THE USE OF BLOOD FLOW RESTRICTION TO ENHANCE HIGH-INTENSITY ENDURANCE PERFORMANCE AND SKELETAL MUSCLE ADAPTATION

By

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ABSTRACT

Post-exercise blood flow restriction (BFR) is a novel training method which, through alterations to the haemodynamic, metabolic and hypoxic stimulus, could augment skeletal muscle adaptation in endurance trained individuals. The studies described in this thesis investigated the combined effect of post-exercise BFR and interval training on angiogenic and mitochondrial biogenic molecular and adaptive responses and high-intensity endurance performance. It was initially demonstrated that a very strong correlation exists between critical power (CP), an important parameter of endurance performance, and indices of skeletal muscle capillarity (r = 0.94), implying that skeletal muscle capillarity is an important determinant of CP (Chapter 3). Chapter 4 investigated the efficacy of combining post-exercise BFR with 4 weeks of sprint interval training (SIT) (4-7 x 30 s maximal bouts) in trained individuals. SIT increased CP by ~3.5% in trained individuals, but the addition of BFR did not enhance this further. In contrast, neither skeletal muscle capillarity or mitochondrial protein content increased following SIT with or without BFR. However, there was a trend (P = 0.06) for an increase in proliferating endothelial cells after four weeks of SIT only with post-exercise BFR, tentatively suggesting that post-exercise BFR could elicit an enhanced angiogenic response when undertaken with a greater training duration and/or volume. This was investigated in Chapter 6 in which the time course of transient transcriptional changes during two weeks of post-exercise BFR combined with a higher volume high intensity interval training (HIIT) modality was examined. BFR did not enhance the transient increases in VEGF, PGC-1α of HIF-1α mRNA following HIIT during two-weeks of training and did not increase VEGF protein content. There was however, an increase in resting eNOS mRNA after two weeks only when HIIT was combined with post-exercise BFR, suggesting BFR elicited an increased shear stress stimulus. There was no increase in mitochondrial protein content or citrate synthase activity following 2 weeks of HIIT with or without post-exercise BFR. Collectively the findings from these studies imply that post-exercise BFR does not increase mitochondrial content, but although there was no increase in skeletal muscle capillarity, there were suggestions that post-exercise BFR could elicit an enhanced angiogenic stimulus when undertaken with higher training volumes and could subsequently increase performance.

Key words: Blood flow restriction, angiogenesis, mitochondrial biogenesis, critical power interval training, trained individuals.

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PUBLICATIONS

The publications that have arisen from this thesis are as follows:

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AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATP	Adenosine Triphosphate
BFR	Blood flow restriction
BSA	Bovine serum albumin
CaMKII	Ca^{2+}/Calmodulin-dependent kinase II
CC type I	Capillary contacts around type I fibres
CC type II	Capillary contacts around type II fibres
CON	Control
COXII	Cytochrome c oxidase subunit II
COXIV	Cytochrome c oxidase subunit IV
CP	Critical power
CS	Citrate synthase
CSA	Cross sectional area
eNOS	Endothelial nitric oxide synthase
FXYD1	Phospholemman-1
GET	Gas exchange threshold
HDAC4	Histone deacetylase-4
HHb	Deoxyhaemoglobin
HIF-1α	Hypoxia inducible factor-1 alpha
HIIT	High intensity interval training
HR	Heart rate
MAP	Maximal aerobic power
MEF2	Myocyte enhancer factor-2
MHC	Myosin heavy chain
MMP	Matrix metalloproteinase
MPO	Mean power output
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NIRS	Near-infrared spectroscopy
NO	Nitric oxide

NOS	Nitric oxide synthase
NRF	Nuclear respiratory factor
O_2Hb	Oxyhaemoglobin
p38 MAPK	p38 mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PPO	Peak power output
RF	Rectus femoris
RM	Repetition maximum
RPII	RNA polymerase II
RPE	Rating of perceived exertion
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SIT	Sprint interval training
TBST	Tris buffered saline with tween
Tfam	Mitochondrial transcription factor
TOI	Tissue oxygenation index
Type I fibre %	%proportion of type I fibres
Type II fibre %	%proportion of type II fibres
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VL	Vastus lateralis
$\dot{V}O_2$	Oxygen uptake
$\dot{V}O_{2max}$	Maximal oxygen uptake
$\dot{V}O_{2peak}$	Peak oxygen uptake
W'	Curvature constant

CHAPTER 1

INTRODUCTION

Skeletal muscle demonstrates remarkable plasticity in its adaptive remodelling and functional response to a range of physiological stimuli (Hoppeler, 2016). These include: hypoxia (Hoppeler *et al*. 2008), disuse (Bodine, 2013), nutritional modulations (Hawley *et al*. 2011) and not least endurance training (Holloszy *et al* 1977; Saltin *et al.* 1977). The adaptations elicited are specific to the stimuli imposed, allowing the individual better defence against future disturbances to homeostasis (Coffey & Hawley, 2007; Egan & Zierath, 2013). Specifically, endurance training elicits adaptations supporting oxygen delivery and utilisation, such as increased capillary supply and mitochondria (Andersen & Henriksson, 1977; Klausen *et al*. 1981; Holloszy *et al*. 1977; Holloszy & Coyle, 1984). These adaptations are critical for optimising oxidative capacity and demonstrate close correlations with parameters of endurance performance (Flueck, 2010; Iaia *et al*. 2011; Saltin *et al*. 1977; Tonkonogi & Shalin, 1997).

At the periphery multiple physiological and metabolic signals are induced during endurance exercise, such as mechanical stretch, calcium release and changes in the adenosine monophosphate (AMP) to adenosine triphosphate (ATP) ratio, that activate a cascade of molecular pathways (Coffey & Hawley, 2007; Hoppeler, 2016). These pathways elicit transient increases in the transcription of genes and translocation of proteins involved in skeletal muscle adaptation (Hoier *et al*. 2012; 2013b; Little *et al*. 2010a; Perry *et al.* 2010). The cumulative effect of these transient responses elicits a gradual increase in protein content and phenotypic remodelling, ultimately leading to functional performance enhancements (Egan & Zierath, 2013; Hoppeler 2016; Perry *et al*. 2010).

It is generally considered that further adaptations are more difficult to elicit in endurance trained individuals, who through years of training already possess highly adapted physiology, including capillary to fibre ratios and oxidative enzyme activities double that of untrained individuals (Hermansen & Wachtlova, 1971; Ingjer, 1979; Saltin *et al.* 1977; Tonkonogi & Sahlin, 1997). Indeed, as training progresses there is an attenuation in the magnitude of performance improvements (Hoppeler *et al*. 1985) alongside diminished increases in oxidative enzymes and capillary supply (Saltin *et al*. 1977). Moreover, training methods demonstrated to

elicit potent increases in oxidative capacity in untrained individuals, do not induce these adaptions in endurance trained athletes (Weston *et al*. 1997). This blunting of the adaptive scope is reflected at the molecular level. As individuals become accustomed to a specific exercise bout there is a blunting of the acute angiogenic and mitochondrial biogenic transcriptional response (Hoier *et al*. 2012; Perry *et al*. 2010; Richardson *et al*. 2000; Schmutz *et al*. 2006). Accordingly, an inverse relationship exists between mitochondrial content and the expressional response of mitochondrial and angiogenic transcripts (Flueck, 2010). The challenge for the endurance athlete therefore, is to identify training methods that are able to overcome this blunted response and promote continued adaptation.

Blood flow restriction (BFR) achieved through the application of inflatable cuffs proximal to the working muscle, is a potential strategy to enhance the endurance training stimulus in trained individuals. The use of BFR has received extensive focus in the literature as tool to augment the adaptative response to low-load resistance training and has been demonstrated to be an effective method to enhance strength and hypertrophic adaptations (Pope *et al*. 2013; Scott *et al*. 2015; Slysz *et al*. 2016). In contrast, the use of BFR during endurance training has received little attention and is currently an underexplored training method. Some studies have highlighted the potential of BFR, observing enhancements to maximal oxygen uptake ($\dot{V}O_{2max}$) and exercise capacity when combined with 'low'-intensity training (Abe *et al*. 2010a; de Oliviera *et al*. 2016; Park *et al*. 2010; Paton *et al*. 2017). Moreover, the application of BFR can increase multiple primary contractile stimuli including: shear stress (Takarada *et al*. 2000; Paiva *et al*. 2016), skeletal muscle hypoxia (Karabulut *et al*. 2011; Tanimoto *et al*. 2005), metabolic stress (Takarada *et al*. 2000; Suga *et al*. 2009; 2012) and oxidative stress (Christiansen *et al*. 2018), all of which are important stimuli of angiogenesis (Egginton, 2009) and/or mitochondrial biogenesis (Hood, 2009; Ljubicic *et al*. 2010). Indeed, the addition of BFR to low-intensity exercise can increase the acute transcriptional response of several key angiogenic and mitochondrial biogenic genes (Christiansen *et al*. 2018; Ferguson *et al*. 2018; Larkin *et al*. 2012).

For individuals to be able to tolerate a combined stimulus, the application of BFR during exercise requires a reduction in training intensity. Accordingly, current studies have only investigated the use of BFR during low-intensity (≤40% VO_{2max}) continuous (Abe *et al*. 2010a; Park *et al*. 2010) or reduced intensity interval type training (de Oliveira *et al*. 2016; Paton *et al*. 2017). This model of BFR training may have applications during specific circumstances such as periods of rehabilitation following injury when high loads are contraindicated.

However, the maintenance of a high training intensity is critical to optimise adaptations and endurance performance in athletes (Laursen *et al*. 2002; 2005; Mujika, 2010), suggesting a limited application of these 'reduced' intensity protocols during pre-competition training.

In an alternative approach a recent investigation provided preliminary support for the potency of post-exercise BFR. Whereby the BFR stimulus is applied as an additional stimulus in the recovery intervals during an interval training program, without requiring a reduction in the training intensity (Taylor *et al*. 2016a). This study combined post-exercise BFR with a classic sprint interval training (SIT) program. 20 trained cyclists undertook 4 weeks of SIT either alone or with 2 min of post-exercise BFR during the recovery intervals. They observed a potent increase in $\dot{V}O_{2max}$ of 4.5% after just 4 weeks in the BFR group, with no change following SIT alone. However, despite preliminary signalling data supporting the angiogenic potential of the combined stimulus, the effect on skeletal muscle adaptation and endurance performance have yet to be established.

In the context outlined above, this thesis herein outlines a series of experiments designed to further examine the potential of post-exercise BFR as an endurance training tool for trained individuals. The series of experiments investigated responses from the molecular to the whole-body level to assess the potential of post-exercise BFR to overcome the blunted molecular response in the trained state, augment skeletal muscle adaptations favourable to performance and ultimately enhance endurance performance.

CHAPTER 2

LITERATURE REVIEW

The performance of endurance training, either involving continuous low intensity sustained efforts or high intensity intervals interspersed with periods of recovery, elicits central and peripheral adaptations when performed for sufficient durations and intensities. The adaptations elicited enhance delivery and utilisation of oxygen and substrates and ultimately enhance an individual's ability to defend homeostasis and resist fatigue (Andersen & Henriksson, 1977; Egan & Zierath, 2013; Ekblom *et al*. 1968; Holloszy *et al*. 1977; Holloszy & Coyle 1984). Thereby, enabling the individual to maintain a higher power output or speed for a given duration.

2.1 PERIPHERAL ADAPTATIONS TO ENDURANCE TRAINING

Multiple peripheral adaptations are elicited by endurance training that are advantageous for endurance performance, including: mitochondrial biogenesis (Holloszy, 1967), angiogenesis (Anderssen & Henrikssen, 1977; Klausen *et al*. 1981; Jensen *et al*., 2004a), muscle fibre type conversion from type IIx to IIa (Bamman *et al*. 2007; Fitzsimons *et al*. 1990), increased muscle glycogen content (Greiwe *et al*. 1999; Hickner *et al*. 1997) and alterations to substrate metabolism at submaximal workloads (Holloszy & Coyle, 1984; Saltin *et al*. 1977). This review, however, will focus upon angiogenesis and mitochondrial biogenesis.

August Krogh's pioneering work first recognised the capillary bed to be the critical site of exchange for oxygen between the vasculature and the working skeletal muscle (Krogh, 1919a; 1919b). It is now acknowledged that a greater capillary bed enhances respiratory gas and substrate and metabolite exchange and consequently the capillary-muscle interface is considered as a potential limiting factor for aerobic exercise (Egginton, 2009; Prior *et al*. 2004). Accordingly, muscle capillarity has been implied as an important determinant of endurance performance (Coyle, 1995; Joyner & Coyle, 2008). Endurance trained individuals have consistently been reported to possess skeletal muscle with enhanced capillary supplies, with

capillary to fibre ratios up to double that of untrained individuals (Brodal *et al*. 1977; Hermansen & Wachtlova, 1971; Ingjer, 1979; Saltin *et al*. 1977).

Studies have provided evidence for the importance of the capillary bed to aerobic function and endurance performance. Indeed, the number of capillaries surrounding each muscle fibre has been shown to be proportional to the oxidative activity of that fibre (Romanul, 1964) and a close correlation has also been observed between the number of capillaries per fibre of the vastus lateralis and cycling $\dot{V}O_{2max}$ (Saltin *et al*. 1977). Moreover, Coyle *et al*. (1988) observed a correlation of $r = 0.74$ between capillary density and time to exhaustion during exercise at 88% of $\dot{V}O_{2max}$ and Iaia *et al*. (2011) reported positive correlations between capillary density and capillary to fibre ratio with time to task failure during performance times of ~1-20 minutes.

A high capillary supply is key to enhancing oxygen extraction. Oxygen extraction is a function of muscle oxygen diffusion capacity and muscle blood flow (Roca *et al*. 1992; Wagner, 1992), the former of which is primarily determined by the number of red blood cells in contact with the contracting skeletal muscle fibres (Federspiel & Popel, 1986). This is therefore, facilitated by a high capillary network, given that the majority of capillaries are believed to support red blood cell flux (Poole *et al*. 2013). Furthermore, capillary supply also facilitates the removal of fatigue inducing muscle metabolites, therefore sustaining exercise tolerance (Joyner & Coyle, 2008). Capillarity is correlated to blood lactate accumulation and pH during and upon completion of an exercise bout (Iaia *et al*. 2011; Tesch & Wright 1983). Iaia *et al*. (2011) further demonstrated a positive correlation between capillary density and the mean net rate of plasma potassium (K^+) accumulation, suggesting an increased transfer of K^+ from the extracellular space to the circulation. This would delay extracellular accumulation of K^+, which has been implicated to contribute to skeletal muscle fatigue and limiting high intensity exercise performance (Allen *et al*. 2008; McKenna *et al*. 2008).

Capillary supply is typically expressed as: capillary density (the number of capillaries per mm^{-2}), capillary to fibre ratio (the number of capillaries divided by the number of fibres) and capillary contacts (the number of capillaries in contact with each fibre), to give an overall picture of skeletal muscle capillarity. It is important to note that capillary density is limited as a marker of capillarity to determine the extent of exercise-induced angiogenesis as it is also a function of muscle fibre cross sectional area (CSA), which is similarly modulated in response to training (Olfert *et al*. 2016). Furthermore, capillary to fibre ratio may be of more importance to enhancing oxygen extraction than capillary density. Hepple *et al*. (2000) demonstrated that

a decrease in capillary density through short-term immobilization, which left capillary to fibre ratio unchanged, did not increase muscle oxygen diffusing capacity. This is further supported by observations that a group of endurance trained individuals possessed higher capillary to fiber ratios but had similar capillary densities as a group of untrained individuals (Hermansen & Wachtlova, 1971).

Endurance training is also well known to elicit increases in mitochondrial content and mitochondrial function (Holloszy, 1967; Holloszy *et al*. 1977; Hood, 2001; Bishop *et al*. 2014). Trained individuals have consistently been shown to have higher markers of mitochondrial protein content, enzyme activity and higher mitochondrial functions than untrained individuals (Befroy *et al*. 2008; Hoppeler, 1986; Larsen *et al*. 2009; Mogensen *et al*. 2006; Saltin *et al*. 1977), with elite level athletes possessing even higher values (Jacobs & Lundby, 2013). Mitochondria are a crucial component of skeletal muscle and have several key functions including cell apoptosis, cell signalling and are often referenced as the 'power houses' of the cell as the fundamental organelle of ATP production, the energy currency of living organisms (Hood, 2001). Within mitochondria ATP resynthesis occurs during the reactions of the Krebs cycle in the matrix and the electron transport chain at the inner mitochondrial membrane. Given the pivotal role of the mitochondria in providing ATP, it is not surprising adaptations to the mitochondria have been associated with endurance performance. Studies have demonstrated a close correlation between markers of mitochondrial density and $\dot{V}O_{2max}$ (Flueck, 2010; Jacobs & Lundby, 2013; Tonkonogi & Sahlin, 1997). Jacobs *et al*. (2011) further observed that mitochondrial function, as assessed by mitochondrial respiration, accounted for 47% of the variance in the performance of a 26 km time trial in a group of well-trained cyclists ($\dot{V}O_{2max}$ = 70.5 ± 5.6 $ml.min^{-1}.kg^{-1}$).

2.2 MECHANISMS OF THE ADAPTIVE RESPONSE TO ENDURANCE TRAINING

Skeletal muscle demonstrates remarkable plasticity in response to exercise. The molecular mechanisms that induce the adaptive response of angiogenesis and mitochondrial biogenesis involve a gradual modification of protein content and enzyme activity as a result of the activation of specific signalling pathways (Egan & Zierath, 2013). Following an acute bout of exercise in response to primary physiological and metabolic signals (such as mechanical stretch, calcium release, changes in AMP:ATP ratios, production of reactive oxygen species (ROS) etc; Coffey and Hawley, 2007) there is a transient increase in gene transcription of selected genes (Gustafsson *et al*. 1999; Perry *et al*. 2010; Pilegaard *et al*. 2000; 2003), alterations to protein stability and subcellular localisation of transcription factors (Ameln *et al*. 2005; Hoier *et al*. 2013b; Little *et al*. 2010a). With continued training these repeated transient changes lead to the changes in protein content and phenotypic remodelling demonstrated with training (Egan *et al*. 2013; Perry *et al*. 2010). An overview of the key signalling pathways for angiogenesis and mitochondrial biogenesis is displayed in Figure 2.1.

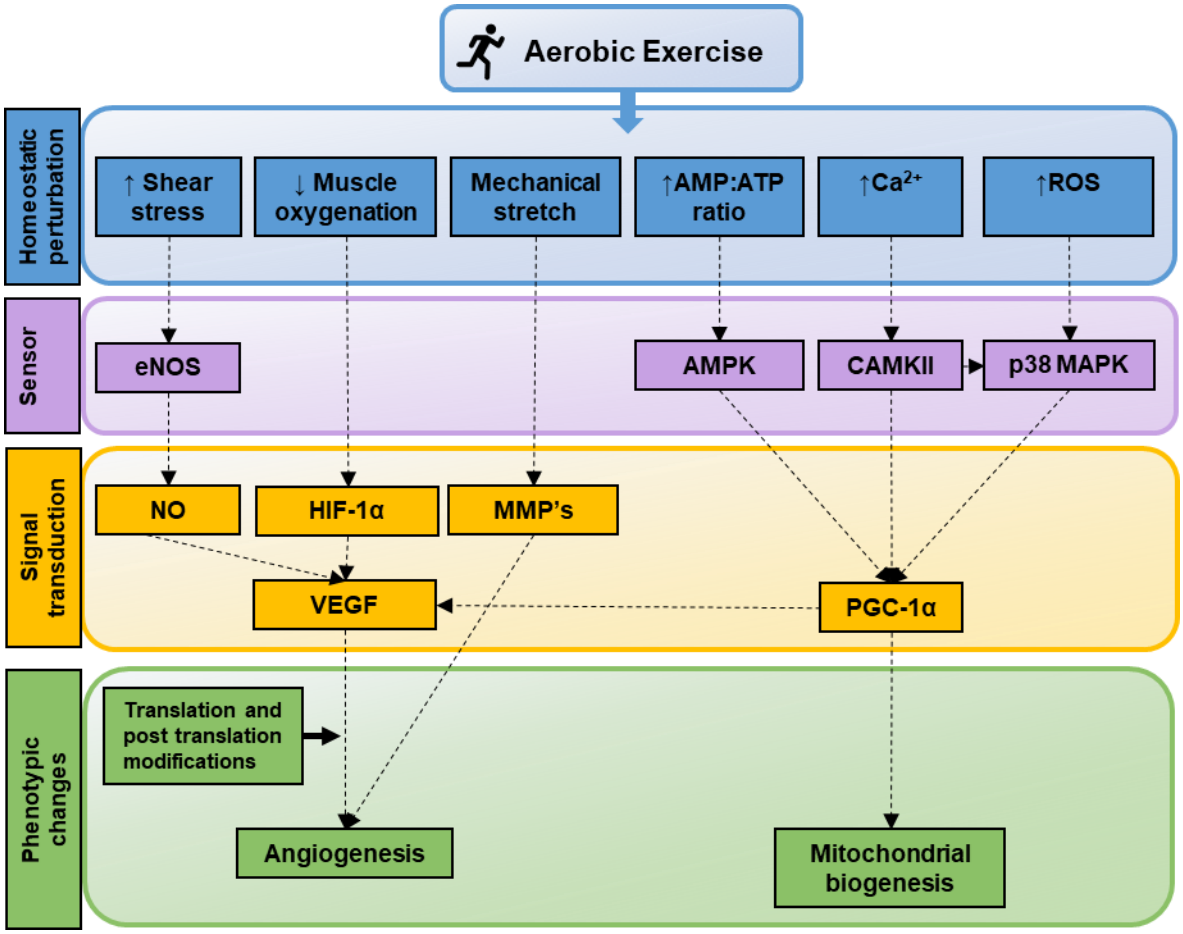

Figure 2.1. Schematic of the key components of the known signalling pathways likely eliciting exercise-induced angiogenesis and mitochondrial biogenesis (Adapted from Egan & Zierath, 2013).

Angiogenesis

Angiogenesis is the process of growth of new capillaries from an existing capillary bed (Egginton, 2011). Whilst in animals angiogenesis has been reported to occur in as little as four days with continuous low frequency electrical stimulation (Hudlicka 1998), with exercise training in humans the process appears to take longer, with significant changes in capillarity being reported only after 4-5 weeks of training (Andersen & Henriksson, 1977; Jensen, *et al*. 2004a). This process is tightly regulated by a combination of pro- (e.g. Vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), matrix metalloproteinases (MMP), angiopoietin-2) and anti-angiogenic factors (e.g. angiopoietin-1, tissue inhibitors of metalloproteinases, angiostatin), which appear to both be required for well controlled angiogenesis (Egginton, 2009; Hoier *et al*. 2012; Olfert & Birot, 2011).

Exercise-induced angiogenesis occurs through two processes: sprouting angiogenesis and intussusception. Sprouting angiogenesis refers to the process in which activated endothelial cells branch out from an existing capillary (Prior *et al.* 2004). Here, following the initial proteolytic breakage of the basement membrane, typically quiescent endothelial cells extend laterally from a capillary through the extracellular matrix to form abluminal sprouts (Egginton, 2009; Hoier & Hellsten, 2014). These sprouts then elongate and develop lumen, before re-entering the capillary bed by joining with existing vessels or other sprouts (Egginton, 2009; Prior *et al*. 2004). This newly formed capillary is initially leaky but becomes functional when it is invested by pericytes and the basement membrane is reconstructed (Hoier & Hellsten, 2014). Intussusception, also referred to as longitudinal splitting, is a luminal process where opposing endothelial cells extend into the lumen of the capillary and make contact dividing the vessel in two (Hoier & Hellsten, 2014, Prior *et al*. 2004). The inter-endothelial pillar-like structure is stabilised through the depositing of collagen by pericytes and/or myofibroblasts, forming a new capillary in the same direction as the parent vessel (Hoier & Hellsten, 2014). This process appears to be more efficient than sprouting angiogenesis, requiring less endothelial cell proliferation and the capillary is also not functionally compromised during the process (Egginton, 2009; Hoier & Hellsten, 2014).

VEGF is well recognised as the most central pro-angiogenic factor in skeletal muscle (Neufeld, *et al*. 1999; Olfert *et al*. 2010; 2016; Wagner *et al*. 2006). VEGF exerts its effects through endothelial cell specific receptor tyrosine kinases, vascular endothelial growth factor receptor -1 (VEGFR-1) and VEGFR-2, of which VEGFR-2 appears to be the most important (Neufeld

et al. 1999). The binding of VEGF to its receptors is known to stimulate the proliferation and migration of endothelial cells, promote endothelial cell survival and differentiation and induce capillary permeability (Egginton, 2009). Studies on rodents have elucidated the importance of the role of VEGF for exercise-induced angiogenesis. Early evidence observed VEGFR blockade partially inhibited the training induced increase in capillarisation in exercising rats (Lloyd *et al*. 2005). More recent studies have demonstrated that muscle specific VEGF knockout in mice markedly reduces basal skeletal muscle capillary supply (Olfert *et al*. 2009) and skeletal myocyte-specific VEGF gene-deletion abolishes exercise induced angiogenesis (Delavar *et al*. 2014; Olfert *et al*. 2010), providing evidence that VEGF is essential for exercise induced-angiogenesis.

Increased expression of VEGF messenger RNA (mRNA) has been reported following an acute bout of a range of exercise modalities including; single (Gustaffson *et al*. 1999; Richardson *et al*. 1999) and two legged knee extensor exercise (Hiscock *et al*. 2003; Jensen *et al*. 2004b), submaximal cycling exercise (Gavin *et al*. 2004; Hoier *et al*. 2012; Hoier *et al*. 2013b; Rullman *et al*. 2007; Ryan *et al*. 2006) and both high intensity interval training (HIIT) (Hoier *et al*. 2013a) and sprint interval training (SIT) (Taylor *et al*. 2016a; 2016b). The increased expression of VEGF mRNA has been demonstrated between zero and six hours after the completion of exercise, with levels returning to baseline within 20 – 24 hours (Hiscock *et al*. 2003; Jensen *et al*. 2004b), and is attenuated following a period of training (Hoier *et al*. 2012; Jensen *et al*. 2004b; Richardson *et al*. 2000). In comparison to mRNA expression the muscle VEGF protein response to an acute bout of exercise and period of training has not been well elucidated. Varied results have been observed following an acute bout of submaximal continuous cycling with reports showing a decrease (Gavin *et al*. 2004) or no change in protein levels immediately (Rullman *et al*. 2007) and up to two hours post exercise (Hoier *et al*. 2013b), whereas, other studies have demonstrated an increase in protein levels 2-4 hours post exercise (Rullman *et al*. 2007; Ryan *et al*. 2006). It could therefore be speculated that an increase in content of VEGF protein typically takes longer. However, in humans no increase in basal VEGF protein content have been reported following 4-8 weeks of either continuous or HIIT (Gliemann *et al*. 2015; Hoier *et al*. 2012; Hoier *et al*. 2013a). Nevertheless, Gustafsson *et al*. (2002) did identify an increase in VEGF protein following short term (10 days) one legged knee extensor exercise. Therefore, VEGF protein levels may be upregulated in the early phase of a training period before returning to baseline as training continues, as has been reported in rodents (Olenich *et al*. 2013).

In addition to the transcriptional response, during and immediately after exercise there is also an increase of VEGF protein content in interstitial fluid (Hellesten *et al*. 2008; Hoffner *et al*. 2003; Hoier *et al*. 2010; 2012; 2013b). For muscle derived VEGF to stimulate angiogenesis, secretion into the interstitial fluid is required (Olfert *et al*. 2016). In human skeletal muscle there is localisation of VEGF in small vesicles located in subsarcolemmal regions and between the contractile elements (Hoier *et al*. 2013b). Following an exercise bout, there is an increase in these VEGF-containing vesicles close the sarcolemma. This suggests that muscle contraction stimulates these vesicles to redistribute to the sarcolemma and to secrete VEGF into the extracellular fluid (Hoier *et al*. 2013b). Muscle interstitial fluid obtained during exercise induces a several-fold greater rate of endothelial cell proliferation *in vitro* than that obtained during rest (Hoffner *et al*. 2003; Hoier *et al*. 2010; Hoier *et al*. 2013a). VEGF appears to contribute to the proliferative effect, since the extent of this effect is paralleled by the content of VEGF protein (Hoffner *et al*. 2003; Hoier *et al*. 2013a). With the increase in VEGF transcription occurring only after exercise, it appears that VEGF synthesis is not required for secretion and therefore, the transcriptional response following an exercise bout may play a primary role in replenishing lost stores (Hoier & Hellesten, 2014)

Physiological signals for angiogenesis

Several primary signals are considered to stimulate exercise induced angiogenesis, these include mechanical factors: shear stress and mechanical stretch of the tissue and reduced oxygen tension/increased metabolic activity (i.e. hypoxia) (Egginton, 2009). In an exercise model it is, however, hard to discriminate between these signals given that all are increased during exercise (Hoier & Hellsten, 2014).

Shear stress

Shear stress is the tangential frictional force exerted on the luminal side of endothelial cells as blood flows along their surface (Prior *et al.* 2004). Therefore, increased velocity of blood flow, as occurs during exercise, increases shear stress (Hudlicka & Brown, 2009). To study the effects of shear stress upon angiogenesis *in vivo* investigations have administered vasodilators, such as the α_1-adrenergic receptor blocker prazosin, or have undertaken chronic electrical stimulation in rodent models, which increase shear stress approximately three- and two-fold respectively (Hudlicka *et al*. 2006; Ziada *et al.* 1989). Utilising these models shear stress has been observed to induce increases in the expression of angiogenic factors, including VEGF, (Hudlicka *et al*. 2006; Milkiewicz *et al*. 2001; 2005; Rivilis *et al*. 2002) as well increase skeletal

muscle capillary supply (Rivilis *et al*. 2002; Williams *et al*. 2006; Ziada *et al*. 1989; Zhou *et al*. 1998).

Both nitric oxide (NO) and eNOS expression are increased in response to shear stress and are considered as important mediators of shear-induced angiogenesis (Baum *et al*. 2004; Egginton *et al*. 2016; Hudlicka & Brown, 2009). NO is thought to play an important role in mediating VEGF (Egginton, 2009). Indeed, it has been demonstrated that the administration of the nitric oxide synthase (NOS) inhibitor $_{L}$-MNNA in rats, which blocked the activity of eNOS, supressed the increased expression of VEGF and VEGFR-2 and abolished early endothelial cell proliferation and the increased capillary to fibre ratio observed in chronic electrically stimulated muscle (Milkiewicz *et al*. 2005). Furthermore, the increase in capillary supply evident in wild-type mice treated with prazosin for 14 days was abolished in eNOS knockout mice (Baum *et al*. 2004).

Studies in humans on the impact of shear stress for angiogenesis are limited. However, an increase eNOS protein has been demonstrated following 4 weeks of continuous aerobic training (Hoier *et al*. 2012) and after six weeks of SIT (Cocks *et al*. 2013). Moreover, studies have utilised a passive movement model of the lower leg, which increases blood flow to the muscle without a significant impact upon muscle activity and metabolism. An acute bout of passive exercise has been demonstrated to promote an angiogenic response, inducing an increase in interstitial VEGF protein concentration and a greater proliferative effect of interstitial fluid (Hellsten *et al*. 2008; Hoier *et al*. 2010) and moreover, 4 weeks of passive training elicited an increase in capillary contacts and proliferating endothelial cells (Hoier *et al*. 2010).

Mechanical Stretch

During muscle contraction the sarcomere length of skeletal muscle fibres is altered, and tensile forces act upon the tissue. The role of this mechanical stretch in angiogenesis has been confirmed by rodent and cells models. Stretch of endothelial cells in cell cultures elicited an upregulation of both VEGF mRNA and protein content and also increased endothelial cell migration and tube formation (Zheng *et al*. 2001). In accordance, rat muscles which were stretch-stimulated for 2 weeks also demonstrated increased VEGF protein content, endothelial cell proliferation and capillary to fibre ratios (Rivilis *et al*. 2002). Angiogenesis induced by mechanical stretch appears to occur via sprouting angiogenesis (Pior *et al*. 2004). Structural analysis revealed that the progressive increase in capillary to fibre ratio in stretch induced rat muscle was accompanied by abluminal sprouts and basement membrane breakage (Egginton

et al. 2001). Furthermore, models of mechanical stretch have also demonstrated elevated activity of MMP2 and MT1-MMP (Rivilis *et al*. 2002; Prior *et al*. 2004). Matrix MMPs act to modify the extracellular matrix and degradation of the basement membrane and are therefore important regulators of sprouting angiogenesis (Prior *et al*. 2004; Egginton, 2009; Haas *et al*. 2000; Hoier & Hellsten, 2014). Indeed, the inhibition of MMPs during a muscle stimulation model was observed to abolish endothelial cell migration (Haas *et al*. 2000). Given the difficulty in isolating muscle stretch in humans, with even passive movement models also increasing shear stress, limited evidence exists for the role of mechanical stretch for exercise induced angiogenesis in humans, however, an acute bout of exercise has been demonstrated to increase mRNA and protein levels of MMP-9 (Hoier *et al*. 2012; Rullman *et al*. 2007).

Reduced oxygen tension/increased metabolic activity

The regulation of angiogenesis by reduced oxygen tension, arising from either decreased oxygen supply or high levels of oxygen utilisation, is important to match the cardio-pulmonary-vascular oxygen supply to metabolic demand in local tissue (Hirota & Semenza, 2006). Hypoxia is considered to be one of the more potent angiogenic stimuli which appears to act primarily through the upregulation of VEGF (Egginton, 2009). Indeed, endothelial cell migration and increased VEGF mRNA are induced under exposure to hypoxic conditions, a response which is reversed upon return to normoxic conditions (Shweiki *et al*. 1992; Toffoli *et al*. 2009). The angiogenic response to reduced oxygen tension is likely mediated by the increase in hypoxia inducible factor-1 (HIF-1), in particular its subunit HIF-1α (Ameln *et al*. 2005; Toffoli *et al*. 2009). It has been demonstrated that when HIF-1α was inhibited, the increase in endothelial cell migration recorded in hypoxia was completely prevented (Toffoli *et al*. 2009). HIF-1α is highly sensitive to oxygen levels and increases exponentially as cellular oxygen concentration is reduced (Jiang *et al*. 1996). Upon activation in hypoxic conditions HIF-1α translocates to the nucleus where it then activates the expression of multiple gene targets by binding to DNA within the hypoxia response element (Ameln *et al*. 2005; Ke & Costa, 2006; Toffoli *et al*. 2009), with VEGF shown to be one of these target genes (Forsythe *et al*. 1996).

Ameln *et al*. (2005) first reported an increase in multiple factors of the HIF-1 angiogenic pathway in response to an acute exercise bout undertaken under normal oxygen delivery conditions in humans. They demonstrated that HIF-1α protein content increased immediately after 45 minutes of one-legged knee extension by 83%, which remained elevated for 6 hours, and also observed increased myonuclear staining of HIF-1α following exercise. Moreover, they

reported an increase in VEGF mRNA expression. However, HIF-1α mRNA levels were not increased at any time point following exercise. This is consistent with other investigations, demonstrating no increase in HIF-1α mRNA following 45-minutes of one-legged knee extension (Gustafsson *et al*. 1999) or SIT (Taylor *et al*. 2016a). In contrast Lundby *et al*. (2006) did report an increase in HIF-1α mRNA expression but this occurred only after 6 hours following a 3 hour two-legged knee extension exercise protocol. Therefore, it appears that under conditions of normal oxygen supply the early HIF-1α upregulation in response to exercise is not due to increased transcription.

Chronic hypoxia does not appear to promote angiogenesis in humans, with increases in capillary density only reported alongside decreased muscle fibre CSA (Hoppeler *et al*. 1990; Lundby *et al*. 2004). However, undertaking training sessions in hypoxic conditions can induce increases in capillarisation. In the study of Desplanches *et al*. (1993) participants undertook 3 weeks of continuous cycling exercise at the same relative intensity of ~75% $\dot{V}O_{2max}$ under either normoxic or hypoxic (FIO_2 = 10-12.7%) conditions. They reported a significant increase in capillary to fibre ratio following training in hypoxic conditions only. Furthermore, Geiser *et al.* (2001) also observed an increase in capillary supply following 6 weeks of continuous cycling training for 30 minutes at ~80% $\dot{V}O_{2max}$ undertaken in hypoxia, whereas there was no change after the same training regime performed in normoxia.

Role of PGC-1α

More recently, in addition to its role as a master regulator of mitochondrial biogenesis (see below), peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) has been established as an important pro-angiogenic factor (Arany *et al*. 2008; Chinsomboon *et al*. 2009; Geng *et al*. 2010; Leick *et al*. 2009; Tadaishi *et al*. 2011). Overexpression of PGC-1α has been observed to elicit increases in VEGF mRNA and VEGF protein secretion in cell cultures (Arany *et al*. 2008) and increase the basal capillary network in transgenic mice (Arany *et al*. 2008; Tadasihi *et al*. 2011). Further evidence for the importance of PGC-1α has been established from PGC-1α knockout mice. Leick *et al.* (2009) observed that the increase in VEGF mRNA and protein content following an acute exercise bout and five weeks of exercise training, respectively, was eliminated in PGC-1α knockout mice. Furthermore, the exercise induced increase in capillary supply with wheel running is abolished in knockout mice (Chinsomboon *et al*. 2009; Geng *et al*. 2010). Interestingly it was also observed that there was no difference in the capillary supply when mice did not undertake any wheel running,

demonstrating that PGC-1α is specifically involved in the angiogenic response to exercise (Chinsomboon *et al*. 2009). Fentz *et al*. (2015) also later investigated the effect of upstream 5' AMP-activated protein kinase (AMPK) knockout on VEGF signalling. They observed that the increased expression of VEGF mRNA in wild-type mice following an acute bout of exercise was abolished in AMPK knockout mice. Therefore, also implicating the importance of the AMPK signalling pathway in exercise induced angiogenesis.

Exercise modality and angiogenesis

A range of exercise training modalities are known to induce angiogenesis, including continuous moderate intensity exercise (Andersen & Henriksson, 1977; Cocks *et al*. 2013; Hoier *et al*. 2012; Hoier *et al*. 2013a; Klausen *et al*. 1981), HITT (Frandsen *et al*. 2000; Jensen *et al*. 2004a) and SIT (Bonafiglia *et al*. 2017; Cocks *et al*. 2013). Current data however, supports the importance of training volume over training intensity to promote angiogenesis in trained individuals (Gliemann *et al*. 2016). Hoier *et al*. (2013a) reported that four weeks of continuous moderate intensity (65% of $\dot{V}O_{2max}$) cycling exercise promoted an increase in capillary supply in previously untrained healthy participants. Following this initial preconditioning period, the participants then undertook four weeks of HIIT (~117% $\dot{V}O_{2max}$), which did not induce any additional increases in skeletal muscle capillarity. Furthermore, they demonstrated that an acute session of the HIIT elicited a lower increase in muscle interstitial VEGF protein concentration, and the interstitial fluid produced a lower proliferative effect than acute moderate intensity exercise (2.5- vs 6-fold increase compared to rest). Moreover, Gliemann *et al.* (2015) demonstrated that when trained runners replaced two of three weekly endurance training sessions with 10-20-30 HIIT training (which briefly involves one-minute intervals of 30, 20 and 10 s at an intensity of ~30, 60 and 90-100% of maximal running speed), thereby reducing their total training volume for a period of 8 weeks, muscle VEGF protein concentration was decreased by 22% and there was no increase in capillarisation.

