MANIPULATING EXERCISE AND RECOVERY TO ENHANCE ADAPTATIONS TO SPRINT INTERVAL TRAINING

By

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ABSTRACT

Highly-trained athletes are accustomed to varied and high-volume based exercise stimuli and eliciting adaptation in individuals already possessing the necessary physiology to compete at the highest level is difficult. Therefore, identifying novel, potent and time efficient methods of achieving cumulative training stress is a continual quest for coaches and exercise scientists. This thesis examined the acute and chronic effects of manipulating exercise and recovery during brief 'all-out' sprint cycling on adaptive responses favouring enhanced endurance capacity. Chapter 3 highlighted that low-volume non-work matched 'all-out' sprint cycling, whether it be interval- (4 x 30 s bouts) or continuous–based (1 x 2 min bout) provides a similarly potent stimulus for the acute induction of cell signalling pathways and key growth factors associated with mitochondrial biogenesis and angiogenesis in trained individuals. In line with manipulating recovery and in attempting to identify a novel and potent exercise intervention capable of giving athletes more return on their training investment, Chapters 4-6 investigated the efficacy of combining sprint interval training with post-exercise blood flow restriction (BFR). Firstly, it was demonstrated that BFR potentiates HIF-1α mRNA expression in response to SIT, tentatively suggesting an enhanced stimulus for hypoxia- and/or metabolic-mediated cell signalling associated with mitochondrial biogenesis and angiogenesis over SIT alone. Secondly, four weeks of SIT combined with post-exercise BFR provides a greater training stimulus over SIT alone in trained individuals to enhance $\dot{V}O_{2max}$ (4.7 v 1.1 % change) and MAP (3.8 v 0.2 % change), but not 15-km TT performance. Finally, in response to four weeks of SIT combined with post-exercise BFR, an international female track sprint cyclist increased her CP and W' by 7 and 2 % and $\dot{V}O_{2max}$ and absolute MAP by 3 and 4 %, respectively. Through a combination of an acute in vivo molecular experiment, a training study and an athlete case study, this thesis has introduced a potentially potent and novel training concept that appears capable of augmenting aerobic capacity.

Key words: sprint interval training; angiogenesis; blood flow restriction; skeletal muscle; maximal oxygen consumption; cell signalling; trained individuals

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PREFACE

The work presented in this thesis has been peer reviewed as follows:

Peer reviewed publications

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACC	phospho-acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
Ang1	angiopoietin-1
Ang2	angiopoietin-2
ATP	adenosine triphosphate
HAD	B-Hydroxyacyl-CoA-dehydrogenase
BFR	blood flow restriction
CaMK	calmodulin-dependent protein kinase
C:F	capillary-to fibre ratio
CD	capillary density
CON	continuous-based ‘all-out’ cycling protocol
COX	cytochrome c oxidase
CP	critical power
CS	citrate synthase
CV	coefficient of variation (standard deviation/mean)
EC	endothelial cells
eNOS	endothelial nitric oxide synthase
ERRα	estrogen-related receptor-α
HIF-1α	hypoxia inducible factor-1 alpha
HI	high intensity exercise condition
HIT	high-intensity interval training
HK-II	hexokinase-II
INT	interval-based ‘all-out’ cycling protocol
LDH	lactate dehydrogenase

LO	low intensity exercise condition
MAP	maximal aerobic power
MMP-2	matrix metalloproteinase 2
MMP-9	matrix metalloproteinase 9
MPO	mean power output
mRNA	messenger RNA
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
PFK	phosphofructokinase
PGC-1α	peroxisome proliferator-activated γ receptor co-activator 1 alpha subunit
PPO	peak power output
p38 MAPK	p38 mitogen-activated protein kinase
RNA	ribonucleic acid
rpm	revolutions per minute (cadence)
RPII	RNA polymerase II
RT-PCR	reverse transcriptase polymerase chain reaction
SDH	succinate dehydrogenase
SIT	sprint interval training
SE	standard error
Tie2	angiopoietin receptor-2
TIMP-1	tissue inhibitor of matrix metalloproteinase 1
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
VEGF-R2	vascular endothelial growth factor receptor 2
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_{2max/peak}$	maximal/peak oxygen uptake
W	Watt
W’	curvature constant of the power-duration relationship

CHAPTER 1 - INTRODUCTION

A primary objective of a full-time athlete's training programme is to strike the balance between maximising the magnitude of event-specific performance adaptation whilst minimising injury risk and/or illness through over-training and inadequate recovery. Considering a single exercise stress and the consequent physiological adaptive response is therefore important when constructing the weekly training micro-cycle and the broader periodised macro-cycle. The acute adaptive response to a single exercise bout and the structural and functional remodelling that transpires after a period of training are highly specific and serve to better prepare the individual to cope with future overload-induced metabolic, mechanical, neurological and hormonal stress (Hoppeler 2016).

Skeletal muscle is a malleable tissue, exhibiting unique properties enabling it to respond rapidly to a variety of stimuli. These include, but are not limited to, cold (Puigserver et al. 1998; van den Berg et al. 2011) and hot exposure (Tamura et al. 2014), disuse (Bodine 2013), nutritional modulation (Hawley et al. 2011) and of course physical exercise. The resulting divergent adaptations in response to the latter are governed by a multitude of factors including the initial training status of the individual (e.g. untrained vs trained) and the nature of the contractile activity undertaken (Saltin et al. 1977; Coffey and Hawley 2007; Flueck 2010; Coffey and Hawley 2016). In this regard, exercise intensity or load, duration and frequency (i.e. which together make up the training volume) are the primary factors that govern the peripheral remodelling and central adaptations that transpire over time. At one end of the exercise/training stimulus continuum sits resistance or 'strength' training as it is more commonly referred to. Strength training typically constitutes high load, low repetition demands on the working muscles and imposes concomitant increases in neurological and

mechanical stress (Folland and Williams 2007). Repeated exposure to this type of contractile activity promotes increases in muscle-fibre cross sectional area (CSA) and improved intra-muscular co-ordination. Alongside favourable architectural modifications (e.g. changes to the pennation angle of the muscle), these serve to enhance muscle force output (Baar and Esser 1999; Balagopal et al. 2001). In contrast, traditional endurance training, characterised by low load, high repetition continuous exercise, promotes central and peripheral adaptations that together enhance fatigue resistance during tasks relying primarily on aerobic metabolism for energy provision (Holloszy 1967; Holloszy and Booth 1976; Holloszy et al. 1977). Positioned somewhat centrally on this continuum are the physical activities that require high force outputs and that stress both aerobic and anaerobic energy producing pathways (for example the majority of high-intensity intermittent team and combat sports).

It is generally considered that the above phenotypic adaptations are more difficult to elicit in well-trained athletes who, through a genetic predisposition and years of specified training, already possess the necessary physiology to compete at the highest level. Early research has highlighted the reduced plasticity of skeletal muscle in the trained state (Saltin et al. 1977; Hoppeler et al. 1985) and indeed a body of literature exists demonstrating that an increase in traditional endurance training *volume* alone is insufficient to improve aerobic performance or associated physiological determinants in well-trained and 'physically active' individuals (Daniels et al. 1978; Hickson and Rosenkoetter 1981; Denis et al. 1982; Costill et al. 1988; Lake and Cavanagh 1996; Westgarth-Taylor et al. 1997; Weston et al. 1997; Londeree 1997). This blunting of the adaptive scope in trained individuals is reflected at a molecular level, as demonstrated by an attenuation of acute skeletal muscle mitochondrial transcript expression after endurance training and the inverse relationship between transcript expression levels and mitochondrial volume (Schmutz et al. 2006; Flueck 2010). Furthermore, it has recently been demonstrated that a continual reduction in the magnitude of exercise-induced gene expression

of mitochondrial and angiogenic protein regulators occurs in the early recovery period following successive identical exercise bouts (Perry et al. 2010).

The above poses the question: what interventions and exercise stimuli can be used to further augment the acute adaptive response to training and, if repeated over time, enhance performance in trained populations? Highly trained individuals are typically well accustomed to varied and volumous exercise stimuli. Therefore identifying novel and time efficient methods of achieving cumulative training stress is a continual quest for coaches and exercise scientists. However the challenges exercise physiologists face in trying to persuade highly trained athletes and their coaches to accommodate periods of experimental work in and around their own training programme, has meant that the majority of acute in vivo molecular investigations have been conducted on healthy recreationally active or sedentary male cohorts. Coupled with the often invasive nature of investigating the adaptive response to training, which involve collected blood and muscle samples are of paramount to understanding the associated mechanisms involved, it is unsurprising that highly-trained athletes are reluctant to participate in these types of investigations. The body of work herein is the product of a collaborative venture between Loughborough University and the English Institute of Sport in attempting to enable such investigations to take place and in doing so contribute in some capacity to bridging the gap between the academic and applied worlds of sports and exercise science.

This thesis describes a series of experiments that examined, both at a molecular and whole body level, the acute and chronic effects of manipulating exercise and recovery during brief maximal sprint cycling on adaptive responses that favour enhanced endurance performance/capacity. The thesis comprises four experimental chapters that have been conducted on trained individuals. The first of these investigated the consequences of manipulating the ‘pattern’ of brief maximal sprint exercise (whether that be interval or

continuous in nature) on the acute intramuscular cell signalling responses that regulate endurance adaptations. Here, light is shed on the potential for high-intensity exercise, specifically 'all-out' sprint interval exercise, to promote capillary growth. Moreover, it has challenged the importance (or seemingly lack of) of the 'pulsatile' nature of intermittent exercise in initiating the acute adaptive response to this type of stimulus, instead providing further support for exercise intensity as being the key driver. In line with manipulating recovery and in attempting to identify a novel training intervention that could potentially give trained individuals more 'bang for their buck', the subsequent three experimental chapters investigated the use of a practical model of restricting blood flow and combining this with sprint interval training to enhance adaptation of an endurance phenotype. Through a combination of an acute in vivo molecular experiment, a training study and an athlete case study, this thesis introduces a potentially potent and certainly novel training concept that appears capable of augmenting aerobic capacity.

CHAPTER 2 - LITERATURE REVIEW

This chapter provides a critical review of the literature considering the importance of incorporating training of an intense interval nature into the endurance athlete's programme and then the current understanding of how peripheral adaptations to this type of training are regulated through highly orchestrated molecular events. Moreover, this review introduces the potential importance of training variety in the trained individual's programme and how using a novel training method, utilising a practical method of blood flow restriction during intense interval exercise may provide an enhanced stimulus for greater activation of these molecular events and over time enhanced aerobic capabilities.

2.1 Skeletal muscle adaptations favouring enhanced endurance performance

Traditional endurance training is typically comprised of long duration, low to moderate intensity exercise that is continuous or 'steady state' in nature. If undertaken frequently at adequate intensities and for appropriate durations this training stimulus will drive central and peripheral adjustments that improve oxygen and substrate delivery and augment an individual's capacity to resynthesize ATP at the working muscles (Holloszy and Booth 1976; Holloszy and Coyle 1984). Whilst maximal oxygen transport and uptake appear to be largely governed by central factors (i.e. cardiac output) (Andersen and Saltin 1985), peripheral factors (i.e. capillary and mitochondrial density) are also key physiological determinants underpinning endurance performance, including maximal oxygen uptake ($\dot{V}O_{2max}$), the anaerobic threshold and sustainable velocity/power during exercise (fractional utilisation of

$\dot{V}O_{2max}$), as well as the economy of locomotion (mechanical efficiency) (Joyner and Coyle 2008).

At the periphery, endurance exercise enhances oxidative potential through increasing mitochondrial size and density (mitochondrial biogenesis) and the maximal activity of oxidative enzymes (Holloszy, 1967; Gollnick & King, 1969). The importance of mitochondrial biogenesis and increased mitochondrial enzyme activity for endurance performance is of no surprise given the mitochondrion are the site of cellular respiration and the fundamental rate limiting organelle in ATP synthesis (Hood, 2001). Indeed, a strong correlation exists between $\dot{V}O_{2max}$, power output (PO) and the mitochondrial density of the vastus lateralis muscle (Flueck 2010) and trained skeletal muscle can exhibit three- to four-fold greater oxidative enzyme activity than untrained muscle (Henriksson 1992). In addition to the above, muscle fibre conversion from type IIb to type IIa (Fitzsimons et al. 1990), increased basal glycogen content (Saltin and Rowell 1980), alterations in substrate metabolism for energy provision at a given workload (Holloszy et al. 1977), elevated myoglobin content and increased capillarity (Andersen and Henriksson 1977; Svedenhag et al. 1984; Hudlicka et al. 1992) are key peripheral adaptations that play a significant role in enhanced endurance capability.

August Krogh's seminal work in the early 20th century (Krogh 1919) identified the importance of the microvasculature for oxygen delivery and that the capillary network surrounding the active muscle may contribute as a limiting factor to aerobic metabolism by virtue of limiting intramuscular oxygen diffusion path-lengths. It is now well accepted that a greater capillary density (CD) improves the diffusive exchange of oxygen and nutrients between the vascular space and the intracellular volume of the fibres (Prior et al. 2004). As such, well-trained endurance skeletal muscle has a greater capillary supply compared to

untrained muscle to meet the increased oxygen demands of intensified work. This is evidenced by the superior capillary : fibre ratios and total number of capillaries surrounding a muscle fibre in endurance trained populations (Brodal et al. 1977). In fact, endurance trained individuals exhibit a capillary-to-muscle fibre ratio of more than 200% that of muscle from untrained individuals (Ingjer 1979). These, coupled with the correlation between exercise capacity, $\dot{V}O_{2max}$ and capillarity of the vastus lateralis (Coyle et al. 1988; Vock et al. 1996; Iaia et al. 2011), emphasize the importance of the capillary bed to aerobic performance. Moreover, the observations that just a few weeks (~ 4-5 weeks) of aerobic exercise can increase capillarity by 10-30% in healthy active individuals (Andersen and Henriksson 1977; Hoier et al. 2012; Hoier et al. 2013a; Hellsten et al. 2015) highlight the health implications of an enhanced vascular network. At this point it is worth briefly highlighting a methodological factor concerning assessment of capillary growth. The number of capillary profiles per cross-sectional area of muscle fibres (capillaries/mm^2) is used to determine CD. However, as the mean cross-sectional area of muscle fibres alters in response to changes in the physical demand and metabolic status of the tissue, this measure does not provide the most reliable indication of changes in capillarity. A method less sensitive to scaling issues and thus more reliable in tracking changes in capillarity is the calculation of capillary-to fibre ratio (C:F), a process that involves quantifying the number of muscle fibres and capillary profiles within a given area. As such, the above information requires consideration when interpreting correlations between capillarity and exercise performance and data related to capillary growth after training. For a more detailed discussion on methods surrounding quantification of capillary adaptation, the reader is referred to a recent review by Olfert and colleagues (2016).

Regarding exercise performance, Coyle et al. (1988) have previously reported that over 92% of the variance in cycle time to exhaustion at 88% $\dot{V}O_{2max}$ in competitive cyclists could be explained by muscle capillarity. More recently, Iaia and colleagues (2011) have reported that

in exhaustive cycling trials lasting ~30 s to 3 min, 50-80% of the estimated total variance in performance could be explained by differences in CD as measured by capillaries/mm^2. The aerobic system was contributing 40–80% of the total energy production in these trials and as such a higher number of capillaries would facilitate an enhanced oxygen transport (and thus uptake) of oxygen at the contracting muscles. A higher CD also likely facilitates greater removal of metabolic-end products, thereby reducing the fall in muscle pH and in turn sustaining exercise tolerance (Joyner and Coyle 2008). In this regard, the exercise intensity at which blood lactate accumulation occurs is correlated ($r = 0.83$) with the capillary : fibre ratio of the exercising muscle (Tesch et al. 1982), as is peak blood lactate concentration ($r = 0.64$) and its clearance rate ($r = 0.69$) following repeated maximal voluntary knee-extension exercise (Tesch and Wright 1983). Moreover, Iaia et al. (2011) observed an inverse relationship between CD and the net rate of plasma K^+ accumulation ($r^2 = 0.68$) and muscle pH ($r^2 = 0.80$) during and after 3-min exhaustive exercise. Indeed, a greater capillary supply may accelerate the transfer of K^+ from the muscle interstitium to the circulation, delaying the accumulation of K^+ in muscle interstitium, and lowering the concentration of H^+ outside of the muscle cells favouring release of protons from the muscle cell (McKenna et al. 2008). In support of the relationship between capillarity and exercise performance in humans, maximal aerobic capacity also correlates strongly with skeletal muscle CD in rodents (Hudlicka et al. 1992). In addition to its direct role in both aerobic and anaerobic performance, there is also evidence from rodent studies that increased capillarity precedes muscle fibre type conversion from IIb to IIa fibres in response to voluntary exercise (Waters et al. 2004) and the increase in mitochondrial enzyme activity after electrical stimulation (Skorjanc et al. 1998). These raise the question as to whether capillary growth is initiated first to support other skeletal muscle re-modelling such as fibre type transformation and mitochondrial biogenesis.

2.2 Importance of intense interval training for the endurance athlete

In trained endurance athletes who already possess a highly developed aerobic capacity, an increase in traditional submaximal endurance training *volume* alone does not appear, at least in the short term, to further enhance endurance performance or associated physiological determinants such as $\dot{V}O_{2max}$, the anaerobic threshold, and economy of motion (Daniels et al. 1978; Denis et al. 1982; Costill et al. 1988; Lake and Cavanagh 1996; Westgarth-Taylor et al. 1997; Weston et al. 1997; Londeree 1997). Conversely, when trained endurance cyclists supplement or replace their typical volume-based aerobic training with a relatively brief period (2-8 weeks) of high-intensity interval training (HIT), this provides a potent stimulus to elicit physiological adaptation and subsequent performance improvements (Table 1).

Although its definition varies throughout the scientific community, HIT can be broadly characterised as repeated, intermittent bouts of short to moderate duration exercise performed at an intensity close to or greater than $\dot{V}O_{2max}$, interspersed by brief periods of low-intensity work or inactivity that allows a partial, but often incomplete recovery (Daniels and Scardina 1984; Laursen and Jenkins 2002; Gibala 2009). Similarly, Buchheit and Laursen (2013) have defined HIT as "repeated short (<45 s) to long (2–4 min) bouts of rather high- but not maximal-intensity exercise, or short (<10 s) or long (>20–30 s) 'all-out' sprints, interspersed with recovery periods". In this sense HIT is infinitely variable in its prescription and subtle differences in the intensities and durations between protocols need careful consideration when interpreting the findings from acute and chronic exercise studies, as is highlighted throughout this chapter.

Whilst an overwhelming amount of literature has been published over the past four decades demonstrating the effectiveness of HIT in promoting beneficial skeletal muscle re-modelling and exercise performance in untrained individuals, this is by no means a new concept in

athletic training and sports and exercise research. As far back as the early 1900s, ‘repetition training’ was being prescribed to athletes according to international running coach Peter Thompson in Athletics Weekly (Thompson, 2005) and investigations of intermittent exercise in humans were being conducted by AV Hill in the 1920s (Hill et al. 1924). Decades later, eminent Scandinavian physiologists performed ground-breaking research that paved the way for further research attempting to uncover the adaptive responses to interval exercise and the effects manipulating ratios of work to rest duration have on skeletal muscular and vascular physiology (Astrand et al. 1960; Christensen et al. 1960). The more recent investigations conducted over the past four decades have reinforced the long-standing thoughts and practices of coaches; HIT is a highly potent and effective stimulus that should be integrated into the endurance athlete’s long-term training plans.

Table 1 - Evidence demonstrating HIT improves endurance performance and associated physiological parameters in trained endurance athletes

Study	Subjects	Experimental Design	Intervention	Results	Conclusion
Lindsay et al. (1996)	8 competitive male cyclists	MAP and TT40 were determined before and after a 4-wk HIT intervention	Cyclists replaced ~15 % of their ~300-km per week training with HIT (total of 6 sessions), consisting of 6 to 8 x 5 min repetitions at 80% of MAP, with 60-s recovery between work bouts.	HIT significantly improved TT40 (56.4 ± 3.6 vs. 54.4 ± 3.2 min; $P < 0.0001$), and absolute (301 ± 42 vs. 326 ± 43 W; $P < 0.0001$) and relative (72.1 ± 5.6 vs. 75.0 ± 6.8 % of MAP; $P < 0.05$) MAP.	A 4-wk program of HIT increased the MAP and fatigue resistance of competitive cyclists and improved their 40-km time trial performances.
Westgarth-Taylor et al. (1997)	8 endurance-trained male cyclists	MAP and 40 km time-trial performance TT40 were determined before and after a 6-wk HIT intervention	Cyclists replaced ~15 % of their ~300-km per week endurance training with HIT (a total of 12 sessions), consisting of 6- to 9-5 min repetitions at 80% of MAP, with 60-s recovery between work bouts.	HIT increased MAP from 404 (40) to 424 (53) W ($P < 0.01$) and improved TT40 speeds from 42.0 (3.6) to 43.0 (4.2) km.h-1 ($P < 0.05$).	Faster TT40 performances were due to increases in both the absolute work rates from 291 (43) to 327 (51) W ($P < 0.05$) and the relative work rates from 72.6 (5.3) % of pre-HIT PPO to 78.1 (2.8) % of post-HIT MAP ($P < 0.05$).
Weston et al. (1997)	6 competitive male cyclists	Skeletal muscle buffering capacity (beta m), PFK and CS activity and TT40 were determined before and after a 4-wk HIT intervention	Cyclists replaced a portion of habitual endurance training with HIT (a total of 6 sessions), consisting of 6- to 8-5 min bouts at 80% of MAP, with 60-s recovery between work bouts.	beta m increased from 206.6 (17.9) to 240.4 (34.1) mumol H+.g muscle dw-1.pH-1 after HIT ($P < 0.05$). PPO and TT40 significantly improved after HIT ($P < 0.05$). There was no change in PFK or CS activity after HIT.	Skeletal muscle buffering capacity may be an important determinant of relatively short-duration (< 60 min) endurance cycling activity and responds positively to just six sessions of HIT.
Stepto et al. (1999)	20 endurance trained male cyclists	MAP, 25-kJ sprint cycle performance and TT40 were determined before and after a 3-wk HIT intervention.	Cyclists were assigned to complete one of five HIT interventions (a total of 6 sessions in each) in addition to their usual aerobic base training: 12 x 30 s at 175% MAP, 12 x 60 s at 100% PPO, 12 x 2 min at 90% MAP, 8 x 4 min at 85% MAP, or 4 x 8 min at 80% MAP.	Intervals performed at 80 and 100% MAP did not appear to enhance performance. The cubic trend was strong and statistically significant ($r = 0.70$, $P < 0.05$) and predicted greatest enhancement when intervals performed at 85% (2.8%, 95% CI = 4.3-1.3%) and at 175% MAP (2.4%, 95% CI = 4.0-0.7%).	HIT performed close to and above race pace enhanced 1-h endurance performance.
Laursen et al. (2002)	14 highly-trained male cyclists	VO_{2peak}, VT1, VT2 and MAP were determined before and after a 2-wk HIT intervention.	Subjects were divided equally into a HIT group, who performed 4 HIT sessions (20 x 60 s at MAP separated by 120 s of recovery) and a control group who maintained their regular training program. Both groups were reassessed on the same timeline.	There was no change in VO_{2peak} for either group. However, the HIT group showed a significantly greater increase in VT1 (+22% vs. -3%), VT2 (+15% vs. -1%), and MAP (+4.3 vs. -.4%) compared to controls (all $P < 0.05$).	HIT appears capable of improving VT1, VT2, and MAP, following only 4 sessions in already highly trained cyclists.
Laursen et al. (2005)	38 well-trained male cyclists	VO_{2peak}, MAP, VT1 and VT2, TTE at MAP, TT40 and PV were determined before and after 2 and 4 weeks of 3 different HIT interventions.	Cyclists were assigned to complete 1 of 4 training interventions: 8 x 60% TTE at MAP, 1:2 work-recovery ratio; 8 x 60% TTE at MAP, recovery at 65% maximum heart rate; 12 x 30 s at 175% MAP, 4.5-minute recovery; control (no training).	TT40, VO_{2peak}, VT1 and VT2 significantly increased in all training interventions ($p < 0.05$) except the control group. PV did not change in response to training. Changes in TT40 were modestly related to the changes in VO_{2peak}, VT1 and VT2 ($r = 0.41$, 0.34, and 0.42, respectively; all $p < 0.05$).	Improvements in TT40 appear related to significant increases in VO_{2peak}, VT1 and VT2. Peripheral adaptations rather than central adaptations appear likely responsible for the improved performance after HIT in well-trained endurance athletes.

Abbreviations: PPO, peak sustained power output; PFK, phosphofructokinase; CS, citrate synthase; TT40, 40-km cycle time trial performance; $VO_{2peak,}$ peak oxygen uptake; VT1, ventilatory threshold 1; VT2, ventilatory threshold 2; PV, plasma volume; HIT, high intensity interval training; TTE, exercise time to exhaustion; MAP, maximal aerobic power; beta m, buffering capacity.

In addition to the studies conducted on trained cyclists (Table 1), Kohn and colleagues (2011) assessed the adaptive response of well-trained runners to 6 weeks of HIT running sessions twice per week, comprising 6 efforts at 94% of pre-determined peak treadmill speed (~2.7 min each). In attempting to provide a mechanistic insight into the runners' increase in peak treadmill speed after training, they reported a significant increase in lactate dehydrogenase (LDH) activity (notably in the type II muscle fibres), suggesting an up-regulation of intracellular lactate metabolism. In contrast, the authors reported no improvements in $\dot{V}O_{2max}$ and no differences in capillary supply or maximal activity of the mitochondrial enzymes citrate synthase (CS) and the rate limiting enzyme in beta oxidation, 3-hydroxyacetyl CoA dehydrogenase (3-HAD), after six weeks of HIT running. Taken together with the studies in Table 1, the above demonstrates that although performance improvements observed in trained individuals in response to a period of HIT may primarily result from an overriding central or peripheral physiological enhancement, it is more likely that several adaptations underpin these performance improvements. Moreover, the specific adaptations will no doubt reflect the duration, intensity and frequency of work prescribed. Importantly, through furthering our understanding of how subtle manipulations to work-rest periods can change the physiological adaptations that transpire, recent investigations have helped to optimise HIT prescription.

2.3 Low-volume sprint interval training (SIT): Adaptations and performance benefits

Brief 'all-out' (i.e. non-paced maximal effort) sprint interval training (SIT) has become a well-researched form of low-volume HIT over recent decades given its ability to promote rapid (i.e. 2-6 weeks) physiological remodelling comparable to that observed after an identical period of high-volume traditional endurance training (Gibala et al. 2006;

Burgomaster et al. 2008; Rakobowchuk et al. 2008; Gibala et al. 2012; Cocks et al. 2013; Scribbans et al. 2014). In the majority of studies examining SIT, the protocols have typically comprised 4-6 x 30 s cycle sprints interspersed by 4-5 min of passive recovery (i.e. a total of 15-20 min session duration and 2-3 min total exercise time). Three to four sessions per week of this training can increase muscle buffering capacity, capillarity, and muscle oxidative potential (as evidenced by increases in the maximal activity of mitochondrial enzymes CS, pyruvate dehydrogenase (PDH) complex and cytochrome c oxidase (COX) subunits II and IV) to a similar extent to 40-120 min of moderate intensity (~ 65% $\dot{V}O_{2peak}$) continuous exercise (Burgomaster et al. 2006; Gibala et al. 2006; Burgomaster et al. 2008; Cocks et al. 2013). Moreover, just 6 sessions of SIT cycling can increase resting muscle glycogen content and reduce muscle glycogen utilisation during work-matched exercise (Burgomaster et al. 2006), which has implications for athletic performance over a range of durations/distances. In support of these data in humans, similar increases in both COX and CS maximal activity have also been observed in rats subjected to six weeks of low-volume intense interval running or prolonged moderate intensity continuous running (Dudley et al. 1982) and after just 8 days of intense interval swimming or low-intensity continuous swimming (Terada et al. 2001).

In accordance with the above re-modelling, as little as 2-6 weeks of SIT has been demonstrated to increase $\dot{V}O_{2max}$ in recreationally active or non-trained, sedentary individuals (Burgomaster et al. 2008; Whyte et al. 2010; Cocks et al. 2013). A recent study published by MacPherson and colleagues (2011) compared the effects of six weeks of run SIT (4-6 × 30-s 'all-out' sprints interspersed by 4 min recovery) and 30-60 min continuous running at 65 % $\dot{V}O_{2max}$ on performance and cardiac output in healthy active men and women. Performance, as quantified by 2000 m run time trial performance and $\dot{V}O_{2max}$, increased similarly between groups, however cardiac output only increased following traditional endurance training,

suggesting the improvements in aerobic performance observed following low-volume SIT in that study were likely the result of primarily enhanced peripheral and not central factors (Macpherson et al. 2011). Nevertheless, in line with the aforementioned central and peripheral adaptations, SIT has consistently shown to significantly improve exercise performance in healthy untrained individuals as further evidenced by 7-10% and 100% improvements in time to complete a given work load and cycle time to exhaustion at a fixed workload (i.e. exercise capacity), respectively (Burgomaster et al. 2005; Burgomaster et al. 2006; Gibala et al. 2006).

It is a common finding that HIT performed below $\dot{V}O_{2max}$ improves $\dot{V}O_{2max}$ in well trained individuals (Tabata et al. 1996; Helgerud et al. 2007; Gunnarsson et al. 2012). However, studies evaluating the effect of Wingate–based SIT or HIT involving exercise intensities at or above 90% of $\dot{V}O_{2max}$ or peak power output (PPO) have reported unchanged or smaller improvements in $\dot{V}O_{2max}$ in trained individuals (Stepto et al. 1999; Hawley and Stepto 2001; Stepto et al. 2001; Laursen et al. 2002a; Laursen et al. 2002b; Millet et al. 2014) and in some cases untrained cohorts (Burgomaster et al. 2005). This suggests that shorter maximal efforts may not be optimal or in some cases insufficient to enhance this physiological parameter in this population when compared to longer, less intense interval-based protocols. A recently published meta-analysis by Bacon and colleagues (2013) comprising 40 distinct training groups appears to support this in the untrained population. These authors concluded that interval training alone or combined with continuous training can increase $\dot{V}O_{2max}$ in untrained individuals by up to 0.6 $L.min^{-1}$ and by up to 0.9 $L.min^{-1}$ in response to protocols that feature longer (i.e. 3-5 min) exercise periods (Bacon et al. 2013). In addition to differences in exercise protocol, when interpreting physiological and performance changes in response to

HIT and SIT it is important to consider the varied lengths of the training periods (i.e. 2-8 weeks) as these can also influence the post-training outcomes.

A number of studies on untrained people have shown increased glycolytic and oxidative enzyme activity after training comprising of short brief 'all-out' exercise bouts (5–30 s) interspersed with longer rest periods (45 s–20 min) (Costill et al. 1979; Roberts et al. 1982; Parra et al. 2000; Rodas et al. 2000; Burgomaster et al. 2005). In contrast, studies enrolling well-trained subjects have not been able to show changes in oxidative enzyme expression (notably CS and HAD) or enzymes related to anaerobic energy production when training volume is reduced (Iaia et al. 2008; Bangsbo et al. 2009; Christensen et al. 2015). In the most recent of these studies, Christensen and colleagues (2015) subjected 8 trained cyclists to a 7 week period of SIT (30 s cycle sprints with 4 min rest intervals) with 50% reduced training volume. After the intervention, the maximal activity of CS, HAD and PFK, as well as capillary to fibre ratio was unaltered. This could be the result of a relatively large reduction in training volume and perhaps in trained individuals a certain amount of volume is required to supplement training of a low-volume, 'all-out' nature. Nevertheless, despite unaltered levels of enzymes related to aerobic and anaerobic energy production in the above study, other studies (Iaia et al. 2008; Bangsbo et al. 2009) have observed enhanced short-term performance in trained populations. These results suggest there may be other important mechanisms, such as actions of the Na^+-K^+ pump, that support improved performance.

In summary, HIT and SIT play a seemingly important role in the endurance athlete's training programme and appear necessary to drive numerous peripheral adaptations that enhance an individual's anaerobic and aerobic capabilities. Clearly, these stimuli need to be well-planned into any training programme but especially that of the highly-trained athlete. The disparity in certain adaptive responses (e.g. levels of increase in oxidative enzymes) in untrained versus

trained individuals after similar interval protocols appears to indicate that as an individual becomes trained, a significant amount of low- to moderate-intensity training alongside intense interval work is required to maintain specific structural adaptations and perhaps enable the athlete to tolerate progressive training overload and intensification. Indeed, strong evidence exists that an ~80:20 ratio of low-intensity training to HIT yields impressive long-term results over time among endurance athletes training daily (Seiler and Tønnessen 2009). Moreover, the aforementioned data remind us that differences in the intensity, volume and frequency of the exercise stimulus, as well as the initial fitness level of the individual require great consideration when examining the physiological responses observed across studies. Manipulations of these factors alter the relative demands on metabolic pathways at a cellular level as well as oxygen delivery at a systemic level, and subsequently long term adaptation at a whole body level.

