or the inherent 'hard-easy' intermittent contraction pattern. Higher intensities of exercise induce greater disturbances in skeletal muscle homeostasis (Sahlin *et al.*, 1987, 1989; Constantin-Teodosiu *et al.*, 1991), and the magnitude of changes in some indices of metabolic stress during INT exercise correspond to acute skeletal muscle responses related to the upregulation of mitochondrial biogenesis (Fiorenza *et al.*, 2018). The fluctuations in metabolism that occur as a result of an

intermittent pattern of exercise may also promote mitochondrial responses following exercise. Indeed, greater phosphorylation of signalling proteins proposed to be involved in mitochondrial biogenesis was found following INT compared to work and intensity-matched CONT exercise (Combes *et al.*, 2015). With respect to low volumes of exercise, 6 weeks of brief 'all-out' CONT training (i.e., cycling for ~4 min at 1.0 to 1.25 kJ/kg body weight as quickly as possible) did not increase the maximal activity of citrate synthase (CS) (Cochran *et al.*, 2014), a marker of mitochondrial content (Larsen *et al.*, 2012), despite other studies finding 'all-out' INT training-induced increases in mitochondrial content following a similar training period and total amount of work (Burgomaster *et al.*, 2008; Gillen *et al.*, 2014; Scribbans *et al.*, 2014). These limited data suggest that an intermittent pattern of contraction may contribute to exercise-induced increases in mitochondrial content, independent of exercise intensity. However, it is unknown whether INT training would enhance mitochondrial adaptations compared to work- and absolute intensity-matched CONT training within the same group of participants.

The primary purpose of the present study was to investigate the effect of short-term SL CONT and INT training, matched for total work, absolute intensity, and session duration, on mitochondrial content. We assessed mitochondrial content using transmission electron microscopy (TEM) in addition to commonly measured biomarkers including CS maximal activity and cytochrome *c* oxidase IV (COXIV) protein content (Larsen *et al.*, 2012). Given that mitochondrial signalling responses are reported to be enhanced following an acute bout of INT compared to work and absolute intensity-

matched CONT exercise (Combes *et al.*, 2015), we hypothesized that INT training would elicit greater increases in mitochondrial content compared to work- and absolute intensity-matched CONT training.

METHODS

Participants and ethical approval

Ten healthy young healthy men (n=5) and women (n=5) were recruited for the study [age 21 ± 2 years; peak oxygen uptake ($\dot{V}O_{2peak}$) 43 ± 5 ml/kg/min; body mass index 23 ± 2 kg/m^2]. All subjects were habitually active but not specifically training for any sport. All experimental procedures were approved by the Hamilton Integrated Research Ethics Board and the study conformed with the *Declaration of Helsinki*. All participants provided written informed consent before study participation.

Pre-training procedures

An overview of the experimental protocol is depicted in Figure 1. Participants performed a double-legged incremental ramp test to exhaustion on an electronically braked cycle ergometer (Excalibur Sport, version 2.0; Lode, Groningen, The Netherlands) to determine whole-body $\dot{V}O_{2peak}$ and peak power output (W_{peak}). Following a 2-min warm-up at 50 W, workload was increased 1 W every 2 s until the participant reached volitional exhaustion or cadence decreased below 60 revolutions per minute (rpm). Expired gases were analyzed using a metabolic cart (Moxus modular oxygen uptake

system: AEI Technologies, Pittsburgh, PA, USA) and the $\dot{V}O_{2peak}$ was defined as the highest average oxygen consumption over a 30-s period.

The cycle ergometer was modified for SL cycling by attaching an 11.4 kg counterweight to the contralateral pedal (Abbiss *et al.*, 2011; MacInnis *et al.*, 2017*c*). The counterweight reduces ipsilateral hip flexion and permits a muscle recruitment pattern that is more similar to double-leg cycling, particularly in comparison to unassisted SL cycling (Burns *et al.*, 2014; Bini *et al.*, 2015; Elmer *et al.*, 2016). On a separate visit, participants were familiarized with the SL cycling technique. Participants pedalled using one leg, with the non-exercising leg resting on a stationary platform. Following familiarization, participants performed an incremental test to exhaustion with each leg. The rate at which the workload increased was reduced by half for the SL tests compared to the double-leg tests (i.e., 1 W every 4 s). Participants rested for 10 minutes before the contralateral leg was tested in an identical manner.

At least 2 d following the SL incremental tests to exhaustion, participants arrived at the laboratory following an overnight fast and completed SL cycling tests to exhaustion. Following a 5 min warm-up at 50W, participants cycled at 70% of the average W_{peak} obtained with each leg during the ramp incremental tests. Participants were instructed to maintain a cadence of 80 rpm and the test ended when the cadence dropped below 60 rpm. Other than cadence, participants received no verbal or physiological feedback during the tests. Participants rested for 45 minutes before the contralateral leg was tested in an identical manner.

At least 3 d after the final baseline testing session, a resting muscle biopsy was obtained from the *vastus lateralis* of a randomly selected leg. The other leg was not biopsied, as the activity of mitochondrial enzymes are reported to be similar between legs prior to a training intervention (Saltin *et al.*, 1976; Henriksson, 1977; Lindholm *et al.*, 2014, 2016; MacInnis *et al.*, 2017*c*). Participants were instructed to refrain from exercise, alcohol and food for a minimum of 48, 24 and 10 h, respectively. Biopsies were collected under local anaesthesia (1% xylocaine) using a Bergström needle modified for suction (Tarnopolsky *et al.*, 2011). Muscle biopsies were immediately cleaned of excess blood, sectioned into several pieces, frozen in liquid nitrogen, and stored at -80°C until analysis.

Training intervention

The training intervention consisted of three sessions per week over 4 weeks, for a total of 12 sessions. One participant completed 11 sessions over 4 weeks. The legs of each participant were randomly assigned to perform either CONT or INT cycling. Exercise was prescribed based on the average SL W_{peak} obtained from the participants' left and right legs during the ramp incremental tests. The INT protocol involved 10 x 3 min at 50% W_{peak}, interspersed with 1 min at 10% W_{peak}, whereas to match for total time at each intensity, the CONT protocol consisted of 30 min at 50% W_{peak} followed by 10 min at 10% W_{peak}. The training protocols were based on previous investigations in our laboratory (MacInnis *et al.*, 2017*b*, 2017*c*) which demonstrated that 30 min of SL cycling at 50% W_{peak} is feasible in recreationally active individuals and elicits an increase in metabolic stress, as evidenced by a blood lactate concentration of ~5 mM at the end of the

protocol. Both protocols began with a 2 min warm-up at 25 W. Training loads were increased by 2.5% W_{peak} every 4^{th} session if participants could tolerate the progression in workload. The two legs were trained consecutively on the same day, in an alternating order, with 10 minutes of rest between sessions. Participants were instructed to maintain a cadence of 80 rpm during the training sessions. Heart rate was measured continuously during training (A300, Polar Electro Oy, Kempele, Finland). Blood lactate was measured via finger prick half-way and during the final minute of the first protocol completed in the first two training sessions (Lactate Plus, Nova Biomedical, Mississauga, ON, Canada).

Post-training procedures

A resting muscle biopsy was obtained from each leg ~72 hours following the final training session. Approximately 3 and 5 d following the muscle biopsy, respectively, the SL ramp incremental tests and time to exhaustion tests were repeated.

Muscle analyses

Transmission electron microscopy (TEM)

TEM samples were prepared by a technician in the Electron Microscopy Facility at McMaster University, as previously described (Nilsson *et al.*, 2015). Muscle tissue samples were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were rinsed twice in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour and then dehydrated in ascending ethanol concentrations, followed by two propylene oxide baths. Samples were infiltrated

with Spurr's resin through a graded series with rotation of the samples in between solution changes. Samples were then rotated and embedded in 100% fresh Spurr's resin and polymerized overnight in a 60°C oven.

Thin sections (90 nm) were cut on a Leica UCT ultramicrotome (Leica Microsystems GmbH, Vienna, Austria), picked up onto Cu grids and stained with both uranyl acetate and lead citrate. The grids were viewed on a transmission electron microscope (JEOL 1200 EX TEMSCAN, Peabody, MA, USA) at the x10,000 magnification. Images were acquired with an AMT 4-megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA). Four micrographs, two from the intermyofibrillar area and two from the subsarcolemmal area, from eight different random muscle fibres were taken (i.e., a total of 32 images per muscle biopsy).

Mitochondrial area was estimated by point counting using Image J software (Version 1.51, National Institute of Health, Bethesda, Maryland, USA). A grid size of 500 x 500 nm (0.25 μm^2) was superimposed on each micrograph (Broskey *et al.*, 2013). The ratio of points (i.e., intersecting grid lines) that touched mitochondria to the number of points that touched muscle fibre was used to calculate total mitochondrial area. We also assessed intermyofibrillar and subsarcolemmal mitochondrial area by calculating the ratio of points that touched population-specific mitochondria to the number of points that touched muscle fibre. Mitochondria that were not separated by myofibrils from the sarcolemma were identified as subsarcolemmal mitochondria (Devries *et al.*, 2013; Meinild Lundby *et al.*, 2018).