Mitochondrial biogenesis

It is well-known that regular endurance training, at a sufficient intensity and frequency, can induce an increase in mitochondrial content in less than a week (Egan *et al*. 2013; Perry *et al*. 2010) and increases can range from ~50-100% within 6 weeks (Hood, 2001). The process by which there is an increase in the mitochondrial reticulum is referred to as mitochondrial biogenesis (Hood, 2009). The increase in mitochondria does not happen *de novo*, rather new proteins are recruited into the organelle, which subsequently divides by fission (Ryan & Hoogenraad, 2007). Although the majority of DNA is located within the nucleus, mitochondria also possess multiple copies of their own circular DNA, typically referred to as mitochondrial DNA (mtDNA). mtDNA codes for 13 proteins, 22 transfer RNA and 2 ribosomal RNA which are essential for protein synthesis within the mitochondria. Therefore, mitochondrial biogenesis requires the coordination of both nuclear and mitochondrial genomes (Hood *et al*. 2006; Hood, 2001). In addition to an increase in mitochondrial content, mitochondrial biogenesis can also be associated with a change in mitochondrial function (Bishop *et al*. 2014) i.e. the ability of the mitochondria to maintain cellular energetic homeostasis (Johnson *et al*. 2013), which is also an important determinant for endurance performance (Jacobs *et al*. 2011).

At the molecular level the process of exercise-induced mitochondrial biogenesis is initiated by perturbations to homeostasis at the onset of activity, including; increased AMP:ATP ratio, mechanical stretch, calcium release and production of ROS (Coffey & Hawley, 2007; Hood, 2009). These putative signals subsequently activate kinases (such as AMPK, p38 mitogen-activated protein kinases (p38 MAPK) and Ca^{2+}/Calmodulin-dependent kinases II (CaMKII) which in turn phosphorylate downstream targets such as transcription factors or transcriptional coactivators which are involved in the regulation of DNA transcription (Hood, 2009). These events trigger a transient increase in mRNA of these sensor enzymes and downstream proteins (Hood *et al*. 2006; Ljubicic *et al*. 2010). Translation then occurs, and the subsequent precursor proteins formed are imported into the mitochondria (Hood, 2001; Ljubicic *et al*. 2010). These proteins undergo post translational modifications and become functionally active and act as metabolic enzymes (such as those of the Krebs cycle), form part of electron transport chain complexes or serve as transcription factors for mtDNA-encoded proteins (Hood, 2009). mtDNA transcription and translation subsequently increases, and the resulting mitochondrial encoded proteins combine with nuclear derived proteins to form multi-subunit complexes of the electron transport chain (Hood, 2009).

PGC-1α is considered as the 'master regulator' of mitochondrial biogenesis (Wu *et al*. 1999; Puigserver & Spiegelman, 2003). PGC-1α is a transcriptional coactivator, it therefore lacks the ability to bind to DNA, but acts by binding to and co-activating DNA binding transcription factors (Ljubicic *et al*. 2010). Of importance to mitochondrial biogenesis is the interaction of PGC-1α with the nuclear respiratory factors NRF-1 and NRF-2 (Hood, 2009), which mediate the expression of multiple nuclear encoded proteins, including cytochrome c components of the electron transport chain complexes and mitochondrial import machinery (Hood, 2009; Ljubicic *et al*. 2010). The nuclear respiratory factors, in particular NRF-1, also activate transcription factors of mtDNA, such as mitochondrial transcription factor (Tfam) (Virbasius & Scarpulla, 1994; Wu *et al*. 1999). Tfam is a nuclear encoded protein which, upon import into the mitochondria, binds to the D-loop region of mtDNA and upregulates mtDNA transcription and replication (Hood *et al*. 2006; Ljubicic *et al*. 2010). Through this NRF-1: Tfam axis PGC-1α thus also activates mtDNA transcription. In addition, PGC-1α has also been demonstrated to translocate into mitochondria (Safdar *et al*. 2011; Smith *et al*. 2013) and form a complex with Tfam in the mtDNA D-loop region (Safdar *et al*. 2011) suggesting that PGC-1α may also play a more direct role in upregulating the mitochondrial genome. Therefore, through these mechanisms PGC-1α is able to coordinate both nuclear and mitochondrial gene expression.

The potency of PGC-1α to induce mitochondrial biogenesis has been demonstrated by PGC-1α overexpression in cells and transgenic mice. For example, overexpression of PGC-1α in myotubes from C2C12 muscle cells increased the mRNA content of electron transport proteins cytochrome c oxidase subunits II (COXII) and IV (COXIV) and cytochrome c and transcription factors NRF-1 and Tfam and increased mitochondrial density by 57% (Wu *et al*. 1999). Transgenic mice expressing upregulated skeletal muscle PGC-1α, also have enhanced exercise capacity, mtDNA and citrate synthase (CS) activity than wild-type mice (Lin *et al*. 2002; Calvo *et al*. 2008). Furthermore, PGC-1α knockout mice possess reduced mitochondrial enzyme content, mitochondrial densities, exercise capacity and $\dot{V}O_{2max}$ than their wild-type counterparts (Adhihetty *et al*. 2009; Leick *et al*. 2008; Leone *et al*. 2005). Although the evidence above clearly highlights the importance of PGC-1α for regulating and optimising mitochondrial adaptations, there appears to be some redundancy in the pathways and PGC-1α is not essential for exercise induced mitochondrial biogenesis. Indeed, PGC-1α knockout mice still demonstrated training induced increases in mRNA and protein content of mitochondrial enzymes (Leick *et al*. 2008) and mitochondrial content (Adhihetty *et al*. 2009; Rowe *et al*. 2012).

PGC-1α mRNA expression is upregulated in response to multiple training modalities; studies have reported increased expression following two-legged knee extension exercise (Pilegaard *et al*. 2003), continuous exercise (Bartlett *et al*. 2012; Coffey *et al*. 2006; Egan *et al*. 2010; Mathai *et al*. 2008; Pilegaard *et al*. 2005), HIIT (Bartlett *et al*. 2012; Nordsborg *et al*. 2010; Perry *et al*. 2010) and SIT (Gibala *et al*. 2009; Little *et al*. 2011; Niklas *et al*. 2010; Taylor *et al*. 2016a). Taylor *et al*. (2016b) even observed that just 2 minutes of 'all-out' continuous cycling was sufficient to elicit an increase in PGC-1α mRNA after 3 hours by 5.5. fold. PGC-1α mRNA levels appear to peak between 2 and 5 hours post exercise and return to baseline within 24 hours (Little *et al*. 2011; Mathai *et al.* 2008; Nordsborg *et al*. 2010 Pilegaard *et al.* 2003; 2005). Current evidence suggests PGC-1α mRNA may be increased in an intensity dependent manner (Egan *et al*. 2010; Popov *et al*. 2014), at least at submaximal intensities; Edgett *et al.* (2013) compared work matched HIIT at 73%, 100% and 133% of peak work rate and observed the greatest increase in PGC-1α mRNA at 100%, leading them to conclude that supramaximal exercise may not elicit any additional increases. Although typically unchanged within in the first hours following an acute exercise bout (Bartlett *et al*. 2012; Gibala *et al*. 2009; Little *et al*. 2010a; Little *et al*. 2011; Perry *et al*. 2010), increases in protein content of ~20-60% have consistently been reported 24 hours following an acute bout of exercise (Egan *et al*. 2013; Little *et al*. 2011; Mathai *et al.* 2008; Perry *et al*. 2010). PGC-1α protein has also been demonstrated to translocate to the cell nucleus and to subsarcolemmal mitochondria following an acute exercise bout (Little *et al*. 2010a; Little *et al*. 2011; Smith *et al*. 2013). It has been suggested that it is this rapid activation and translocation of PGC-1α, as opposed to increased protein content, that enables the initial rapid stimulation of mitochondrial biogenesis (Little *et al*. 2010a; Wright *et al*. 2007b). This is supported by the observation that several mitochondrial constituents were increased prior to any changes in PGC-1α protein content (Wright *et al*. 2007b).

In addition to the acute response, PGC-1α protein content has been reported to increase by ~16 to 90% following 12 days to 6 weeks of training, ranging from continuous exercise to SIT (Burgomaster *et al*. 2008; Egan *et al*. 2013; Granata *et al*. 2016; Gurd *et al*. 2010; Perry *et al*. 2010; Russell *et al*. 2003; Stepto *et al*. 2012). Some studies have not observed changes in PGC-1α protein content following an endurance training period (Edgett *et al*. 2016; Granata *et al*. 2016; Little *et al*. 2010b; Vincent *et al*. 2015). This variation in response is likely attributable at least in part to the training methodologies selected. For example, Granata *et al*. (2016) investigated the effect of 3 different training modalities; including sub-lactate threshold

continuous cycling, HIIT and SIT. They observed an increase in PGC-1α protein content only following SIT, suggesting training intensity may be an important factor. However, Burgomaster *et al*. (2008) reported a similar increase in PGC-1α protein following both SIT and continuous cycling exercise at 65% $\dot{V}O_{2max}$. This continuous exercise was undertaken at a similar exercise intensity as that in the study of Granata *et al*. (2016) but was continued for longer (40-60 min vs 20-36 min), it could therefore be suggested that volume of training in both the continuous and HIIT groups (which were matched for total work done) in the study of Granata *et al*. (2016) were too low to induce an increase in PGC-1α protein content.

Physiological signals of mitochondrial biogenesis

As mentioned above multiple perturbations to homeostasis in response to muscular contraction have been evidenced to activate signalling cascades which initiate mitochondrial biogenesis.

AMP:ATP Ratio and AMPK

Any cellular stress which either sufficiently raises ATP consumption or reduces ATP re-synthesis, e.g. muscle contraction at the onset of exercise, results in an increase in the AMP:ATP ratio (Coffey & Hawley, 2007). This is detected by a increase in AMPK, which is often referred to as an energy sensing enzyme (Hood, 2009). AMPK is activated in three ways: through allosteric activation, increasing the binding of upstream kinases and increased phosphorylation by inhibiting the dephosphorylation of the 172-threonine residue (Thr172), whilst high concentrations of ATP reduces this activation (Hardie *et al*. 2004a; 2004b). AMPK activation and phosphorylation are increased immediately following exercise in an intensity-dependent manner (Egan *et al*. 2010; Wojtaszewski *et al*. 2000), returning within minutes to near resting values (Rasmussen *et al*. 1998). In addition to regulating many of the acute metabolic responses that occur in response to a bout of exercise, AMPK also plays a role in stimulating mitochondrial biogenesis by altering gene expression and transcriptional regulation largely through the activation of PGC-1α (Canto & Auwerx, 2010). AMPK enhances PGC-1α promotor activity (Irrcher *et al*. 2008) and activates PGC-1α through the direct phosphorylation of PGC-1α at threonine-177 and serine-538 (Jager *et al*. 2007). In support of the role of AMPK activation for mitochondrial biogenesis, studies have observed that the direct activation of AMPK in rodent models leads to an increase in cytochrome-c protein content, NRF-1 DNA binding and mitochondrial densities (Bergeron *et al*. 2001; Zong *et al*. 2002). These increases and an increase in PGC-1α mRNA expression were not evident when the same treatment was undertaken upon mice with dominant negative AMPK (Zong *et al*. 2002).

ROS and p38 MAPK

During exercise ROS are produced from mitochondria, where a small fraction of oxygen undergoes a one-electron reduction (Ljubicic *et al*. 2010) and from non-mitochondrial sources, which include xanthine oxidases and NADPH oxidases (Merry & Ristow, 2016). Although significant focus has been placed around tissue damage resulting from ROS production, a growing body of literature has emerged supporting the role of ROS in activating signalling pathways involved in mitochondrial biogenesis (Camera *et al*. 2016; Hood, 2009). For example, chemically induced increases in ROS in myoblasts lead to increased PGC-1α promoter activity and mRNA, a response which is blocked with the addition of antioxidants (Irrcher *et al*. 2009). Although a direct link between ROS production during exercise and mitochondrial biogenic response has yet to be made in human skeletal muscle (Petrick *et al.* 2018), it has been demonstrated that antioxidant supplementation can interfere with key mitochondrial signalling intermediates including p38 MAPK and AMPK in rodents (Merry & Rostow, 2016; Wadley *et al*. 2013) and can attenuate the increase in PGC-1α mRNA and protein and COXIV protein following a period of training in humans (Paulsen *et al*. 2014; Ristow *et al* 2009). p38 MAPK appears to play a role in linking cellular ROS levels and skeletal muscle mitochondrial biogenesis, with phosphorylation of p38 MAPK increased in response to ROS (Egan & Zierath, 2013; Powers *et al*. 2010). p38 MAPK is an important signalling protein in PGC-1α regulated mitochondrial biogenesis. In cell cultures p38 MAPK induces PGC-1α transcription (Akimoto *et al*. 2005) through direct phosphorylation (Puigserver *et al*. 2001). Increases in phosphorylation of p38 MAPK alongside increased PGC-1α mRNA and PGC-1α nuclear abundance have also been reported following an acute exercise bout in humans (Bartlett *et al*. 2012; Little *et al*. 2010a; Little *et al*. 2011). In addition to p38 MAPK, AMPK has further been implicated in regulating the effect of ROS given that AMPK activation and phosphorylation were increased in cell cultures following treatment with ROS. This increase in AMPK may be elicited due to the ROS induced decrease in ATP levels (Irrcher *et al*. 2009).

Calcium Release and CAMKII

Neural activation of skeletal muscle elicits an action potential which leads to the transient release of Ca^{2+} from the sarcoplasmic reticulum (Coffey & Hawley, 2007). These calcium oscillations during muscle contraction appear to act as a potent primary signal which can be translated into a transcriptional response leading to mitochondrial biogenesis. This is evidenced by studies utilising cultured myocytes, which have demonstrated that elevated Ca^{2+} leads to

increased expression of mRNA and protein content of mitochondrial enzymes and key mitochondrial biogenesis transcription factors such as PGC-1α, Tfam, NRF-1 and NRF-2 (Chin, 2005; Ojuka *et al*. 2002). At the onset of elevations in intracellular Ca^{2+} calmodulin (CaM), an intermediate binding protein, binds with Ca^{2+} (Chin, 2005), this complex then acts on downstream targets which includes the Ca^{2+}/CaM-dependent phosphatase calcineurin and a family of CaM dependent kinases (CaMK's) (Chin, 2010), of which CaMKII is the dominant isoform in skeletal muscle (Rose *et al.* 2006). On elevation of Ca^{2+}, the Ca^{2+}/calmodulin complex binds with CaMKII which initiates intramolecular autophosphorylation, this results in the maintenance of the increase in the kinase activity when Ca^{2+} returns to basal levels (Chin, 2005). CaMKII appears to elicit mitochondrial biogenesis in part via the upregulation of PGC-1α through the phosphorylation of p38 MAPK; the inhibition of CaMKII and p38 MAPK blocked the increased expression of key transcription factors (PGC-1α, Tfam, NRF-1 and NRF-2) and PGC-1α and COXI respectively, induced by raising Ca^{2+} (Wright *et al*. 2007a). Furthermore, CaMKII can also act to upregulate mitochondrial biogenesis by dissociating myocyte enhancer factor-2 (MEF2) from histone deacetylase-4 (HDAC4), allowing MEF2 to undertake its transcriptional activities on downstream targets, which includes PGC-1α (Chin, 2005; Liu *et al*. 2005; Egan & Zierath, 2013).

Exercise modality and mitochondrial biogenesis

Multiple training modalities have been shown to induce increases in a range of markers of mitochondrial content (Hoppeler *et al*. 1985; Gibala *et al*. 2006; Perry *et al*. 2010; Shepherd *et al*. 2013). Granata *et al*. (2018) compiled data from available cycling training studies that assessed CS activity (a valid marker of mitochondrial content) to investigate the effect of intensity and training volume on the magnitude of adaptations. They reported that there was no relationship between relative exercise training intensity and CS activity. In agreement with this, studies directly comparing SIT to continuous cycle training at ~65% $\dot{V}O_{2max}$ reported similar increases in the maximal activity of COX and protein content of COX and COX subunits II and IV (Gibala *et al*. 2006; Shepherd *et al*. 2013). Granata *et al*. (2018) did however observe a correlation of $r = 0.59$ between changes in CS activity and training volumes and when studies employing SIT (which demonstrated large variability in changes in CS) were removed this correlation was increased to $r = 0.71$. This led the authors to conclude that training volume was an important determinant of training induced increases in mitochondrial content when relative exercise intensities are ≤ 100% of maximal aerobic power (MAP). Although fewer studies were available, they did report relative training intensity to be an important determinant of training

induced increases in mitochondrial function, with the highest magnitude of increases occurring when at least one third of training was undertaken at an intensity higher than 90% MAP. Interestingly, however, they did report a reduced increase in mitochondrial function when SIT was undertaken alone, leading to the suggestion that this could be attributed to the significantly reduced training volume.

2.3 REDUCED PLASTICITY OF THE TRAINED MUSCLE

So far, this literature review has outlined skeletal muscle angiogenesis and mitochondrial biogenesis as peripheral adaptations that are key to optimising endurance performance, and has overviewed the key signalling and molecular pathways eliciting these adaptations. Despite the adaptability of skeletal muscle described above, it is generally accepted that eliciting further adaptations in well-trained individuals, who through continued years of high volumes of training possess well adapted physiology for competition, becomes challenging.

Early research established the reduced plasticity of trained individuals. Hoppler *et al*. (1985) demonstrated that the magnitude of increases in maximal power individuals could maintain for 30 minutes gradually decreased as training progressed during a six-week period. Moreover, Saltin *et al*. (1977) combined data from longitudinal and cross-sectional studies to demonstrate that the large increases elicited during the first few weeks of endurance training in both $\dot{V}O_{2max}$, and skeletal muscle adaptations, including capillarity and oxidative enzymes, are progressively attenuated as training continues for several months. More recently HIIT, which is a potent method to enhance both oxidative and glycolytic enzyme activity in untrained and recreationally active individuals (Laursen & Jenkins, 2002), was demonstrated not to increase the activity of CS or phosphofructokinase in trained cyclists who had a $\dot{V}O_{2max}$ greater than 60 $ml.min^{-1}.kg^{-1}$ (Weston *et al*. 1997). Furthermore, a body of literature exists demonstrating that an increase in traditional endurance training volume alone appears to be insufficient to further enhance aerobic performance or associated physiological determinants in endurance trained individuals (Costill *et al*. 1988; Daniels *et al*. 1978; Londeree, 1997).

This blunting of the adaptive scope in trained individuals is also reflected at the molecular level. The expression of the mitochondrial transcript in response to an acute exercise bout was observed to be blunted following six weeks of endurance training in untrained individuals (Schmutz *et al.* 2006). Moreover, just 10 days of endurance training in previously sedentary individuals was enough to abolish a ~10-fold increase in AMPK activity during 120 min of cycling at ~66% $\dot{V}O_{2max}$ (McConell *et al*. 2005). Perry *et al*. (2010) later investigated the time course of the mitochondrial signalling response over 14 days of a HIIT protocol in recreationally active individuals. They observed a continual reduction in the magnitude of PGC-1α mRNA following each subsequent training session, despite an increase in the absolute training intensity (Fig. 2.2). Whilst PGC-1α protein content was increased relative to the pre-training value throughout the training period, this peaked following 10 days and although not

significantly different slightly decreased after 15 days of training. A blunted molecular response has also been observed in angiogenic factors, with the transient expression of VEGF mRNA following an acute exercise bout reduced after a period of 4-8 weeks of endurance training (Richardson *et al*. 2000; Hoier *et al*. 2012).

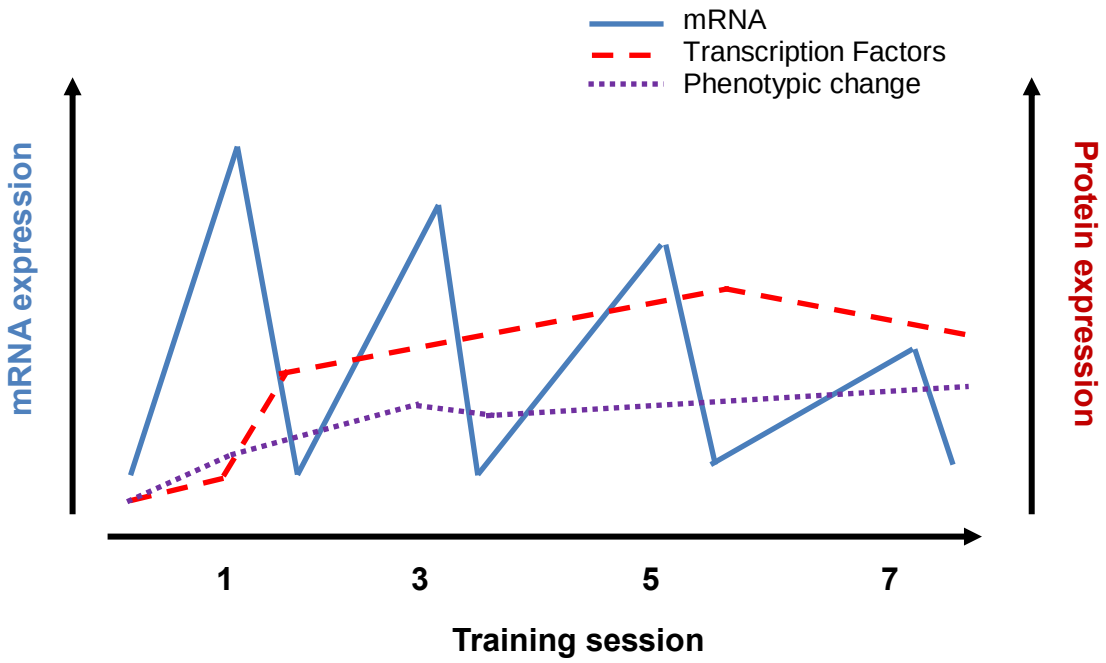

Figure 2.2. Schematic of the blunted molecular response with continued training. Adapted from Perry *et al.* (2010), with data specific to PGC-1α mRNA and protein content and CS activity.

In accordance with the blunted molecular response, structural constraints may impose limitations on the myocellular adaptive responses. This is supported by the inverse relationship observed between mitochondrial content and the expressional response of mitochondrial and angiogenic transcripts; specifically, inverse relationships have been observed between mitochondrial content and basal levels of phosphorylation of MAPKs including p38 MAPK (Flueck, 2010). As such individuals with the highest mitochondrial densities display diminished regulation of functionally implicated gene transcripts during recovery from an exercise stimulus (Flueck, 2010). This has also been demonstrated in a desensitisation of trained individuals to primary training signals; highly trained endurance athletes were observed

to display a smaller increase in shear stress-dependent blood flow in comparison with habitually active individuals in response to passive movement (Hellsten *et al*. 2015).

Therefore, this body of literature highlights the need to identify effective novel training methods for trained athletes, who are well-accustomed to high volumes of training over a range of exercise intensities, that are able to augment the primary signals elicited in response to exercise outlined in the previous section, and overcome the blunted molecular response.

2.4 TRAINING WITH BLOOD FLOW RESTRICTION (BFR)

BFR has emerged in recent literature as a popular intervention to manipulate the training stimulus and potentially enhance performance. This training strategy is typically achieved through the application of an inflatable cuff (Abe *et al*. 2006), tourniquet (Shinohara *et al*. 1998) or elastic banding (Loenneke *et al*. 2010) proximal to the working muscle, restricting blood flow into and occluding blood flow out of the muscle (Takano *et al*. 2005).

Commonly, the application of BFR has been applied during low load (< 50% of one repetition maximum [RM]) resistance exercise. In order to elicit strength gains and promote hypertrophy, it is generally recommended that resistance exercise with loads of at least 60% of 1-RM are required (ACSM, 2009; Kraemer & Ratamess, 2004). However, it has been consistently demonstrated that the addition of BFR to low load resistance exercise can; induce strength gains (Madarame *et al*. 2008; Moore *et al*. 2004; Patterson & Ferguson, 2010; Takarada *et al.* 2000, 2002; Yamanaka *et al*. 2012) promote hypertrophy (Manimmanakorn *et al*. 2013; Takarada *et al*. 2000; 2002) and increase muscle endurance (Manimmanakorn *et al.* 2013; Takarada et al. 2002). The use of this training modality has typically been advocated for populations where high mechanical loads may not be feasible or contraindicated, including the frail and elderly (Abe *et al*. 2010b; Patterson & Ferguson, 2010) and rehabilitation patients (Leonneke *et al*. 2013), however, its efficacy has also been supported for athletes with a history of resistance training (Takarada *et al*. 2002).

BFR in endurance training

In addition to its use within resistance exercise, research has also started to explore the use of BFR to enhance the adaptations achieved through aerobic training. Early studies utilised pressure chambers during one-legged cycling exercise (Kaijser *et al*. 1990; Sundberg *et al*. 1993). In these studies participants undertook four training sessions a week, for a period of four weeks at an intensity of ~50% $\dot{V}O_{2max}$ for 45 minutes. One leg was trained under normal atmospheric conditions, and the other with a pressure set to 50 mmHg above atmospheric pressure, inducing a moderate BFR of ~20%. They reported a greater increase in exercise capacity, $\dot{V}O_{2max}$ and CS activity in the leg trained under BFR in comparison to the leg trained with a normal blood supply (Kaijser *et al*. 1990; Sundberg *et al*. 1993).

More recent studies have investigated the potential of BFR, achieved through the application of inflatable cuffs during endurance-type exercise. These studies have provided support that

BFR can enhance endurance adaptations to submaximal exercise training in recreationally active individuals. Indeed, investigations have demonstrated that the addition of BFR at a pressure of 160-210 mmHg during low intensity cycling or walk training (~40% $\dot{V}O_{2max}$) can induce increases in $\dot{V}O_{2max}$ and exercise capacity, which were not observed after the same intensity of training performed alone (Abe *et al*. 2010a; Park *et al*. 2010). This was even observed when BFR exercise was maintained for only 15 minutes compared to 45 minutes in the exercise alone condition (Abe *et al*. 2010a). Paton *et al* (2017) reported similar findings during HIIT, whereby BFR was applied during repeated 30 s efforts at 80% of peak running velocity and induced small but potentially worthwhile greater improvements in $\dot{V}O_{2max}$ and exercise capacity than exercise alone. Furthermore, de Oliveira *et al* (2016) demonstrated that the addition of BFR during low intensity interval cycle exercise (30% of peak power) induced similar improvements in the onset of blood lactate accumulation, $\dot{V}O_{2max}$ and peak power as the same protocol performed at 95-110% of peak power under normal blood flow conditions. In contrast, other studies have found that BFR applied during both low intensity cycle training and HIIT induced no benefits to aerobic parameters (Keramidas *et al*. 2012; Kim *et al.* 2016). The lack of effect could, however, be attributable to the methodology employed whereby Keramidas *et al.* (2012) utilised a pressure of 90 mmHg above atmospheric pressure, resulting in a pressure <110 mmHg, which is well below the 160-210 mmHg utilised in the aforementioned studies. Furthermore, Kim *et al.* (2016) utilised heart rate reserve to set the intensity for their investigation, the control group exercised at 60-70% and the BFR at 30% of heart rate reserve. Due to the increased heart rate with the application of BFR, this resulted in the BFR group exercising at a lower relative intensity as demonstrated by a reduced RPE compared to the control group. Therefore, these studies may not have elicited a sufficient training/restriction stimulus to induce adaptations.

2.5 PHYSIOLOGICAL SIGNALS FROM BFR

As summarised in Figure 2.3, BFR could be hypothesised to elicit both angiogenesis and mitochondrial biogenesis through the manipulation of multiple primary training signals and the activation of the downstream signalling pathways.

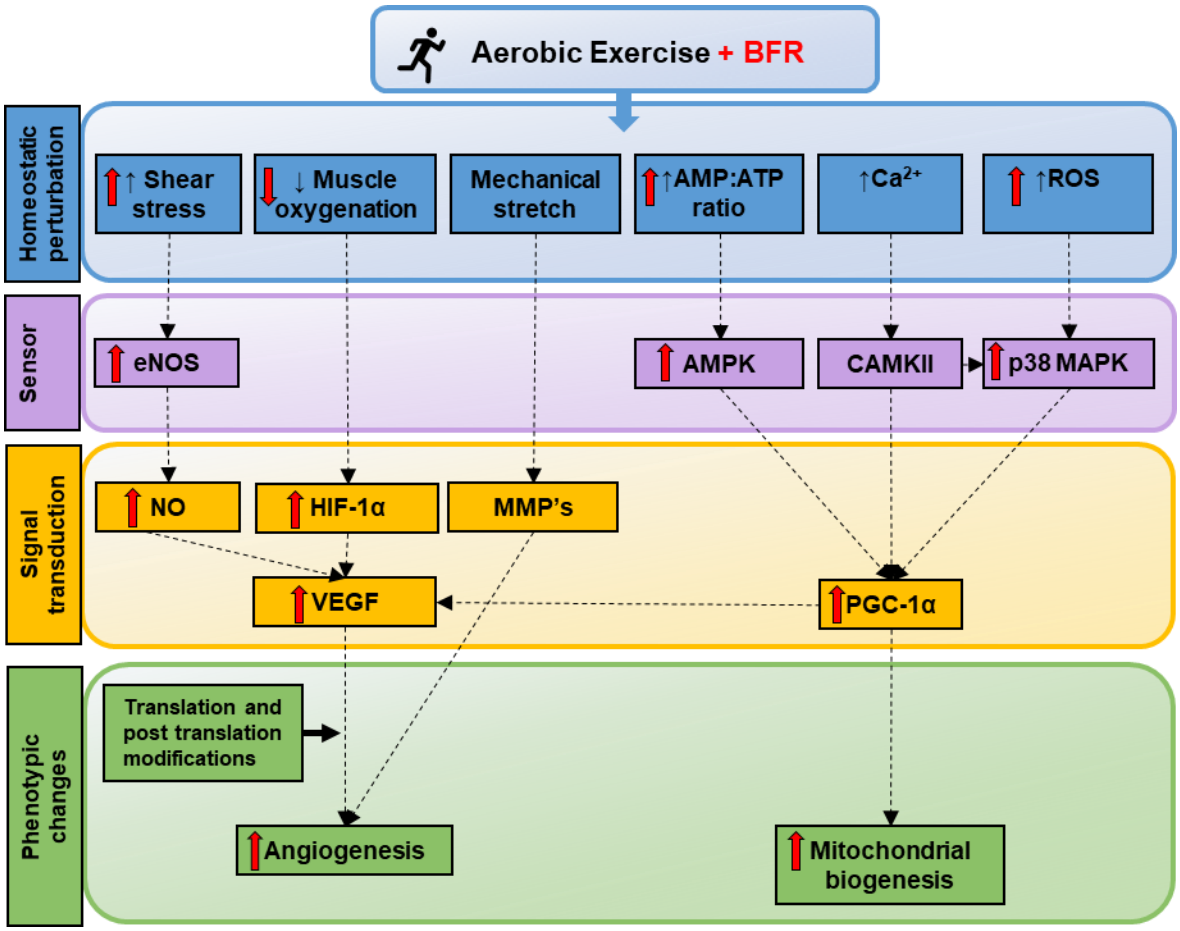

Figure 2.3. Hypothesised effects of blood flow restriction (BFR) (demonstrated by red arrows) on the signalling pathways of angiogenesis and mitochondrial biogenesis

Haemodynamic Response –shear stress

The compression of blood vessels induced by the application of BFR, results in a distorted haemodynamic profile immediately distal to the area of compression with the induction of reciprocating shear stress (Chiu & Chien, 2011). At rest, the application of BFR by an inflatable cuff increases retrograde shear rate in the absence of changes to antegrade shear rate (Credeur *et al*. 2010; Jenkins *et al*. 2013; Thijssen *et al*. 2009), which increases in a dose-dependent

response to the level of pressure applied (Thijssen *et al*. 2009). Although this isolated increase in retrograde shear rate has been shown to impair endothelial function (Thijssen *et al*. 2009), the increase in retrograde shear rate evident during BFR combined with low load exercise is also accompanied by an increase in antegrade shear rate (Credeur *et al*. 2010). This oscillatory pattern of increased antegrade and retrograde flow has been suggested to elicit increased shear stress-mediated endothelial NO production (Green *et al*. 2005).

Furthermore, upon cuff deflation following low intensity BFR exercise vessels are further exposed to reactive hyperaemia, with post-exercise blood flow shown to be higher than that of low intensity exercise undertaken alone (Paiva *et al*. 2016; Takarada *et al*. 2000). This response has been demonstrated from pressures as low as 100 mmHg and an increase in blood flow can be maintained up to one-hour post deflation (Gundermann *et al*. 2012; Takarada *et al*. 2000). Therefore, this suggests the induction of a sustained shear stimulus following BFR exercise.

Muscle oxygenation

The application of BFR reduces muscle oxygen saturation in a dose-response manner with the level of restriction pressure applied at rest (Karabulut *et al*. 2011). Accordingly, multiple studies have observed reduced muscle oxygenation during low load resistance exercise or low intensity cycling combined with BFR to levels equal to, or even lower than that achieved during higher load intensity exercise under normal blood flow conditions (Corvino *et al*. 2017; Downs *et al*. 2014; Ganesan *et al*. 2015; Tanimoto *et al*. 2005). For example, Downs *et al*. (2014) observed that leg press exercise performed to task failure at 20% and 80% of 1-RM reduced thigh muscle oxygen saturation by 35% and 20% respectively, when BFR was applied during the same exercise at 20% of 1-RM muscle oxygen saturation was reduced by approximately 50%. This state of hypoxia is also sustained during the rest intervals if the restriction is maintained (Downs *et a*l. 2014; Ganesan *et al*. 2015).

Consequently, studies have assessed the effect of BFR upon the acute transcriptional response of HIF-1α mRNA. HIF-1α mRNA has been demonstrated to be upregulated following an acute bout of low load (20-40% 1 rep max) knee extensions when combined with BFR, with no change in HIF-1α mRNA when exercise was performed alone (Ferguson *et al*. 2018; Larkin *et al*. 2012) Similarly, Taylor *et al*. (2016a) reported an increase in HIF-1α mRNA 3 hours after a single session of SIT only when BFR was undertaken during the recovery intervals.

Metabolic stress

BFR can also increase the metabolic stress imposed by an exercise bout. Indeed, increased lactate accumulation has been observed when BFR was added to low load resistance exercise (20-40% 1RM) in comparison to the same exercise undertaken alone (Fujita *et al*. 2007; Poton & Polito, 2015; Takano *et al*. 2005; Takarada *et al*. 2000). Further investigations have also demonstrated greater decreases in pH and phosphocreatine (PCr) and increase in Pi with the addition of BFR (Suga *et al*. 2009; 2012). These increased metabolic perturbations have further been demonstrated to be to the same magnitude as induced by higher load exercise (65-80% 1RM) (Neto *et al*. 2017; Poton & Polito, 2015; Reeves *et al*. 2006; Suga *et al*. 2012). Under such conditions it could be hypothesised that there would be an increase in AMPK and its associated downstream signalling.

ROS

Both exposure to hypoxia (Clanton, 2007) and reperfusion (Granger and Kvietys, 2015) can increase ROS. On this basis it could therefore by hypothesised that BFR would provide a sufficient stimulus to increase ROS and enhance the associated downstream mitochondrial biogenic signalling. In support of this hypothesis Christiansen *et al.* (2018) observed that the addition of BFR during the work periods of a HIIT running protocol, where BFR was removed during the rest phases to allow multiple instances of reperfusion, elicited increases in HSP27 protein, and *catalase* and HSP70 mRNA, markers of the occurrence of oxidative stress. The addition of BFR also led to the increased expression of mRNA of PGC-1α isoforms PGC-1α1 and PGC-1α4 which was associated with the markers of oxidative stress.

Current signalling evidence

In support of the above hypothesised mechanisms several studies have reported enhanced acute angiogenic and mitochondrial signalling responses when BFR was undertaken during a range of exercise modalities. Within the study of Larkin *et al*. (2012) participants undertook 2 sessions of unilateral knee extensions at 40% of 1-RM with and without BFR at a pressure of 220 mmHg. They observed the addition of BFR enhanced mRNA expressions of VEGF, VEGFR-2, HIF-1α, nNOS and iNOS at 2 and 4 hours post exercise. In a similar design Ferguson *et al.* (2018) additionally observed enhanced mRNA expression of PGC-1α and eNOS when BFR at 110 mmHg was added to bilateral knee extension performed at 20% of 1RM. Furthermore, Taylor *et al*. (2016a) demonstrated enhanced HIF-1α mRNA expression

following a typical SIT session only when combined with post-exercise BFR, however, they did not report any enhancements in either PGC-1α, VEGF or eNOS mRNA expression 3 hours post-exercise with the addition of BFR. Christiansen *et al*. (2018) further investigated the addition of BFR to intermittent running training, undertaken at an intensity of 105% of the lactate threshold. They observed that BFR enhanced mRNA expression of PGC-1α and also the Na^+, K^+-ATPase complex subunit ancillary protein phospholemman-1 (FXYD1). In an interesting design, participants also undertook an additional trial under hypoxic conditions, whereby the level of muscle hypoxia, as assessed by near-infrared spectroscopy, was matched to the BFR condition. In this group there was no increase in either PGC-1α or FXYD1 signalling, suggesting that this enhanced signalling was not related to hypoxia or CaMKII signalling. However, the enhanced signalling with BFR was associated with fibre type-specific AMPK downstream signalling and markers of oxidative stress.

Moreover, the angiogenic potential of the addition of BFR to low load resistance training has further been inferred with studies reporting increased calf filtration capacity, an indirect measure of capillarisation following 4-6 weeks of training (Evans *et al*. 2010; Hunt *et al*. 2013).

2.6 POST-EXERCISE BFR

The ability of BFR to manipulate the training stimulus to submaximal intensity exercise could have useful applications during periods of rehabilitation and to control the training load of athletes. Nevertheless, maintaining a high training intensity is important to optimise adaptations and endurance performance in trained individuals (Laursen *et al*. 2002; 2005; Mujika, 2010) and therefore the application of BFR as an additional stimulus whilst maintaining the 'typical' intensity of training protocols is arguably of more interest to well-trained endurance athletes. In a novel approach Taylor *et al*. (2016a) investigated the use of post-exercise BFR during a typical SIT program, with the aim to add a BFR stimulus in the absence of a reduction in training intensity. 20 trained cyclists undertook 4 weeks of progressive SIT, involving two sessions a week of 4-7 maximal 30 s sprints interspersed with 4.5 min recovery, either alone or with post-exercise BFR. In the control group the cyclists remained stationary on the bike during the recovery period. In the BFR group participants dismounted the bike and lay in a semi-supine position on a couch and BFR was rapidly applied for 2 min at ~130 mmHg, which was ascertained as the highest tolerable pressure. After the 4 weeks of training there was a potent increase in $\dot{V}O_{2max}$ of 4.5% in the trained cyclists ($\dot{V}O_{2max}$ = 61.2 ± 4.0 $ml.min^{-1}.kg^{-1}$) in the BFR group, whereas there was no change in the control group. However, there was no change in MAP or performance during a 15 km time trial. Although further investigation is required to establish if post-exercise BFR can elicit improvements in exercise performance, this study clearly highlights the potential potency of post-exercise BFR as an additional training stimulus for endurance athletes.

