# HUMAN SKELETAL MUSCLE MITOCHONDRIAL RESPONSE TO INTERVAL TRAINING

# HUMAN SKELETAL MUSCLE MITOCHONDRIAL RESPONSE TO INTERVAL TRAINING: ROLE OF EXERCISE INTENSITY

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## LAY ABSTRACT

Mitochondria are an important component of cells that use oxygen to convert fuels such as sugars and fats into energy. One of the factors that determines the amount of mitochondria in skeletal muscle is physical activity. Aerobic exercise training can be performed over a range of intensities, from relatively easy to very hard, and in an intermittent or continuous manner. This thesis examined the effect of short-term, intermittent exercise training performed at two different intensities on the content of mitochondria in human skeletal muscle. It found that both high-intensity interval training (HIIT) and sprint interval training (SIT) increased mitochondrial content. The increase was greater after SIT compared to HIIT, even though the total "dose" or amount of exercise was lower in the former compared to the latter. These results suggest that intensity is an important determinant of skeletal muscle remodeling induced by intermittent exercise in humans.

## ABSTRACT

It has been proposed that intermittent exercise can differentially affect mitochondrial responses to training, with training volume being more important than intensity for increasing skeletal muscle mitochondrial content and with intensity playing a greater role in mitochondrial respiration. To test this hypothesis, we examined markers of skeletal muscle mitochondrial content and respiration in response to two different interval training protocols performed using single-leg cycling, which permitted a within-subjects design. Ten healthy active adults [6 males / 4 females, 22±4 y, peak oxygen uptake ($VO_{2peak}$) = 42±4 ml/kg/min] were recruited. Each leg was randomized to either a HIIT [4 × (5 min at 65% $W_{peak}$ and 2.5 min at 20% $W_{peak}$)] or SIT [4 x (30-s "all-out" sprints and 4 min active recovery)] protocol and completed three exercise sessions/wk over 4 wk for a total of 12 exercise sessions/leg. The mean work performed during each session was 133±32 and 44±8.0 kJ for HIIT and SIT respectively, and the average workload during intervals was 95±25 W and 322±77 W for HIIT and SIT respectively. Citrate synthase (CS) maximal activity increased compared to baseline after training interventions, with the change being greater after SIT vs HIIT (42±25% vs 16±13%, interaction $p=0.01$). COXIV protein content and succinate-supported state 3 were unchanged. Single-leg $VO_{2peak}$ and time to exhaustion (TTE) increased to a similar extent in both HIIT and SIT (main effect of time, $p<0.05$). These data suggest that, in contrast to what has been proposed by others, training intensity is more important than volume for increasing mitochondrial content during short-term interval training in human skeletal muscle.

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| | |
|---|---|
| ACC | Acetyl-coa carboxylase |
| ADP | Adenosine diphosphate |
| AMP-K | Adenosine monophosphate- activated protein kinase |
| ATP | Adenosine triphosphate |
| β-HAD | 3-hydroxyacyl coa dehydrogenase |
| bpm | Beats per minute |
| CAMKII | Calcium-calmodulin kinase II |
| $Ca^{2+}$ | Calcium |
| CS | Citrate synthase |
| COXIV | Cytochrome c oxidase subunit IV |
| h | Hour |
| $H^+$ | Hydrogen ion |
| HIIT | High intensity interval training |
| HR | Heart rate |
| kJ | Kilojoule |
| Km | Michaelis constant |
| MICT | Moderate-intensity continuous training |
| min | Minute |
| mitoPS | Mitochondrial protein synthesis |
| ml $kg^{-1}$ $min^{-1}$ | Milliliters/kilogram/minute |
| mRNA | Messenger ribonucleic acid |
| PGC-1α | Peroxisome proliferators-activated receptor-γ coactivator-1α |
| P38-MAPK | Mitogen-activated protein kinase p38 |
| r | Correlation coefficient |
| $R_c$ | Lin's concordance coefficient |
| RPE | Rating of Perceived Exertion |
| rpm | Revolutions per minute |
| RS-SIT | Repeated Sprint-sprint interval training |
| s | Second |
| SDH | Succinate dehydrogenase |
| SE-SIT | Spring endurance-sprint interval training |
| SIT | Sprint interval training |
| T2D | Type 2 diabetes |
| TEM | Transmission electron microscopy |
| TTE | Time to exhaustion |
| $VO_{2peak}$ | Peak oxygen uptake |
| W | Watts |
| $W_{peak}$ | Peak power output |
| y | Year |

## DECLARATION OF ACADEMIC ACHIEVEMENT

**Format and Organization of Thesis:**

This thesis is prepared in the standard format as outlined in the School of Graduate Studies' Guide for the Preparation of Master's Theses. The first chapter is a literature review and the second chapter is a draft of a manuscript for submission to a journal.

**Contributions to Content of Thesis:**

E.M. Jenkins, L.E. Skelly, C. McGlory, S.Y. Ng, D.G. McCarthy, V. Ljubicic, M.J. MacInnis, D.L. Richards, M.J. Gibala

**Contribution**

E.M. Jenkins and M.J. Gibala conceptualized and designed the study with input from L.E. Skelly, M.J. MacInnis and V. Ljubicic. E.M. Jenkins screened potential participants and facilitated participant recruitment. E.M. Jenkins facilitated data collection with assistance from M.J. Gibala, D.G. McCarthy, C. McGlory, S.Y. Ng, D.L. Richards and L.E. Skelly. E.M. Jenkins performed the data analyses with assistance from S.Y. Ng and guidance from L.E. Skelly. E.M. Jenkins and M.J. Gibala interpreted the results. E.M. Jenkins drafted the thesis version of the manuscript, with input from M.J. Gibala. All coauthors will be involved with subsequent revision of the manuscript prior to eventual submission for publication.

# Chapter 1: Literature Review

## 1.1 Introduction

Aerobic exercise training stimulates mitochondrial responses in human skeletal muscle, which facilitates an increased capacity for substrate oxidation and improved performance and metabolic health (Holloszy & Coyle, 1984). The mitochondrial reticular network continually remodels in response to various cellular stressors including those generated during skeletal muscle contraction. The initiation of a signalling cascade in skeletal muscle leads to increased expression of genes involved in mitochondrial biogenesis concurrent with increases in catabolic activity, continuously turning over the pool of mitochondria to maintain an optimal functioning network (Perry *et al.*, 2010; Egan & Zierath, 2012; Hood *et al.*, 2019). The biosynthetic pathway increases the expression of genes encoding structural and functional proteins of the mitochondria, including the enzymatic machinery of aerobic metabolism (i.e., electron transport chain) which is responsible for producing adenosine triphosphate (ATP), the energy currency of cellular activities in the human body (Granata *et al.*, 2018*a*).

The increase in enzyme concentration enhances aerobic exercise capacity, by increasing the reliance of oxidative over anaerobic metabolism and sparing glycogen stores, subsequently reducing unfavourable metabolic by-products associated with fatigue (Joyner & Coyle, 2008; Lundby & Robach, 2015). Moreover, in more aerobically trained individuals, the greater mitochondrial capacity requires less of a cellular stress signal to stimulate aerobic metabolism at a given workload, leading to higher ATP production per unit of fuel compared to anaerobic metabolism (Henriksson, 1977; Dudley *et al.*, 1987; Holloszy & Coyle, 1984). This mitochondrial response and subsequent enhancement in aerobic metabolism is associated with improvements in athletic performance (Bassett & Howley, 2000; Joyner & Coyle, 2008; Hawley *et al.*, 2018). Performance is a product of $VO_{2peak}$, lactate threshold and efficiency (Joyner &

Coyle, 2008) and although these variables are regulated by a myriad of factors, greater mitochondrial response is associated with higher $VO_{2max}$ (Jacobs & Lundby, 2013) and lactate threshold (Bassett & Howley, 2000). Therefore, individuals are able to exercise at higher percentages of their $VO_{2peak}$.

Training-induced mitochondrial responses are also associated with improvements in metabolic health. Contrarily, during periods of disuse or muscle loss the mitochondria reverse to a more fragmented state, decreasing the content and function and increasing their reliance on anaerobic metabolism (Granata & Little, 2017; Hood *et al.*, 2019). Furthermore, many pathological conditions including type 2 diabetes (T2D) demonstrate dysfunctional mitochondrial phenotypes (Berria *et al.*, 2003; Hawley, 2004; Booth *et al.*, 2012; Hood *et al.*, 2019). Skeletal muscle insulin resistance is a hallmark feature of T2D and affects metabolic processes including glucose transport, breakdown and oxidation (Hawley, 2004). Impaired oxidative capacity is associated with higher insulin resistance as evident by greater dysregulations between mitochondrial oxidative capacity and capacity for glycolysis in individuals with insulin resistance compared to healthy controls (Simoneau & Kelley, 2017). Aerobic exercise may be beneficial as training-induced increase in oxidative capacity may contribute to improved insulin sensitivity that is seen following training (Little *et al.*, 2011). Therefore, mitochondria play a pivotal role in health and performance, and its dynamic nature enables it to continuously adapt to its environment. The purpose of this review is to explore the molecular events regulating training-induced mitochondrial responses and the potential outcomes in response to different variables of training.

## 1.2 Skeletal muscle mitochondrial response to aerobic exercise

During aerobic exercise the increased cellular stress imposes a metabolic demand on mitochondria for an increase in ATP production (Perry *et al.*, 2010; Egan *et al.*, 2013). Ultimately this demand is met by increasing the content and the functional capacity of the mitochondria. This is done via expansion of pre-existing mitochondrial networks through an increase in the transcription of genes that encode proteins involved in structural and functional components of the mitochondria (Perry *et al.*, 2010; Lundby *et al.*, 2018). The expansion of the mitochondrial network induced by training requires the integration of both the nuclear and mitochondrial genomes. The mitochondrial hosts its own genome which encodes 13 proteins within the mitochondria, with the vast majority of mitochondrial proteins encoded in the nuclear genome. Following translation in the cytosol, the proteins are chaperoned and incorporated into the mitochondria (Hood *et al.*, 2006).

### 1.2.1 Effect of acute exercise on markers of mitochondrial biogenesis

Skeletal muscle contraction perturbs metabolic homeostasis and changes the concentration of signalling molecules, which include: AMP, formed through the adenylate kinase reaction under conditions of increased ATP turnover; calcium ($Ca^{2+}$), which is released from the sarcoplasmic reticulum; and reactive oxygen species, generated from muscle contraction and aerobic respiration (Hood *et al.*, 2006; Perry *et al.*, 2010). These signals activate kinases including AMP- activated protein kinase (AMPK), calcium-calmodulin kinase II (CAMKII), and mitogen-activated protein kinase p38 (p38-MAPK) (Hood, 2009). These kinases interact with peroxisome proliferators-activated receptor-$\gamma$ coactivator-1$\alpha$ (PGC-1$\alpha$), a transcriptional coactivator that serves as a critical coordinator of mitochondrial biogenesis (Hood *et al.*, 2006). PGC-1$\alpha$ interacts with transcription factors to increase expression of nuclear genes encoding

mitochondrial proteins. With repetitive training the accumulation of these transient increases in gene expression result in corresponding increases in mitochondrial protein content (Perry *et al.*, 2010; Egan *et al.*, 2013).

### 1.2.2 Exercise training-induced changes in biomarkers of mitochondrial remodelling

Training-induced mitochondrial responses can be assessed in many different ways including measuring the rate of mitochondrial protein synthesis (mitoPS) (Miller & Hamilton, 2012; Bishop *et al.*, 2019). However, the increase synthesis of mitochondrial proteins occurs in conjunction with catabolic events (i.e., mitochondrial breakdown, mitophagy, and apoptosis) and fusion and fission processes (Perry *et al.*, 2010; Hood *et al.*, 2019). Therefore, to determine a more holistic impact of training on the mitochondrial reticulum, it is helpful to assess measures of mitochondrial content and function (Bishop *et al.*, 2019). The gold standard for measuring mitochondrial content is transmission electron microscopy (TEM) to assess mitochondrial volume density (Larsen *et al.*, 2012). However the time consuming nature, expertise required, and equipment needed poses barriers for many laboratories (Larsen *et al.*, 2012). Surrogate measures include the maximal activities or protein content of mitochondrial enzymes involved in the tricarboxylic acid cycle [citrate synthase (CS)], beta-oxidation [3-hydroxyacyl CoA dehydrogenase (β-HAD)], and the electron transport chain (complexes I-V) (Larsen *et al.*, 2012).