Citrate synthase (CS) maximal activity

One piece of muscle (~20 mg) was homogenized in 20 volumes of buffer (70 mM sucrose, 220 mM mannitol, 10 mM Hepes and 1 mM EGTA) supplemented with protease inhibitors (Complete Mini, Roche Applied Science, Laval, PQ, Canada) using Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA). Enzyme activity was determined, as previously described (Wyckelsma *et al.*, 2017) using a spectrophotometer (Cary Bio-300; Varion Inc., Palo Alto, CA, USA). The slope between 30 s and 90 s was used to calculate CS maximal activity. Enzyme activity was expressed relative to total protein measured with a BCA assay kit (Pierce, Rockford, IL, USA).

Western blotting

A second piece of muscle (~25 mg) was homogenized in 20 volumes of radioimmunoprecipitation assay (RIPA) buffer (Sigma Aldrich, Oakville, Ontario, Canada) supplemented with protease (Roche Applied Science) and phosphatase (PhosSTOP, Roche Applied Science, Laval, Quebec, Canada) inhibitors using Lysing Matrix D tubes (MP Biomedicals). Samples were diluted to 2.5 µg wet weight/µL in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). A small amount of whole muscle homogenate from each sample was mixed together to generate a pooled sample for calibration curves.

Samples were separated on 26 well, 4-15% pre-cast Criterion TGX Stain-Free gels (Bio-Rad) and run for 40 min at 200 V in running buffer (25 mM Tris, 182 mM glycine, 0.1% SDS, pH 8.3, Bio-Rad). Protein was semi-dry transferred to a nitrocellulose

membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% skimmed milk in 1x Tris-buffered saline-Tween (TBST) for 1 h and then incubated in primary antibody overnight at 4°C and 2 h at room temperature. After washing in blocking buffer, membranes were incubated in secondary antibody for 1 h at room temperature and then washed in 1x TBST. Membranes were incubated in chemiluminescent substrate (Clarity or Clarity Max, Bio-Rad), imaged using a Chemidoc MP (Bio-Rad) and analyzed using Image Lab software (Version 6.0, Bio-Rad). Primary antibodies from the Total OXPHOS Blue Native WB Antibody Cocktail (Abcam, mouse, monoclonal, 1:1000, ab110412) were used: NADH:ubiquinone oxidoreductase subunit A9 (NDUFA9; complex I, ab14713), succinate dehydrogenase subunit A (SDHA; complex II, ab14715), ubiquinol-cytochrome *c* reductase core protein 2 (UQCRC2, complex III, ab14745), cytochrome *c* oxidase subunit IV (COXIV; complex IV, ab14744) and ATP synthase α-subunit (ATP5A; complex IV, ab14748).

A protein ladder (Precision Plus Protein All Blue Prestained Protein Standards, Bio-Rad) and a calibration curve (e.g., 2, 4, 8, 16 µL) of pooled samples were run on every gel. Ultraviolet (UV) activation of each gel was used to visualize total protein loaded and analyzed using Image Lab software (Bio-Rad). On each gel, the abundance of a given protein was expressed relative to the calibration curve and then normalized to total protein, which was also expressed relative to the calibration curve (Murphy & Lamb, 2013).

Statistical analyses

Data were assessed for normality using the Shapiro-Wilk test and the majority of data were normally distributed. Muscle data were analyzed using a one-way repeated measures analysis of variance (ANOVA) to compare biopsy samples (pre-training, CONT, INT). A two-factor repeated measures ANOVA with one factor of time (pre-training, post-training) and one factor of group (CONT, INT) was used to analyze data from the SL ramp incremental and time to exhaustion tests. Post hoc testing, involving Holm-Sidak's multiple comparisons tests, was performed. Pearson's correlation coefficients were calculated to investigate the correlation between absolute values and training-induced changes in total mitochondrial area assessed using TEM and biomarkers of mitochondrial content. Data from all biopsy time points were combined for the correlational analyses. Mean heart rate, peak heart rate and mean blood lactate during training sessions were analyzed using a paired t-test. Analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $p<0.05$ and all data are presented as mean ± SD.

RESULTS

Descriptive Exercise Data

Mean power output was 74 ± 6 W during the work period and 15 ± 1 W during the recovery periods in both training protocols. The average total work performed in each session, including warm-up, was similar in CONT and INT training (145 ± 11 vs. 145 ± 11 kJ; $p = 0.59$). Mean heart rate was higher during CONT compared to INT training (131 ± 8 vs. 128 ± 9 bpm; $p = 0.045$) and corresponded to 69 ± 4 and 68 ± 4 % of double-leg

maximal heart rate, respectively. Mean blood lactate was similar during CONT and INT training (4.2 ± 1.6 vs. 4.6 + 1.4 mM; $p = 0.24$; n=9).

Skeletal Muscle Mitochondrial Content

There was no change in total mitochondrial area following CONT or INT training (Pre: 6.1 ± 1.0%, CONT: 7.1 ± 1.1%, INT: 6.4 ± 1.4%; ANOVA, $p=0.13$). Intermyofibrillar mitochondrial area did not change following either training protocol (ANOVA, $p=0.24$; Fig. 2A). There was an increase in subsarcolemmal mitochondrial area following CONT (ANOVA, $p=0.04$; post hoc $p<0.01$; Fig. 2B) but not INT training (post hoc $p=0.34$).

The maximal activity of CS increased by 23% following CONT training (ANOVA, $p=0.03$; post hoc, $p=0.03$) but was unchanged following INT training (post-hoc, $p=0.20$; Fig. 3A). The protein content of COXIV increased following CONT training only (+35%; ANOVA, $p<0.001$; post hoc, $p<0.01$) and was greater in the CONT group compared to the INT group following training (post hoc, $p=0.045$; Fig. 3E). The protein content of Complex III UQCRC2 also increased following CONT training only (ANOVA, $p=0.01$; post hoc $p<0.01$; Fig. 3D). A significant effect of treatment was detected for the protein content of Complex V ATP5A (ANOVA, $p=0.03$), however, the post hoc tests revealed no significant differences between treatments ($p>0.05$; Fig. 3F). There were no changes in the protein content of Complex I NDUFA9 (ANOVA $p=0.12$; Fig. 3B) or complex II SDHA (ANOVA $p=0.19$; Fig. 3C).

There was a significant positive correlation between absolute mitochondrial area and the protein content of COXIV (Fig. 4A; r=0.61, p<0.001; n=30). The correlation between absolute values of mitochondrial area and the maximal activity of CS was not significant (Fig. 4B; r=0.32, p=0.08; n=30). Training-induced changes in mitochondrial area and COXIV protein content were significantly correlated (Fig. 4C; r=0.57, p=0.01; n=20); however, training-induced changes in mitochondrial area and CS maximal activity were uncorrelated (Fig. 4D; r=0.07, p=0.77; n=20).

Single-Leg Exercise Tests

SL W_{peak} increased in the CONT group (146 ± 10 to 157 ± 13 W) and the INT group (144 ± 13 to 158 ± 14 W) to a similar extent (main effect for time p<0.01). There were no changes in SL $\dot{V}O_{2peak}$ following training (CONT: 2.2 ± 0.2 to 2.3 ± 0.2 L/min; INT: 2.2 ± 0.2 to 2.2 ± 0.4 L/min; main effect for time p=0.95). SL time to exhaustion improved similarly after CONT (8.4 ± 1.3 to 13.6 ± 6.4 min) and INT training (8.9 ± 2.9 to 16.4 ± 7.4 min; main effect p<0.01; n=9).

DISCUSSION

The present study examined skeletal muscle adaptations to 4 weeks of SL moderate-intensity CONT and INT training. Contrary to our hypothesis, we found increases in mitochondrial content following CONT training only. Specifically, subsarcolemmal mitochondrial area, the maximal activity of CS and the protein content of COXIV and UQCRC2 increased in response to CONT training, whereas there were no

increases in these measures following INT training. These findings demonstrate that the intermittent pattern of INT training is not a major stimulus for increasing mitochondrial content, suggesting that the potency of high-intensity INT training to improve mitochondrial content is primarily related to a high absolute intensity. Furthermore, when compared based on absolute values and training-induced changes, total mitochondrial area was positively correlated with COXIV protein content, supporting the use of COXIV protein content as a biomarker of mitochondrial content over a short-term training intervention. Lastly, both CONT and INT training improved SL W_{peak} and exercise performance during a time to exhaustion test.