2.4 The molecular basis of peripheral skeletal muscle adaptations to intense exercise

The underlying factors that regulate skeletal muscle re-modelling and consequent improvements in exercise performance after HIT, SIT and traditional, continuous-based endurance training protocols are becoming better understood. However, it is perhaps less well understood what makes HIT/SIT such as potent stimulus for enhancing maximal aerobic capacity and associated physiological adaptations akin to that observed following far greater total work. In addition to differences in total work done, exercise duration and intensity, clearly the interval or 'pulsatile' nature of HIT and SIT is a distinguishing factor between protocols. Nevertheless, the comparable responses between these drastically different protocols are perhaps unsurprising given the significant initial reliance on anaerobic metabolism (Table 2; Jones et al. 1985; Cheetham et al. 1986; Nevill et al. 1989; Greenhaff et al. 1994; Bogdanis et al. 1995; Bogdanis et al. 1996; Parolin et al. 1999) and subsequently aerobic metabolism to resynthesize ATP during repeated intermittent high-intensity exercise (Bangsbo et al. 2001).

Table 2 - Estimated total anaerobic contribution and ATP production during a 30 s 'all-out' effort in cycling and running

Reference	Exercise modality	Duration (s)	ATP produced (mmol/kg dry wt)	% contribution	
				PCr	Glycolysis
Bogdanis et al. (1995)	Cycle	0-30	255	24	70
Boobis et al. (1983)	Cycle	0-6	63	47	53
		0-30	189	30	64
Cheetham et al. (1986)	Run	0-30	184	32	63
Jones et al. (1985)	Cycle	0-10	168	42	58
		0-30	291	21	79
Nevill et al. (1989)	Run	0-30	186	33	67

This increase in aerobic metabolism at the expense of anaerobic contribution is indeed reflected in the reduction of peak and average power outputs, as well as reduced rates of glycogenolysis during repeated, successive 'all-out' (i.e. non-paced) 30 s sprints (Bogdanis et al. 1996; Parolin et al. 1999). Moreover, the potency of SIT is no doubt a reflection of its potential to recruit and stress all muscle fibre types, and in particular type II fibres at the very onset of exercise. In support of this statement, Greenhaff and colleagues have demonstrated that whilst ATP utilisation is similar between type I and II fibres in response to a 30 s 'all-out' treadmill sprint in healthy individuals, phosphocreatine and glycogen degradation is markedly greater in the type II fibres (Greenhaff et al. 1994).

As an oxidative phenotype is promoted in response to SIT, it would seem highly likely that similar adaptations in response to other forms of HIT and traditional endurance protocols are at least partly mediated via similar cell signalling pathways. Whilst evidence supporting this is presented below, it should be appreciated that the activation of a signalling pathway is a complex process that occurs in response to diverse stimuli and challenges to homeostasis such as hypoxic, shear and mechanical stress, ATP turnover, changes in calcium flux and substrate availability to name but a few. As Egan and Zierath (2013) state "cell signalling pathways will demonstrate some degree of dependence, crosstalk, interference, and redundancy in their regulation, making the exact contribution of each signalling pathway to measured changes in gene expression difficult to isolate".

At a muscle cellular level, homeostatic perturbations at the onset of exercise activate several key protein kinases (i.e. sensors) involved in signal transduction. Two notable kinases are the 5'-AMP-activated protein kinase (AMPK) and the p38 mitogen-activated protein kinase (p38MAPK). When phosphorylated, and thus activated, they converge upon the cell nucleus and directly activate downstream putative targets, in particular peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α), which has been repeatedly cited as the 'master regulator' of mitochondrial biogenesis (Hood 2001; Jäger et al. 2007) and has more recently been identified as a key player in capillary growth (Arany et al. 2008; Chinsomboon et al. 2009; Geng et al. 2010).

AMPK is an energy-sensing, heterotrimeric enzyme formed from three protein subunits, the catalytic α subunit and the regulatory β and γ subunits. It plays an important role during contraction-induced metabolic disturbances, serving primarily to restore cellular energy status. AMPK is activated in response to an increased AMP:ATP ratio and low muscle glycogen availability, such as during previously discussed intense intermittent exercise or

continuous running events of greater than 200m when aerobic metabolism supersedes anaerobic metabolism for ATP resynthesis (Hardie 2011). There is a wealth of evidence from cell and in vivo rodent studies that strongly implicates the involvement of AMPK in regulating mitochondrial biogenesis, exerting its effects by directly phosphorylating PGC-1α (Zong et al. 2002; Jäger et al. 2007; Irrcher et al. 2008). Zong et al. (2002) observed that in genetically bred mice expressing an inactive dominant-negative (DN) AMPK, neither PGC-1α, mitochondrial DNA, nor mitochondrial density were affected following injection of β-guanidinoproprionic acid used to mimic a state of chronic phosphocreatine depletion in skeletal muscle. Conversely, identical treatment to wild-type mice expressing normally-functioning AMPK was associated with increased protein levels of PGC-1α (Zong et al. 2002). Additional animal research has shown that chronic pharmacological activation of AMPK using 5-aminoimidazole-4-carboxamide riboside (AICAR) results in increases in the mitochondrial enzymes CS and malate dehydrogenase (Winder et al. 2000), as well as increases in PGC-1α mRNA and total protein content in skeletal muscle (Irrcher et al. 2008).

AMPK has also received considerable attention over that past decade in human studies combining exercise with muscle biopsy sampling and analysis techniques. These have observed increased phosphorylation of AMPK following various intense cycling and running protocols (Yeo et al. 2010; Little et al. 2011; Bartlett et al. 2012). Employing a 'real-world' model of exercise in the form of 90 min endurance cycling, Little and colleagues observed a 5 fold increase in acetyl CoA carboxylase (ACC) phosphorylation, a recognized marker of AMPK activation (Little et al. 2010a). The same research group later reported ~100% increase in cytosolic ACC phosphorylation immediately post-exercise in healthy men subjected to 4 × 30s Wingate tests interspersed by 4 min of recovery, as well as an increased PGC-1α protein content at 3 h post-exercise (Little et al. 2011). Of importance is that exercise-induced activation of AMPK occurs in an intensity-dependant manner as

demonstrated by a 2.8-fold increase in AMPK phosphorylation in response to cycling at 80% of $\dot{V}O_{2peak}$ compared to no increase in response to cycling at 40% $\dot{V}O_{2peak}$ (Egan et al. 2010).

p38MAPK has emerged as an important signalling protein implicated in regulating contraction-induced PGC-1-α and adaptations of an oxidative phenotype, with increases in the phosphorylation status of human and rodent skeletal muscle p38MAPK occurring in response to exercise-induced mechanical stress (Chan et al. 2004; Akimoto et al. 2005; Wright et al. 2007; Little et al. 2010a; Little et al. 2011; Bartlett et al. 2012). Wright and colleagues (2007) raised cytosolic calcium levels in rat skeletal muscle with caffeine to activate CaMK (to mimic contractile activity) and observed increases in phosphorylation of p38MAPK and activation of its downstream target, activating transcription factor-2 (ATF2). These authors also observed attenuated expression of PGC-1α and consequent mitochondrial biogenesis by deactivating the CaMK-p38MAPK signalling pathway through inhibition of CaMK and p38 MAPK by the protein kinase inhibitors KN93 and SB202190, respectively (Wright et al. 2007). Moreover, in wild-type sedentary mice subjected to a single bout of voluntary running, a transient increase in PGC-1α mRNA expression in skeletal muscle occurred simultaneously to increased activation of the p38MAPK pathway (Akimoto et al. 2005).

In general, p38MAPK has received less attention over recent years in comparison to AMPK, however data from randomised control trials in humans suggests this protein kinase also plays an important role in initiating the signalling events resulting in adaptations of an endurance phenotype. Little et al. (2010) subjected healthy males to an acute bout of 90 min endurance cycling at 65% $\dot{V}O_{2peak}$. They reported a several fold increase in p38MAPK phosphorylation and a 50% increase in nuclear PGC-1α protein expression immediately post-exercise (Little et al. 2010a). The aforementioned findings provide evidence implicating the

activation of the p38MAPK pathway as an integral component of the intracellular signalling network initiating skeletal muscle adaptations to endurance exercise and in particular mitochondrial biogenesis through its regulatory control of PGC-1α gene expression. A more recent study conducted by this research group, observed increased p38MAPK activation immediately after 4 bouts of all-out 30 s maximal intensity cycling interspersed with 4 min of recovery, as well as increased nuclear PGC-1α protein expression 3 h after exercise (Little et al. 2011). Cochran et al. (2010) reported a 4-fold increase in p38MAPK immediately after 5 x 4 min bouts of cycling at ~90-95% of heart rate reserve. Additionally, they reported an ~8-fold increase in PGC-1α mRNA at 3 h after exercise, providing further implication for the role of p38MAPK being partly responsible for activating mitochondrial biogenesis through a PGC-1α mediated signalling cascade. In support of these data, similar increases in p38MAPK and PGC-1α mRNA have been also observed in humans immediately and 3 h after interval and continuous running protocols, respectively (Bartlett et al. 2012).

PGC-1α co-activates and increases the expression of several transcription factors involved in the regulation of nuclear genes, most notably nuclear respiratory factor -1 (NRF-1) and -2 (NRF-2), which subsequently encode mitochondrial proteins (Handschin and Spiegelman 2006). Knockout of the PGC-1α protein in genetically bred mice has been shown to result in decreased mitochondrial gene expression, such as ATP synthase and decreased function of mitochondrial respiratory chain proteins, for example succinate dehydrogenase (SDH) (Handschin et al. 2007). Although PGC-1α activates NRF-1 and -2 for transactivation of genes involved in the respiratory chain such as CS and SDH, PGC-1α is also indirectly involved in regulating the expression of mitochondrial DNA (mtDNA) transcription through exercise-induced NRF-1 co-activation of mitochondrial transcription factor A (Tfam) (Handschin and Spiegelman 2006; Aquilano et al. 2010; Safdar et al. 2011). There is evidence that an attenuation of PCG-1α transcriptional activity translates to impaired physical

performance in vitro, as mice deficient in PGC-1α protein display reduced exercise capacity and fatigue resistance (Leone et al. 2005). Moreover, mice that over-express the PGC-1α protein exhibit enhanced peak oxygen uptake (~20%) and improvements in physical performance during voluntary and forced exercise (Calvo et al. 2008).

In humans, Gibala and colleagues (2009) reported a 2-fold increase in PGC-1α mRNA 3 h after an acute bout of the SIT protocol repeatedly studied in their laboratory (i.e. 4 x 30 s 'all-out' sprints). Despite these increases in post-exercise PGC-1α mRNA, there was no change in whole-muscle PGC-1α protein content after 3 h of recovery. However in a later study conducted by Little et al. (2011), an increase in nuclear PGC-1α protein abundance at 3 h post this exercise protocol was observed and coincided with an increased (76-83%) mRNA expression of mitochondrial enzymes including CS, COX II and COX IV. This data, along with the previous observations of the absence of an increase in total PGC-1a protein content from whole muscle homogenates at 3 h post-SIT (Gibala et al. 2009), suggests that translocation of PGC-1α from the cytosol to the nucleus might be an important molecular mechanism responsible for activation of this transcriptional co-activator in the early recovery phase following intense exercise in human skeletal muscle. Perry et al. (2010) obtained muscle biopsies from healthy active males in a time-course manner over seven successive identical HIT sessions that resulted in a 12% increase in $\dot{V}O_{2peak}$ over a 2 week period. Specifically, they sampled muscle tissue 4 h and 24 h after every second training session to examine the temporal response of PGC-1α mRNA and protein content to training. The authors observed a progressive increase in PGC-1α total protein content over the seven successive HIT sessions. Moreover, they observed 4- to 10-fold increases in PGC-1α mRNA at 4 h post exercise, which preceded the increase in protein content observed at 24 h. These date suggest that the training-induced increases in PGC-1α protein content may result from the cumulative effects of transient bursts of increased PGC-1α mRNA expression after

successive bouts of training (Figure 3). This temporal and cumulative pattern, which is supported by previous data (Pilegaard et al. 2000; Pilegaard et al. 2003), is indeed specific to a given gene and exercise challenge (Atherton et al. 2005; Wilkinson et al. 2008) and is thought to represent the kinematic basis of phenotypic skeletal muscle adaptation to divergent exercise training stimuli (Egan and Zierath 2013; Camera et al. 2016)

There is growing evidence that PGC-1α is only activated in response to exercise if the intensity of work is sufficient (Egan et al. 2010; Nordsborg et al. 2010; Oliveira et al. 2014). This intensity-dependant regulation akin to AMPK was neatly illustrated by Egan and co-workers who subjected healthy sedentary males to two bouts of energy expenditure matched cycling at ~40% (~70 min) and ~80% (~36 min) of $\dot{V}O_{2peak}$. At 3 h post-exercise, PGC-1α mRNA abundance had increased 3.8 fold after the low intensity bout and 10.2 fold after the high intensity bout suggesting that exercise intensity regulates PGC-1α mRNA abundance in human skeletal muscle (Egan et al. 2010). In support of this, Nordsborg and colleagues (2010) provide data suggesting that the relative intensity of exercise appears important in activating PGC-1α. In their study, trained and untrained subjects performed exhaustive intermittent cycling exercise at 85% $\dot{V}O_{2peak}$ (4 x 4 min bouts interspersed by 3 min). Importantly, they also had the same trained cohort perform the protocol at 70% $\dot{V}O_{2peak}$ (i.e. the same absolute workload of the untrained subjects). A similar increase in PGC-1 mRNA expression was observed between the trained and untrained individuals at the higher relative intensity. However, when comparing the magnitude of increase in PGC-1a mRNA between 70 and 85% $\dot{V}O_{2peak}$ in the trained muscle, this was markedly higher in response to the higher relative workload (Nordsborg et al. 2010).

Whilst much of the research to date on skeletal muscle adaptation to HIT and low-volume SIT has centred on mitochondrial biogenesis and the potency of these to augment the activity

of mitochondrial enzymes, far less is known of its effects on remodelling the microvasculature at the periphery. Of the sparse research available, there are still questions over whether intense interval training supports capillary growth. Jensen and colleagues observed an increased in capillary : fibre ratio in healthy active men following ~ 4 weeks of intermittent one-legged knee extensor exercise at 150 and 90 % of leg $\dot{V}O_{2peak}$ (Jensen et al. 2004a). Moreover, Cocks and colleagues have suggested that a period of exposure to the 'classic' SIT protocol is equally effective as prolonged endurance training in enhancing capillary growth in untrained sedentary men (Cocks et al. 2013). In contrast, high-intensity intermittent cycling (24 x 1 min bouts above $\dot{V}O_{2peak}$) has been demonstrated to present a weaker stimulus for capillary growth in young healthy subjects (with a mean $\dot{V}O_{2peak}$ of 42 $ml \cdot kg^{-1} \cdot min^{-1}$) in comparison to moderate intensity continuous cycling (Hoier et al. 2013a). Insights into the effects of SIT and other intense exercise protocols on capillary growth in trained individuals is remarkably sparse given the previously highlighted importance of the capillary network to performance (Coyle et al. 1988; Iaia et al. 2011). Whilst research in this area is clearly lacking, of the limited data available it appears supplementing a sub-elite soccer players' training regime with additional speed endurance work does not provide a sufficient stimulus to promote capillary expansion (Gunnarsson et al. 2012) and various HIT running protocols are seemingly less effective in improving capillary growth in comparison to longer duration, less intense running protocols (Nybo et al. 2010; Kohn et al. 2011; Gliemann et al. 2014). A recent study by Gliemann and colleagues, recruited a cohort of 160 recreational runners who were subsequently assigned into either a 10-20-30 training group (replacing 2 of 3 weekly training sessions with 10-20-30 training for 8 weeks) or a group that continued their regular training. The 10-20-30 training consisted of 1-min intervals comprised of 30, 20, and 10 s at ~30%, ~60%, and ~90–100% of maximal running speed, respectively, a HIT protocol that was previously developed with recreationally active runners (Iaia et al.

2008; Bangsbo et al. 2009). They examined the effect of this training concept on 5-km run performance, $\dot{V}O_{2max}$ and capillary growth. Whilst the 10-20-30 training increased run performance and $\dot{V}O_{2max}$, it did not influence capillary growth and actually resulted in a reduced total protein expression of the primary capillary growth factor, vascular endothelial growth factor (VEGF) by 22%. Conversely, no changes at all were observed in the group that continued to engage in their regular training. Their results suggest that whilst the 10-20-30 intervention was more effective than the regular training in improving endurance performance and $\dot{V}O_{2max}$, it did not provide a sufficient stimulus for enhanced structural re-modelling of skeletal muscle (Gliemann et al. 2014). The aforementioned reports not only suggest that HIT might be sub-optimal for capillary growth, but perhaps could even have a blunting effect on microvascular re-modelling. Thus, until additional evidence is made available, special care and attention needs to be paid to the distribution of HIT and continuous moderate intensity training depending on the individuals long term goals.

Angiogenesis is the formation of capillaries from existing blood vessels and is fundamental to tissue repair following injury and skeletal muscle adaptation after exercise (Olfert et al. 2016). For exercise-induced angiogenesis to occur, a sufficient intensity and duration of isometric or isotonic contractile activity is required (Egginton 2009). This re-modelling of the microvasculature is believed to occur through two primary mechanisms: sprouting and intussusception angiogenesis. Sprouting angiogenesis has been understood for far longer than intussusception angiogenesis and refers to the process by which activated endothelial cells sprout out from existing capillaries to form new tree like branches. In intussusception (or splitting) angiogenesis, activated endothelial cells extend and initiate longitudinal divide on the luminal side of the capillary, resulting in the formation of two tubes through which the blood can pass. Like mitochondrial biogenesis, the regulation of angiogenesis is controlled via complex processes involving multiple intramuscular signalling pathways (Figure 1).

These are tightly regulated by many putative pro- (including eNOS, MMPs, HIFs and VEGF) and anti-angiogenic factors (including Ang1, Ang2 and Tie2), that destabilise the existing capillary and stabilise the newly formed capillary, respectively (Egginton 2009).

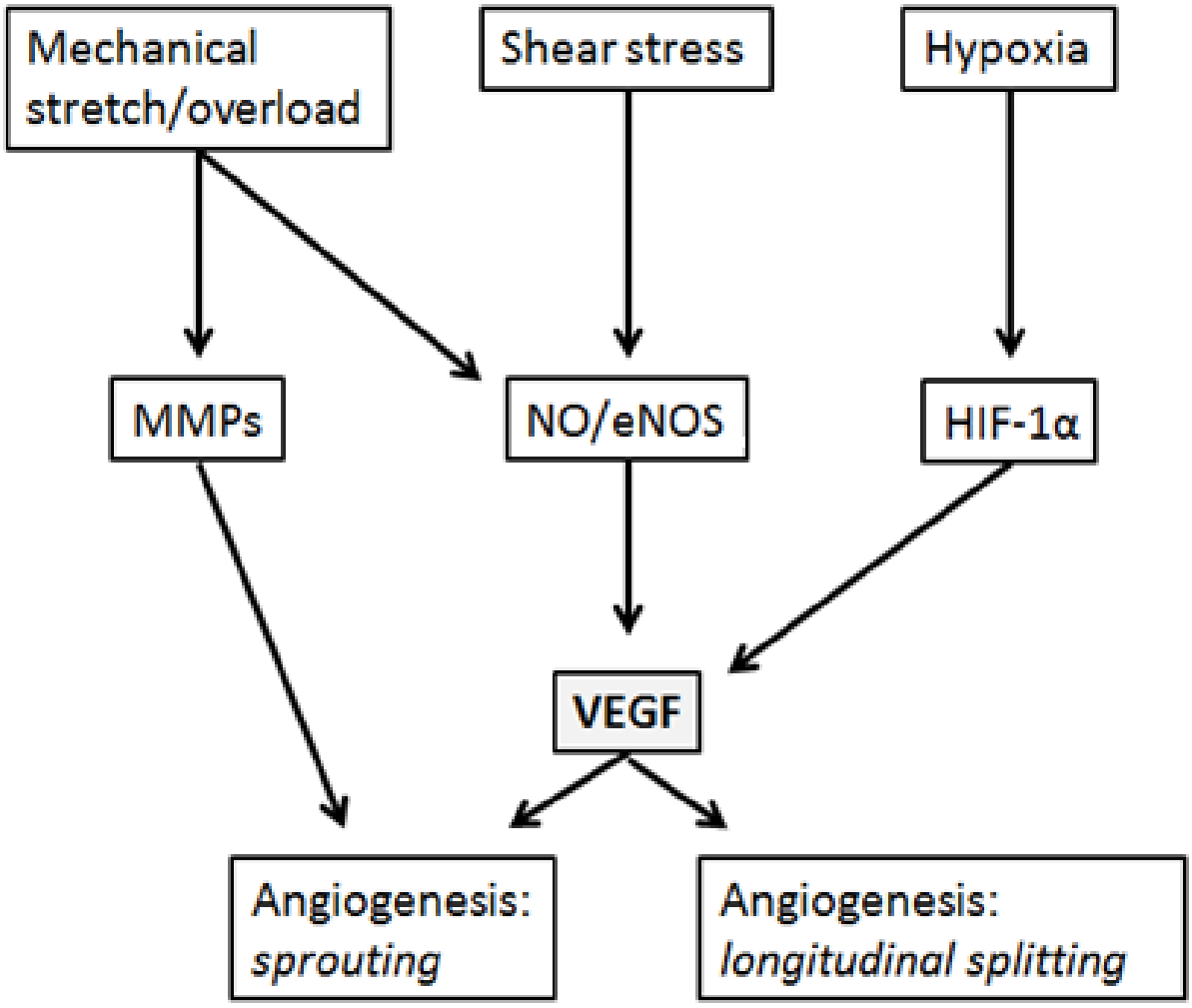

Figure 1 - A simplified schematic of the physiological signals and associated factors evidently important in exercise-induced differential capillary growth.

Vascular endothelial growth factor (VEGF) is strongly associated with the angiogenic response to increases in mechanical stretch (Brown and Hudlicka 2003), shear stress (Zou et al. 1998) and hypoxia (Shweiki et al. 1992). VEGF plays an important role in promoting

endothelial cell production and migration through its receptor VEGF-R2, expressed predominantly by vascular endothelial cells (Brown and Hudlicka 2003). Whilst it should be appreciated that numerous growth factors are involved in the angiogenic process, it is widely accepted that VEGF activation of VEGF-R2 elicits the complete angiogenic response (Conway et al. 2001).

In support of the general acceptance that VEGF plays an essential role in angiogenesis, a high association has been reported between VEGF-R2 protein expression and contractile activity–induced capillary proliferation (Milkiewicz et al. 2003). Moreover, lifelong deletion of VEGF in mice muscle has shown to reduce capillarity by 65% with no compensatory angiogenic pathways available to restore capillarity (Wagner et al. 2006). Moreover, in these mice, run time to exhaustion was only 20% of what these mice could typically achieve with normal VEGF protein content (Wagner et al. 2006). A single bout of endurance exercise is sufficient to induce VEGF mRNA expression in humans (Gustafsson et al. 1999; Ameln et al. 2005; Hoier et al. 2012) and rats (Breen et al. 1996), peaking between 2 and 3 h after exercise before returning to baseline thereafter. It could therefore be expected that VEGF protein levels are increased after a period of training (due to cumulative transient-increases in VEGF mRNA) although this has not always held true with some studies reporting increased VEGF protein content after a period of regular endurance training (Gustafsson et al. 2001; Gustafsson et al. 2002) and others not (Hoier et al. 2012).

As mentioned above, the exercise-induced up- and down-regulation of the aforementioned angiogenic factors is under the influence of changes in shear stress, mechanical stretch, and hypoxia, and their co-ordinated activity is likely to determine when capillary growth is ‘turned on and off’. Whilst it is still not fully understood which of these stimuli are responsible for intussusception and sprouting angiogenesis and which process dominates in

exercise-induced angiogenesis, it has been proposed that intussusception angiogenesis provides a more efficient process to increase the capillary supply as it requires less endothelial cell proliferation (Djonov et al. 2003).

Shear stress

Shear stress is widely considered a primary driver of exercise-induced capillary growth and refers to the frictional drag experienced by endothelial cells as blood flows along its surface or the frictional force of blood applied to the luminal side of the blood vessel (Prior et al. 2004). In rodents, acute administration of prazosin on the microcirculation increases blood flow, can result in a 4-fold increase in shear stress and can increase skeletal muscle C:F by 30% (Dawson and Hudlická 1989).

Nitric oxide (NO) is upregulated in response to shear stress and is believed to play an important role in mediating the effects of VEGF and stimulating endothelial cell proliferation (Williams et al. 2006b; Williams et al. 2006a). While NO may stimulate dilation and activate VEGF (Tsurumi et al. 1997), activation of VEGF-R2 stimulates NO production (Morbidelli et al. 1996), suggesting that there is likely some important reciprocity between these angiogenic factors. In addition to an increase in VEGF, high shear stress induced by prazosin upregulates endothelial NO synthase (eNOS) mRNA and protein expression (Baum et al. 2004), further demonstrating this reciprocity. Moreover, aerobic training has been shown to up-regulate eNOS mRNA in rats (Lloyd et al. 2003) and given that angiogenesis is blunted in the skeletal muscle of eNOS-KO mice after prazosin administration (Baum et al. 2004), these observations provide further evidence for eNOS and in turn NO as vital players in shear stress-mediated angiogenesis induced by exercise and/or pharmacological intervention.

In humans, studies investigating the effects of shear stress on angiogenesis are lacking and a direct role for NO in angiogenesis is yet to be determined. However, eNOS protein content is increased in human skeletal muscle following 4 weeks of 60 min cycling at ~ 60% $\dot{V}O_{2max}$, 3 times per week (Hoier et al. 2012) and 6 weeks of SIT in sedentary individuals (Cocks et al. 2013). Moreover, 90 min of one-legged passive movement exercise (which resulted in a 2.8-fold increase in blood flow and thus increased shear stress and little accompanying O_2 uptake) has been demonstrated to enhance eNOS mRNA and interstitial VEGF protein concentration (Hellsten et al. 2008). More recently these authors observed upregulated eNOS and increased capillary growth after 4 weeks of passive leg movement training for 90 min 4 times per week (Høier et al. 2010).

Recent data also supports an important role for p38MAPK in angiogenesis and in particular shear-stress induced angiogenesis (Pogozelski et al. 2009; Gee et al. 2010). In rodent muscle, pharmaceutical inhibition of p38MAPK prevents shear-stress induced VEGF production and increases in C:F ratio (Gee et al. 2010) and transgenic deletion of p38MAPK blocks endurance-exercise induced PGC-1α mRNA expression and subsequent VEGF mRNA expression (Pogozelski et al. 2009). It is therefore possible that the aforementioned role of p38MAPK in mitochondrial biogenesis could be extended to supporting angiogenesis.

Mechanical stretch/overload

Whilst VEGF appears to be vital for all forms of exercise-induced angiogenesis, sprouting angiogenesis in particular relies on proteolysis of the basement membrane, which is made possible by several families of enzymes, most notably the matrix metalloproteinases (MMPs) which are activated in response to mechanical stretch/overload. This particular stimulus refers to the physical stretch of the vasculature caused by the deformation of cells during

contractions (Prior et al. 2004). MMPs modulate cell-matrix interactions, and with regards to angiogenesis, initiate degradation of the basement membrane, allowing activated EC proliferation and migration so that new branch-like sprouts can form from existing capillaries (Haas et al. 2000; Egginton 2009). A rodent study conducted by Haas and colleagues (2000) demonstrated the importance of MMPs for capillary growth. It was found that by inhibiting the MMPs in rat skeletal muscle, the expansion of the capillary network induced by chronic muscle stimulation was eliminated. Further evidence from rodent studies highlight the importance of increased MMP activity (notably MMP-2 and MMP–9) in regulating exercise-induced angiogenesis, with data supporting roles for ischaemia (Muhs et al. 2004) and muscle-overload/stretching (Rivilis et al. 2002) in increasing the expression of these proteins. In humans, a single bout of aerobic exercise increases MMP-9 mRNA and activates MMP-9 protein (Rullman et al. 2007; Rullman et al. 2009). In their later study, Rullman et al. (2009) subjected 10 healthy men to 5 weeks of 45 min constant load one-legged cycling with and without restricted blood flow, thereby imparting a similar mechanical stress on both legs, yet greater ischaemia and associated metabolic stress (see Esbjörnsson et al. 1993) on the restricted limb. In contrast to MMP-9, MMP-2 mRNA did not increase after a single bout of exercise. Moreover, restricting blood flow had no additional effect on the mRNA expression or activity of either MMPs over that induced by the non-restricted leg, suggesting that metabolic stress did not play a major mediating role in exercise-induced increases in MMP activation in their investigation (Rullman et al. 2009). There appears a dose-dependent relationship between exercise intensity, duration and expression of MMPs. In this regard, Carmeli et al. (2005) observed that 2 weeks of treadmill running at low intensity (18 min at ~ 50% $\dot{V}O_{2max}$) did not alter the expression of MMP-2 in rat skeletal muscle, whilst high intensity exercise (32 min at ~ 70% $\dot{V}O_{2max}$) increased both mRNA and protein levels of MMP-2 in skeletal muscles containing a high percentage of type II fibres. Nevertheless, it

appears the MMPs play an important role in sprouting angiogenesis and exercise of a sufficient duration and/or intensity is required for their upregulation.

Hypoxia

Hypoxia is believed to be one of the most potent stimuli to promote angiogenesis, primarily through up-regulating VEGF (Prior et al. 2004). Lowering the partial pressure of oxygen in culture stimulates endothelial cell proliferation, migration and tube formation (Shweiki et al. 1992). Conversely, returning the partial pressure of oxygen to higher values reduces VEGF expression and suppresses endothelial cell activation (Shweiki et al. 1992). It is becoming increasingly evident that increased oxygen consumption and/or lowered tissue oxygen tension mediate their effects on angiogenesis through the heterodimeric transcription factor, hypoxia inducible factor-1, and in particular it's α subunit (HIF-1α) (Gustafsson et al. 1999; Ameln et al. 2005; Lundby et al. 2006; Semenza 2006; Lindholm and Rundqvist 2015). Ameln and colleagues (2005) observed an increase in HIF-1α protein content and more prevalent staining of nuclear HIF-1α immediately after 45min of one-legged knee extensor exercise in healthy male subjects. Moreover, they observed an increase in VEGF mRNA after exercise, reaching a peak expression at 2 h into recovery, a time-course which appears necessary for exercise-induced transcriptional activation (Pilegaard et al. 2000) and yet further evidence of transcriptional activation of VEGF through HIF-1α. An interesting finding taken from the study by Ameln et al. (2005) was that they observed an increased skeletal muscle HIF-1α protein content immediately after exercise. This could suggest that even during submaximal exercise where blood flow is adequate, the PO_2 of the capillary network in the active muscle may be low enough to promote a hypoxic-induced adaptive response.

Various endurance and resistance based exercise protocols have been demonstrated to increase HIF-1α mRNA (Lundby et al. 2006; Drummond et al. 2008; Larkin et al. 2012)

whilst others have not (Gustafsson et al. 1999; Ameln et al. 2005). Lundby et al. (2006) examined the time-course of HIF-1α mRNA expression in response to a 3 h two-legged knee extensor protocol, whereby one leg had been trained for four weeks (5 days/week) and the other untrained. In the untrained leg, the exercise bout induced a 10-fold increase in HIF-1α mRNA 6 h into recovery, demonstrating for the first time that exercise in normoxic conditions could elevate HIF-1α mRNA and in doing so provided further insight into the likely importance of this transcription factor in regulating exercise-induced adaptive responses. Prior to this, one study had investigated the effects of acute endurance exercise (45 min one-legged kicking protocol) on HIF-1α mRNA content 30 min post-exercise and observed no increase (Gustafsson et al. 1999). Moreover, in the study by Ameln and co-workers (2005) no increase was observed in HIF-1α mRNA expression at immediately post or at 30 min, 2 or 6 h after the 45min one-legged knee extensor exercise. Clearly, differences in exercise protocols and biopsy sampling time-points need to be considered when interpreting these results. With regards to the time course response post-exercise, HIF-1α mRNA was unchanged immediately at termination of exercise and also at 2 h into recovery in the study by Lundby and colleagues, suggesting the mRNA expression of this protein occurs later into recovery from exercise, possibly to replenish lost stores and over time increase basal HIF-1α protein content. Indeed, a recent rodent study conducted by Abe and colleagues observed increased basal levels of HIF-1α following training of a high intensity nature (Abe et al. 2015). Nevertheless, the aforementioned in vitro and in vivo studies suggests VEGF induction by the exercise-induced increases in HIF-1α likely plays a role in promoting endurance adaptations and in particular, capillary growth. The above observations also raise the question as to what is the stimulus associated with the exercise-induced HIF gene responses.

Involvement of PGC-1α and upstream kinases in angiogenesis

In addition to its previously discussed master regulatory role in mitochondrial biogenesis, evidence from transgenic mice studies suggests PGC-1α plays an important functional role in the angiogenic response to exercise, likely through coactivation of estrogen-related receptor-α (ERRα) (Arany et al. 2008; Leick et al. 2009; Chinsomboon et al. 2009; Geng et al. 2010; Tadaishi et al. 2011). Firstly, Arany and colleagues (2008) demonstrated that muscle-specific deletion of the PGC-1α gene impaired angiogenesis in response to hypoxia. Subsequently, Leick et al. (2009) subjected mice with whole body knockout of PGC-1α and wild-type controls to 5 weeks of exercise training, observing lower skeletal muscle VEGF protein content and C : F ratio by ~ 60-80 and 20%, respectively, in the transgenic mice compared to the wild-type controls. Moreover , whilst basal VEGF expression was similar between the knock-out and wild type mice, acute exercise and AICAR treatment (used to pharmacologically activate AMPK), as well as 4 weeks of chronic AICAR injection (without exercise) increased VEGF expression by ~ 15% in the wild-type mice only (Leick et al. 2009). In another study published that year, Chinsomboon et al. (2009) demonstrated that in response to exercise, mice lacking PGC-1α exhibit blunted acute VEGF expression and failed to increase their skeletal muscle capillary density compared to wild-type controls. The importance of PGC-1α for capillary growth was soon further supported by Geng and co-workers who observed a significant attenuation of exercise-induced capillary growth and COX subunit IV protein expression in PGC-1α knock-out mice compared to wild-type controls. Most recently, Tadaishi et al. (2011) reported increased VEGF and eNOS protein expression in skeletal muscle of mice bread to overexpress PGC-1α protein. Furthermore, these authors observed an increase in C : F ratio, $\dot{V}O_{2peak}$ (20%), and exercise capacity (35%, expressed by maximum treadmill speed) in these mice compared to wild-type controls (Tadaishi et al. 2011).