Using a cross-sectional design, Larsen et al. (2012) sought to determine which surrogate measures exhibited the strongest correlations with mitochondrial volume measured by TEM. Cardiolipin, a component of the inner mitochondrial membrane, showed the strongest association with mitochondrial fractional area as demonstrated by an $R_C$ of 0.85 defined as "almost perfect concordance" followed by citrate synthase (CS) and complex I activities Rc= 0.80 and 0.77 respectively which meet the criteria of "substantial concordance" (Larsen *et al.*,

2012). Other markers that demonstrated a substantial concordance included complex I, II and IV activity and complex II and V protein content. Lundby et al. (2018) sought to determine whether these biomarkers were valid as measures for mitochondrial volume in response to exercise interventions. Mitochondrial volume density is typically ~4-5% of the muscle fibre area (Montero *et al.*, 2015; Lundby *et al.*, 2018). It can increase by ~40-50% following training in untrained individuals (Montero *et al.*, 2015; Lundby & Jacobs, 2013; Lundby *et al.*, 2018). Following 6 weeks of endurance training mitochondrial volume as measured by TEM increased by 52.5±12.4% in healthy males (Lundby *et al.*, 2018). Citrate synthase activity also increased; however, cytochrome c oxidase activity (COX), a marker of the inner mitochondrial membrane, did not increase. Correlations between mitochondria volume and markers of content were made at pre and post-training time points, whereby only CS activity and volume were correlated at both time points (Lundby *et al.*, 2018). However, there was no correlation for the exercise-induced change between mitochondrial volume and markers of content.

Aerobic exercise training also induces increases in skeletal muscle mitochondrial respiratory function (i.e., the rate of oxygen consumption/gram of tissue) due to the biosynthesis of mitochondrial enzymes involved in oxidative metabolism (Jacobs *et al.*, 2013). However, training-induced increases in respiratory function have also been established independent of increases in the content of mitochondria (Hood *et al.*, 2019; Jacobs & Lundby, 2013). Numerous methods have been used to measure mitochondrial respiration, including isolated mitochondria in vitro analysis, however the process of isolation impacts the ability to analyze the mitochondria with the surrounding environment (Kuznetsov *et al.*, 2008). Contrarily, *in vivo* analyses of intact cells enable measurements of mitochondria and their environment. Although the scope of analysis is limited as the cell membrane is not penetrated and mitochondrial reactions regarding

various substrates cannot be studied (Kuznetsov *et al.*, 2008). Analysis of mitochondria in situ by selective plasma membrane permeabilization through the addition of specific substrate and inhibitors overcomes the aforementioned limitations, as physiologically relevant processes within the mitochondria can be analyzed while limiting disruption to the surrounding area. Mitochondrial respiratory measurements of the in situ model are not limited to but include; ADP affinity, membrane integrity, measurement of complex specific electron flux, maximal state 3 respiration oxidative phosphorylation capacity, electron transport system capacity (Jacobs *et al.*, 2013; MacInnis *et al.*, 2017*c*; Lundby *et al.*, 2018). Mitochondrial respiration can be represented as mass-specific, or it can be normalized to a marker of mitochondrial protein content known as mitochondria-specific to determine improvements in mitochondria function independent of changes in content. The ability to use multiple techniques to measure acute and chronic measures of mitochondrial content and respiration allow for a holistic approach and view of the mitochondrial response to exercise.

## 1.3 The nature of the exercise stimulus and skeletal muscle mitochondrial responses

### 1.3.1 Terminology: volume, intensity, duration and frequency

There are multiple variables of aerobic training that can be manipulated to facilitate mitochondrial remodelling. The total volume of training is a product of the frequency, intensity and duration of training and facilitates mitochondrial remodelling when increased (Bishop *et al.*, 2014; Granata & Little, 2017; MacInnis & Gibala, 2017; Jacobs & Lundby, 2018). Recently, research has focused on the impact of intensity on training-induced mitochondrial remodelling. Interval training, which involves alternating periods of intense exercise and recovery, has been used to examine the role of intensity on training-induced responses. Two common forms of interval training are high intensity interval training (HIIT) and sprint interval training (SIT). HIIT

and SIT are commonly compared to traditional endurance training, often referred to as moderate-intensity continuous training (MICT), which consists of continuous exercise carried out below 75% $W_{peak}$ (Bishop *et al.*, 2019). HIIT is generally defined as submaximal exercise that elicits a heart rate of at least 80% of age-predicted maximal heart rate ($HR_{max}$) (Macinnis & Gibala, 2017) and/or eliciting intensities above 75% $W_{peak}$ (Bishop *et al.*, 2019). HIIT is often matched to MICT for total volume of work completed. Whereas SIT involves 'all-out' or 'supramaximal' efforts that exceed the absolute workload that elicits $VO_{2peak}$. Due to the all-out nature of SIT, it often involves a lower total volume of work compared to MICT and HIIT (Weston *et al.*, 2013; MacInnis & Gibala, 2017; Bishop *et al.*, 2019).

1.3.2 The role of intensity on acute markers of mitochondrial remodelling

Intensity is a key factor that influences the mitochondrial response to training. Egan et al. (2010) demonstrated an intensity-dependent regulation of the underlying signaling pathway involved in mitochondrial biogenesis (Egan *et al.*, 2010). On two separate occasions eight sedentary males cycled at either 80% $VO_{2peak}$ (HI) or 40% $VO_{2peak}$ (LO) with the two trials matched for total work. The HI condition elicited a greater increase in PGC-1α mRNA expression compared to the LO condition despite being matched for total volume. The authors speculated that the HI condition elicited a greater cellular stress signal as supported by a greater increase in the phosphorylation of AMPK. Furthermore, the HI condition elicited a greater increase in CAMKII phosphorylation, which was attributable to a greater intensity of skeletal muscle contraction and $Ca^{2+}$ release. The authors demonstrated that CAMKII and AMPK are regulated in an intensity dependent manner and may contribute to the increase in PGC-1α mRNA expression. Nevertheless, inconsistencies within the literature remain regarding the effect of intensity on acute signaling molecules.

A study employing three different intensities of 73, 100 and 133% $W_{peak}$ all matched for total volume of work, demonstrated that PGC-1α mRNA expression was significantly greater following exercise performed at 100%$W_{peak}$ condition compared to 73 and 133%$W_{peak}$, suggesting that the intensity-dependent regulation of PGC-1α mRNA expression does not hold true for supramaximal exercise (Edgett *et al.*, 2013). In support of these findings, a SIT protocol consisting of 8x20-s at 170%$W_{peak}$ had similar increases in the phosphorylation of acetyl-CoA carboxylase (ACC), an indicator of AMPK activity, compared to higher volume MICT (Scribbans *et al.*, 2014). However, in contrast to Edgett et al (2013), the SIT protocol required a lesser total training volume demonstrating that the intensity of exercise may compensate for lower training volume. While an acute bout of SIT consisting of 4×30-s "all-out" efforts elicited greater molecular signaling compared to higher-volume MICT, as measured by the phosphorylation of p53, a transcription factor involved in mitochondrial biogenesis (Granata *et al.*, 2017). Which demonstrates that supramaximal exercise may be a greater stimulus for inducing molecular events involved in training-induced mitochondrial remodelling compared to lower intensity higher volume exercise (Granata *et al.*, 2017). Overall, comparing the results of these studies is complicated by the use of different training protocols, the manipulation of different variables of training (intensity and/or volume) and measuring different markers of mitochondria remodelling. Interestingly, Fiorenza et al. (2018) examined whether the extent of exercise-induced metabolic perturbation/cellular stress (i.e., muscle lactate and $H^+$ accumulation) effects the molecular response. In a randomized cross-over manner trained cyclists performed three conditions: two SIT protocols matched for work but aimed to elicit differing levels of cellular stress, and a higher volume MICT session. Sprint endurance (SE-SIT) which consisted of 6 × 20-s "all-out" efforts elicited greater metabolic stress than work matched repeated sprints

(RS-SIT) which involved 18 × 5-s "all-out", and MICT carried out at 70%$VO_{2peak}$ for 50-min. SE-SIT led to greater exercise-induced increases of p-CAMKII and p38-MAPK compared to RS-SIT and MICT. Their results suggested that for a given volume and intensity of exercise, greater metabolic stress mediates events of mitochondrial biogenesis and that intensity may compensate for lesser volume (Fiorenza *et al.*, 2018).

1.3.3 The role of training intensity on markers of mitochondrial remodelling

When total volume of work is matched, HIIT has been shown to elicit superior (Daussin *et al.*, 2008; Tjønna *et al.*, 2008; Macinnis *et al.*, 2017*c*) or similar (Henriksson & Reitman, 1976; Baekkerud *et al.*, 2016) mitochondrial responses compared to MICT, based on measures of content (i.e., CS, PGC-1α protein content) and function (mass- and mitochondria-specific respiration). MacInnis et al. (2017c) examined the effect of intensity using a single-leg cycling protocol, with each leg subjected to 6 sessions of either HIIT (4x5-min at 65% $W_{peak}$) or work-matched MICT (30-min at 50%$W_{peak}$) in a randomized manner. They demonstrated a greater increase in CS maximal activity and mass-specific respiration following HIIT compared to MICT, suggesting that intensity is a regulating factor for training-induced mitochondrial remodelling. This is supported by two studies examining the impact of work-matched HIIT and MICT. They demonstrated greater increases in mitochondrial content measured by PGC-1α protein content (Tjønna *et al.*, 2008) and respiration measured by maximal ADP stimulated respiration (Daussin *et al.*, 2008). However, two studies also employing work-matched protocols demonstrated equivalent responses between work-matched HIIT and MICT, potentially due to differences in participant training status (Baekkerud *et al.*, 2016), and small sample size (Henriksson & Reitman, 1976).

SIT protocols often involve a lower total volume of work compared to HIIT and MICT. Despite the reduced volume, similar increases in markers of mitochondrial content have been reported is studies involving comparisons of SIT vs MICT (Gibala *et al.*, 2006; Burgomaster *et al.*, 2008; Gillen *et al.*, 2016). The findings from these studies support that intensity is a mediating factor in training-induced mitochondrial remodelling, and that higher intensity exercise may compensate for the lower volume in inducing mitochondrial adaptations (Fiorenza *et al.*, 2018). However, few studies have examined whether supramaximal SIT further augments training-induced mitochondrial remodelling compared to near maximal HIIT. Granata et al. (2016) directly compared between two interval protocols of varying intensity on markers of mitochondrial content and respiration. In a between-subject design, twenty-nine healthy active males were randomly assigned to one of three training groups: MICT; 20-36-min at ~65 $\%W_{peak}$, HIIT; 4–7 x 4-min intervals at ~90$\%W_{peak}$ or SIT; 4–10 x 30-s all-out bouts at~200% $W_{peak}$. Each group performed 12 training sessions over 4 weeks and the MICT and HIIT group were matched for total volume of work. The authors reported that CS maximal activity remained unchanged throughout in all groups whereas mitochondrial respiration (mass- and mitochondrial specific) increased only after 4 weeks of SIT (Granata *et al.*, 2016). They suggested that there is a dissociation between mitochondrial content and respiration in response to intense exercise, such that intensity plays a greater role in altering mitochondrial respiration compared to content.

### 1.3.4 Intensity vs Volume: the potential diverging effect on mitochondrial content and respiration

Early work from Holloszy et al. (1984) demonstrated that the increase in the activity of respiratory chain enzymes was due to an increase in mitochondrial enzyme content. However, work since then has shown that increases in respiratory capacity is not always dependent on changes in mitochondrial content but due to improvements in the intrinsic function of the

organelle (Granata *et al.*, 2016; Holloway, 2017; Jacobs & Lundby, 2013). Enhanced mitochondrial respiratory functions may be due to improvements in specific control points for substrate and ADP transport across the mitochondrial membrane (Holloway, 2017), improved ADP sensitivity (lower Km) (Miotto *et al.*, 2018), and an increase in mitochondrial coupling efficiency (Holloway, 2017). As mentioned previously, it is speculated that intensity plays a greater role in mitochondrial function, whereas volume plays a greater role in increases in content (Granata *et al.*, 2016). Studies investigating intensity on mitochondrial respiration have demonstrated an intensity dependent relation. Work-matched HIIT elicited increases in mass-specific respiration, whereas there was no change observed in MICT (Macinnis *et al.*, 2017*c*). SIT protocols have also demonstrated superior increases in mitochondrial function assessed by succinate dehydrogenase (SDH) (Daussin *et al.*, 2008) and mass/mitochondrial- specific respiration (Granata *et al.*, 2016) despite lesser volume, supporting an intensity dependent regulation on mitochondrial respiration.

Granata et al. (2018) examined the relation between training volume and changes in mitochondrial content as assessed by CS activity. They discovered a moderate correlation (r=0.71) between training volume and CS when exercise was carried out at submaximal intensities ($<W_{peak}$). However, studies demonstrating greater increases in mitochondrial content in HIIT vs work matched MICT are in contrast to the thought that volume is the prominent factor below maximal intensities (Tjønna *et al.*, 2008; MacInnis *et al.*, 2017*c*). Furthermore, the correlation was weakened to r=0.59 when including training studies eliciting intensities $>W_{peak}$ and no correlation was exhibited (r=-0.19) when comparing only studies of intensities $>100\%W_{peak}$ (Granata *et al.*, 2018*b*). This is supported by low-volume SIT protocols that lead to comparable increases in mitochondrial content to MICT despite a lesser volume (Gibala *et al.*,

2006; Burgomaster *et al.*, 2008; Gillen *et al.*, 2016). On the other hand, they did not find a significant correlation between intensity and training induced changes in mitochondrial content. Once again, this contrasts studies which demonstrate greater increases in markers of mitochondrial content following HIIT compared to work-matched MICT (Tjønna *et al.*, 2008; MacInnis *et al.*, 2017*c*). It is difficult to interpret the findings from different studies as each comparison was unique regarding differing intensities and volumes used, thus it remains equivocal whether intensity plays a mediating role in enhancing mitochondrial content.