2.7 THE CRITICAL POWER CONCEPT

The critical power (CP) concept was first introduced by Monod and Scherrer (1965) and was used to describe the hyperbolic relationship between work rate and time to exhaustion in a single muscle group. They determined two parameters that would denote this hyperbolic relationship; the power asymptote, CP and a finite amount of work which can be completed above CP, i.e. the curvature constant, which was later termed *W′* (Equation 1). This concept has later been demonstrated to apply during whole body exercise including cycling (Moritani *et al*. 1981), running (Hughson *et al*. 1984) and swimming (Wakayoshi *et al*. 1992). Together, CP and *W′* describe cycling performance within the severe-intensity domain (equation 1) and are therefore important performance parameters (Vanhatalo *et al*. 2011a).

$$t = W' / (P - CP) \qquad (1)$$

CP was originally proposed to represent the power associated with the highest rate of aerobic energy supply that can be sustained for a prolonged time in the absence of fatigue (Monod & Scherrer, 1965; Moritani *et al*. 1981), typically around 30 min (Brickley *et al*. 2002). CP has subsequently been established as the demarcation between the heavy- and severe-intensity exercise domains (Jones *et al*. 2008; Poole *et al*. 1988; Whipp, 1996). Below CP, i.e. within the heavy-intensity exercise domain, $\dot{V}O_2$, intramuscular substrates (PCr) and metabolites (e.g. Pi and H^+) achieve submaximal steady state values. In contrast during severe-intensity exercise, i.e. above CP, pulmonary $\dot{V}O_2$, intramuscular substrates and metabolites fail to reach a steady state and continue to increase/decrease until they attain their respective maxima/minma and task failure ensues (Jones *et al*. 2008; Poole *et al*. 1988; Vanhatalo *et al*. 2016). CP is therefore considered to reflect the greatest sustainable rate of oxidative metabolism in the absence of a progressive loss of muscle metabolic homeostasis (Jones *et al*. 2010).

CP has been well established to be a parameter of aerobic function. For example, CP has been observed to be negatively correlated with the fundamental time constant of the oxygen uptake response to constant load exercise in the severe intensity domain (Murgatroyd *et al*. 2011) and positively correlated with type I fibre proportion (Vanhatalo *et al*. 2016). CP is also sensitive to alterations in oxygen delivery; CP is increased in hyperoxia (Vanhatalo *et al*. 2010) and decreased in hypoxia (Dekerle *et al*. 2012; Simpson *et al*. 2015) and with the application of BFR (Broxterman *et al*. 2015). Furthermore, CP is increased following both continuous (Gaesser & Wilson, 1988; Jenkins & Quigley, 1992) and HIIT (Gaesser & Wilson, 1988; Poole *et al*. 1990; Vanhatalo *et al*. 2008), which are known to increase both oxidative capacity

(Burgomaster *et al*. 2008) and capillary supply (Andersen & Henriksson, 1977; Jensen *et al*. 2004a).

In comparison to CP the physiological underpinnings of W' are less clear. Classically W' was considered to represent a fixed anaerobic energy store, representing energy derived from high-energy phosphates and anaerobic glycolysis and a small contribution from aerobic stores of myoglobin and haemoglobin-bound O_2 stores (Moritani *et al*. 1981; Fukuba & Whipp, 1999; Morton, 2006). However, this has been challenged by the observation that W' is reduced under conditions of hyperoxia (Vanhatalo *et al*. 2010). Instead W' may be linked to the development of the $\dot{V}O_2$ slow component, with strong positive correlations observed between W' and the $\dot{V}O_2$ slow component (Murgatroyd *et al*. 2011; Vanhatalo *et al*. 2011b), and the attainment of critical levels of intramuscular pH, PCr and Pi, with consistent 'critical' values demonstrated at the point of task failure in the severe-intensity domain irrespective of the work rate or the inspired O_2 fraction (Vanhatalo *et al*. 2010). Furthermore, a recent study demonstrating a positive correlation between W' and gross thigh volume and maximum torque production of the knee extensors, suggests that there may be a neuromuscular contribution to the mechanistic underpinning of W' (Kordi *et al*. 2018).

2.8 SUMMARY AND AIMS

This literature review has outlined the ability of skeletal muscle to adapt to the demands elicited from a specific exercise bout and has highlighted the importance of optimising adaptations to skeletal muscle capillarity and the mitochondria for enhancing endurance performance in trained individuals. However, it has also identified the reduced adaptive scope of this population. Whereby the acute molecular signalling response of both angiogenic and mitochondrial biogenic factors are attenuated as individuals become accustomed to an exercise stimulus and subsequently structural and functional adaptations are diminished. The review further highlighted the potential for post-exercise BFR as a training tool to overcome these attenuated responses and given the current lack of research clearly identified the need for further investigation. Finally, this literature review described the CP concept as an important performance model and with CP likely being an important parameter of aerobic function, it would be expected for CP to be sensitive to peripheral adaptations favouring endurance performance. In light of the above, the primary aims of the experimental chapters of this thesis were as follows:

Chapter 3:

To establish if there is a relationship between the parameters of the power-duration relationship and indices of skeletal muscle capillarity and muscle fibre morphology in trained cyclists, which would imply them as determinants of CP and/or W'. It was hypothesised that CP would be positively correlated to skeletal muscle capillarity and the proportion and CSA of type I fibres, whereas W' would not be associated with fibre type composition or capillarity.

Chapter 4:

To determine if 4 weeks of SIT combined with post-exercise BFR would increase the parameters of the CP concept, and therefore severe-intensity performance, skeletal muscle capillarity and mitochondrial protein content more than SIT alone in trained cyclists. It was hypothesised that the addition of post-exercise BFR to SIT would enhance CP, angiogenesis and mitochondrial protein content.

Chapter 5:

Firstly, to establish the level of skeletal muscle oxygenation during SIT with and without post-exercise BFR and secondly, to investigate if different BFR protocols altered the skeletal muscle

oxygenation response. It was hypothesised that post-exercise BFR would reduce skeletal muscle oxygenation during the recovery period and that a higher restriction period would decrease this further.

Chapter 6:

To investigate if post-exercise BFR would enhance and better maintain the magnitude of transient transcriptional responses of key angiogenic and mitochondrial biogenic genes and increase the translational and phenotypic response to two weeks of higher volume HIIT. It was hypothesised that post-exercise BFR would increase and maintain the attenuating acute transcriptional response of angiogenic and mitochondrial biogenic genes (VEGF, PGC-1α, HIF-1α and eNOS) and enhance VEGF protein content and CS activity.

CHAPTER 3

CRITICAL POWER IS POSITIVELY RELATED TO SKELETAL MUSCLE CAPILLARITY AND TYPE I MUSCLE FIBRES IN ENDURANCE TRAINED INDIVIDUALS

The study presented in this experimental chapter has been published and has the following citation:

Mitchell EA, Martin NRW, Bailey SJ, Ferguson RA (2018). Critical power is positively related to skeletal muscle capillarity and type I muscle fibers in endurance-trained individuals. *J Appl Physiol* **125**: 737–745.

This chapter presents the published manuscript.

3.1 ABSTRACT

The asymptote (CP) and curvature constant (*W′*) of the hyperbolic power-duration relationship can predict performance within the severe-intensity exercise domain. However, the extent which these parameters relate to skeletal muscle morphology is less clear, particularly in endurance trained individuals who, relative to their lesser trained counterparts, possess skeletal muscles that can support high levels of oxygen transport and oxidative capacity i.e. elevated type I fiber proportion and CSA and capillarity. Fourteen endurance trained males performed a maximal incremental test to determine peak oxygen uptake ($\dot{V}O_{2peak}$; 63.2 ± 4.1 ml.min^{-1}.kg^{-1}), maximal aerobic power (MAP) (406 ± 63 W), and 3-5 constant load tests to task failure for the determination of CP (303 ± 52 W) and *W′* (17.0 ± 3.0 kJ). Skeletal muscle biopsies were obtained from the vastus lateralis and analyzed for % fiber type proportion, CSA and indices of capillarity. CP was positively correlated with the % proportion (r = 0.79; P = 0.001) and CSA (r = 0.73; P = 0.003) of type I fibers, capillary to fiber ratio (r = 0.88; P < 0.001) and capillary contacts around type I fibers (r = 0.94; P < 0.001) and type II fibers (r = 0.68; P = 0.008). *W′* was not correlated with any morphological variables. These data reveal a strong positive association between CP and skeletal muscle capillarity. Our findings further support the assertion that CP is an important parameter of aerobic function.

3.2 NEW AND NOTEWORTHY

This investigation demonstrated very strong positive correlations between CP and skeletal muscle capillarity, particularly around type I fibers, and type I fiber composition. These correlations were demonstrated in endurance trained individuals expected to possess well-adapted skeletal muscles, such as high levels of oxygen transport structures and high oxidative capacities, supporting the view that CP is an important parameter of aerobic function. In contrast, the curvature constant *W′* was not associated with fiber type composition or capillarity.

3.3 INTRODUCTION

The hyperbolic relationship between time to exhaustion and power output during high-intensity exercise is defined by a power asymptote, CP, and curvature constant, *W′* (Monod & Scherrer, 1965; Morton, 2006; Poole *et al*. 1988). Together, these parameters determine exercise performance capabilities within the severe-intensity exercise domain (Jones *et al*. 2010; Vanhatalo *et al*. 2011a) or alternatively termed the very-heavy intensity domain (Whipp, 1996). These parameters are, therefore, of significance to athletes, coaches and exercise physiologists interested in fatigue development and its underpinning mechanisms (Burnley *et al*. 2012; Jones *et al*. 2010; Poole *et al*. 2016; Vanhatalo *et al*. 2010; Vanhatalo *et al*. 2011b). It is well established that CP demarcates the heavy and severe exercise intensity domains (Jones *et al*. 2008; Poole *et al*. 1988; Whipp, 1996). During heavy-intensity exercise, pulmonary $\dot{V}O_2$ and intramuscular substrates (e.g. phosphocreatine (PCr)) and metabolites (e.g. Pi and H^+) achieve sub-maximal steady-state values. In contrast, during severe-intensity exercise, i.e. above CP, pulmonary $\dot{V}O_2$, and intramuscular substrates and metabolites continue to increase/decrease until their respective maxima/minima are obtained and task failure occurs (Jones *et al*. 2008; Poole *et al*. 1988; Vanhatalo *et al*. 2016). CP is, therefore, considered to reflect the greatest sustainable rate of oxidative metabolism in the absence of a progressive loss of muscle metabolic homeostasis, and is an important determinant of endurance exercise performance (Jones *et al*. 2010).

Whilst the relationship between CP and the broad parameters of aerobic function, such as oxygen delivery and $\dot{V}O_2$ kinetics, have been well established (Dekerle *et al*. 2012; Murgatroyd *et al*. 2011; Vanhatalo *et al*. 2010; Whipp, 1996), the association between CP and aspects of skeletal muscle morphology is not fully understood. Vanhatalo *et al.* (2016) previously reported a positive relationship between CP and the proportion of type I muscle fibers in recreationally active individuals. Since type I skeletal muscle fibers possess a superior phenotype for oxidative metabolism and enhanced fatigue resistance compared to type II fibers (for review see Schiaffino and Reggiani, 2011), this observation is compatible with the interpretation that CP is principally a parameter of oxidative metabolism. Another aspect of skeletal muscle morphology that will influence oxidative metabolism and fatigue resistance is capillarity (Joyner & Coyle, 2008). Muscle capillarity is an important determinant of oxygen extraction which itself is a function of the muscle oxygen diffusion capacity and muscle blood flow (Roca *et al*. 1992; Wagner, 1992). The former is primarily determined by the number of

red blood cells that are in contact with the contracting skeletal muscle fibers (Federspiel & Popel, 1986) which is facilitated by a high capillary network and the likelihood that most capillaries support red blood cell flux (Poole *et al*. 2013). Taken together, therefore, it seems logical, that CP would be related to skeletal muscle capillary supply. Iaia *et al*. (2011) have previously demonstrated a positive relationship between capillary supply and time to task failure over a performance range of ~1-20 minutes. Although this study did not partition the exercise intensity domains as defined by CP, these exercise durations would be expected to fall close to or within the severe intensity domain. Therefore, the correlation between time to task failure, over a range that spans the severe-intensity exercise domain, and skeletal muscle capillarity could be linked to the CP, but the relationship between skeletal muscle capillarity and CP has yet to be assessed.

Compared to CP, the physiological understanding of *W′* is less clear. Classically, *W′* was considered to represent a fixed anaerobic energy store (Morton, 2006). However, more recent observations have challenged this interpretation since *W′* appears to be sensitive to changes in oxygen delivery (Vanhatalo *et al*. 2010). Instead, *W′* appears to be linked to the development of the $\dot{V}O_2$ slow component, and the attainment of critical levels of intramuscular pH, PCr and Pi (Jones *et al*. 2008; Vanhatalo *et al*. 2011b), both of which are dependent on muscle fiber composition (Krustrup *et al*. 2004; Pringle *et al*. 2003). Moreover, as exercise intensity exceeds CP and the utilization of *W′* ensues, muscle blood flow is preferentially distributed to type II muscle fibers (Copp *et al*. 2010) suggesting a potential dependence of *W′* utilization on type II skeletal muscle fiber recruitment and perfusion. However, Vanhatalo *et al.* (2016) did not report any relationship between the magnitude of *W′* and the proportion of type II fibers in recreationally active individuals.

It has long been established that endurance trained individuals possess well-adapted skeletal muscles with a significantly greater capillary supply (Hermansen & Wachtlova, 1971; Ingjer, 1979; Saltin *et al*. 1977) and oxidative capacity (Costill *et al*. 1976; Saltin *et al*. 1977) compared to untrained individuals. This permits athletes to achieve high levels of oxygen extraction and, consequently, very high values of leg $\dot{V}O_2$ (>600 $ml.min^{-1}.kg^{-1}$) have been observed (Richardson *et al*. 1993). Therefore, the expected high level of oxidative capacity and oxygen transport structures in endurance trained athletes suggest this population would achieve greater values of CP. Indeed, CP typically occurs at 80-90% of $\dot{V}O_{2max}$ in athletes compared to 70-80% of $\dot{V}O_{2max}$ in healthy young individuals (Poole *et al*. 2016). However, despite findings of a higher CP and skeletal muscle capillarity and type I fiber percentage in athletes, a direct

relationship between capillary supply and muscle fiber composition and CP in endurance trained individuals has yet to be established.

Therefore, the aim of the current study was to assess the relationship between parameters of the power-duration relationship (CP and *W′*) and indices of capillarity and muscle fiber morphology in endurance trained individuals. It was hypothesized that CP would be positively related to indices of skeletal muscle capillarity and the proportion and cross-sectional area of type I skeletal muscle fibers.

3.4 METHODS

Participants

Fourteen healthy males (Table 3.1) volunteered to take part in the study. Participants were competitive cyclists or triathletes and had to achieve the inclusion criteria of $\dot{V}O_{2peak}$ approximately 60 $ml \cdot min^{-1}kg^{-1}$. All completed health and muscle biopsy screening questionnaires prior to participation to mitigate for contraindications to maximal exercise and muscle biopsy procedures. Participants did not have a history of neuromuscular, hematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period. All experimental procedures were approved by the Loughborough University Ethics Approvals (Human Participants) Sub-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.

Experimental protocol

Participants attended the laboratory on five to seven occasions over a period of approximately 10 days. $\dot{V}O_{2peak}$ was initially tested to ensure participants attained the appropriate inclusion criteria. Following a minimum of 48 hours a muscle biopsy was then obtained. After a further 48 hours participants undertook a series of 3-5 constant load tests to the limit of tolerance, each separated by a minimum of 24 hours, to determine CP and *W'*.

All performance tests were conducted upon an electronically braked cycle ergometer (Lode Excalibur Sport, Lode B.V. Gronigen, The Netherlands). Ergometer saddle and handlebar dimensions were recorded for each participant during preliminary testing and remained standardized for the remainder of the testing period. Participants were instructed to maintain a normal diet during the testing period and refrain from ingesting alcohol and caffeine during the 48 h preceding testing. All tests were conducted in constant laboratory ambient conditions (19-21°C, 40-50% humidity).

Performance measures

$\dot{V}O_{2peak}$ and maximal aerobic power

Participants performed an incremental test to exhaustion to establish $\dot{V}O_{2peak}$ and MAP. Participants began cycling, at a freely chosen, constant pedal cadence for 1 min at 50 W, after which power increased 25 W every 60 s until volitional exhaustion or when cadence fell 10%

below the chosen cadence for more than 5 s, despite strong verbal encouragement. Pulmonary gas exchange was measured continuously throughout exercise (Cortex MetaLyzer 3B, Leipzig, Germany). $\dot{V}O_{2peak}$ and MAP were defined as the highest $\dot{V}O_2$ and power output achieved for a 30 and 60 s period during the test, respectively.

Table 3.1. Parameters of performance and skeletal muscle morphology

	Mean ± SD	**Range**
Participant characteristics		
Age (yr)	25 ± 6	20 – 41
Height (m)	1.82 ± 0.06	1.70 – 1.95
Mass (kg)	76.5 ± 9.0	64.2 – 90.4
Performance parameters		
$\dot{V}O_{2peak}$ ($ml.min^{-1}.kg^{-1}$)	63.2 ± 4.1	58.7 – 72.2
$\dot{V}O_{2peak}$ ($l.min^{-1}$)	4.86 ± 0.68	3.93 – 5.86
MAP (W)	406 ± 63	295 – 485
CP (W)	303 ± 52	207 – 376
W′ (kJ)	17.0 ± 3.0	13.9 – 22.9
Skeletal muscle morphology		
Type I fiber %	56.6 ± 11.9	41.2 – 83.9
Type II fiber %	43.4 ± 11.9	16.1 – 58.8
CSA fiber type I (μm^{-2})	5937 ± 1333	3835 – 8568
CSA fiber type II (μm^{-2})	5967 ± 1294	4024 - 7997
Capillary density ($cap.mm^{-2}$)	424 ± 55	314 – 489
Capillary to fiber ratio	2.84 ± 0.63	1.90 – 4.22
CC type I	6.9 ± 1.4	4.8 – 9.4
CC type II	6.1 ± 1.1	4.6 – 8.4
Sharing index type I	2.43 ± 0.16	2.20 – 2.64
Sharing index type II	2.19 ± 0.24	1.74 – 2.62

Abbreviations: CC type I, capillary contacts around type I fibers; CC type II, capillary contacts around type II fibers; CP, critical power; CSA fiber type I, cross sectional area of type I fibers; CSA fiber type II, cross sectional area of type II fibers; MAP, maximal aerobic power; Sharing index type I, sharing index of type I fibers; Sharing index type II, sharing index of type II fibers; Type I fiber %, % proportion of type I fibers; Type II fiber %, % proportion of type II fibers %; $\dot{V}O_{2peak}$, peak oxygen uptake; *W′,* curvature constant.

CP and *W′*

Participants performed a minimum of 3 constant-load tests that were continued until the limit of tolerance at between 75-100% of MAP, the sequence of which was randomized. These were designed to elicit exhaustion within 2- to 15-min (Poole *et al*. 1988). Each test was preceded with an initial warm-up at 50 W for 5 min. Time to exhaustion (t) was recorded to the nearest second and was taken as either volitional exhaustion or when pedal cadence fell 10% below the freely chosen cadence for more than 5 s, despite strong verbal encouragement. No feedback regarding the power output or times achieved were provided, however participants were permitted to view pedal cadence throughout. To enhance the accuracy of parameter estimates, when the standard error (SE) of CP was >5% and *W′* >10% an additional test was performed (Hill & Smith, 1994).

The parameters of the power-duration relationship, CP and *W′*, were calculated using the inverse linear model (equation 1), the linear work-time model (equation 2) and the hyperbolic model (equation 3). The equation associated with the lowest combined standard error was selected and used for all further analysis.

$$P = W' \cdot (1/t) + CP \qquad (1)$$

$$W = CP \cdot t + W' \qquad (2)$$

$$t = W' / (P - CP) \qquad (3)$$

Muscle sampling and analysis

Muscle biopsies were obtained from the lateral portion of the vastus lateralis muscle under local anaesthesia (1% lidocaine) using the percutaneous needle biopsy technique with suction. Muscle samples were immediately embedded in mounting medium (Tissue-Tek OCT Compound, Sakura Finetek Europe, The Netherlands) and frozen in liquid-cooled isopentane. All samples were then stored at -80°C until analysis.

Immuno-histochemistry

Transverse serial sections (8 μm) were obtained using a cryotome and placed onto poly L-lysine coated glass slides. Sections were fixed for 10 min in 3.7% formaldehyde at room temperature and blocked with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 5% goat serum for 1 h at room temperature. Serial muscle sections were

then incubated with either primary antibodies for CD-31 (ab119339, abcam, Cambridge, UK) diluted 1:100 and MHC II (ab91506, abcam, Cambridge, UK) diluted 1:1000 in PBS-2% BSA or MHC I (A4.951, DSHB, Iowa, USA) diluted 1:500 in PBS-2% BSA for 1 h at room temperature. Sections were then incubated for 2 h at room temperature with the appropriate secondary antibodies; goat anti-mouse Alexa Fluor 488, (CD-31, MHC I) and goat anti-rabbit Alexa Fluor 594 (MHC II) diluted 1:500 in PBS-2% BSA. Following incubation cover slips were mounted with fluoromount aqueous mounting medium (F4680, Sigma-Aldrich, Dorset, UK). Specificity of staining was assessed with no primary antibody negative controls.

Images were captured using a fluorescence microscope (Leica DM2500) at 20x magnification. A minimum of 7 images were taken from across the entire cross-sectional area of the sample to avoid bias towards smaller fibers, with at least 6 cryo-sections per participant analyzed. The number of fibers counted equated to nearly 200 per participant (type I = 93 ± 18 and type II; 80 ± 33). Images analysis was undertaken using Fiji (ImageJ) software and the investigator was blinded to the participant code of each sample. Only transverse fibers were included in the analysis which was assessed primarily by the presumption of circularity. Any fibers which were clearly oblique or not transverse to the long axis of the fiber were excluded from analysis. Cross-sectional area (CSA) of fibers was assessed by manually drawing around the perimeter of each fiber and was calculated as the cumulative area of each fiber type divided by the number of fibers analyzed. Although absolute fiber size may be over-estimated due to fiber swelling during thawing of frozen sections, this should be consistent between participants. Fiber type composition was expressed as a percentage of the number of fibers of each type relative to the total number of fibers counted. Capillarity was expressed as; capillary density, capillary to fiber ratio, number of capillaries in contact with type I (CC type I) and type II fibers (CC type II) and sharing index of type I and type II fibers (calculated as CC/capillary-to-fiber ratio). Coefficients of variation for intra-rater reliability of image analysis were: % fiber type composition = 2.4%; fiber CSA = 2.2%; capillary density = 2.2%; capillary to fiber ratio = 1.7% and capillary contacts = 1.7%.

Statistics

Data were initially checked for normality using Shapiro-Wilk tests and relationships were analyzed using the Pearson's product-moment correlation coefficient. Data are displayed as mean ± SD unless otherwise stated. Significance was accepted at $P \leq 0.05$ and a statistical trend as $P \leq 0.10$.

3.5 RESULTS

The parameter estimates of the power-duration relationship for all three equations are displayed in Table 3.2. The linear inverse relationship produced the lowest combined standard error for CP and *W′* and was therefore used for further analyses. The ranges of times-to-exhaustion for the shortest and longest trials were 127 – 218 s and 512 – 1050 s, respectively. Power-duration relationship parameters were established from; 3 trials n = 8, 4 trials n = 5, and 5 trials n = 1. Representative images for immuno-histochemical staining are displayed in Figure 3.1 Performance and skeletal muscle morphology characteristics are displayed in Table 3.1.

Table 3.2. Parameter estimates of the power-duration relationship

	CP (W)	CP SE (%)	*W′* (kJ)	*W′* SE (%)	R^2
Inverse model	303 ± 52	1.8 ± 1.0	17.0 ± 3.0	8.9 ± 5.2	0.982 ± 0.019
Work-time model	302 ± 52	1.8 ± 1.0	17.4 ± 2.3	14.5 ± 8.5	0.999 ± 0.001
Hyperbolic model	299 ± 52	1.8 ± 1.3	19.0 ± 3.0	16.8 ± 10.1	-

Abbreviations: CP, critical power; SE, standard error; *W′*, curvature constant.

CP correlates

CP was positively correlated with the % proportion of type I fibers, and inversely correlated with the % proportion of type II fibers. The correlation remained when type I fibers were expressed as CSA but was eliminated when type II fibers were expressed as CSA (Fig. 3.2). CP was positively correlated with capillary to fiber ratio and CC type I and CC type II. There was a modest (non-significant, $P = 0.07$) correlation between CP and capillary density (Fig 3.2). CP was negatively correlated with the sharing index of type II fibers ($r = -0.69$, $P = 0.006$), but was not correlated with the sharing index of type I fibers ($r = -0.16$, $P = 0.59$).

W′ correlates

There were no correlations between *W′* and any measures of fiber type composition and capillarity (Fig 3.3).

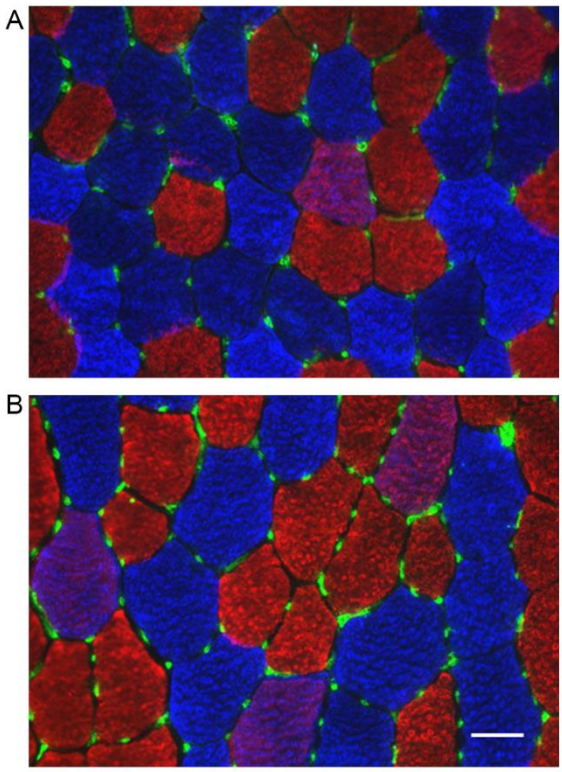

Figure 3.1. Representative images of immuno-histochemical staining of muscle samples from two participants; A) CP = 207 W, $\dot{V}O_{2peak}$ = 3.93 l.min^{-1}, capillary to fiber ratio = 1.90; B) CP = 353 W, $\dot{V}O_{2peak}$ = 5.38 l.min^{-1}, capillary to fiber ratio = 3.40. Type I fibers = red, type II fibers = blue, and capillaries = green. Scale Bar = 50 µm.

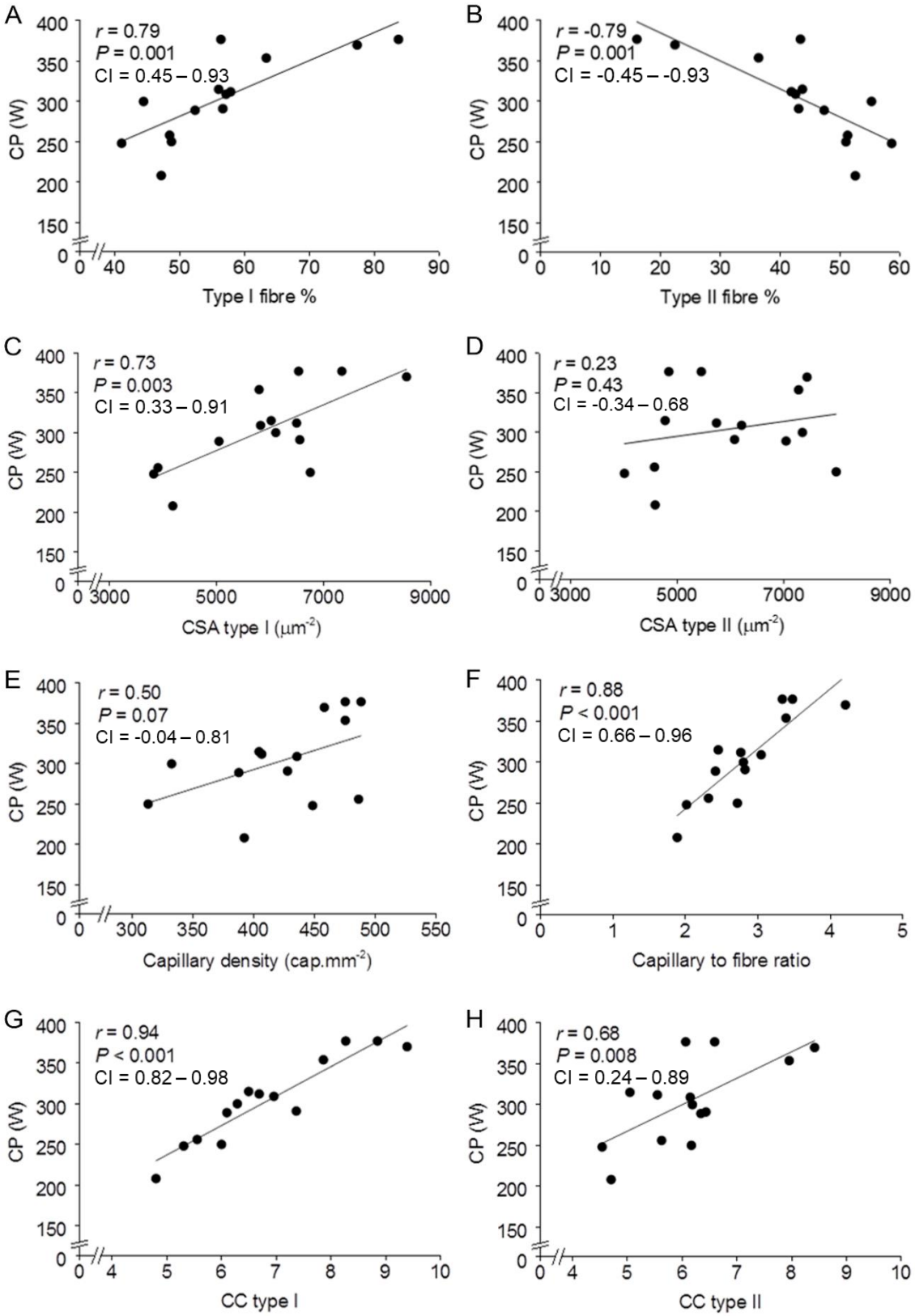

Figure 3.2. Correlations between CP and; A) % proportion of type I fibers, B) % proportion of type II fibers, C) CSA of type I fibers, D) CSA of type II fibers, E) capillary density, F) capillary to fiber ratio, G) capillary contacts around type I fibers, H) capillary contacts around type II fibers. CI = 95% confidence interval of the correlation coefficient.

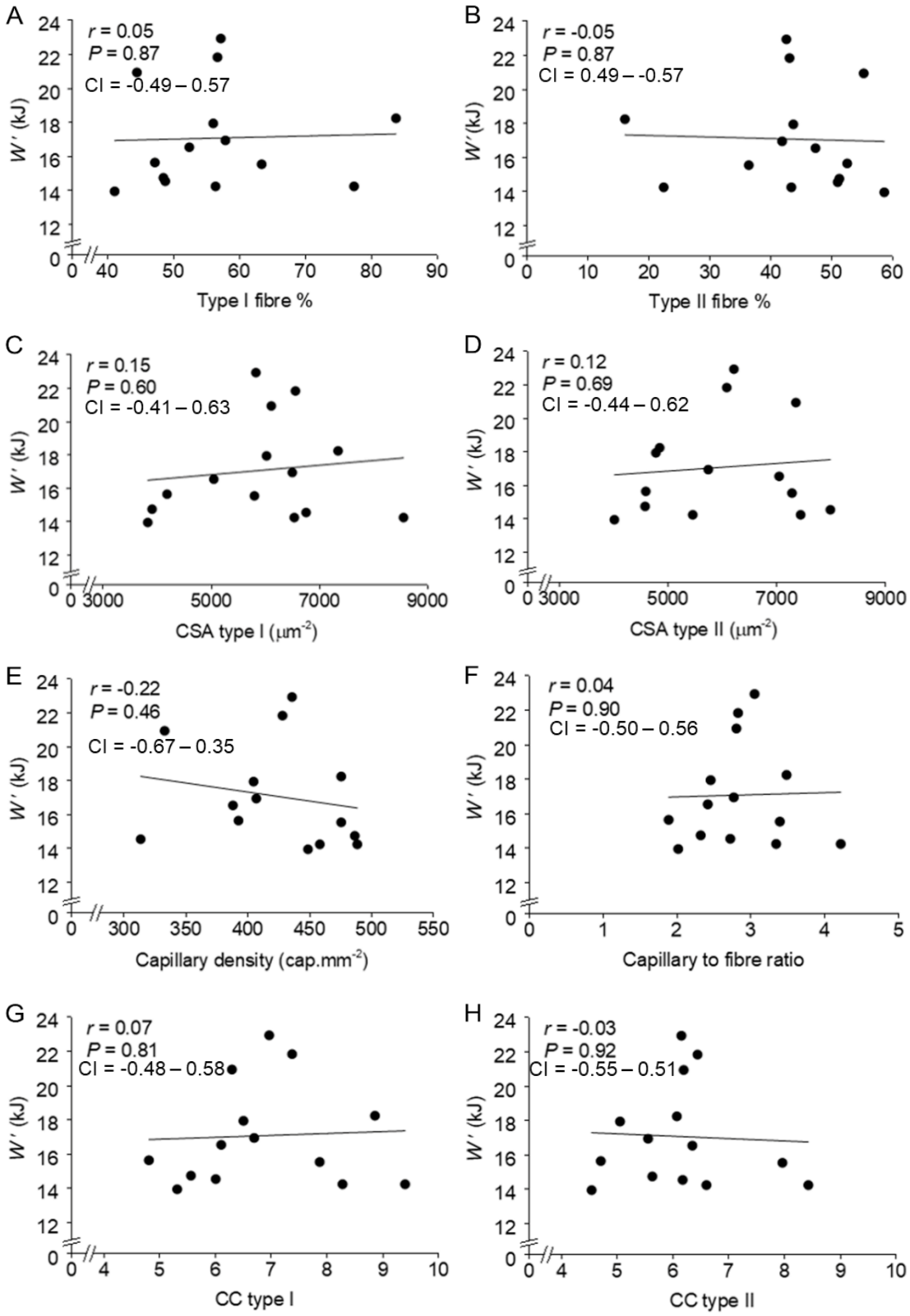

Figure 3.3. Correlations between W' and; A) % proportion of type I fibers, B) % proportion of type II fibers, C) CSA of type I fibers, D) CSA of type II fibers, E) capillary density, F) capillary to fiber ratio, G) capillary contacts around type I fibers, H) capillary contacts around type II fibers. CI = 95% confidence interval of the correlation coefficient.

$\dot{V}O_{2peak}$ correlates

Absolute $\dot{V}O_{2peak}$ was positively correlated with capillary to fiber ratio and CC type I and CC type II. In contrast, there was no correlation between $\dot{V}O_{2peak}$ and capillary density. $\dot{V}O_{2peak}$ was also positively correlated with % proportion and CSA of type I fibers and negatively correlated with % proportion of type II fibers. $\dot{V}O_{2peak}$ was not correlated with CSA of type II fibers (Table 3.3).

MAP correlates

MAP was positively correlated with all measures of capillarity; capillary density, capillary to fiber ratio, CC type I and CC type II. MAP was also positively correlated with % proportion and CSA of type I fibers and negatively correlated with % proportion of type II fibers. MAP was not correlated with CSA of type II fibers (Table 3.3).

Table 3.3. Correlations between $\dot{V}O_{2peak}$ and MAP and markers of skeletal muscle capillarization and muscle fiber composition

	$\dot{V}O_{2peak}$ (l.min^{-1})	**$\dot{V}O_{2peak}$ (ml.min^{-1}.kg^{-1})**	**MAP (W)**
Type I fiber %	*r* = 0.82 *P* = 0.002 CI = 0.43 – 0.95	*r* = 0.33 *P* = 0.32 CI = -0.34 – 0.78	*r* = 0.74 *P* = 0.002 CI = 0.34 – 0.91
Type II fiber %	*r* = -0.82 *P* = 0.002 CI = -0.43 – -0.95	*r* = -0.33 *P* = 0.32 CI = 0.34 – -0.78	*r* = -0.74 *P* = 0.002 CI = -0.34 – -0.91
CSA fiber type I (μm^{-2})	*r* = 0.81 *P* = 0.003 CI = 0.41 – 0.95	*r* = 0.19 *P* = 0.58 CI = -0.46 – 0.71	*r* = 0.69 *P* = 0.01 CI = 0.25 – 0.89
CSA fiber type II (μm^{-2})	*r* = 0.47 *P* = 0.14 CI = -0.18 – 0.83	*r* = 0.22 *P* = 0.52 CI = -0.44 – 0.72	*r* = 0.21 *P* = 0.46 CI = -0.36 – 0.67
Capillary density (cap.mm^{-2})	*r* = 0.43 *P* = 0.19 CI = -0.23 – 0.82	*r* = 0.42 *P* = 0.20 CI = -0.24 – 0.81	*r* = 0.55 *P* = 0.04 CI = -0.26 – 0.81
Capillary to fiber ratio	*r* = 0.94 *P* < 0.001 CI = 0.78 – 0.98	*r* = 0.40 *P* = 0.23 CI = -0.26 – 0.81	*r* = 0.86 *P* < 0.001 CI = 0.61 – 0.95
CC type I	*r* = 0.95 *P* < 0.001 CI = 0.81 – 0.99	*r* = 0.40 *P* = 0.22 CI = -0.26 – 0.81	*r* = 0.92 *P* < 0.001 CI = 0.76 – 0.97
CC type II	*r* = 0.81 *P* = 0.003 CI = 0.41 – 0.95	*r* = 0.49 *P* = 0.13 CI = -0.16 – 0.84	*r* = 0.68 *P* = 0.01 CI = 0.23 – 0.89

Abbreviations: CC type I, capillary contacts around type I fibers; CC type II, capillary contacts around type II fibers; CSA fiber type I, cross sectional area of type I fibers; CSA fiber type II, cross sectional area of type II fibers; MAP, maximal aerobic power; Type I fiber %, % proportion of type I fibers; Type II fiber %, % proportion of type II fibers %; $\dot{V}O_{2peak}$, peak oxygen uptake. CI = 95% confidence interval of the correlation coefficient.

3.6 DISCUSSION

The novel findings of this study are the very strong positive correlations between CP and indices of capillarity in a homogenous group of endurance trained individuals. The findings of the current study also confirm previous observations, undertaken on recreationally active individuals, of a positive association between the proportion of type I skeletal muscle fibers and CP and extend these observations by indicting that CP is also positively associated with type I muscle fiber CSA. In contrast, there were no correlations between W' and any index of skeletal muscle fiber type or capillarity. These observations add further support to our understanding of the physiological mechanisms that likely underpin CP and, by extension, endurance exercise performance.

CP correlates

In the current study CP was positively correlated with indices of skeletal muscle capillarity, in particular the number of capillary contacts with type I fibers, which displayed a correlation coefficient >0.9. These novel findings extend previous observations of significant correlations between capillary to fiber ratio and time to task failure during exercise trials lasting ~2-20 minutes (Iaia *et al*. 2011), which span the spectrum of the tolerable duration of exercise within the severe exercise intensity domain (Jones *et al*. 2010; Poole *et al.* 2016). The strong positive correlations between CP and capillarity in the group of endurance trained individuals who participated in the present study imply that skeletal muscle capillary supply could be an important determinant of CP.