The lack of change in mitochondrial content following INT training despite increases in response to work and intensity-matched CONT training does not align with evidence that a single session of INT exercise augments the phosphorylation of mitochondrial signalling proteins compared to work and intensity-matched CONT exercise (Combes *et al.*, 2015). It is possible that the previously observed differences in the phosphorylation of signalling proteins does not reflect the acute responses of downstream processes, including mitochondrial protein transcription and translation. The incongruent findings between studies may also relate to disparities in the INT and CONT exercise protocols studied. The double-leg INT exercise protocol utilized in Combes *et al.* (2015) involved three-fold more intervals, passive recovery, and a greater relative intensity of ~70% W_{peak} and therefore, elicited a greater number and degree of metabolic fluctuations compared to the present study. We employed an INT exercise protocol consisting of 10 bouts with light active recovery because these parameters are comparable

to INT training protocols that have elicited increases in mitochondrial content to a similar extent as CONT training (Burgomaster *et al.*, 2008; Bækkerud *et al.*, 2016). It is difficult to compare relative exercise intensity across SL and double-leg cycling modes, in part, because greater workloads per leg are obtained during SL incremental tests to exhaustion compared to double-legged tests and cardiovascular responses are reduced during SL *versus* double-leg cycling (Gleser, 1973; Neary & Wenger, 1986; Burns *et al.*, 2014; MacInnis *et al.*, 2017*b*).

Considering we found no evidence that INT training augments training adaptations compared to work and intensity-matched CONT training, the superior changes in mitochondrial content relative to exercise volume reported following INT compared to CONT training (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2013, 2017; Scribbans *et al.*, 2014; Bækkerud *et al.*, 2016; Gillen *et al.*, 2016; MacInnis *et al.*, 2017*c*) are likely primarily related to differences in absolute intensity rather than the pattern of contraction. This assertion is reasonable, as many key components involved in the molecular cascade linked to training-induced increases in skeletal muscle mitochondrial content are sensitive to exercise intensity. Higher intensity exercise elicits larger exercise-induced increases in metabolites, including muscle lactate and creatine (Kristensen *et al.*, 2015), the phosphorylation of signalling proteins, specifically AMPK and CaMKII (Wojtaszewski *et al.*, 2000; Egan *et al.*, 2010), the mRNA expression of *PGC-1α* (Egan *et al.*, 2010) and mitochondrial protein synthesis (Di Donato *et al.*, 2014) compared to work-matched lower intensity exercise. An interval pattern of exercise may simply contribute to training adaptations by providing recovery periods that are necessary to

sustain repeated high-intensity efforts. It is also possible that an intermittent pattern of exercise has a role in mediating mitochondrial adaptations when exercise intensity and the corresponding metabolic fluctuations are higher *versus* the present study. This notion may apply to lower volumes of intense exercise in particular, as we have previously reported that 6 weeks of brief 'all-out' CONT training did not increase the maximal activity of CS (Cochran *et al.*, 2014) despite other studies finding training-induced increases in mitochondrial content following 'all-out' INT training involving a similar amount of work (Burgomaster *et al.*, 2008; Gillen *et al.*, 2014; Scribbans *et al.*, 2014). Nonetheless, our data suggest that without the additional metabolic stress induced by a high exercise intensity, INT training does not lead to augmented skeletal muscle responses compared to work-matched CONT training.

TEM is regarded as the gold-standard technique for measuring mitochondrial content (Medeiros, 2008; Larsen *et al.*, 2012); however, surrogate measures of mitochondrial content, including the activity and protein content of mitochondrial enzymes, are more widely utilized by laboratories owing to their greater accessibility, lower cost and time efficiency. We assessed mitochondrial content using TEM and biomarkers, including the maximal activity of CS and the protein content of electron transport chain complexes. These biomarkers have demonstrated positive correlations with TEM-based mitochondrial content in a cross-sectional comparison (Larsen *et al.*, 2012). Investigations that have assessed exercise training-induced changes in mitochondrial content in healthy adults using TEM and the maximal activity of CS and/or COXIV protein content have generally shown consistent mean group responses among

the different measures (Tarnopolsky *et al.*, 2007; Samjoo *et al.*, 2013; Devries *et al.*, 2013; Meinild Lundby *et al.*, 2018). The shorter training intervention and smaller relative changes in mitochondrial content may partially explain the inconsistent findings among measures of mitochondrial content in the present study.

We also conducted correlational analyses between the absolute and training-induced changes in our key measures of mitochondrial content. While CS maximal activity and complex IV protein content demonstrate good correlations with TEM-based mitochondrial volume at rest (Larsen *et al.*, 2012), these associations may not remain consistent over periods of skeletal muscle remodelling such as an exercise intervention. One study examining both the maximal activity of CS and TEM-determined mitochondrial content found positive correlations between the two measures before and after 6 weeks of CONT training, but no associations between training-induced changes (Meinild Lundby *et al.*, 2018). We also found no relationship between changes in total mitochondrial area and the maximal activity of CS. In contrast, total mitochondrial area was positively correlated with COXIV protein content, when compared based on absolute values and training-induced changes. These associations provide further support for utilizing COXIV protein content as a biomarker of mitochondrial content over a short-term training intervention.

In order to gain insight into subcellular location-specific changes in mitochondrial content, we assed subsarcolemmal and intermyofibrillar mitochondrial area. The observed preferential increase in subsarcolemmal mitochondrial area following CONT training is consistent with other investigations demonstrating greater training-induced increases in

subsarcolemmal compared to intermyofibrillar mitochondrial area in human skeletal muscle (Hoppeler *et al.*, 1985; Howald *et al.*, 1985; Nielsen *et al.*, 2010; Montero *et al.*, 2015; Koh *et al.*, 2018; Meinild Lundby *et al.*, 2018). Mitochondria form a reticular network (Ogata & Yamasaki, 1997) and subsarcolemmal and intermyofibrillar mitochondria have shown to be physically interconnected rather than existing in separate populations (Dahl *et al.*, 2015). Mitochondria in closer proximity to the sarcolemma and/or other components in skeletal muscle fibres close to the sarcolemma (e.g., capillaries, nuclei) may be more responsive to exercise training. A limitation of our study is that we did not determine the fibre types of the 8 random fibres that were analyzed in each muscle biopsy. Differences in mitochondrial content between fibre types may have increased variability within our measures and contributed to our inability to detect changes in total or intermyofibrillar mitochondrial area following CONT training.

Consistent with a previous SL cycling study in our laboratory (MacInnis *et al.*, 2017*c*), short-term CONT and INT training increased SL W_{peak} but SL $\dot{V}O_{2peak}$ was unchanged. Longer or higher intensity SL training interventions may be required to elicit improvements in SL $\dot{V}O_{2peak}$. Exercise performance during a time to exhaustion test also improved to a similar extent following CONT and INT training. The similar improvements in exercise performance following CONT and INT training do not align with our observation that skeletal muscle measures improved following CONT training only. Multiple physiological factors, in addition to mitochondrial content, impact exercise performance and may have contributed to the comparable improvements following

CONT and INT training (Basset & Howley, 2000). Overall, the pattern of contraction did not influence performance adaptations to SL cycling.

In summary, the present study examined changes in mitochondrial content following SL work- and intensity-matched CONT and INT training. Subsarcolemmal mitochondrial area, CS maximal activity and the protein content of COXIV and UQCRC2 increased following CONT training; however, INT training elicited no changes in measures of mitochondrial content. Therefore, contrary to our hypothesis, INT training did not augment mitochondrial adaptations compared to work- and intensity-matched CONT training. These findings suggest that an intermittent pattern of exercise is not a primary stimulus for training-induced changes in mitochondrial content.

ADDITIONAL INFORMATION

Competing interests

The authors have no competing interest to declare.

Author contributions

All human experimental trials, exercise training and analyses were completed at McMaster University. LES, MJM, MAT, and MJG contributed to the conception and design of the work. LES, MJM, WB, DGM, EMJ, LRA, MAT and MJG collected samples, performed analyses, and/or interpreted data. LES and MJG wrote the first draft of the manuscript and all authors critically revised the manuscript for intellectual content.