### 1.4 Single-leg cycling as a model to study skeletal muscle response to exercise

In exercise training studies, a within-subject experimental designs is advantageous, as the same population is exposed to all conditions, decreasing unsystematic variation (variation due to random factors unrelated to experimental manipulation) (Fields, 2000). Within-subject designs in training studies, however, can be challenging owing to large time commitments and the need for subjects to effectively "detrain" after the first intervention to a similar baseline level. Unilateral exercise training models — while not without some limitations — allow for both conditions to be examined simultaneously, decreasing time commitments and other potential confounding effects of repeated measures designs (Macinnis *et al.*, 2017*a*).

#### 1.4.1 Single-leg cycling to examine the impact of intensity

During whole body exercise that requires large muscle groups (i.e., double-leg cycling, running etc.), the muscle cells capacity to utilize oxygen from the blood exceeds the capacity of the central circulation to deliver blood (Perfusion *et al.*, 1985; Klausen *et al.*, 2017). With single-leg cycling, a lower total amount of muscle volume is recruited therefore a greater proportion of blood is directed toward the active muscle compared to double-leg cycling (Andersen & Saltin, 1985; Burns *et al.*, 2014). This increases the normalized intensity of the cycling leg (i.e., power

output/number of active legs) (Abbiss *et al.*, 2011; MacInnis *et al.*, 2017b) and subsequently the potential for greater mitochondrial adaptation compared to regular cycling (Abbiss *et al.*, 2011). This provides an ideal mode of exercise to measure the impact of intensity on mitochondrial adaptation.

Single-leg cycling has been used previously to examine the effect of different intensities of training on mitochondrial adaptation. Saltin et al. (1976) examined the impact of two types of training; SIT (20-30 x 40-50s at 150% single-leg $VO_{2peak}$ with 90-sec rest) and MICT (35-45 min at 75% single-leg $VO_{2peak}$), which both demonstrated increases in SDH activity. Furthermore, they demonstrated a lowering of submaximal heart rate and blood lactate values, and increase in single-leg $VO_{2peak}$ in the trained leg compared to the untrained leg demonstrating an interplay between the peripheral and central system (Andersen & Saltin,1976). MacInnis et al (2017c) examined whether work-matched HIIT and MICT led to differences in mitochondrial adaptation. The authors concluded that HIIT led to superior changes compared to MICT, and demonstrated that the single-leg model is suitable in investigating the role of intensity on training induced mitochondrial adaptation.

### 1.4.2 Counter-weight single-leg cycling

Single-leg cycling can be performed on a traditional single-leg cycling model (Bell & Wenger, 2004; nersen & Saltin, 1976), however modifications have been made to better emulate double-leg cycling. During traditional single-leg cycling, the lack of weight from the opposite leg to bring the foot back up to top dead center leads to greater recruitment of the hip flexor muscle group. This elicits greater ratings of perceived exertion, HR, and $VO_2$ compared to double-leg cycling (Burns *et al.*, 2014). The addition of the counter-weight on the contralateral crank helps preserve similar biomechanics of double-leg cycling (Abbiss *et al.*, 2011; Burns *et*

*al.*, 2014; MacInnis *et al.*, 2017*b*). Specifically, it helps with the upward phase of the active leg reducing the reliance on the hip-flexors, subsequently leading to similar $VO_2$, RPE and HR values to double-leg cycling. Furthermore, counter-weighted single-leg cycling was perceived as more tolerable compared to traditional single-leg cycling (Burns *et al.*, 2014). The counter-weight model has also been used in single-leg modified Wingate's, demonstrating its application at all intensity levels (Draper, 2018).

Purpose

Few studies have compared the effect of different intensities of exercise on mitochondrial responses, and specifically whether SIT and HIIT differentially affect markers of mitochondrial content and respiration. There are limited and equivocal data regarding the potential for different interval training protocols to differentially affect changes in mitochondrial content and respiration. This may be owing in part to different methodological practices between studies (e.g., with respect to protocol intensities or the training status of participants) as well as inter-individual differences in responses related to between-subject designs. Further research comparing different intermittent exercise protocols, ideally using a within-subject design, would enhance our understanding of whether the intensity of exercise leads to a dissociated response between mitochondrial content and respiration. The primary purpose of this study is to compare markers of skeletal muscle mitochondrial content and respiration in response to two training protocols performed at different intensities (i.e., HIIT and SIT). We will use a counter-weighted single-leg cycling model previously established in our laboratory that facilitates a within-subject designs and eliminates inter-individual variability in assessing the response (MacInnis *et al.*, 2017*a*).

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# Chapter 2: Human Skeletal Muscle Mitochondrial Response To Interval Training: Role Of Exercise Intensity

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## Introduction

Regular aerobic exercise stimulates mitochondrial biogenesis and subsequent remodeling of human skeletal muscle, which facilitates improved performance and metabolic health owing to an enhanced capacity for oxidative metabolism (Holloszy & Coyle, 1984; Hood *et al.*, 2019). Training-induced responses are commonly assessed by measuring mitochondrial content, with the gold standard measure being transmission electron microscopy to determine mitochondrial volume density (Larsen *et al.*, 2012; Lundby *et al.*, 2018). However the need for specialized equipment, expertise and cost poses a barrier for many laboratories (Larsen *et al.*, 2012). Citrate synthase maximal activity (CS) is commonly used as a surrogate measure for mitochondrial content, as it is strongly associated with mitochondrial volume in human skeletal muscle (Larsen *et al.*, 2012). Another biomarker involves the analysis of mitochondria *in situ* by selective plasma membrane permeabilization to determine mitochondrial respiration. This measure can be expressed as mass-specific, or it can be normalized to mitochondrial content to assess potential improvements in mitochondria-specific function, independent of changes in content.

Training-induced mitochondrial responses are highly dependent on the nature of the exercise impulse, which in turn depends on duration, frequency and intensity as well as the total volume of work performed (Macinnis & Gibala, 2016; Granata & Little, 2017). Intensity has been shown to be a key factor involved in the activation of signaling pathways associated with mitochondrial biogenesis (Egan *et al.*, 2010*b*). Interval training, which involves alternating periods of relatively intense exercise and recovery within a given session, has also been used to examine the role of intensity on training-induced responses (MacInnis & Gibala, 2017). Two common forms of interval training are high intensity interval training (HIIT), which is commonly described as submaximal efforts performed at an intensity that elicits at least 80% of age-predicted maximal

heart rate, and sprint interval training (SIT), a more intense version characterized by 'all-out' or 'supramaximal' efforts that exceed the absolute workload that elicits $VO_{2max}$ (Weston *et al.*, 2013; Macinnis & Gibala, 2016; Bishop *et al.*, 2019). HIIT and SIT are commonly compared to traditional endurance training, often referred to as moderate-intensity continuous training (MICT), which typically involves exercise performed below 75% of peak power achieved on a graded exercise test ($W_{peak}$) (Bishop *et al.*, 2019). HIIT is often matched to MICT for total volume of work completed, whereas SIT often involves a lower total volume due to the all-out nature of the sprints (MacInnis & Gibala, 2017; Bishop *et al.*, 2019). MacInnis et al. (2017b) demonstrated a greater increase in CS maximal activity and mass-specific respiration following HIIT compared to work-matched MICT using a within subject design. Their results suggest that intensity is a key regulating factor for mitochondrial remodelling when total volume of work is matched, a suggestion supported by others (Daussin *et al.*, 2008; Tjønna *et al.*, 2008). It has also been demonstrated that low-volume SIT protocols lead to increases in CS comparable to higher-volume MICT despite a lower volume of work (Gibala *et al.*, 2006; Burgomaster *et al.*, 2008; Gillen *et al.*, 2014). These results are in line with MacInnis et al. (2017) that intensity is a determining factor in mitochondrial adaptation.

Granata et al. (2016) further examined the effect of intensity on mitochondrial content and respiration. In a between-subject design, participants were randomly assigned to one of three training groups of varying intensities: MICT, work-matched HIIT, or low-volume SIT (Granata *et al.*, 2016). The authors reported that citrate synthase activity remained unchanged throughout in all groups whereas mitochondrial respiration (mass- and mitochondrial specific) increased only after 4 weeks of SIT (Granata *et al.*, 2016). It was suggested that there is a dissociation between mitochondrial content and respiration in response to intense exercise, such that intensity

plays a greater role in altering mitochondrial respiration compared to content (Granata *et al.*, 2016).

Few studies have directly compared the effect of different intensities of exercise on mitochondrial content and respiration. The inconsistencies in the literature may be due to different methodological practices between studies (i.e., protocol intensities/volume, participants training status). Furthermore, the influence of inter-individual difference in between-subject designs may potentially limit the ability to detect changes in mitochondrial adaptation. Further research comparing different intensities within-subjects is needed to determine whether there is a dissociation between mitochondrial content and respiration in response to the intensity of exercise. The primary purpose of this study is to compare markers of skeletal muscle mitochondrial content and respiration, to two training protocols performed at different intensities (i.e. HIIT and SIT). We hypothesized that HIIT and SIT will induce similar increases in markers of mitochondrial content and mitochondrial respiration. That is, there would not be a divergent response of mitochondrial content and mitochondrial respiration to the two interventions, as suggested by others (Granata, 2016). We employed a counter-weighted single-leg cycling model previously established in our laboratory to facilitate a within-subject design (Macinnis *et al.*, 2017*b*) and thus eliminate the potential interindividual variation in response, which is a potential confounding variable inherent to between-group designs.

**Methods:**

*Participants:*

Ten healthy active adults were recruited (22±4 y;  6 men, 4 women). All participants were habitually active but not training specifically for any sport. Participants completed a Get Active Questionnaire and provided written informed consent prior to their participation. The Hamilton Integrated Research Ethics Board approved the protocol.

*Pre-training procedures*

Participants initially performed a ramp test to exhaustion on an electronically-braked cycle ergometer (Excalibur Sport, version 2.0; Lode, Groningen, The Netherlands) to determine whole-body peak oxygen uptake ($VO_{2peak}$) and peak power output ($W_{peak}$) as we have previously described (Macinnis *et al.*, 2017*b*). Briefly, after a 2-min warm-up at 50 W, workload was increased 1 W every 2 s until the participant reached volitional exhaustion or cadence fell below 60 rpm. Expired gases were collected and analyzed using an online system (Cosmed Quark CPET; Rome, Italy) and $VO_{2peak}$ was determined from the highest 30-s average. The $VO_{2peak}$ and $W_{peak}$ for the group was 42 ±4 mL $kg^{-1}$ $min^{-1}$ and 268 ± 61 W, respectively.

At least 48 h following the double-leg $VO_{2peak}$ test, participants returned to the laboratory to perform a single-leg $VO_{2peak}$ during counter-weighted single-leg cycling performed on an electronically braked cycle ergometer (Veletron; RacerMate, WA, USA) as we have previously described (Macinnis *et al.*, 2017*b*). Briefly, one crank on the ergometer was fitted with a custom-machined pedal that held an 11.4 kg counterweight while the non-exercising leg rested on a stationary platform. The single-leg test was conducted in the same manner as the double-legged test, except the ramp workload phase involved an increase of 1W every 4 s. One test was conducted on each leg separated by a 10-min break. The order of legs was randomized using an allocation concealment procedure (Macinnis *et al.*, 2017*b*), such that half of the legs to be allocated to each of the two training conditions were tested first, i.e., 5 for HIIT and 5 for SIT. Baseline single-leg $VO_{2peak}$ values were 29.9±5.9 and 30.1±6.2 ml/kg/min for the HIIT and SIT conditions respectively, with no difference between legs (p=0.96). The corresponding single-leg $W_{peak}$ values were 144±38 and 143±36 W for HIIT and SIT respectively, with no difference between conditions (p=0.85)

At least 48-h following the single-leg ramp test, participants performed single-leg time to exhaustion tests (TTE) at a workload corresponding to 70% $W_{peak}$ based on the mean of the two baseline single-leg $VO_{2peak}$ tests. TTE was defined by volitional exhaustion or the point when cycling cadence dropped below 60 rpm. Single-leg time to exhaustion was 1042±571 s and 1013±423 s for HIIT and SIT, respectively, and not statistically different between conditions ($p=0.95$).

At least 24-h following the single-leg $VO_{2peak}$ and TTE tests, subjects completed a familiarization session that involved both HIIT and SIT protocols, with each leg performing half of the total work to be performed during the main training session.