A high capillary supply would be hypothesized to be beneficial to CP, and therefore the ability to sustain high rates of oxidative phosphorylation, through enhancing oxygen extraction, via improved muscle oxygen diffusion capacity (Roca *et al*. 1992; Wagner, 1992), and an enhanced ability to remove metabolites considered to be involved in skeletal muscle fatigue, such as H^+ and K^+ (Allen *et al*. 2008). In support of the latter, Iaia *et al*. (2011) demonstrated positive correlations between capillary supply and the rate of plasma K^+ accumulation and muscle pH recovery. In contrast to the other markers of capillarity, there was only a modest (non-significant) correlation between CP and capillary density. This is perhaps not surprising as it is important to note that capillary density is also a function of muscle fiber CSA (Olfert *et al*. 2016) which was also positively correlated with CP, at least in type I fibers. Given that oxygen extraction is primarily determined by the number of red blood cells in contact with the contracting skeletal muscle fibers (Federspiel & Popel, 1986; Roca *et al*. 1992; Wagner, 1992)

this would suggest that the number of capillaries as opposed to capillary density *per se* would be important to oxygen extraction. This interpretation is supported by previous observations by Hepple *et al*. (2000) which demonstrated that an increase in capillary density following short-term immobilization did not increase muscle oxygen diffusing capacity, and supports the premise that a high capillary to fiber ratio and capillary contacts are more important determinants of CP.

We have also demonstrated a positive correlation between CP and % proportion of type I fibers in endurance trained athletes, which is consistent with previous observations in recreationally active participants (Vanhatalo *et al*. 2016). We have further extended this observation by demonstrating that CP was also positively related to the CSA of type I fibers. These observations are in keeping with the notion that CP is a parameter largely dictated by facets of oxidative metabolism since type I fibers possess characteristics that facilitate high rates of oxidative metabolism including higher mitochondrial content, density and enzyme activity and a higher capillary supply, as well as greater fatigue resistance compared to type II fibers (Saltin *et al*. 1977; Schiaffino & Reggiani, 2011).

It should be noted that the correlations do not establish causation. Indeed, there are multiple other adaptations that are also elicited through endurance training and associated with an 'endurance-trained' phenotype, that could determine CP and would indirectly be associated with capillary supply and fiber composition. Nevertheless, there is a significant body of experimental evidence demonstrating CP to be a parameter of aerobic function. Given that capillary supply in particular is a critical determinant of oxygen extraction (Poole *et al*. 2013) this evidence would support that these correlations could be causative. For example, CP is negatively correlated with the fundamental time constant of the oxygen uptake response to constant load exercise within the severe intensity domain (Murgatroyd *et al*. 2011). CP is also sensitive to changes in oxygen delivery and has been demonstrated to decrease under systemic hypoxia (Dekerle *et al*. 2012) and with blood flow restriction (Broxterman *et al*. 2015). Moreover, CP has been shown to increase with both continuous (Gaesser & Wilson, 1988; Jenkins & Quigley, 1992) and HIIT (Gaesser & Wilson 1988; Poole *et al*. 1990; Vanhatalo *et al*. 2008), which increase both skeletal muscle capillarity (Andersen & Henriksson, 1977; Jensen *et al*. 2004a) and oxidative capacity (Burgomaster *et al*. 2008).

We also demonstrated a negative correlation between CP and % proportion of type II fibers which supports the observation by Vanhatalo *et al*. (2016). In contrast, we have demonstrated

that there was no correlation when type II fiber composition was expressed relative to CSA, however, it is important to acknowledge that we did not distinguish between type IIa and IIx fibers. It has been demonstrated that type IIa fibers are larger than type IIx and type I fibers (Andersen & Henriksson, 1977; Ingjer, 1979; Saltin *et al*. 1977) and therefore the relative proportions of type IIa and type IIx fibers may affect this relationship. Furthermore, given that type IIa fibers possess greater oxidative capacity relative to type IIx fibers (Schiaffino & Reggiani, 2011), it could also be speculated that type IIx but not type IIa fiber proportion and CSA would be negatively correlated with CP. Indeed, Vanhatalo *et al*. (2016) demonstrated a negative correlation only between CP and type IIx fiber proportion, whereas CP was not related to type IIa fibers. Moreover, the mean CSA area of type I and type II fibers were similar in the present study. The endurance trained status of our participants is likely to explain this observation which is consistent with previous reports that fiber CSA is comparable between type I and type II fibers in a group of well-trained middle- and long-distance runners (Tesch & Karlsson, 1985) supporting the notion that preferential hypertrophy of type I fibers may occur with prolonged endurance training.

W′ correlates

There were no correlations between *W′* and any of the skeletal muscle morphological measurements in the present study. The lack of correlation with the % proportion or CSA of type II muscle fibers may perhaps seem surprising given that type II fibers possess a greater resting content of PCr (Sahlin *et al*. 1997; Sant' Ana Pereira *et al*. 1996) and glycolytic capacity (Greenhaff *et al*. 1993; Schiaffino & Reggiani, 2011), both of which has been suggested to play an integral role in determining *W′* i.e. the so-called "anaerobic capacity" (Moritani *et al*. 1981; Morton, 2006). Moreover, individuals with a greater proportion of type II fibers possess a greater $\dot{V}O_2$ slow-component (Pringle *et al*. 2003), which has been demonstrated to have a strong relationship with the magnitude of *W′* (Murgatroyd *et al*. 2011; Vanhatalo *et al*. 2011b). However, Vanhatalo *et al*. (2016) also demonstrated that there was no significant correlation between the magnitude of *W′* and the proportion of either type IIa or type IIx muscle fibers, suggesting that the determinants of *W′* are not specific to type II muscle fibers *per se*. It could be speculated that the absence of a correlation between *W′* and muscle morphology is attributable to the muscle fiber recruitment patterns that would be observed during exhaustive exercise in the severe intensity domain. For example, maximal sprint exercise has been shown to activate all muscle fiber types, as demonstrated by large reductions in PCr concentrations in type I and II fibers (including IIA and IIAX hybrid fibers; Karatzaferi *et al*. 2001; Sant' Ana

Pereira *et al*. 1996), whereas during submaximal exercise at 75% of $\dot{V}O_{2max}$ muscle fiber recruitment was shown to reach a steady state during 45 minutes of exercise, with only around 55% of type II fibers recruited (Altenburg *et al*. 2007). Therefore, it is possible that during whole body exhaustive exercise in the severe intensity domain not all type II fibers would be fully recruited when task failure and complete *W′* utilization occur. This might account for the lack of a correlation between *W′* and skeletal muscle fibre type reported in the current study and elsewhere (Vanhatalo *et al*. 2016) and suggests that *W′* might be more closely linked to other physiological events. For example, a recent study has demonstrated, in a group of elite track cyclists, that *W′* is positively correlated with the maximum force generating capacity of the knee extensors and gross thigh volume (Kordi *et al*. 2018). Clearly, further research is required to resolve the physiological bases of *W′*.

Limitations

Whilst common practice, there are limitations to the skeletal muscle analysis methods utilised in the present investigation. Firstly, only a single biopsy, taken from one site of the vastus lateralis muscle was analysed. Previous investigations have elucidated that capillary supply, fiber type proportion and fiber CSA can display a heterogeneous distribution within the vastus lateralis muscle (Boman *et al*. 2015; Dwyer *et al*. 1999; Lexel & Taylor, 1989). Therefore, whilst a high number of fibers were analysed for each individual sample (analysis of the data from the present study demonstrated that there was a levelling off in the mean values of capillary contacts and muscle fiber CSA within the mean number of fibers analysed for each sample) it cannot be assumed that the samples taken are truly representative of the whole vastus lateralis muscle. Furthermore, the samples were only analysed in 2D. A 2D analysis of capillaries assumes that capillaries are structured parallel to the longitudinal axis of muscle fibers. However, capillaries are not arranged directly linear to the fibers but demonstrate a degree or tortuosity and branching anastomoses (Olfert *et al*. 2016). As a result, the 2D quantification of capillarity can underestimate the true capillary network (Cebask *et al*. 2010; Olfert *et al*. 2016).

Conclusion

This investigation has demonstrated very strong positive correlations between CP and skeletal muscle capillarity, particularly in relation to type I fibers, in endurance trained individuals. Moreover, CP was positively correlated with type I skeletal muscle fiber proportion and CSA. In contrast, there were no correlations between *W′* and capillarity or fiber type. Collectively,

these results add support to the notion that CP is a parameter of aerobic function and is associated to physiological processes that support oxidative metabolism.

CHAPTER 4

THE COMBINED EFFECT OF SPRINT INTERVAL TRAINING AND POST-EXERCISE BLOOD FLOW RESTRICTION ON CRITICAL POWER, CAPILLARY GROWTH AND MITOCHONDRIAL PROTEINS IN TRAINED CYCLISTS.

The study presented in this experimental chapter has been published and has the following citation:

Mitchell EA, Martin NRW, Turner MC, Taylor CW, Ferguson RA (2018). The combined effect of sprint interval training and post-exercise blood flow restriction on critical power, capillary growth, and mitochondrial proteins in trained. *J Appl Physiol* **126**: 51–59.

This chapter presents the published manuscript.

4.1 ABSTRACT

Sprint interval training (SIT) combined with post-exercise BFR is a novel method to increase $\dot{V}O_{2max}$ in trained individuals, and also provides a potent acute stimulus for angiogenesis and mitochondrial biogenesis. The efficacy to enhance endurance performance has however yet to be demonstrated. 21 trained male cyclists ($\dot{V}O_{2max}$; 62.8 ± 3.7 $ml.min^{-1}.kg^{-1}$) undertook 4 weeks of SIT (repeated 30 s maximal sprints) either alone (CON; n = 10) or with post-exercise BFR (n = 11). Before and after training $\dot{V}O_{2max}$, CP and *W′* were determined and muscle biopsies obtained for determination of skeletal muscle capillarity and the content of mitochondrial proteins. CP increased (*P* = 0.001) by a similar extent following CON (287 ± 39 W to 297 ± 43 W) and BFR (296 ± 40 W to 306 ± 36 W). $\dot{V}O_{2max}$ increased following BFR by 5.9% (*P* = 0.02) but was unchanged after CON (*P* = 0.56). All markers of skeletal muscle capillarity and the content of mitochondrial proteins were unchanged following either training intervention. In conclusion, 4 weeks of SIT increased CP, however this was not enhanced further with BFR. SIT was not sufficient to elicit changes in skeletal muscle capillarity and the content of mitochondrial proteins with or without BFR. However, we further demonstrate the potency of combining BFR with SIT to enhance $\dot{V}O_{2max}$ in trained individuals.

4.2 NEW AND NOTEWORTHY

This investigation has demonstrated that 4 weeks of SIT increased CP in trained individuals; however, post-exercise BFR did not enhance this further. SIT, with or without BFR, did not induce any changes in skeletal muscle capillarity or the content of mitochondrial proteins in our trained population. We do, however, confirm previous findings that SIT combined with BFR is a potent stimulus to enhance maximal oxygen uptake.

4.3 INTRODUCTION

Well-trained individuals are typically accustomed to high training volumes across a broad spectrum of intensities. However, it is generally accepted that eliciting further adaptations within this population becomes challenging (Laursen & Jenkins, 2002; Londeree, 1997). Early research has highlighted the reduced plasticity of skeletal muscle in the trained state (Hoppeler *et al*. 1985; Slatin *et al*. 1977) and 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 (Costill *et al*. 1988; Daniels *et al*. 1978). This blunting of the adaptive scope in trained individuals is also reflected at a molecular level (Flueck, 2010; Perry *et al*. 2010) as demonstrated by an attenuated acute transcriptional response as individuals become accustomed to a specific exercise bout (Perry *et al*. 2010). Therefore, the development of effective novel training methods is of particular relevance to this population.

We have recently demonstrated the potency of combining SIT with BFR in enhancing the adaptive responses in well-trained individuals (Taylor *et al*. 2016a). The addition of BFR elicited an increase in $\dot{V}O_{2max}$ of ~4.5%, compared to no change with SIT alone. Alongside this we presented preliminary mechanistic evidence that SIT combined with BFR led to enhanced angiogenic signalling, suggesting the potential for a greater capillary growth with this novel intervention. Increased muscle capillarity is a critical adaptation to enhance oxygen and substrate delivery (Joyner & Coyle, 2008). Classic work in skeletal muscle has shown that the number of capillaries per fibre is proportional to the oxidative activity of that fibre (Romanul, 1964) and subsequently demonstrates a strong correlation with $\dot{V}O_{2max}$ (Saltin *et al*. 1977). An enhanced capillary network also facilitates the greater removal of metabolic end products, which would improve sub-maximal exercise tolerance (Joyner & Coyle, 2008). Both SIT combined with BFR and SIT alone were also potent in upregulating PGC-1α expression suggesting the potential for mitochondrial biogenesis (Taylor *et al*. 2016a). Therefore, it was surprising that despite the gains in $\dot{V}O_{2max}$ Taylor *et al*. (2016a) did not observe any improvements in exercise performance, measured through a 15 km time trial. This might be because the relative contribution of central and peripheral factors to performance of such a self-paced exercise is task dependent, with increased contribution of central fatigue within longer low intensity time trials (>30 min) and a greater degree of peripheral fatigue after shorter high intensity efforts (approximately 6 min) (Thomas *et al*. 2015). Therefore, the peripheral

adaptations that we have hypothesised may not be reflected in the performance measure selected but would be more relevant to shorter high intensity efforts.

Since its introduction by Monod and Scherrer (1965) the CP concept has been used to describe the relationship between the tolerable duration that high intensity exercise can be maintained at a given power output for whole body exercise, (i.e. the power-duration relationship) (Poole *et al*. 1988). This hyperbolic relationship can be described by two constants; the asymptote CP, which is considered to represent the greatest rate of oxidative metabolism that can be maintained in the absence of a progressive loss of muscle metabolic homeostasis; and the curvature constant (*W′*) representing the capacity for work performed above CP (Jones *et al*. 2010; Morton, 2006; Vanhatalo *et al*. 2016). Accordingly, performance within the severe intensity domain is a function of CP and *W′*, which makes them important determinants of performance (Jones *et al*. 2010; Vanhatalo *et al*. 2011a). CP is enhanced under conditions of increased oxygen delivery and is linked to the ability to maintain a metabolic steady state (Poole *et al*. 2016; Vanhatalo *et al*. 2016), therefore CP could be hypothesised to be sensitive to changes in skeletal muscle capillarity. Indeed, we recently demonstrated a strong positive association between CP and skeletal muscle capillarity in well-trained individuals (Mitchell *et al*. 2018). Furthermore, since CP represents the highest sustainable rate of oxidative metabolism, it is likely to be closely related to mitochondrial content and could therefore be sensitive to an increase in the content of mitochondrial enzymes.

Therefore, the present investigation assessed the potency of SIT combined with BFR in enhancing CP. Furthermore, the angiogenic and mitochondrial biogenic potential of SIT combined with BFR was also assessed. It was hypothesised that SIT combined with BFR would result in a greater increase in CP, which would be associated with a greater increase in skeletal muscle capillarity and the content of mitochondrial proteins, compared to SIT alone.

4.4 METHODS

Participants

21 healthy males (age 23 ± 5 yr, height, 179.5 ± 6.4 cm, body mass, 75.5 ± 7.9 kg) volunteered to take part in the study. Participants were competitive cyclists or triathletes and had to achieve the inclusion criteria of $\dot{V}O_{2max} \geq 60$ ml.min^{-1}kg^{-1}. All completed health and biopsy screening questionnaires prior to participation to mitigate for contraindications to maximal exercise, muscle biopsy procedures and BFR. Participants did not have a history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period. All experimental procedures were approved by the Loughborough University Ethics Approvals (Human Participants) Sub-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.

Experimental protocol

The study used an independent-groups design whereby participants were assigned to one of two groups to perform four weeks of SIT either on its own (CON, n = 10) or combined with post-exercise BFR (BFR, n = 11). Participants were pair matched between groups based upon initial $\dot{V}O_{2max}$, maximal aerobic power (MAP) and CP. Participants were initially familiarized to the testing and training procedures during preliminary visits. Pre-training outcome measures were assessed over a period of 10 days. $\dot{V}O_{2max}$ was tested initially to ensure participants attained the appropriate inclusion criteria. After approximately two days the pre-training muscle biopsy was then obtained. After a further two days the power-duration relationship for determination of CP and W' was assessed, with a minimum of 24 hours separating each test. Participants then embarked on the four-week supervised training programme. After a maximum of four days following the final training session the post-training outcome measures were assessed in the same order and over a similar time period

All performance tests were conducted on an electronically braked cycle ergometer (Lode Excalibur Sport, Lode B.V. Gronigen, The Netherlands). Ergometer saddle and handlebar dimensions were recorded for each participant during preliminary testing and remained standardised for the rest of the study. Participants were instructed to maintain a normal diet during the pre-training testing and replicate that diet during the post-training measures.

Participants were instructed to refrain from ingesting alcohol and caffeine during the 48 hours preceding testing. Exercise trials were undertaken at approximately the same time each day (± 2 hours). Laboratory conditions during pre- and post-training exercise measurements remained constant (19-21°C, 40-50% humidity).

Pre and post training outcome measures

$\dot{V}O_{2max}$ and MAP

Participants performed an incremental test to exhaustion to establish $\dot{V}O_{2max}$ and MAP. Participants began cycling, at a freely chosen, constant pedal cadence for 1 min at 50 W, after which power increased 25 W every 60 s until volitional exhaustion or when cadence fell 10% below the freely chosen cadence for more than 5 s, despite strong verbal encouragement. Pulmonary gas exchange was measured continuously throughout exercise (Cortex MetaLyzer 3B, Leipzig, Germany). $\dot{V}O_{2max}$ and MAP were defined as the highest $\dot{V}O_2$ and power output achieved for a 30 and 60 s period during the test, respectively.

CP and W'

Participants performed a series of 3-5 constant-load tests that were continued until the limit of tolerance at between 70-100% of MAP, the sequence of which was randomised. These were designed to elicit exhaustion within 2 to 15 min (Poole *et al*. 1988). Time to exhaustion (t) was recorded to the nearest second and was taken as either volitional exhaustion or when cadence fell 10% below the freely chosen cadence for more than 5 s, despite strong verbal encouragement. No feedback regarding the power output or times achieved were provided, however participants were permitted to view pedal cadence throughout. To enhance the accuracy of parameter estimates, when the standard error of CP was >5% and W' >10% an additional test was performed.

The parameters of the power-duration relationship, CP and W', were calculated using the inverse linear relationship (equation 1), the linear work-time model (equation 2) and the hyperbolic relationship (equation 3). The equation associated with the lowest combined standard error was selected and used for all further analysis.

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

$$W = CP \,.\, t + W' \quad (2)$$

$$t = W' / (P - CP) \quad (3)$$

Muscle sampling and analysis

16 participants consented to provide muscle biopsy samples (CON, n = 7; BFR, n = 9). Muscle biopsies were obtained, at rest, from the lateral portion of the vastus lateralis muscle under local anaesthesia (1% lidocaine) using the percutaneous needle biopsy technique with suction. Pre- and post-training samples were obtained through separate incisions 2 cm apart on the same leg. Muscle samples were split into two portions. One portion was immediately embedded in mounting medium (Tissue-Tek OCT Compound, Sakura Finetek Europe, The Netherlands) and immediately frozen in liquid nitrogen-cooled isopentane. The other portion was snap-frozen in liquid nitrogen. All samples were then stored at -80°C until analysis.

Immunohistochemistry

Transverse serial sections (8 μm) were obtained using a cryotome and placed onto poly L-lysine coated glass slides. Sections were fixed for 10 min in 3.7% formaldehyde at room temperature and blocked with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 5% goat serum for 1 h at room temperature. Serial muscle sections were then incubated with either primary antibody CD-31 (ab119339, abcam, Cambridge, UK) diluted 1:100 and MHC II (ab91506, abcam, Cambridge, UK) diluted 1:1000 in PBS-2% BSA or MHC I (A4.951, DSHB, Iowa, USA) diluted 1:500 in PBS-2% BSA for 1 h at room temperature. A separate slide was also incubated in CD-31 as described above and was subsequently incubated with Ki-67 (ab92742, abcam, Cambridge, UK) diluted 1:250 in PBS-2% BSA overnight at room temperature. Sections were then incubated for 2 h at room temperature with the appropriate secondary antibodies; goat anti-mouse Alexa Fluor 488, (CD-31, MHC I) and goat anti-rabbit Alexa Fluor 594 (MHC II, Ki-67) diluted 1:500 in PBS-2% BSA. Following incubation cover slips were mounted with fluoromount aqueous mounting medium (F4680, Sigma-Aldrich, Dorset, UK). Specificity of staining was assessed with no primary antibody negative controls.

Images were captured with a fluorescence microscope (Leica DM2500) at 20x magnification. Images were taken across the entire CSA of the sample to avoid bias toward smaller fibres. Camera exposure time and gain were adjusted and kept consistent for all images captured for each participant. An average of 117 ± 43 type I and 90 ± 49 type II fibres were analysed per sample. Capillarity was expressed as; capillary to fibre ratio, capillary density and number of capillary contacts around type I (CC type I) and type II fibres (CC type II). Ki-67 positive nuclei co-localised within capillaries were expressed per fibre (all Ki-67 data are CON n = 6 and BFR n = 9, with one participant missing due to insufficient tissue sample size). Only transverse fibres were included in the analysis, which was assessed primarily by the presumption of circularity. Any fibres that were clearly oblique or not transverse to the long axis of the fibre were excluded from analysis. Cross sectional area (CSA) of fibres was assessed by manually drawing the perimeter of each muscle fibre with the image analysis software Fiji (ImageJ). Although absolute fibre size may be overestimated because of fibre swelling during thawing of frozen sections, this should be consistent between all samples. The investigator was blinded to the exercise training status and condition of samples for all analysis.

Western blot analysis

Muscle tissue was homogenised in cold lysis buffer containing PBS-0.2% Triton X-100 and protease and phosphatase inhibitor cocktail (Fisher Scientific, Loughborough, UK). Samples were blitzed using a tissue lyser (Qiagen, UK) twice for 2 min at 20 Hz and centrifuged at 12000 g for 10 min to pellet insoluble material. The supernatant was transferred to a fresh Eppendorf tube and protein concentrations were determined by Pierce 660 protein assay according to the manufacturer's instructions (Fisher Scientific, Loughborough, UK). Samples were mixed with dH_2O, 4x LDS sample buffer (Invitrogen, Loughborough, UK) and 0.1% β-mercaptoethanol (Sigma, Dorset, UK) to a concentration of 1.5 μg.μl and were boiled for 5 min at 95°C. 15 μg of protein was loaded on to 4-20% TGX polyacrylamide gels (Bio-Rad, Herts, UK) and separated by electrophoresis at 100 V for 80 minutes. All samples were run in duplicate to establish coefficients of variation for a measure of technical variability. Proteins were transferred onto PVDF membrane at 30 V for 90 minutes (Bio-Rad, Herts, UK) and washed for 5 min in Tris buffered saline with tween (TBST) before being blocked in 5% blotting grade milk (Bio-Rad, UK) for 1 h at room temperature. Membranes were washed three times for 5 min in TBST and were incubated overnight with the primary antibodies; citrate synthase (CS) (ab129095, abcam, Cambridge, UK), cytochrome c oxidase (COX) subunit II (ab110258, abcam, Cambridge, UK) and COX IV (ab33985, abcam, Cambridge, UK) diluted

1:1000 in 3% blotting grade milk at 4°C. Membranes were then washed three times for 5 min in TBST and incubated with the appropriate secondary antibody; anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako, Stockport, UK) or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Herts, UK) diluted 1:10,000 in 3% blotting grade milk for 1 h at room temperature. Following three 5 min washes in TBST, membranes were incubated with enhanced chemiluminescence substrate (ClarityMax, Bio-Rad, Herts, UK) for 5 min. Membranes were visualised using image analysis (ChemiDocTM XRS+, BioRad, Herts, UK) and band densities determined using image analysis software (Quality One 1-D analysis software v 4.6.8, Bio-Rad, Herts, UK). GAPDH was used as a loading control, as it remained stable across time for both conditions (CON $P = 0.30$; BFR $P = 0.39$; Fig. 4.4). Protein content was expressed in arbitrary units relative to GAPDH. All protein content data are CON n = 7 and BFR n = 7, with two participants missing due to insufficient tissue sample size. The coefficients of variation for CS, COXII and COXIV were 5.1 ± 3.9%, 6.8 ± 5.8% and 4.3 ± 3.2% respectively.

Exercise training

Participants completed a four-week supervised SIT programme (2 sessions per week) each session being separated by a minimum of 48 h. Participants were encouraged to maintain their regular training regime with the exception of performing any form of interval training. This was to ensure a substantial reduction in training volume was avoided. Each training session consisted of repeated 30 s maximal sprints 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 sprints in weeks 1, 2, 3 and 4, respectively. Each sprint was separated by a 4.5 min recovery period, during which participants immediately dismounted the cycle ergometer and lay in a semi-supine position upon a couch. In BFR participants were subjected to BFR (applied within 25 s of each sprint). This was achieved by rapidly applying pneumatic pressure cuffs (Hokanson SC12L) as high up as possible on the proximal portion of each thigh, which were inflated (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA) to a pressure of ~ 120 mmHg for 2 min (this pressure was kept constant throughout the four-week training period). The cuffs were then rapidly deflated and participants remained in the supine position until 30 s prior to the next sprint where they re-mounted the ergometer in time for the subsequent sprint which began precisely 4.5 min after the previous sprint ended. In CON participants remained in the semi-supine position before re-

mounting the ergometer in time for the subsequent sprint. Pre- and post-training measurements of peak power output (PPO) and mean power output (MPO) were obtained (Monark software) from the best sprint within the first and last training sessions respectively. Total work completed throughout the training period was calculated as a sum of the product of the MPO from each sprint and sprint duration.

Statistics

Training data were analysed using an unpaired *t*-test. Two-factor repeated-measures ANOVA, with one within factor (time; pre vs post) and one between factor (condition; CON vs BFR) were utilised to undertake all subsequent analysis. Where significant interaction effects were observed Bonferroni-corrected *post hoc* paired *t*-tests were used to locate differences. Data are presented as mean ± SD. Significance was accepted at $P \leq 0.05$.

4.5 RESULTS

Performance measures

There were no differences in physiological and performance measures prior to training between groups (Table 4.1). Participants completed 99% of the assigned training sessions without any complications (one participant missed one training session). The total amount of work done throughout the training was not different (interaction; $P = 0.75$) between CON (815 ± 88 kJ) and BFR (830 ± 129 kJ).

Physiological and performance variables measured before and after CON and BFR are presented in Table 4.1. There were significant interactions for absolute and relative $\dot{V}O_{2max}$. Subsequent *post hoc* tests revealed both absolute (Fig. 4.1A) and relative $\dot{V}O_{2max}$ increased following BFR (absolute $P = 0.02$; relative $P = 0.01$) but not in CON (absolute $P = 0.56$; relative $P = 0.88$). Absolute MAP (Fig. 4.1B) was unchanged with training in either group. Relative MAP increased with training (main effect for time; $P = 0.03$), however there was no difference between CON and BFR.

The inverse linear relationship produced the lowest combined standard error for CP (CON, Pre; 1.8 ± 1.0%, Post; 1.1 ± 0.7%, BFR, Pre; 1.7 ± 0.8%, Post; 1.9 ± 1.1%) and W' (CON, Pre; 7.4 ± 4.4%, Post; 4.9 ± 3.5%, BFR, Pre; 8.4 ± 5.7%, Post; 9.1 ± 5.9%) and therefore this equation was used to calculate parameter estimates. CP (Fig. 4.1C) increased with training, however there was no difference between CON and BFR. W' (Fig. 4.1D) was unchanged with training in either group.

Absolute (Fig. 4.1E) and relative PPO and absolute (Fig. 4.1F) and relative MPO increased with training, however there were no differences between CON and BFR.

Table 4.1. Physiological and performance variables before and after control (CON) and blood-flow restriction (BFR) training interventions.

Parameter	CON			BFR			ANOVA interaction *P* value	ANOVA main effect of time *P* value	ANOVA main effect of condition P value
	Pre	Post	Percentage change	Pre	Post	Percentage change			
Body Mass (kg)	74.6 ± 7.7	74.3 ± 8.5	-0.3	76.3 ± 8.3	75.6 ± 7.7	-0.8	0.59	0.24	0.67
$\dot{V}O_{2max}$ ($l.min^{-1}$)	4.59 ± 0.46	4.56 ± 0.48	-0.8	4.70 ± 0.63	4.98 ± 0.72 *	5.9	0.02	0.08	0.45
$\dot{V}O_{2max}$ ($ml.min^{-1}.kg^{-1}$)	63.2 ± 4.4	63.0 ± 5.9	-0.3	62.2 ± 3.4	65.2 ± 4.4 *	4.9	0.04	0.06	0.79
MAP (W)	393 ± 47	397 ± 51	1.0	402 ± 51	412 ± 41	2.6	0.44	0.09	0.56
MAP ($W.kg^{-1}$)	5.3 ± 0.5	5.4 ± 0.6	1.5	5.3 ± 0.4	5.5 ± 0.4	3.5	0.40	0.03	0.81
CP (W)	287 ± 39	297 ± 43	3.6	296 ± 40	306 ± 36	3.3	0.93	0.001	0.58
***W′* (kJ)**	18.1 ± 5.1	16.2 ± 4.3	-10.4	17.9 ± 6.5	17.4 ± 5.1	-3.0	0.53	0.26	0.80
PPO (W)	1057 ± 174	1112 ± 179	5.2	1065 ± 245	1142 ± 262	7.2	0.62	0.008	0.85
PPO ($W.kg^{-1}$)	14.3 ± 2.3	15.0 ± 2.2	5.4	13.8 ± 2.7	14.9 ± 2.8	8.3	0.53	0.004	0.80
MPO (W)	686 ± 73	692 ± 62	0.8	701 ± 113	722 ± 112	3.1	0.23	0.05	0.60
MPO ($W.kg^{-1}$)	9.2 ± 0.6	9.3 ± 0.6	1.4	9.1 ± 1.0	9.5 ± 1.1	4.2	0.22	0.01	0.99

Values are mean ± SD. Abbreviations: CP, critical power; MAP, maximal aerobic power; MPO, mean power output during 30 s sprint; PPO, peak power output during 30 s sprint; VO_{2max}, maximal oxygen uptake and *W′*, curvature constant. All data are CON n = 10 and BFR n = 11, except for $\dot{V}O_{2max}$ where n = 8 and PPO and MPO where n = 10. * Denotes significantly different to Pre-training (Bonferroni-corrected *post hoc* paired *t*-test; $P < 0.05$).

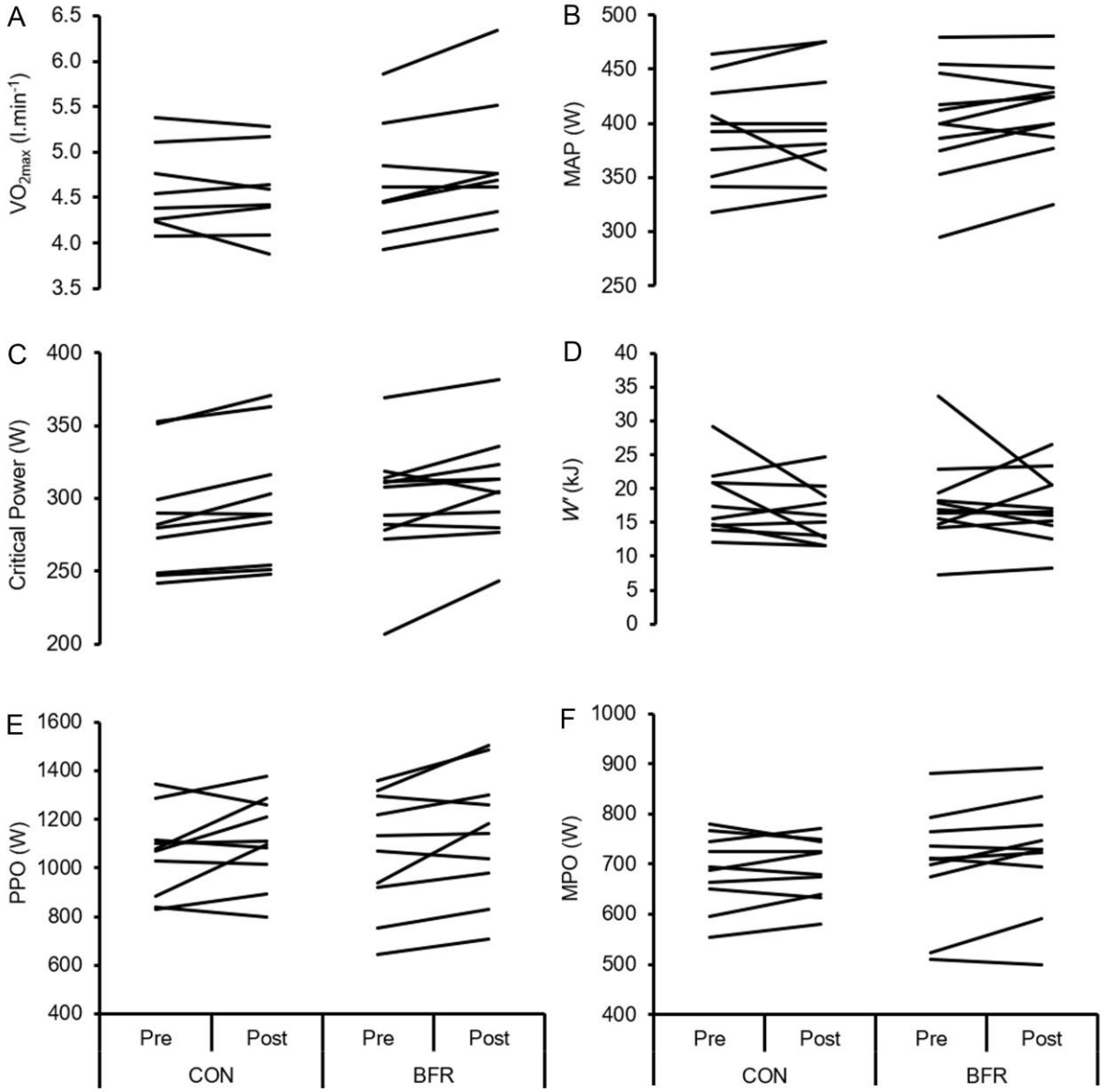

Figure 4.1. Individual responses of $\dot{V}O_{2max}$ (A), MAP (B), CP (C), *W′* (D), PPO (E) and MPO (F) before and after control (CON) and blood flow restriction (BFR) training interventions.

Capillarisation and muscle morphology

All measures of capillarisation (Fig. 4.2) were unchanged with training in either group (Table 4.2). The number of Ki-67 proliferating cells co-localised with capillaries was not significantly changed (interaction; $P = 0.06$) with training in either group (Fig. 4.3). CSA of type I and type II fibres were unchanged with training in either group (Table 4.2).

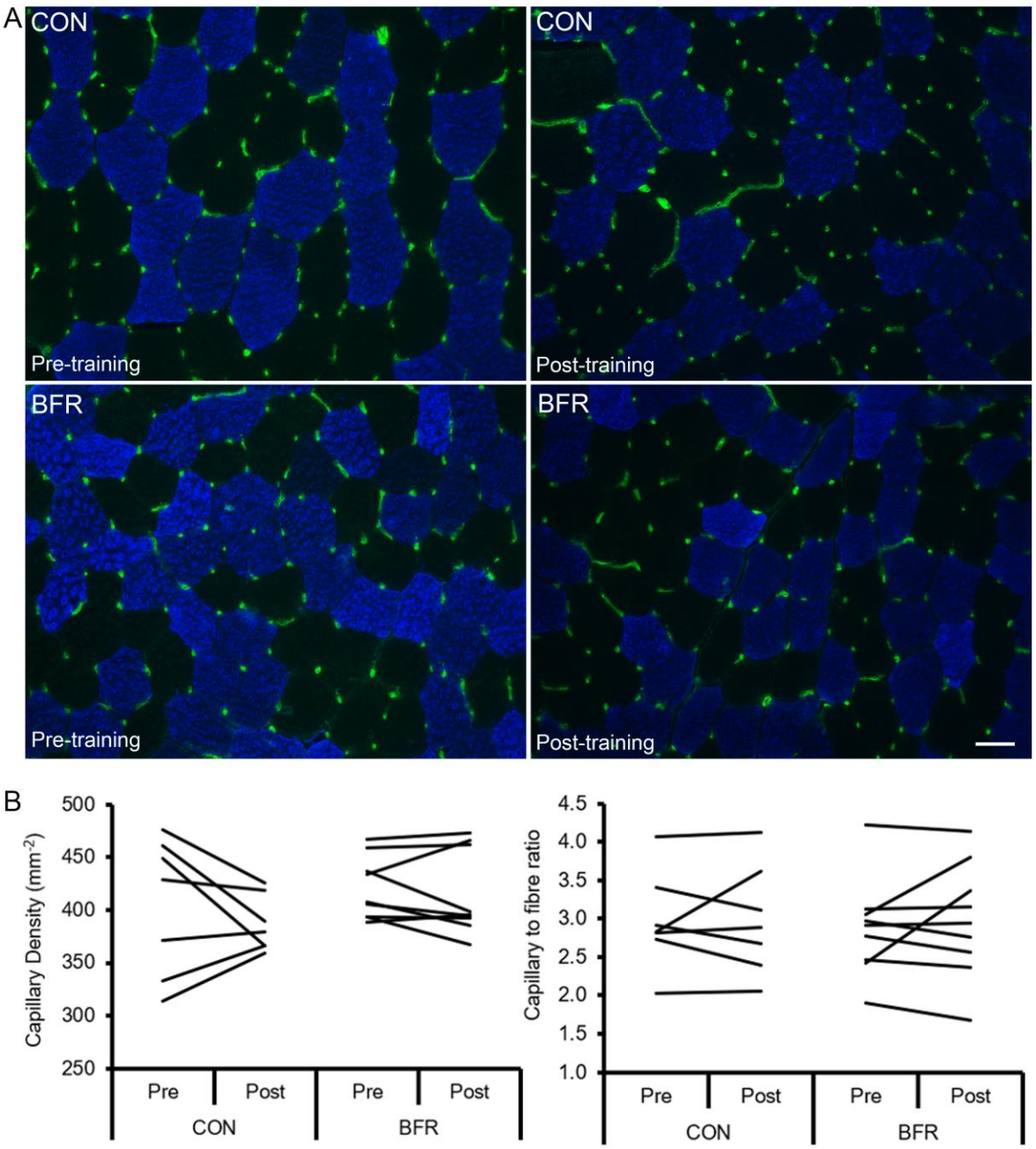

Figure 4.2. Muscle capillaries before and after control (CON) and blood-flow restriction (BFR) training interventions. A) Representative images of muscle capillaries stained with CD-31 (green) and type II fibres (blue). Scale Bar = 50 µm. B) Individual responses of capillary density and capillary to fibre ratio. Data are CON n = 7; BFR n = 9.