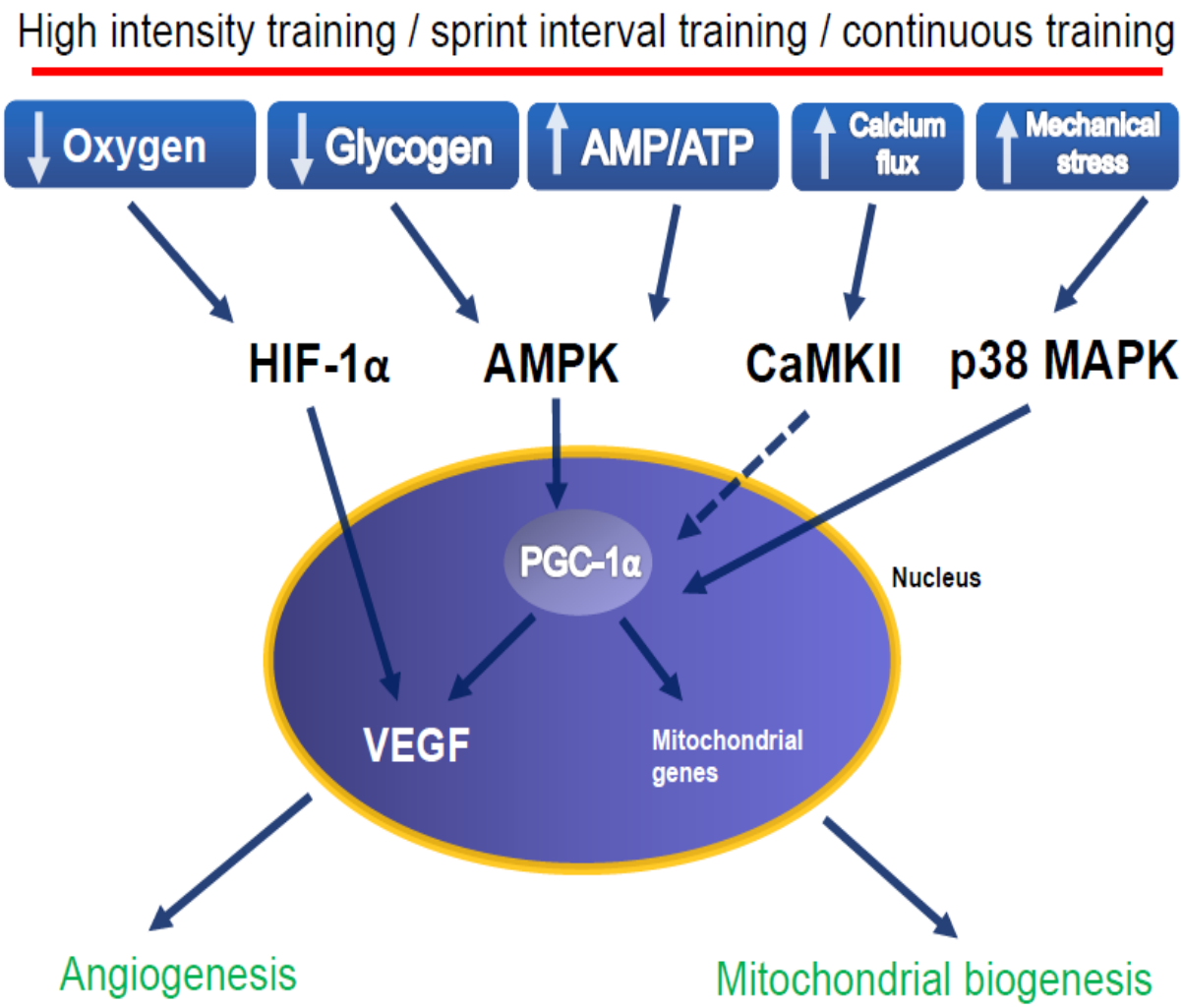

Figure 2 - Schematic representation of the common cell signalling pathways that likely regulate skeletal muscle angiogenesis and mitochondrial biogenesis in response to exercise. Skeletal muscle remodelling in response to HIT, SIT, moderate-intensity continuous training and subsequent activation of the above cell-signalling cascades is not limited to angiogenesis and mitochondrial biogenesis. Increases in fat oxidative capacity, glucose transporters, resting muscle glycogen content and type I muscle fibres are also mediated by common cell-signalling cascades in response to the above exercise stimuli.

The body of research presented thus far provides strong evidence that under conditions of increased cellular energy stress and mechanical stretch, activation of AMPK, p38MAPK and their downstream target PGC-1α likely play an important role in mediating mitochondrial biogenesis and angiogenesis in response to SIT, HIT and traditional endurance training,

possibly through a common intramuscular signalling cascade (Figure 2). However, what is also evident is that the vast majority of research in humans, either performance or mechanistic based, has been conducted on healthy recreationally active individuals. Indeed, the efficacy of SIT in particular has yet to be established in well-trained athletes. Furthermore, whilst the angiogenic response to traditional endurance exercise in humans is relatively well established, somewhat surprisingly, there remains a sparsity of research investigating whether SIT also provides an angiogenic potential in non-sedentary, athletic populations and whether this training stimulus is capable of augmenting the expression of angiogenic growth factors in these populations. Given the aforementioned importance of exercise intensity on upregulating molecular markers implicated in both mitochondrial biogenesis and angiogenesis, it is plausible that this type of training presents angiogenic potential and this warrants further investigation.

2.5 The adaptive scope of trained muscle: the need to adopt novel training methods

Sport and exercise research involving molecular techniques and analysis is in its relative infancy. Seiler and Tønnessen (2009) have stated that ‘about 85% of all publications involving gene expression and exercise are less than 10 years old’ and therefore we do not currently have the capabilities to inform athlete training from molecular snapshots in the field. Light can be shed, however, on the potency of an exercise stimulus to induce adaptation through examining the acute signalling response to exercise. As alluded to in Chapter 1, the challenges scientists face in trying to persuade highly trained athletes and their coaches to participate in investigations of an invasive nature and manipulate their training accordingly, has meant that the majority of the above human investigations into the effects of diverse

training stimuli on acute cellular responses and whole body metabolism have been conducted on healthy, recreationally active male cohorts and sedentary populations. Well-trained individuals are typically accustomed to varied and volumous exercise stimuli and thus it is generally considered more difficult to enhance adaptations in these populations, a notion that is repeatedly reflected at a molecular level. The attenuated adaptive scope of muscle as it becomes more highly trained is nicely demonstrated by a blunting of the acute response of mitochondrial transcript expression in recruited muscle after 6 weeks of endurance exercise training (Schmutz et al. 2006). Furthermore, in the study by Perry and colleagues on untrained active males, although the authors observed a progressive increase in PGC-1α total protein content over the seven successive identical HIT sessions, the magnitude of increase for PGC-1α mRNA expression decreased with every subsequent training session (Perry et al. 2010). Similarly, the 9-fold increase in AMPK activity in untrained muscle from rest after 120 min of cycling (~66 % $\dot{V}O_{2max}$) is almost completely blunted after only 10 identical training sessions (McConell et al. 2005). This attenuated response is illustrated in Figure 3. Not only do these observations highlight the plasticity of skeletal muscle, it demonstrates the necessity for a progressively intensified and/or varied training programme to prevent stagnation of cell-signalling responses that drive adaptation. Indeed, as the athletes training history increases this likely becomes even more important.

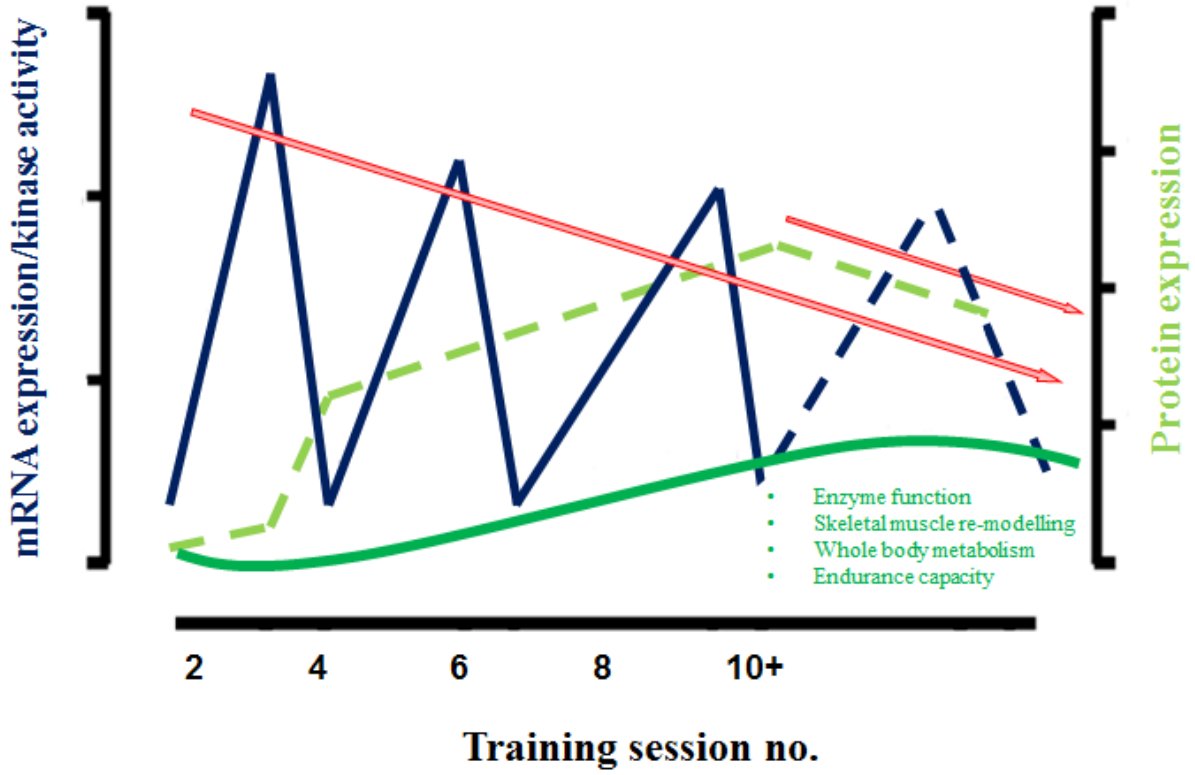

Figure 3 – Schematic representation of the transient and blunted exercise-induced increases in protein kinase activity, mRNA expression and total protein content of important key regulators of structural and functional adaptation to training. This temporal pattern is specific to a given gene and exercise challenge. The red arrows highlight the reduced acute cell signalling response to successive exercise bouts and thus the importance of progressively intensified and/or varied and novel training stimuli to drive continual adaptation (adapted from Perry et al. 2010).

The correlation between the reduced rate of exercise-induced mitochondrial remodelling in endurance trained muscle and associated transcript expression suggests that structural constraints limit myocellular adaptive scope (Flueck 2010). Indeed, there exists an inverse relationship between mitochondrial content and the expression response of mitochondrial and angiogenic transcripts to exercise (Schmutz et al. 2006), such that muscle with the highest mitochondrial densities show reduced regulation of functionally implicated gene transcripts during recovery from exercise. This reduced plasticity of trained muscle beyond a specific morphology and architecture (Saltin et al. 1977; Hoppeler et al. 1985) suggests that either 1)

adaptations in highly trained individuals primarily serves to maintain their level of aerobic performance or 2) that the individual's training has been insufficient to promote further adaptation, perhaps lacking the required exercise intensity, duration and/or training frequency. Training should in theory combine an appropriate mix of these variables as well as adequate variety/novelty of exercise stimuli to prevent stagnation of the acute training stimulus and with this in mind, identifying novel methods of further enhancing the adaptive response to training is a continual quest for coaches and exercise scientists.

2.6 Manipulating exercise and recovery to enhance adaptations in trained individuals

As discussed earlier in this chapter, it is common for athletes to undertake HIT and although less documented and certainly less researched, it is also not unusual for athletes involved in sprint and endurance sports to undertake training sessions consisting of 'all out' interval and continuous efforts, especially during the competition preparation period (i.e. 'taper period') prior to a major competition/event (Mujika 2010). Observations from the author who at the time of completing this thesis provided physiological support to full-time Olympic track cyclists, has confirmed that at various times in the training macrocycle, performing low-volume, 'all-out' sprint interval and continuous cycling form integral sessions within the cyclists' weekly training programmes. However, since the training status of the muscle is considered an important factor in determining the contraction-induced adaptive response to exercise (Coffey and Hawley 2007), the acute and chronic adaptive responses that typically transpire following this low-volume, 'all out' sprint interval training in sedentary or recreationally trained individuals, may not manifest to the highly-trained athlete and thus warrants further investigation. A greater understanding of the physiological responses to this

exercise stimulus in highly-trained athletic populations may help our mechanistic understanding of why it proves such a potent training method for improving health and performance parameters in the untrained. To date only one study has investigated the effect of an acute bout of SIT on PGC-1α and other genetic markers of mitochondrial biogenesis in highly trained athletes. Psilander et al. (2010) observed a 6-fold increase in PGC-1α in national level cyclists subjected to 7 × 30-s 'all out' efforts of sprint cycling and 3 × 20-min efforts at ~87% $\dot{V}O_{2peak}$. However, as the downstream target of PGC-1α, Tfam, increased only following the 'all-out' sprint cycling, it was concluded that in their highly-trained study cohort this specific type of training may prove a more potent stimulus to induce mitochondrial adaptation than the less intense, yet greater volume cycling (Psilander et al. 2010). Interestingly, the 6-fold increase in PGC-1 α is a similar magnitude of change observed in response to the 'classic' SIT protocol in healthy, recreationally active muscle (Gibala et al. 2009). This highlights that brief 'all-out' interval cycling also provides a potent stimulus for mitochondrial biogenesis in highly-trained muscle. Indeed, it should be recognised that the similar magnitude of fold-change between cohorts could simply be due to the fact the trained cyclists performed 3 additional efforts (i.e.7 efforts in total) to those in the studies on untrained individuals. Additionally, there is arguably a greater chance of sampling highly active tissue in trained individuals who are not only typically leaner but also more likely to recruit a larger muscle mass during a given effort.

Clearly there is scope for further investigation into SIT in clinical populations and more so trained cohorts. Firstly, it has not yet been assessed whether this type of training increases the expression of angiogenic factors in trained muscle. Moreover, it remains unclear whether the intermittent nature of this training strategy (and the characteristic alternating hard–easy pattern) is a fundamental aspect related to the potency of SIT to induce comparable cell-signalling responses to traditional endurance training, despite the large difference in total

work done. In addition to differences in total work done, exercise duration and intensity, the 'pulsatile' nature of SIT and HIT is clearly a distinguishing factor between these and traditional endurance exercise.

Given the aforementioned importance of metabolic, hypoxic and shear stress related signals in upregulating transcription and growth factors governing exercise-induced cellular adaptations such as angiogenesis, a plausible method of enhancing the acute and chronic adaptive response to training would be to combine exercise with blood flow restriction (BFR). Using inflatable blood pressure cuffs has become a practical and popular method of restricting blood flow in and around exercise, specifically in combination with low load resistance training to augment muscle hypertrophy and strength (Fujita et al. 2007; Patterson and Ferguson 2010; Loenneke et al. 2012; Wilson et al. 2013; Lowery et al. 2014; Scott et al. 2015). Moreover, 8 weeks of this type of training increases resting muscle glycogen content (Burgomaster et al. 2003) and there is indirect evidence of increased muscle capillarity after 6 weeks of plantar flexion exercise combined with BFR in healthy males (Hunt et al. 2013).

One-legged endurance cycling performed with even moderate blood flow restriction (~ 20 %) in healthy individuals has been shown to promote greater improvements in exercise capacity (assessed via time to exhaustion protocols) and $\dot{V}O_{2max}$, as well as peripheral metabolic and structural changes (i.e. increases in glycogen content, capillary density, type I muscle fibres and the maximal activity of CS) compared to exercising legs with a normal blood supply to the active muscles (Kaijser et al. 1990; Eiken et al. 1991; Sundberg et al. 1993; Esbjörnsson et al. 1993; Nygren et al. 2000). In these series of investigations, blood flow was restricted to the lower legs during exercise by placing the individual's legs inside a large pressure chamber, a method first described by Eiken and Bjurstedt (1987). The chamber is subsequently increased to 50mmHg, which when exercising at the workloads prescribed,

elicits a ~ 20% decrease in leg blood flow (Sundberg and Kaijser 1992). Using these methods, Esbjornsson et al. (1993) subjected 8 healthy men (who were accustomed to physical training) to a 4 week period of 45 min load-matched BFR and non-BFR one legged cycling 4 times per week. Although in this study both the ischaemic and control trained legs improved their one-legged exercise capacity (as measured by a time to exhaustion test), the leg that undertook ischaemic training improved by 4% greater than that of the control leg. Moreover, when exercise capacity was evaluated under ischaemic conditions, the ischaemic trained leg had a further 13% improvement over that reported in the non-BFR trained leg. In alignment with their performance data, a greater increase in resting glycogen content and C : F ratio, as well as a tendency for increased CS and PFK activity was observed after training in the ischaemic leg. Prolonged exercise of this nature has likely been made possible due to the relatively small reduction in leg blood flow (20%) elicited by the pressure chamber. The research that has examined the effects of combining BFR with resistance exercise have traditionally had participants perform bilateral or unilateral knee extension activity with blood pressure cuffs placed on the upper limbs and inflated to pressures that elicit reductions in arterial blood flow by 40-60%. Given the difference in exercise duration and method/extent of reduced blood flow, employing BFR with blood pressure cuffs in combination with exercise may present distinct findings.

Combining intense interval exercise with this practical method of BFR could present a potentially intensified training stimulus (beyond that of interval exercise alone) to augment the above homeostatic perturbations and subsequently activate associated cellular regulatory pathways. Whilst this could plausibly have implications for athletic performance, the efficacy of this novel approach remains to be studied. BFR alters the shear stress response during (Hudlicka and Brown 2009) and post-exercise (Gundermann et al. 2012). Moreover, increased shear stress elicited by reactive hyperaemia following cuff deflation (Gundermann

et al. 2012) could provide an increased stimulus for shear mediated angiogenesis in response to SIT and BFR. Alternatively, BFR exercise may stimulate angiogenesis through signals of reduced oxygen tension and higher metabolic stress (Kawada 2005; Tanimoto et al. 2005; Semenza et al. 2006; Semenza 2006). Utilising the aforementioned pressurised chamber, previous studies have investigated the regulation of exercise-induced angiogenesis in response to BFR and non-BFR single-leg knee extension exercise (45 min at ~24% one leg peak load) (Norrbom et al. 2004; Gustafsson et al. 2007), demonstrating greater expression of the mitochondrial and pro-angiogenic factors, PGC-1α and VEGF-R2, during recovery from ischaemic exercise. More recently, Larkin and colleagues demonstrated that combining BFR with low-load resistance exercise using blood pressure cuffs results in greater exercise-induced increases in VEGF, VEGF-R2 and HIF-1α at 4 h into recovery (Larkin et al. 2012).

Whilst the above demonstrates that combining low-load resistance exercise with BFR using blood pressure cuffs is a practical and thus popular approach for enhancing favourable adaptations in skeletal muscle for clinical and athletic populations, it also highlights scope to investigate the efficacy of combining SIT with BFR. Indeed, this concept presents a potentially potent and novel training strategy to further augment the aforementioned acute and chronic adaptive responses to intense interval training, with clear implications for athletes.

2.7 Summary

This literature review has highlighted the importance of intense interval training and clear scope for further research into optimising the prescription of this exercise stimulus in trained individuals. In particular, it has highlighted the paucity of studies on the acute and chronic effects of SIT in trained individuals and a lack of knowledge on the angiogenic potential of SIT. This is in stark contrast to the well understood effects of SIT on mitochondrial adaptations and somewhat surprising given the importance of the capillary network to physical performance. This review has also identified the need to assess the importance of the 'pulsatile nature' of intense interval-based exercise and whether it is fundamental to the adaptive response to this type of training. Finally, the review has highlighted the attenuated adaptive response in trained individuals already highly accustomed to intense and volumous training regimes, leading to the need for identifying novel methods to enhance the adaptive response to training in these cohorts. The above gaps in the literature have been examined through the following experimental chapters:

Chapter 3:

This investigation examined the importance of the 'interval' effects of acute low-volume, exercise-duration matched interval and continuous 'all-out' cycling on mitochondrial and angiogenic cell signalling in trained individuals. It was hypothesised that given total work done and the pattern of exercise (i.e. whether interval or continuous in nature) appear to have little effect on acute cell signalling responses, brief 'all-out' interval and continuous cycling would induce similar increases in cell signalling cascades linked to mitochondrial biogenesis and angiogenesis in trained skeletal muscle.

Chapter 4:

The aim of this investigation was to assess the adaptive potency of combining 'all-out' sprint interval cycling combined with a practical method of post-exercise BFR on acute mitochondrial and angiogenic cell signalling in trained individuals, compared to sprint interval cycling alone. It was hypothesized that combining sprint interval cycling with post-exercise BFR would present an amplified training stress and thus would induce greater expression of all measured genes and proteins that regulate mitochondrial biogenesis and angiogenesis.

Chapter 5:

In this investigation we attempted to ascertain whether the combination of sprint interval training with post-exercise BFR enhances maximal aerobic physiology and performance in trained individuals to a greater magnitude than sprint interval training alone. It was hypothesized that BFR combined with sprint interval training would result in a greater increase in $\dot{V}O_{2max}$ and 15 km time-trial (TT) performance than sprint interval training alone.

Chapter 6:

The final experimental chapter comprises an athlete case study. Our aim was to incorporate the BFR protocol used in the previous two experimental chapters into the training of a national level sprint cyclist to enhance her aerobic physiology and performance. It was hypothesised that despite a reduction in overall training volume and frequency, a 4 week block of BFR sprint interval training would increase the cyclist's aerobic capacity and performance.

CHAPTER 3 - THE EFFECTS OF EXERCISE DURATION-MATCHED INTERVAL AND CONTINUOUS 'ALL-OUT' SPRINT CYCLING ON ACUTE ACTIVATION OF MITOCHONDRIAL AND ANGIOGENIC CELL SIGNALLING IN TRAINED INDIVIDUALS

3.1 Introduction

The classic SIT protocol, comprising 4-6 x 30 s 'all-out' efforts interspersed by 4 min of recovery, has repeatedly proven as effective as traditional, high-volume endurance training (e.g. > 40 min continuous moderate intensity training) in increasing muscle oxidative potential, buffering capacity, resting muscle glycogen content and muscle capillarity (Gibala et al. 2006; Burgomaster et al. 2008; Cocks et al. 2013). However, these studies have, to date, only been conducted on untrained, recreationally active cohorts whereby this time-efficient training model presents health benefits for sedentary and clinical populations. Indeed, such training interventions present a potentially potent stimulus for enhancing athletic performance, if incorporated correctly into the athletes training programme. Endurance athletes are typically accustomed to high-volume training methods and thus novel, work- and time- efficient exercise stimuli to further enhance training adaptation are desirable for certain times in the macro and micro training cycles.

The underlying factors responsible for the comparable skeletal muscle re-modelling and exercise performance after SIT and high-volume endurance training remain unclear (Baar et al. 2002; Pilegaard et al. 2003; Gibala et al. 2009; Lee-Young et al. 2009). In addition to differences in the initial fitness levels of the aforementioned study cohorts and differences in

total work done, exercise duration and intensity, the interval nature of SIT is a distinguishing factor between the training protocols prescribed. Recent studies (Bartlett et al. 2012; Cochran et al. 2014) have examined the effects of this 'pulsatile' exercise pattern on the acute activation of cell signalling cascades that, if repeatedly stressed over time, regulate endurance based skeletal muscle adaptations and in particular mitochondrial biogenesis (Perry et al. 2010).

In this regard, homeostatic perturbations within skeletal muscle (e.g. increased AMP/ATP ratios and reductions in muscle glycogen) phosphorylate and activate protein kinases such as the adenosine monophosphate-activated protein kinase (AMPK). Upon activation, AMPK converges on the cell nucleus and is implicated in the regulation of peroxisome proliferator-activated γ receptor co-activator (PGC-1α) (Jäger et al. 2007; Cantó and Auwerx 2010). Via interactions with downstream transcription factors and nuclear receptors, PGC-1α is considered to play a "master" regulatory role in exercise-induced mitochondrial biogenesis (Puigserver and Spiegelman 2003) and angiogenesis through an interaction with vascular endothelial growth factor (VEGF) (Leick et al. 2009; Chinsomboon et al. 2009; Geng et al. 2010). Indeed, similar increases in AMPK phosphorylation and PGC-1α mRNA expression have been observed after work/duration matched (~50 min) interval and continuous high-intensity running in recreationally active men (Bartlett et al. 2012). More recently, a study by Cochran and colleagues (Cochran et al. 2014) observed similar increases in acetyl-CoA carboxylase (ACC) phosphorylation (a surrogate marker of AMPK activation) and PGC-1α mRNA expression in response to work-matched SIT and maximal continuous (~ 4 min) cycling. However, whilst acute SIT has previously been shown to increase upstream genetic markers of mitochondrial biogenesis in both recreationally active individuals (Gibala et al. 2009) and 'elite level' cyclists (Psilander et al. 2010), its effects on regulators of exercise-

induced angiogenesis (e.g. VEGF, HIF-1α, eNOS, MMP-9) have not been reported in untrained or trained muscle.

Since work done and the pattern of exercise (i.e. interval vs. continuous) have previously been demonstrated to have little effect on acute cell signalling responses, it was hypothesised that despite differing in total work done, brief 'all-out' cycling, be it repeated intervals or a continuous single effort, would induce similar increases in cell signalling cascades linked to mitochondrial biogenesis and angiogenesis in trained skeletal muscle.

3.2 Methods

Participants

Eight healthy males (mean ± SD: age, 30 ± 5 yr; stature, 180 ± 9 cm; body mass, 79 ± 11 kg; PPO, 16 ± 2 W.kg; $\dot{V}O_{2peak,}$ 4.5 ± 0.5 L.min^{-1}; 57 ± 7 ml.kg^{-1}.min^{-1}; MAP, 347 ± 27 W) volunteered to take part in the investigation. All were involved in cycling activities three to six times per week and on at least one occasion per week also performed resistance training consisting of lower body bilateral compound exercises (e.g. squats, Olympic lifting). Although all participants had previous experience of undertaking high-intensity training, none were engaging in this type of training during the study period. Participants completed a medical questionnaire and biopsy screening document prior to participation to mitigate for maximal exercise and biopsy contraindications. Participants had no history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period. The participants were fully informed of the purposes, risks and discomforts associated with the investigation before providing written consent. The investigation conformed to current local guidelines and the Declaration of Helsinki and was

approved by the Ethics Approval (Human Participants) Sub-Committee of the Loughborough University Ethical Advisory Committee.

Experimental design

In a fully randomized, repeated measures cross-over design, participants performed 'all-out' interval (INT) or continuous (CON) cycling protocols, which were separated by 14-21 days. Muscle biopsies were obtained before, immediately and 3 h post-exercise. Participants recorded all food consumed and physical activity performed during the 24 h prior to their first experimental trial and were instructed to replicate these dietary and activity patterns prior to their second experimental trial as well as refrain from ingesting alcohol and caffeine containing substances during the 48 h preceding each trial. Participants were instructed to arrive at the laboratory by identical means of travel for both experimental trials. All arrived by car and made a short walk to the ground floor laboratory in which all testing took place (i.e. very minimal work was performed in getting to the laboratory). All cycle protocols were performed on an SRM ergometer (Schroberer Rad McBtechink, Weldorf, Germany) calibrated according to the manufacturer guidelines.

Preliminary testing

Two weeks prior to the first experimental trial, participants reported to the laboratory on three occasions, separated by at least 3 days to complete preliminary measurements and protocol familiarisation. During the first visit optimal pedal cadence for peak power output (PPO) was determined by performing maximal sprints (< 12 s) at multiple fixed cadences. The optimal pedal cadence, defined as the zenith of the cadence-peak power relationship, was subsequently used as the fixed (isokinetic) cadence for the subsequent experimental trials. A maximal incremental cycle test was also performed during this visit to establish $\dot{V}O_{2peak}$ and maximal aerobic power (MAP). Following a 5 min warm-up at 120 W, at a freely chosen but

constant pedal cadence, work rate was increased by 20 W every 60 s until volitional exhaustion (8-10 min). Pulmonary gas exchange was measured breath by breath throughout exercise (Oxycon Pro, Carefusion, UK) and $\dot{V}O_{2peak}$ determined as the highest 30 s recording of $\dot{V}O_2$ during the test. MAP was defined as the highest average 60 s power recorded during the test. On the two subsequent visits participants performed familiarisation trials during which they performed the INT and CON protocols. The cycle ergometer saddle and handlebar configuration was consistent for each participant during all exercise testing.

Experimental protocols

Participants attended the laboratory in the morning (~0800 h) of each experimental trial following an overnight fast. On arrival, participants rested in a supine position for 20 min whilst the muscle biopsy sites were prepared. After a resting muscle biopsy was taken, participants performed a standardised warm-up, consisting of cycling at 120 W for 5 min, immediately after which they performed either the INT or CON cycling trials. The INT protocol consisted of 4 × 30 s 'all-out' efforts at the predetermined fixed (isokinetic) pedal cadence. Each bout was interspersed by 4 minute of recovery during which participants remained seated on the cycle ergometer and were permitted to cycle at a cadence of ~60 revs.min^{-1} against a resistance of < 20 W. This resulted in total session duration of 14 min, excluding the 5 min warm-up. The CON protocol consisted of a single 2 min bout of continuous 'all-out' cycling at the same isokinetic pedal cadence. To ensure each protocol was performed in an 'all-out' manner, participants were verbally instructed and encouraged before commencing and during each protocol to attain PPO within the first few seconds of each exercise bout and apply maximal force through the pedals until the end of each exercise bout. Immediately after both protocols, participants quickly dismounted the ergometer and were helped onto an adjacent plinth where the immediate post-exercise muscle biopsy was taken within 1 min of the cessation of each protocol. During this time a finger-prick capillary

blood sample was obtained and then every consecutive minute and immediately analysed for lactate concentration (Biosen HBAC1 Analyser, EKF Diagnostics, UK) until a peak lactate concentration was determined (within 10-15 minutes). Participants then rested before a final muscle biopsy was taken 3 h post-exercise. Biopsies for a given trial were obtained from the same leg through separate incisions ~ 3cm apart. In the subsequent experimental trial, biopsies were obtained from the alternate leg in a randomised, cross-over fashion to avoid any potential order bias. The consumption of food was prohibited at all times during each experimental trial. Water was consumed freely but the volume and pattern ingested was noted during the first trial and replicated for the subsequent trial. Laboratory conditions remained constant for both experimental trials (19-21°C, 40-50% humidity). Power output during preliminary testing and both experimental protocols were sampled at 0.5 Hz and analyzed using SRM software (v6.40.05, Schoberer Rad Messtechnik, Germany) which was calibrated before each trial by recording the zero offset without any force/load on the cranks. Peak power output (PPO) was defined as the highest single power output value recorded during each sprint. Mean power output (MPO) was defined as the average value of all measures of power output throughout each sprint.