Approximately 72-h following the familiarization session, a needle biopsy sample was obtained from the vastus lateralis muscle of one thigh, which was selected in a manner to ensure an equal distribution of baseline biopsies from the HIIT and SIT condition. Participants were instructed to refrain from exercise, alcohol and food for a minimum of 48, 24 and 10-h, respectively and participants recorded a 24-h diet log before biopsy procedure. Samples were collected under local anaesthesia (1% xylocaine) using a Bergström needle modified for manual suction as described previously (Tarnopolsky *et al.*, 2011). Each sample was immediately sectioned into several pieces. One piece placed in ice-cold buffer for the measurement of mitochondrial respiration and the remaining pieces were immediately frozen in liquid nitrogen and stored at –80°C for subsequent analyses.

*Exercise Training Intervention*

All training was performed on the same cycle ergometer adapted for single-leg cycling. Participants completed 3 sessions per week over 4-wk for a total of 12 training sessions per leg. Legs were trained on the same day in an alternating order with 10-min in between sessions. The SIT protocol consisted of a 5-min warm-up, followed by 4 x 30-s bouts of all-out cycling against

resistance set at 0.054 kg/kg body mass interspersed with a 4-min active recovery followed by a 2-min cool-down at 25W. Fifteen seconds prior to the onset of the sprint, participants were encouraged to increase their cadence to reach a maximal value in the 5 seconds preceding the onset of the sprint. The HIIT protocol consisted of a 5-min warm up, followed by 4 x 5-min intervals at 65% of the average single leg $W_{peak}$, interspersed with 2.5-min of active recovery (at 20% $W_{peak}$). Heart rate was measured continuously (Polar Electro, Kempele, Finland) and ratings of perceived exertion were measured following each interval (Borg Rating of Perceived Exertion Scale, 6–20) (Borg, 1990). Blood lactate was measured via finger prick before exercise, and following each interval during the first training session for each protocol (Lactate Plus;Nova Biomedical, Mississauga, ON, Canada).

*Post-training Measurements*

Bilateral muscle biopsies were obtained approximately 72-h following the last training session. Approximately 48-72-h after the biopsy procedure, participants performed single-leg $VO_{2peak}$ test with each leg, and this was followed ~48-h later by two single-leg TTE tests using the same workload as for the pre-training tests. All post-training testing conditions were conducted in a manner identical to baseline testing.

**Muscle Analysis**

*Citrate synthase activity:*

One piece of muscle (~25 mg) was homogenized in Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA) using the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, USA) for 6 x 5-s cycles at a speed of 4 m/s with samples placed on ice for 5-min in between cycles. Samples were homogenized in 20 volumes of buffer containing 70 mM sucrose, 220 mM mannitol, 10 mM HEPES supplemented with protease inhibitors (Complete Mini, Roche Applied Science, Laval, PQ, Canada) and used to determine the maximal activity of citrate synthase as

we have previously described (MacInnis *et al.*, 2017*b)*. Protein concentration of homogenates was determined using a commercial assay (BCA Protein Assay, ThermoFisher Scientific, Waltham, MA, USA).

*Western blotting:*

A piece of muscle (~30 mg) was homogenized in RIPA buffer using Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA) with a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, USA). Samples were processed for 6x20-s cycles at 4.0 m/s, with samples placed on ice for 5-min in between cycles. All samples were run on 4–15% Criterion TGX Stain-Free protein gels (BioRad, Hercules, CA, USA) at 200 V for 45-min. A protein ladder (Fermentas PageRuler Prestained Ladder, ThermoFisher Scientific, Waltham, MA,USA) and a calibration curve (e.g. 2,4,8,16 µl) of pooled whole 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 5.2.1 (Bio-Rad, Hercules, CA, USA). Proteins were then transferred using the Trans-Blot Turbo transfer system in transfer buffer (Bio-Rad). Proper transfer was visualized with UV activation of the gel and membrane post-transfer (StainFree Imager; Bio-Rad). Membranes were then rocked at medium speed in blocking buffer (5% skim milk in Tris-buffered saline-Tween; TBST) for 1-h at room temperature. After rinsing in TBST, membranes were placed in solutions of primary antibodies (Total OXPHOS Human WB Antibody Cocktail; dilution 1:1000; 4844, lot no. 0003; abcam lot K1862, Cambridge, UK) and then incubated for 2-h at room temperature and overnight at 4°C on rockers. After washing in blocking buffer, membranes were incubated in goat anti-mouse IgG HRP secondary antibody (7076; dilution 1:10 000 in blocking buffer; Cell Signaling) for 1-h at room temperature. Following a final wash in TBST, membranes were exposed to Supersignal West Femto (Pierce), imaged (ChemiDoc MP; Bio-Rad) and analysed (ImageLab, version 5.2.1; Bio-Rad).

*Mitochondrial respiration in permeabilized muscle fibres:*

Mitochondrial respiration from the left and right legs of subjects were concurrently determined with Oxygraph-2K respirometer (Oroboros, Innsbruck, Austria). Following the muscle biopsy, ~10mg of muscle tissue was sectioned and immediately placed in ice-cold biopsy preservation solution (BIOPS; 10mM Ca-EGTA solution, 5.77mM ATP, 6.56mM $MgCl_2$, 20mM taurine, 15mM phosphocreatine, 20mM imidazole, 0.5mM dithiothreitol, and 50mM MES hydrate, pH 7.2). Muscles fibers were mechanically separated into fiber bundles underneath a microscope with fine tip antimagnetic forceps. To permeabilize the samples, ~2mg fiber bundles were incubated in BIOPS solution with saponin (30µg/µl) for 30-min on a mechanical rotor. Following permeabilization, samples were incubated in mitochondrial respiration medium (MiR05; 0.5mM EGTA, 3mM $MgCl_2$, 60mM lactobionate, 20mM taurine, 10mM $H_2PO_4$, 20mM HEPES, 110mM Sucrose, 1g/L bovine serum albumin, pH 7.2) for 15-30-min at 4ºC until measurements were initiated.

Samples were blotted dry on a Kimwipe with a wet weight of ~2mg and then added to the respirometer chamber containing 2ml of MiR05 and blebbistatin (5µM). All measurements were performed at 37ºC and at oxygen concentrations >200nmol/ml. For ADP-stimulated respiratory kinetics, 5mM pyruvate and 2mM malate were added to stimulate complex 1 followed by titrations of ADP (25µm, 250µM, 500µM, 2.5mM, 5mM, 7.5mM, and 10mM). Succinate (20mM) was then added to saturate electron entry into complex II to demonstrate complex I and II supported respiration. Cytochrome c (10µM) was added to verify mitochondrial membrane integrity, which induced a <10% increase in respiration in all trials, suggesting that mitochondrial membranes were intact.

For the analysis of the respiration data, oxygen flux was determined from the derivative of the oxygen concentration of the chamber using DatLab 6.1.0.7 (Oroboros) and expressed as mass-

specific (pmol $O_2$/s/mg wet weight). During certain experimental trials, an absence of complex I specific respiration was observed and excluded from the analysis. Succinate-supported state 3 respiration has been previously reported (Hughes et al., 2015) and determined by the difference between the addition of succinate and the previous substrate addition. These values, presented in the current study, were corrected by subtracting the residual oxygen consumption value (leak respiration) for each sample. Respiration values were also normalized to CS activity to determine mitochondria-specific oxygen flux (MacInnis et al., 2017b; Jacob and Lundby, 2013; Jacobs et al., 2013).

**Statistical Analysis:**

Muscle data were analyzed using a one-way analysis of variance (ANOVA) with Pre, HIIT and SIT as the three conditions. TTE, single-leg $VO_{2peak}$ data were measured using a two-way repeated measures ANOVA with condition (SIT vs HIIT) and time (pre- and post-training) as the two factors. HR, RPE and total work collected during each training session were averaged across sessions and analyzed using a two-way, repeated measures ANOVA with condition and interval (1-4) as the factors. Other data (blood lactate concentrations, total work) was compared across groups using paired Student's *t* test. Statistical significance was set at $P \leq 0.05$. Significant effects based on ANOVA were analyzed further using Holm-Sidak post hoc test. All data analyses were performed with Prism, version 6.0 (GraphPad Software, La Jolla, CA, USA). All data are reported as mean±SD. All data represent n=10, except CS (n=9) owing to the removal of one participant who was deemed a statistical outlier (> ±2 SD).

**Results:**

*Training characteristics*

*Work and intensity*

Mean work per session was 133±32 and 44±8.0 kJ for HIIT and SIT respectively ($p<0.01$). Total work completed was 28.4±7.3 kJ during HIIT intervals 1-4, the amount of work completed was 10.2 ±2.5, 9.6±2.5, 9.4±2.2, 9.3±2.2 kJ during SIT intervals 1-4 respectively. The amount of work completed during HIIT was greater than SIT ($p<0.01$). Mean workload was 95±25 W during the 5-min HIIT intervals. Mean workload during the 30-s SIT intervals was 343±84, 320±84, 314±73, 311±745W during interval 1-4 respectively. Mean workload was greater during the 30-s SIT intervals compared to 5-min HIIT intervals ($p<0.01$). Mean workload and work completed during HIIT and SIT intervals are summarized in Table 1. Figure 1 displays a typical mean HR and work during a representative HIIT and SIT session.

*Blood lactate*

Mean lactate was 5.2±1.7, 8.1±3.0, 7.8±3.0, 8.8±3.0 mM during HIIT intervals 1-4 respectively and 7.2±1.9, .3±2.3, 11.7±3.3, 10.8±3.6 mM during SIT intervals 1-4 respectively. Blood lactate was higher during SIT intervals compared to HIIT ($p<0.01$) (n=9). Lactate values increased across the training session in both HIIT and SIT ($p<0.01$) (Table 1).

*Ratings of perceived exertion*

Mean RPE was 14.1±1.4, 14.9±1.1, 15.4±0.93, 15.7±0.87 during HIIT intervals 1-4 respectively and 16.2±1.1, 17.4±0.97, 17.9±0.93, 18.3±0.92 during SIT intervals 1-4 respectively. SIT elicited greater RPE compared to HIIT ($p<0.01$) (n=10). RPE increased across the session in both HIIT and SIT ($p<0.01$) (Table 1).

*Heart rate*

Heart rate is displayed as a percentage of $HR_{max}$ achieved on the double leg $VO_{2peak}$ test. Mean heart rate represents the average HR across the interval. Mean HR was 78±5, 80±5, 81±4,

81±4% during HIIT intervals 1-4 respectively, mean HR was 83±5, 84±5, 86±6, 86±6% during SIT. Mean HR was significantly greater during SIT compared to HIIT ($p<0.01$). Peak HR was the highest HR attained during an interval. Peak HR was 84±5, 86±5, 86±4, and 87±4% during HIIT intervals 1-4 respectively, peak HR was 89±4, 90±5, 90±5, and 91±5% during SIT intervals 1-4 respectively. Peak HR attained during each interval was significantly greater during SIT compared to HIIT ($p<0.01$). HR increased across the session in both HIIT and SIT ($p<0.01$) (Table 1, Figure 1).

Table 1: Training Characteristics

| Variable | Condition | Bout 1 | Bout 2 | Bout 3 | Bout 4 | p values |
|---|---|---|---|---|---|---|
| Heart Rate, mean | HIIT | 78±5% | 80±5% | 81±4% | 81±4% | <0.01, <0.01, |
| | SIT | 83±5% | 84±5% | 86±6% | 86±6% | <0.26 |
| Heart Rate, peak | HIIT | 84±5% | 86±5% | 86±4% | 87±4% | <0.01, <0.01, |
| | SIT | 89±4% | 90±5% | 90±5% | 91±5% | <0.41 |
| RPE (6-20) | HIIT | 14.1±1.4 | 14.9±1.1 | 15.4±0.93 | 15.7±0.87 | <0.01, <0.01, |
| | SIT | 16.2±1.1 | 17.4±0.97 | 17.9±0.93 | 18.3±0.92 | <0.01 |
| Intensity (W) | HIIT | 95±24 | 95±24 | 95±24 | 95±25 | <0.01, <0.01, |
| | SIT | 343±84 | 320±84 | 314±73 | 311±75 | <0.01 |
| Work (kJ) | HIIT | 28.4±7.3 | 28.4±7.3 | 28.4±7.3 | 28.4±7.3 | <0.01, <0.01, |
| | SIT | 10.2 ±2.5 | 9.6±2.5 | 9.4±2.2 | 9.3±2.2 | <0.01 |
| Lactate (mM) | HIIT | 5.2±1.7 | 8.1±3.0 | 7.8±3.0 | 8.8±3.0 | <0.01, <0.01, 0.16 |
| | SIT | 7.2±1.9 | 9.3±2,3 | 11.7±3.3 | 10.8±3.6 | |

P values denote main effect of bout, main effect of condition, and interaction, respectively.