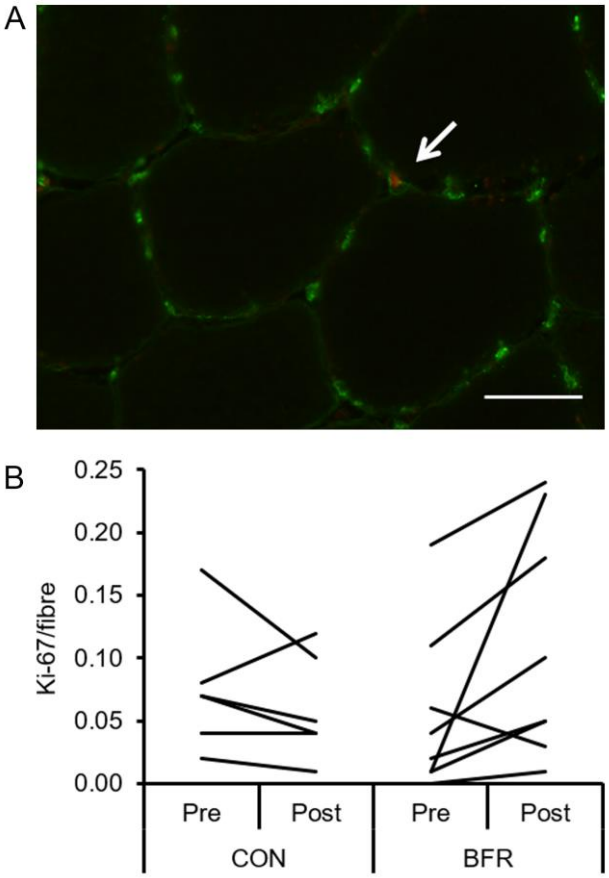

Figure 4.3. Presence of proliferating endothelial cells before and after control (CON) and blood-flow restriction (BFR) training interventions. A) Representative image of Ki-67 (red) positive endothelial cell (green). Scale Bar =50 µm. B) Individual responses of Ki-67 positive endothelial cells per fibre. Data are CON n = 6; BFR n = 9. One bar for BFR is hidden as data are pre =0.00 and post = 0.00.

Table 4.2. Capillarisation and muscle morphology before and after control (CON) and blood-flow restriction (BFR) training interventions.

	CON			BFR					
Parameter	**Pre**	**Post**	**Percentage change**	**Pre**	**Post**	**Percentage change**	**ANOVA interaction *P* value**	**ANOVA main effect of time *P* value**	**ANOVA main effect of condition P value**
Capillary Density (mm^{-2})	405 ± 65	386 ± 27	-4.6	420 ± 30	415 ± 40	-1.3	0.49	0.22	0.27
Capillary to fibre ratio	2.97 ± 0.63	2.98 ± 0.71	0.4	2.87 ± 0.64	2.97 ± 0.75	3.5	0.68	0.60	0.88
CC Type I	7.03 ± 1.21	6.92 ± 1.54	-1.6	6.99 ± 1.25	7.19 ± 1.43	2.7	0.44	0.85	0.86
CC Type II	6.45 ± 1.09	6.63 ± 1.40	2.8	6.01 ± 1.06	6.32 ± 1.53	5.1	0.73	0.20	0.56
CSA Type I (μm^{-2})	6456 ± 1651	6379 ± 1764	-1.2	6146 ± 1242	6375 ± 1472	3.7	0.62	0.81	0.83
CSA Type II (μm^{-2})	6908 ± 1463	6998 ± 1415	1.3	5896 ± 963	6364 ± 1639	7.9	0.47	0.29	0.23
Ki-67/fibre	0.07 ± 0.05	0.06 ± 0.04	-19.7	0.05 ± 0.06	0.10 ± 0.09	103.4	0.06	0.29	0.85

Values are mean ± SD. Abbreviations: CC Type I, capillary contacts of type I muscle fibres; CC Type II, capillary contacts of type II muscle fibres; CSA Type I, cross sectional area of type I muscle fibres; CSA Type II, cross sectional area of type II muscle fibres and Ki-67/fibre, Ki-67 positive capillaries per fibre. All data are CON n = 7 and BFR n = 9, except Ki-67/fibre which is CON n = 6 and BFR n = 9.

Content of mitochondrial enzyme proteins

Protein content of CS, COXII and COXIV (Fig. 4.4) were all unchanged with training in either group.

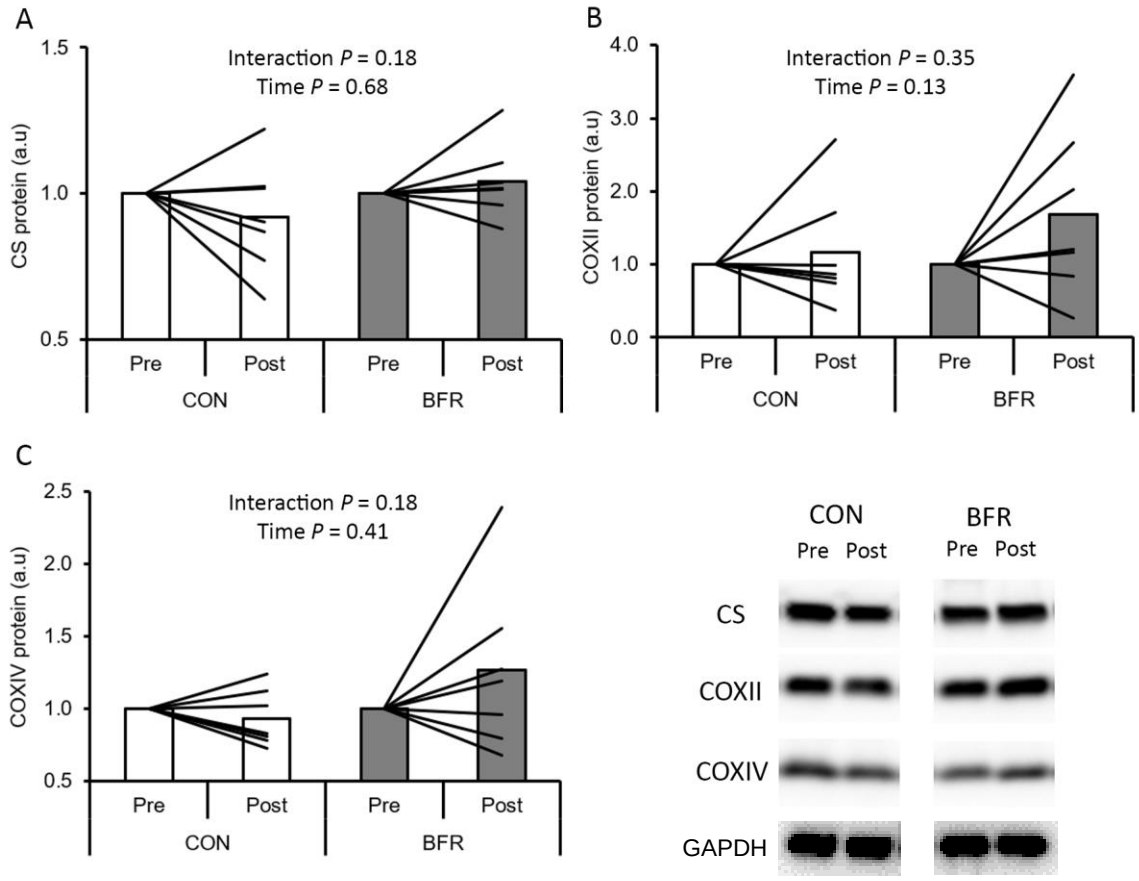

Figure 4.4. Content of CS (A), COXII (B) and COXIV (C) protein before and after control (CON) and blood flow restriction (BFR) training interventions. Values are expressed as fold changes relative to pre-training values. Bars represent the mean and lines represent individual responses. Data are CON n = 7; BFR n = 7.

4.6 DISCUSSION

This study has demonstrated that 4 weeks of SIT increased CP in trained individuals, however the addition of BFR did not enhance this further. Furthermore, SIT, with or without BFR, did not induce any changes in skeletal muscle capillarity or the content of mitochondrial proteins in our trained population. The study has, however, confirmed the potency of SIT combined with BFR in increasing $\dot{V}O_{2max}$ to a greater extent than SIT alone.

We had hypothesised that the increase in CP following SIT would be further enhanced with BFR. In contrast to this hypothesis, whilst there was an increase in CP of 3.6% with SIT, there was no greater enhancement with BFR which had a similar magnitude of increase of 3.3%. The improvement in CP is in line with previous research which has demonstrated increases in CP following HIIT (Gaesser & Wilson, 1988; Poole *et al*. 1990). The increases of 10-15% in the aforementioned studies are notably greater than that of the present study; however, considering our shorter training period and well-trained participants this is not surprising. Nevertheless, it is important to note that the gains of ~3.5% are greater than the smallest worthwhile change in power for well-trained individuals of 1% (Paton & Hopkins, 2001) and therefore represents a meaningful observation.

Given the established relationship between capillarity and high intensity exercise performance (Iaia *et al*. 2011), particularly CP (Mitchell *et al*. 2018), together with the potency of the acute angiogenic stimulus our novel training intervention provides (Taylor *et al*. 2016a), it was surprising that we did not observe an increase in any measure of capillarity in either training group. The lack of increase in capillarity could be due to the volume of training undertaken, given that, in comparison to rodents where angiogenesis can occur within a week (Waters *et al*. 2004), in humans it is generally considered that angiogenesis manifests later than other training adaptions, typically occurring after 4-5 weeks of training (Andersen & Henriksson, 1977; Jensen *et al*. 2004a). Whilst the current training protocol was 4 weeks in duration it involved only 8 low volume training sessions. Although SIT has been shown to increase skeletal muscle capillarity, this was in untrained individuals ($\dot{V}O_{2max}$ of 41.9 ± 1.8 $ml.min^{-1}.kg^{-1}$) and was after 18 sessions over a longer period of 6 weeks (Cocks *et al*. 2013), resulting in a training volume more than double that of the present study. Volume of training seems to play a greater role than intensity in stimulating angiogenesis (Gliemann, 2016). For example, HIIT has been shown to produce a lower acute angiogenic response in interstitial fluid than moderate intensity training (Hoier *et al*. 2013a) and an increase in training intensity at the expense of the

volume of training in trained individuals has been demonstrated to reduce skeletal muscle VEGF protein content (Gliemann *et al*. 2015) and stunt increases in capillarisation (Hoier *et al*. 2013a). Therefore, in spite of our hypothesis, the 8 sessions may still have been an insufficient stimulus to induce an increase in capillarisation. This is further exacerbated by the trained nature of our participants in which the baseline capillary to fibre ratio of 2.9 is around double that previously reported in untrained participants of ~1.4 (Andersen & Henriksson, 1977; Jensen *et al*. 2004a).

Nevertheless, the angiogenic potential of the present training intervention was further explored by investigating the presence of proliferating endothelial cells. The antibody Ki-67 detects a proliferation-associated nuclear antigen whereby its co-localisation within endothelial cells in skeletal muscle allows the identification of proliferating endothelial cells and thus location of growing capillaries (Hoier *et al*. 2010; Jensen *et al*. 2004a) and is therefore a measure that would precede increases in skeletal muscle capillarity. There was no significant change in Ki-67 positive endothelial cells following training in either group, although the interaction effect was $P = 0.06$ and there was an approximate 100% increase in Ki-67 positive endothelial cells following BFR, which was not present after CON. Indeed, there was a greater presence of the number of Ki-67 positive endothelial cells in 7 out of 9 participants of the BFR group compared to only one participant demonstrating any evidence of increased EC proliferation in CON and a calculation of Cohen's d revealed a medium effect size of 0.63. These observations suggest the potential of post-exercise BFR to enhance angiogenesis is worth further exploration and to induce measurable further adaptation in this already well adapted population may require a greater training period or a higher training volume.

There were no changes in the content of mitochondrial proteins CS, COXII and COXIV following either training group. This contrasts with previous findings which have consistently demonstrated increases in markers of mitochondrial biogenesis following SIT, including increased activity of CS and COX (Burgomaster *et al*. 2005; 2006; 2008; Gibala *et al*. 2006), increased protein content of COXII and COXIV (Burgomaster *et al*. 2007; Gibala *et al*. 2006) and increased maximal mitochondrial respiration (Granata *et al*. 2016). These previous investigations have, however, only been undertaken on untrained or recreationally active populations; therefore, it seems likely that the lack of effect in the present study is attributable to our already well adapted trained population.

In the absence of any observable angiogenesis and mitochondrial biogenesis the improvement in CP in both groups could be attributable to multiple factors related to the so-called anaerobic capacity of skeletal muscle. For example, increases in skeletal muscle buffering capacity (Weston *et al*. 1997) and monocarboxylate transporter protein (Bickham *et al*. 2006) have been demonstrated following HIIT in trained individuals. These adaptations, which would enhance the ability for the removal of fatigue inducing metabolites and thus be expected to increase the power at which the loss of metabolic homeostasis occurs, could be hypothesised to increase CP. Such adaptations may also be expected to increase W' which, as the second parameter of the power-duration relationship, has classically been considered to represent an anaerobic component (Morton, 2006). W' was, however, unchanged in the present study, as has typically been reported in many high-intensity training studies that have reported an increase in CP (Gaesser & Wilson, 1988; Poole *et al*. 2016; Vanhatalo *et al*. 2008). It is important to consider the interrelated nature of CP and W' which have regularly been reported to change in opposite directions in response to multiple interventions (Poole *et al*. 2016) and as such the observed increase in CP may have offset any increases in W'.

Although CP increased in both groups, $\dot{V}O_{2max}$ increased in the BFR group only. This supports our previous work (Taylor *et al*. 2016a) which initially demonstrated the potency of this novel training intervention, with an increase in $\dot{V}O_{2max}$ of ~4.5%, and agrees with further studies which have demonstrated an increase in $\dot{V}O_{2max}$ with the addition of BFR to low intensity (~40% of $\dot{V}O_{2max}$) exercise (Abe *et al*. 2010a; Park *et al*. 2010). Improvements in $\dot{V}O_{2max}$ have been demonstrated to precede increases in skeletal muscle capillaries (Andersen & Henriksson, 1977) and while submaximal thresholds are predominantly determined by peripheral mechanisms (Joyner & Coyle, 2008), central components, in particular maximal cardiac output, are considered to be the principal limiting factors of $\dot{V}O_{2max}$, at least within whole body exercise such as cycling (Bassett & Howley, 2000; Joyner & Coyle, 2008). Therefore, on the basis we have not observed any increase in capillarity, the increase in $\dot{V}O_{2max}$ following BFR is perhaps more likely to be attributable to central adaptations, i.e. increased cardiac output, with SIT alone not presenting a sufficient challenge within our trained population. The addition of BFR during and after exercise poses a significant challenge to the central cardiovascular system through the induction of the exercise pressor reflex (Alam & Smirk, 1937; Spranger *et al*. 2015) that results in an increase in HR and systolic blood pressure (Bull *et al*. 1989; Iida *et al*. 2007; Poton & Polito, 2015; Renzi *et al*. 2010; Staunton *et al*. 2015). Central cardiovascular adaptations have previously been reported with BFR exercise whereby Park *et al*. (2010)

demonstrated that 2 weeks of walk training combined with BFR increased stroke volume by 21.4%. Although no assessment of stroke volume was made in the control group of that study, so the effect of exercise alone is unknown, it seems plausible that the pressor reflex related stimulus of BFR could induce a central adaptive response that contributes to an increase in maximal cardiac output and thus $\dot{V}O_{2max}$.

The present study is not without its limitations. Whilst the use of trained individuals clearly enhances the validity of the application of the study findings to elite training practice, this resulted in a reduced sample size, in particular of participants who consented to muscle biopsies. Although typical of the current literature, the sample size must therefore be considered when interpreting the results, which is likely to have reduced the chances of correctly accepting or rejecting the null hypotheses. This is perhaps reflected in the present study with several parameters which displayed *P* values close to the critical value. An absolute BFR cuff pressure of 120 mmHg was also utilised for all participants. It is known that there is a variation between individuals in the level of BFR imposed by a standard absolute external cuff pressure (Hunt *et al*. 2016) which will affect the level of muscle oxygenation and muscle metabolite accumulation (Karabulut *et al*. 2011; Takarada *et al*. 2000). Indeed, unpublished work in our laboratory using the same standard cuff pressure suggests that the decrease in muscle oxygenation imposed by the post-exercise BFR in the present training protocol varies between 11 and 43% (Mitchell EA, Bailey SJ & Ferguson RA, unpublished observations). It is therefore possible that the physiological signals imposed with the addition of post-exercise BFR were not consistent between individuals and may have impacted the extent of any adaptations. Furthermore, the present study only focused on the peripheral adaptations to the current intervention. As discussed above it seems likely that the observed increase in $\dot{V}O_{2max}$ could be attributable to central adaptations, such as increased cardiac output. The central adaptive responses to BFR exercise clearly require further investigation.

In conclusion, the addition of post-exercise BFR did not enhance the increase in CP observed after 4 weeks of SIT in trained individuals. SIT with or without BFR did not induce any changes in skeletal muscle capillarity or the content of mitochondrial proteins. The study has, however, confirmed previous findings of the potency of combining post exercise BFR during SIT in enhancing $\dot{V}O_{2max}$ in trained individuals.

CHAPTER 5

THE EFFECT OF COMBINED SPRINT INTERVAL TRAINING AND POST-EXERCISE BLOOD FLOW RESTRICTION ON SKELETAL MUSCLE OXYGENATION

5.1 INTRODUCTION

A reduction in skeletal muscle oxygenation elicits an accumulation of the oxygen-sensing subunit HIF-1α and its translocation into the nucleus (Ameln *et al*. 2005; Jiang *et al*. 1996). In the nucleus HIF-1α binds to DNA and activates the expression of multiple target genes, one of which is the pro-angiogenic factor VEGF (Forsythe *et al*. 1996; Ke & Costa, 2006). Accordingly, skeletal muscle hypoxia is considered as a potent angiogenic stimulus (Egginton, 2009). Indeed, it has been demonstrated that undertaking training sessions under systemic hypoxia can elicit increases in skeletal muscle capillary supply which do not arise when the same exercise training is performed in normoxia (Desplanches *et al*. 1993; Geiser *et al*. 2001; Kon *et al*. 2014).

BFR reduces skeletal muscle oxygenation during low-load exercise (Tanimoto *et al*. 2005; Ganesan *et al*. 2015; Downs *et al*. 2014; Corvino *et al*. 2017). Moreover, when BFR is maintained during rest intervals the reductions in muscle oxygenation are sustained (Downs *et al*. 2014; Ganesan *et al*. 2015). Post-exercise BFR could therefore, have the potential to elicit a hypoxic environment which may be an important factor for optimising the angiogenic potential. Taylor *et al*. (2016) provided preliminary evidence to support this by demonstrating that HIF-1α mRNA expression was enhanced 3 hours post SIT with the addition of post-exercise BFR. Acute exercise elicits transient increases in HIF-1α mRNA expression when muscle is exposed to a sufficient hypoxic stimulus (Vogt *et al*. 2001). Therefore, the increase in HIF-1α mRNA expression observed likely reflects a reduction in skeletal muscle oxygenation during the restriction periods. This suggests that post-exercise BFR applied for only short periods (4 x 2 min) is adequate to reduce skeletal muscle oxygenation, however this has yet to be established.

In Chapter 4, it was observed that post-exercise BFR did not enhance performance, as assessed by CP, or skeletal muscle capillarity following 4 weeks of SIT. Given that training volume appears to be a more important factor than training intensity in eliciting angiogenesis in trained individuals (Gliemann *et al*. 2015; 2016; Hoier *et al*. 2013a), it seems likely the training volume may have been too low to elicit an increase in skeletal muscle capillarity in the trained population. Nevertheless, increasing the post-exercise BFR stimulus may also enhance the adaptive potential. BFR has previously been shown to reduce skeletal muscle oxygenation in a dose-response manner with the level of restriction pressure applied (Karabulut *et al*. 2011). Moreover, the extent of HIF-1α accumulation is increased with increasing reductions in muscle oxygenation (Jiang *et al*. 1996). Therefore, the hypoxia related stimulus of post-exercise BFR may be enhanced with different restriction protocols.

Therefore, the aim of the present study was to establish the level of skeletal muscle oxygenation using near-infrared spectroscopy (NIRS) during SIT with and without post-exercise BFR of the vastus lateralis (VL) and rectus femoris (RF) muscles (Study 1) and secondly to investigate whether the level of skeletal muscle oxygenation would be altered with different restriction protocols (Study 2). It was hypothesised that post-exercise BFR would reduce skeletal muscle oxygenation during the recovery periods and that this reduction would be greater with a higher cuff pressure.

5.2 METHODS

Study 1

Experimental Design

Six healthy males, all of whom undertook regular endurance training, volunteered to participate in the study. In a repeated measures design, participants undertook two sessions of SIT; alone (CON) and with post-exercise BFR (BFR) in a randomised order. The testing sessions were separated by a minimum of 72 hours. All testing protocols were undertaken on a mechanically braked cycle ergometer (SE-780 50, Monark, Stockholm, Sweden). Seat dimensions were adjusted during the initial visit and were kept consistent for the second session. Participants were instructed to arrive in a rested state, having refrained from strenuous exercise for 24 hours and abstained from caffeine and alcohol for 12 and 24 hours prior to the sessions, respectively.

Experimental Protocols

Following a 5 min warm-up at a frictional resistance of 1 kg, participants performed four repeated 30 s maximal sprints against a manually applied resistance equivalent to 0.075 kg/kg body mass. Each sprint was interspersed by a 4.5 min recovery period during which participants immediately dismounted the bike and lay in a semi-supine position upon a couch. In BFR, lower limb BFR was applied within 25 s of finishing each sprint. This was achieved through the rapid application of a pneumatic pressure cuff (13.5cm wide, Hokanson SC12L) to the proximal portion of each thigh, rapidly inflated (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source; Hokanson, Bellevue, WA, USA) to a pressure of ~120 mmHg for 120 s. The cuffs were then rapidly deflated and participants remained in the supine position until 30 s prior to the next sprint where they re-mounted the ergometer. In CON participants remained in the semi-supine position before re-mounting the ergometer in time for the subsequent sprint.

Study 2

In a case study format one participant undertook four sessions of SIT, which were undertaken as described above but with variations to the recovery protocol. These sessions were separated by a minimum of 6 days to minimise any training effects. The sessions the participant undertook were: 1) SIT with post-exercise BFR at 120 mmHg for 120 s (BFR120); 2) SIT alone (CON); 3) SIT with post-exercise BFR at 160 mmHg for 90 s (BFR160) and 4) SIT with post-

exercise BFR at 120 mmHg for 60 s, deflated for 30 s and re-inflated for a further 60 s (BFR2min).

Experimental Measures

A NIRS (NIRO-200NX, Hamamatsu Photonics KK) was utilised to monitor changes in muscle oxygenation status throughout the testing protocols. Two NIRS probes were affixed to the left leg; upon the RF and the VL, in line with two-thirds of the distance between the top of the patella and the inguinal crease, using double-sided adhesive tape. Participants wore elastic shorts which covered the probes to help prevent movement and reduce extraneous light exposure during data collection. Light was emitted at wavelengths of 735, 810 and 850 nm, with a light source-detector separation distance of 3 cm. The relative contribution of haemoglobin and myoglobin to the NIRS signal is unresolved; the changes in the concentrations of deoxy-haemoglobin and myoglobin (HHb) and oxy-haemoglobin and myoglobin (O_2Hb) were calculated using the modified Beer-Lambert method, and the tissue oxygenation index (TOI) was used to describe the oxygenation of the muscle during the protocol. All variables were sampled at 2 Hz and were interpolated into 1-s intervals. In study 1 data was expressed as a mean over the sprint (30 s), restriction (2 min when blood flow restriction was applied in BFR) and rest (final 30 s in supine position) periods for each repetition. In study 2, data is presented as 10 s averages, averaged across the four sprint repetitions for each session.

Statistics

For study 1 total work done was analysed using a paired samples *t* test and three factor (condition; BFR vs CON, repetition 1-4 and part; sprint vs restriction vs rest) repeated measures ANOVAs were utilised to analyse all subsequent analysis. When significant effects were identified paired samples *t* tests corrected with the Holm-Bonferroni step-wise method were used to locate differences (Atkinson, 2002). Data are displayed as mean ± SD. Significance was accepted at $P \leq 0.05$, and a statistical trend was accepted as $P \leq 0.10$.

5.3 RESULTS

Study 1

Total word done during each session was not different between CON (72 ± 3 kJ) and BFR (72 ± 3 kJ) conditions (P =0.33). All participants completed the full protocol in CON, however, two participants were unable to undertake the final sprint and restriction within BFR.

O_2Hb in RF (Fig 5.1A) and VL (Fig 5.1B) decreased during the sprints ($P < 0.01$) and increased during the recovery periods ($P < 0.001$; main effect of part $P < 0.001$). There was no difference between groups throughout the protocol in RF (main effect of group $P = 0.65$; group*part interaction effect; $P = 0.16$), although there was a trend for lower O_2Hb in BFR than CON in VL (main effect of group $P = 0.07$; group*part interaction effect; $P = 0.06$).

HHb in both RF (Fig. 5.1C) and VL (Fig. 5.1D) (main effect of part: $P < 0.001$) in BFR ($P < 0.05$), and in VL in CON increased during the sprints ($P < 0.05$) and tended to increase in the RF in CON ($P < 0.10$). HHb decreased during the restriction period in CON in the VL ($P < 0.01$) and tended to decrease in the RF ($P < 0.10$), however, in BFR HHb increased in RF ($P < 0.05$) and VL ($P < 0.05$; group*part interaction effect $P = 0.001$ and $P < 0.001$, respectively). During the restriction period HHb was 19 ± 8 and 15 ± 5 higher in BFR than CON in RF and VL respectively (main effect of group $P = 0.005$ and $P = 0.003$). During the rest period HHb decreased in BFR in RF and VL ($P < 0.05$) and in CON in VL ($P < 0.05$) and tended to decrease in RF ($P < 0.10$).

TOI in both RF (Fig. 5.1D) and VL (Fig. 5.1E) decreased during the sprints (main effect of part: $P < 0.001$) in both CON and BFR ($P < 0.05$). Following the sprint in CON TOI increased in both RF and VL during the initial recovery period ($P < 0.05$), however, excluding the initial sprint in VL, in BFR TOI in RF and VL was unchanged from the sprint during the restriction period ($P > 0.05$; group*part interaction effect $P = 0.006$, $P < 0.001$, respectively). During the restriction period TOI was 20 ± 10% and 11 ± 5% lower in BFR than CON in RF and VL respectively (main effect of group $P = 0.003$ and $P = 0.001$, respectively). TOI increased in the final recovery period in CON and BFR in RF ($P < 0.05$) and tended to increase in VL ($P < 0.10$).

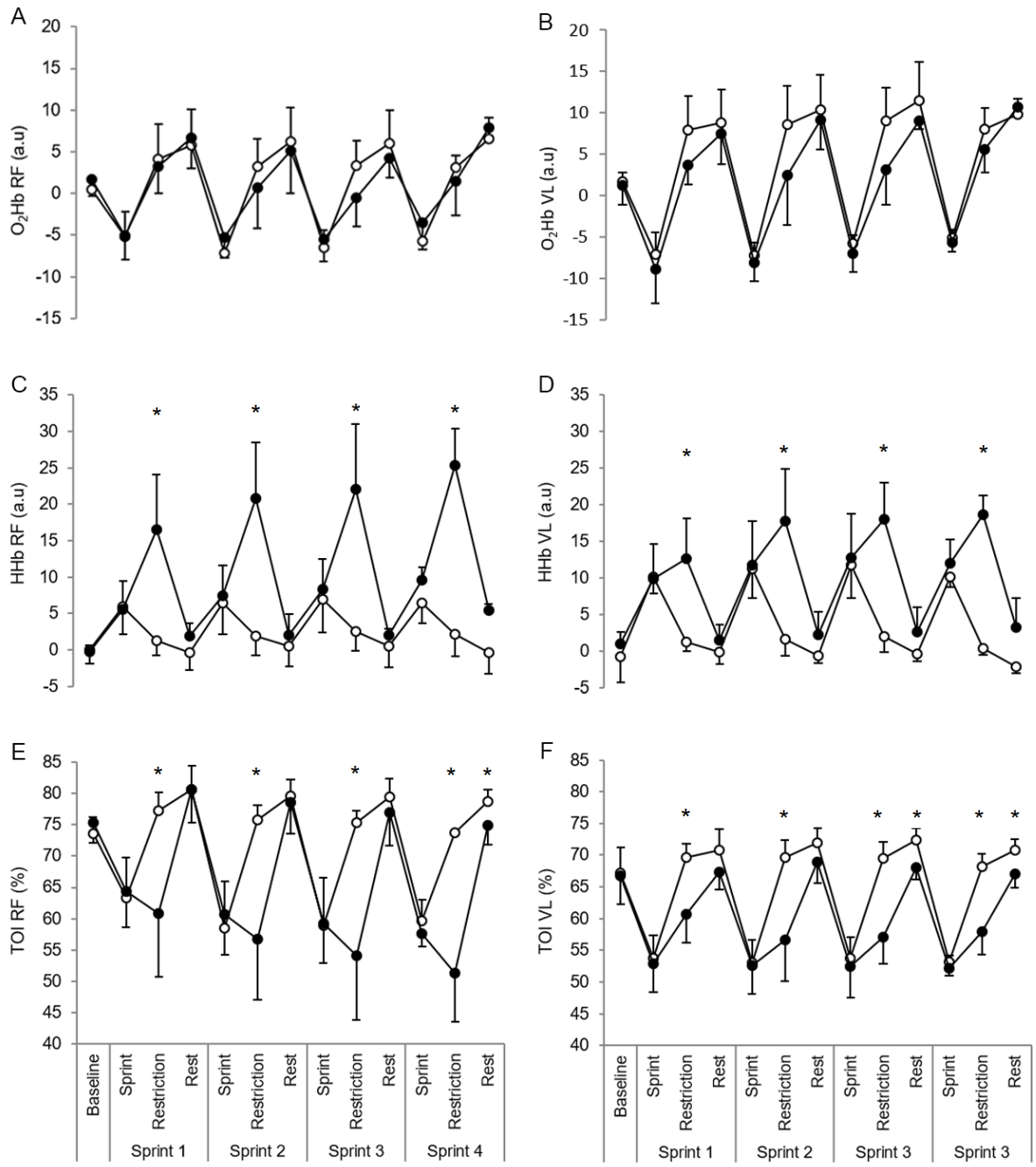

Figure 5.1. O_2Hb in RF (A) and VL (B), HHb in RF (C) and VL (D) and TOI in RF (E) and VL (F) during control (CON) (open circles) and blood flow restriction (BFR) (closed circles) sprint interval training sessions. * denotes significantly different to CON, $P < 0.05$.

Study 2

O_2Hb in RF (Fig. 5.2A) was similar between all four protocols at the end of the initial recovery period. In contrast in the VL (Fig. 5.2B) O_2Hb was lower in all three restriction protocols than CON during the initial recovery period. Following the initial cuff deflation in BFR2 O_2Hb increased to levels similar to CON and was only slightly reduced (2.0 a.u) upon the second restriction.

During the initial recovery period HHb decreased in RF (Fig. 5.2C) and VL (Fig. 5.2D) in CON but increased in all restriction protocols. BFR160 increased during the restriction period by a greater extent (256.9% in RF and 61.9% in VL) than BFR120 (184.4% in RF and 42.3% in VL) and BFR2min (126.0% in RF and 23.5% in VL). In BFR2 HHb was reduced during the 30 s deflation period but did not reach baseline values. The peak during the second restriction period was 14.2% and 24.0% lower in the RF and VL, respectively, than the first 60 s restriction period.

In RF (Fig. 5.2E) during the first 60 s of the restriction period mean TOI was similar in all three restriction protocols (BFR120 = 46.9%; BFR160 = 45.0%; BFR2 = 48.4%), but was notably lower than CON (67.7%). During the 30 s reperfusion period in BFR2 TOI increased towards baseline but was still 7.1% lower than CON at the same time point. The TOI during the second 60 s restriction period was 11.8% lower than the first restriction. TOI in the VL (Fig. 5.2F) was lower during the restriction protocols (BFR120 = 53.6; BFR160 = 57.9; BFR2 = 55.7) than CON (60.4%), but demonstrated much smaller differences than RF.

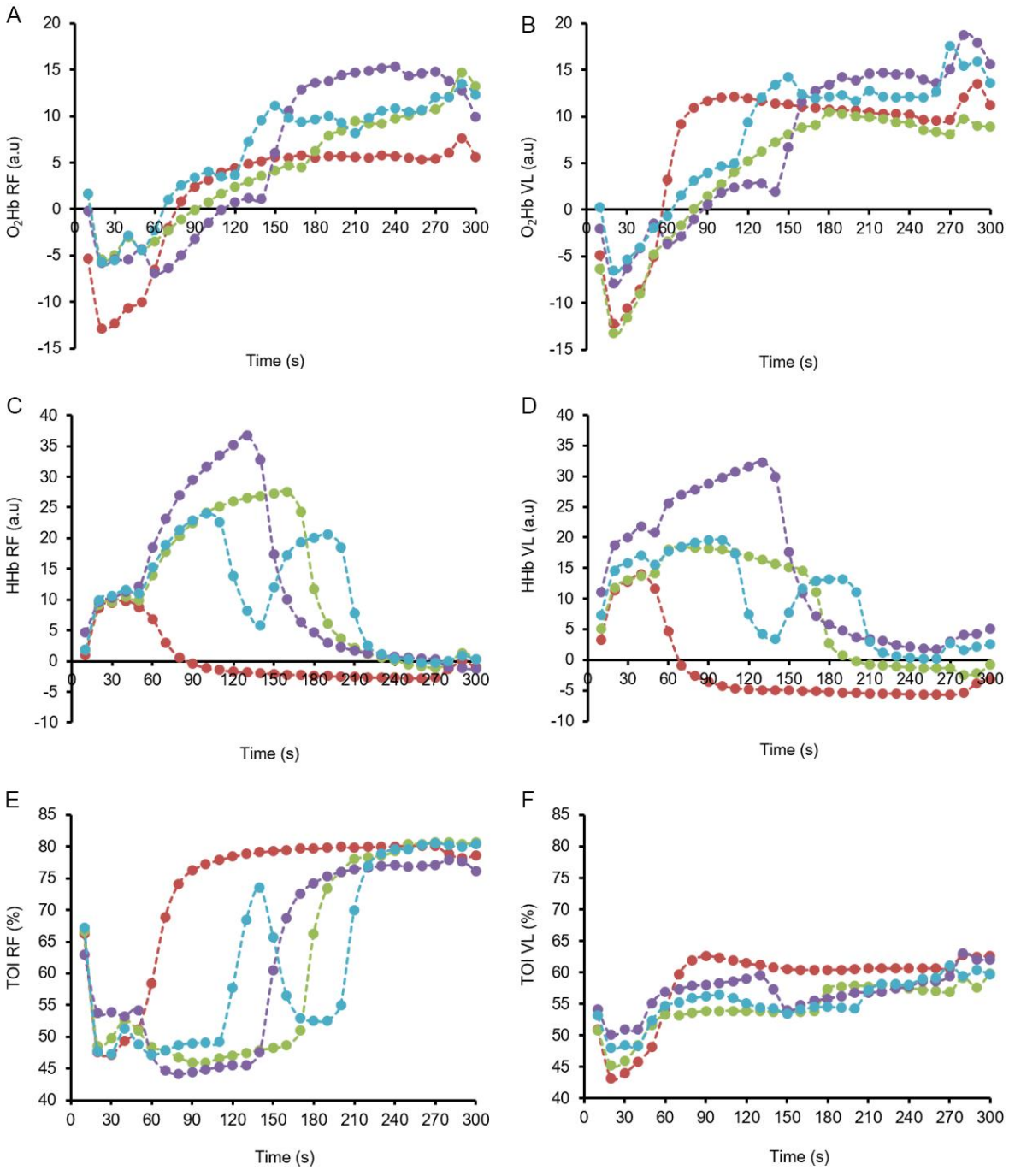

Figure 5.2. O_2Hb in RF (A) and VL (B), HHb in RF (C) and VL (D) and TOI in RF (E) and VL (F) during a single sprint and recovery period in CON (red), BFR120 (green), BFR160 (purple) and BFR2 (blue) sprint interval training sessions.

5.4 DISCUSSION

This study has demonstrated that the addition of post-exercise BFR to SIT reduces skeletal muscle oxygenation and increases HHb during the restriction periods. Whilst the restriction protocol had little effect on the extent of the reduction in TOI, a higher restriction pressure enhanced the increase in HHb.

In agreement with the hypothesis, post-exercise BFR combined with SIT reduced TOI during the recovery periods by ~20 and ~11% in the RF and VL muscle, respectively, compared with SIT alone. In addition, post-exercise BFR also substantially increased HHb. The HHb signal from NIRS is considered to provide a non-invasive index of local oxygen extraction and therefore reflects the balance between muscle oxygen delivery and utilisation (DeLorey *et al*. 2003; Grassi *et al*. 2003). Thus, the increase in HHb could be attributed to both the reduced arterial inflow and the occluded venous outflow with BFR application (Takano *et al*. 2005). Indeed, it is possible that a reduction in the rate of blood flow due to the venous occlusion facilitated oxygen extraction of the available blood supply (Ganesan *et al.* 2015) contributing to the continual rise in HHb. Perhaps surprisingly, there was no concomitant significant decrease in O_2Hb with post-exercise BFR. This is, however, consistent with previous observations in BFR exercise (Ganesan *et al*. 2015). The lack of change could, at least in part, be attributable to the fact that O_2Hb is more sensitive to changes in blood volume and total haemoglobin levels (Grassi & Quaresima, 2016) which are altered during BFR (Ganesan *et al*. 2015).

The reduction in TOI and increase in HHb are consistent with previous observations of enhanced HIF-1α mRNA following SIT combined with post-exercise BFR (Taylor *et al*. 2016a), and confirm that short periods of post-exercise BFR at a relatively low restriction pressure are sufficient to reduce skeletal muscle oxygenation of the quadriceps muscle. Hypoxic conditions are known to elicit angiogenic signalling (Ameln *et al*. 2005; Forsythe *et al*. 1995). Previous investigations have also demonstrated that undertaking exercise training sessions under conditions of systemic hypoxia can elicit increases in indices of skeletal muscle capillarity following both continuous endurance-type (Desplanches *et al*. 1993; Geiser *et al*. 2001) and resistance-type exercise (Kon *et al*. 2014). Therefore, these findings tentatively suggest that the local hypoxic conditions elicited with post-exercise BFR could contribute to an enhanced angiogenic response to interval training. Whilst this was not confirmed by the findings of Chapter 4 of this thesis, which demonstrated no increase in any indices of skeletal

muscle capillarity following SIT with or without post-exercise BFR, as discussed in Chapter 4, it seems likely that this could have been attributable in part to a training volume that was too low to elicit angiogenesis (Gliemann, 2016).

Alongside raising the volume of training, it is also possible that alterations to the post-exercise BFR protocols used in Chapter 4 could enhance the adaptive stimulus of future post-exercise BFR training protocols. Therefore, the aim of the second part of the present study was to investigate the effect of different BFR protocols on the hypoxic response of post-exercise BFR. In contrast to the hypothesis, a higher restriction pressure did not notably further reduce TOI. The small variations observed were perhaps more likely to be attributable to the variation arising from the replacement of the NIRS probes between the different protocols. However, HHb was considerably increased at 160 mmHg compared to 120 mmHg. This suggests that there was a greater imbalance between muscle oxygen delivery and utilisation during post-exercise BFR (DeLorey *et al*. 2003; Grassi *et al*. 2003) and therefore implies an increased hypoxic stimulus.