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FIGURES

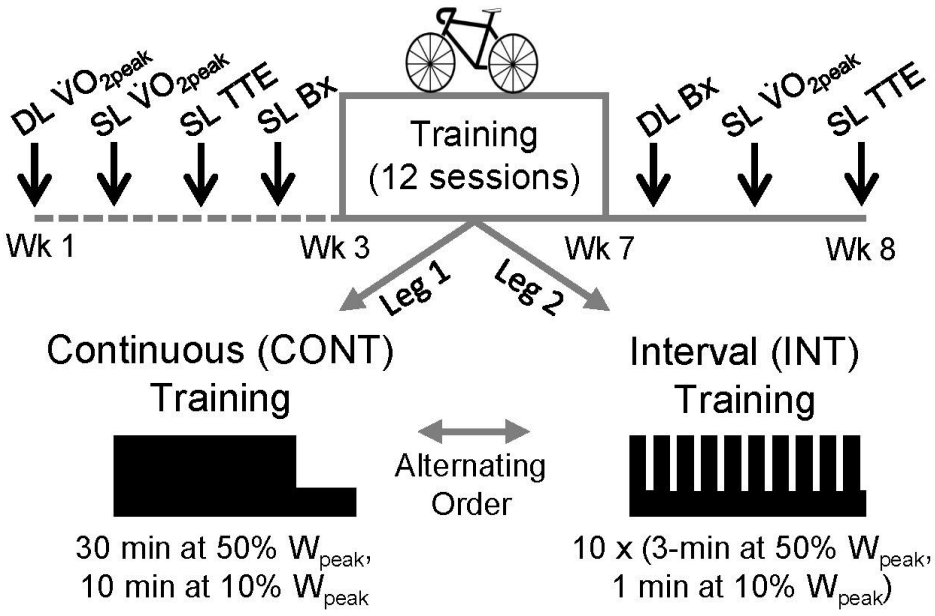


Figure 1. A schematic illustration of the experimental protocol. Double leg (DL); single leg (SL); peak power output (W_{peak}); time to exhaustion test at 70% W_{peak} (TTE); Bx (muscle biopsy); week (wk).

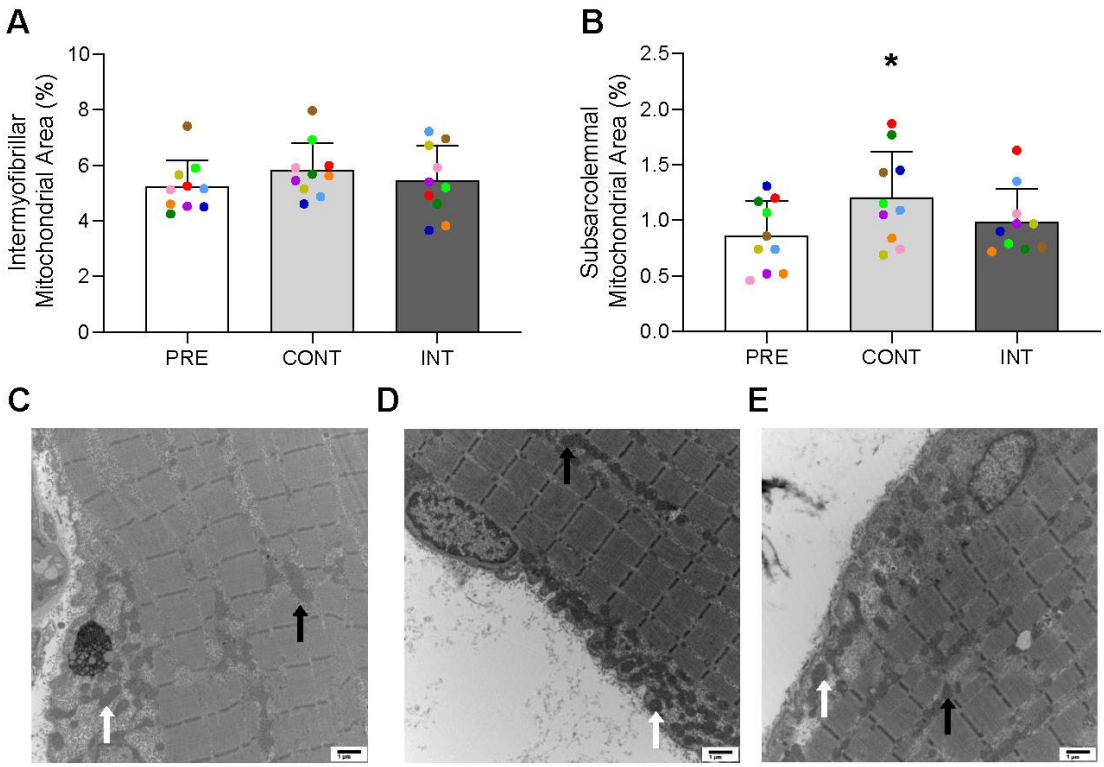
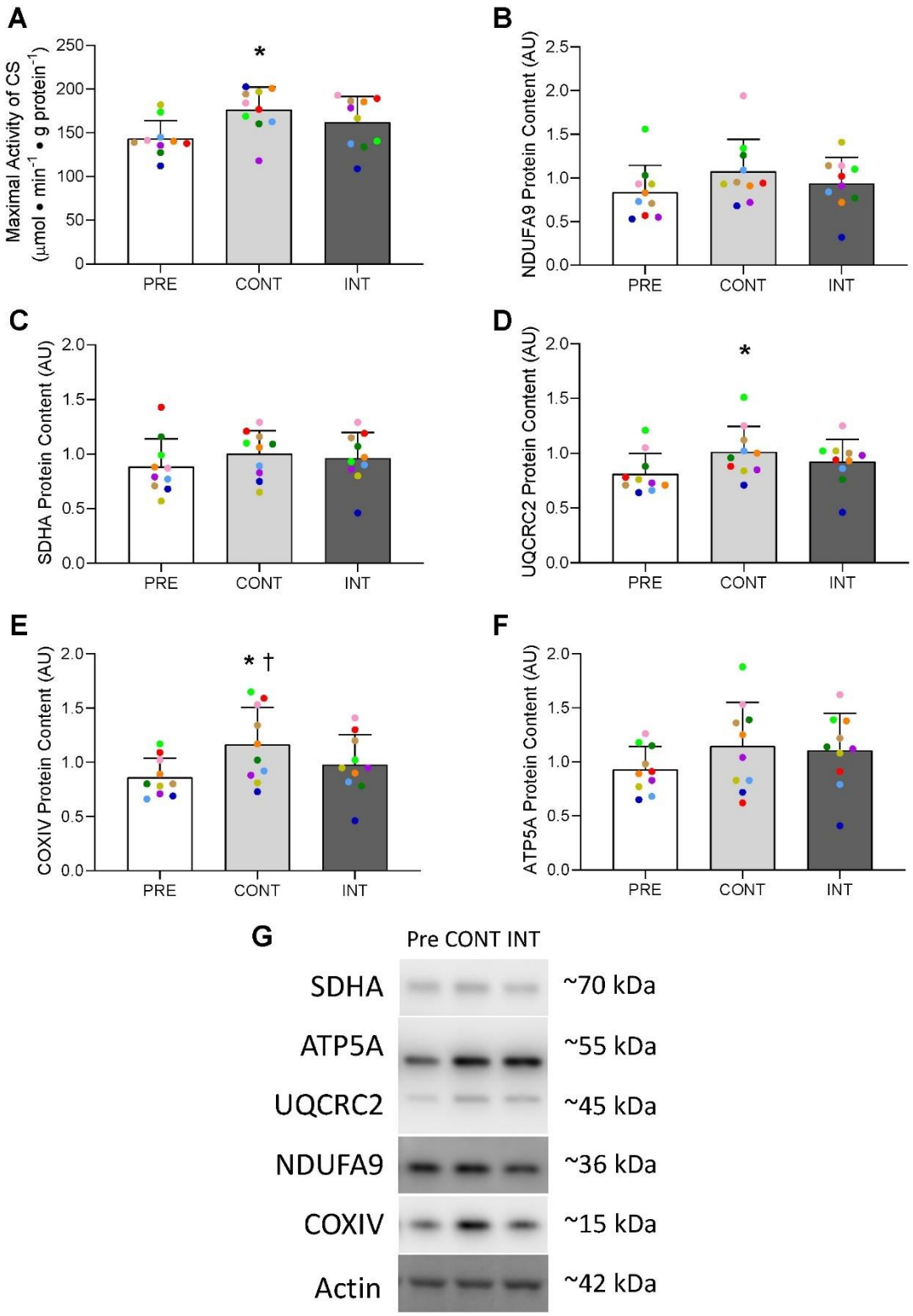


Figure 2. Mitochondrial content assessed using transmission electron microscopy before (white bars) and following single-leg CONT training (light grey bars) and INT training (dark grey bars). Intermyofibrillar mitochondrial area (panel A), subsarcolemmal mitochondrial area (panel B), and micrographs from a pre-training biopsy (panel C), as well as post-training biopsies from the CONT training (panel D) and INT training (panel D) legs of the same participant. The symbol indicates a significant difference between PRE and CONT (*p=0.04). Each colour within the individual data points represents one individual and is consistent across panels and figures. Black arrows point to intermyofibrillar mitochondria and white arrows point to subsarcolemmal mitochondria. Scale bar, 1 µm.