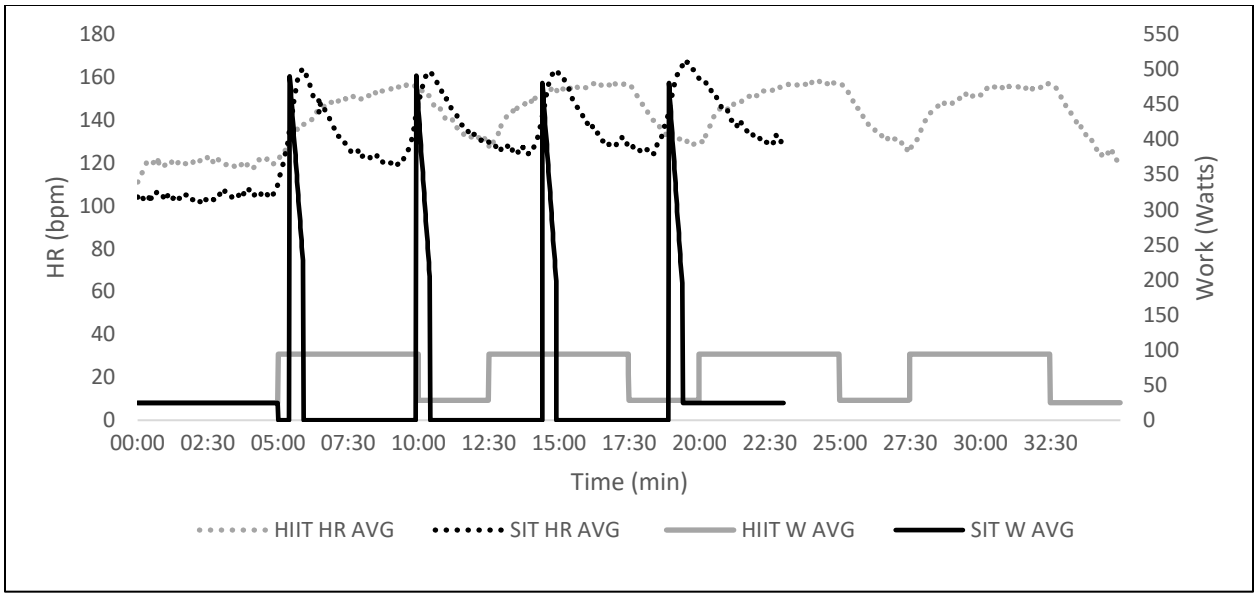
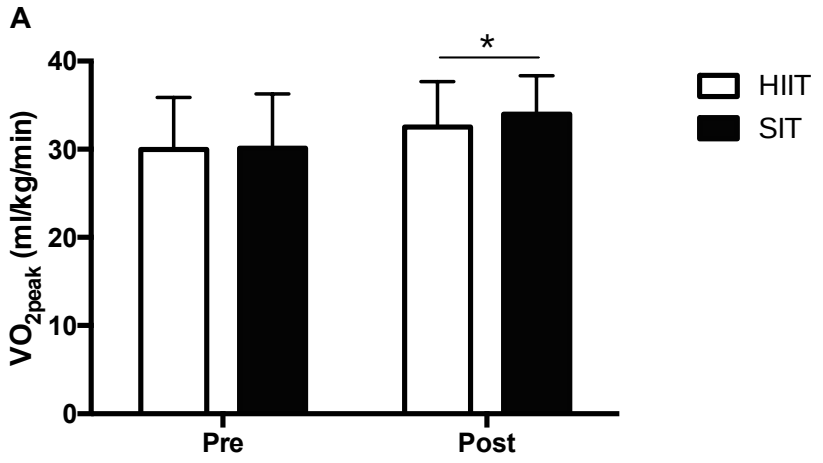



Figure 1: Typical mean HR and work during a representative mid-training session (n=10).

*Cardiorespiratory response and performance*

Single-leg $VO_{2peak}$ increased from 29.9±5.9 and 30.1±6.2 to 32.5±5.2 and 34.0±4.4 ml/kg/min for HIIT and SIT, respectively (main effect of training $p<0.01$). $W_{peak}$ similarly increased from 144±38 and 143±36 to 156±35 and 156±34 W for HIIT and SIT respectively (main effect of training $p<0.01$). Single-leg TTE increased from 1042±571 and 1013±423 to 1485±662 and 1458±496 s in HIIT and SIT respectively (main effect of training $p<0.01$) (figure 2A-C).



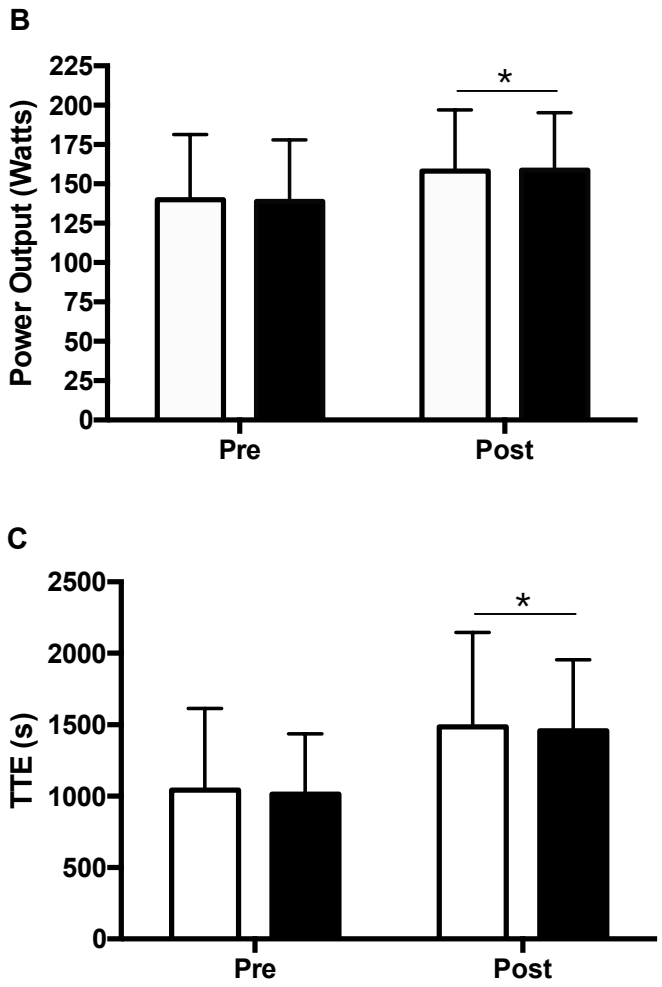



Figure 2.Single leg $VO_{2peak}$ and TTE in response to twelve training sessions. Bars in figures A-C represent mean values for single-leg $VO_{2peak}$ ml/kg/min, single-leg $VO_{2peak}$ power output, single-leg TTE respectively. Symbols represent a significant difference within group from pre (*) ($p<0.05$). Error bars represent SD (n=10).

*Muscle data*

CS maximal activity was higher compared to baseline after both HIIT (16%, $p=0.01$) and SIT (42%, $p<0.01$), with the increase being greater in SIT versus HIIT (main effect of training; ($p<0.01$) (Figure 3).

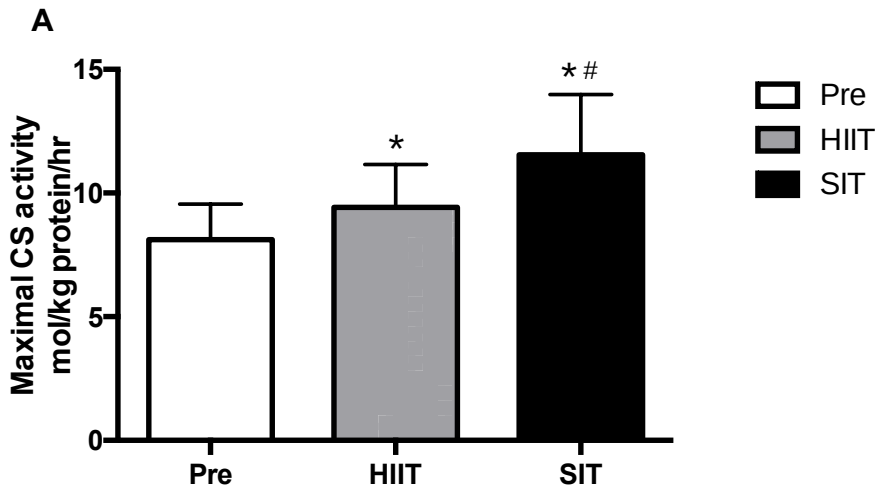



Figure 3: Maximal CS activity in resting skeletal muscle biopsies in response to twelve sessions of HIIT and SIT. Bars represent mean data, symbols represent a significant difference within group from pre (*), and a significant difference between groups at post-training (#) ($p<0.05$). Error bars represent SD (n=9).

COXIV protein content was not different between conditions (p=0.1) (Figure 4).

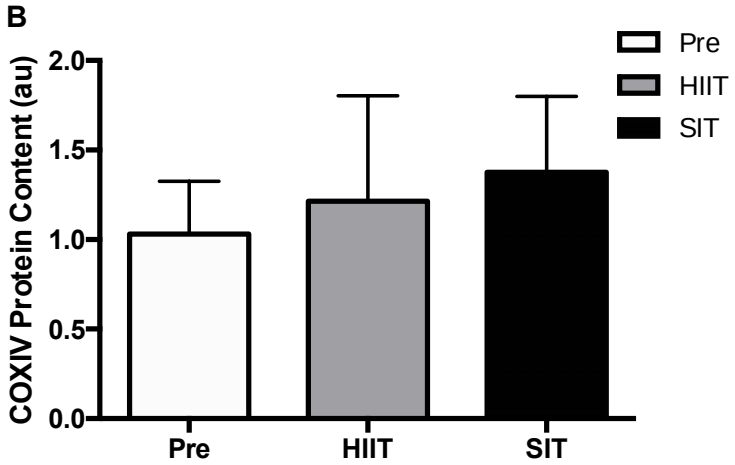



Figure 4: COXIV protein content in resting skeletal muscle biopsies in response to twelve sessions of HIIT and SIT. Bars represent mean values. Muscle samples were normalized to pre-training samples. Error bars represent SD (n=10).

Mass-specific and mitochondrial respiration were also unchanged (both p=0.06; Figure 5A and 5B, respectively).

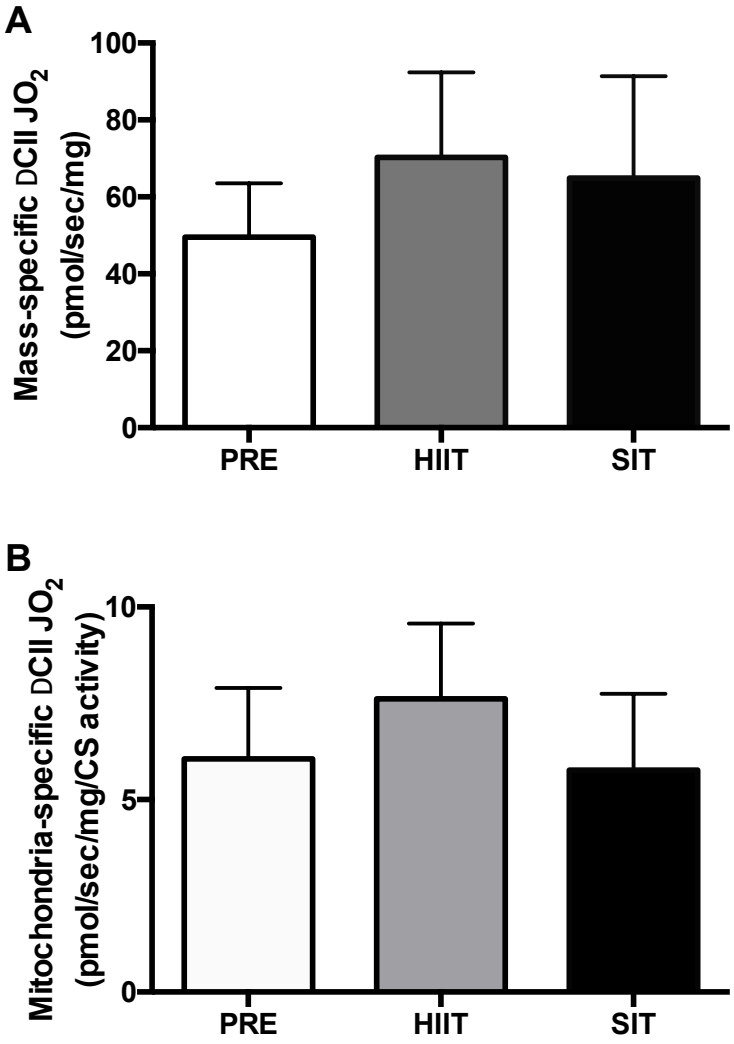



Figure 5: Mass- and mitochondrial-specific respiration in resting skeletal muscle biopsies in response to twelve sessions of HIIT and SIT. Bars represent mean values of succinate supported state 3 respiration for mass- and mitochondrial-specific respiration in A and B respectively. Error bars represent SD (n=10).