In addition to investigating different cuff pressures, the BFR2 protocol was selected to increase the number of BFR applications and reperfusions. Following cuff deflation there is a sustained hyperaemic response (Takarada *et al*. 2000; Gundermann *et al*. 2012) that would be expected to increase the level of shear stress, which is a key stimulus of angiogenesis (Egginton, 2009; Williams *et al*. 2006; Zhou *et al*. 1998). Therefore, an increased number of reperfusions could be hypothesised to enhance any shear stress mediated angiogenic stimulus of post-exercise BFR (Egginton, 2009; Williams *et al*. 2006; Zhou *et al*. 1998). The present study, however, demonstrated that following the initial 30 s reperfusion period that the magnitude of decrease in TOI and increase in HHb was reduced during the second 60 s restriction period. Therefore, although this protocol could enhance the shear stress stimulus, these findings suggest it may concomitantly reduce the level of hypoxia and subsequent signals induced.

NIRS is a non-invasive method used to give an indirect assessment of oxygenation of skeletal muscle, therefore it is important to note that there are limitations to this method. Firstly, the NIRS only measures the area directly beneath the probes. It is known that NIRS measurements can demonstrate spatial heterogeneity across the quadriceps muscle group (Koga *et al*. 2007). Both the RF and VL were investigated in the present investigation to account for this, nevertheless, it is still unclear whether these sites measured are reflective of muscle oxygenation across the whole quadriceps. It is also important to acknowledge that the NIRS

signal reflects a combination of microvascular haemoglobin and intracellular myoglobin, with the true relative contribution to the NIRS signal currently unknow. Indeed, current estimates suggest that myoglobin may contribute anywhere in a range of 60-90% to the NIRS signal (Barstow, 2019). Furthermore, the relative contribution of the arterioles, veins and capillaries to the haemoglobin signal is also unknown, with little or no information available on the relative PO_2 gradients between the capillaries and both the arterioles and veins during exercise and occlusion protocols (Barstow, 2019). Moreover, the interpretation of the NIRS device can be complicated by high adipose tissue thickness and increased skin blood flow, as occurs during exercise (Barstow, 2019). However, the NIRS derived HHb and TOI, which the conclusions of the present investigation are largely based on, have been shown not to be substantially altered by increased blood flow (Grassi & Quaresima, 2016; Koga *et al*. 2015; Messere & Roatta, 2013). In addition, the participants in the present investigation were all lean endurance trained individuals. It is therefore expected that these individuals did not possess sufficient adipose tissue thickness that would prevent the NIRS signal, which penetrates a distance of half of the source detector distance (Barstow, 2019), penetrating into the muscle.

In conclusion, this study has demonstrated that short periods of post-exercise BFR during SIT reduced TOI and increased HHb during the recovery intervals. Furthermore, the present results demonstrated that the increase in HHb with the addition of post-exercise BFR was increased with a higher cuff pressure, suggesting to optimise the hypoxic stimulus of post-exercise BFR protocols that where tolerable for sufficient durations, higher restriction pressures should be used.

CHAPTER 6

TIME COURSE OF THE TRANSCRIPTIONAL, TRANSLATIONAL AND PHENOTYPIC RESPONSE TO HIGH INTENSITY INTERVAL TRAINING COMBINED WITH POST-EXERCISE BLOOD FLOW RESTRICTION

Chapter 4 demonstrated that 4 weeks of SIT with or without post-exercise BFR did not elicit an increase in skeletal muscle capillarity. There was, however, a trend for an increase in proliferating endothelial cells after 4 weeks only when SIT was combined with post-exercise BFR. Given the importance of training volume for eliciting angiogenesis in trained individuals (Gliemann *et al*. 2015; Hoier *et al*. 2013a), it is therefore possible that the training volume of the SIT protocol was too low to elicit an increase in capillarity and that with a higher training volume post-exercise BFR could have elicited an increased capillary supply. Moreover, training volume also appears to be an important stimulus for increasing mitochondrial content (Granata *et al*. 2018).

In order to increase the training volume above that of the SIT protocol utilised in Chapter 4, this chapter investigated the use of post-exercise BFR during a higher volume interval training protocol, i.e. HIIT. In addition, given that Chapter 5 demonstrated that an increased restriction pressure enhanced the level of hypoxia, the restriction pressure was increased from ~120 mmHg to ~160 mmHg, which was possible for participants to tolerate given the reduced training intensity of HIIT vs SIT.

The main purpose of this chapter was to investigate the addition of post-exercise BFR to two weeks of HIIT on the magnitude and maintenance of the transient transcriptional response of angiogenic and biogenic genes and the translational and phenotypic response.

6.1 INTRODUCTION

Skeletal muscle demonstrates remarkable plasticity in its adaptive response to endurance exercise training, including increases in capillary supply and mitochondrial content (Hoppeler, 2016). Following an acute bout of exercise there is a transient increase in the mRNA expression of genes involved in the adaptive response which typically peak after several hours (Hoier *et al*. 2012; Jensen *et al*. 2004b; Pilegaard *et al*. 2003; 2005; Richardson *et al*. 1999; 2000; Hoier *et al*. 2012) as well translocation (Little *et al*. 2010a; Hoier *et al*. 2013b) and post-translational modification of key adaptive proteins (Egan & Zierath, 2013; Granata *et al*. 2017). Overtime, the cumulative effect of repeated training sessions elicits gradual increases in protein content, enzyme activity and phenotypic remodelling, ultimately leading to functional performance enhancements (Egan & Zierath, 2013; Perry *et al*. 2010).

As individuals become accustomed to an exercise stimulus there is a blunting of both the acute angiogenic (Richardson *et al*. 2000; Hoier *et al*. 2012) and mitochondrial biogenic (McConnel *et al*. 2005; Schmutz *et al*. 2006) molecular response. For example, Perry *et al*. (2010) clearly demonstrated an early attenuated transcriptional response to a specific exercise bout of the transcriptional coactivator PGC-1α, which is considered as the 'master regulator' of mitochondrial biogenesis (Hood, 2009; Puigserver & Spiegelman, 2003; Wu *et al*. 1999). They took muscle biopsies 4 hours after 4 training sessions at 5-day intervals over a 15-day training period of HIIT and observed a continual reduction in the acute increase in PGC-1α mRNA expression. This attenuation of gene expression was observed even with a continued progression in absolute training intensity. This blunting of the molecular response is reflected with attenuated increases in protein content and functional and structural adaptation in the trained state (Hoppeler *et al*. 1985; Perry *et al*. 2010; Saltin *et al*. 1977). Therefore, to promote continued adaptation in endurance trained individuals, who are accustomed to high training volumes across a range of exercise intensities, novel training methods capable of overcoming and better maintaining the attenuated molecular response are required.

Post-exercise BFR is a potential training tool that could enhance both the angiogenic and mitochondrial biogenic stimulus to training. Recent studies have demonstrated the potential potency of this novel training method, observing SIT combined with post-exercise BFR to increase $\dot{V}O_{2max}$ by ~4.5-6% in already trained individuals following just 4 weeks (Chapter 4; Taylor *et al*. 2016a). BFR can elicit multiple signals upon its application i.e. reduced muscle oxygenation (Karabulut *et al*. 2011) and increased shear (Gundermann *et al*. 2012), metabolic

(Suga *et al*. 2009; 2012) and oxidative stress (Christiansen *et al*. 2018), all of which are considered key stimuli of exercise-induced angiogenesis (Egginton, 2009) and/or mitochondrial biogenesis (Hood, 2009; Ljubicic *et al*. 2010).

The angiogenic and mitochondrial biogenic potential of both SIT combined with post-exercise BFR and SIT alone has been demonstrated with acute increases both in PGC-1α and VEGF mRNA (Taylor *et al*. 2016a), which is well regarded as the most central pro-angiogenic factor in skeletal muscle (Neufeld, *et al*. 1999; Olfert *et al*. 2016). Therefore, it was surprising that in Chapter 4 there was no increase in skeletal muscle capillarity or content of mitochondrial proteins following 4 weeks of SIT with BFR or SIT alone. This might have been due to the short training volume of the SIT intervention, being only 8 short training sessions in total, given that current evidence supports the importance of training volume over intensity for eliciting both angiogenesis (Gliemann *et al*. 2015; 2016; Hoier *et al*. 2013a) and increases in mitochondrial content (Granata *et al*. 2018). Therefore, it is possible that combining post-exercise BFR with a lower intensity HIIT protocol, which would allow for a greater training volume than SIT and an increased number of BFR applications and reperfusions, could elicit an enhanced adaptive response.

Therefore, the purpose of the present study was to assess the effect of post-exercise BFR during two-weeks of HIIT on the magnitude and maintenance of the transient transcriptional response of key angiogenic and mitochondrial biogenic genes and the translational and phenotypic response across the training period. It was hypothesised that the addition of post-exercise BFR would enhance and better maintain the acute transcriptional response of VEGF, PGC-1α and HIF-1α and elicit greater increases in VEGF protein and mitochondrial protein content and enzyme activity.

6.2 METHODS

Participants

Eleven healthy males (age; 22 ± 1 yr, height; 182.5 ± 6.8 cm, body mass; 84.6 ± 10.7 kg) volunteered to participate in the study. Participants were physically active, but none undertook regular endurance training. All completed health and muscle biopsy screening questionnaires prior to participation to mitigate for contraindications to maximal exercise, muscle biopsy procedures and BFR. Participants did not have a history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period. All experimental procedures were approved by the Loughborough University Ethics Approvals (Human Participants) Sub-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.

Experimental design

The study used an independent groups design, where participants were assigned to one of two groups to perform seven HIIT sessions over a two-week period either without (CON = 5) or combined with post-exercise BFR (BFR = 6). Muscle biopsies were obtained before and 3 hours after the first, fourth and seventh training sessions (Fig. 6.1). Prior to training participants were familiarised to training procedures and undertook a maximal ramp incremental test a minimum of 4 days before the first training session. Following a minimum of 2 days after completing the training, participants repeated the ramp incremental test.

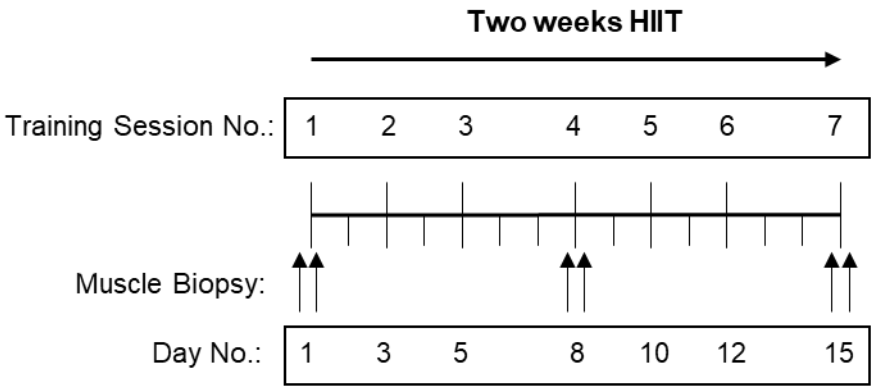

Figure 6.1. Schematic of training schedule and muscle biopsy timings

All performance tests and training sessions were conducted on an electronically braked cycle ergometer (Lode Excalibur Sport, Lode B.V. Gronigen, The Netherlands). Ergometer saddle and handle bar dimensions were recorded for each participant during preliminary testing and remained standardised for the rest of the study. Participants were instructed to maintain a normal diet throughout the study and to record dietary intake 24 hours prior to the maximal incremental ramp tests and the muscle biopsy experimental sessions. Participants were asked to replicate these leading up to the repeated assessments. Participants were further instructed to refrain from ingesting alcohol and caffeine 24 hours preceding the ramp incremental tests. Participants were instructed to abstain from any strenuous exercise 24 hours prior to the ramp incremental tests and any physical activity 48 hours prior to pre-training muscle biopsies. Ramp incremental tests and muscle biopsies were undertaken at approximately the same time each day (± 2 hours). Laboratory conditions during pre- and post-training exercise measurements remained constant (19-21°C, 40-50% humidity).

Maximal ramp incremental test

Participants performed a ramp incremental test to exhaustion to establish $\dot{V}O_{2max}$, maximal aerobic power (MAP) and the gas exchange threshold (GET). Participants began cycling, at a freely chosen, constant pedal cadence at 25 W, which was increased at a rate of 25 $W.min^{-1}$ in a linear fashion (0.4 $W.sec^{-1}$) until either volitional exhaustion or when cadence fell 10% below the freely chosen cadence for more than 5 s, despite strong verbal encouragement. Pulmonary gas exchange was measured continuously throughout exercise (Jaeger CPX, San Diego, USA). $\dot{V}O_{2max}$ and MAP were defined as the highest $\dot{V}O_2$ achieved for a 30 s period and the final power reached during the test, respectively. GET was determined using a cluster of measurements: the first disproportionate increase in carbon dioxide production vs $\dot{V}O_2$, an increase in expired ventilatory equivalent of oxygen in the absence of a concomitant increase in the expired ventilatory equivalent of carbon dioxide and an increase in end-tidal oxygen tension in the absence of a decrease in end-tidal carbon dioxide tension (Beaver *et al*. 1986; Whipp *et al*. 1986).

Exercise training

Participants completed seven supervised HIIT training sessions over a 15-day period, sessions were separated by a minimum of 48 hours. Each training session consisted of ten 3 min intervals performed at a power output equivalent to 60% Δ of the power achieved at GET and MAP. In the few instances when participants were unable to maintain the set power output for

10 intervals during the first session, the power output was lowered slightly so participants could complete the full training session. The mean power output undertaken during the training intervals was calculated, and participants then performed all subsequent training sessions at this power output. Each interval was separated by a 3 min recovery period, during which participants immediately dismounted the cycle ergometer and lay in a semi-supine position upon a couch. In BFR, pneumatic pressure cuffs (Hokanson SC12L) were applied as high up as possible on the proximal portion of each thigh and were rapidly inflated (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA) within 20 s to a pressure of ~160 mmHg. The cuffs remained inflated for 2 min before being rapidly deflated and participants remained in the supine position until 30 s prior to the next interval where they re-mounted the ergometer. In CON, participants remained in the semi-supine position until remounting the ergometer in time for the subsequent interval. Heart rate (HR) and the Borg rating of perceived exertion (RPE; 6-20 scale) were recorded at 2.5 min in each training interval. HR was also recorded 2 min into each recovery period. HR and RPE were averaged across the 10 intervals to give a mean HR and RPE for each training session. The intensity of training and pressure were kept constant throughout the training period. Total work completed during the training sessions was calculated as a sum of the product of the power output of each interval and interval duration.

Muscle biopsy procedure

Muscle biopsies were obtained, at rest, before and exactly 3 hours following completion of the first, fourth and seventh training session from the lateral portion of the vastus lateralis muscle. The post-training muscle biopsies were sampled at 3 hours post-exercise to coincide within the period peak mRNA expression of VEGF and PGC-1α have previously been observed post-exercise (Hoier *et al*. 2012; Jensen *et al*. 20014b; Little *et al*. 2011; Pilegaard *et al*. 2003) and close to the time HIF-1α mRNA has been demonstrated to be increased after BFR exercise (Larkin *et al*. 2012; Taylor *et al*. 2016a). Biopsies were performed under local anaesthesia (1% lidocaine) using the percutaneous needle biopsy technique with suction. Pre- and post-training biopsies during each experimental training session were taken on the same leg from separate incisions 2 cm apart. The leg biopsies were taken from were alternated between each experimental training condition. Muscle samples were snap-frozen in liquid nitrogen and subsequently stored at -80°C until analysis.

Muscle analysis

Real-time RT-PCR

One-step quantitative RT-PCR was used to determine muscle mRNA expression, with primer sequences designed by Sigma-Aldrich (PGC-1α, HIF-1α, eNOS and RPII) or Primer design (VEGF) (Table 6.1). Snap frozen muscle samples were homogenised in TRIzol reagent using a handheld TissueRuptor probe (Qiagen). Chloroform was added and following centrifugation the aqueous phase was separated. RNA was isolated following precipitation with 2-Propanol. The subsequent RNA pellet was washed once with 75% ethanol and was air-dried and resuspended in 50 µl of RNA storage solution (Fisher Scientific, Loughborough, UK). Sample RNA concentration (230 ± 117 ng/µl) and purity (260/280: 1.9 ± 0.1) were analysed using spectrophotometry (Nanodrop 2000, Thermo Scientific, Loughborough, UK). 10 µl PCR reactions were made as follows in 384 well plates: 18 ng of RNA in 4.5 µl of RNAase-free water, 5 µl of Precision PLUS One Step RT-qPCR Master Mix premixed with SYBR green (Primer Design, Southampton, UK) and 0.1 µl of both forward and reverse primers and 0.3 µl of RNAase-free water for Sigma-Aldrich primers or 0.5 µl of primer mix for Primer design primers. All reactions were run in triplicate. Following preparation of PCR plates, they were transferred to the qPCR cycler (ViiA 7 Real-Time PCR System, Applied Biosystems, Carlsbad, USA), with cycling conditions of one cycle at 55°C for 10 min and 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. Fluorescence was assessed at the end of each repeated cycle. mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, with RNA polymerase II (RPII) used as the reference gene. At the end of each PCR, melt curve analysis was undertaken to assess the specificity of primers. Values are reported as fold changes relative to the values from the pre-training biopsy.

Table 6.1. Primers used for real-time RT-PCR analyses

Target gene		Primer sequence
VEGF	Forward	CACTGAGGAGTCCAACATCAC
	Reverse	TGTGCTGTAGGAAGCTCATCTC
PGC-1α	Forward	ACTCTCGCTTCTCATACTC
	Reverse	CCTCTTCAAGATCCTGCTA
HIF-1α	Forward	AATCTGTGTCCTGAGTAGAA
	Reverse	TCACCTGAGCCTAATAGTC
eNOS	Forward	TGTGAAGGCTGTAGGTTAT
	Reverse	CAAGTTGGAATCTCGTGAA
RPII	Forward	AAGGCTTGGTTAGACAACAG
	Reverse	TATCGTGGCGGTTCTTCA

Abbreviations: eNOS, endothelial nitric oxide synthase; HIF-1α, hypoxia inducible factor 1α; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; RNA polymerase II; VEGF, vascular endothelial growth factor.

Preparation of whole muscle homogenate

Approximately 20–50 mg of frozen muscle tissue was homogenised in cold lysis buffer (1:10 Wet weight/volume) containing PBS, 0.2% Triton X-100 and protease and phosphatase inhibitor cocktail (Fisher Scientific, Loughborough, UK). Samples were blitzed using a tissue lyser (Qiagen, UK) for 4 min at 20 Hz and centrifuged at 12000 g for 10 min to pellet insoluble material. The supernatant was then transferred to a fresh Eppendorf tube. Protein concentrations were determined by Pierce 660 protein assay according to the manufacturer's instructions (Fisher Scientific, Loughborough, UK). Muscle homogenates were subsequently analysed for protein content (Western blotting) and CS enzyme activity.

Western blot analysis

Samples were mixed with dH_2O, 4x LDS sample buffer (Invitrogen, Loughborough, UK) and 0.1% β-mercaptoethanol (Sigma, Dorset, UK) and were boiled for 5 min at 95°C. 15-60 µg of protein was loaded on to TGX polyacrylamide gels (Bio-Rad, Herts, UK) and separated by electrophoresis at 100 V for 80 minutes. Proteins were transferred onto PVDF membrane at 30 V for 90 minutes (Bio-Rad, Herts, UK) and washed for 5 min in Tris buffered saline with tween (TBST) before being blocked in 5% blotting grade milk (Bio-Rad, UK) for 1 h at room temperature. Membranes were washed three times for 5 min in TBST and were incubated overnight at 4°C with the primary antibodies; CS (ab129095, abcam, Cambridge, UK), COXII

(ab110258, abcam, Cambridge, UK), COX IV (ab33985, abcam, Cambridge, UK) and VEGF (sc-7269, Santa Cruz Biotechnology, CA, USA) diluted at a concentration of 1:1000 in 2-3% milk. Membranes were then washed three times for 5 min in TBST and incubated with the appropriate secondary antibody; anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako, Stockport, UK) or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Herts, UK), diluted 1:10,000 in 2-3% blotting grade milk for 1 h at room temperature. Following three 5 min washes in TBST, membranes were incubated with enhanced chemiluminescence substrate (ClarityMax, Bio-Rad, Herts, UK) for 5 min. Membranes were visualised using image analysis (ChemiDocTM XRS+, BioRad, Herts, UK) and band densities determined using image analysis software (Quality One 1-D analysis software v 4.6.8, Bio-Rad, Herts, UK). GAPDH was used as a loading control and protein content was expressed in arbitrary units relative to GAPDH.

Enzyme activity analysis

CS activity was analysed on a 96 well plate. Each well contained: 150 µl 100 mM TRIS buffer (pH 8.3), 10 µl of 1 mg/ml muscle homogenate, 25 µl of 1 mM 5,5-dithio-bis (2-nitrobenzoic acid), 40 µl of 3 mM Acetyl coenzyme A and 10 µ of 1% Triton X-100. 15 µl of 10 mM oxaloacetate (pH 8.3) was added to each well and the plate was immediately placed in a spectrophotometer (Varioskan Flash, Thermo Scientific, Loughborough, UK) maintained at 30℃. Following 30 s of linear agitation, absorbance at 412 nm was measured every 15 s for 3 min. Values were corrected for the pathlength of the plate and were expressed as $mol.hr^{-1}.kg^{-1}$. The coefficient of variation for CS activity was 4.8 ± 3.1%.

Statistics

Training data was analysed using an unpaired t-test. Two-factor repeated measures ANOVAs with one within factor (time; pre vs post, 1-3, 1-6 or 1-7) and one between factor (condition; CON vs BFR) were utilised to analyse all subsequent analysis. Where significant effects were observed LSD *post hoc* analysis was undertaken to locate differences. Data are presented as mean ± SD unless otherwise stated. Significance was accepted at $P \leq 0.05$ and a statistical trend as $P \leq 0.10$.

6.3 RESULTS

There were no differences in physiological and performance measures prior to training between groups (Table 6.2). Participants completed 100% of the assigned training sessions without any complications, with the exception that one participant was unable to complete ten repetitions in the initial training session so undertook nine intervals for each session. The total amount of work done throughout the seven training sessions was not different (P = 0.82) between CON (2855 ± 268 kJ) and BFR (2779 ± 667 kJ).

mRNA expression

There was a main effect of time for VEGF mRNA (P = 0.005) (Fig. 6.2a). *Post hoc* tests revealed that VEGF mRNA increased 3 h post-exercise compared to rest in training session 1, 4 and 7 (P < 0.05). VEGF mRNA 3 h post-exercise was lower in training session 7 than session 4 (P = 0.03). There was however, no difference between CON and BFR (interaction effect P = 0.82; main effect of group P = 0.83).

There was a main effect of time for PGC-1α mRNA (P = 0.001) (Fig. 6.2b). PGC-1α mRNA increased 3 h post-exercise compared to rest in training session 1, 4 and 7 (P < 0.01). PGC-1α mRNA 3 h post-exercise was lower following training session 7 than 4 (P = 0.007) and tended to be lower following training session 7 than 1 (P = 0.09). There was however, no difference between CON and BFR (interaction effect P = 0.57; main effect of group P = 0.25).

There was a main effect of time for HIF-1α mRNA (P < 0.001) (Fig. 6.2c). HIF-1α mRNA expression increased 3 hours post-exercise compared to rest following training session 1, 4 and 7 (P < 0.01). There was a trend for resting HIF-1α mRNA to be higher on day 15 compared with day 8 (P = 0.07). There was however, no difference between CON and BFR (interaction effect P = 0.40; main effect of group P = 0.70).

There was no main effect of time (P = 0.10) or interaction effect (P = 0.14) for eNOS mRNA, however, there was a main effect of group (P = 0.03) (Fig. 6.2d). *Post hoc* tests revealed that eNOS mRNA was significantly higher in BFR pre (P < 0.001) and 3 h post (P = 0.03) training session 7 than CON; all participants in BFR had higher resting eNOS mRNA on day 15 compared to day 1, whereas all participants in CON had lower resting eNOS mRNA on day 15 compared to day 1.

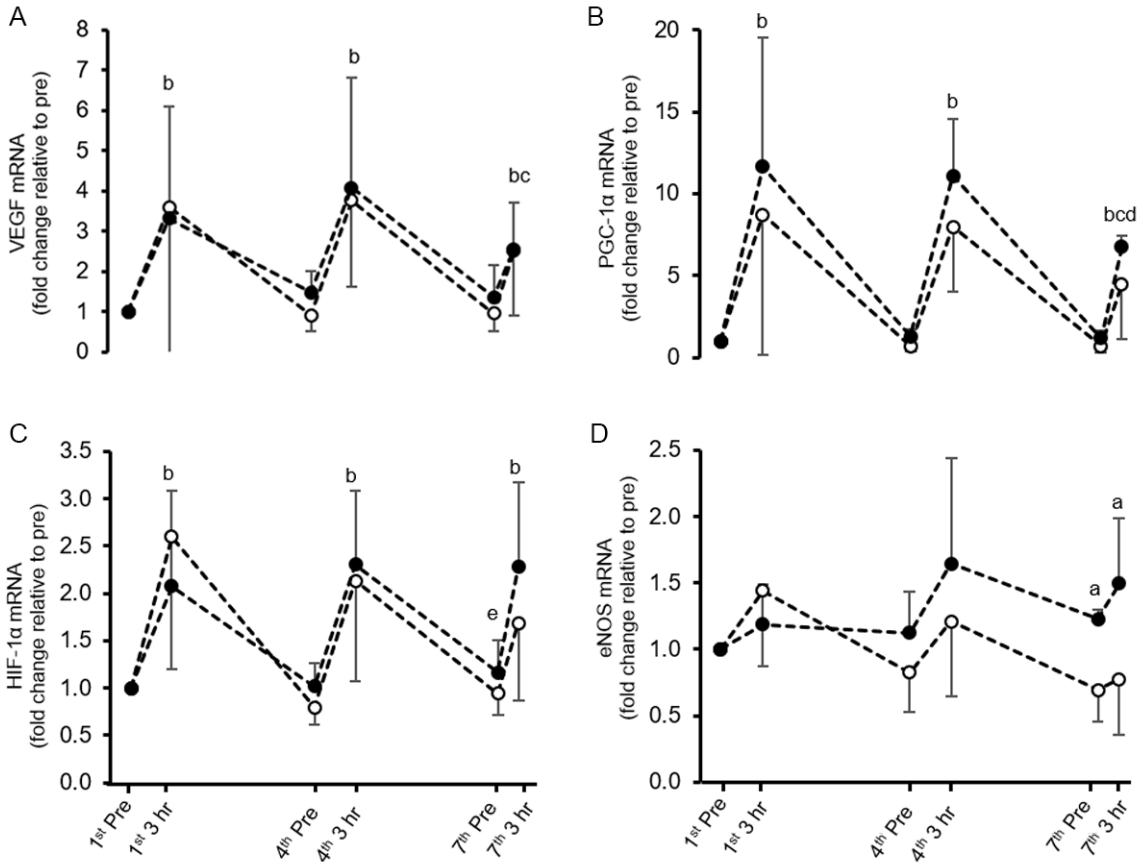

Figure 6.2. mRNA expression of VEGF (A), PGC-1α (B), HIF-1α (C) and eNOS (D) pre and 3 hours post-training sessions 1, 4 and 7 in control (CON) (open circles) and blood flow restriction (BFR) (closed circles). Values are expressed as fold changes relative to pre-training values. Data are mean ± SD. [a]$P < 0.05$ vs CON, [b]$P < 0.05$ vs pre, [c]$P < 0.05$ vs 4th 3 hr, [d]$P < 0.10$ vs 1st 3 hr pre, [e]$P < 0.10$ vs 4th pre.

Protein content

VEGF protein content (Fig. 6.3A) was unchanged with training (main effect of time $P = 0.26$) and was not different between CON and BFR (interaction effect $P = 0.29$; main effect of group $P = 0.28$). There was a near-significant trend for an increase in COXII (Fig. 6.3B), COXIV (Fig. 6.3C) and CS protein content (Fig. 6.3D) with training (main effect of time; $P = 0.09$, $P = 0.06$, $P = 0.06$, respectively), however there was no difference between CON and BFR (interaction effect and main effect of group $P > 0.10$). A Western blot at the correct molecular weight could not be attained for PGC-1α (described in detail in Chapter 8), therefore PGC-1α protein content was not assessed.

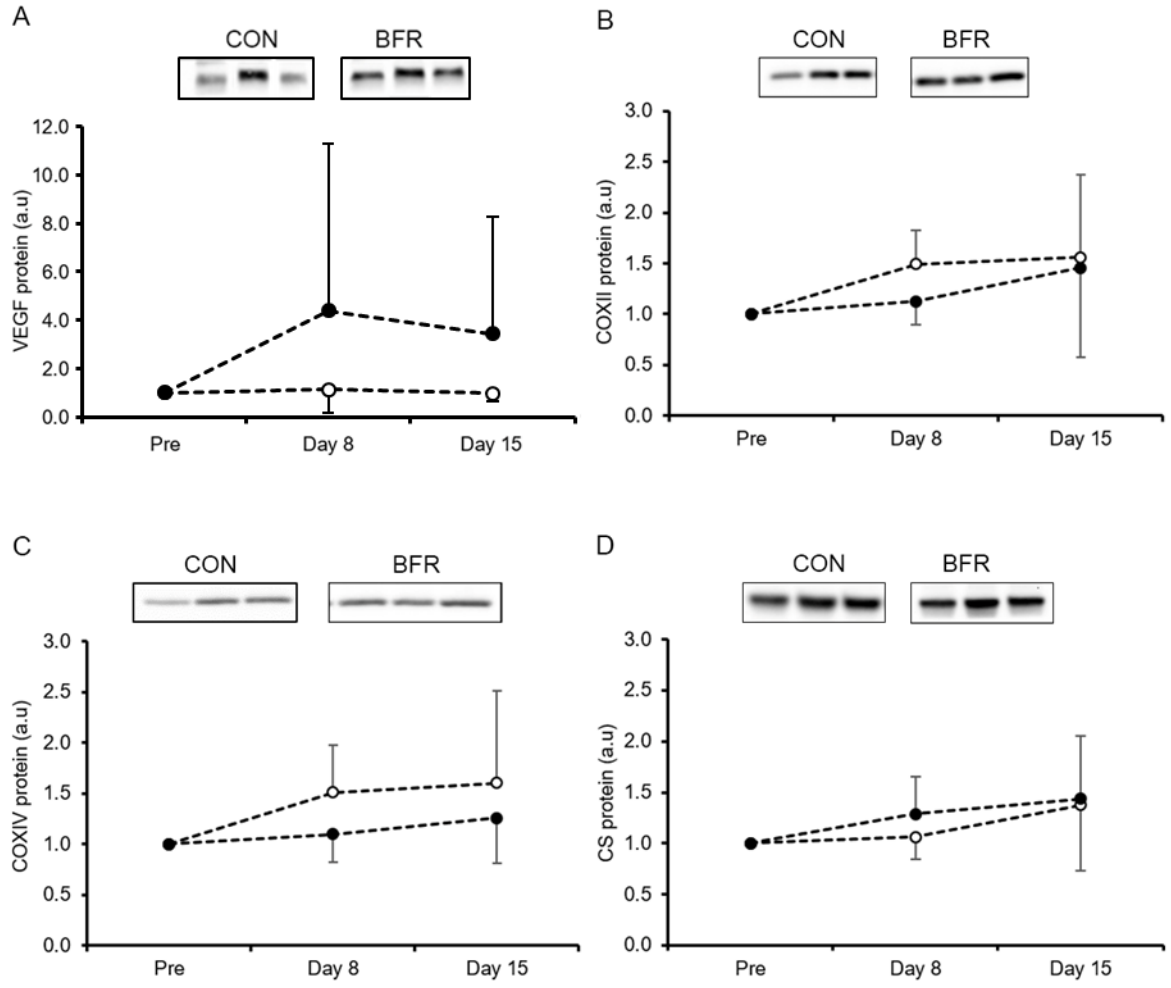

Figure 6.3. Protein content of VEGF (A), COXII (B), COXIV (C) and CS (D) in control (CON) (open circles) and blood flow restriction (BFR) (closed circles) training interventions. Values are expressed as fold changes relative to pre-training values. Data are mean ± SD.

CS activity

CS activity (Fig. 6.4) was unchanged with training (main effect of time $P = 0.42$) and was not different between CON and BFR (interaction effect $P = 0.58$; main effect of group $P = 0.61$).

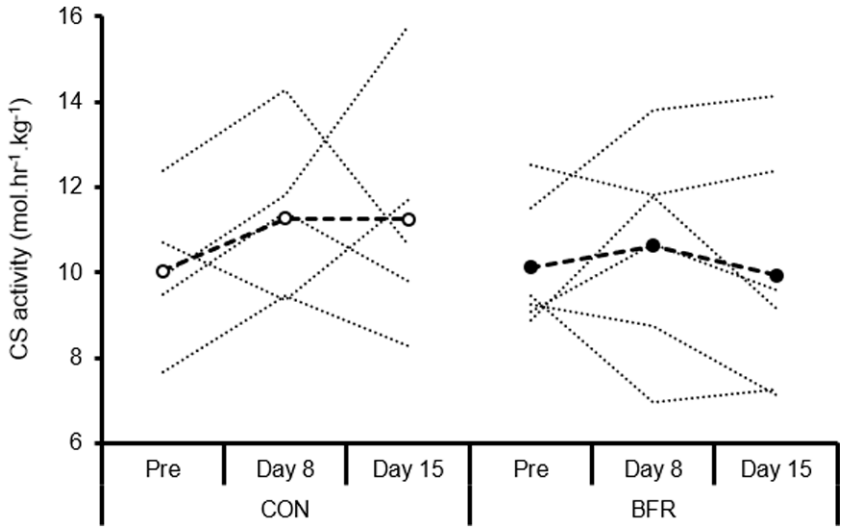

Figure 6.4. Citrate synthase (CS) activity in control (CON) (open circles) and blood flow restriction (BFR) (closed circles) training interventions. Markers with thick dashed lines represent means and thin dotted lines represent individual responses.

Performance variables

Absolute $\dot{V}O_{2max}$, relative $\dot{V}O_{2max}$, MAP and GET (Table 6.2) all increased with training, however, there were no differences between CON and BFR for any of the variables.

Table 6.2. Performance variables before and after control (CON) and blood-flow restriction (BFR) training interventions.

	CON			BFR					
Parameter	**Pre**	**Post**	**Percentage change**	**Pre**	**Post**	**Percentage change**	**ANOVA interaction *P* value**	**ANOVA main effect of time *P* value**	**ANOVA main effect of condition P value**
Body Mass (kg)	88.7 ± 6.8	88.8 ± 7.0	0.1	81.1 ± 12.6	80.8 ± 12.2	-0.4	0.63	0.74	0.24
$\dot{V}O_{2max}$ ($l.min^{-1}$)	3.70 ± 0.16	3.79 ± 0.16	2.5	3.50 ± 0.64	3.61 ± 0.62	3.3	0.80	0.04	0.63
$\dot{V}O_{2max}$ ($ml.min^{-1}.kg^{-1}$)	40.3 ± 2.0	41.3 ± 2.1	2.4	44.0 ± 9.5	45.4 ± 9.5	3.3	0.61	0.03	0.52
MAP (W)	327 ± 25	337 ± 20	3.0	326 ± 64	338 ± 58	3.6	0.72	0.004	0.99
GET (W)	102 ± 20	110 ± 16	8.0	111 ± 32	124 ± 34	11.9	0.36	0.003	0.52

Values are mean ± SD. Abbreviations: GET, gas exchange threshold; MAP, maximal aerobic power; $\dot{V}O_{2max}$, maximal oxygen uptake.

Training HR & RPE

There was a main effect of time on mean HR during exercise (P = 0.002). Mean HR during exercise was significantly reduced from training session 1 by training session 4 (P < 0.02) but did not significantly decrease further after training session 4 (P > 0.10) (Fig. 6.5a). There was no difference between CON and BFR (interaction effect P = 0.28; main effect of group P = 0.14). There was no main effect of time (P = 0.16) or interaction effect (P = 0.831) of mean HR during the recovery intervals, however there was a significant effect of group (P = 0.049) (Fig. 6.5b). *Post hoc* tests revealed that mean HR during the recovery intervals was significantly higher in BFR than CON during training session 4 (P = 0.02) and 7 (P = 0.04) and tended to be higher in BFR than CON during training session 2 (P = 0.08) and 6 (P = 0.06).

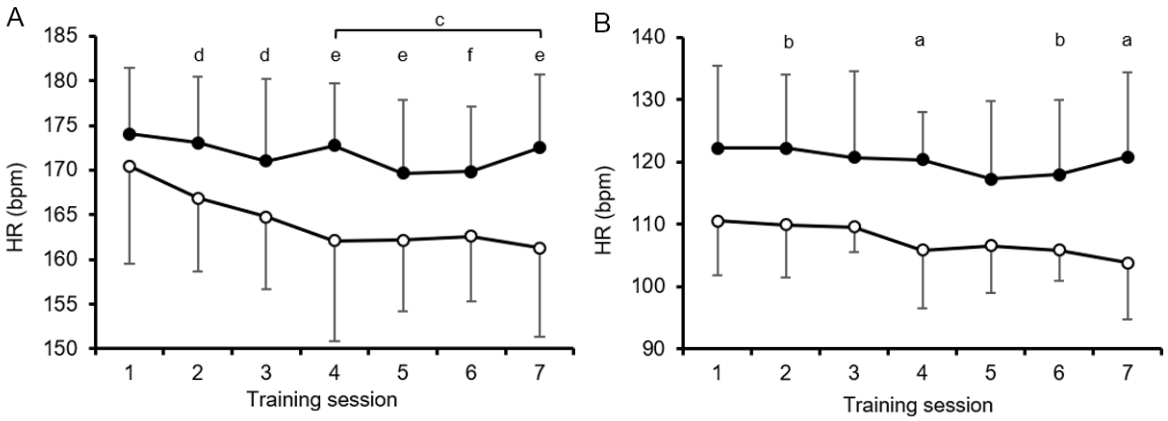

Figure 6.5. HR during exercise (A) and recovery (B) during control (CON) (open circles) and blood flow restriction (BFR) (closed circles) training interventions. Data are mean ± SD. ^{a}P < 0.05 vs CON, ^{b}P < 0.10 vs CON, ^{c}P < 0.05 vs 1, ^{d}P < 0.10 vs 1, ^{e}P < 0.05 vs 2, ^{f}P < 0.10 vs 2.

There was a main effect of time for RPE (P = 0.01). RPE was significantly reduced from training session 1 by training session 3 (P < 0.05) but did not significantly decrease further until training session 7 (Fig. 6.6). There was no difference between CON and BFR (interaction effect P = 0.14; main effect of group P = 0.76).