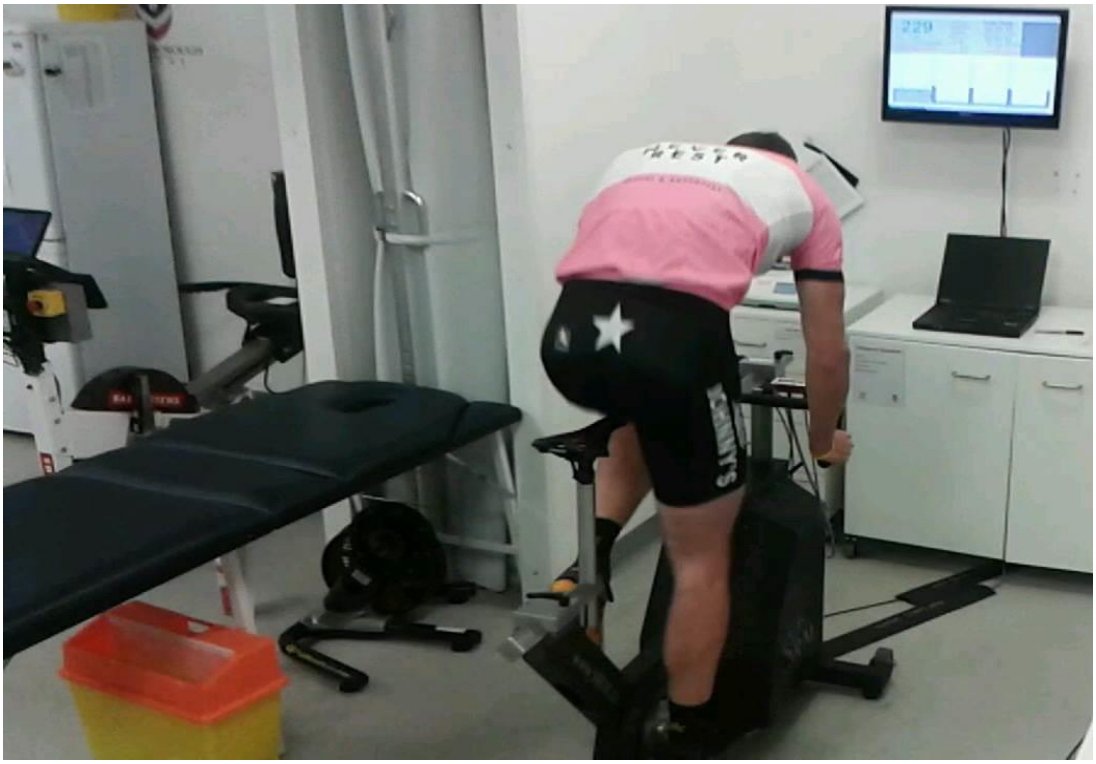

Figure 4 - Participant performing the INT protocol (protocol can be seen displayed on the monitor in the background). The plinth adjacent to the SRM ergometer in the background is where particpants lay to have muscle biopsies taken.

Muscle biopsy sampling and analysis

Muscle biopsies were obtained from the medial portion of the vastus lateralis muscle under local anaesthesia (1% lidocaine) using the micro-biopsy technique (Acecut 11G Biopsy Needle, TSK). Muscle samples at each time point were obtained through separate incisions with two needle insertions and thus two samples (each ~ 25 mg) being taken from each incision. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses.

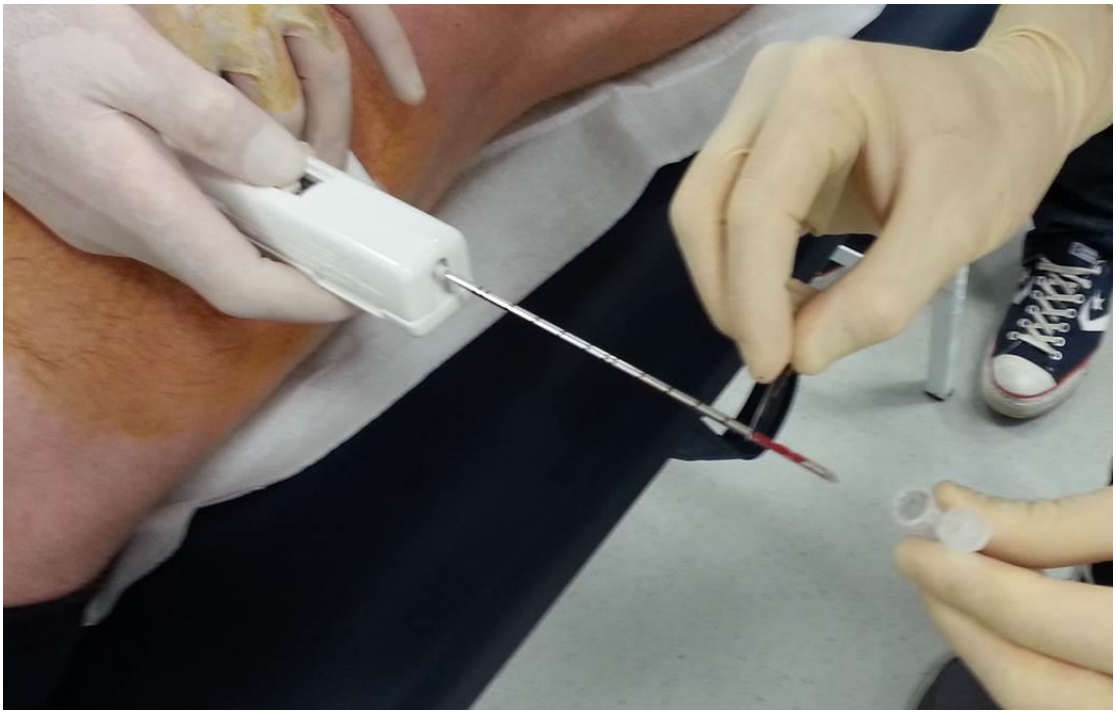

Figure 5 - Muscle tissue being sampled using the micro-biopsy technique.

Western blot analysis

Approximately 20 mg of frozen muscle was ground to powder under liquid nitrogen using a laboratory grade pestle and mortar before being homogenised in 120 µl of ice cold lysis buffer (25 mM Tris/HCl [pH 7.4], 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-Pyrophosphatase, 1 mM Na_3VO_4, 0.27 M sucrose, 1% Triton X-100, 0.1% 2-mercaptoethanol) supplemented with a Pierce™ Protease Inhibitor Tablet (Thermo Scientific, UK). Homogenates were centrifuged at 13,500*g* for 10 min at 4˚C and the supernatant collected. Protein content of the supernatant was determined using a Pierce™ 660 Protein Assay (Thermo Scientific, UK). Each sample was solubilized for 5 min at 100˚C with an equal volume of sample buffer containing 1 M Tris-HCl (pH 6.8), 8% glycerol, 10% sodium dodecyl sulphate, 0.4% 2-β-mercaptoethanol and 0.05% bromophenol blue. For each blot a negative control was loaded along with 25 µg of each sample and then separated (~100 V for ~2 h) in Tris-glycine running buffer using self-cast 4% stacking and 10% separating polyacrylamide gels. Gels were transferred wet onto nitrocellulose membranes for 2 h at 35

mA in a 1 × transfer buffer (0.3% Tris base, 1.4% glycine, 20% methanol). Membranes were then blocked for 1 h at room temperature in Tris-buffered saline (TBST: 0.19 M Tris [pH 7.6], 1.3 M NaCl, 0.1% Tween-20) with 5% non-fat blocking grade milk. Membranes were washed for 3 x 5 min in TBST before being incubated overnight at 4˚C with anti-phospho^{Thr172} (cat no. 2531) and anti-total AMPK (cat no. 2532) antibody (both from Cell Signalling, UK), at a concentration of 1:1000 in 1 X TBST. The following morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with anti-species horseradish peroxidise-conjugated secondary antibody (Bio-Rad, UK) for 1 h at room temperature. After a further 3 x 5 min washes in TBST, membranes were saturated in chemiluminescence (SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA) for 5 min prior to exposure. Membranes were visualised using image analysis (ChemiDoc^{TM} XRS+, Bio-Rad, Herts, UK), and band densities determined (Quality One 1-D analysis software v 4.6.8, Bio-Rad, Herts, UK). Samples from each participant for both exercise protocols were run on the same gel and all gels were run in duplicate to verify responses. All raw densitometry data were used for statistical analysis purposes so as to compare the within-subject responses to both the SIT and CON protocols. However, because it is technically incorrect to compare densitometry data between gels and thus subjects, for graphical purposes each subject's pre-exercise values of phosphorylation relative to total for each participant in both trials was normalised to 1 with post-exercise and 3 h post-exercise values subsequently expressed as fold-change relative to pre-exercise values (hence there are no error bars for this time-point). This approach has been used previously by a number of other researchers (Clark et al. 2004; Perry et al. 2010; Bartlett et al. 2012).

Real-time RT-PCR

One-step quantitative RT-PCR was used to determine skeletal muscle mRNA levels of genes of interest. Primer sequences (Table 3) were designed by Sigma-Aldrich (Sigma-Aldrich Co. Ltd., Haverhill, UK) ideally with 40-60% GC content and spanning exon-exon boundaries. Primer specificity was determined by performing BLAST and melt curve analysis at the end of each PCR run. Total RNA was isolated from muscle tissue using TRIzol® according to the manufacturer's instructions (Life Technologies/Invitrogen, USA). Briefly, once the tissue (~25mg) was homogenised in TRIzol®, chloroform (1:5 v/v) was added followed by RNA precipitation using isopropanol. The resultant RNA pellet was washed in 75% absolute ethanol and air dried prior to re-suspension in 50µL of 1mM sodium citrate. RNA concentration (232 ± 73 ng/µl) and purity (260/280: 1.9 ± 0.1) was confirmed using spectrophotometry (Nanodrop) before being stored at -80 °C for future use. 20 µl PCR reactions were made up as follows in a 96 well plate; 70 ng of RNA in 9.5 µl of nuclease free water, 0.2 µl of Quantifast Reverse Transcriptase mix (Qiagen, Crawley, UK), 0.15 µl of both forward and reverse primers at 100 µM concentrations, and 10 µl of SYBR green mix (Qiagen). All reactions were performed in triplicate. Once PCR plates were prepared, they were transferred to the mx3005p qPCR cycler (Stratagene MX3005P, Agilent Technologies, Berkshire, UK), which was programmed to perform the following steps; 50°C for 10 min (reverse transcription), followed by a 5 min hold at 95°C, and then 40 cycles at 95°C for 10 s and 60°C for 30 s. Fluorescence was detected at the end of each cycle, and expression levels were determined using the $2^{-\Delta\Delta Ct}$ method using RNA polymerase II (RPII) as the reference gene. Stability of this reference gene in response to both protocols is demonstrated in Figure 6. The mRNA expression was

calculated according to Livak and Schmittgen (2001). Post-exercise values are reported as a fold-change relative to pre-exercise values for each individual participant as described previously (Pilegaard et al. 2000; Norrbom et al. 2004; Lundby et al. 2006; Psilander et al. 2010).

Table 3 - Primers used for real-time RT-PCR analyses

Target Gene		Primer sequence	No. in Gene bank
VEGF	Forward	CTGCTCTACCTCCACCAT	NM_001171630
	Reverse	ATGAACTTCACCACTTCGT	
PGC-1α	Forward	CCTCTTCAAGATCCTGCTA	NM_013261
	Reverse	ACTCTCGCTTCTCATACTC	
eNOS	Forward	CAAGTTGGAATCTCGTGAA	NM_001160111
	Reverse	TGTGAAGGCTGTAGGTTAT	
MMP-9	Forward	GGCACCTCTATGGTCCTC	NM_004994
	Reverse	AGTAGTGGCCGTAGAAGG	
HIF-1α	Forward	TCACCTGAGCCTAATAGTC	NM_181054
	Reverse	AATCTGTGTCCTGAGTAGAA	
RPII	Forward	GAGTCAACGGATTTGGTC	NM_000938.1
	Reverse	GGTGGAATCATATTGGAACAT	

VEGF, vascular endothelial growth factor; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; eNOS, endothelial nitric oxide synthase; MMP-9, membrane metalloproteinase-9; HIF-1α, hypoxic inducible factor-1α; RPII, RNA polymerase II

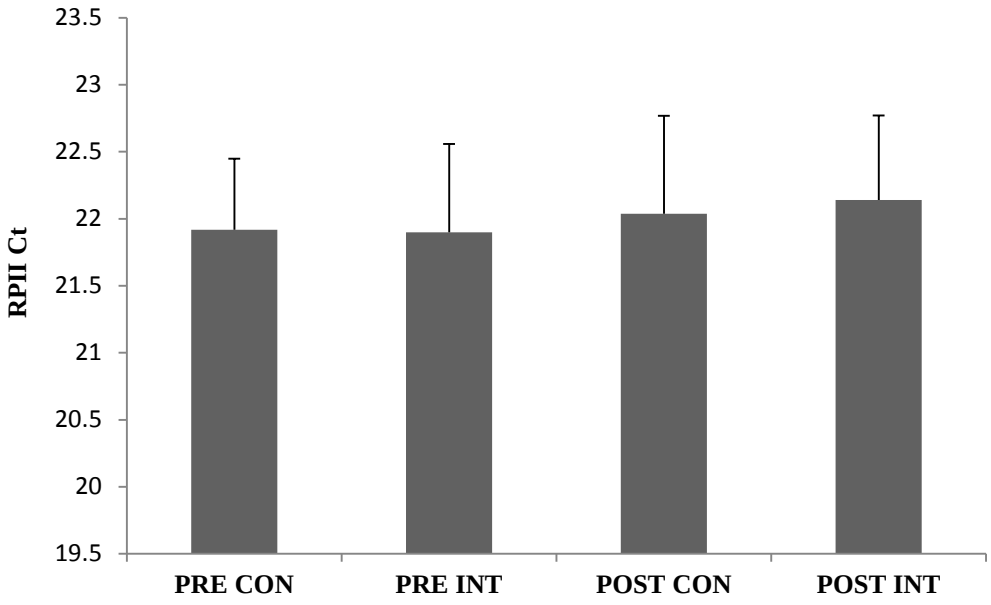

Figure 6 - Ct values for RPII housekeeping gene across conditions and time points.

Statistical analysis

Protein phosphorylation and mRNA data were analysed using a two-way ANOVA. Where significant main effects were observed, Bonferroni corrected post-hoc t-tests were used to locate differences. Student's t-test for paired samples was also used to compare differences in physiological and performance variables between time points and protocols. All data are presented as mean ± SE. Significance was accepted at $P < 0.05$.

3.3 Results

Physiological and performance variables

The optimal cadence for PPO was 118 ± 5 revs.min^{-1}. There was no difference (P = 0.93) in PPO attained during INT and CON (1217 ± 257 vs. 1215 ± 201 W, respectively). However, there was a greater MPO (Figure 7) during INT compared to CON (593 ± 61 vs. 386 ± 23 W, P = 0.01) resulting in 53% greater total work done in the INT compared to CON (71.2 ± 7.3 vs. 46.3 ± 2.7 kJ, P = 0.01). Peak blood lactate concentration was higher (P = 0.04) following INT compared to CON (19.2 ± 1.0 vs. 17.4 ± 2.1 mmol.L^{-1}, respectively).

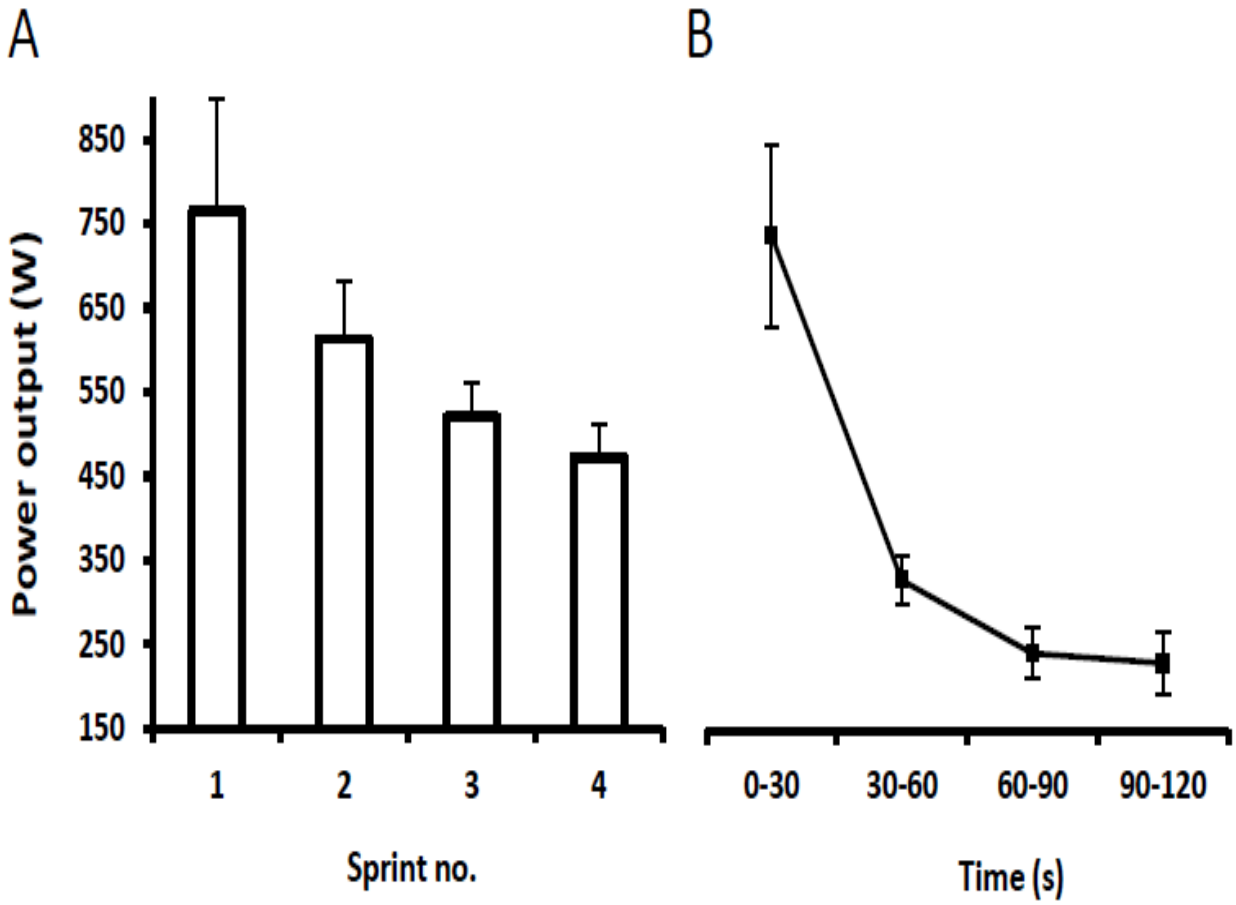

Figure 7 - MPO achieved for each of the 4 x 30 s 'all-out' sprints during INT (A) compared to the MPO achieved in each consecutive 30 s period of the continuous 2 min 'all-out' effort in CON (B).

AMPK activation

Phosphorylation of AMPK^{Thr172} increased (P = 0.011) 1.6- and 1.3-fold immediately following INT and CON, respectively, before returning to baseline at 3 h post-exercise (Figure 8). The magnitude of phosphorylation immediately post-exercise was not different between protocols (P = 0.347).

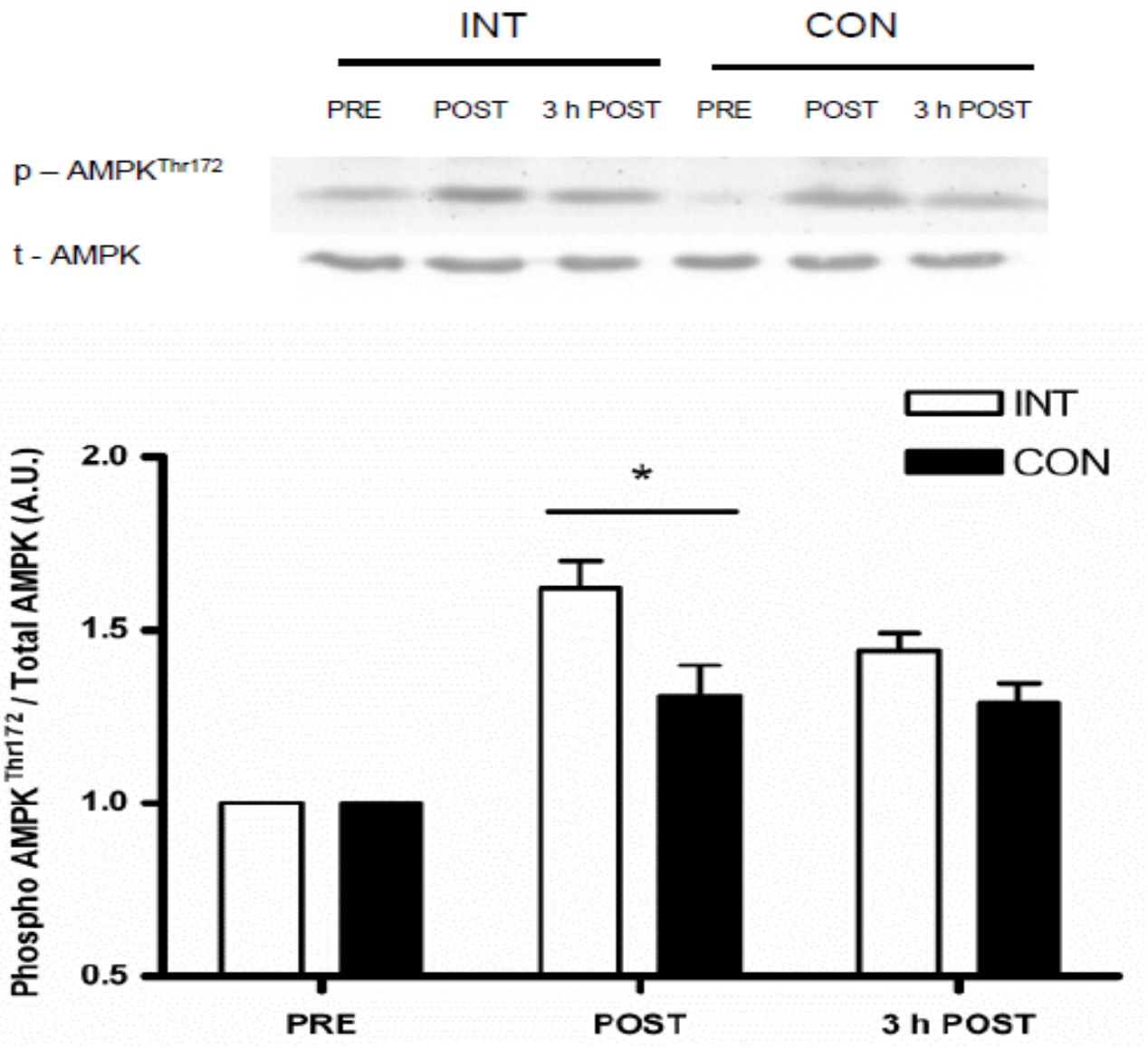

Figure 8 - Phosphorylation of AMPKThr172 expressed relative to total AMPK immediately before (PRE), after (POST) and 3 h after (3 h POST) the INT and CON protocols. Representative Western blots above figure. p-, phosphorylated; t- total. Each participant's PRE values have been normalised to 1 and thus the POST and 3 h POST values are expressed as fold change relative to PRE values. Values are means ± SE (n=8). *Significant difference from Pre and 3 h Post (P < 0.05).

mRNA expression

There was an increase in mRNA expression of PGC-1α (5.5- vs. 7.0-fold, P <0.01) and VEGF (4.3- vs. 3.5-fold, P = 0.02) from pre to 3 h post-exercise in CON and INT, respectively, the magnitude of which were not different between trials (Figure 9). HIF-1α mRNA expression increased (1.5- vs. 2-fold, P = 0.04), and there was a trend for MMP-9 mRNA expression (1.4 vs. 1.3-fold, P = 0.06) to increase from pre to 3 h post-exercise in CON and INT, respectively, the magnitude of which were not different between trials (Figure 10). There was no change in eNOS mRNA expression (P = 0.6) in either CON or INT (Figure 10).

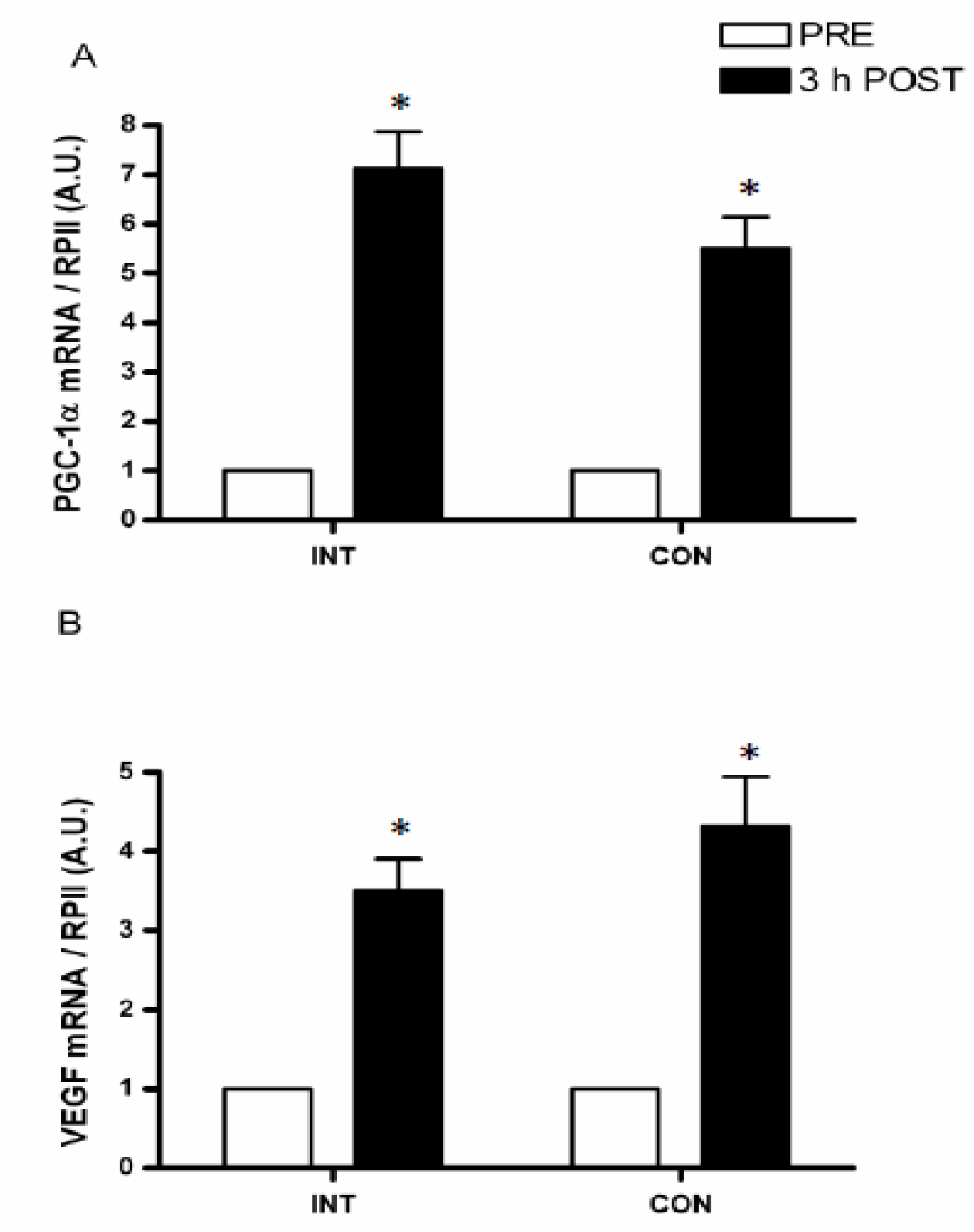

Figure 9 - PGC-1α (A) and VEGF (B) mRNA expression immediately before (open bars) and at 3 h after (closed bars) the INT and CON protocols normalised to RPII mRNA content and expressed relative to PRE. Values are means ± SE (n=8). *Significant difference from PRE ($P < 0.05$).

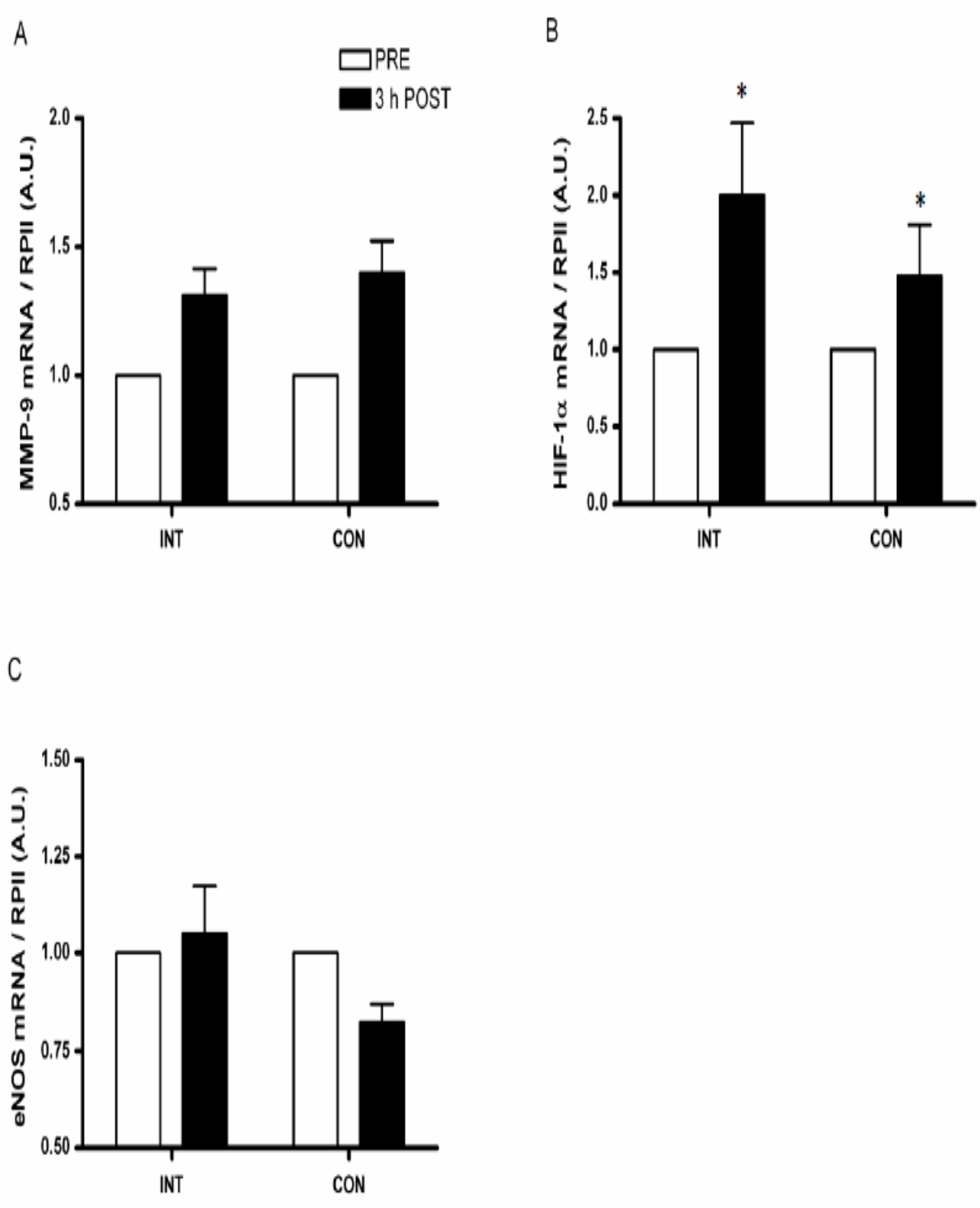

Figure 10 - MMP-9 (A), HIF-1α (B) and eNOS (C) mRNA expression immediately before (open bars) and at 3 h after (closed bars) the INT and CON protocols normalised to RPII mRNA content and expressed relative to PRE. Values are means ± SE (n=8). *Significant difference from PRE (P < 0.05).

3.4 Discussion

This study has demonstrated that low-volume interval and continuous 'all-out' sprint cycling, both totalling 2 min in duration but differing in total work done, induce comparable AMPK phosphorylation and increases in PGC-1α, VEGF and HIF-1α mRNA expression in trained individuals.

The similar increases in AMPK phosphorylation observed immediately after both protocols in the present study, are consistent with those previously reported in trained (Yeo et al. 2010; Little et al. 2010b) and untrained (Gibala et al. 2009; Little et al. 2011) human muscle after various cycling protocols. The capability of INT and CON to induce comparable acute cell signalling responses (despite 53% more work being performed in INT) and similar magnitudes of increase to that observed after traditional high-volume endurance training appears related to the 'all-out' nature of the exercise. This possibly reflects the rapid and significant depletion of ATP and muscle glycogen content that occurs in all fibre types and in particular type II muscle fibres during 'all-out' sprint exercise (Greenhaff et al. 1994; Bogdanis et al. 1996; Hargreaves et al. 1998; Karatzaferi et al. 2001). Indeed, in human skeletal muscle the highly glycolytic type IIX fibres display the greatest basal and exercise-induced increases in AMPK phosphorylation (Lee-Young et al. 2009) and the high rates of glycogenolysis observed during this type of exercise (Hargreaves et al. 1998) is likely to play a role given the importance of muscle glycogen in regulating AMPK activity (Wojtaszewski et al. 2003; Yeo et al. 2008; Philp et al. 2012).

In accordance with the role of upstream AMPK activation in regulating PGC-1α (Jäger et al. 2007), the similar magnitudes of fold changes in PGC-1α mRNA observed at 3 h after INT and CON are consistent with magnitudes and time courses previously reported in response to cycling and running in trained (Little et al. 2010b; Psilander et al. 2010) and untrained

(Gibala et al. 2009; Egan et al. 2010; Bartlett et al. 2012) individuals. Whilst our data supports previous observations (Gibala et al. 2009), we also provide novel data that a single 2 min continuous 'all-out' effort provides an equal stimulus for PGC-1α expression. As with AMPK, PGC-1α mRNA abundance is increased in an intensity-dependent manner in the hours after exercise, (Egan et al. 2010; Nordsborg et al. 2010; Tobina et al. 2011). Increases in PGC-1α protein content however typically occur in the days following exercise (Baar et al. 2002), probably as a result of cumulative transient increases in mRNA transcripts encoding new protein after successive training bouts (Perry et al. 2010). Further research is required to determine whether both protocols used here would result in comparable PGC-1α protein increases after an identical number of successive training sessions in both trained and untrained individuals.