## Discussion

The main finding of the present study was that a short-term SIT protocol elicited greater increases in measures of mitochondrial content compared to a similarly structured HIIT protocol, despite involving 30% of the total training volume. This observation runs counter to the suggestion by others that training volume is more important for determining increases in mitochondrial content in response to interval exercise (Bishop *et al.*, 2014; Granata *et al.*, 2016, 2018*a*). A strength of the present design is that we utilized a single-leg protocol that facilitated a within-subject comparison of mitochondrial adaptive responses to SIT and HIIT. The present results are supported by previous work that has demonstrated higher intensity exercise augments training-induced markers of mitochondrial content when work is matched (Tjønna *et al.*, 2008; Macinnis *et al.*, 2017b). Studies examining markers of mitochondrial content between low-volume SIT and MICT have reported similar increases in content despite the reduced volume (Gibala *et al.*, 2006; Burgomaster *et al.*, 2008; Gillen *et al.*, 2016). To our knowledge, only one previous study has directly compared the differences between HIIT and SIT on markers of mitochondrial content and respiration, and found no change in markers of mitochondrial content after 4 weeks of training using a between-subjects design (Granata *et al.*, 2016). In the present study, we measured CS maximal activity and COXIV protein content to assess mitochondrial content, as both biomarkers are associated with the gold standard metric of mitochondrial content, mitochondrial volume assessed by TEM (Larsen *et al.*, 2012*a*). CS maximal activity increased following SIT and HIIT compared to baseline, with the increase being greater in SIT compared HIIT. Our other measure of mitochondrial content, COXIV protein content, was unchanged; this could reflect differences in the sensitivity of the various methods (i.e., activity assay versus western blotting) to detect training-induced changes given our sample size.

We speculate the greater responsiveness to SIT compared to HIIT may be due in part to a greater metabolic stress and subsequent activation of signalling molecules involved in mitochondrial biogenesis. This interpretation is supported by Fiorenza et al (2018), who demonstrated that the molecular response to intense exercise is dependent on metabolic-stress. The study involved two SIT protocols, termed sprint endurance SIT (SE-SIT) which consisted of 6 × 20-s "all-out" efforts, and repeated SIT (RS-SIT) which involved 18 × 5-s "all-out". The protocols were designed to be matched for total work and intensity, but elicit a different amount of metabolic stress, as measured by muscle lactate and $H^+$ accumulation. SE-SIT induced higher metabolic stress and led to a greater metabolic response, measured by PGC-1α mRNA, CaMKII and p38 MAPK phosphorylation (Fiorenza *et al.*, 2018). Other studies have shown that higher intensity exercise has a more pronounced effect on key markers of the mitochondrial biosynthetic pathway, as evident by greater increases in PGC-1α mRNA, AMPK and CaMKII phosphorylation in a high-intensity protocol compared to a work-matched low intensity protocol (Egan *et al.*, 2010). In our study, SIT elicited greater subjective and objective markers of metabolic stress compared to HIIT. Ratings of perceived exertion were higher following each interval in SIT compared to HIIT. Additionally, peak and mean heart rates during each interval were greater in the SIT condition. Lastly, blood lactate measures indicated a greater metabolic stress in the SIT condition. Although we did not measure any acute muscle markers of the molecular response to training (i.e., mRNA and/or phosphorylation of proteins), the greater metabolic stress and higher intensity in SIT may have triggered greater perturbations of the molecular pathways involved in mitochondrial biogenesis, which resulted in greater increase in markers of mitochondrial content. Potentially, this could be related to differences between interventions in the magnitude of the transient, exercise-induced increases in mRNA expression

that eventually lead to increases in mitochondrial content, as measured by various markers including CS maximal activity and COXIV protein content (Perry *et al.*, 2010).

Our results differ from the findings of Granata et al. (2016), although it is unclear why the authors did not detect any changes in CS maximal activity following SIT or HIIT, as this lack of a training effect contrasts with many previous studies that have shown increases following HIIT (Macinnis *et al.*, 2017b) and SIT (Gibala *et al.*, 2006; Burgomaster *et al.*, 2008). The use of different exercise protocols and training status of participants impacts our ability to compare between studies. A strength of the present study was the counterweighted single-leg cycling ergometer which allowed for a within-subject comparison, and thus obviated the potential confounding effect of interindividual differences in the responses to the two conditions.

To evaluate the hypothesis that intensity plays a regulating role in mitochondrial respiration, we also examined complex I and II mass- and mitochondria-specific respiration in permeabilized muscle fibres. However, during certain trials an absence of complex I specific respiration was observed, resulting in a limited data set and incomplete analyses as compared to what was initially envisioned. We were able to observe succinate-supported state 3 respiration determined by the difference between the addition of succinate and the previous substrate addition, which has been reported elsewhere (Hughes *et al.*, 2015). There was no apparent change in mass- or mitochondrial-specific respiration in either condition, contrasting the thought that intensity is a primary regulating factor for training-induced changes in respiration. Furthermore the lack of change in mass-specific respiration following training does not align with previous studies that have displayed coinciding increases in mitochondrial content and mass-specific respiration (MacInnis et al. 2017*b*, Jacobs et al., 2013) and the thought that the

increase in mass-specific respiration is a result of the expanding mitochondrial content (Jacobs *et al.*, 2013; Holloszy & Coyle, 1984).

SIT led to greater increases in CS maximal activity compared to HIIT, suggesting an important role of intensity on training-induced changes in mitochondrial content. Our findings contrast that of Granata et al. (2016); however, it is important to note that their study and our present study are the only two that have directly compared HIIT versus SIT while measuring both mitochondrial content and respiration. Further research is needed to determine whether intensity and volume lead to divergent responses with respect to training-induced mitochondrial remodelling. Additionally, we are mindful of our limitations in making conclusions of the change in mitochondrial respiration as we were only able to assess one marker of mitochondrial respiration. There are numerous other measurements including but not limited to: complex I, III, IV respiration, ADP sensitivity, index of uncoupling, and membrane integrity that would provide a more holistic view of mitochondrial function. The inclusion of more measurements in future investigations is warranted to provide a more encompassing analysis of training-induced changes in mitochondrial respiration.

There was a similar increase in the SIT and HIIT leg for both single-leg $VO_{2peak}$ and TTE, despite the divergent response in mitochondrial remodelling following the two conditions. $VO_{2peak}$ is determined by a host of factors including "central" cardiovascular variables that affect oxygen delivery to the muscle in addition to peripheral changes within the muscle cell (Bassett & Howley, 2000). $VO_{2peak}$ is believed to be mainly centrally-limited during whole-body exercise, however exercise using smaller muscle mass (i.e., single leg cycling) is not limited by central factors, i.e., cardiac output. The lower absolute muscle mass recruited during exercise allows for a greater amount of blood flow to the working muscles (Andersen & Saltin, 1985), resulting in

higher normalized power outputs and greater mitochondrial responses compared to double leg cycling (Abbiss *et al.*, 2011). It is difficult to discern as to why there was no apparent differences in metrics of performance between conditions despite differences in mitochondrial responses between the two conditions. However, it is highly plausible that other variables are contributing to the improvements in performance. Although not limiting during single-leg exercise, the contribution of the central system is not obsolete and there is potential that our training protocol modulated variables relating to blood delivery. Muscle capillarization is a central component of the delivery of oxygen to the muscle cell. Mechanical factors including shear stress (i.e., increase blood flow) and passive stress of the muscle during exercise stimulate angiogenic factors which induce capillarization (Hoier *et al.*, 2013; Gliemann, 2016). Of interest is the "Janus-faced" role intensity plays in inducing capillarization, such that higher intensities are weaker stimulators of capillarization (Gliemann, 2016). Shear stress results in the release of pro-angiogenic factors in a dose dependent manner. The amount of shear stress is a product of the duration and magnitude, therefore MICT is associated with greater amounts of shear stress compared to lower volume more intense exercise (Hoier *et al.*, 2013). Additionally, higher intensity exercise is associated with the release of anti-angiogenic factors (Hoier *et al.*, 2013). Consequently this leads to greater increases in training-induced capillarization following higher volume lower intensity exercise compared to low-volume higher intensity exercise (Daussin *et al.*, 2008; Hoier *et al.*, 2013). These findings suggest a potential for greater capillarization following our HIIT condition, as the greater volume and lower intensities of training would result in greater shear stress and pro-angiogenic factors and lower anti-angiogenic factors compared to SIT.

There are limitations to this study that must be acknowledged: first, the sample size of ten was powered for, and allowed us to detect changes in our primary variable of interest,

mitochondrial content as reflected by CS maximal activity. This relatively small sample size, however, may be a factor impacting our ability to detect differences in performance outcomes between conditions. Other limitations include the fact that we did not conduct fibre-specific analysis on our muscle homogenates (i.e., type I and II fiber types). We are assuming a similar composition within our muscle samples at each time point. It is important to note that there are fibre-specific responses (i.e., mitochondrial markers) to aerobic training (Howald et al., 1985; Kristensen et al., 2015; Macinnis et al., 2017b), therefore if muscle samples are of varying compositions at each time points, it may bias the results. Furthermore, whether protocols of varying exercise intensity elicit different fibre-specific adaptations remains equivocal in the literature (Scribbans et al., 2014; Kristensen et al., 2015; Macinnis et al., 2017b). Lastly, due to our inability to obtain a complete data set of all measures from our mitochondrial respiration protocol we are limited in our analysis of mitochondrial respiration. Results from the various measurements (i.e., ADP affinity, membrane integrity, measurement of complex specific electron flux, maximal state 3 respiration oxidative phosphorylation capacity, electron transport system capacity) are warranted to provide a holistic analysis of the changes in mitochondrial respiration.

In conclusion, we compared two training protocols of varying intensity and volume on different markers of mitochondrial remodelling using a within-subject design. SIT led to greater increases in mitochondrial content as measured by CS maximal activity as compared to HIIT despite requiring a lower volume of work. The increase in content was supported by a greater metabolic stimulus seen by higher HR, RPE and lactate responses in the SIT group. However, when examining markers of mitochondrial respiration no change was observed. Due to our limited assessment of mitochondrial respiration, we warrant further investigation in determining

the impact of intensity and volume on training induced increases in mitochondrial respiration. Overall the present study supports prior research that suggested higher intensity exercise augments mitochondrial content when work is matched, and furthering this area of research we were able to demonstrate that intensity can augment responses even when a lower volume of work is performed. These results suggest that intensity is an important regulating factor inducing mitochondrial content when comparing between a supramaximal low-volume SIT and higher-volume HIIT protocol.

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## Appendix A: Participant Characteristics

**Table 1: Participant Characteristics**

| | |
|---|---|
| **Age (years)** | 22.2 ± 4 |
| **Sex (m/f)** | 6/4 |
| **Weight (kg)** | 67.3 ± 13 |
| **Body fat (%)** | 20.1 ± 9 |
| **Lean mass (%)** | 79.9 ± 9 |
| **$VO_{2peak}$ (ml/kg/min)** | 41.5 ± 4 |

Data are mean ± SD (n=10)

**Table 2: Individual Participant Characteristics**

| **Participant** | Age (years) | Weight (kg) | Sex | $VO_{2peak}$ (ml/kg/min) | $HR_{peak}$ (bpm) | $W_{peak}$ (W) | Fat (%) | Fat Free Mass (%) |
|---|---|---|---|---|---|---|---|---|
| **01** | 26.0 | 58.0 | M | 42.4 | 202 | 218 | 15.5 | 84.5 |
| **02** | 22.0 | 83.3 | M | 36.44 | 180 | 275 | 25.8 | 74.2 |
| **03** | 32.0 | 89.4 | M | 40.3 | 179 | 305 | 22.1 | 77.9 |
| **04** | 19.0 | 57.9 | M | 45.9 | 203 | 275 | 8.6 | 91.4 |
| **05** | 23.0 | 50.5 | F | 42.3 | 192 | 212 | 19.7 | 80.3 |
| **06** | 19.0 | 69.9 | M | 46.0 | 195 | 286 | 4.0 | 96 |
| **07** | 20.0 | 67.8 | F | 44.6 | 182 | 293 | 21.1 | 78.9 |
| **08** | 20.0 | 81.4 | M | 45.1 | 199 | 388 | 20.6 | 79.4 |
| **09** | 21.0 | 62 | F | 35.4 | 177 | 186 | 34.5 | 65.5 |
| **10** | 20.0 | 53.1 | F | 36.7 | 184 | 200 | 28.7 | 71.3 |

**Table 3: Baseline Performance Data**

| Participant | SL $VO_{2PEAK}$ HIIT (ml/kg/min) | SL $VO_{2PEAK}$ SIT (ml/kg/min) | SL $W_{PEAK}$ HIIT (W) | SL $W_{PEAK}$ SIT (W) | TTE HIIT (s) | TTE SIT (s) |
|---|---|---|---|---|---|---|
| **01** | 29.1 | 30.6 | 107 | 114 | 867 | 1012 |
| **02** | 26.8 | 24.3 | 162 | 161 | 904 | 1071 |
| **03** | 24.6 | 25.9 | 150 | 156 | 1105 | 1301 |
| **04** | 33.7 | 34.6 | 146 | 146 | 625 | 684 |
| **05** | 32.4 | 33.3 | 123 | 117 | 1910 | 1245 |
| **06** | 30.5 | 31.3 | 144 | 143 | 741 | 725 |
| **07** | 30.1 | 33.4 | 174 | 172 | 695 | 686 |
| **08** | 41.5 | 39.4 | 224 | 214 | 2248 | 1993 |
| **09** | 19.2 | 17.2 | 90 | 88 | 635 | 592 |
| **10** | 32.0 | 31.6 | 118 | 115 | 688 | 819 |