A
Maximal Activity of CS
(µmol • min-1 • g protein-1)
250
200
150
100
50
0
*
PRE
CONT
INT
B
NDUFA9 Protein Content (AU)
2.5
2.0
1.5
1.0
0.5
0.0
PRE
CONT
INT
C
SDHA Protein Content (AU)
2.0
1.5
1.0
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PRE
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D
UQCRC2 Protein Content (AU)
*
2.0
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COXIV Protein Content (AU)
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ATP5A Protein Content (AU)
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G
Pre CONT INT
SDHA
~70 kDa
ATP5A
~55 kDa
UQCRC2
~45 kDa
NDUFA9
~36 kDa
COXIV
~15 kDa
Actin
~42 kDa

Figure 3. Biomarkers of mitochondrial content before (white bars) and following single-leg CONT training (light grey bars) and INT training (dark grey bars). The maximal activity of citrate synthase (CS; panel A) and protein content of NADH:ubiquinone oxidoreductase subunit A9 (NDUFA9; panel B), succinate dehydrogenase subunit A (SDHA; panel C), ubiquinol-cytochrome *c* reductase core protein 2 (UQCRC2, panel D), cytochrome *c* oxidase subunit IV (COXIV; panel E) and ATP synthase α-subunit (ATP5A; panel F). Representative blots for mitochondrial proteins assessed using Western blotting (panel G) are displayed separately. The symbols indicate a significant difference between PRE and CONT (*$p<0.05$) and CONT and INT (†$p=0.045$). Each colour within the individual data points shown represents one individual and is consistent across panels and figures.

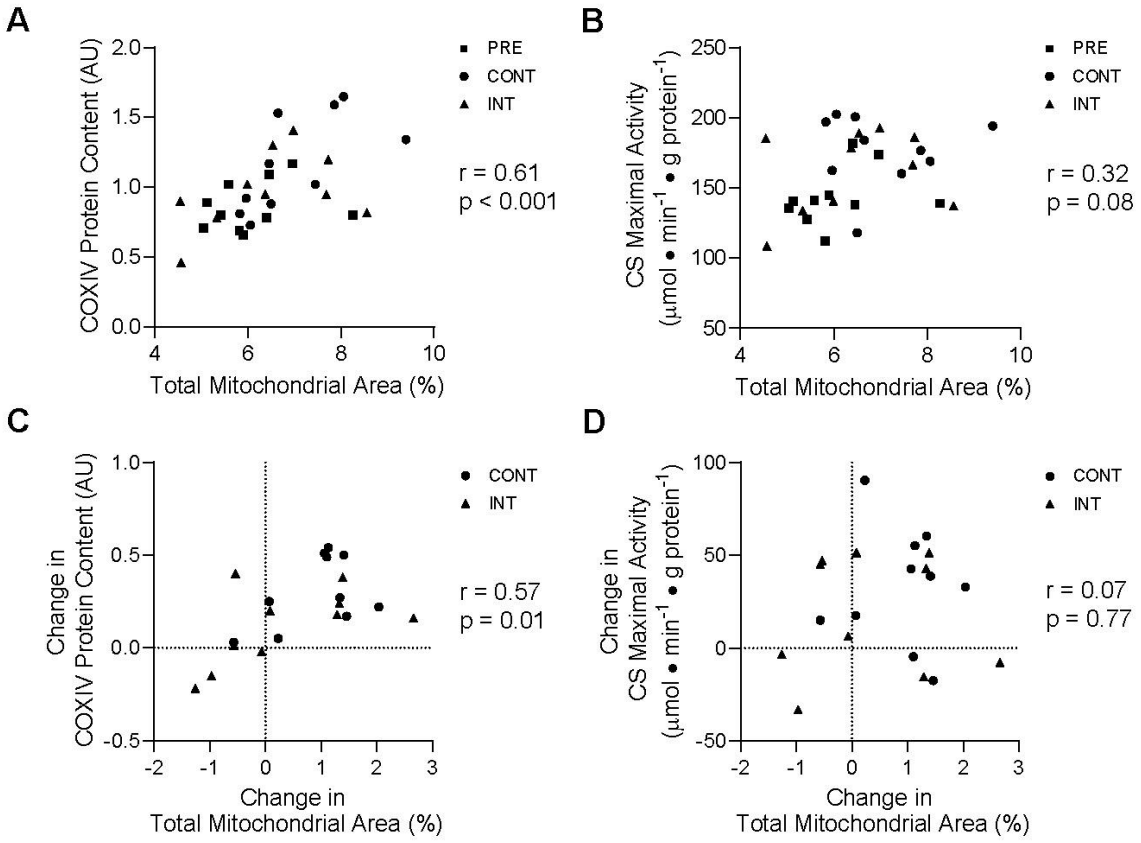

Figure 4. Correlations between mitochondrial area and biomarkers of mitochondrial content. Correlations between absolute values and training-induced changes in total mitochondrial area and the protein content of cytochrome *c* oxidase subunit IV (COXIV; panels A and C) or the maximal activity of citrate synthase (CS; panels B and D) before (squares) and following single-leg CONT (circles) and INT (triangles) training are displayed separately. Dashed lines indicate an x and y value of 0.

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CHAPTER 5: GENERAL DISCUSSION

5.1. Introduction

The present thesis sought to examine the influence of three potential factors on skeletal muscle responses to INT training: sex (Chapter 2), fibre type-specific changes (Chapter 3) and contraction pattern (Chapter 4). The first study (Chapter 2) investigated whether sex-based differences existed in the acute skeletal muscle response to a low-volume SIT protocol consisting of 3 x 20-sec 'all-out' sprints. Despite evidence of lower rates of mitochondrial biogenesis in women compared to men during 3 weeks of SIT (Scalzo *et al.*, 2014), we found that the exercise-induced increases in the mRNA expression of genes involved in mitochondrial biogenesis, including *PGC-1α*, were largely similar between sexes. We also reported similar SIT-induced glycogen utilization in men and women. Study 2 (Chapter 3) compared fibre type-specific responses to the same low-volume SIT intervention as employed in study 1 and compared responses to a more traditional higher-volume MICT protocol. The first experiment in study 2 found that an acute session of SIT and MICT induced similar increases in the phosphorylation of mitochondrial signalling proteins in mixed muscle and pooled type I and IIa fibres. In contrast, the second experiment in study 2, which involved 12 weeks of exercise training, revealed that MICT elicited greater increases in markers of mitochondrial content in type I fibres and similar increases in type IIa fibres, compared to SIT. In the final study (Chapter 4), we assessed changes in mitochondrial content following single-leg moderate-intensity CONT and INT training. The CONT and INT exercise protocols were matched for work, intensity and duration such that only the contraction pattern differed. Training-induced increases in mitochondrial content were observed following CONT only,

suggesting that an intermittent pattern of contraction is not a primary stimulus for increasing mitochondrial content. The present chapter will integrate findings from all studies and highlight the contribution of the thesis to the larger field. Potential limitations and future directions will be discussed throughout.

5.2. Discordance between acute and chronic responses

It is generally accepted a single session of exercise elicits acute responses within skeletal muscle and over repeated exercise sessions throughout a training intervention, these acute responses accumulate over time to induce phenotypic changes and improve exercise performance (Egan & Zierath, 2013). The disturbances in skeletal muscle homeostasis that occur during a single session of exercise activate signalling pathways that co-ordinate an increase in the transcription of target genes (Coffey & Hawley, 2007; Egan & Zierath, 2013; Hawley *et al.*, 2014; Perry & Hawley, 2018). The repeated and transient increases in the mRNA expression of proteins related to skeletal muscle remodelling following multiple exercise sessions precede and lead to increases in corresponding protein content (Perry *et al.*, 2010; Egan *et al.*, 2013). The present thesis utilized a variety of methods to examine different markers and time-points to characterize responses to acute exercise and training. Specifically, the studies assessed the phosphorylation of signalling proteins linked to mitochondrial biogenesis (study 2; immediately post-exercise) and the mRNA expression of mitochondrial genes (study 1; immediately and 3 h post-exercise) following a single session of exercise, and markers of

mitochondrial content following short-term exercise training (study 2 and 3; 4 and 12 weeks).

Results from the present thesis are consistent with other reports that acute responses to a single session of exercise do not necessarily align with training-induced changes (Cochran *et al.*, 2014; Mitchell *et al.*, 2014). Despite sex influencing rates of mitochondrial protein synthesis after 3 weeks of SIT (Scalzo *et al.*, 2014), we found no sex-based differences in the mRNA expression of mitochondrial genes following a single session of SIT. In study 2, MICT elicited greater training-induced increases in mitochondrial content in type I fibres *versus* SIT; however, acute responses to the two training approaches were similar within type I fibres. Lastly, while an acute bout of INT exercise was reported to enhance the phosphorylation of AMPK, p38 MAPK and CaMKII compared to work- and intensity-matched CONT (Combes *et al.*, 2015), we did not observe greater changes in mitochondrial content following single-leg INT compared to CONT training. There are many potential explanations for the discordance between acute and chronic measures observed in the present thesis as considered further below.