PGC-1α is implicated in a signalling cascade that results in the induction of VEGF (Chinsomboon et al. 2009; Geng et al. 2010), the most important angiogenic growth factor, integral to both longitudinal splitting and sprouting angiogenesis (Olfert et al. 2010; Egginton 2011). It is possible that PGC-1α played a role in increasing the VEGF mRNA expression observed after both 'all-out' exercise protocols given the regulation of PGC-1α by post-translational modifications (i.e. phosphorylation and deacetylation) (Puigserver et al. 2001; Jäger et al. 2007). The similar magnitudes of VEGF mRNA increase that we report here are consistent with those observed in the early hours of recovery after acute exercise of varied intensities and durations (Gustafsson et al. 1999; Jensen et al. 2004b; Hoier et al. 2012; Hoier et al. 2013b), and to our knowledge are the first reported in response to both interval and continuous 'all-out' cycling. Although clearly important, it remains to be fully established whether VEGF mRNA is increased during recovery from exercise primarily to replenish secreted VEGF protein or is in fact vital to trigger VEGF secretion.

Distinct angiogenic phenotypes occur as a result of exposure to different stimuli which is affected by the mode and intensity of exercise (Egginton 2011). HIF-1α is a key regulator of the tissue to hypoxia and thus metabolic stress (Semenza et al. 2006; Semenza 2006), during which it is stabilized and functions in regulating angiogenesis through targeted activation of VEGF in human skeletal muscle (Lee et al. 2004; Ameln et al. 2005). Acute exercise results in an increase in skeletal muscle HIF-1α mRNA expression provided that the exercise intensity is sufficient or the tissue is exposed to a hypoxic stimulus (Vogt et al. 2001; Zoll et al. 2006). Thus, the comparable increases in HIF-1α mRNA expression observed in the early recovery after exercise in the present study may tentatively suggest INT and CON provide a similar stimulus for hypoxia-mediated angiogenesis. Neither of the protocols used here increased eNOS mRNA which has been implicated in high-shear stress mediated angiogenesis (Egginton 2009). However, there was a trend ($P = 0.06$) in the present study for comparable increases in MMP-9 mRNA after INT and CON. MMP-9 is activated in response to muscle overload or mechanical stretch during contractions and plays a role in initiating proteolysis of the endothelial cell (EC) basement membrane, thus facilitating EC migration and the formation of new capillaries (Egginton 2009). Increased MMP-9 mRNA expression has previously been observed 2 hours into recovery from moderate intensity endurance exercise in healthy human skeletal muscle (Rullman et al. 2007; Rullman et al. 2009) and such transcriptional activation appears important in the regulation of MMP-9 activity (Van den Steen et al. 2002).

The present study is not without limitations. Notably, assessments of exercise-induced changes in muscle glycogen content and skeletal muscle protein expression of mitochondrial and angiogenic factors were not made. Moreover, measures of interstitial angiogenic protein content were not assessed in the present study. Indeed, although previous studies have demonstrated that the acute angiogenic response can help to inform the magnitude of

capillary growth with training (Hellsten et al. 2008; Høier et al. 2010; Hoier et al. 2012), temporal factors relating to the timing and number of biopsies, as well as the training status of the examined muscle, need to be considered when predicting chronic adaptations based on transient molecular responses to acute exercise. Intermittent high-intensity exercise has been previously shown to induce greater elevations of angiogenic growth factors than moderate intensity continuous exercise, however the latter has been demonstrated to result in greater acute elevations in interstitial levels of VEGF (Hoier et al. 2013a). Moreover, whilst increased capillarity has been observed after 4 weeks of high-intensity intermittent knee extensor exercise in young healthy individuals (Jensen et al. 2004a), others have provided data suggesting high-intensity intermittent training provides a weak stimulus for capillary growth in a similar population (Hoier et al. 2013a). Although it is plausible from our acute data that trained skeletal muscle exposed to repeated bouts of INT and CON over time would promote comparable increases in mitochondrial biogenesis and capillarity further work is required to directly assess these changes. Finally, future work should also consider whether this 2 min all-out effort repeated over a period of time has the capacity to improve overall fitness. Cochran and colleagues (2014) have recently demonstrated in untrained individuals, that whilst 'all-out' SIT and work-matched continuous cycling (~ 4 min) induce similar acute cell signalling responses, when performed regularly over 6 weeks, the continuous protocol did not augment the maximal activity or protein content of mitochondrial markers as has been observed after a similar exposure to SIT in earlier work by this group (Gibala et al. 2006; Burgomaster et al. 2008). That said, in their relatively untrained cohort, the continuous protocol (which should be noted was not 'all-out' in nature as evidenced by the significantly lower mean peak power output attained in this trial in comparison to SIT) was sufficient to increase $\dot{V}O_{2peak}$ (Cochran et al. 2014).

In conclusion, low-volume ‘all-out’ sprint interval and continuous cycling, matched for total exercise duration but not work done, provides an equally potent stimulus to activate cell signalling pathways associated with exercise-induced mitochondrial biogenesis and angiogenesis in trained skeletal muscle. These findings add to existing data demonstrating that in addition to the amount of total work done, the interval/continuous nature of a training session appears to have little effect on the observed cell signalling response. Whilst these data have implications for exercise prescription and implicate exercise intensity as the key driver in regulating adaptation to low-volume exercise, training studies employing similar ‘all-out’ exercise protocols are warranted to investigate their effects on structural re-modelling, whole body metabolism and exercise performance.

CHAPTER 4 - THE EFFECTS OF SIT AND SIT COMBINED WITH POST-EXERCISE BFR ON ACUTE MITOCHONDRIAL AND ANGIOGENIC CELL-SIGNALLING IN TRAINED INDIVIDUALS

4.1 Introduction

The previous chapter demonstrated that manipulating the pattern of low-volume 'all-out' exercise (i.e. whether that be interval or continuous in nature) does not impact on the acute activation of cell signalling pathways associated with exercise-induced mitochondrial biogenesis and angiogenesis in trained skeletal muscle. A potential strategy to exacerbate the cell-signalling responses to SIT and over repeated training sessions present an intensified stimulus for re-modelling of skeletal muscle would be to combine SIT with post-exercise BFR using blood pressure cuffs. The efficacy of this concept has not been explored yet by exercise scientists as a viable novel, and thus potent, training stimulus. As discussed in Chapter 2, the signals for capillary growth differ depending on the exercise modality and the angiogenic response is graded according to the stimulus intensity (Egginton et al. 1998). Indeed SIT with the addition of post-exercise BFR during the recovery periods between bouts may provide an augmented stimulus for angiogenesis through signalling associated with reduced oxygen tension and higher metabolic stress (Gustafsson et al. 2007). For example, the decrease in muscle oxygen levels during low-load resistance exercise combined with BFR (Kawada 2005) has been demonstrated to increase HIF-1α mRNA, VEGF, and VEGF-R2 mRNA, whilst the same exercise non-restricted has not (Larkin et al. 2012). Moreover, exercise induced VEGF mRNA expression appears to be graded by metabolic stress (Gustafsson et al. 1999; Takano et al. 2005) and BFR has been previously demonstrated to

enhance the metabolic perturbations during low intensity exercise (Sundberg 1994; Takano et al. 2005; Reeves et al. 2006; Suga et al. 2009; Krustrup et al. 2009). It is plausible that cell-signalling cascades upregulated in response to metabolic stress to regulate mitochondrial biogenesis (i.e. the AMPK/p38MAPK-PGC-1α pathway) are also involved in the angiogenic response to BFR and non-BFR exercise. In addition to its hypoxic and metabolic effects on the skeletal muscle, BFR alters the shear stress response during (Hudlicka and Brown 2009) and post-exercise (Gundermann et al. 2012). These stimuli play a predominate role in up-regulating eNOS and MMP-9 expression, which are likely key factors involved in longitudinal splitting and sprouting angiogenesis (Haas et al. 2000; Brown and Hudlicka 2003; Prior et al. 2004; Williams et al. 2006a; Williams et al. 2006c). It is plausible that increased shear stress induced by addition of BFR during exercise, and in particular the likely increased reactive hyperaemia upon cuff deflation, would provide a greater stimulus compared with SIT alone to upregulate the above genes and drive shear stress-induced angiogenesis. Establishing this angiogenic response to acute exercise can inform the morphology and extent of capillary growth with repeated training (Høier et al. 2010; Hoier et al. 2012).

The purpose of this investigation was therefore to assess the potency of combining SIT with a practical method of post-exercise BFR in trained individuals on acute intramuscular cell signalling that regulates remodelling favouring enhanced aerobic capacity, in particular angiogenesis and mitochondrial biogenesis. It was hypothesized that combining SIT with post-exercise BFR would present an amplified training stress compared to SIT alone, as examined by an increased expression of recognised markers of mitochondrial biogenesis and ischemic, metabolic, shear and mechanical stress induced angiogenesis, notably PGC-1α, VEGF, VEGF-R2, HIF-1α, eNOS and MMP-9 mRNA.

4.2 Methods

Participants

Eight trained participants (age, 32 ± 7 years; height, 180 ± 10 cm; body mass, 75.3 ± 9.1 kg; $\dot{V}O_{2max}$, 4.3 ± 0.4 L.min^{-1}, 58.2 ± 3.9 ml.kg^{-1}.min^{-1}) took part in this study. All experimental procedures were approved by the Loughborough University Ethics Advisory Committee and conformed in all respects with the Declaration of Helsinki. Participants were fully informed of the risks and discomforts associated with all experimental trials before providing written, informed consent. All completed a medical questionnaire and biopsy screening document prior to participation to mitigate for maximal exercise and biopsy contraindications. Participants did not have a history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period.

Experimental design

Using a repeated measures cross-over design, participants performed an acute bout of sprint interval training with (BFR) or without (CON) post-exercise blood flow restriction. Muscle biopsies were obtained before, immediately post- exercise and 3 h post-exercise.

Experimental protocols

Preliminary testing

Participants reported to the laboratory on three separate occasions prior to their first experimental trial. On their first visit they performed an incremental test to exhaustion as outlined in the previous chapter to establish their $\dot{V}O_{2max}$. They also performed a series of randomised maximal sprints (<12 s) on a SRM cycle ergometer at multiple fixed cadences

(90, 100, 110, 120, 130 rpm) which was achieved by placing the ergometer into 'isokinetic mode'. These were performed to determine each participant's optimal cadence for peak power output (PPO) which was subsequently used as the fixed cadence at which each participant performed their experimental trials. On two subsequent visits, participants were familiarised with the experimental protocols.

Experimental trials

Participants attended the laboratory in the morning (~ 0800 h) of each experimental trial following an overnight fast. On arrival, participants rested supine for 20 min whilst muscle biopsy sites were prepared, before a resting biopsy sample was obtained. Participants then performed a standardised warm-up, consisting of cycling at 120 W for 5 minutes. Immediately after the warm-up, they performed 4 × 30 s 'all out' sprints at the previously determined fixed pedal cadence, each sprint separated by 4.5 minutes of recovery. In CON participants remained seated and stationary on the ergometer between each sprint. In BFR participants immediately dismounted the ergometer after each sprint and lay supine on a couch, upon which they were subjected to lower limb BFR, within 15 s of each sprint. This was achieved by rapidly applying a pneumatic pressure cuff (Hokanson SC12L) as high up as possible on the proximal portion of each thigh, inflated (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA) to a pressure of ~ 130 mmHg for 2 min. After 2 min the cuff was rapidly deflated and the participants remained in a supine position for a further 2 min. The participants subsequently re-mounted the ergometer in time for the subsequent sprint which began precisely 4.5 min after the previous sprint ended. The cuff pressure of 130 mmHg was used following months of preliminary work that demonstrated this was the highest cuff pressure that could be tolerated in combination with the current sprint training protocol in similar participant cohorts to that used in this investigation and those that follow. Trials had been performed with higher pressures, however often this

resulted in the cuffs needing to be removed prematurely due to participants experiencing high levels of pain and at times vomiting occurred. Nevertheless, an identical size cuff and cuff location inflated to a similar cuff pressure (~ 130 mmHg) to that used throughout this thesis has been demonstrated in our lab to restrict blood flow through the male popliteal artery by approx. 70% in the resting state (Hunt et al. 2016). At the cessation of each protocol, participants dismounted the ergometer, and were helped onto an adjacent couch where a muscle biopsy was taken within 1 min (Figure 11). Participants then rested passively before having a final muscle biopsy 3 h post-exercise. Muscle biopsies were obtained at these time points as mRNA expression for the genes of interest measured have previously been shown to peak 1-6 h post intense exercise (Hoier et al. 2012; Bartlett et al. 2012; Hoier et al. 2013a; Hoier et al. 2013b) and post-exercise phosphorylation of p38MAPK$^{Thr180/Tyr182}$ has previously been demonstrated to peak and return to baseline within this time-course (Gibala et al. 2009; Bartlett et al. 2012).

Participants recorded all food consumed and physical activity during the 24 h prior to their first experimental trial and were instructed to replicate these dietary and activity patterns prior to their second experimental trial, which were separated by 14-21 days. Whilst all the participants had previous experience of undertaking high-intensity training, none of the participants were currently engaging in high-intensity training outside of that performed during the study. They were also instructed to refrain from ingesting alcohol and caffeine during the 48 h preceding each trial. Whilst the consumption of food was prohibited at all times during each experimental trial, water was consumed *ad libitum.* Laboratory conditions remained constant (19-21°C, 40-50% humidity) for both experimental trials.

Figure 11 - Participant undergoing BFR between sprints.

Muscle sampling and analysis

Muscle biopsies were obtained from the medial portion of the vastus lateralis muscle under local anaesthesia (1% lidocaine) using the micro-biopsy technique (Acecut 11G Biopsy Needle, TSK, Japan). All muscle samples were obtained through separate incisions >2 cm apart with two samples (~ 30 mg each) taken from each incision at each time point. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses.

Western Blotting

Approximately 20 mg of frozen muscle was ground to powder under liquid nitrogen using a laboratory grade pestle and mortar before being homogenised in 120 µl of ice cold lysis buffer (25 mM Tris/HCl [pH 7.4], 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-Pyrophosphatase, 1 mM Na_3VO_4, 0.27 M sucrose, 1% Triton X-100, 0.1% 2-mercaptoethanol) supplemented with a Pierce™ Protease Inhibitor Tablet (Thermo Scientific,

UK). Homogenates were centrifuged at 13,500*g* for 10 min at 4˚C and the supernatant collected. Protein content of the supernatant was determined using a Pierce™ 660 Protein Assay (Thermo Scientific, UK). Each sample was solubilized for 5 min at 100˚C with an equal volume of sample buffer containing 1 M Tris-HCl (pH 6.8), 8% glycerol, 10% sodium dodecyl sulphate, 0.4% 2-β-mercaptoethanol and 0.05% bromophenol blue. For each blot a negative control was loaded along with 10 µg of each sample and then separated (~2 h at ~100 V) in Tris-glycine running buffer using self-cast 4% stacking and 10% separating polyacrylamide gels. Gels were transferred wet onto nitrocellulose membranes for 2 h at 35 mA in a 1 × transfer buffer (0.3% Tris base, 1.4% glycine, 20% methanol). Membranes were then blocked for 1 h at room temperature in Tris-buffered saline (TBST: 0.19 M Tris [pH 7.6], 1.3 M NaCl, 0.1% Tween-20) with 5% non-fat blocking grade milk. Membranes were washed for 3 x 5 min in TBST before being incubated overnight at 4˚C with anti-phospho$^{Thr180/Tyr182}$ and anti-total p38MAPK antibody (Cell Signalling, UK), at a concentration of 1:1000 in 1 X TBST. The following morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with anti-species horseradish peroxidise-conjugated secondary antibody (Bio-Rad, UK) for 1 h at room temperature. After a further 3 x 5 min washes in TBST, membranes were saturated in chemiluminescence (SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA) for 5 min prior to exposure. Membranes were visualised using image analysis (ChemiDoc™ XRS+, Bio-Rad, Herts, UK), and band densities determined (Quality One 1-D analysis software v 4.6.8, Bio-Rad, Herts, UK). Samples from each participant for both exercise protocols were run on the same gel and all gels were run in duplicate to verify responses. Pre-exercise values of phosphorylation relative to total for each participant were normalised to 1 with post-exercise and 3 h post-exercise values subsequently expressed as fold-change relative to pre-exercise values.

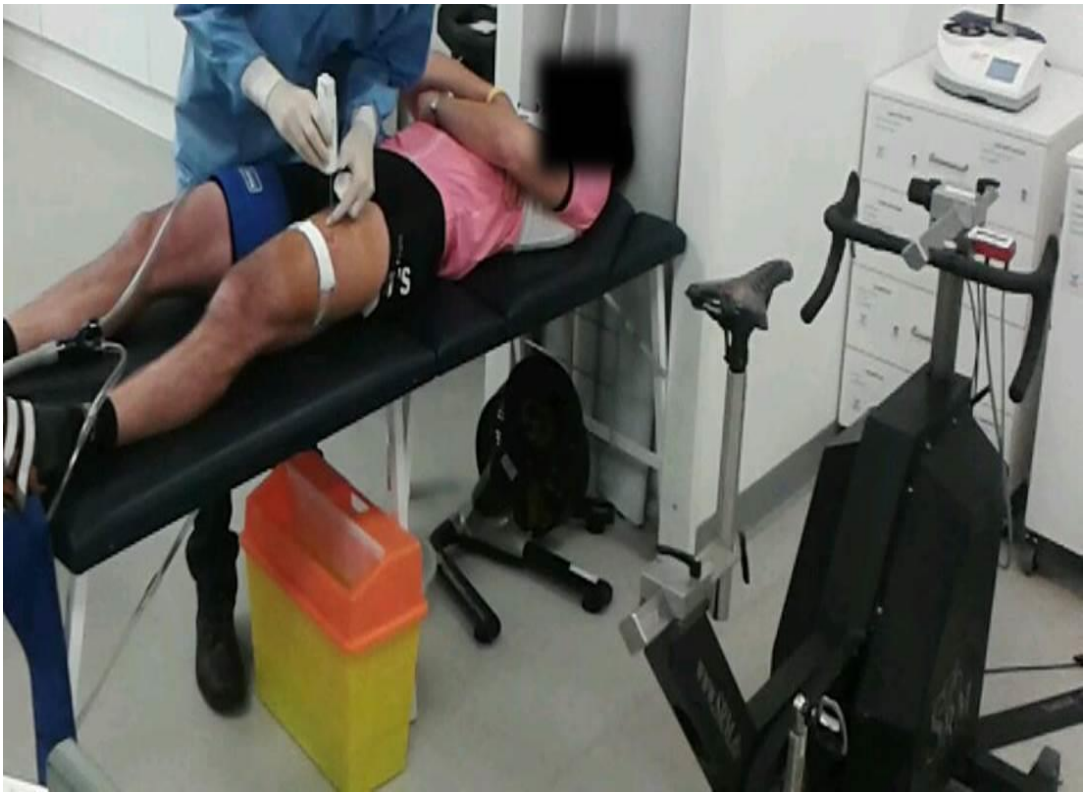

Figure 12 - Participant having muscle biopsy taken during BFR trial. Pressure cuff can be seen on the opposite leg, deflated but in the correct position, and the SRM ergometer used for testing in the foreground.

Real-time RT-PCR

One-step quantitative RT-PCR was used to determine skeletal muscle mRNA levels of genes of interest. Primer sequences were designed by Sigma-Aldrich (Sigma-Aldrich Co. Ltd., Haverhill, UK) ideally with 40-60% GC content and spanning exon-exon boundaries. Primer specificity was determined by performing BLAST and melt curve analysis at the end of each PCR run. Total RNA was isolated from muscle biopsies (~ 30 mg) using a pestle and mortar and TRIzol® reagent (Life Technologies/Invitrogen, USA), according to the manufacturer's protocol. Sample RNA concentration (232 ± 73 ng/µl) and purity (260/280: 1.9 ± 0.1) was confirmed using spectrophotometry (Nanodrop) before being stored at -80 °C for future use. 20 µl PCR reactions were made up as follows in a 96 well plate; 70 ng of RNA in 9.5 µl of nuclease free water, 0.2 µl of Quantifast Reverse Transcriptase mix (Qiagen, Crawley, UK),

0.15 µl of both forward and reverse primers at 100 µM concentrations, and 10 µl of SYBR green mix (Qiagen). All reactions were performed in triplicate. Once PCR plates were prepared, they were transferred to the mx3005p qPCR cycler (Stratagene MX3005P, Agilent Technologies, Berkshire, UK), which was programmed to perform the following steps; 50 °C for 10 minutes (reverse transcription), followed by a 5 minute hold at 95°C, and then 40 cycles at 95°C for 10 seconds and 60°C for 30 s. Fluorescence was detected at the end of each cycle, and expression levels were determined using the $2^{-\Delta\Delta Ct}$ method using RNA polymerase II (RPII) as the reference gene. Post-exercise values are reported as a fold-change relative to pre-exercise values.

Statistics

Protein phosphorylation and mRNA data were analysed using a two-way repeated measures ANOVA. Where significant main effects were observed, Bonferroni corrected post-hoc t-tests were used to locate differences. Data are presented as mean ± SD unless stated otherwise. Significance was accepted at $P < 0.05$.

4.3 Results

The optimal cadence for PPO was 122 ± 13 revs.min^{-1}. There was no difference (t-test P = 0.91) in PPO achieved during BFR and CON (1147 ± 171 W vs 1149 ± 179 W, respectively). Total work done was similar (t-test P = 0.11) between protocols (BFR; 67.1 ± 9.8 vs. CON; 68.3 ± 10.4 kJ).

p38MAPK activation

Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ increased (main effect for time; P = 0.02) 3.2- and 4.1-fold immediately following CON and BFR, respectively, before returning to baseline at 3 h post-exercise (Figure 13). The magnitude of phosphorylation immediately post-exercise was not different between protocols (interaction; P = 0.52).

mRNA expression

mRNA expression data is presented in Figure 14. PGC-1α, VEGF, and VEGFR-2 increased (main effects for time; all P = 0.01) at 3 h in both CON and BFR. There was, however, no difference in the magnitude of fold-change for either of these genes between protocols. HIF-1α mRNA expression increased (interaction; P = 0.04) at 3 h only after BFR. There was no change in eNOS (interaction; P = 0.25), MMP-9 (interaction; P = 0.71) or Ang2 (interaction; P = 0.52) mRNA expression in response to either protocol (Figure 14).

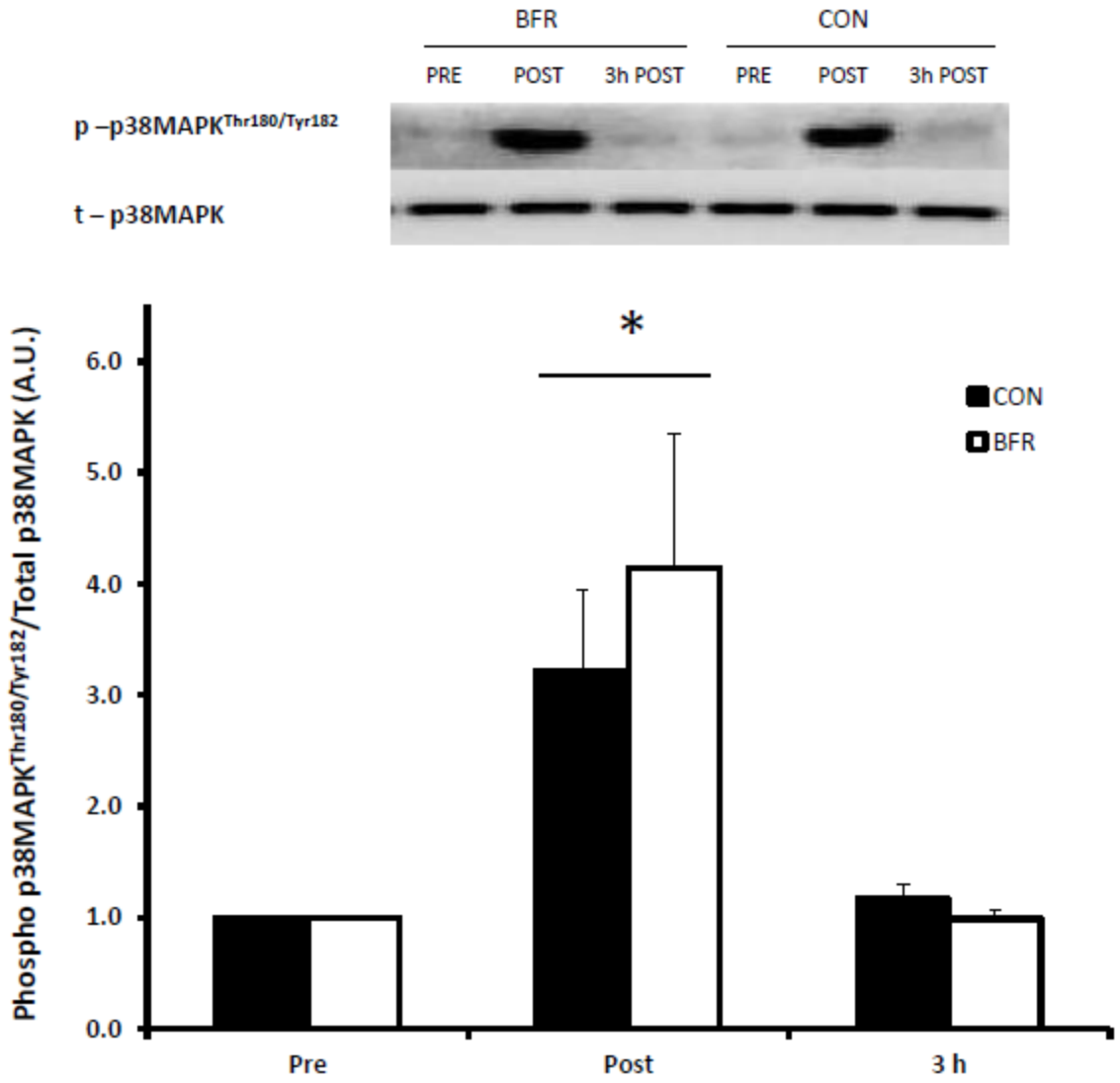

Figure 13 - Phosphorylation of p38MAPK$^{Thr182/Tyr182}$ expressed relative to total p38MAPK immediately before (Pre), after (Post) and 3 h after (3h) the CON and BFR protocols. Each subject's Pre values have been normalised to 1 (hence no error bars are shown for this time-point) such that POST and 3 h values are subsequently expressed as fold-change relative to PRE values. Values are means ± SE (n=8). *denotes significant difference from Pre ($P < 0.05$).

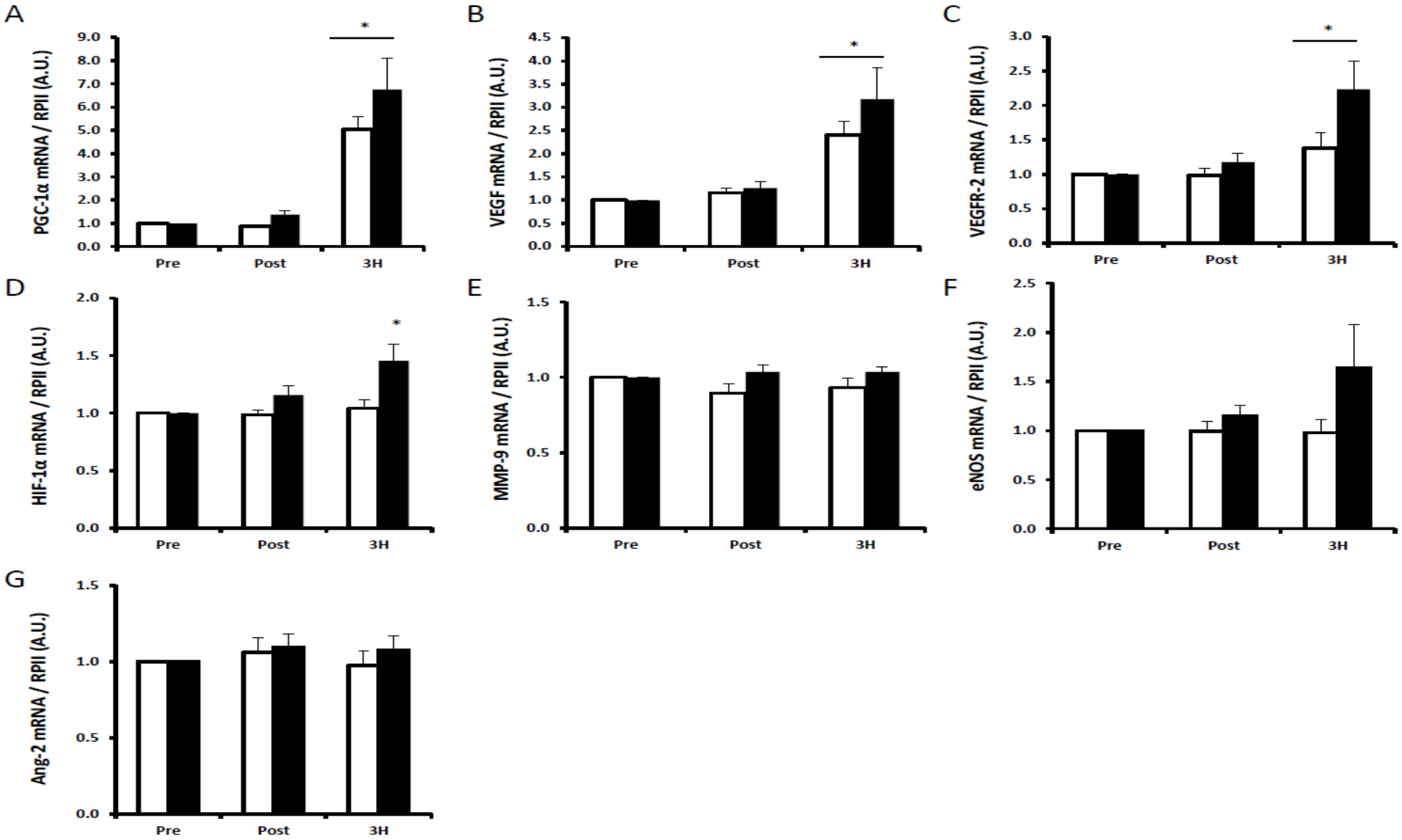

Figure 14 - A) PGC-1α, B) VEGF, C) VEGFR-2, D) HIF-1α , E) MMP-9, F) eNOS, and G) Ang-2 mRNA expression immediately before (Pre), after (Post) and 3 h after (3h) the CON (open bars) and BFR (closed bars) protocols. Gene expression is normalised to RPII mRNA expressed relative to Pre. Values are means ± SE (n=8). * denotes significant difference from Pre (P < 0.05).

4.4 Discussion

This study has demonstrated that post-exercise BFR potentiates HIF-1α mRNA expression in response to 'all-out' interval-based cycling in trained individuals, tentatively suggesting an enhanced hypoxia-targeted angiogenic stimulus in response to BFR combined with SIT. PGC-1α, VEGF and VEGFR-2 mRNA expression increased after both BFR and CON, and although seemingly to a greater extent for all these in response to BFR, the magnitude of increase was not statistically different between conditions. Nevertheless, the increased expression of these genes suggests that SIT indeed holds an angiogenic potential in trained individuals, as has been demonstrated previously in sedentary individuals (Cocks et al. 2013), based on the findings here and in the previous chapter.

HIF-1α is a key regulator of muscle tissue in response to a reduction in oxygen tension and thus metabolic stress (Semenza et al. 2006; Semenza 2006), during which it is stabilized and functions in regulating angiogenesis (Lee et al. 2004) via increased protein accumulation and translocation into the nucleus for targeted VEGF activation (Ameln et al. 2005). Acute exercise results in an increase in skeletal muscle HIF-1α mRNA expression provided that the exercise intensity is sufficient and/or the tissue is exposed to an hypoxic stimulus (Vogt et al. 2001; Lundby et al. 2006; Zoll et al. 2006; Larkin et al. 2012). Larkin et al. (2012) have observed increased VEGF, VEGF-R2 and HIF-1α mRNA expression at 4 h into recovery when low-load resistance exercise was combined with BFR (Larkin et al. 2012). In contrast, other resistance exercise studies employing BFR have observed similar increases in HIF-1α expression, regardless of whether BFR was employed or not (Gustafsson et al. 1999; Ameln et al. 2005; Drummond et al. 2008; Fry et al. 2010) which could suggest regulation by HIF-1α is not mandatory for the augmented VEGF expression in response to this stimulus. The greater HIF-1α mRNA expression after BFR and not CON in our investigation, however,

possibly reflects a greater metabolic stress and magnified/prolonged decreases in intramuscular oxygen levels during the periods of restriction in BFR. Furthermore, the HIF-1α data presented here suggests BFR possibly provides an intensified stimulus for hypoxic- and metabolic-mediated angiogenesis and an increased stress to anaerobic metabolism (Kawada 2005; Tanimoto et al. 2005; Larkin et al. 2012). With specific reference to the latter, Mason and colleagues have reported blunted exercise-induced increases in the expression and activity of glycolytic enzymes in HIF-1α KO mice (Mason et al. 2004). The observations of greater basal HIF-1α mRNA and protein levels in more glycolytic muscle compared with more oxidative muscle (Pisani and Dechesne 2005) and the observation fibre shifts from a slow to a fast, glycolytic profile, in HIF-1α overexpressed rats (Lunde et al. 2011) further supports its role in anaerobic energy turnover.