## Appendix B: Training Characteristics

**Table 4: Individual Heart Rate Data**

| Participant | Condition | Bout 1 | | Bout 2 | | Bout 3 | | Bout 4 | |
|---|---|---|---|---|---|---|---|---|---|
| | | Avg | Peak | Avg | Peak | Avg | Peak | Avg | Peak |
| **P01** | HIIT | 78% | 84% | 82% | 87% | 82% | 87% | 81% | 87% |
| | SIT | 82% | 86% | 86% | 96% | 85% | 88% | 86% | 90% |
| **P02** | HIIT | 80% | 85% | 84% | 88% | 84% | 88% | 85% | 88% |
| | SIT | 86% | 95% | 88% | 82% | 90% | 96% | 90% | 97% |
| **P03** | HIIT | 70% | 77% | 73% | 78% | 75% | 80% | 75% | 82% |
| | SIT | 77% | 85% | 76% | 84% | 75% | 81% | 71% | 80% |
| **P04** | HIIT | 70% | 77% | 71% | 79% | 73% | 80% | 76% | 80% |
| | SIT | 77% | 85% | 77% | 97% | 81% | 87% | 81% | 87% |
| **P05** | HIIT | 85% | 92% | 86% | 94% | 86% | 95% | 87% | 95% |
| | SIT | 93% | 96% | 93% | 88% | 96% | 98% | 94% | 98% |
| **P06** | HIIT | 74% | 80% | 77% | 83% | 77% | 84% | 77% | 83% |
| | SIT | 81% | 86% | 83% | 87% | 86% | 89% | 86% | 90% |
| **P07** | HIIT | 78% | 84% | 81% | 87% | 81% | 87% | 81% | 86% |
| | SIT | 79% | 85% | 80% | 93% | 82% | 87% | 82% | 88% |
| **P08** | HIIT | 79% | 86% | 83% | 87% | 83% | 88% | 84% | 89% |
| | SIT | 81% | 88% | 86% | 92% | 88% | 92% | 88% | 93% |
| **P09** | HIIT | 80% | 88% | 82% | 88% | 82% | 88% | 82% | 88% |
| | SIT | 88% | 90% | 88% | 90% | 89% | 92% | 90% | 93% |
| **P10** | HIIT | 81% | 85% | 83% | 88% | 83% | 87% | 84% | 88% |
| | SIT | 85% | 91% | 87% | 92% | 89% | 93% | 88% | 93% |
| **AVG** | HIIT | 78% | 84% | 80% | 86% | 82% | 86% | 82% | 87% |
| | SIT | 83% | 89% | 84% | 90% | 86% | 90% | 86% | 91% |
| **SD** | HIIT | 4.8% | 4.7% | 4.9% | 4.7% | 4.2% | 4.34% | 4.1% | 4.2% |
| | SIT | 5.1% | 4.2% | 5.4% | 4.9% | 5.8% | 5.0% | 6.4% | 5.2% |

**Table 5: Average Individual Work (kJ)**

| Participant | Condition | Bout 1 | Bout 2 | Bout 3 | Bout 4 |
|---|---|---|---|---|---|
| **P01** | HIIT | 22.0 | 22.0 | 22.0 | 22.0 |
| | SIT | 9.2 | 8.6 | 8.1 | 8.0 |
| **P02** | HIIT | 32.5 | 32.5 | 32.5 | 32.5 |
| | SIT | 13.7 | 13.0 | 12.2 | 11.8 |
| **P03** | HIIT | 31.1 | 31.1 | 31.1 | 31.1 |
| | SIT | 12.6 | 11.5 | 10.9 | 11.1 |
| **P04** | HIIT | 28.7 | 28.7 | 28.7 | 28.7 |
| | SIT | 9.8 | 9.4 | 9.2 | 8.9 |
| **P05** | HIIT | 23.6 | 23.6 | 23.6 | 23.6 |
| | SIT | 8.0 | 6.6 | 7.7 | 7.7 |
| **P06** | HIIT | 28.9 | 28.9 | 28.9 | 28.9 |
| | SIT | 11.5 | 9.9 | 9.8 | 9.5 |
| **P07** | HIIT | 34.0 | 34.0 | 34.0 | 34.0 |
| | SIT | 9.5 | 9.5 | 9.4 | 9.5 |
| **P08** | HIIT | 43.2 | 43.2 | 43.2 | 43.2 |
| | SIT | 13.7 | 13.6 | 13.2 | 13.4 |
| **P09** | HIIT | 17.6 | 17.7 | 17.6 | 17.5 |
| | SIT | 6.5 | 6.0 | 6.1 | 6.0 |
| **P10** | HIIT | 23.0 | 23.0 | 23.0 | 23.0 |
| | SIT | 8.2 | 7.8 | 7.7 | 7.4 |
| **AVG** | HIIT | 28.5 | 28.5 | 28.5 | 28.5 |
| | SIT | 10.3 | 9.6 | 9.4 | 9.3 |
| **SD** | HIIT | 7.3 | 7.3 | 7.3 | 7.4 |
| | SIT | 2.5 | 2.5 | 2.2 | 2.2 |

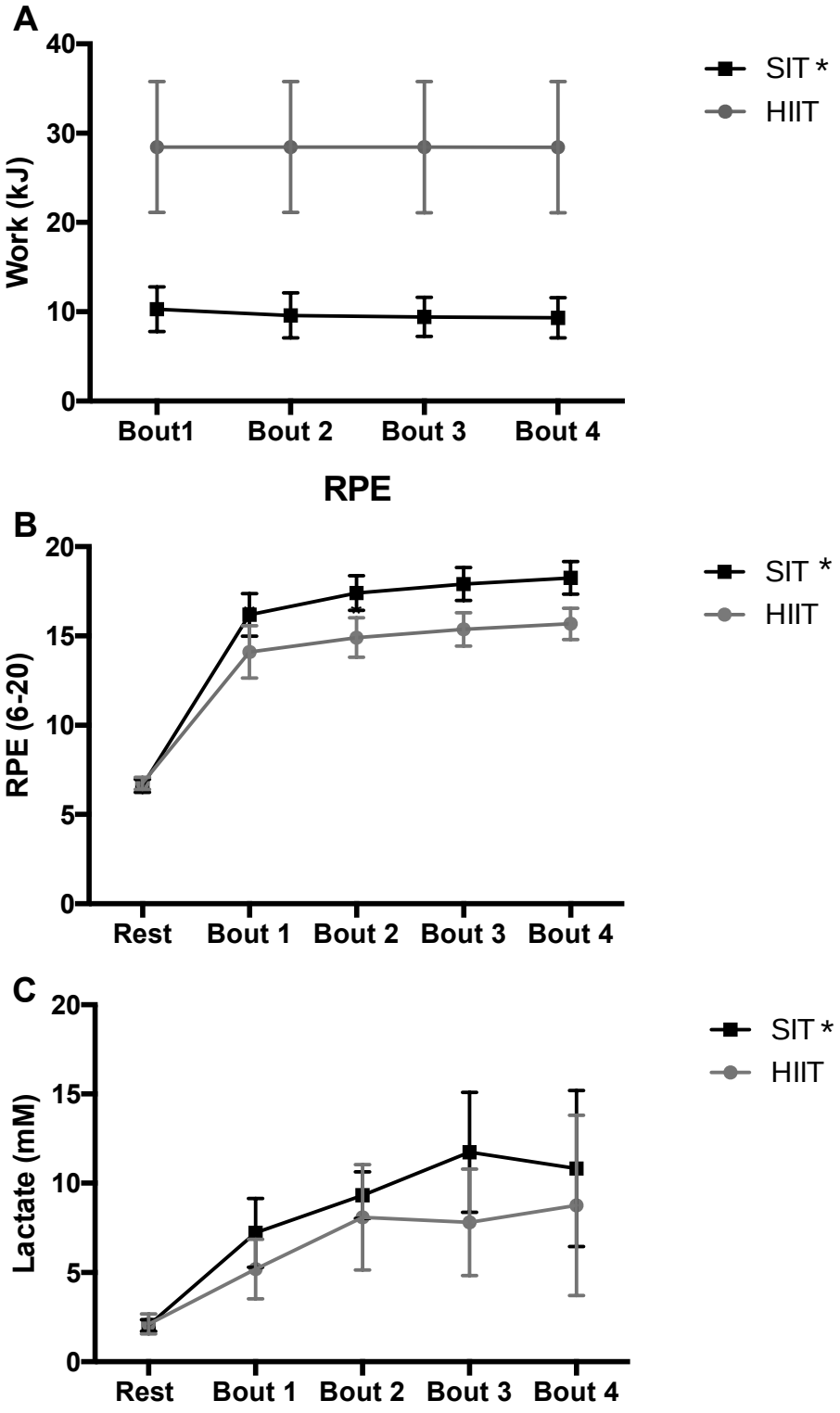



Figure 1: Work, RPE, and lactate levels during each interval (bout) in a HIIT (grey) and SIT (black) training session. Lines represent mean work and RPE for all participants over all twelve

sessions in A and B respectively (n=10). Lactate levels represent the first training session for each protocol (n=9) in figure C. Symbols indicate a significant difference between groups (*) ($p<0.05$).

**Appendix C: Muscle Individual Data Points**

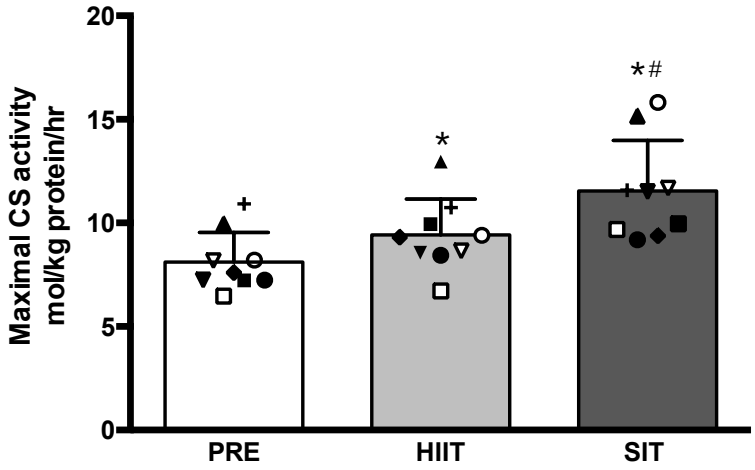



Figure 2: Individual data points of maximal CS activity in resting skeletal muscle biopsies in response to twelve sessions of HIIT and SIT. Bars represent mean data, symbols represent individual data points, (*) represents a significant difference within group from pre, and (#) represents a significant difference between groups at post-training ($p<0.05$). Error bars represent SD (n=9).).

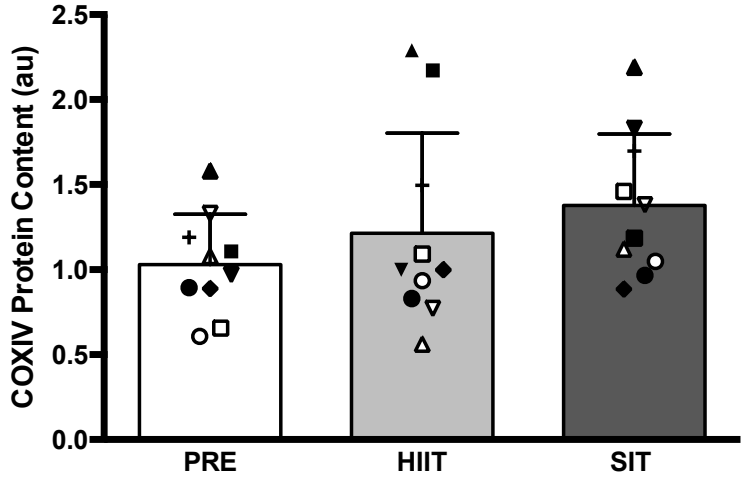



Figure 3: Individual data points of COXIV protein content in resting skeletal muscle biopsies in response to twelve sessions of HIIT and SIT. Bars represent mean values symbols represent individual data points. Muscle samples were normalized to pre-training samples Error bars represent SD (n=10).

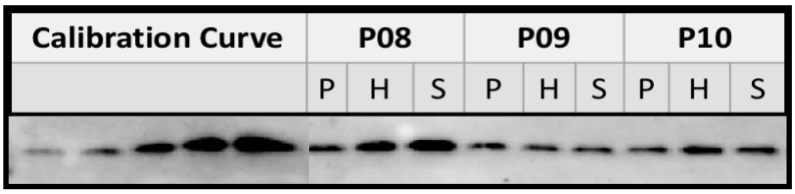


Figure 4: Representative blot of COXIV protein content for P08-P10. Protein content was normalized to total protein loaded in each lane, a calibration curve (e.g. 1, 2, 4, 8 µl) comprised of pooled muscle samples was run on each gel.Pre (P), HIIT (H), SIT (S).