5.2.1. Additional measurements of signalling pathways linked to mitochondrial biogenesis

Firstly, the regulation of exercise-induced mitochondrial biogenesis involves multiple overlapping and redundant signalling pathways. While the present thesis selected key markers of acute responses to a single session of exercise that are well-supported within the literature (i.e., ACC, p38 MAPK, PGC-1α), it would be worthwhile to examine

other proteins that are linked to mitochondrial biogenesis. With respect to signalling molecules, we were unable to obtain data for the ratio of phosphorylated to total protein content of CaMKII and AMPK due to technical difficulties. We measured the phosphorylation of ACC as an indicator of AMPK activity (Winder *et al.*, 1997; Chen *et al.*, 2003) but the inclusion of CaMKII and AMPK measurements would have strengthened our study and may have provided an explanation for the divergent training responses found in type I fibres. Another potential regulator of mitochondrial biogenesis is tumour repressor protein p53 (Saleem *et al.*, 2011, 2014; Bartlett *et al.*, 2014). A single session of SIT and HIIT has shown to elicit increases in the phosphorylation of p53 during the first few hours of recovery (Bartlett *et al.*, 2012, 2013; Broatch *et al.*, 2017; Granata *et al.*, 2017). Low-volume SIT did not induce increases in p53 mRNA expression in study 1; however, our biopsy time-points of immediately post-exercise and 3 h into recovery may have been too early to observe an increase (Granata *et al.*, 2018*a*). It may have also been worthwhile to assess subcellular location-specific protein content of phosphorylated p53 and PGC-1α in study 2, as translocation of these two proteins to the nucleus may provide an early indication of acute adaptive responses to exercise (Hood *et al.*, 2016). Increased nuclear protein content of PGC-1α and p53 is reported following both MICT and SIT in humans (Little *et al.*, 2010, 2011; Tachtsis *et al.*, 2016; Granata *et al.*, 2017).

The major rationale for measuring exercise-induced changes in the phosphorylation of ACC, p38 MAPK and the mRNA expression of *PGC-1α* in the present thesis was to gain insight into the acute activation of PGC-1α, owing to its central

role in upregulating mitochondrial biogenesis (Fernandez-Marcos & Auwerx, 2011). PGC-1α is proposed to be a critical regulator of mitochondrial biogenesis by activating transcription factors that increase the expression of nuclear- and mitochondrial-encoded mitochondrial genes, as transcripts from both genomes are required for mitochondrial content to increase (Fernandez-Marcos & Auwerx, 2011; Hood *et al.*, 2016). In study 1, the similar post-exercise increase in *PGC-1α* mRNA expression in men and women was consistent with no apparent sex-based differences in the expression of other nuclear-encoded genes related to mitochondrial biogenesis, including *PRC, PPARD, TFAM, CS* and *COXIV*. A recent review highlighted that while there is strong evidence that PGC-1α has a large role in upregulating both nuclear- and mitochondrial-encoded genes in cellular and animal models, this coordinated response does not appear to occur following exercise in humans (Islam et al., 2018). There also appears to be PGC-1α-independent mechanisms of exercise-induced mitochondrial biogenesis. Increases in markers of mitochondrial content were observed following short-term exercise training in PGC-1α knockout mice (Leick *et al.*, 2008; Rowe *et al.*, 2012; Ballmann *et al.*, 2016). Thus, while the present thesis focused on examining acute responses known to converge on PGC-1α, there may be other pathways contributing to mitochondrial biogenesis (Islam *et al.*, 2019*b*) that could explain the discordance between acute and chronic results. Other proteins with potential roles in exercise-induced mitochondrial biogenesis include nuclear factor erythroid 2-related factor 2 and estrogen-related receptor gamma (reviewed in Islam *et al*. (2019*b*)).

5.2.2. Differences in the initial vs. prolonged response during recovery: post-transcriptional processes

Discrepancies between acute and chronic findings may also be related to post-transcriptional modifications. Exercise-induced changes in the phosphorylation state of signalling molecules and the mRNA expression of mitochondrial genes provide insight into the initial phases of mitochondrial biogenesis; however, additional biological processes that occur thereafter impact changes in mitochondrial content (Hood *et al.*, 2016). Post-transcriptional processes or regulators are generally less studied following an acute bout of INT exercise in human skeletal muscle but may represent an area where sex, fibre type-specific responses or contraction pattern could impact responses. For example, steady-state mRNA concentrations, which are underpinned by changes in transcription and degradation, may be influenced by RNA-binding proteins (Miller *et al.*, 2016; Van Pelt *et al.*, 2019). RNA-binding proteins can influence mRNA stability and have shown to change in abundance following exercise training in rodents (Lai *et al.*, 2010; Matravadia *et al.*, 2013). MicroRNAs (miRNAs) can also induce mRNA degradation or impede protein translation (Selbach *et al.*, 2008; Zacharewicz *et al.*, 2013) and alterations in the expression of skeletal muscle microRNAs have been demonstrated following a single session of SIT and MICT, and short-term MICT in men (Nielsen *et al.*, 2010*b*; Keller *et al.*, 2011; Russell *et al.*, 2013; D'Souza *et al.*, 2018) however, investigations in women appear limited. Protein import and assembly into functional enzyme complexes in mitochondria are other additional processes downstream of the acute measures employed in the thesis that may explain discrepancies between acute and chronic measures.

Mitochondrial content is a static measure that reflects the net balance between mitochondrial protein synthesis (MitoPS) and mitochondrial protein breakdown. Measuring the rate of MitoPS is proposed to be a superior measure for assessing mitochondrial biogenesis compared to acute signalling measures (Miller & Hamilton, 2012). The rate of MitoPS can be measured using stable isotope tracers (Rennie *et al.*, 1994; Wilkinson *et al.*, 2008; Di Donato *et al.*, 2014; Robinson *et al.*, 2017) and more recently, oral administration of deuterium oxide has been utilized to measure cumulative rates of MitoPS over short-term exercise training (Scalzo *et al.*, 2014). Given the limited data regarding rates of MitoPS following INT training (Scalzo *et al.*, 2014; Robinson *et al.*, 2017), this measure will be valuable to employ in future studies.

The acute measures in the thesis aimed to examine mitochondrial biogenesis related responses to exercise and thus, focused on only one component determining mitochondrial content. The process of mitophagy breaks down damaged and dysregulated mitochondria and is essential for maintaining a healthy reticulum (Hood *et al.*, 2016). It is probable that an acute bout of exercise up-regulates mitophagy to remove dysfunctional mitochondrial in humans (Ogborn *et al.*, 2015; Laker *et al.*, 2017; Hood *et al.*, 2019). In addition to regulating mitochondrial biogenesis, PGC-1α also may have a role in mediating exercise-induced mitophagy, as evidenced by PGC-1α knockout mice displaying reduced rates of mitophagy following an acute bout of MICT (Vainshtein *et al.*, 2015). This dual role of PGC-1α further underscores the importance of examining measures downstream in addition to PGC-1α activation to gain more comprehensive insight into acute differences in the balance between mitochondrial biogenesis and

mitophagy. Directly measuring rates of muscle protein breakdown requires several biopsy time-points and is more technically challenging than measuring rates of muscle protein synthesis. To our knowledge, there are no techniques to directly measure mitochondrial protein breakdown rates in humans (Bishop *et al.*, 2019).

5.2.3. Experimental design considerations

Discrepancies between acute and chronic findings may also stem from differences characteristic to their respective experimental designs. Unless the factor being examined is specific to two or more unique groups of participants (e.g., sex as a factor in study 1), acute investigations often utilize cross-over designs because the wash-out period can be short between trials. In contrast, and unless a unilateral exercise model is employed, training studies more frequently utilize a parallel group design because a long and inconvenient washout period would be required between treatments (MacInnis *et al.*, 2017*a*). Therefore, acute investigations may involve a more controlled environment within a shorter time frame that affords less opportunity for external factors such as changes in diet, sleep or habitual physical activity, to impact responses to exercise compared to chronic studies. There are also some controls often implemented in acute experiment designs that may not be present over a training intervention. For example, pre-exercise diet can influence acute responses related to mitochondrial biogenesis (Guerra *et al.*, 2010; Stocks *et al.*, 2019) and is often controlled for prior to acute exercise trials (e.g., study 1 and 2); however, participants in training studies typically determine their dietary intake prior to training sessions (e.g., study 2 and 3).