There is compelling evidence that VEGF is also regulated independently of HIF-1α through PGC-1α and downstream co-activation of ERR-α (Arany et al. 2008; Chinsomboon et al. 2009; Geng et al. 2010). The signals operating upstream of PGC-1α are continually being researched in the field however, likely involve activation of the regulatory protein kinases AMPK and p38MAPK (Olesen et al. 2010) given their role in exercise-induced mitochondrial biogenesis. Although p38MAPK phosphorylation increased after both protocols, there was no difference in the magnitude between trials. Perhaps this was the result of both protocols inducing a similar degree of mechanical- and shear-stress. Unfortunately, AMPK phosphorylation was undetectable by western blotting after numerous troubleshooting attempts.

BFR resulted in a narrowly, but non-significantly, greater increase in PGC-1α mRNA expression compared to CON. It is plausible that greater transient increases in PGC-1α mRNA transcript following each BFR exercise bout over time would accumulate to augment

basal levels of PGC-1α above CON, which in turn can enhance angiogenesis and exercise capacity (Tadaishi et al. 2011). Indeed, future research utilising a study design similar to that employed by Perry and colleagues (2010), whereby successive training sessions are followed by muscle biopsy sampling, is needed to shed light on this.

Similar to PGC-1α, BFR resulted in a non-significant, although slightly augmented VEGF and VEGF-R2 mRNA expression compared to CON. Activation of VEGF occurs principally through binding to its primary EC expressed receptor VEGFR-2. VEGF is an important index of angiogenic potential and is repeatedly increased in response to acute endurance exercise and HIT, and as we have now shown for the first time in this thesis, SIT. The similar magnitude of increase in VEGF mRNA expression (5- to 7-fold) at 3 h post exercise after both conditions is similar to that reported after longer duration (~ 45 min) BFR protocols (Gustafsson et al. 1999; Ameln et al. 2005; Gustafsson et al. 2007) and those reported in the previous chapter. VEGF protein concentration is likely to increase as a consequence of the enhanced VEGF transcription. Although previous studies have failed to capture this acute/transient translational response after BFR exercise (Gustafsson et al. 2007; Larkin et al. 2012) basal VEGF protein levels are found to increase with BFR training alongside indices of enhanced capillarity (Gustafsson et al. 2007).

MMP-9 mRNA expression was unchanged following BFR and CON, possibly reflecting limited development of muscular tension/stretch during SIT, with or without BFR. Indeed, this is further supported by the lack of increase in MMP-9 observed in the previous chapter after both exercise conditions. The finding that MMP-9 mRNA expression was unchanged following both protocols is likely a consequence of the protocols short duration (~5-8 min) and resultant minimal dynamic stretch of the tissue. Moreover, the fact neither protocol elicited a change in MMP-9 mRNA perhaps supports the previously suggested negligible role

of metabolic stress in exercise induced MMP-9 activity (Rullman et al. 2009). The absent increase in MMP-9 expression may prevent proteolysis of the capillary basement membrane which facilitates endothelial cell migration, thought to be an essential component for the progression of sprouting angiogenesis (Haas et al. 2000; Williams et al. 2006c; Egginton 2011).

Although the failure to up-regulate MMP-9 in response to BFR and CON may hinder the progression of sprouting angiogenesis in response to this exercise stimuli, capillary growth by longitudinal splitting is a process requiring reduced matrix remodelling that can occur independent of MMP involvement. The pro-angiogenic actions of shear stress and activation of eNOS stimulates endothelial cell proliferation and is essential for angiogenesis by longitudinal splitting (Williams et al. 2006a). Although Figure 12(F) depicts eNOS mRNA was elevated after BFR exercise, there was no statistical increase in eNOS mRNA expression in response to either protocol. This may reflect the modest shear stress, circumferential strain (stretch) (Price and Skalak 1994) and/or mechanical compression of the vasculature (Chen et al. 2002) as a result of raised capillary pressure (venous occlusion), associated with short duration SIT, that was unable to be compensated for with the addition of BFR as might have been expected (either whilst the cuffs were inflated or after rapid deflation). Indeed, enhanced eNOS expression following BFR might have been expected a consequence of the shear stimulus elicited by reactive hyperaemia following cuff deflation (Gundermann et al. 2012). Whilst our data suggests a lack of activation of the NO pathway, there are limitations of conducting only gene analysis as post-translational modifications of the eNOS protein underline the dynamic regulation of enzymatic activity and in turn angiogenesis (Sessa 2004; Michel and Vanhoutte 2010). Moreover, it is possible that the peak exercise-induced expression of this protein was missed with the chosen biopsy sampling time points.

This study is of course not without limitations. Primarily, this study largely centres on predicting the potency of exercise protocols and possible adaptations that would likely transpire over repeated exposure from acute gene expression data as mentioned in the previous chapter. Indeed, studies have demonstrated that VEGF, VEGFR-2 expression are associated with a significant capillarisation after 4 weeks of moderate intensity endurance training (Hellsten et al. 2008; Høier et al. 2010; Hoier et al. 2012), however the same remains to be determined for SIT and SIT combined with BFR. Additionally, the current study did not involve the quantification of actual percentage of blood flow restriction induced by the cuff pressure of 130 mmHg. This chosen occlusion pressure, was the highest cuff pressure that could be tolerated in combination with SIT and work in our laboratory has demonstrated this cuff pressure around the mid-thigh restricts resting blood flow to the popliteal artery by ~70% (Hunt et al. 2016).

In summary, the greater HIF-1α mRNA expression after SIT combined with BFR possibly reflects a magnified and prolonged metabolic and/or hypoxic exposure of the muscle tissue during the periods of restriction in BFR. This tentatively suggests BFR provides an intensified stimulus for activation of hypoxia- and/or metabolic-mediated adaptations such as mitochondrial biogenesis and angiogenesis (Kawada 2005; Tanimoto et al. 2005; Larkin et al. 2012). Clearly further work is required to support this hypothesis, including immunoblot and immunohistochemical analysis of VEGF regulation via HIF-1α protein stabilization and nuclear translocation, as well as direct changes in skeletal muscle capillary:fibre ratio after a period of prolonged and repeated exposure to both exercise interventions.

CHAPTER 5 - CHRONIC EFFECTS OF SIT COMBINED WITH POST-EXERCISE BFR ON ENDURANCE PERFORMANCE AND ASSOCIATED AEROBIC PHYSIOLOGY

5.1 Introduction

Despite observed benefits of SIT in promoting improvements in exercise capacity and associated physiological parameters, there is always increasing scope to identify and adopt novel, low volume training methods that result in greater adaptive responses, especially in trained individuals. In Chapter 4 it was demonstrated that post-exercise BFR potentiates HIF-1α mRNA expression in response to 'all-out' interval-based cycling in trained individuals, possibly indicating an enhanced hypoxic and/or metabolic stimulus in response to SIT combined with BFR. These findings coupled with the previous observations that endurance exercise performed with BFR promotes greater improvements in exercise capacity, $\dot{V}O_{2max}$ and peripheral metabolic and structural changes compared to exercising with a normal blood supply (Kaijser et al. 1990; Eiken et al. 1991; Sundberg et al. 1993; Esbjörnsson et al. 1993; Nygren et al. 2000), suggests that aerobic capacity might also be enhanced in response to SIT combined with BFR.

Therefore, the aim of this investigation was to assess the potency of combining SIT with post-exercise BFR in enhancing maximal aerobic physiology and performance in trained participants who already possess enhanced physiological performance capabilities. Given the acute signalling data reported in the previous investigation, it was hypothesised that BFR

combined with SIT would provide a greater training stimulus than SIT alone and thus would result in greater increases in VO_{2max} and 15km TT performance compared to SIT alone.

5.2 Methods

Participants

Twenty participants who were cycling 120 ± 66 km per week took part in this study. There was no significant difference in pre-training age, height, body mass and all other baseline outcome measures between the two intervention groups, respectively (Table 4). All experimental procedures were approved by the Loughborough University Ethics Advisory Committee and conformed in all respects with the Declaration of Helsinki. Participants were fully informed of the risks and discomforts associated with all experimental trials before providing written, informed consent. All participants completed a medical questionnaire prior to participation to mitigate for maximal exercise contraindications and having blood flow restricted. Participants did not have a history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period.

Experimental design

Using a between groups design, participants performed four weeks of sprint interval training either combined with post-exercise blood flow restriction (BFR) or sprint interval training alone (CON). Physiological and performance assessments were made before and after training.

Table 4 - Participant characteristics at baseline prior to training

	CON (*n* = 10)	BFR (*n* = 10)	*P* value
Age (yrs)	27 ± 7	26 ± 5	0.639
Height (cm)	181 ± 9	178 ± 8	0.555
Body mass (kg)	75.5 ± 8.4	74.1 ± 7.4	0.589
$\dot{V}O_{2max}$ **(L.min^{-1})**	4.6 ± 0.3	4.5 ± 0.4	0.305
$\dot{V}O_{2max}$ **(mL.kg^{-1}.min^{-1})**	61.8 ± 4.7	61.2 ± 4.0	0.643
MAP (W)	373 ± 31	377 ± 37	0.721
MAP (W.kg^{-1})	5.0 ± 0.4	5.1 ± 0.3	0.489

Values are mean ± SD. $\dot{V}O_{2max}$, maximal oxygen uptake; MAP, maximum aerobic power; CON, control group; BFR, blood flow restriction group

Experimental protocols

Preliminary testing

Participants were initially familiarized to the testing and training procedures during preliminary visits, before being randomly assigned to either the control (CON) or blood flow restricted (BFR) training intervention. Pre-training outcome measures were assessed on two occasions separated by at least 2 days. Post-training outcome measures were assessed in the same order, each separated by 2 days within 1 week. Laboratory conditions during pre- and post-training measurements remained constant (19-21°C, 40-50% humidity) and all measurements were conducted at the same time of day for each participant. Ergometer saddle and handle bar dimensions recorded for each participant during preliminary testing were standardised for all post-training measures. Participants recorded dietary intake and physical activity performed during the 24 h prior to each of their pre-training tests and replicated these dietary and activity patterns for the 24h prior to all post-training tests. Participants were

instructed to refrain from strenuous exercise 24h prior to all testing visits and from ingesting alcohol and caffeine during the 48 h preceding testing.

Pre and post training assessments

All pre and post training measures were conducted on an SRM cycle ergometer, calibrated according to the manufacturer guidelines (Schroberer Rad McBtechink, Weldorf, Germany). Participants performed an incremental test to exhaustion to establish maximal oxygen uptake ($\dot{V}O_{2max}$) and maximal aerobic power (MAP). Participants began cycling, at a freely chosen, constant pedal cadence for 5 min at 120 W, after which power increased 20 W every 60 s. Pulmonary gas exchange was measured breath by breath throughout exercise (Oxycon Pro, Carefusion, UK). $\dot{V}O_{2max}$ and MAP were defined as the highest $\dot{V}O_2$ and power output achieved for a 30 and 60s period during the test, respectively. The cycling time trial (15km-TT) involved completing 15km as quickly as possible. Except for total distance covered, no visual or verbal technical or motivational feedback was given (Figure 15). To better resemble a time trial on the road, the ergometer was placed into 'hyperbolic mode' enabling the participants to manually select their own gear, pedal cadence and thus power output throughout. Power output was recorded (2 Hz sample rate) and averaged throughout the entire duration of the 15km-TT performance trials. Participants consumed water *ad libitum* during their familiarisation trial with the volume and timings of consumption recorded and replicated during subsequent trials. Prior to commencing training, participants completed a total of three 15km-TT efforts each separated by a week, to establish familiarity. Reproducibility for the 15km-TT (i.e. the coefficient of variance which expresses the typical error as a percentage (Hopkins 2000) when performed at the same time of day and having consumed an identical diet for the 24 h preceding each trial was determined between the 2nd and 3rd TT efforts and was 4.9 %. The 3rd TT effort was reported as the pre-training outcome measure.

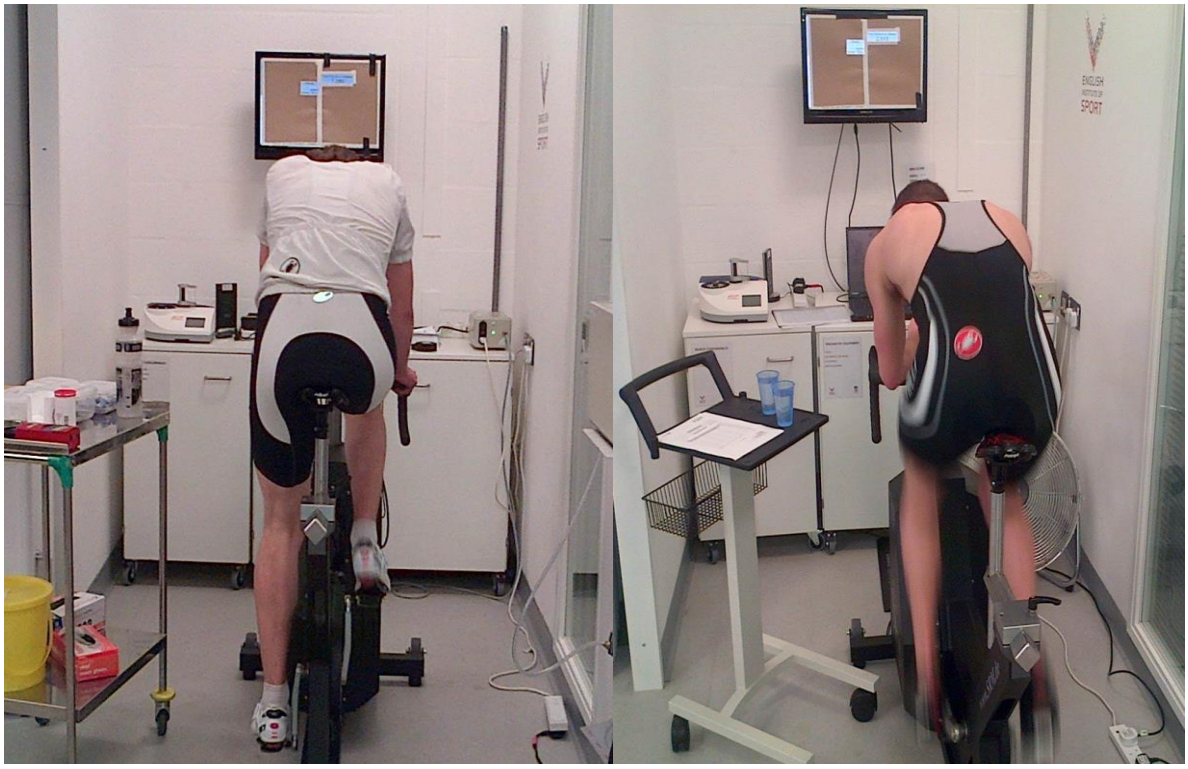

Figure 15 - Participants performing the 15km-TT. Note monitor on the wall only displaying distance covered.

Exercise training

Participants completed a four-week supervised sprint interval training programme (2 sessions per week). Each training session consisted of repeated 30 s maximal sprint cycling bouts performed on a mechanically braked cycle ergometer (SE-780 50, Monark, Stockholm, Sweden) against a manually applied resistance equivalent to 0.075 kg/kg body mass. The training was progressive whereby all participants performed a total of 4, 5, 6 and 7 maximal 30 s cycling bouts in weeks 1, 2, 3 and 4, respectively, with each bout separated by a 4.5 min recovery period. In CON participants remained seated and stationary on the ergometer and refrained from pedalling during recovery between each sprint. At times participants would get out of the saddle and stretch their legs and pedal backwards but this was minimised. In the BFR condition participants immediately dismounted the ergometer after each sprint and lay supine on a couch, upon which they were subjected to lower limb BFR, within 15s of each

sprint, in the exact manner described in the previous chapter. The cuff pressure of ~ 130 mmHg was kept constant through the four-week training period. Participants were instructed to continue normal dietary practices throughout the training period and were instructed to complete daily training diaries to ensure that non-prescribed training remained constant throughout the entire study. Thus the only manipulation to the training structure in both experimental groups was the increased number of intervals performed each week. Whilst all the participants had previous experience of undertaking high-intensity training, none of the participants were currently engaging in high-intensity training outside of that performed during the study and were instructed to replicate all other non-prescribed weekly training to ensure the SIT or SIT and BFR were the only training change experienced. Pre-training measurements of peak power output (PPO) and mean power output (MPO) were obtained from the first training session after a standardised warm-up. Post-training PPO and MPO were taken from the best of two maximal sprints performed separately during the week of post-training outcome measurements.

Statistics

Outcome measures and training data were analysed using independent t-tests. Subsequent analysis was performed with two-factor repeated measures analysis of variance (ANOVA) with one within factor (time: pre versus post) and one between factor (group: CON versus BFR).

5.3 Results

Participants completed 100% of the assigned training sessions without any complications. Training data is presented in Figures 16 and 17. The average power output produced over the 8 training sessions was greater (t-test $P < 0.01$) in CON (642 ± 10W) compared to BFR (618 ± 11W). Consequently, the average total work done throughout each training session was greater (t-test $P < 0.01$) in CON (106 ± 22 kJ) compared to BFR (102 ± 21 kJ).

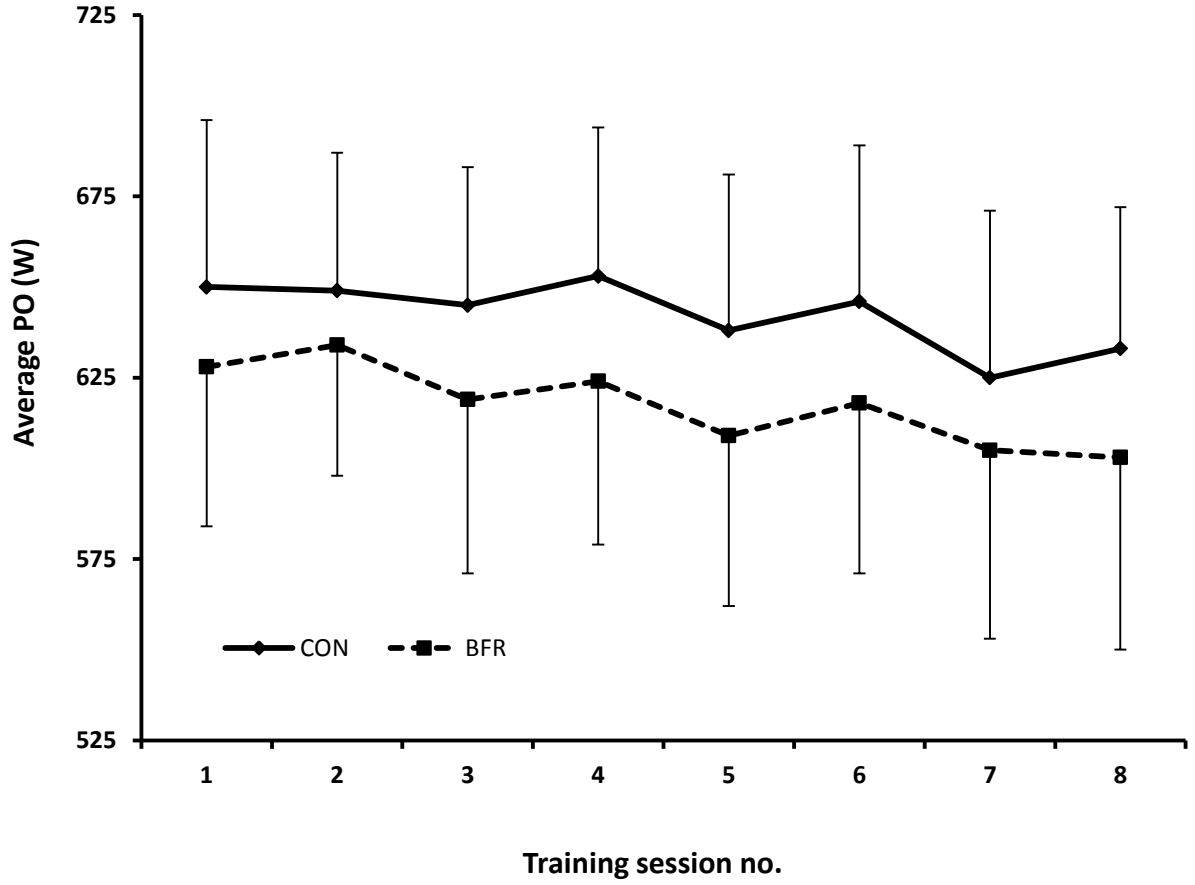

Figure 16 - Average power output produced across all sprints in each training session for all participants in the CON (solid line) and BFR (broken line) condition. Error bars are SD.

Physiological and performance variables measured before and after CON and BFR are presented in Table 6. Absolute and relative $\dot{V}O_{2max}$ (Table 6) increased after BFR but were unchanged after CON (interaction; both $P = 0.01$). There was a trend for absolute and relative MAP (Table 6) to increase after BFR, with no changes after CON, however these did not reach statistical significance (interaction; $P = 0.11$ and $P = 0.09$, respectively). Absolute and relative sprint PPO increased (main effect for time; both $P = 0.02$) but there were no difference between training groups. There were no changes in absolute or relative sprint MPO after CON and BFR. 15km-TT performance time (Table 6) and average power output during the TT were unchanged after CON and BFR.

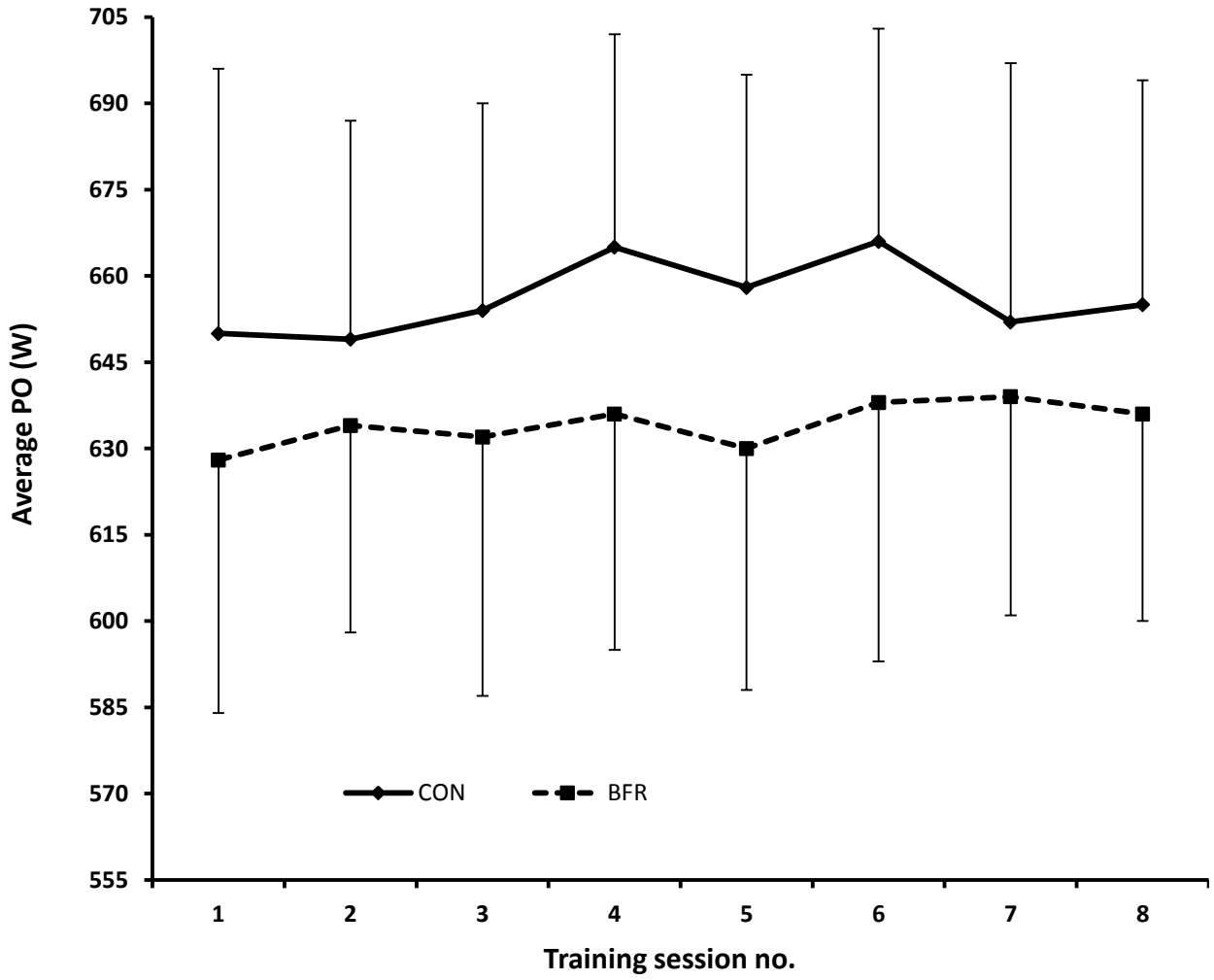

Figure 17 - Average power output produced across the first 4 sprints in each training session for all participants in the CON (solid line) and BFR (broken line) condition. Error bars are SD.

Table 5 - Physiological and performance variables before and after CON and BFR training interventions

	CON			BFR			ANOVA
	PRE	**POST**	**% change**	**PRE**	**POST**	**% change**	**(interaction P value)**
Body mass (kg)	75.5 ± 8.4	75.7 ± 7.9	**0.3**	74.1 ± 7.4	74.0 ± 7.6	**0.3**	**0.55**
$\dot{V}O_{2max}$ **(l.min^{-1})**	4.6 ± 0.3	4.6 ± 0.4	**0.7**	4.5 ± 0.4	4.7 ± 0.4 *	**4.5**	**0.01**
$\dot{V}O_{2max}$ **(ml.min^{-1}.kg^{-1})**	61.8 ± 4.7	61.1 ± 4.9	**1.1**	61.2 ± 4.0	64.1 ± 4.1 *	**4.7**	**0.01**
MAP (W)	373 ± 31	374 ± 37	**0.2**	377 ± 37	392 ± 38	**3.8**	**0.11**
MAP (W.kg^{-1})	5.0 ± 0.4	5.0 ± 0.4	**0.2**	5.1 ± 0.3	5.3 ± 0.5	**4.4**	**0.09**
PPO (W)	1065 ± 214	1137 ± 259	**6.8**	938 ± 168	998 ± 203	**6.4**	**0.80**
PPO (W.kg^{-1})	14.0 ± 1.9	15.0 ± 2.9	**7.0**	12.6 ± 1.5	13.5 ± 2.3	**7.1**	**0.95**
MPO (W)	709 ± 99	711 ± 102	**0.2**	676 ± 97	696 ± 102	**2.9**	**0.19**
MPO (W.kg^{-1})	9.4 ± 0.8	9.4 ± 0.9	**0.3**	9.1 ± 0.7	9.4 ± 0.9	**3.3**	**0.14**
15 km-TT (s)	1363 ± 50	1361 ± 59	**0.1**	1347 ± 81	1339 ± 76	**0.6**	**0.66**
Ave TT power (W)	280 ± 26	288 ± 29	**2.8**	282 ± 39	285 ± 37	**1.1**	**0.53**

Values are mean ± SD. $\dot{V}O_{2max}$, maximal oxygen uptake; MAP, maximal aerobic power; PPO, peak power output during 30 s sprint; MPO, mean power output during 30 s sprint; TT, time trial. * $P < 0.05$, compared to PRE (Bonferroni corrected post-hoc t-test). All data are n = 10, except for Ave TT power which is n = 7 in each group.

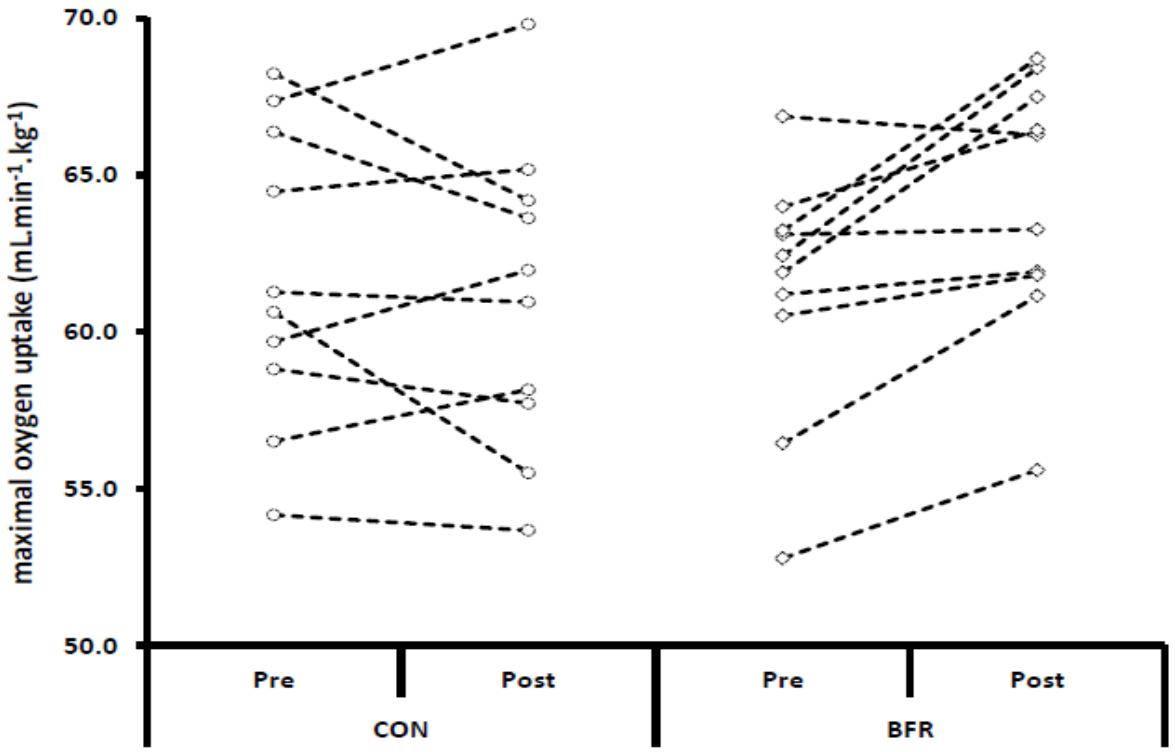

Figure 18 - Individual relative maximal oxygen uptake values for all participants before and after the 4-wk CON and BFR training interventions

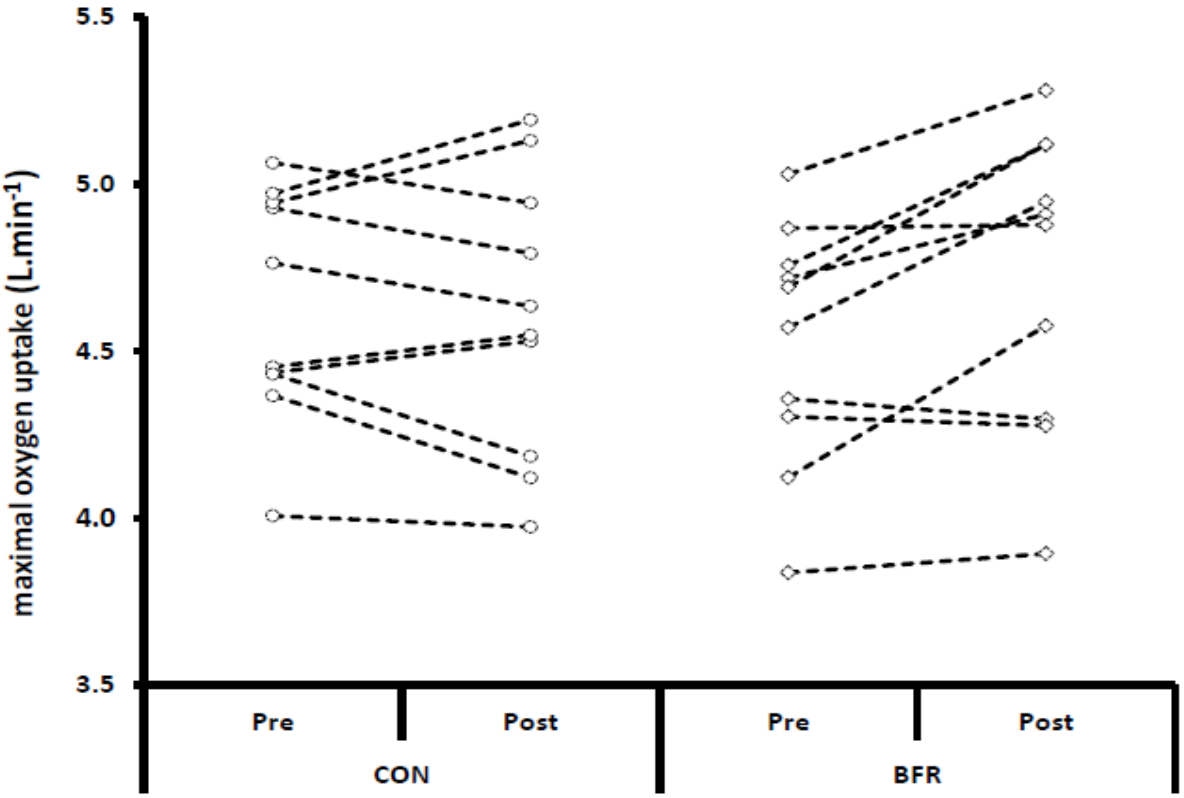

Figure 19 - Individual absolute maximal oxygen uptake values for all participants before and after the 4-wk CON and BFR training interventions