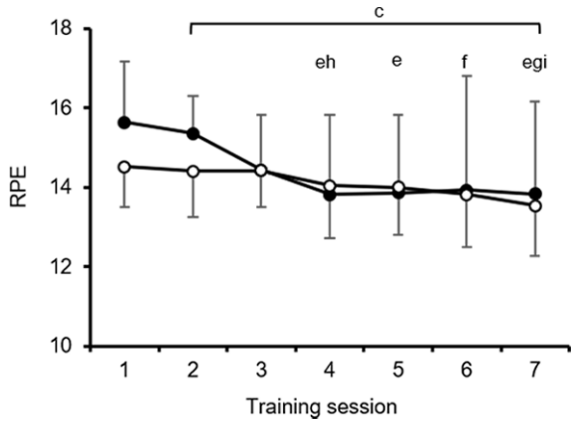

Figure 6.6. RPE during control (CON) (open circles) and blood flow restriction (BFR) (closed circles) training interventions. Data are mean ± SD. ^{c}P < 0.05 vs 1, ^{d}P < 0.10 vs 1, ^{e}P < 0.05 vs 2, ^{f}P < 0.10 vs 2, ^{g}P < 0.05 vs 3, ^{h}P < 0.10 vs 3, ^{i}P < 0.10 vs 5.

6.4 DISCUSSION

This study has confirmed previous findings by demonstrating that the transient increases in PGC-1α mRNA following single HIIT bouts are progressively attenuated over a two-week training period. These findings have been extended by demonstrating that similar responses occur in VEGF and HIF-1α mRNA. The addition of post-exercise BFR, however, did not enhance or better maintain these transient responses. In contrast, resting eNOS mRNA expression was increased only in BFR, suggesting that post-exercise BFR elicited an enhanced shear stress stimulus. There was no increase in mitochondrial protein content or CS activity following two weeks of HIIT with or without BFR.

Consistent with previous observations (Perry *et al*. 2010), the present investigation demonstrated an increased acute transcriptional response of PGC1-α 3 h following single HIIT sessions that was progressively attenuated during two weeks of training as individuals became accustomed to the specific HIIT session. This study has further demonstrated that a similar 'sawtooth' response to HIIT also occurs in VEGF and HIF-1α mRNA expression. Although peaking after one week, similar to PGC-1α, VEGF mRNA expression 3 h post-exercise was also significantly attenuated after two weeks of training. This is consistent with previous findings that have observed attenuated transient increases in VEGF mRNA following 4-8 weeks of training (Richardson *et al*. 2000; Hoier *et al*. 2012).

BFR applied during exercise and at rest has been shown to elicit multiple adaptive signals, i.e. increased shear stress, reduced muscle oxygenation and increased markers of oxidative stress (Christiansen *et al*. 2018; Gundermann *et al*. 2012; Karabulut *et al*. 2011), all of which are considered key stimuli of exercise-induced angiogenesis (Egginton, 2009) and/or mitochondrial biogenesis (Hood, 2009; Ljubicic *et al*. 2010). It was hypothesised that post-exercise BFR could also elicit similar signals and therefore would increase and better maintain the transient increases in transcription of key angiogenic and mitochondrial biogenic genes. However, in agreement with previous research investigating post-exercise BFR within a SIT protocol (Taylor *et al*. 2016a), there was no difference in VEGF or PGC-1α mRNA at rest or 3 h after training between CON and BFR. This suggests that post-exercise BFR is not a sufficient stimulus to enhance transcription of these genes. These findings are in contrast to BFR applied during low-load resistance-type or low intensity endurance-type exercise, which has been shown to enhance the acute expression of both PGC-1α and VEGF mRNA (Christiansen *et al*. 2018; Ferguson *et al*. 2018; Larkin *et al*. 2012). The exercise protocols in

the aforementioned studies undertaken alone did not elicit increases in VEGF mRNA and only elicited modest increases in PGC-1α mRNA expression of ~2-fold, compared to the 5-fold increase following SIT in Taylor *et al*. (2016a) and an initial increase of 9-fold in CON in the present study. Therefore, it seems likely that when combined with standard intensity training protocols, which are already potent stimuli of these genes, there is reduced capacity of BFR protocols to further increase the transcriptional response.

Furthermore, there was no difference in HIF-1α mRNA between CON and BFR. This is not consistent with Taylor *et al*. (2016a) who observed an increase in HIF-1α mRNA expression 3 hours post SIT only when combined with post-exercise BFR, or with the findings from Chapter 5 which demonstrated post-exercise BFR reduced muscle oxygenation by 11-20% during the recovery intervals of SIT. The increase in HIF-1α mRNA in the present study was, however, greater in both groups than that reported in the BFR group in Taylor *et al*. (2016a) (2.2 fold vs 1.5 fold). It is therefore possible that the increase in HIF-1α mRNA arising from training masked any effect of the BFR. Of note, in CON the magnitude of transient increases in HIF-1α mRNA decreased as training progressed but were maintained in BFR. Therefore, a BFR mediated hypoxic stimulus could have manifested with continued training. Moreover, the acute increase in HIF-1α mRNA expression can take longer to manifest, with one study demonstrating an increase in HIF-1α mRNA only after 6 hours post-exercise (Lundby *et al*. 2006), therefore the 3 h post biopsy may have missed the true peak in the transcriptional expression.

There was an increase in resting eNOS mRNA expression only in BFR. Indeed, at day 15 all participants in BFR had higher resting eNOS mRNA than at day 1, whereas in CON eNOS mRNA had decreased in all participants. eNOS expression is raised in response to increased levels of shear stress (Baum *et al*. 2004; Egginton *et al*. 2016) and therefore this finding suggests BFR increased the shear stress stimulus. This could have been elicited through two mechanisms: firstly, due to an increase in retrograde blood flow distal to the cuff arising from vessel compression (Credeur *et al*. 2010) and secondly through reactive hyperaemia upon cuff deflation (Gundermann *et al*. 2012). Although capillary growth was not assessed in the present study, given the importance of shear stress in exercise-induced angiogenesis (Egginton, 2009) and that eNOS is an important mediator of angiogenesis (Baum *et al*. 2004; Williams *et al*. 2006), tentatively it could be hypothesised that BFR elicited an increased angiogenic response. In support of this a previous study also observed an increase in resting eNOS mRNA following 4 weeks of passive knee extensor training in humans (Hoier *et al*. 2010), which increases shear

stress in the absence of alterations to either EMG activity or muscle oxygen uptake (Hellesten *et al*. 2008). Alongside the increase in eNOS mRNA, they also observed an increase in proliferating endothelial cells and capillary contacts. Further analysis of samples from the present study for proliferating endothelial cells would clearly help to confirm this hypothesis.

Despite the increases in VEGF mRNA expression in both groups, there was no change in skeletal muscle VEGF protein content during either CON or BFR. Previous studies have also not observed an increase in VEGF protein following a period of training after either continuous or HIIT (Hoier *et al*. 2012; 2013; Gliemann *et al*. 2015) even when there was an increase in capillarisation (Hoier *et al*. 2012), and varied responses have also been reported following an acute exercise bout (Gavin *et al*. 2004; Rullman *et al*. 2007; Hoier *et al*. 2013b). The importance of increased VEGF protein content for inducing angiogenesis is therefore not well-understood. For muscle-derived VEGF protein to promote angiogenesis secretion into the interstitial fluid is however required (Olfert *et al*. 2016). Indeed, increases in VEGF concentration in interstitial fluid have consistently been observed during and after exercise (Hoffner *et al*. 2003; Hoier *et al*. 2012; 2013b). The transient increases in muscle VEGF transcription observed in the present study may therefore have had a primary purpose to replenish lost stores rather than increase basal VEGF protein content (Hoier & Hellsten, 2014). Nevertheless, we did observe an increase in VEGF protein content in four participants in BFR and two participants in CON, suggesting VEGF protein content can be increased in the early stages of exercise training in some individuals.

Although GET increased in both groups, neither content of mitochondrial proteins CS, COXII and COXIV or CS activity, a biomarker of mitochondrial content (Larsen *et al*. 2012), were significantly increased following 2 weeks of HIIT with or without post-exercise BFR. The lack of change in CS activity is consistent with some studies (Cochran *et al*. 2014; Granata *et al*. 2016), but is in contrast to the majority of studies which have demonstrated large increases in CS activity of 20-30% in untrained individuals following a range of exercise modalities (Burgomaster *et al*. 2008; Granata *et al.* 2018; Gurd *et al*. 2010; Perry *et al*. 2008; 2010; Spina *et al*. 1996). The reason for this discrepancy could be attributable to methodological differences, including the assay method. Perry *et al*. (2010) utilised a very similar HIIT protocol to that of the present study and reported an increase of 17% in CS activity after just three training sessions. Given that training volume appears to be an important stimulus of CS activity (Bishop *et al*. 2014; Granata *et al*. 2018), this could perhaps be a factor. However, the volume of training was only minimally different to that of Perry *et al*. (2010) (10 x 3 min intervals vs

10 x 4 min intervals). Due to difficulty in controlling between the two conditions in the present study, in contrast to Perry *et al.* (2010), we selected not to increase the training intensity throughout the two weeks. This lack of progression could have failed to have induced a sufficient continued stimulus to increase mitochondrial content.

Previous studies have demonstrated that the addition of post-exercise BFR during classic SIT enhanced $\dot{V}O_{2max}$ by up to ~6% in trained individuals (Chapter 4; Taylor *et al*. 2016a). In contrast, in the present study post-exercise BFR did not augment the increase in $\dot{V}O_{2max}$ following HIIT. This discrepancy could be attributable to the shorter length of the present investigation at only 2 weeks long, compared to 4 weeks in the aforementioned studies. It seems plausible that this length of intervention may have been too short for any additional training effect of BFR to manifest. Moreover, this may have been exacerbated by the relatively untrained participants in the present study, meaning the exercise stimulus undertaken alone was a greater stimulus. Indeed, in contrast to the increase of ~2.5% in $\dot{V}O_{2max}$ after two weeks in the present study, 4 weeks of SIT failed to elicit any increase in $\dot{V}O_{2max}$ in the trained populations (Chapter 4; Taylor *et al*. 2016a).

The findings of the present study must be interpreted within the limitations of the current participant number of 11 (BFR n = 6 and CON n = 5). The observed powers for the majority of interaction effects and group effects were only ~0.2 and therefore, there is currently a high chance of type II errors in some of the outcomes. Clearly further participants are required to make firm conclusions of the effectiveness of the intervention. A further limitation of the study is the lack of measurement of the protein content of eNOS and PGC-1α (PGC-1α protein was not assessed due to technical difficulties in attaining a valid blot as described later in Chapter 8) and assessment of capillary growth (i.e. proliferating endothelial cells). This means implications can only be made from the responses at the transcriptional level. Moreover, there was only an assessment of markers of mitochondrial content (CS activity and the content of mitochondrial proteins) and not mitochondrial function. Previous investigations have demonstrated that an increase in mitochondrial function can occur in the absence of changes to mitochondrial content (Granata *et al*. 2016; Groennebaek *et al.* 2018). Therefore, it is possible that the interventions in the present investigation could have elicited mitochondrial adaptations that resulted in increased mitochondrial function.

In conclusion the present study demonstrated a ‘sawtooth’ pattern in the transcriptional response of PGC-1α, VEGF and HIF-1α to two weeks of HIIT. These transient increases 3 h

post-exercise in PGC-1α and VEGF mRNA were attenuated following the two-week training period. The addition of post-exercise BFR did not enhance or maintain these transcriptional responses. However, BFR may have elicited an increased shear stress mediated angiogenic stimulus, with an increase in resting eNOS mRNA expression observed after two weeks of HIIT only with post-exercise BFR. This hypothesis could be confirmed through the assessment of proliferating endothelial cells. Despite the potent increase in PGC-1α mRNA expression, CS activity and mitochondrial protein content were not changed following two weeks of HIIT, with or without post-exercise BFR.

CHAPTER 7

GENERAL DISCUSSION

7.1 SUMMARY

The studies described in this thesis have investigated the efficacy of combining post-exercise BFR with interval training as a potential training tool for endurance trained athletes. The main purpose of this thesis was to assess if this model of BFR could, in the absence of a reduction in training intensity, be utilised to overcome the blunted adaptive response observed in trained individuals. This was addressed with particular reference to augmenting angiogenesis and mitochondrial biogenesis and whether ultimately endurance performance could be enhanced, in particular within the severe-intensity exercise domain.

The main findings of the thesis are summarised below.

1. There was a very strong correlation between critical power (CP) and indices of skeletal muscle capillarity in trained individuals, in particular capillary contacts with type I fibres (r = 0.94). This implies that skeletal muscle capillarity is an important determinant of CP. On the other hand, W' was not related to skeletal muscle capillarity (Chapter 3).

2. Four weeks of 'classic' SIT (4-7 x 30 s maximal bouts) increased CP by ~3.5% in trained individuals, but the addition of post-exercise BFR did not enhance this further. However, consistent with previous observations the addition of post-exercise BFR was potent in increasing $\dot{V}O_{2max}$ (increase of 5.9%), with no change following SIT alone (Chapter 4).

3. Skeletal muscle capillarity did not increase following four weeks of SIT in trained individuals with or without post-exercise BFR. Tentatively, post-exercise BFR could elicit an enhanced angiogenic response when undertaken with a greater training

duration and/or volume, as suggested by a trend (P = 0.06) for an increase in proliferating endothelial cells after four weeks of SIT only with post-exercise BFR (Chapter 4).

4. The addition of post-exercise BFR to two weeks of lower intensity, higher volume HIIT did not enhance VEGF mRNA expression or protein content. However, a greater angiogenic stimulus from post-exercise BFR may be elicited by increased shear stress, implicated by an increase in resting eNOS mRNA after two weeks of HIIT only when combined with post-exercise BFR (Chapter 6). Furthermore, post-exercise BFR reduced skeletal muscle oxygenation during SIT restriction periods (Chapter 5) and could therefore enhance hypoxia mediated signalling.

5. Post-exercise BFR did not enhance or maintain the attenuating acute transcriptional response of PGC-1α during two weeks of HIIT (Chapter 6). Moreover, post-exercise BFR did not elicit increases in markers of oxidative capacity after 4 weeks of SIT in trained individuals (Chapter 4) or mitochondrial content after 2 weeks of HIIT in recreationally active individuals (Chapter 6).

7.2 CRITICAL POWER

This thesis has extended our current knowledge of the physiological underpinnings of CP, the asymptote of the power-duration relationship, and by extension high-intensity endurance performance. The primary purpose of Chapter 3 was to assess the relationship between skeletal muscle morphology and the parameters of the power-duration relationship in endurance trained individuals, specifically indices of skeletal muscle capillarity and proportion and CSA of type I and type II muscle fibres.

The key novel finding was the very strong positive correlations between CP and indices of skeletal muscle capillarity in trained individuals. In particular capillary contacts around type I fibres, which demonstrated a correlation coefficient of r = 0.94. This extends a previous observation of a significant correlation between capillary to fibre ratio and time to task failure during performance times of ~2-20 min (Iaia *et al*. 2011), which would be expected to span the duration of the severe-intensity domain. Although correlation does not directly establish causation, these very strong correlations imply that skeletal muscle capillarity is likely an important determinant of CP.

Specifically, the results suggest that the number of capillaries as opposed to diffusion distance *per se* are important for CP, given that only capillary to fibre ratios and capillary contacts were strongly correlated with CP and no significant correlation was observed with capillary density. This is perhaps not surprising given that oxygen extraction is determined primarily by the number of red blood cells in contact with contracting muscle fibres (Federspiel & Popel, 1986; Roca *et al*. 1992; Wagner, 1992). This assertion is also supported by previous observations demonstrating that an increase in capillary density following short-term immobilization did not enhance muscle diffusion capacity (Hepple *et al*. 2000).

Chapter 3 also confirmed previous findings in recreationally active individuals (Vanhatalo *et al*. 2016), demonstrating a positive correlation between the proportion of type I fibres and CP in endurance trained individuals. Furthermore, it was observed that the CSA of type I fibres was also positively correlated with CP.

A high skeletal muscle capillary supply enhances skeletal muscle oxygen extraction (Roca *et al*. 1992; Wagner, 1992) and type I fibres possess characteristics to facilitate high rates of oxidative phosphorylation including a higher mitochondrial content and increased fatigue resistance compared to type II fibres (Saltin *et al*. 1997; Schiaffino & Reggiani, 2011). Therefore, the positive correlations between these parameters and CP adds support to a previous body of literature implicating CP as a parameter of aerobic function. For example, previous investigations have demonstrated CP to be sensitive to changes in oxygen delivery, decreasing under conditions of systemic hypoxia (Dekerle *et al*. 2012) and BFR (Broxterman *et al*. 2015), and to be negatively correlated with the fundamental time constant of the oxygen uptake response to constant-load submaximal exercise (Murgatroyd *et al*. 2011). Moreover, CP has been observed to increase following training interventions also known to increase skeletal muscle capillarisation and mitochondrial biogenesis (Gesser & Wilson, 2007; Jenkins & Quigley, 1992; Poole *et al*. 1990).

Chapter 4 was the first investigation to demonstrate that SIT enhances CP. Previous investigations have demonstrated HIIT to increase CP by 10-15% following 4-7 weeks (Gaesser & Wilson, 1988; Poole *et al*. 1990; Vanhatalo *et al*. 2008). Whilst the enhancements of CP of ~3.5% observed in Chapter 4 are lower than the aforementioned studies, this is not surprising given the reduced training volume (total of just 8 sessions) and endurance trained status of the participants ($\dot{V}O_{2max}$ of ~63ml.min^{-1}.kg^{-1}). Moreover, the gains of ~3.5% are greater than the smallest worthwhile change in power for well-trained individuals of 1% (Paton

& Hopkins, 2001). Together CP and *W′*, the curvature constant of the power-duration relationship, describe cycling performance within the severe intensity domain (Monod & Scherrer, 1965). Therefore, with the absence of a decrease in *W′*, Chapter 4 demonstrates SIT to be an effective training protocol for endurance athletes competing within the severe-intensity domain.

Chapter 4 also demonstrated that despite the very strong correlations between CP and indices of skeletal muscle capillarity (Chapter 3), CP was enhanced without any concomitant increases in skeletal muscle capillary supply or mitochondrial protein content. Therefore, the improvements in CP could be attributable to factors classically related to the so-called anaerobic capacity of skeletal muscle, such as increases in skeletal muscle buffering capacity and monocarboxylate transporter proteins. Such skeletal muscle adaptations have been demonstrated to increase following HIIT in trained individuals (Bickham *et al*. 2006; Weston *et al*. 1996) and would enhance the ability for the removal of fatigue inducing metabolites. Since CP represents the highest sustainable power output in the absence of a progressive loss of muscle metabolic homeostasis, it therefore seems possible that these adaptations could be another determinant of CP. Furthermore, despite the fact that changes in CP were observed with training in the absence of any increase in capillary supply in Chapter 4, capillary supply is also likely to be important to CP by enhancing the removal of fatigue inducing metabolites such as H^+ and K^+ (Joyner & Coyle, 2008). Indeed, positive correlations between capillary supply and K^+ accumulation and muscle pH recovery have previously been observed (Iaia *et al*. 2011). Clearly the importance of these 'anaerobic' adaptations to CP requires further investigation.

CP and *W′* often change in opposite directions in response to multiple interventions (Poole et al. 2016), therefore it was not surprising that in contrast to CP, *W′* was not correlated to skeletal muscle capillary supply (Chapter 3). Moreover, consistent with previous observations in recreationally active individuals (Vanhatalo *et al*. 2016) *W′* was not correlated with type II fibre proportion or CSA (Chapter 3), despite *W′* being associated with characteristics also associated with type II fibres, including the VO_2 slow component (Murgatroyd *et al*. 2011; Vanhatalo *et al*. 2011b) and greater end-exercise muscle metabolic perturbations (creatine and lactate) (Vanhatalo *et al*. 2016). In the absence of these correlations, this suggests that *W′* is more closely related to other physiological events, for example there may be a neuromuscular contribution to the mechanistic underpinning of *W′* (Kordi *et al*. 2018).

7.3 ANGIOGENIC POTENTIAL OF POST-EXERCISE BFR

As discussed above, Chapter 3 implies that a high skeletal muscle capillary supply may be a key determinant of CP, and thus performance in the severe intensity domain. This finding highlights the importance of optimising the angiogenic potential of training protocols for endurance athletes. As outlined in the literature review (Chapter 2) it was suggested that post-exercise BFR could increase the extent of shear stress, metabolic stress and level of hypoxia induced by training, all of which are key stimuli of exercise-induced angiogenesis (Egginton, 2009). Therefore, it was hypothesised that post-exercise BFR would increase skeletal muscle capillarity.

Using a between groups experimental design, Chapter 4 assessed the potential of the addition of post-exercise BFR during a typical SIT programme (4 weeks of 4-7 repeated 30 s maximal sprints) to enhance skeletal muscle capillarity in a group of trained cyclists, with a $\dot{V}O_{2max}$ of a minimum of around 60 $ml.min^{-1}.kg^{-1}$. However, there was no increase in any index of skeletal muscle capillarity following 4 weeks of SIT with or without post-exercise BFR. This observation is in contrast to previous findings in a group of sedentary males ($\dot{V}O_{2max}$ of 41.9 $ml.min^{-1}.kg^{-1}$), where SIT was demonstrated to be a potent angiogenic stimulus, eliciting increases in indices of skeletal muscle capillarisation of 20-30% (Cocks *et al*. 2013). This disparity in findings suggests that in comparison to untrained individuals, SIT may not be a sufficient stimulus to elicit angiogenesis in endurance-trained individuals, who possess baseline skeletal muscle capillary supplies around double that of their sedentary counterparts (Andersen & Henriksson, 1977; Jensen *et al*. 2004a). However, the acute angiogenic stimulus of SIT with and without post-exercise BFR has been demonstrated in trained individuals, with the observation that VEGF, VEGFR-2 and PGC-1α mRNA increased 3 hours after a single training session (Taylor *et al*. 2016a). Therefore, as discussed in Chapter 4, given that current evidence supports the importance of training volume over training intensity for stimulating angiogenesis in already trained individuals (Gliemann *et al*. 2015; Hoier *et al*. 2013a), it seems likely that the training volume of the 4 weeks of SIT, which was just 8 sessions in total, may have been too low to elicit an increase in capillary supply in the trained population.

Nonetheless, there was still a suggestion of an enhanced angiogenic stimulus from the addition of post-exercise BFR in Chapter 4. To further investigate the angiogenic stimulus, proliferating endothelial cells were assessed by determining the co-localisation of the proliferation marker Ki-67 in CD31 positive endothelial cells. As a marker of capillary growth that would be

expected to proceed any measurable changes in skeletal muscle capillary supply (Jensen *et al*. 2004a; Hoier *et al*. 2010), this may have been more sensitive given the short intervention. There was a near significant interaction effect (P = 0.06), indicating an increase in Ki-67 positive endothelial cells in the BFR group only, with an increase in proliferating endothelial cells in 7 out of 9 participants in BFR, whereas only one participant demonstrated an increase in CON. This trend tentatively suggests that the addition of post-exercise BFR may have elicited an angiogenic stimulus which could have manifested had training been continued for a greater duration or had been undertaken with a higher training volume. In support of this suggestion, preliminary data on 5 participants from a follow-up study currently being conducted demonstrates an increase in capillary to fibre ratio and capillary contacts around type I fibres of 13.7% and 11.5%, respectively, following six weeks of the same SIT combined with post-exercise BFR training in trained cyclists.

Angiogenic signalling from post-exercise BFR

The angiogenic potential of post-exercise BFR was further explored in Chapter 6 during the first two weeks of a HIIT protocol. In a thorough approach, muscle biopsies were obtained pre and 3 hours post the first, middle and final training sessions across a two-week period. This allowed the investigation of whether post-exercise BFR would not only enhance the initial transient angiogenic transcriptional response following a single session, but also whether post-exercise BFR could better maintain the attenuated transient mRNA expressions expected at the end of the training period (Hoier *et al*. 2012; Richardson *et al*. 2000) and subsequently increase VEGF translation during the two week period. With a view to increase the training volume from Chapter 4 to enhance the angiogenic stimulus, post-exercise BFR was combined with a lower intensity, higher volume HIIT protocol. In a between groups design individuals undertook 7 training sessions, over a 14-day period, each involving 10 repetitions of 3 min cycling bouts at an intensity of 60%Δ of the GET and MAP interspersed with 3 min recovery periods. In the BFR group, this permitted a total of 10 applications of BFR (2 min at ~160 mmHg) and reperfusions compared with only 4 or 5 (2 min at ~120 mmHg) in the first two weeks of the SIT protocol in Chapter 4. Indeed, Taylor *et al*. (2016) previously established that the addition of post-exercise BFR to SIT did not enhance the increase in VEGF or VEGFR-2 mRNA 3 hours following a single training session.

HIIT elicited transient increases in VEGF transcription following single sessions that were attenuated after two weeks, however, there was no difference in VEGF mRNA at any timepoint

between the control and BFR group. This is consistent with the findings of Taylor *et al*. (2016), suggesting that post-exercise BFR is not a sufficient stimulus to increase VEGF transcription. Moreover, there was no increase in VEGF protein content following HIIT with or without post-exercise BFR. VEGF is well regarded as the most central pro-angiogenic factor in skeletal muscle (Neufeld, *et al*. 1999; Olfert *et al.* 2010; Wagner *et al*. 2006; Olfert *et al*. 2016) and is critical for exercise-induced angiogenesis (Olfert *et al*. 2010; Delavar *et al*. 2014). Therefore, the lack of change in the protein content of VEGF may suggest a failure to alter the angiogenic stimulus of the training intervention.

Nevertheless, the importance of increased baseline VEGF protein content for eliciting angiogenesis is not currently well established (Hoier & Hellesten, 2014). In contrast, for muscle-derived VEGF protein to promote angiogenesis, VEGF secretion into the extracellular space is known to be required (Olfert *et al*. 2016). The concentration of VEGF in interstitial fluid appears to parallel the proliferative effect on endothelial cells (Hoffner *et al*. 2003; Hoier *et al*. 2013a; Hoier & Hellsten, 2014) and therefore, the magnitude of VEGF secretion may be more important for the magnitude of angiogenesis. Whilst in healthy individuals, training does not appear to alter the exercise-induced increase in muscle interstitial VEGF (Hoier *et al*. 2012; Olfer *et al*. 2016), this can vary between exercise training protocols (Hoier *et al*. 2013a). Therefore, in the absence of immunohistochemical analysis of VEGF localisation and analysis of interstitial fluid for VEGF concentration, the full effect of the training intervention on VEGF signalling cannot be confirmed.

A reduction in muscle oxygenation elicits an accumulation of the oxygen-sensing subunit HIF-1α protein content and translocation into the nucleus (Ameln *et al*. 2005), where it binds to DNA and activates the expression of multiple target genes, one of which is VEGF (Forsythe *et al*. 1996; Ke & Costa, 2006). The finding that post-exercise BFR during SIT increased HIF-1α mRNA (Taylor *et al*. 2016a) therefore, provided preliminary evidence that BFR decreases muscle oxygenation which may lead to enhanced downstream hypoxia mediated angiogenic signalling. In support of this Chapter 5 established the muscle oxygenation response using NIRS during SIT with and without BFR. During the recovery periods post-exercise BFR reduced TOI, an overall indication of muscle oxygenation, by 20% and 11% in the RF and VL muscles, respectively, and increased HHb, which is considered to reflect the imbalance between muscle oxygen delivery and utilisation (DeLorey et al. 2003; Grassi et al. 2003) by 176% and 44% in the RF and VL respectively.

Unexpectedly, in contrast to SIT, post-exercise BFR did not enhance the increases in HIF-1α mRNA following a single HIIT session, with a similar mean increase of ~2.2-fold in both the control and BFR group (Chapter 6). This was even in despite of an increased cuff pressure of ~160 mmHg, which as demonstrated by Chapter 5, would be expected to increase HHb further. In contrast to HIIT, Taylor *et al*. (2016) reported SIT alone did not increase HIF-1α mRNA, and the increase in HIF-1α mRNA expression following SIT combined with post-exercise BFR was still lower (1.5-fold) than that observed with HIIT alone in Chapter 6. Therefore, post-exercise BFR may only increase HIF-1α transcription when this is not elicited by the exercise stimulus alone. Perhaps in support of this was the observation that in the control group in Chapter 6 the magnitude of transient increases in HIF-1α mRNA decreased as training progressed but was maintained in BFR. It is also important to note, that HIF-1α protein levels can be increased in response to a single exercise bout in the absence of any change in HIF-1α mRNA expression (Ameln *et al*. 2005), with HIF-1α protein levels largely regulated at the post-translational level (Semenza, 2000). Therefore, increased HIF-1α signalling with BFR in Chapter 6 cannot be ruled out in the absence of the analysis of HIF-1α protein content.

Arguably the finding of greatest interest in Chapter 6 was the observation that resting eNOS mRNA was increased following 2 weeks of HIIT only when combined with post-exercise BFR. Since eNOS expression is raised under conditions of increased shear stress (Baum *et al*. 2004; Egginton *et al*. 2016), this observation suggests that the addition of post-exercise BFR may have elicited an increased shear stress stimulus. Given that shear stress can be potent at eliciting angiogenesis (Rivilis *et al*. 2002; Williams *et al*. 2006; Zhou *et al*. 1998) and eNOS appears to be critical to shear stress-stimulated angiogenesis (Baum *et al*. 2004 Milkiewicz et al. 2005), tentatively, this suggests that the addition of post-exercise BFR may have elicited an increased shear stress-mediated angiogenic stimulus. Although, no measure of capillary growth was measured in Chapter 6 to confirm this, a previous investigation provides some support of the above hypothesis. Four weeks of passive knee extension training in humans, which increases shear stress in the absence of an increase in muscle activation or metabolic activity (Hellesten *et al*. 2008), was similarly demonstrated to increase resting eNOS mRNA expression and also increased proliferating endothelial cells and capillary contacts after 2 weeks (Hoier *et al*. 2010).

The potential of post-exercise BFR to increase the shear stress stimulus during HIIT could have implications for training prescription. HIIT forms an important part of the training regime of endurance athletes, with strong evidence demonstrating that the inclusion of HIIT is required to optimise adaptations and performance (Laursen *et al*. 2002; 2005; Lindsay *et al*. 1996;

Westgarth-Taylor *et al*. 1997; Weston *et al*. 1997). Moreover, during a tapering phase HIIT can be a key training method when athletes reduce training load through a decrease in their training volume (Mujika, 2010; Spilsbury *et al*. 2015). However, as mentioned above current evidence suggests that training volume as opposed to training intensity is a greater stimulus for angiogenesis in already trained individuals, at least in the short term. Studies have demonstrated a greater proliferative effect arising from continuous moderate intensity exercise than intense interval training (Hoier *et al*. 2013a) and a reduction in skeletal muscle VEGF protein content when trained runners increased bouts of intense exercise at the expense of total training volume (Gliemann *et al*. 2015). Gliemann (2016) proposed that one reason for a reduction in the angiogenic stimulus with a reduced training volume was the reduction in the shear stress stimulus. Shear stress is a product of both magnitude and duration, but although high levels of shear stress are elicited during intense intervals, it seems likely that this is outweighed by the prolonged stimulus continuous moderate intensity exercise elicits (Gliemann, 2016). Therefore, if post-exercise BFR does increase shear stress, either during the restriction periods with an increase in retrograde blood flow while vessels are under compression (Credeur *et al*. 2010) or through reactive hyperaemia following the cuff deflations (Gundermann *et al*. 2012), it could falsely increase this aspect of 'the volume stimulus' of training helping to maintain the angiogenic stimulus.

PGC-1α can also play a role in mediating exercise-induced angiogenesis (Chinsomboon *et al*. 2009; Geng *et al*. 2010) and is upregulated in response to multiple stimuli, including ROS and increased metabolic stress (Olesen *et al*. 2010), which were both hypothesised to be increased by post-exercise BFR. However, the addition of post-exercise BFR to either SIT (Taylor *et al*. 2016a) or HIIT (Chapter 6) did not enhance the transient increases in PGC-1α mRNA.

In conclusion, although Chapter 4 did not demonstrate an increase in skeletal muscle capillarity following SIT combined with post-exercise BFR, the angiogenic potential of post-exercise BFR was still suggested by the trend towards enhanced proliferating endothelial cells (Chapter 4), an increase in resting eNOS mRNA (Chapter 6) and reduced muscle oxygenation (Chapter 5) with the addition of post-exercise BFR. Therefore, the effect of post-exercise BFR protocols during lower intensity, higher volume HIIT on skeletal muscle capillarity warrants further investigation.

7.4 MITOCHONDRIAL BIOGENIC POTENTIAL OF POST-EXERCISE BFR

In addition to enhancing angiogenesis, it was also hypothesised that post-exercise BFR would augment the mitochondrial biogenic stimulus to interval training. It was postulated that as observed with BFR applied during exercise, that post-exercise BFR could also increase metabolic stress (Suga *et al*. 2009; 2012; Takano *et al*. 2005; Takarada *et al*. 2000) and production of ROS (Christiansen *et al*. 2018) and increase downstream signalling of PGC-1α, which is considered as the 'master regulator' of mitochondrial biogenesis (Wu *et al*. 1999; Puigserver & Spiegelman, 2003).

Consistent with previous observations (Perry *et al*. 2010), in Chapter 6 the acute increase in PGC-1α mRNA expression following single HIIT sessions was progressively attenuated throughout two weeks of training. In contrast to the aforementioned hypothesis, however, as discussed above, post-exercise BFR did not enhance or better maintain the attenuating 'saw-tooth' response of PGC-1α mRNA. This is in agreement with observations in SIT, with no difference in PGC-1α mRNA also demonstrated following a single SIT session with and without post-exercise BFR (Taylor *et al*. 2016a). These findings suggest that in contrast to the addition of BFR applied during moderate-intensity (105% of lactate threshold) running interval training, which was demonstrated to enhance PGC-1α mRNA expression 3 hours post-exercise (Christiansen *et al*. 2018), that post-exercise BFR is not a sufficient stimulus to augment PGC-1α transcription. It seems likely that there is a limited capacity to further elicit increases in PGC-1α mRNA when using BFR alongside training protocols undertaken at a standard training intensity. Indeed, in the study of Christiansen *et al*. (2018) the increase in PGC-1α mRNA expression following the exercise protocol undertaken alone was only ~2-fold compared to the potent 9-fold increase following the initial training session in the control group of Chapter 6. Unfortunately, due to methodological issues outlined in Chapter 8, analysis of PGC-1α protein content to assess the timecourse of the translational response was not possible.

In accordance with the PGC-1α mRNA data, post-exercise BFR did not enhance the content of mitochondrial proteins COXII and COXIV following 4 weeks of SIT (Chapter 4) or 2 weeks of HIIT (Chapter 6), and also did not increase CS activity, a valid biomarker of mitochondrial content (Larsen *et al*. 2012) during 2 weeks of HIIT (Chapter 6). This thesis therefore, does not support the hypothesis that post-exercise BFR enhances mitochondrial biogenesis.

Nevertheless, it is important to note that neither the SIT intervention in Chapter 4 or the HIIT intervention in Chapter 6 elicited increases in the markers of mitochondrial biogenesis assessed

without BFR. These findings are surprising given the potent PGC-1α mRNA responses observed following these training protocols (Chapter 6; Taylor *et al.* 2016a). SIT has consistently been demonstrated to increase markers of oxidative capacity, including increases in the protein content of COXII and COXIV (Burgomaster *et al*. 2007; Gibala *et al.* 2006). These studies have only been undertaken in untrained or recreationally active participants, however, therefore it seems likely that the lack of effect in Chapter 4 was attributable to the well-trained population that was tested. Perry *et al*. (2010) utilised a very similar HIIT protocol in participants of a similar fitness level to Chapter 6, and observed an increase in CS activity after just 3 training sessions. As discussed in Chapter 6 it is possible that the failure to progress the training intensity throughout the two weeks of training, as was undertaken in Perry *et al*. (2010), meant the training intervention did not elicit a sufficient continued stimulus to increase mitochondrial content. Therefore, it is possible that these factors prevented any adaptations of post-exercise BFR manifesting. Furthermore, it is also possible that the training interventions elicited increases in mitochondrial function which were not assessed in this thesis. Indeed, previous investigations have observed an increase in maximum coupled mitochondrial respiration through complex I and II as assessed by high-resolution respirometry following SIT and resistance training in the absence of changes to CS activity (Granata *et al*. 2016; Groennebaek *et al*. 2018).

7.5 THE EFFECT OF POST-EXERCISE BFR ON ENDURANCE PERFORMANCE

It was hypothesised that CP would be enhanced with the addition of post-exercise BFR. However, in Chapter 4 a similar increase in CP was observed following 4 weeks of SIT alone (3.6%) and SIT with post-exercise BFR (3.3%). The lack of increase in performance is consistent with Taylor *et al*. (2016) who observed no enhancement in 15 km time trial performance with the addition of post-exercise BFR to SIT. In addition to being an important performance parameter, CP was selected as the main performance measure in Chapter 4 as it was hypothesised to be sensitive to the peripheral adaptations that post-exercise BFR was postulated to elicit, i.e. angiogenesis and mitochondrial biogenesis. Therefore, given that post-exercise BFR also failed to elicit increases in skeletal muscle capillarity and mitochondrial protein content, it is perhaps not surprising that no enhancement in CP was observed after this 4-week intervention.

Moreover, the addition post-exercise BFR to SIT did not enhance *W′*, the second parameter of the power-duration relationship, or mean power output (MPO) to 4 weeks of SIT (Chapter 4), parameters which may give more of an indication of the 'anaerobic capacity' (Smith & Hill, 1991; Vanhatalo *et al*. 2016). BFR could have been hypothesised to have increased anaerobic capacity. For example, HIF-1α is known to stimulate increases in the activity of glycolytic enzymes (Semenza, 2009) and training in hypoxia can enhance mRNA expression and activity of glycolytic enzymes (Puype *et al*. 2013; Vogt *et al*. 2001; Zoll *et al*. 2006). Whilst not significantly different the percentage changes with post-exercise BFR were more favourable than SIT alone. *W′* decreased by 10.4 and 3.0% and MPO increased by 0.8 and 3.1% following SIT alone and SIT with post-exercise BFR, respectively. Although it is important to note for *W′* this pattern largely emerged from robust responses from just a few individuals.

In contrast, chapter 4 did confirm previous findings (Taylor *et al*. 2016a) further demonstrating the potency of post-exercise BFR during SIT to enhance $\dot{V}O_{2max}$, with a 5.9% increase in trained individuals in just four weeks. Given that no increases in skeletal muscle capillarity or mitochondrial protein content were observed, this improvement seems more likely to be attributable to central adaptations, in particular cardiac output. Whilst SIT alone does not elicit increases in cardiac output (MacPherson *et al*. 2011), the addition of BFR could pose a greater challenge to the central cardiovascular system through the induction of the exercise pressor reflex (Alam & Smirk, 1937; Bull *et al*. 1937; Spranger *et al*. 2015), as evidenced by an increase in heart rate and systolic blood pressure with the addition of BFR during and after exercise (Chapter 6; Brandner *et al*. 2015; Poton & Polito, 2015; Renzi *et al*. 2010; Staunton *et al*. 2015; Takano *et al*. 2005). In addition to the reductions in stroke volume during BFR application (Renzi *et al*. 2010; Staunton *et al*. 2005; Takano *et a*l. 2005), it seems plausible that the pressor reflex related stimulus could induce a central adaptive response, i.e. increased stroke volume and cardiac output. Having currently received little attention in the literature, these findings highlight that the central adaptive responses to BFR exercise require further investigation.

7.6 LIMITATIONS

The studies in this thesis were not without their limitations, many of which have been addressed in the discussion sections of the individual experimental chapters. One of the main limitations throughout this thesis was the lack of quantification of the level of BFR imposed in individuals.