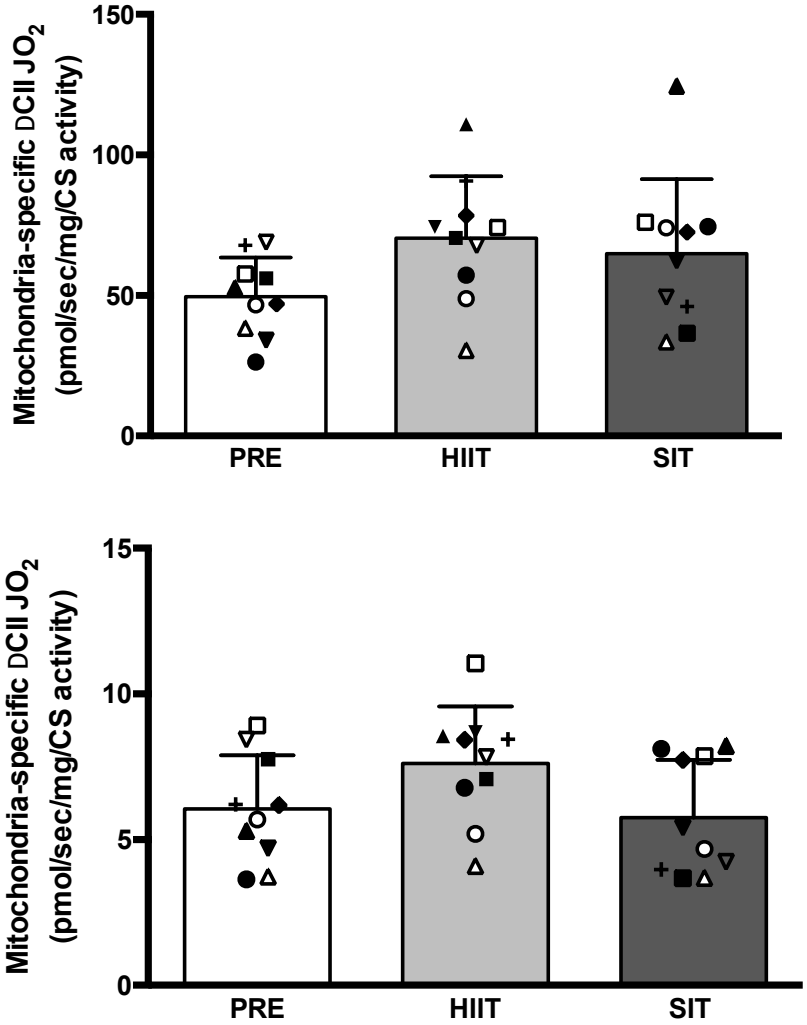
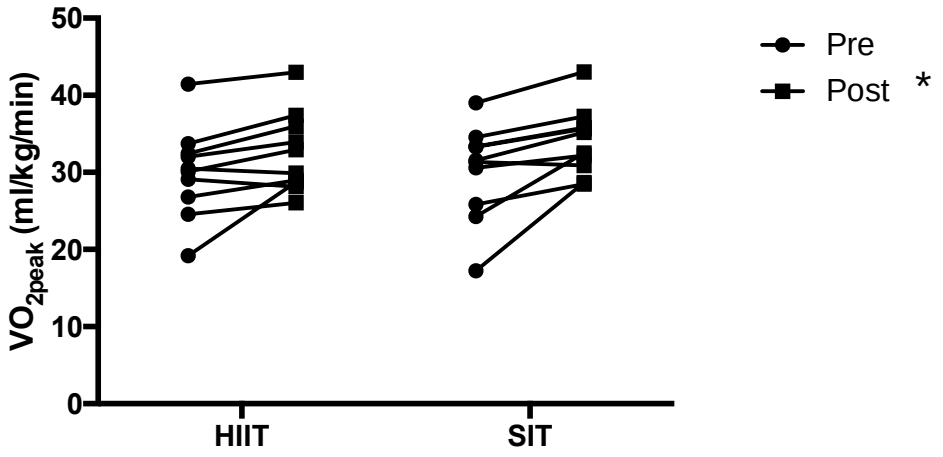
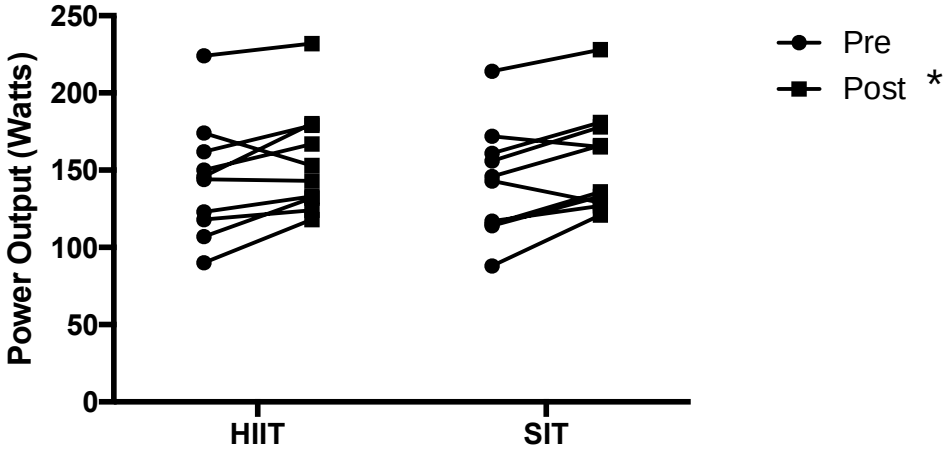


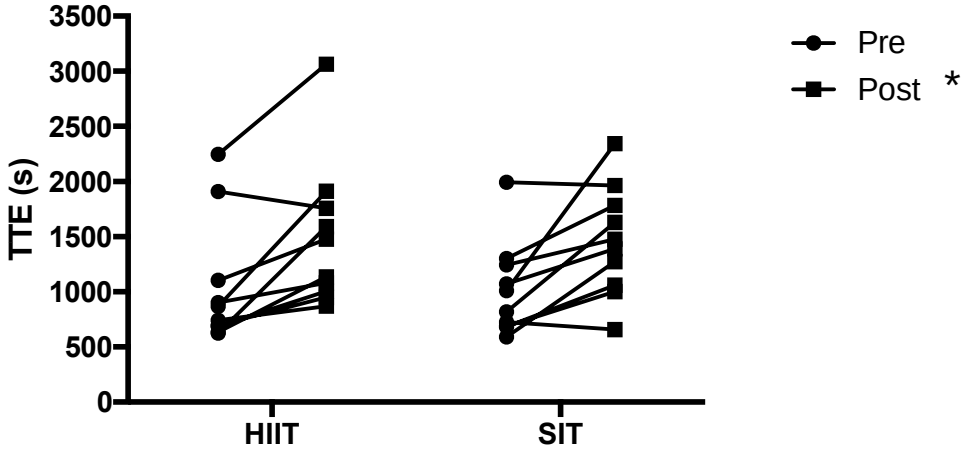

Figure 5: Individual data points of mass- and mitochondrial-specific respiration in resting skeletal muscle biopsies in response to twelve sessions of HIIT and SIT. Bars represent mean values of succinate supported state 3 respiration for mass- and mitochondrial-specific respiration in A and B respectively. Error bars represent SD (n=10).




A)




B)




C)

Figure 6: Individual data points of single leg VO2peak and TTE in response to twelve training sessions. Bars in figures A-C represent mean values for single-leg $VO_{2peak}$ ml/kg/min, Single-leg $VO_{2peak}$ Watts, Single-leg TTE respectively. Symbols represent a significant difference within group from pre (*) ($p<0.05$). Error bars represent SD (n=10).

## Appendix D: Randomization Key

**Table 6: Randomization Key**

| PARTICIPANT | Left | Right | 1st Biopsy | 1st Perf |
|---|---|---|---|---|
| **1** | HIIT | SIT | SIT (R) | HIIT (L) |
| **2** | HIIT | SIT | SIT (R) | SIT (R) |
| **3** | HIIT | SIT | HIIT (L) | SIT (R) |
| **4** | SIT | HIIT | SIT (L) | HIIT (R) |
| **5** | HIIT | SIT | SIT (R) | HIIT (L) |
| **6** | SIT | HIIT | HIIT (R) | SIT (L) |
| **7** | SIT | HIIT | HIIT (R) | HIIT (R) |
| **8** | HIIT | SIT | HIIT (L) | HIIT (L) |
| **9** | SIT | HIIT | HIIT (R) | SIT (L) |
| **10** | SIT | HIIT | SIT (L) | SIT (L) |

Right leg (R), left leg (L).

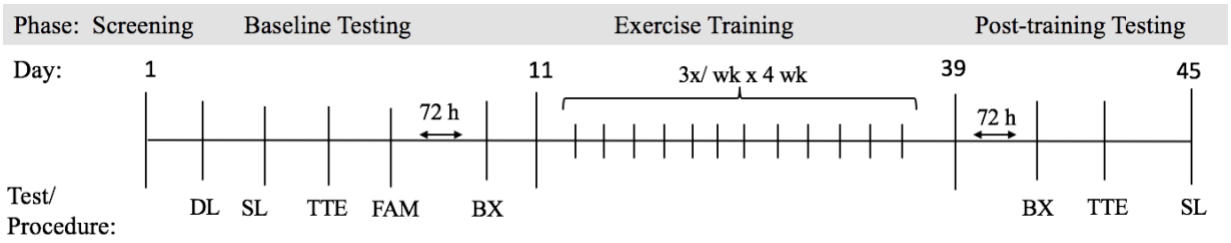



Figure 7: Overview of experimental design. Double-leg $VO_{2peak}$ (DL), single-leg $VO_{2peak}$ (SL), time to exhaustion (TTE), familiarization (FAM), biopsy (Bx).

## Appendix E: ANOVA Outputs for Muscle Data

### Citrate Synthase Activity

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 15015 | 2 | 7508 | F (1.415, 11.32) = 17.00 | P = 0.0008 |
| Individual (between rows) | 17420 | 8 | 2178 | F (8, 16) = 4.930 | P = 0.0033 |
| Residual (random) | 7068 | 16 | 441.7 | | |
| Total | 39503 | 26 | | | |

| Holm-Sidak's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value | |
|---|---|---|---|---|---|
| PRE vs. HIIT | -21.70 | Yes | * | 0.0128 | A-B |
| PRE vs. SIT | -57.21 | Yes | ** | 0.0033 | A-C |
| HIIT vs. SIT | -35.52 | Yes | * | 0.0134 | B-C |

### COXIV Protein Content

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 0.6009 | 2 | 0.3005 | F (1.659, 14.94) = 2.880 | P = 0.0948 |
| Individual (between rows) | 3.622 | 9 | 0.4025 | F (9, 18) = 3.857 | P = 0.0071 |
| Residual (random) | 1.878 | 18 | 0.1043 | | |
| Total | 6.102 | 29 | | | |

| Holm-Sidak's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value | |
|---|---|---|---|---|---|
| Pre vs. HIIT | -0.1840 | No | ns | 0.4788 | A-B |
| Pre vs. SIT | -0.3465 | Yes | * | 0.0301 | A-C |
| HIIT vs. SIT | -0.1624 | No | ns | 0.4788 | B-C |

Mass-Specific Respiration

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 2321 | 2 | 1161 | F (1.513, 13.62) = 3.669 | P = 0.0631 |
| Individual (between rows) | 6732 | 9 | 748.0 | F (9, 18) = 2.364 | P = 0.0574 |
| Residual (random) | 5695 | 18 | 316.4 | | |
| Total | 14748 | 29 | | | |

| Holm-Sidak's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value | |
|---|---|---|---|---|---|
| PRE vs. HIIT | -20.77 | Yes | * | 0.0303 | A-B |
| PRE vs. SIT | -15.35 | No | ns | 0.2891 | A-C |
| HIIT vs. SIT | 5.416 | No | ns | 0.4627 | B-C |

Mitochondrial-Specific Respiration

| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
|---|---|---|---|---|---|
| Treatment (between columns) | 19.87 | 2 | 9.933 | F (1.442, 12.98) = 4.021 | P = 0.0546 |
| Individual (between rows) | 55.79 | 9 | 6.199 | F (9, 18) = 2.509 | P = 0.0462 |
| Residual (random) | 44.47 | 18 | 2.470 | | |
| Total | 120.1 | 29 | | | |

| Holm-Sidak's multiple comparisons test | Mean Diff. | Significant? | Summary | Adjusted P Value | |
|---|---|---|---|---|---|
| PRE vs. HIIT | -1.557 | Yes | * | 0.0419 | A-B |
| PRE vs. SIT | 0.2989 | No | ns | 0.7458 | A-C |
| HIIT vs. SIT | 1.856 | Yes | * | 0.0419 | B-C |