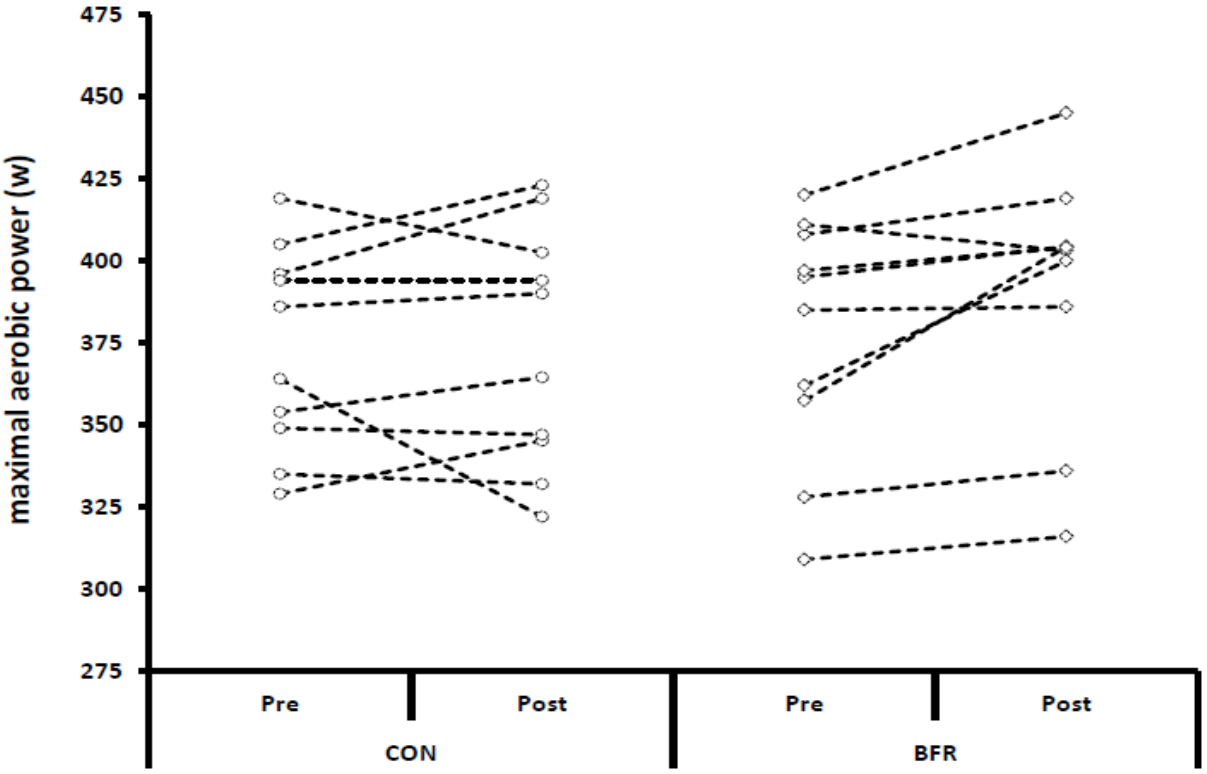

Figure 20 - Individual absolute maximal aerobic power (MAP) for all participants before and after the 4-wk CON and BFR training interventions

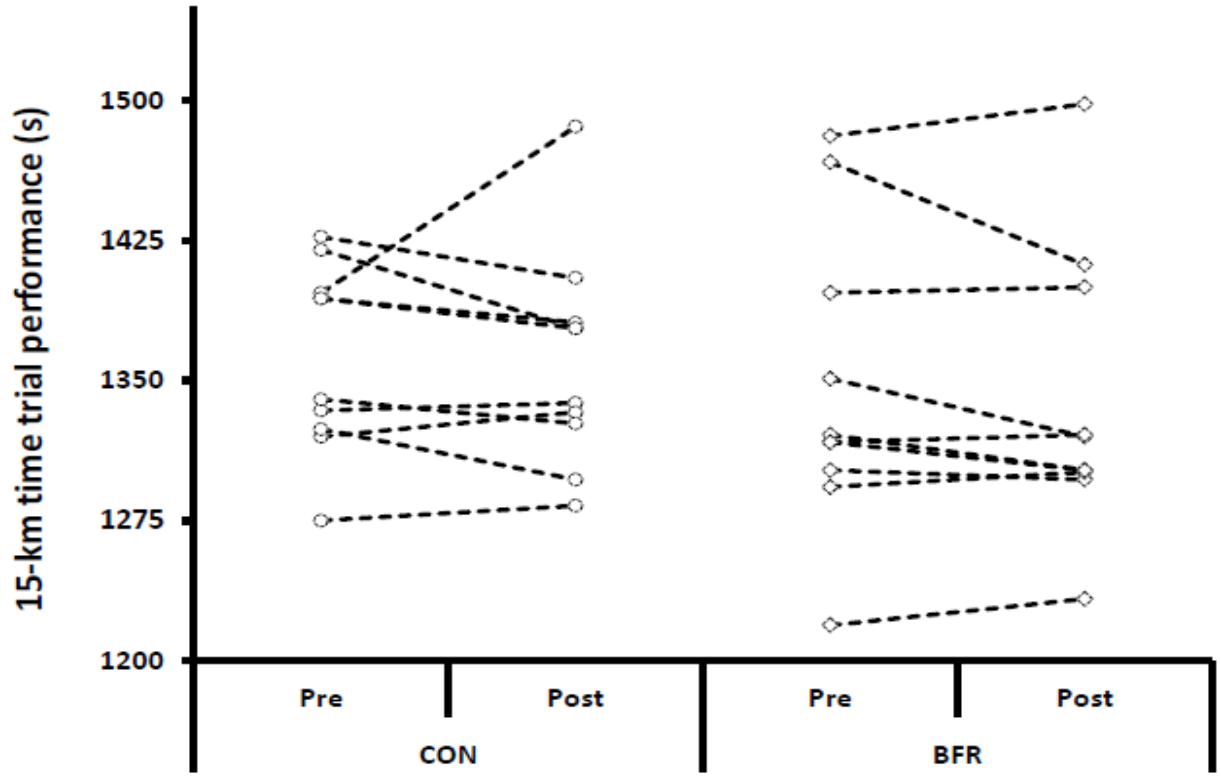

Figure 21 - Individual 15km-TT performance values for all participants before and after the 4-wk CON and BFR training interventions

5.4 Discussion

This study has demonstrated the potency of combining blood flow restriction with sprint interval training (SIT) in increasing $\dot{V}O_{2max}$ in trained individuals. The mechanisms responsible for this adaptive response are unclear however the previous chapter presents preliminary findings suggesting BFR possibly presents an intensified hypoxic and/or metabolic stimulus over SIT alone for enhanced skeletal muscle remodelling, such as increased mitochondrial biogenesis and capillary density.

In the present study, $\dot{V}O_{2max}$ increased by ~4.5% in response to SIT with BFR, compared with 0.7% in CON. Out of the 10 participants in BFR, only one individual did not demonstrate an enhancement in relative maximal oxygen uptake. Moreover, whilst it did not reach statistical significance, the percentage increase in maximal aerobic power was evidently greater for the BFR group. These improvements are in spite of a lower overall work done in the BFR group compared to CON. The potency of combining BFR in enhancing oxygen uptake and the adaptive response to other forms of exercise has previously been demonstrated. For example, one-legged cycle training whereby blood flow was restricted during exercise by approximately 15-20% through the use of a pressure chamber (Sundberg and Kaijser 1992) has previously been demonstrated to promote a greater increase in one-legged $\dot{V}O_{2peak}$ over that performed with normal blood supply (Sundberg et al. 1993). Of course, the use of such pressure chambers is limited by accessibility and ease of use, whereas the use of blood pressure cuffs placed on the proximal portion of the legs and a rapid inflation system seems effective in providing a fixed pressure for a set duration. The feasibility of combining BFR with whole body exercise, specifically SIT has also been demonstrated. This is of particular relevance to well-trained cyclists where there is a requirement to maintain an appropriate load

or training intensity as part of a structured training programme. In pilot work, combining SIT and BFR concurrently proved impractical and even at moderate cuff pressures (100 mmHg) this combination was not tolerable. Either way, this type of training represents a novel way to enhance the adaptive responses in well trained individuals.

There was no improvement in $\dot{V}O_{2max}$ with SIT alone in our group of trained individuals (CON). The effects of SIT on $\dot{V}O_{2max}$ in less well-trained individuals is inconsistent, with some studies demonstrating increases in response to training (Burgomaster et al. 2006; Burgomaster et al. 2008; Cocks et al. 2013) and others not (Burgomaster et al. 2005). Indeed, previous investigations into the effects of HIT (that involve longer duration intervals at sub-maximal intensities rather than 'all out' maximal sprints) have demonstrated positive adaptive responses in indices of aerobic performance (e.g. $\dot{V}O_{2max}$, lactate threshold) in well-trained cyclists with pre-training $\dot{V}O_{2max}$ values of ~ 65 $mL.min^{-1}.kg^{-1}$ (Laursen and Jenkins 2002; Laursen et al. 2005). As highlighted in Chapter 1, differences in the $\dot{V}O_{2max}$ response, and indeed many adaptations after training, between the aforementioned studies could be attributable to a number of factors such as differences in the duration, intensity and frequency of the interval training stimulus, as well as the total training volume prescribed.

The main determinants of $\dot{V}O_{2max}$ are complex. Although it is generally accepted that the vascular bed is not a limiting factor for oxygen transport during one legged knee-extensor exercise (Andersen and Saltin 1985) where approximately 2-3 kg of muscle mass is recruited, performing cycling exercise in which a larger muscle mass (i.e. 5-6kg) is recruited stresses both central and peripheral limitations in oxygen delivery and uptake (Poole and Richardson 1997). As well as the important role the capillary network has on increasing oxygen delivery and reducing diffusion gradients, it also facilitates greater removal of metabolic end products which sustains exercise tolerance (Joyner and Coyle 2008). Moreover, an increased capillary

density specific to type II muscle fibres will improve performance and recovery from maximal and 'all out' exercise (Tesch and Wright 1983) during which these fibres are preferentially recruited (Greenhaff et al. 1994).

$\dot{V}O_{2max}$ clearly holds importance to exercise capacity, as does the $\dot{V}O_2$ and power/velocity at lactate threshold which is also directly influenced by muscle capillary density (Joyner & Coyle, 2008). These measurements, however, are not necessarily direct assessments of performance *per se*. We chose to assess exercise performance with a 15km self-paced time trial (TT). Despite the improvements in $\dot{V}O_{2max}$ with BFR, there was no improvement in 15 km TT performance after BFR or SIT alone. Whilst this is perhaps surprising given HIT has previously been demonstrated to improve 40 km TT performance in well trained athletes (Laursen et al. 2005), the TT distance needs to be considered when considering performance improvements. For example, performance during self-paced exercise is predominantly limited by central or peripheral factors with a greater degree of peripheral fatigue evident after shorter high-intensity (approximately 6 min) TTs and increased contribution of central fatigue after longer lower-intensity TTs (>30 min) (Thomas et al. 2015). It is plausible that the 15 km TT performed in this study lacked sensitivity to at least partly reflect the increases in $\dot{V}O_{2max.}$ In contrast, Craig et al. (1993) examined the relationship between select physiological parameters and 4 km individual pursuit (IP) performance (i.e. ~ 5.6 min) in a group of male high-performance track endurance cyclists and observed a strong relationship between IP performance and $\dot{V}O_{2max}$ (r = -0.79), and power output at lactate threshold (r = -0.86). It is therefore important that future studies continue to include TT tests of performance, both longer and shorter than the duration employed in this study, to better inform the application of training interventions to athletic populations. In addition, participants perhaps required greater familiarisation to the TT prior to commencing training in order to increase

the reliability of this performance test. The CV between familiarisation sessions in the current study was 4.7 %. Indeed, this is contrast to a study by Palmer et al. (1996) who reported a CV of 1.1 % between three 20-km time trials performed by high-trained individuals who also had feedback of distance covered throughout.

In the present study there appeared to be a trend towards significant increases in MAP and MPO (Table 6) after BFR. It is possible that with greater participant numbers the change in these parameters after training would have reached statistical significance and in this regard the present studied may have been 'under powered'. However, increases in the region of 2.9% to 4.4% in these parameters after BFR compared to more marginal changes of 0.2% to 0.3% after CON are more meaningful for athletes, coaches and applied sports scientists rather than achieving a traditional statistical significance. When deciding on whether to adopt such an intervention, it should be noted that 9 of the 10 participants enhanced their relative $\dot{V}O_{2max}$ after BFR whilst only 4 individuals observed improvements after CON.

In conclusion, we have demonstrated the potency of combining BFR with SIT in increasing $\dot{V}O_{2max}$ in trained individuals. Moreover, SIT alone did not induce any observable adaptation. Although the potential mechanisms are not fully understood, it is possible BFR provides an intensified stimulus for hypoxia- and or metabolic- mediated adaptations such as angiogenesis and mitochondrial biogenesis. In particular, the inclusion of muscle protein analysis and immuno-histochemical techniques to evaluate direct changes in capillary:fibre ratio are necessary in future work to support this hypothesis and draw firmer conclusions that such a training stimulus manifests in the above skeletal muscle remodelling.

CHAPTER 6 - ELITE ATHLETE CASE STUDY: THE APPLICATION OF SIT COMBINED WITH BFR AS A NOVEL APPROACH TO ENHANCE PHYSIOLOGICAL AND PERFORMANCE CAPABILITIES

6.1 Introduction

In the previous two chapters the use of BFR combined with SIT has been introduced as a potentially novel and practical training method to upregulate the signals involved in endurance based adaptations and enhance important associated physiological parameters in trained individuals. These results would no doubt be of interest to a range of athletic events, in particular linear energetic events such as rowing, running and cycling, where the athlete's underlying physiology (rather than technique and decision making such as in team based sports) is strongly associated with the outcome of performance. Although further research is required to confirm the performance benefits of this exercise model and gain a greater understanding of the mechanisms of the adaptive responses, there is an equally important requirement to explore whether such a novel training modality transfers to high performance athletes. A full-scale intervention with elite athletes would be the optimum circumstance to explore the effectiveness of such an intervention, however, preparation for the 2016 Olympic games eliminated this as a potential option. However, the opportunity presented itself to intervene with a full-time nationally funded track cyclist who was transitioning away from competing in the sprint events but was considered to hold potential in middle-distance track events. The question posed by the national cycling federation was whether this athlete could enhance her aerobic abilities while maintaining her anaerobic qualities in a compressed time-

scale? This led to the opportunity to explore a novel intervention regimen involving SIT and BFR.

The principal track cycling events currently competed at World Championship and/or Olympic level include the team sprint, the 500 and 1000 m time trials (TT), the match sprint (including a flying 200 m qualification TT) and Keirin, the individual (3-4 km) and team (4 km) pursuits and the Omnium, whereby a rider competes in 6 events over 2 days ranging from a flying lap TT to a 25-40 km points race. Clearly, the predominant physiological determinants that underpin successful performances in these events (and individual rider performances within the team events) will vary depending on the duration of the event and the race tactics/strategy, which together ultimately governs the intensity at which the race is ridden. For example, whilst large absolute and relative maximal power outputs and anaerobic capabilities are critical success factors for the shorter sprint events. Moreover, the primary physiological determinant of a winning performance in the individual and team pursuit and the 25-40 km 'bunch' races is a high aerobic capacity, enabling sustained high average power outputs. For example, it has been suggested that the relative contributions of aerobic and anaerobic metabolism to 4 km individual pursuit are approximately 80 and 20 %, respectively (Neumann, 1992). Moreover, Craig and colleagues (1993) have examined the relationship between select physiological parameters and 4 km individual pursuit (IP) performance in a group of male high-performance track endurance cyclists. The highest correlations between 4 km IP performance and physiological parameters were the athlete's $\dot{V}O_{2max}$ (r = -0.79) and power output at lactate threshold (r = -0.86) (Craig et al. 1993).

After demonstrating, in the previous chapters, the potency for SIT combined with post-exercise BFR to enhance $\dot{V}O_{2max}$ and likely MAP and MPO above SIT alone, the present chapter describes a case study detailing the integration of this training method into an

international track cyclists training programme. The aim was to explore the effectiveness of the SIT and BFR intervention in enhancing the athlete's aerobic capabilities, while maintaining the anaerobic capabilities, given the importance of both to the track cycling events and in particular, individual and team pursuit.

6.2 Methods

The Athlete

A female international track sprint cyclist, subjected to UCI and UKAD out-of-competition and in-competition testing, was the focus of this intervention (age, 20 yrs; height, 170 cm; body mass, 60.4 kg). She had previously competed in the team sprint at the Senior European Track Cycling Championships and medalled at the U23 European Track Cycling Championships. Furthermore, she has previously held the title of track cycling Great Britain National 500 m Time Trial Champion. The athlete's national governing body and coaches provided us with full access to the athlete's training programmes/history and data, and we were given full control over training prescription and monitoring/assessment over the entire period of the intervention.

Pre-intervention training structure

During a 46-day period, which ended 3 weeks before the start of the intervention, the athlete completed 43 training sessions in total (on average 0.9 sessions per day). These comprised of 14 resistance training sessions (comprising lower body bilateral and unilateral weight lifting), 16 road rides ($\geq$ 90 min) and 13 track sessions (involving 6-12 x $\leq$ 30 s maximal sprints per session).

The training intervention

The athlete completed 8 supervised sprint interval sessions over a four-week period (on average 2 sessions per week). As described in Chapters 4 and 5, each training session consisted of repeated 30 s maximal sprint cycling bouts performed on a road bicycle fixed into a turbo trainer (UK Sport Innovation). All sprints across the 4 weeks were performed on a set fixed gear and power was measured at the back wheel hub using a commercially available power meter, calibrated according to the manufactures guidelines (PowerTap, Madison, WI). The training was progressive whereby the athlete performed a total of 4, 5, 6 and 7 maximal 30 s cycling bouts in weeks 1, 2, 3 and 4, respectively, with each bout separated by a 4.5 min recovery period. After each sprint, the athlete immediately dismounted the ergometer and lay supine on an adjacent physio plinth (Figure 22). Within 15 s of each sprint the athlete was then subjected to lower limb BFR, in the exact manner described in the previous two chapters. The cuff pressure of ~ 130 mmHg was kept constant throughout the entire training period. The athlete was instructed to continue normal dietary practices throughout the training period and to complete daily training diaries to ensure that non-prescribed training remained constant throughout the entire study. Thus the only manipulation to the training structure in both experimental groups was the increased number of intervals performed each week. All BFR training sessions as well as pre- and post-training assessments were conducted in a British Association for Sport and Exercise Sciences (BASES) accredited laboratory. Laboratory conditions remained relatively constant for all BFR training sessions and all pre- and post-intervention assessments (19-21°C, 40-50% humidity). In addition to the 8 BFR training sessions, the athlete on one occasion per week also performed a resistance training session and on two days per week performed a 90 min low-intensity road ride (Table 7). These were kept constant so that the only training which adapted each week was the BFR training.

Table 6 – The weekly training structure during the intervention period.

	Mon	Tue	Wed	Thu	Fri	Sat	Sun
AM	Rest	Rest	Road Ride	Rest	Gym	Road Ride	Rest
PM	SIT+BFR	Rest	Rest	SIT+BFR	Rest	Rest	Rest

Figure 22 - Athlete undergoing BFR in between sprints. Device used to restrict blood flow can be seen in the background and the athlete's bike fitted to the turbo trainer in foreground.

Pre- and post-intervention assessments

Body composition

Body composition was assessed using DEXA (dual energy X-ray absorptiometry) alongside anthropometry skinfold measurements. DEXA and anthropometry measurements were conducted at the same time of day (~ 9am) in the fasted state. DEXA scans where conducted according to the manufactures guidelines by a qualified technician at Liverpool John Moores University. Anthropometry skinfold measurements were performed by an International

Standard for Anthropometric Assessment (ISAK) accredited practitioner. Thigh circumference was measured horizontal to the long axis of the femur at the mid- point between the inguinal fold and the anterior aspect of the patella. The horizontal (circumferential) line was marked and extended to intercept with the vertical mid-line of the anterior and posterior surface of the thigh. The sum of 8 skinfold sites (triceps, subscapular, biceps, iliac crest, supraspinale, abdominal, front thigh, medial calf) were measured using a newly calibrated Harpenden Skinfold Caliper (Baty International, UK).

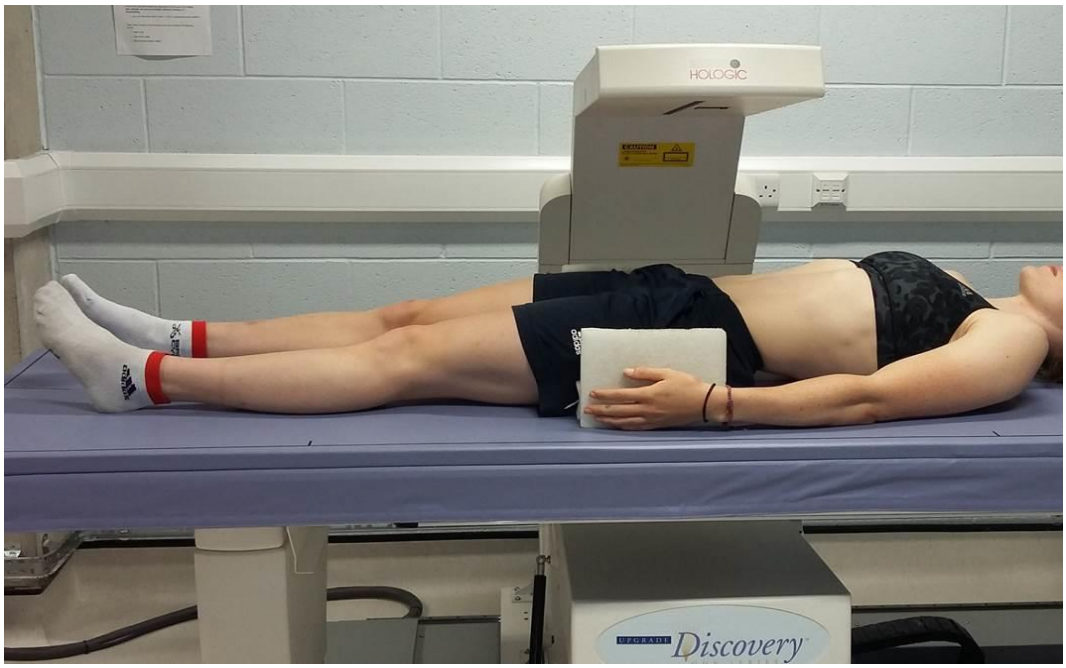

Figure 23 - Athlete undergoing DEXA scan.

*On-track ass**e**ssment of maximal anaerobic capabilities*

Four and ten days after the last BFR training session the athlete attended the National Cycling Centre (Manchester, UK) to perform 3 stationary starts. After performing a standardised warm-up (on cycling rollers) undertaken before every track training session and competition), the athlete performed 3 x ~ 10 s 'all-out' efforts from a stationary position, each effort separated by 10 min recovery. These efforts were performed to assess the change in the athlete's PPO from pre- to post-intervention. All pre and post intervention track efforts were

started from an identical position on the track and on the same track sprint bicycle fitted with an identical fixed gear. The bicycle was instrumented with SRM cranks to provide power measurement, which were initially calibrated before the first effort and then re-calibrated after each subsequent effort according to the manufacturer guidelines (Schroberer Rad McBtechink, Weldorf, Germany). Power output was recorded at a sample rate of 2 Hz. Body mass was collected at every track session to provide an accurate calculation of relative power output ($W.kg^{-1}$). All track training sessions were performed at a similar time of day (approx. 2-3pm) and under similar environmental conditions (approx. 21-24°C).

Maximal Aerobic physiology assessment

The athlete performed an incremental test to exhaustion to establish maximal oxygen uptake ($\dot{V}O_{2max}$) and maximal aerobic power (MAP) ~ 7 days either side of the first and last BFR training session to assess changes in their maximal aerobic physiology. Following a 5 min warm-up at 120 W, at a freely chosen but constant pedal cadence, work rate was increased by 20 $W.min^{-1}$ until volitional exhaustion (8-10 min). Pulmonary gas exchange was measured breath by breath throughout exercise (Oxycon Pro, Carefusion, UK) and $\dot{V}O_{2peak}$ determined as the highest 30 s recording of $\dot{V}O_2$ during the test. MAP was defined as the highest average 60 s power recorded during the test.

Assessments of critical power and W'

Critical power (CP) and the curvature constant of the power-duration relationship (W') were assessed pre- and post-intervention via a series of fixed-duration, self-paced maximal time trials (TT) in accordance with the methods described by Simpson and Kordi (2016). Prior to the training intervention, a number of familiarisation TTs were performed to ensure the athlete was accustomed to the task. The athlete completed three 3-min, two 12-min and one 6-min maximal TTs. All TTs were completed on the athletes own road bicycle fitted to a UK

Sport Innovation turbo trainer with a power meter at the rear wheel in identical configuration to that used during the interval training sessions detailed above.

The athlete was instructed to achieve the highest power output they could for a fixed, known duration of either 3-, 6- or 12-min. The athlete had visual feedback of power output, pedal cadence, heart rate (HR) and elapsed time. All TTs were supervised by the principle investigator, who provided strong verbal encouragement throughout each TT.

Power, cadence and HR data were extracted from the wireless data acquisition device (Garmin Edge 500, Garmin UK, Southampton) for analysis in desktop software (Golden Cheetah, goldencheeth.org). Critical power was determined using two linear models; (i) work vs. time and (ii) power vs. time^{-1} which both conform to equation 1.

$$P = (W'/t) + CP \quad [1]$$

Where P is the power achievable and t is the fixed duration of a given TT. The linear model providing the best fit (highest R^2) and least standard error of the estimate (SEE) for both CP and W′ was use for the parameter estimates.

6.3 Results

A total of 552 KJ of work was performed across the 8 BFR training sessions. The athlete's changes in body composition across the intervention period are presented in Table 8. Body mass recorded during the pre- and post-intervention track sessions were 61.1 and 60.4 kg, respectively. There was a 1 and 2 % increase in the athlete's absolute (1041 vs 1049 W) and relative (17.0 vs. 17.4 W.kg^{-1}) PPO after the intervention. The athlete's changes in maximal

anaerobic and aerobic physiology and performance in response to the intervention are presented below in the tables and figures.

Pre-intervention CP was 195 ± 4 W (SEE). Post-intervention CP was 209 ± 6 W. W′ was 18.36 ± 1.73 and 18.72 ± 2.30 kJ pre- and post-intervention respectively. This represents a 7% increase in CP and a 2% increase in W′.

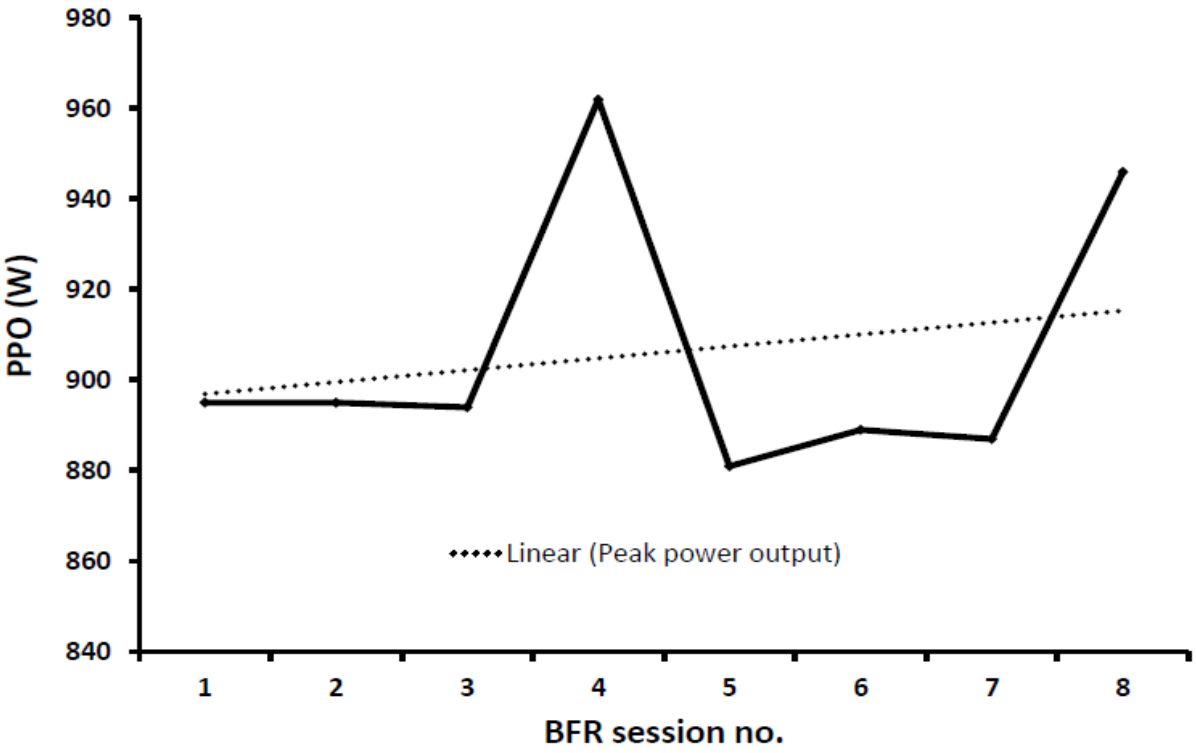

Figure 24 - Peak power output achieved during each BFR training sessions.

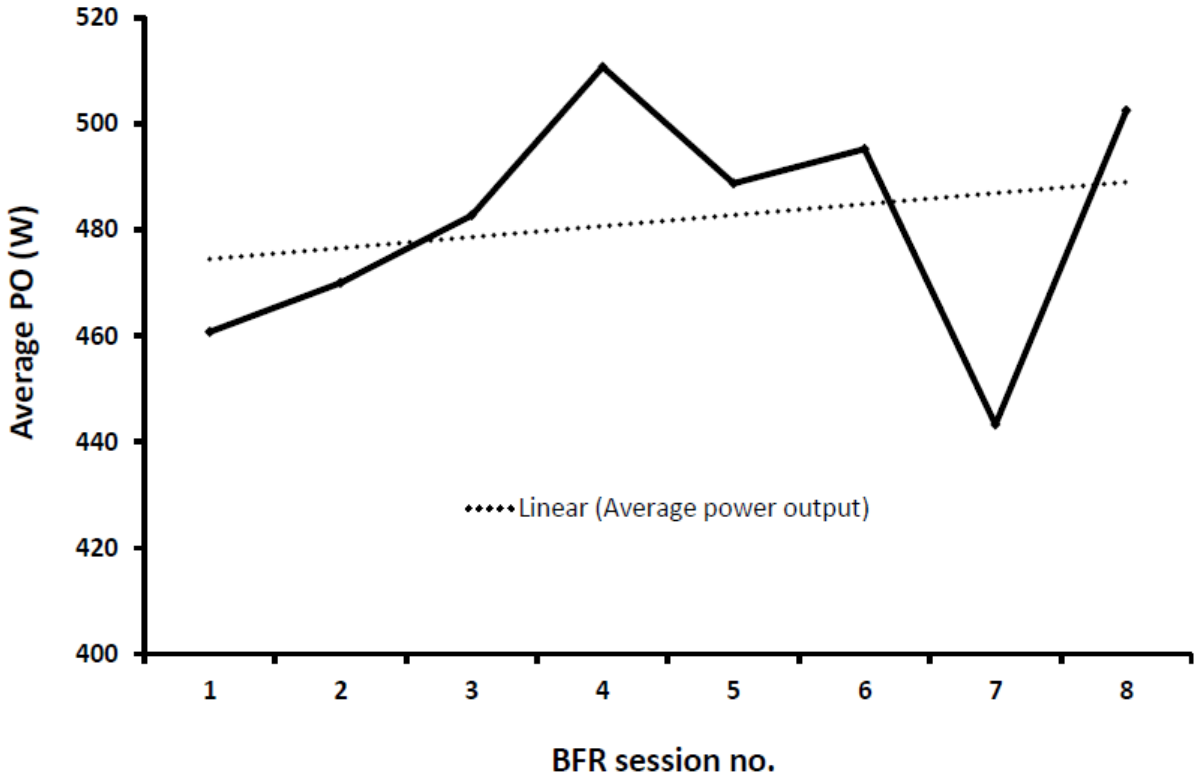

Figure 25 - Average power output achieved for the first 4 sprints of each BFR training session.

Table 7 - Body composition changes measured by anthropometric assessment and DEXA from before to after the training intervention.

	Pre	Post	% change
Body mass (kg)	60.4	60.4	0
Sum of 8 site skinfolds (mm)	78.5	74.5	-4
Mid-thigh R-leg girth (cm)	52.2	51.1	-2
Mid-thigh L-leg girth (cm)	52.3	51.8	-1
Total body fat (%)	13.9	14.9	7
Total body fat mass (g)	8633	8250	-7
Total body lean mass (g)	46772	46325	-1

Table 8 - Aerobic physiology and performance changes from before to after the training intervention.

	Pre	Post	% change
VO_{2max} ($L.min^{-1}$)	3.1	3.2	3
VO_{2max} ($ml.kg^{-1}.min^{-1}$)	51.3	53.0	3
MAP (W)	305	317	4
MAP ($W.kg^{-1}$)	5.0	5.2	4
Max HR (bpm^{-1})	189	193	2
3 min max PO (W)	286	305	7
6 min max PO (W)	250	265	6
12 min max PO (W)	214	234	9

6.4 Discussion

This case study assessed the application of SIT combined with BFR in a full-time athlete's training over a four week period as a potential novel, low-volume strategy to enhance aerobic physiology and performance capabilities. Following four weeks of dedicated SIT combined with post-exercise BFR (2 sessions per week), the athlete significantly improved their aerobic performance and associated physiological parameters, which came at no expense to their maximal anaerobic physiology. Indeed, the methods and results presented here have implications for a number of sports, notably linear energetic sports such as track cycling, in which well-developed aerobic and anaerobic capacities are critical to success.