Due to the invasive nature of muscle biopsy sampling, studies examining acute responses to exercise are also typically limited to a few time-points. Our measures of acute responses to exercise in study 1 and 2 were examined in skeletal muscle biopsies obtained immediately post-exercise and/or 3 h into recovery. There is a paucity of information regarding the time-course of acute mitochondrial responses within a single cohort of participants and it is plausible that the timing of responses are influenced by factors such as sex and fibre-type (Hornberger *et al.*, 2016). The phosphorylation of signalling proteins occurs rapidly, with increases found after a single 30-sec 'all-out' sprint (Birk & Wojtaszewski, 2006; Fuentes *et al.*, 2012) and 1 min of MICT (Rose *et al.*, 2006). Considering the rapid phosphorylation of signalling proteins from the onset of exercise, it may be better to standardize time-points utilized to assess gene expression based on the start of exercise rather than the end (Granata *et al.*, 2018*a*). In support of the potential influence of measurement timing relative to the start of exercise on acute responses, a recent review by Granata *et al.* (2018a) highlighted that the duration of exercise protocols appears to influence the time-course of exercise-induced increases in *PGC-1α* mRNA expression. The authors noted that long duration protocols involving 90 min of exercise demonstrate increases in *PGC-1α* mRNA expression immediately post-exercise but shorter duration protocols generally do not elicit changes until later into recovery (Granata *et al.*, 2018*a*). Sex-specific findings from study 1 would not be influenced by our measurement timing relative to the start of exercise since both men and women performed the same low-volume SIT protocol. Nevertheless, the timing of measurements from the onset of exercise may be an important consideration when

interpreting and linking findings of acute investigations comparing MICT and shorter duration SIT (Granata *et al.*, 2017; Fiorenza *et al.*, 2018; Trewin *et al.*, 2018) to training studies (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2013, 2017; Gillen *et al.*, 2016).

In light of the potential reasons for misalignment of acute and chronic findings, it is not surprising that the value of measuring exercise-induced changes in activation of signaling proteins and gene expression has been questioned (Miller *et al.*, 2016). It is important to note however, that there is a paucity of investigations comparing acute responses to training adaptations within the same group of individuals. Bonafiglia *et al.* (2017) found a strong positive relationship between SIT-induced increases in the mRNA expression of *PGC-1α* and increases in SDH activity over 6 weeks of the same SIT protocol in recreationally active men. While correlational analyses only provide information regarding an association and not a direct connection between two variables, these results suggest that the magnitude of exercise-induced changes in PGC-1α may be related to subsequent training adaptations (Bonafiglia *et al.*, 2017) and provide support for examining acute responses to exercise. Additional research comparing individual responses to a single session of INT exercise and a training period are warranted.

Overall, the acute data in the present thesis provide important insight into whether sex and fibre type influence the initial skeletal muscle responses to a single bout of INT training. The similar acute responses found between sexes and following SIT and MICT within both fibre types do not explain the sex and fibre type-specific responses observed in training studies (Scalzo *et al.*, 2014 and study 2). Our results provide a strong basis for future work to examine whether sex, fibre type-specific responses and contraction pattern

impact other proteins involved in mitochondrial biogenesis (e.g., p53) and measures of post-transcriptional processes (e.g., mitochondrial protein synthesis) in an effort to reconcile the discrepancies between acute and chronic results.

5.3. The potency of low-volume interval training for increasing mitochondrial content

Results from study 1 and study 2 demonstrated robust skeletal muscle responses in response to only 1 min of 'all-out' intermittent exercise within a 10 min exercise session. These findings are consistent with a large body of evidence demonstrating that low-volume INT training is a potent stimulus for eliciting physiological adaptations (Gibala *et al.*, 2012; MacInnis & Gibala, 2017; Granata *et al.*, 2018*b*). An editorial by Vollaard & Metcalfe (2017) proposed that future research should examine SIT protocols involving shorter and a lower total number of sprints compared to more traditionally studied Wingate sprint-based protocols (i.e., repeated 30-sec 'all-out' Wingate sprints interspersed with ~4 min of recovery). The authors suggested that the lower time commitment and perceived exertion of shorter SIT protocols, such as the one examined in study 1 and 2, may be a more acceptable protocol for the general population by removing some of the common barriers to exercise, including "lack of time" (Trost *et al.*, 2002; Vollaard & Metcalfe, 2017). Results from the present thesis support that shorter SIT protocols can induce skeletal muscle responses in sedentary or recreationally active participants; however, whether SIT protocols are likely to be utilized by the general population has been debated within the literature (Hardcastle *et al.*, 2014; Del Vecchio *et*

al., 2015; Astorino & Thum, 2016; Jung *et al.*, 2016; Robertson-Wilson *et al.*, 2017). Additional research examining whether shorter SIT protocols performed in modes that do not require specialized equipment and are more accessible to the public, such as stair climbing-based protocols, improve skeletal muscle oxidative capacity in addition to cardiorespiratory fitness (Allison *et al.*, 2017) may provide important information relevant to this debate.

The greater understanding of potential factors impacting responses to exercise may also provide valuable information regarding the prescription of INT training for improving skeletal muscle health and/or exercise performance. Given the largely similar responses of markers of mitochondrial content to INT training in men and women found in study 1 and by others (Gillen *et al.*, 2014; Scalzo *et al.*, 2014), the currently available data support low-volume INT training as an exercise option for increasing mitochondrial content in women. Findings from study 3 suggest that INT training may need to be performed at high intensity to induce skeletal muscle adaptations in men and women, at least over a short training period. Results from study 2 showed that 12 weeks of low-volume INT training did not change mitochondrial content within type I fibres. This finding may be relevant to individuals seeking to improve their endurance exercise performance, as type I fibres have shown to be primarily utilized during MICT based on glycogen depletion patterns (Gollnick *et al.*, 1973, 1974). In trained runners, Skovgaard *et al.* (2018) found a decrease in the protein content of CS within type I fibres despite no change in type IIa fibres following a 40 d reduced volume training period consisting of 10 sessions of both MICT and low-volume SIT. While 10 km performance was improved

following the training intervention, there was no control group included and the potential impact of reduced type I-specific mitochondrial content on exercise performance is unclear (Skovgaard *et al.*, 2018). The fibre type-specific results from study 2 and Skovgaard *et al.* (2018) suggest that higher volume SIT protocols may be required to improve or maintain mitochondrial content in type I fibres, which may be important knowledge for individuals training for endurance events.

5.3.1. Potential mechanism(s)

The potential for INT training to elicit superior increases in mitochondrial content for a given volume of exercise compared to MICT (Burgomaster *et al.*, 2008; Shepherd *et al.*, 2013; Scribbans *et al.*, 2014; Gillen *et al.*, 2016; MacInnis *et al.*, 2017*b*; Shepherd *et al.*, 2017) may be related to a higher exercise intensity *per se* and/or the inherent intermittent pattern of exercise. Findings from study 3 suggest an intermittent contraction pattern, when performed at a moderate intensity, does not enhance training-induced changes in mitochondrial content compared to work-matched CONT training. A higher exercise intensity may lead to augmented mitochondrial responses to exercise training by recruiting and remodelling type II fibres to a greater extent and/or inducing larger increases in metabolic stress compared to lower intensity exercise. Results from study 2 suggest that the comparable skeletal muscle responses found following low-volume SIT and MICT are not related to enhanced type IIa responses. While direct measurements of disturbances in homeostasis during exercise were not a major focus of the present thesis, a recent investigation by Fiorenza *et al.* (2018) proposed that initial events involved in

upregulating mitochondrial biogenesis are sensitive to the magnitude of metabolic stress induced by INT exercise. Using multiple linear regression, the authors found that SIT-induced changes in skeletal muscle phosphocreatine, hydrogen ions, lactate, glycogen and/or ATP predicted the increases in the phosphorylation of CaMKII and p38 MAPK and the mRNA expression of mitochondrial genes, including *PGC-1α* and *TFAM* (Fiorenza *et al.*, 2018). There were no relationships between markers of metabolic stress and acute responses related to mitochondrial biogenesis following MICT, highlighting that the importance of the magnitude of disturbance in skeletal muscle homeostasis in eliciting downstream responses may be distinct to low-volume SIT (Fiorenza *et al.*, 2018). Our findings from study 1 align with the relationship between SIT-induced increases in metabolic stress and mitochondrial gene expression reported by Fiorenza *et al.* (2018), as the similar SIT-induced glycogen depletion in men and women preceded the comparable increases in the mRNA expression of *PGC-1α*.

There may be diminishing benefits of higher intensities of exercise for increasing mitochondrial content. Supramaximal efforts do not necessarily confer the largest acute skeletal muscle responses, as greater exercise-induced increases in the mRNA expression of *PGC-1α* were shown following INT exercise performed at 100% W_{peak} compared to work-matched INT exercise performed at 73% or 133% W_{peak} (Edgett *et al.*, 2013). Similar increases in markers of mitochondrial content have also been reported in sedentary men following 3 weeks of an INT training protocol performed at either 70% or 100% of W_{peak} (Boyd *et al.*, 2013). In both investigations (Boyd *et al.*, 2013; Edgett *et al.*, 2013) one minute intervals were interspersed with one minute of recovery. Other

investigations comparing various intensities of INT training by examining both HIIT and SIT protocols have shown similar increases (Bækkerud *et al.*, 2016) or no changes in mitochondrial content (Granata *et al.*, 2016). Additional studies that compare skeletal muscle responses to INT exercise protocols that vary in exercise intensity, total duration at a high intensity, and total volume may provide further insight into the mechanisms of skeletal muscle adaptations following INT training. The unilateral model of exercise implemented in study 3 would be an advantageous experimental design in this regard.

5.3.2. Markers of mitochondrial responses to exercise training

The present thesis utilized measures of mitochondrial area, the maximal activity of CS and the protein content of COXIV to determine mitochondrial content (studies 2 and 3). Exercise training-induced increases in mitochondrial content are a result of enlargement of existing mitochondria rather than *de novo* synthesis, as evidenced by an increase in the size of individual mitochondrial but not the number of mitochondrial profiles following short-term MICT (Tarnopolsky *et al.*, 2007; Meinild Lundby *et al.*, 2018). Content is only one facet of mitochondria that is responsive to exercise training and may improve skeletal muscle oxidative capacity (Granata *et al.*, 2018*b*; Bishop *et al.*, 2019; Hood *et al.*, 2019). Mitochondrial fusion and fission processes influence mitochondrial morphology and are important for maintaining a healthy mitochondrial network (Yan *et al.*, 2012; Hood *et al.*, 2019). Study 2 provided novel comparisons of fibre type-specific responses of two key proteins involved in mitochondrial fusion, MFN2 and OPA1, following SIT and MICT. Mitochondrial respiratory capacity and cristae

density are additional measures that are correlated with $\dot{V}O_{2peak}$ and are higher in endurance-trained compared to recreationally active individuals (Jacobs & Lundby, 2013; Nielsen *et al.*, 2017). Studies measuring a range of mitochondrial measures, including dynamics proteins, respiratory capacity and cristae density, are needed to provide a more comprehensive understanding of how INT training influences skeletal muscle oxidative capacity. Determining whether sex, fibre type and contractile pattern also influence changes in these mitochondrial measures following INT training is warranted.

5.4. Examining compartments within mixed skeletal muscle

The methods employed in the present thesis provided deeper insight into skeletal muscle tissue by examining responses to INT training in mixed muscle samples (all studies), pooled single fibre segments (study 2) and subpopulations of mitochondria within muscle fibres (study 3). The majority of research examining skeletal muscle responses to INT training has focused on mixed muscle analyses. Results from study 2 and 3 demonstrate that unique insight into training responses can be gained from examining compartments within the skeletal muscle tissue obtained from a biopsy. Specifically, study 2 found a greater increase in COXIV and NDUFA9 protein content in type I fibres following MICT compared to SIT, despite similar increases in both proteins in mixed muscle samples. In study 3, there were no changes in total mitochondrial area however, subsarcolemmal mitochondrial area increased following CONT training.

Whole muscle analyses may mask divergent responses between different compartments in skeletal muscle (e.g., type I *versus* II fibres, subsarcolemmal *versus*

intermyofibrillar mitochondria). In study 3, a relatively greater mean increase in mitochondrial area following CONT training was observed in the subsarcolemmal region compared to the intermyofibrillar region. Other investigations have also found preferential increases in subsarcolemmal mitochondrial area following exercise training in humans (Hoppeler *et al.*, 1985; Howald *et al.*, 1985; Nielsen *et al.*, 2010*a*; Montero *et al.*, 2015; Koh *et al.*, 2018; Meinild Lundby *et al.*, 2018), although data are equivocal in this regard (Turner *et al.*, 1997; Samjoo *et al.*, 2013). One factor that may have introduced variability in our measure and masked significant training-induced increases in total and intermyofibrillar mitochondrial area is differences in the proportion of fibre types between the 8 fibres analyzed in each sample. Fibres can be identified as type I or II within micrographs obtained using TEM based on the combination of Z-line width and mitochondrial volume (Nielsen *et al.*, 2011; Koh *et al.*, 2017). This may be a worthwhile additional analysis for future studies employing TEM in our laboratory as it would also allow for the determination of fibre type-specific responses. The results could also provide an interesting comparison to Western blotting-determined fibre type-specific responses. Another factor that may have increased variability in our measures of mitochondrial area is the proximity of our micrographs to capillaries, as mitochondria are often distributed near capillaries (Hoppeler *et al.*, 1973; Glancy *et al.*, 2014).

Skeletal muscle is a heterogenous tissue with respect to fibre type and type I fibres contain greater mitochondrial content than type II fibres (Howald *et al.*, 1985). A recent study by Islam *et al.* (2019*a*) examined fibre type distribution in two pieces from a single muscle biopsy. While the mean proportion of type I and IIa fibres were not statistically

different, the measurements were not correlated suggesting poor repeatability of fibre type distribution across two pieces of skeletal muscle tissue (Islam *et al.*, 2019*a*). An advantage of analyzing segments of single fibres is that that the potential influence of differences in fibre type distribution between biopsy samples on training responses is eliminated.

In addition to the mixed muscle analyses, the compartment-specific findings provide valuable and unique information regarding skeletal muscle adaptations to INT training. The use of advanced microscopy techniques that provide three-dimensional mapping of mitochondrial networks (Dahl *et al.*, 2015; Vincent *et al.*, 2019) and single fibre proteomics (Murgia *et al.*, 2015, 2017) in human skeletal muscle will continue to advance our knowledge regarding compartment-specific responses to training. It is fascinating that changes induced by exercise training in human skeletal muscle are measurable in only a few single fibres, or regions within single fibres, taken from a skeletal muscle biopsy.

5.5. Skeletal muscle responses to interval exercise training in females

Female participants are underrepresented in exercise physiology research, and account for ~39% of participants in studies published between 2011-2013 in three major journals in the field (Costello *et al.*, 2014). Sex-based comparisons of responses to INT training are limited and thus, the findings of study 1 are an important contribution to the field. Given the similar responses of mitochondrial genes observed in study 1, the reported similar increases in the maximal activity and protein content of CS following

short-term SIT in men and women (Gillen *et al.*, 2014; Scalzo *et al.*, 2014), and the lower number of female participants in exercise physiology research compared to male participants (Costello *et al.*, 2014), we studied responses to INT training in mixed cohorts for study 2 and 3. We did not examine sex-specific responses in these studies, owing to the small sample size of women (n=4 or 5) and that our purpose was to examine the influence of other factors on our measures. Nonetheless, one reason for examining responses to an exercise training intervention of 4 weeks in length in study 3 was to cover the typical time period of a single menstrual cycle in women (Sims & Heather, 2018) and eliminate the potential influence of menstrual cycle phase on training-induced changes in mitochondrial content.

Interestingly, three recent studies have suggested that sex-based differences exist in human basal mitochondrial bioenergetics, although some of the findings within these reports are conflicting (Cardinale *et al.*, 2018; Miotto *et al.*, 2018; Montero *et al.*, 2018). Greater (Montero *et al.*, 2018), similar (Miotto *et al.*, 2018) and lower (Cardinale *et al.*, 2018) mitochondrial content is reported in women compared to men. Disparities in these findings may be related to different methods used to assess mitochondrial content. It is also important to note that these sex-based comparisons did not match men and women based on $\dot{V}O_{2peak}$ relative to kilogram of fat free mass, which is considered to be best-practice (Tarnopolsky, 2008). Similar mitochondrial content was found in men and women matched for VO_2peak relative to fat free mass (Tarnopolsky *et al.*, 2007). Continued research in basal and INT exercise-induced changes in mitochondrial

measures, including mitochondrial content, in cohorts of participants that include both men and women is critical to ensure findings are generalizable to the population.

5.6. Conclusions

The studies in the present thesis advance our understanding of the potential influence of sex, fibre type-specific changes, and contraction pattern on the acute and chronic skeletal muscle response to INT training. Our findings suggest that sex does not impact SIT-induced increases in mitochondrial gene expression and that similar responses of mitochondrial signalling proteins occur within fibre types following SIT and MICT. Over a 12 week training intervention, potential differences in the response of type I but not type IIa fibres exist following SIT and MICT. Lastly, greater training-induced increases in mitochondrial content were not observed following single-leg cycling performed in an INT compared to a CONT contraction pattern. Collectively, these findings add to our knowledge of the mechanisms by which INT training can elicit increases in mitochondrial content despite a low total volume of exercise. While the present thesis addresses basic research questions, an improved understanding of potential factors impacting responses to exercise may also better inform the prescription of INT training for improving health and fitness. Importantly, when performed at a high intensity, INT training is a potent stimulus for inducing mixed skeletal muscle responses in men and women. Future research that assesses acute and chronic responses to INT training within a single cohort of men and women, includes a comprehensive range of mitochondrial measures related to improved skeletal muscle oxidative capacity and/or

uses unilateral exercise models to investigate potential differences in training responses to two different exercise protocols is warranted.

5.7. References

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