The observed 3 and 4 % increases in the athlete's absolute and relative $\dot{V}O_{2max}$ and MAP, respectively, are slightly lower, but nevertheless consistent with those reported in the BFR group in the previous chapter. Although Sjogaard and colleagues (1985) have reported relative changes in $\dot{V}O_{2max}$ of as much as 22% in a Danish international track cyclist across a whole training year, the magnitude of change for this parameter reported here and in the previous chapter could be of greater interest to coaches and athletes given the brief 4-week period of training and the low total work performed. Chapter 2 highlighted that shorter maximal efforts might not be optimal for enhancing $\dot{V}O_{2max}$ in well trained individuals (this is also supported by the data in the previous chapter) when compared to longer, less intense interval-based protocols performed below $\dot{V}O_{2max}$ (Tabata et al. 1996; Stepto et al. 1999; Hawley and Stepto 2001; Laursen et al. 2002a; Laursen et al. 2002b; Helgerud et al. 2007; Gunnarsson et al. 2012; Millet et al. 2014). The meta-analysis conducted by Bacon and colleagues concluded interval training alone or combined with continuous training can increase $\dot{V}O_{2max}$ in untrained individuals by up to 0.6 $L.min^{-1}$ and by up to 0.9 $L.min^{-1}$ in

response to protocols that feature longer intervals (Bacon et al. 2013). However, we have shown here and in the previous chapter that when combined with post-exercise BFR, SIT provides a potent stimulus for aerobic adaptations in trained individuals. Indeed, differences in training status could account for the more modest increases in absolute $\dot{V}O_{2max}$ observed here compared to those reported above. Additionally, it must be noted that in the majority of the studies included in the review by Bacon and colleagues, participants undertook intense interval training on 3 occasions per week compared to a frequency of 2 sessions per week here and in the previous chapter.

In the previous chapter, despite the observed changes in $\dot{V}O_{2max}$ and MAP in response to BFR, no improvements in 15 km TT performance were observed, irrespective of whether BFR was incorporated into the interval training. This led to the conclusion that the 15 km TT likely lacked sensitivity to reflect an increased $\dot{V}O_{2max.}$ As such, in this case study the athlete performed pre- and post-intervention efforts of different durations (3, 6, 12 min), whereby the athlete aimed to achieve the highest MPO possible for each duration. Whilst these efforts taken as individual performance tests would appear to better reflect changes in $\dot{V}O_{2max}$ (Craig et al. 1993), they were also used to model the athletes changes in critical power and W' across the intervention period. The 7 and 2 % increase in CP and W′, respectively, combined with the athletes changes in PPO relate to a calculated 3.0% increase in average PO achievable over a 30 s period (i.e. similar time taken to complete 500 m TT by international female track cyclists) and a 5.5% increase in power output achievable over a 3.5 min period (3 km IP). Of interest and importance is that the athlete's CP increased at no expense to the W'. It is not uncommon to observe an increase in CP at the expense of W' and vice versa after a period of training (Jenkins and Quigley 1992; Vanhatalo et al. 2008). As such, the data presented in this chapter suggests the training regime prescribed here could have application in specific

scenarios where athletes' competing in sports or tasks highly reliant on anaerobic capabilities, seek rapid gains in aerobic physiology and performance (e.g. a track cyclist transitioning from sprint to endurance disciplines and visa-versa).

Absolute and relative PPO (i.e. power to body mass ratio) are key performance determinants in track sprint cycling. The latter is particularly important to the track cycling TT events (e.g. 500 m TT through to the 4 km team pursuit) as this dictates an athlete's ability to accelerate off the start line. Here the athlete's standing start absolute and relative PPO assessed on the track were maintained throughout the intervention period despite a reduction in gym frequency from ~3–1 session per week and the complete removal of track based training sessions. It is plausible that PPO would have decreased across the intervention period with this change in training focus and in particular the reduced strength stimulus. Moreover, the data from the pre and post intervention DEXA scans would suggest that the athlete reduced and increased their whole body lean and fat mass, respectively, which would indeed more likely support a reduction in maximal PO capabilities, given the role of muscle cross sectional area for strength and power (Folland and Williams 2007). In contrast, and in support of the data obtained from the track assessments, there was also a positive linear trend in PPO achieved during the BFR training sessions over the four weeks.

A positive linear trend for 30 s MPO was also observed across the training period. This is an interesting observation as one might expect that the accumulation of fatigue across the four weeks would result in a negative trend in this training variable. This could possibly reflect the weekly SIT sessions being separated by 72 hrs and perhaps an appropriate balance of training and recovery being struck over the four weeks in this scenario. Also of interest is that in Chapter 5, two sessions per week of SIT without BFR did not result in any changes in physiology or performance. Indeed, the few studies that have reported increases in $\dot{V}O_{2max}$

have done so in recreationally active or non-trained, sedentary individuals and have typically prescribed 3 SIT sessions per week (Burgomaster et al. 2008; Whyte et al. 2010; Cocks et al. 2013). Indeed, training frequency is also a critical variable alongside intensity and duration that is manipulated by the coach and athlete to optimise the specific training response. It is possible that whilst the duration and intensity of the intervals in the SIT group itself were insufficient to induce adaptation in that cohort, the frequency of SIT training may also have been insufficient. In this regard, Billat et al. (1999) have explored the impact of increasing training frequency in a group of middle distance runners who initially trained 6 sessions per week of continuous training only. They found that manipulating the training week to comprise four continuous training sessions, 1 x HIT session, and one lactate threshold (LT) session resulted in improved running economy and speed at $\dot{V}O_{2max}$ (albeit with no enhancement of $\dot{V}O_{2max}$). Interestingly, further intensification of the training week to 2 continuous training sessions, 3 x HIT sessions and one LT session did not result in any additional adaptation (Billat et al. 1999), indicating that approx. 2 sessions of interval-based exercise a week appears an appropriate frequency for this type of training. Nevertheless, of interest to coaches, athletes and scientists is that the athlete performed significantly less work as a result of fewer and less frequent training sessions during the intervention period compared to previous training blocks. Nevertheless, the athlete achieved substantial improvements in endurance capabilities whilst seemingly maintaining their maximal anaerobic physiology. Indeed, the impact of training frequency on adaptation and performance, in particular relating to HIT/SIT, is an area of 'training science' that requires further research attention given the paucity of data on this training variable in well trained athletes.

Perhaps its application could be extended to intermittent team sports athletes as both high aerobic and anaerobic energy provision are necessary to meet the physical demands of these

sports. Clearly the absence of a control condition significantly reduces the scientific rigour of this study and does not allow comparisons between BFR and non-restricted SIT to be made. Unfortunately, retrospective examination of the athlete's adaptive potential to past interval-based training interventions undertaken has been made difficult due to a lack of relevant test data. De-conditioning the athlete to pre-intervention baseline values and then re-intervening with SIT alone would have enabled comparisons to be drawn between the magnitudes of adaptive response to SIT or SIT combined with post-exercise BFR. This, however, was also not possible due to time constraints. This was the athlete's first experience of undertaking BFR exercise and below is a post-intervention self-written reflection of her experiences as she underwent the change in training structure and was exposed to a new type of training intervention:

"The interval training with blood flow restriction was probably the most physically and mentally tough training I have ever done, but at the same time both satisfying and seemingly effective. However, I felt that as the training progressed beyond the first week I began to better cope with the discomfort after each interval. The most mentally challenging aspect of the training was the 'all-out' nature of the efforts and in particular the last couple of efforts in each session where the recovery between efforts seemed almost non-existent. The burning sensation during BFR towards the last few sessions of the training block was just about tolerable. The 2 minutes of occlusion immediately after the 30 second sprint was a strange experience. After the first interval, the occlusion would be mildly uncomfortable but I could have continued for longer than 2 minutes. As we progressed through the intervals the occlusion became more painful, and in the later weeks where I was doing 5 or more intervals, the pain level was on the limit of what I could cope with for the last few efforts."

"The fatigue in the early hours of recovery after the BFR sessions was far more pronounced to what I've experienced from any other training session. I'd find that I often needed to nap

in the immediate hours that followed, which I wouldn't usually after other types of sessions. In the days that followed, I wouldn't experience the same type of painful DOMS that I would from a gym or track session, instead just a general tiredness. Although incredibly tough, overall the training was enjoyable and highly satisfying. I also definitely felt myself coping better with the intervals and blood flow restriction over the short period of time that we were doing them, so would be interested to see the effect of it over a longer period of time".

In conclusion, the findings from this study have implications for athletes and coaches seeking training novelty or variety and/or those with the goal of achieving a potent time- and work-efficient training stimulus. Specifically, it has presented application for the use of SIT combined with post-exercise BFR in future training programmes for cyclists and other linear energetic athletes (e.g. rowers, runners, triathletes) to induce relatively rapid adaptation favouring endurance performance. It is important to consider that whilst HIT/SIT, for the most part, is often associated with rapid adaptations (2-12 weeks), the adaptive response or rate of adaptation to this training is typically quick to plateau (Seiler and Tønnessen 2009). Indeed, in applying these ideas, athletes and coaches should consider the need to be considerate to strike the optimal balance between maintaining athlete health and daily training tolerance and maximising training/competition performance. In this regard, the indications would suggest that the interventions outlined in this thesis could offer a potent stimulus for development, perhaps prescribed as brief training blocks in the context of the athlete's training macrocycle to provide novel training stress.

CHAPTER 7 – GENERAL DISCUSSION

7.1 Summary

This thesis reports a series of studies that examined, both at the whole body and cellular level, the acute and chronic effects of manipulating exercise and recovery during brief 'all-out' cycling on aerobic physiology and performance in trained individuals. In doing so, the primary aim was to identify novel methods of training that could potentially provide athletes, in particular those competing in sports or tasks highly reliant on aerobic metabolism, with a greater adaptive response for their training effort. The main findings of the thesis were as follows:

1. Low-volume 'all-out' sprint interval (SIT; 4 x 30 s bouts) and continuous cycling (CON; 1 x 2 min bout), thereby matched for total exercise duration, but not work done (71.2 vs. 46.4 kJ, respectively), likely provide an equally potent stimulus to acutely activate cell signalling pathways and gene expression of factors associated with exercise-induced mitochondrial biogenesis and angiogenesis.

2. Post-exercise BFR potentiates HIF-1α mRNA expression in response to SIT, tentatively suggesting an enhanced metabolic and hypoxia-targeted angiogenic stimulus over SIT alone. Moreover, the increased expression of PGC-1α, VEGF and VEGFR-2 mRNA after both BFR and non-restricted SIT in trained individuals further supports the angiogenic potential of brief 'all-out' exercise, as has been previously demonstrated in untrained individuals.

3. Four weeks of SIT combined with post-exercise BFR likely provides a greater training stimulus over SIT alone in trained individuals. $\dot{V}O_{2max}$ (4.7 v 1.1 % change) and MAP (3.8 v

0.2 % change) were increased to a greater extent with BFR compared to CON, respectively. However, this did not translate to improving 15 km TT performance.

4. In response to four weeks of SIT combined with post-exercise BFR, an elite female track sprint cyclist increased CP and W' by 7 and 2 %, respectively. She also increased her relative $\dot{V}O_{2max}$ and absolute MAP by 3 and 4 %, respectively. Moreover, these results occurred with little change to the athlete's on-track maximal sprint capacity, as demonstrated by a 1 and 2 % increase in absolute and relative PPO and a positive linear trend in PPO over the successive BFR training sessions.

7.2 Interval and continuous based 'all-out' cycling

The primary aim of the study reported in Chapter 3 was to assess to potency of interval and continuous 'all-out' sprint cycling in order to upregulate acute cell-signalling pathways and molecular markers related to mitochondrial biogenesis and angiogenesis in trained individuals. This chapter employed a repeated measures design to compare the regularly studied 'classic' 4 x 30 s 'all-out' cycling bouts (abbreviated INT in Chapter 3) to a continuous 120 s maximal effort cycling sprint protocol, whereby the recovery periods between efforts were essentially removed (CON). Thus the protocols were matched only in terms of total exercise duration (not session duration). Despite this difference in structure and the 53% greater total work done in INT compared to CON, both protocols induced a similar increase in gene expression related to mitochondrial biogenesis and angiogenesis. This was the first study to compare the expression of mitochondrial and angiogenic markers in response to acute 'all-out' interval and continuous cycling and thus demonstrates that the characteristic 'pulsatile' pattern of SIT might not be as important to the magnitude of adaptive response to 'all-out' exercise as previously considered. Moreover, it was the first study to report increased mRNA expression of primary angiogenic growth factors (notably

VEGF and VEGF-R2) in response to an acute bout of the classic 'SIT' protocol as well as the 'all-out' continuous cycling effort in trained individuals, thereby offering insight into the angiogenic potential of maximal exercise. Finally, it is apparent that the 120 s continuous effort is the least amount of exercise in terms of both duration and work done that has been demonstrated, thus far, to increase PGC-1α and VEGF mRNA expression.

The findings reported in Chapter 3 supports previous studies demonstrating similar cell-signalling responses after work-matched HIT and continuous running and cycling (Wang et al. 2009; Bartlett et al. 2012), and non-work matched HIT based cycling (Psilander et al. 2010). Taken together, these findings offer further insight into the role of exercise intensity in mediating skeletal muscle re-modelling and raise questions over the importance of the characteristic 'pulsatile' nature of SIT and HIT. Indeed, it has been previously suggested that this on-off pattern of interval training could play a part in the potency of this low-volume alternative to traditional endurance training (Cochran et al. 2014). For example, in response to moderate-intensity exercise, Combes et al. (2015) observed greater AMPK phosphorylation when a 30 min exercise session was undertaken at 70% $\dot{V}O_{2max}$ as 1 min 'on' 1min 'off' than a continuous effort. Whilst the acute molecular snapshots from Chapter 3 suggest during 'all-out' exercise, the pattern of exercise is perhaps less important, the aforementioned study by Combes and colleagues appears to suggest that during submaximal exercise, interval-based exercise provides a favourable stimulus for adaptation. Of course this requires evidencing with a long term training study. Similarly, whether the protocols performed in Chapter 3 result in comparable capillary growth and mitochondrial biogenesis over repeated sessions, as could plausibly be hypothesised based on the observed acute molecular snapshots, also requires elucidation.

Only one study to date has attempted to investigate the chronic effects of 'all-out' interval versus 'all-out' continuous exercise on aerobic adaptation. Gibala's research group have recently demonstrated that the 'classic' SIT protocol and an 'all-out' work-matched continuous ~ 4 min cycling protocol (CONT), result in similar acute cell-signalling associated with mitochondrial biogenesis but contrasting chronic effects on mitochondrial adaptation after 6 weeks of training (Cochran et al. 2014). Their study actually comprised two investigations. The first investigation utilised a repeated measures design comparing acute mitochondrial cell-signalling after SIT and CONT. The second was a short term training study where participants undertook 6 weeks of CONT, with pre- and post-training changes in maximal activity and protein content of mitochondrial markers, as well as $\dot{V}O_{2peak}$ and TT performance being compared to previously published studies where SIT was undertaken (Gibala et al. 2006; Burgomaster et al. 2008). Despite CONT and SIT inducing similar acute increases in AMPK, p38MAPK and PGC-1α in the first investigation, 6 weeks of CONT did not increase the maximal activity of CS and total protein content of numerous mitochondrial markers. Nevertheless, CONT did improve $\dot{V}O_{2max}$ and TT performance, albeit in untrained individuals (Cochran et al. 2014). Alongside the difficulties in comparing outcomes between separate studies, the PPO achieved in CONT was 38% lower than during SIT in their acute investigation, suggesting an element of pacing, and so it could be argued as to whether CONT was 'all-out' in nature. In contract, the matched PPO achieved between protocols in Chapter 3 clearly demonstrates that these were non-paced and this is likely of importance to the potency of SIT and perhaps CONT.

As exercise intensity increases, so does ADP and the ratio of AMP:ATP (Howlett et al. 1998; van Loon et al. 2001), resulting in the intensity-dependant activation of AMPK (Egan et al. 2010; Gowans et al. 2013; Kristensen et al. 2015).Whilst exercise intensity seems to be the major regulator of AMPK activity, it is also upregulated in situations of reduced muscle

glycogen content (Steinberg et al. 2006; McBride et al. 2009; Yeo et al. 2010). In the study by Cochran et al. (2014), similar muscle glycogen content was reported in the vastus lateralis after both SIT and CONT and this conceivably could have been a similar case after both protocols in Chapter 3. Unfortunately glycogen content was not measured before, during or after either protocol. These, alongside measurements of CaMK and p38MAPK (which despite significant trouble shooting we were unsuccessful in obtaining satisfactory blots for p38MAPK) would no doubt have added further insight here, especially in light of the differences in work done between protocols. In addition to glycogen content, the rate of glycogenolysis could also be important in the upregulation of AMPK and could explain the similar magnitudes of increased phosphorylation between 'all-out' protocols. Parolin et al. (1999) demonstrated that AMP concentrations were greatest after the first of three 30 s 'all-out' cycle sprints (i.e. Wingate Tests), and glycogenolysis was drastically blunted during the third bout relative to the first bout. Moreover, Gibala et al. (2009) measured AMPK phosphorylation after the first and fourth sprint during SIT. AMPK phosphorylation was increased after the forth sprint but not the first. In light of the above, it's plausible that 60-90 s continuous 'all-out' effort would have been sufficient to induce similar increases in AMPK and PGC-1α to SIT. Indeed, future research is warranted to better understand the minimal effective dose of exercise to upregulate the above signalling pathways.

7.3 SIT combined with post-exercise BFR

The primary aim of Chapter 4 was to assess the potency of combining BFR with SIT by measuring the acute post-exercise cell-signalling response and gene expression of mitochondrial and angiogenic factors and comparing this to the response following SIT alone. It was hypothesised that the repeated blood flow restriction and re-perfusion would

promote intensified hypoxic, metabolic and shear stress stimuli, each of these being key physiological stimuli for angiogenesis (Egginton 2011). The findings tentatively indicated that a greater hypoxic and/or metabolic stimulus might be induced during BFR combined with SIT given the significant increase in HIF-1α mRNA expression during recovery after this protocol. This is the first study to demonstrate that BFR potentiates the mRNA expression of HIF-1α to SIT. HIF-1α mRNA levels have previously been demonstrated to transiently increase in human skeletal muscle in response to acute exercise provided it is of a sufficient intensity and duration, or is combined with BFR (Gustafsson et al. 1999; Lundby et al. 2006; Larkin et al. 2012) and is therefore likely to be an important transcription factor involved in the adaptive response to training. Given that HIF-1α mRNA increased at a time course similar to VEGF mRNA after BFR (Chapter 4), it is possible that regulation by HIF-1α could account for the augmented VEGF mRNA expression in response to BFR and SIT. However, without performing immunoblot and immunohistochemical analysis of HIF-1α protein in skeletal muscle, upregulation of VEGF via HIF-1α cannot be confirmed and factors other than HIF-1α could regulate the exercise-induced responses of VEGF. Another possible role for an acute post-exercise increase in HIFs could be to activate the transcription of target genes, including EPO, HK-II, PFK and LDH. The expression of several of these is increased with acute exercise and training (Koval et al. 1998; Pilegaard et al. 2000). Indeed, measurement of these in all of the studies throughout the thesis could have perhaps provided indirect information about HIF1α involvement.

In addition to hypoxia, it is possible that shear stress is elevated to a greater extent during the post-occlusion periods in the BFR condition. The premise of multiple brief BFR exposures immediately after each exercise bout was a strategy aimed at augmenting the brief shear stress response to SIT through enhancing the reactive hyperaemia with repeated cuff deflations (Gundermann et al. 2012). It was therefore hypothesised that combining SIT with

post-exercise BFR would present an intensified shear stress stimulus and thus greater upregulation of post-exercise eNOS mRNA compared to SIT alone. This however was not the case and the greater mean increase in eNOS mRNA at 3 h after BFR (Figure 14F) was driven by one individual's robust 4-fold increase at this time point. Therefore, future studies should investigate the effects of differing cuff exposure durations and manipulation of cuff pressure and its subsequent effects on mRNA and protein expression, as well as structural and functional adaptation over time.

Perhaps the most interesting finding from the work conducted in this thesis was the disparity in $\dot{V}O_{2max}$ changes between experimental groups in Chapter 5. Whilst 2 sessions per week of SIT over a four week period resulted in no change in $\dot{V}O_{2max}$ in trained individuals, the same SIT protocol combined with 2 min of post-exercise BFR applied immediately after each exercise bout resulted in a substantial (4.7 %) increase in $\dot{V}O_{2max}$ over the same time period in trained individuals. A meta-analyses comparing the effects of HIT or SIT to moderate-intensity continuous training on $\dot{V}O_{2max}$ reported a greater response to intense interval training regardless of whether workload is matched or not (Bacon et al. 2013). With specific reference to low-volume SIT, this is highlighted by the similar magnitude of increase in $\dot{V}O_{2max}$ compared to moderate intensity continuous training despite the drastically different volume in work performed (Weston et al. 2014; Gist et al. 2014). The studies that comprise these meta-analyses, however, have almost entirely been conducted on 'non-athletic' populations and have prescribed HIT/SIT three times per week (typically one day apart, i.e. Mon, Wed, Fri). Taken together, it would therefore appear 2 sessions per week of 'all-out' sprint interval exercise is insufficient training volume and/or frequency to elicit improvements in $\dot{V}O_{2max}$ in trained individuals over a four week period. However, when combined with post-exercise BFR, this appears to provide an amplified stimulus for rapid aerobic adaptations and

performance gains. In line with the favourable effects of BFR on $\dot{V}O_{2max}$ and albeit not statistically significant, there was a trend (P =0.09 interaction) in Chapter 5 for greater increases in relative MAP after 4 weeks of SIT combined with BFR, compared to SIT alone (4.4 v 0.2 % change, respectively). Of course, to coaches and athletes these percent changes bear greater weighting than a P value in deciding on the effectiveness of a training intervention and should be considered, particularly if innovative strategies are required.

Despite this enhanced $\dot{V}O_{2max}$ after SIT combined with post-exercise BFR, there was no improvement in 15-km TT performance; possibly suggesting that this duration of performance test (~ 22.5 min) lacked the sensitivity to reflect the observed changes in aerobic physiology. The case study conducted on the full-time elite track sprint cyclist (Chapter 6), however, provides an insight into the performance changes that may have occurred had Chapter 5 included more sensitive assessments of aerobic (i.e. CP) and anaerobic (i.e. W') physiology. Indeed, the athlete increased their CP and W' by 7 and 2 %, respectively. Modelling CP and W' required the athlete to achieve the highest MPO possible for durations of 3, 6 and 12 min (i.e. a duration/power relationship, rather than a traditional assessment of power/duration). Irrespective of CP and W' analysis, these efforts used independently (in particular the 6 min effort) would likely have also provided better tests of pre- and post-intervention performance in Chapter 5, given the previously highlighted strong association between IP performance (i.e. ~ 5.6 min) and relative $\dot{V}O_{2max}$ (Craig et al. 1993).

Of interest is that in Chapter 6 the increases in aerobic capacity after SIT with BFR came at no expense to the athlete's W' or PPO. As alluded to in Chapter 6, it is not uncommon to observe an increase in CP at the expense of W' and vice versa after a period of training (Jenkins and Quigley 1992; Vanhatalo et al. 2008). Although similar changes in $\dot{V}O_{2max}$ and MAP were observed after SIT and BFR in Chapters 5 and 6, it remains to be investigated

whether those in the BFR condition in Chapter 5 would have experienced similar increases in CP and W'. Nevertheless, the data reported in these two experimental chapters suggests this training regime could have application for endurance athletes or in specific scenarios where athletes' competing in sports highly reliant on anaerobic capabilities, seek rapid gains in aerobic physiology and performance such as an athlete who is transitioning from a sprint to an endurance disciplines. Furthermore, these findings could have implications for the athlete seeking a novel training stimulus to fast track their return from an injury/illness setback.

7.4 Limitations

There are a number of limitations to the studies within this thesis, many of which have been alluded to in the respective experimental chapters and above. The most significant limitations to the thesis are that direct assessments of capillary density were not made before and after the training interventions and that the angiogenic response was measured only at the level of mRNA. Indeed, numerous studies have shown that VEGF mRNA levels are increased in human and rodent skeletal muscle after various exercise protocols, whether blood flow is restricted or intact (Jensen et al. 2004a; Leick et al. 2009; Hoier et al. 2012; Larkin et al. 2012; Hoier et al. 2013b). Whilst exercise-induced VEGF mRNA increases are clearly important, it remains to be elucidated whether this is a necessity for the secretion of VEGF or whether it serves to replenish VEGF protein stores during recovery after exercise (Hoier et al. 2013b). Hoier et al. (2013a) reported that while intermittent high-intensity cycling exercise induced greater mRNA elevations of angiogenic factors (including VEGF) compared to moderate-intensity continuous cycling, the latter protocol resulted in greater acute elevations in muscle interstitial levels of VEGF. Moreover, muscle interstitial fluid obtained during the moderate-intensity protocol induced greater proliferative effects on endothelial cells in vitro

than fluid obtained during intermittent high-intensity protocol. Indeed, measuring the release of VEGF would provide valuable insight into the angiogenic potential of the protocols employed in the studies reported throughout this thesis. In general, a more detailed measurement of pro- and anti-angiogenic factors would have provided valuable information to help determine the modulation and morphology of capillary growth with BFR exercise training. Moreover, given that the induction of VEGF by PGC-1α in the context of ischemia and exercise can occur independent of HIF-1α (Arany et al. 2008), more robust HIF-1α data would have been warranted.

Another potential limitation to the experiments reported in Chapters 3, 4 and 5 is the timings of post-exercise biopsy sampling and the absence of measurements of protein expression reflective of mitochondrial biogenesis and angiogenesis in the hours and days that followed each protocol. It is plausible that trained individuals have the same upregulation of mRNA after training, but smaller changes in mitochondrial and angiogenic protein expression and function compared to less fit individuals and this warrants further investigation. Moreover, it would have been interesting to determine whether cumulative transient greater increases in mRNA transcript (following each BFR exercise bout) can encode new protein to augment basal levels of PGC-1α, as this can enhance angiogenesis and exercise capacity (Tadaishi et al. 2011). Nevertheless, given it has previously been demonstrated that increases in mRNA expression for the vast majority of the genes measured through this thesis peak between 1-6 h after intense exercise (Pilegaard et al. 2003; Nordsborg et al. 2010; Hoier et al. 2012; Bartlett et al. 2012; Hoier et al. 2013a; Hoier et al. 2013b), suggests that the timing of post-exercise biopsies (i.e. 3 h into recovery) were appropriate.

Regarding research design, clearly the absence of a control condition in the elite athlete case study (Chapter 6) significantly reduces the scientific rigour of this case study, preventing

direct comparisons to be made on the effectiveness of BFR compared to non-restricted SIT. Furthermore, a limitation to Chapters 4, 5 and 6 is that the level of BFR was set at an identical pressure for all participants irrespective of thigh circumference. Whilst there is no doubt that the cuff pressures administered throughout this thesis resulted in BFR, the efficacy of BFR exercise is achieved by balancing the level of blood flow restriction, with an appropriate level of muscle activation and fatigue (contractile/metabolic impairment), so as to ensure an appropriate stimulus (Yasuda et al. 2008; Fahs et al. 2012). Indeed, maximum voluntary contraction decreases in proportion to the level of BFR and the level of restriction will influence hemodynamic signals, oxygen and nutrient delivery, and the accumulation, clearance and re-synthesis rate of metabolites during exercise (Sahlin et al. 1979; Takarada et al. 2000; Karabulut et al. 2011). These in turn will have implications on the specific adaptations that transpire to a given BFR training protocol. For example, increases in muscle mass maybe proportional to the level of metabolic stress under ischemic conditions (Takada et al. 2012) and capillary growth appears proportional to metabolic activity (Adair and Montani 2010). Ensuring an equal and appropriate level of BFR between participants is therefore important to achieve consistent gains from exercise training and thus future studies employing BFR using inflatable blood pressure cuffs should administer individualised pressures.

7.5 Practical implications and future directions

As with all research in any field, the investigations undertaken within a doctoral thesis should generate far more questions than they were created to answer and this thesis has indeed paved the way for future research to answer questions we were unable to. Given the novelty of the protocols and interventions employed here, there is clearly scope for future research to be

conducted as a result of the studies herein which should not be limited to athletic populations. Firstly, there is a need to further investigate the importance of the intermittent nature of SIT/HIT on the many beneficial structural and functional adaptations and performance enhancements observed in response to this type of training (MacInnis and Gibala 2016). In order to assess this, a series of randomised controlled trials in the form of training studies employing between-groups research designs are required. One reason to investigate this further is that the less complicated and time-consuming an exercise protocol, and indeed the less work needed to reap beneficial rewards, the more interest a training protocol will surely receive. Indeed, from a health perspective 'a lack of time' is repeatedly cited as the primary barrier preventing the public from achieving physical activity participation guidelines (Stutts 2002; Kimm et al. 2006).

Secondly, this thesis has demonstrated that the 'classic' SIT model and just 2 min of continuous 'all-out' exercise holds angiogenic potential. Indeed, it remains to be elucidated whether, if repeated over time, this brief exercise stimulus is capable of increasing the microvasculature as has been observed after SIT in sedentary males (Cocks et al. 2013). In light of two previous studies reporting that an increase in exercise intensity and reduction in training volume provides a weak stimulus for capillary growth (Hoier et al. 2013a; Gliemann et al. 2014), Gliemann (2016) has suggested there may be a 'Janus-faced' role of exercise intensity in peripheral adaptation, such that high-intensity exercise is required for increases in mitochondrial respiration and content (for support of this see review by Hughes et al. (2017), and high-volume continuous exercise for capillary adaptation. Support for this can be gleaned from observations in rodent models. Rodents who are permitted to exercise at their discretion run between 5-10 km/day, rarely exceeding 25% of maximal effort and experience capillary growth within days. Conversely, rodents who are forced to exercise do so for less than half the duration and at double the intensity when compared to voluntary exercise and yet take

weeks if not months to experience capillary expansion (Olfert et al. 2016). The mechanisms at play that underpin these findings remain to be fully explored, however one possible explanation could be that the shear stress experienced during brief 'all-out' exercise is relatively short-lived (albeit high). Given that shear stress-induced angiogenesis results from the duration and magnitude of shear stress exposure, this would presumably provide a plausible explanation for why higher volume exercise appears superior to higher intensity, lower volume stimuli for promoting capillary growth. Additionally, it is possible that a greater release of anti-angiogenic factors (such as TSP-1 and TIMP-1) following higher-intensity exercise (as has been reported previously by Hoier et al. (2013a)) triggers a halt on expansion of the capillary network. Thus, future studies investigating the impact of HIT and SIT on angiogenesis should explore the role of anti-angiogenic factors. Taken together, the above clearly stresses the need for additional randomised control trials employing both between-groups and repeated measures research designs and combining sophisticated muscle analysis techniques to assess whether 'all-out' exercise can increase the C:F ratio in the working muscles.

The research conducted in this thesis has merely scratched the surface into the potential concept of combining SIT with BFR to promote improvement in athletic performance. There is now a requirement for continued work in this area to further examine central and peripheral adaptive responses to this concept and further our understanding of the time course of adaptation and intensity of this stimulus. In this regard, future research should focus on further optimising the exercise and BFR exposure by manipulating cuff pressures, timing and amount of BFR exposure to ultimately ascertain the optimal blend of exercise and BFR to elicit the greatest training response. Whilst the beneficial impacts of HIT, SIT and BFR on physiology and performance transpire relatively rapidly (i.e. after only a few days in some instances), adaptations to these type of training also appear relatively short-lived and in some

instances, rates of adaptation are quick to plateau (Jensen et al. 2004a). Indeed, a challenge for sports and exercise scientists is the transfer and application of results from short-term training interventions, such as those included in this thesis, to long-term physiological development and training periodisation. In light of this, longer-term (> 4 weeks) investigations into the effects of repeated exercise combined with BFR are warranted to assess rate of adaptation and stagnation to this type of exposure.

In summary, the outcomes reported in this thesis present practical applications for the full-time athlete seeking greater return on their training investment and the recreational athlete aiming to achieve a personal best in their local village TT. The findings hold potential health implications for the sedentary individual who states the previously mentioned 'lack of time' as the primary barrier preventing them from achieving physical activity participation guidelines. Indeed, if manipulated appropriately, these protocols may also present important health benefits for those with metabolic and vascular related disorders, such as those suffering from insulin resistance and essential hypertension. This requires evidencing and thus the research designs, methods and protocols employed in this thesis should in the future be tested across the aforementioned participant cohorts; 1) so that the efficacy of BFR and non-BFR 'all-out' interval and continuous exercise can be examined in these populations and 2) to further our understanding of the potential mechanisms of action involved. The latter reason is of particular importance as it is this that will ensure these protocols and interventions are optimised further for both athletic and clinical application. Nevertheless, Chapters 4, 5 and 6 have introduced and provided preliminary evidence for a new training concept, which in years to come could be utilised by athletes and coaches in a similar manner to, or in conjunction with, other training strategies (e.g. hypoxic training, low-carbohydrate training, heat-stress exposure) targeted at giving athletes more 'bang for their buck'.

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