A consistent absolute BFR cuff pressure was used for all participants in Chapters 4-6 as opposed to an individualised approach. Whilst this may make the application of findings into applied practice more practical for athletes who do not have access to the necessary equipment to measure the level of restriction, it has been demonstrated that there is large variation in the level of BFR imposed by a standard external cuff pressure between individuals (Hunt *et al*. 2016; Loenneke *et al*. 2012). This variation will likely affect the extent of the physiological stimuli elicited including the level of the shear stress response, muscle oxygenation and muscle metabolite accumulation (Karabulut *et al*. 2011; Takarada *et al*. 2000). Indeed, Chapter 5 demonstrated that by using a standard cuff pressure of 120 mmHg for post-exercise BFR during SIT the decrease in muscle oxygenation in the restriction periods varied between 11 and 43% across participants. It is therefore likely that this variation in physiological signals imposed may have impacted the extent of any adaptations elicited, with the possibility that in some participants the level of restriction was too low to elicit sufficient changes in some stimuli.

Both the SIT and HIIT control interventions in Chapter 4 and 6 failed to elicit changes in skeletal muscle capillarity and the markers of mitochondrial biogenesis assessed. These training interventions may therefore have provided an insufficient stimulus to assess the effect of post-exercise BFR on these adaptations. Indeed, as discussed above in sections 7.3 and 7.4, the training volume of the SIT in Chapter 4 may have been too low to elicit adaptations in skeletal muscle capillarity and content of mitochondrial proteins and the failure to progress training in Chapter 6 may have prevented mitochondrial adaptations. Therefore, the potential of post-exercise BFR to elicit these adaptations cannot be ruled out.

Furthermore, for Chapter 4 and 6 there were low samples sizes for the skeletal muscle analysis parameters. These low numbers are largely attributable to the invasive nature of the experiments, particularly in the case of Chapter 4 with the use of well-trained individuals wishing to adhere to strict training regimes. Nevertheless, the reduced participant numbers are likely to have decreased the chances of correctly accepting or rejecting some of the null hypotheses. Indeed, this is perhaps reflected in several parameters which displayed *P* values close to the critical value and low observed powers for interaction effects in Chapter 6. Moreover, these may have been exacerbated by the experimental designs, whereby in the experimental conditions the post-exercise BFR stimulus was introduced alongside a novel exercise stimulus. As alluded to above for some parameters the exercise intervention alone proved a potent stimulus and therefore, in some cases there may have been limited capacity to increase the initial response further. To overcome this future studies could look to precondition

individuals to a specific interval training program, before introducing a post-exercise BFR stimulus once an attenuated adaptive response would be expected.

There are also limitations in the methods used for the assessment of mitochondrial adaptations. As aforementioned, no assessment was made of mitochondrial function. Adaptations to mitochondrial function can be dissociated from those of mitochondrial content (Granata *et al*. 2016). Moreover, cross-sectional data from studies suggest that higher physical activity levels are associated with relatively larger values of mitochondrial function than mitochondrial content (Bishop *et al*. 2014) suggesting mitochondrial function may be more responsive to exercise training. Therefore, it seems plausible that the interventions may have increased mitochondrial function. Moreover, CS enzyme activity provides a more valid biomarker of mitochondrial content than the content of mitochondrial proteins assessed in Chapter 4 (Larsen *et al*. 2012). However, in this chapter, due to the large number of pre and post training visits, more than 24 hours rest prior to muscle biopsies was not possible in all participants. CS activity was therefore not assessed given that it can still be acutely elevated after 24 hours in response to a single exercise bout (Leek *et al*. 2001).

7.7 CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has furthered our knowledge on the potential of post-exercise BFR as a possible training tool for endurance athletes. In contrast to the hypotheses it was demonstrated that combining post-exercise BFR with 4 weeks of SIT is not sufficient to elicit improvements in angiogenesis or mitochondrial biogenesis and does not enhance performance within the severe-intensity domain. Nevertheless, whilst the findings of this thesis suggest that post-exercise BFR utilised during training protocols undertaken at a typical training intensity does not elicit mitochondrial biogenesis (at least in the form of increasing mitochondrial content), there were suggestions that post-exercise BFR could induce an enhanced angiogenic stimulus which may manifest when undertaken with greater training volumes and/or training durations. Indeed, this thesis was the first to observe that post-exercise BFR enhances resting eNOS mRNA, suggesting the potential for enhanced shear stress. Given that the findings also imply that skeletal muscle capillarity is a key determinant of CP, these suggestions clearly warrant further investigation. Future studies should look to establish whether the addition of post-exercise BFR during more than 4 weeks of SIT and higher volume HIIT protocols, such as that utilised in Chapter 6, enhances angiogenesis and high-intensity endurance performance.

Post-exercise BFR also has the potential to elicit further peripheral adaptations than that explored in this thesis, such as enhancing the regulation of ion gradients. For example, Christiansen *et al*. (2018) recently provided preliminary data to suggest BFR could enhance sodium and potassium ion regulation. They demonstrated that BFR undertaken during moderate-intensity running interval training augmented the acute increase in mRNA expression of the Na^+, K^+-ATPase complex ancillary protein FXYD1, which performs a role in the maintenance of transmembrane sodium and potassium ion gradients, which are critical to preserve skeletal muscle membrane excitability and thus to resist fatigue (McKenna et al. 2008). Therefore, in addition to angiogenesis future research should also investigate the effect of post-exercise BFR upon these adaptations.

Should future investigation support the preliminary findings of this thesis that suggest the potential for enhanced angiogenic signalling, by demonstrating post-exercise BFR can enhance capillary supply, post-exercise BFR could also have applications for sedentary and clinical populations. Indeed, not only is increased skeletal muscle capillarity linked with functional improvements, decreased capillarity is associated with reduced health outcomes, including reduced insulin sensitivity (Prior *et al*. 2014) and chronic diseases such as peripheral artery disease (Duscha *et al*. 2011) and diabetes (Kondo *et al*. 2015). Although the demanding protocols in the current thesis may not be suitable for sedentary individuals or individuals with functional limitations, post-exercise BFR could be trialled alongside reduced intensity protocols in a bid to enhance the angiogenic stimulus. It seems likely that this model of BFR may reduce the discomfort and perceived exertion in comparison to continuous BFR during exercise, with intermittent applications previously demonstrated to elicit lower perceived exertions (Neto *et al*. 2017), which may assist in adherence to BFR exercise.

CHAPTER 8
SKELETAL MUSCLE ANALYSIS METHOD DEVELOPMENT

8.1 IMMUNOHISTOCHEMICAL PROTOCOL OPTIMISATIONS

Immunohistochemistry utilises the specific bond between antigens and antibodies for the identification of proteins with a fluoresce microscope to allow the visualisation of muscle tissue structure. Within the thesis this process was used to visualise myosin heavy chain (MHC) I and II, CD31 and Ki-67.

Prior to analysis of experimental samples methods for CD31, MHCII and Ki-67 antibodies were optimised to minimise background staining and optimise positive staining. The MHC I and II antibodies had been previously optimised, however, due to use of a different secondary antibody some optimisation was also undertaken for the MHC II antibody. This previous MHC protocol was used as a starting protocol for all proteins, alongside antibody suppliers recommended dilutions.

CD31 Optimisation

CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1), is a transmembrane glycoprotein located within the endothelium. Immunohistochemical staining of CD31 on cross-sectional muscle tissue sections allows a 2D quantification of skeletal muscle capillarity.

CD31 Antibody: Rabbit monoclonal antibody (Abcam: ab76533)

Initial primary antibody concentration of 1 in 250 and secondary antibody concentration of 1 in 1000 was selected. As shown in Figure 8.1A these concentrations did not produce a clear expression of CD31 positive cells. To establish a positive expression of the primary antibody before proceeding further with the optimisation process both primary and secondary antibody concentration were increased together (Primary = 1 in 100, Secondary = 1 in 500). As shown in Figure 8.1B, the increased concentrations resulted in an identifiable positive stain.

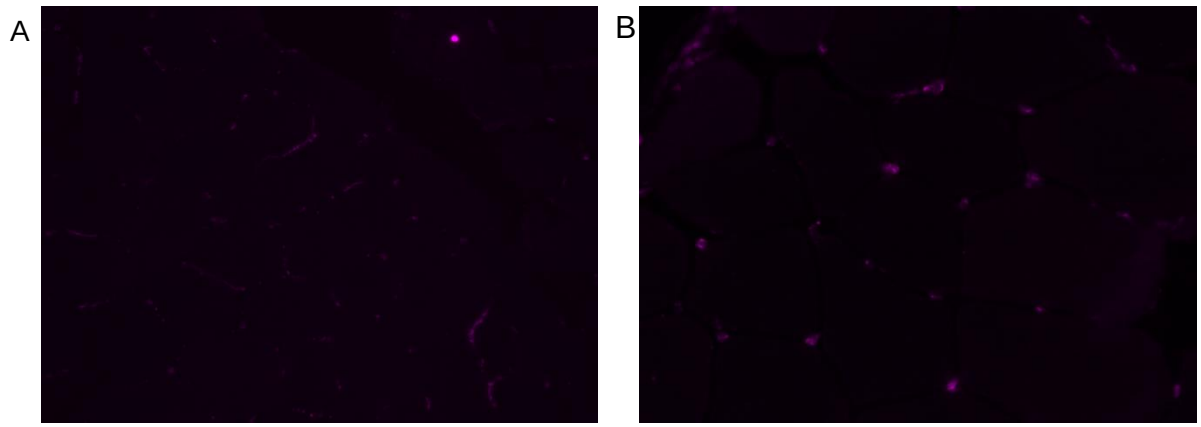

Figure 8.1. CD31 staining. A) Primary antibody concentration 1 in 250 and secondary 1 in 1000. B) Primary antibody 1 in 100 and secondary antibody 1 in 500.

As there is a risk of over fixation, due to modification of amino-acids that form part of the epitope with the use of formaldehyde, two acetone fixing protocols, which have been used in previous literature analysing CD31, were tested against the formaldehyde fixing procedure used in the MHC protocol. The three fixative processes tested were:

1. Incubation of samples in a 3.7% formaldehyde solution for 10 minutes.
2. Immersion of slides in acetone at -20℃ for 5 min.
3. Immersion of slides in acetone at -20℃ for 30 s before they were incubated in a 3.7% formaldehyde solution for 2 min.

As displayed in Figure 8.2, neither acetone protocol enhanced the expression of CD31 positive cells and so the use of the formaldehyde solution was maintained.

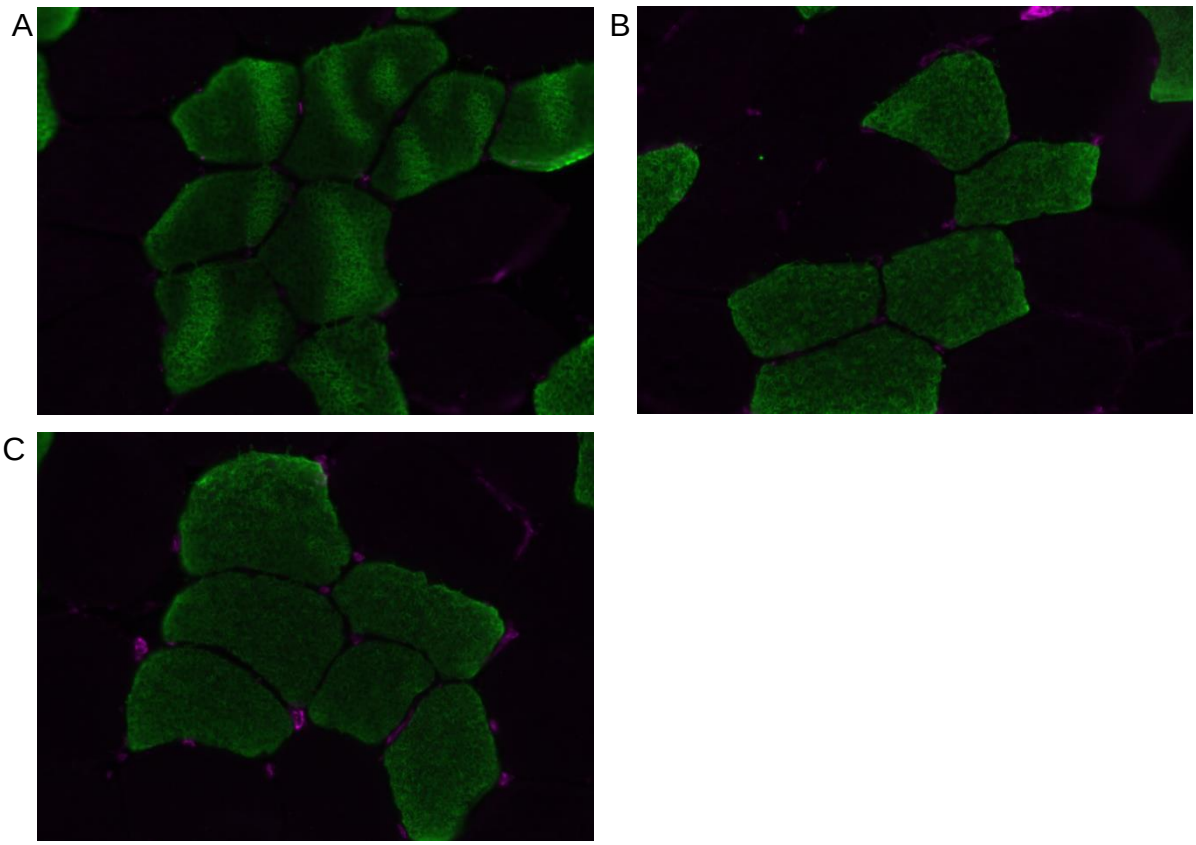

Figure 8.2. CD31 (pink) & MHC I (green) staining. A) Fixed with acetone for 5 min. B) Fixed with acetone for 30 s and P-formaldehyde for 2 min. C) Fixed with P-formaldehyde for 10 min.

Primary antibody concentration was then optimised. Concentrations tested were 1 in 100, 1 in 250 and 1 in 500. Images are displayed in Figure 8.3 (due to an issue with inappropriate storage there was a higher amount of background/non-specific straining in parts). A concentration of 1 in 100 resulted in the brightest stain. A further experiment was conducted at 1 in 50 in an attempt to reduce the high exposure time that was required (>2000 ms) but a higher amount of background staining was visible at this concentration.

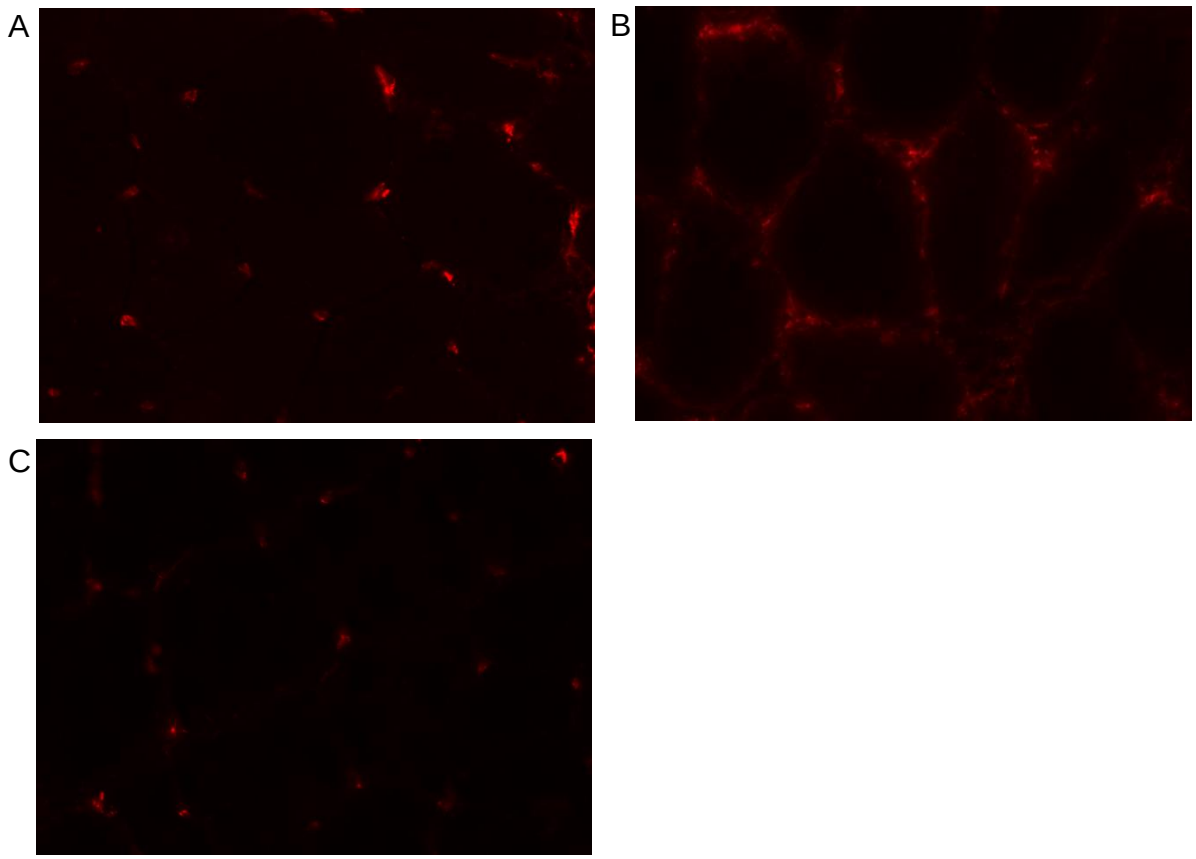

Figure 8.3. CD31 staining (red). Primary antibody concentration: A) 1 in 100; B) 1 in 250; C) 1 in 500.

A higher concentration of seconday antibody (1 in 500 increased to 1 in 250) was then trialled but this did not produce a stronger expression of CD31 positive cells. Following optimisation of this antibody the images produced (Fig 8.3A) still did not produce a clear stain of all capillaries and some background staining was visible at the high required exposure time (~2000 ms), therefore a second antibody was trialled.

Second CD31 Antibody: Mouse monoclonal antibody (Abcam: ab119339)

Initially the new antibody was stained alongside the previous antibody on serial sections using the same optimised protocol. The results from this experiment are displayed in Figure 8.4. Whilst the staining of the capillaries was clearer and more numerous with the new antibody, there was significant background staining of the muscle fibres. Therefore, the incubation of the primary antibody was reduced from overnight to 1 hour. This eradicated the background staining of the muscle fibres (Fig. 8.4C).

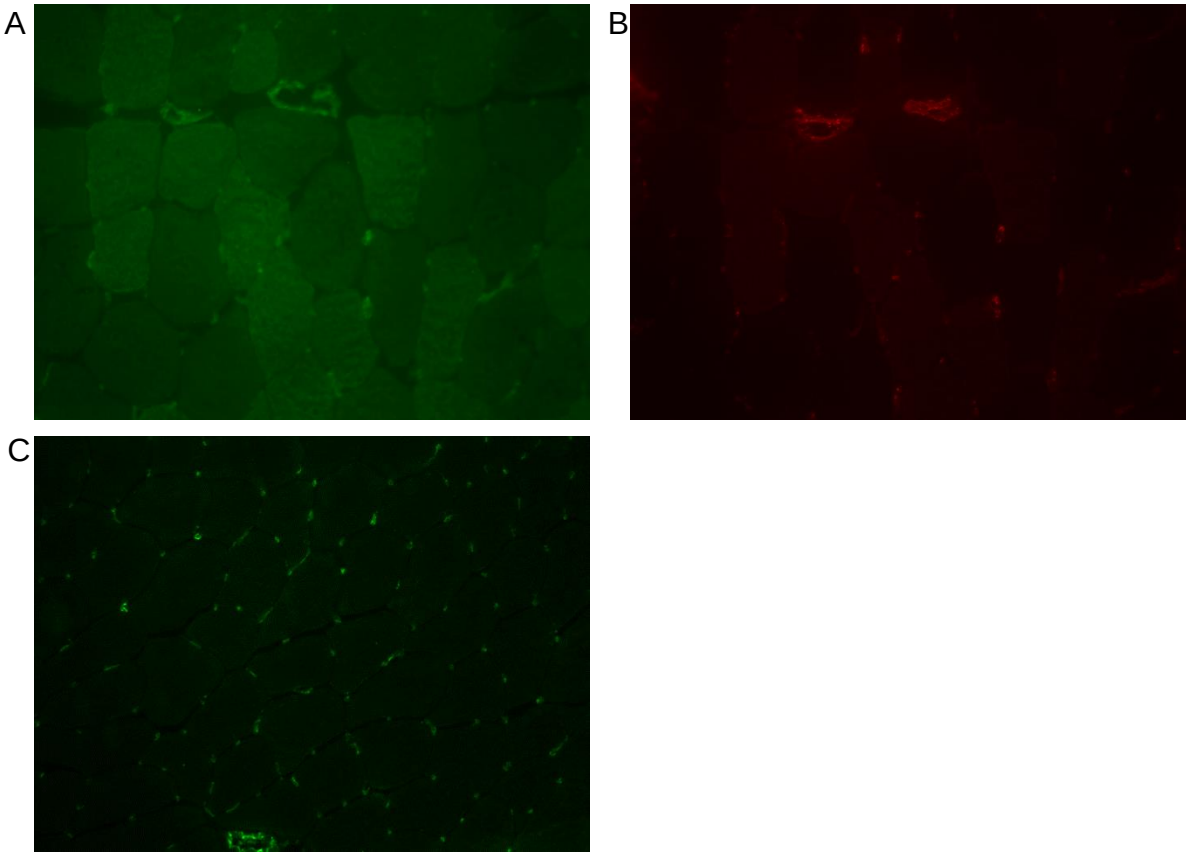

Figure 8.4. A) CD31 (green) new primary antibody incubated overnight, exposure time = 878 ms B) CD31 (red) original primary antibody incubated overnight, exposure time = 1584 ms C) CD31 (green) new primary antibody incubated for 1 hour, exposure time = 623 ms.

The primary antibody concentration was then optimised (Figure 8.5). The concentrations tested were; 1 in 50, 1 in 100 and 1 in 250. A primary antibody concentration of 1 in 250 produced a stain with a lower expression that 1 in 100, however an increased concentration of 1 in 50 did not enhance the positive stain.

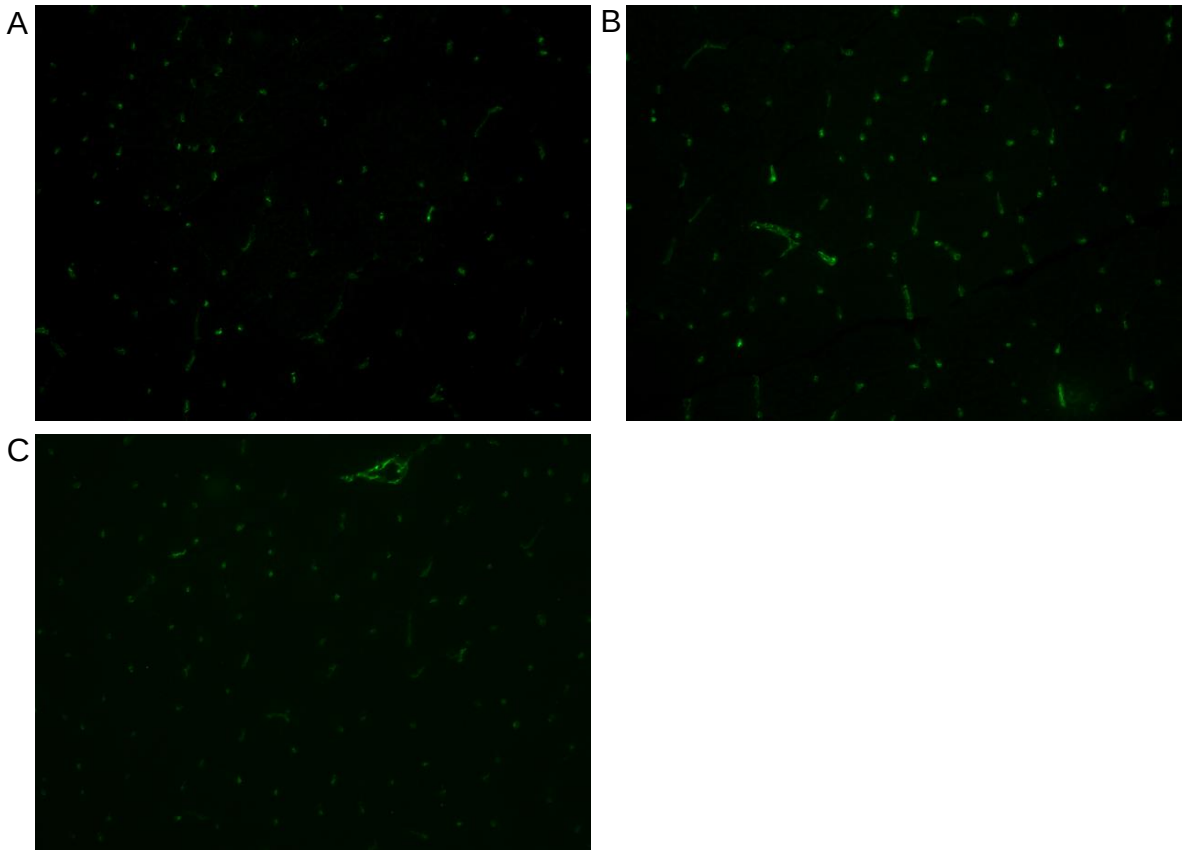

Figure 8.5. CD31 staining (green). Primary antibody concentration: A) 1 in 50 B) 1 in 100 C) 1 in 250.

Finally the secondary antibody concentration was optimised with concentrations of 1 in 250, 1 in 500 and 1 in 1000 tested (Figure 8.6). A concentration of 1 in 1000 produced a low positive expression, but there was no observable difference between 1 in 500 and 1 in 250.

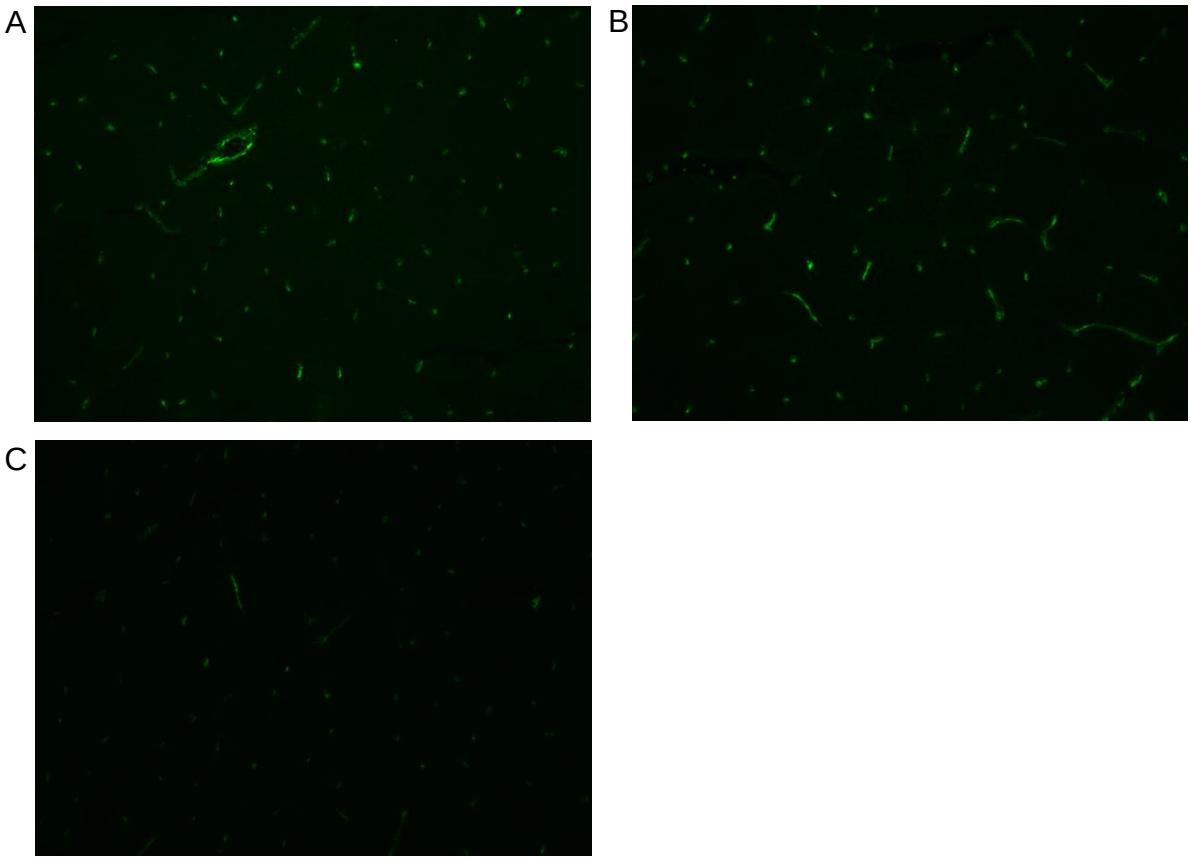

Figure 8.6. CD31 staining (green). Secondary antibody concentration: A) 1 in 250 B) 1 in 500 C) 1 in 1000.

Myosin heavy chain II optimisation

MHCII antibody: Rabbit polyclonal antibody (Abcam: ab91506)

The primary antiobdy concentration was optimised with concentrations of 1 in 1000 and 1 in 2000 trialled (Figure 8.7). A concentration of 1 in 1000 produced a much brighter stain than 1 in 2000.

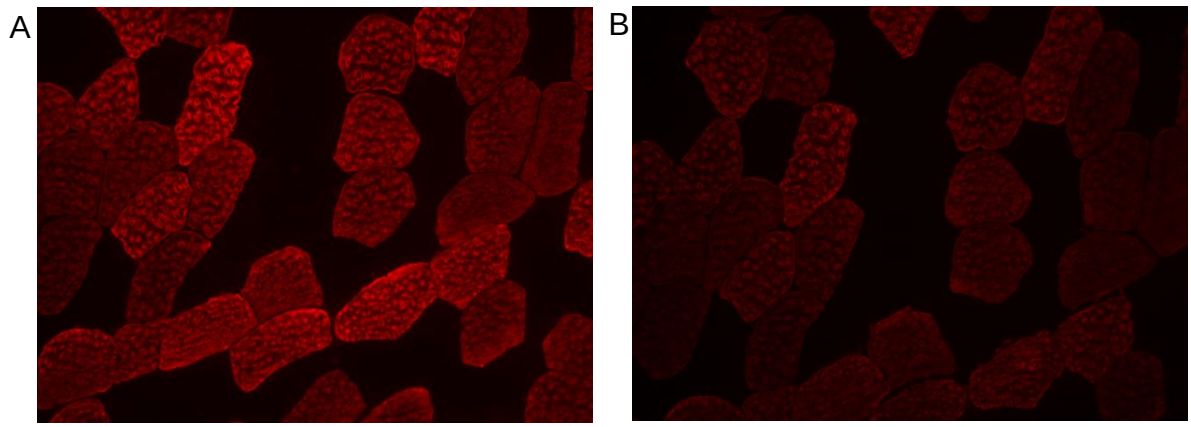

Figure 8.7. MHC II staining (red). Primary antibody concentration: A) 1 in 1000 B) 1 in 2000

Secondary antibody concentrations of 1 in 250, 1 in 500 and 1 in 1000 were then trialled (Figure 8.8). A concentration of 1 in 1000 produced a low positive expression but an increase to 1 in 250 did not make fibres easier to classify than 1 in 500.

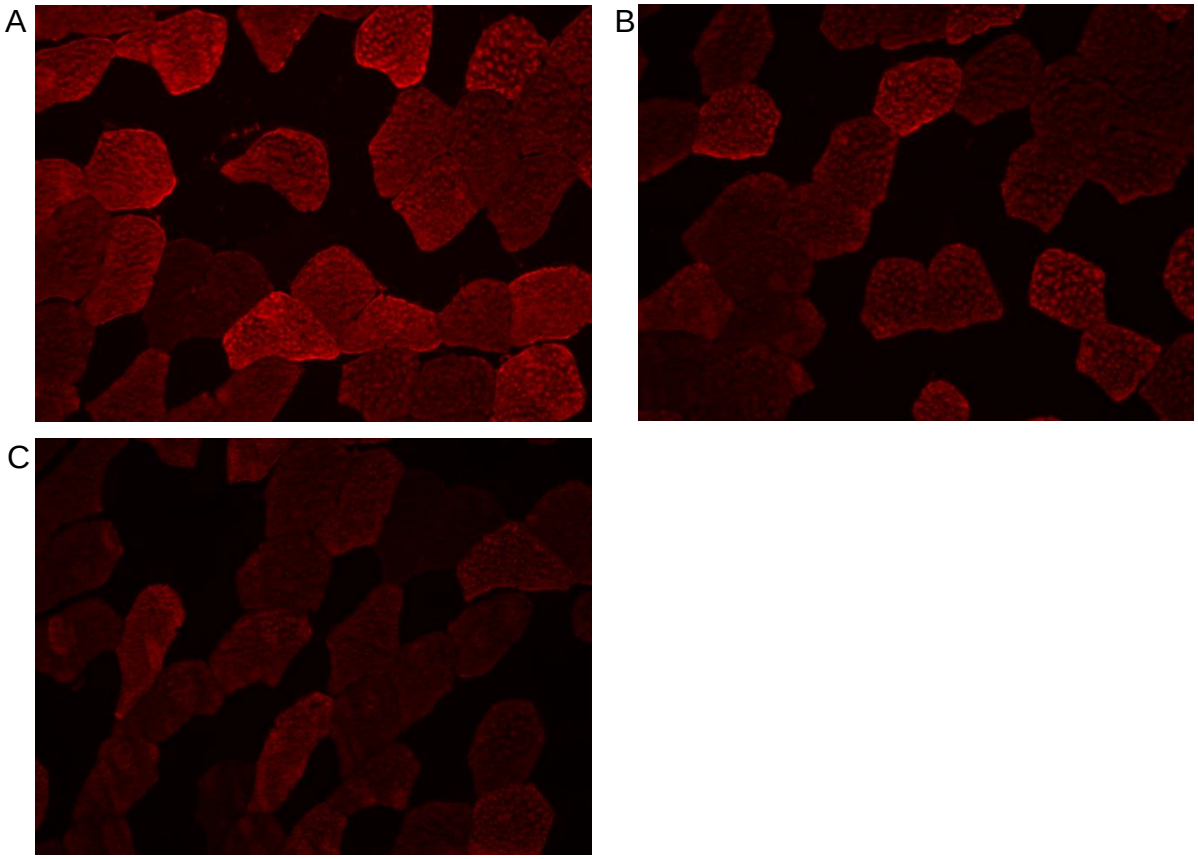

Figure 8.8. MHC II staining (red). Secondary antibody concentration: A) 1 in 250 B) 1 in 500 C) 1 in 1000.

Controls were undertaken with no primary antibodies (Figure 8.9) to ensure specificity of secondrary antibodies.

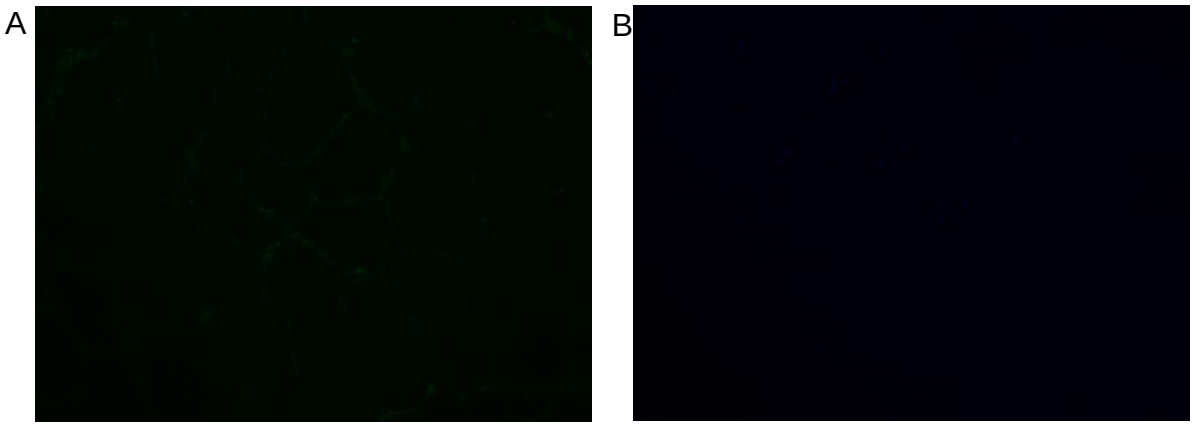

Figure 8.9. No primary antibody controls: A) Alexa Fluor 488 Mouse antibody B) Alexa Fluor 594 Rabbit antibody. Exposure time ~600ms.

Ki-67 antibody optimisation

Ki-67 detects a proliferation-associated nuclear antigen, and its co-localisation within endothelial cells can be used to identify and quantify proliferating endothelial cells (Hoier *et al*. 2010; Jensen *et al*. 2004a).

Ki-67 Antibody: Rabbit monoclonal antibody (Abcam: ab92742)

Due to the infrequency of Ki-67 positive cells, the size of Ki-67 positive staining and level of expression positive staining is not generally visible when printed, therefore, images are not displayed in this section.

Initially the primary antibody concentation was optimised. Concentrations tested were: 1 in 100, 1 in 250 and 1 in 500, these were incubated for 1 hour. The concentrations of 1 in 100 and 1 in 250 were similar, but images were weak in expression. Following this a primary antibody concentration of 1 in 250 was used but primary antibody incubation was continued over night. There was no additional background staining evident so an overnight incubation was continued. A higher secondary antibody concentration of 1 in 250 was then trialled. This did not enhance the expression of positive staining so a concentration of 1 in 500 was maintained. Due to the higher required exposure time (up to 1000 ms) an additional no primary antibody control was performed to confirm seconday antibody speficity at this exposure. After optimisation the signal to noise ratio for some samples remained low. As a result a serial section with a no primary antibody control was stained for all samples concomitantly to confirm if visible staining was Ki-67 positive.

8.2 IHC OPTIMISED PROTOCOL

Sectioning

Preparation

The Cryostat was set to an initial working temperature of -25°C and specimen temperature of -20°C. Samples were removed from the -80°C freezer and were left to equilibrate to the temperature for at least 1 hour.

Cryosectioning

Single sections of 8 μm were sectioned and added to a polysine microscope slide. Cross-sectional orientation was examined under a light microscope, using the assumption of circularity. Microtome height and angle were adjusted as required until a cross-sectional orientation was achieved. 6 or 8 slides were collected (with 18-21 sections per slide) with serial sections depending upon the size and number of mounted samples available. Slides were stored at -80°C until analysis.

Staining

Reagents

1 x PBS (1% Tween-20)

- 2 PBS Tablets
- 0.4 g of Tween-20
- 400 ml of dH_2O

3.7% formaldehyde fixing solution

- 1 ml of 37% formaldehyde
- 9 ml of 1 x PBS (1% Tween-20)

Blocking solution

- 1 g of BSA
- 2.5 ml of goat serum
- 100 μl of Triton X-100
- 46.4 ml of PBS

Immunostaining Protocol

1. Upon removal from -80°C freezer samples were left to dry and slides were circled with a PAP-pen 2-3 times.
2. Muscle samples were fixed within a 3.7% formaldehyde solution, for 10 minutes at room temperature.
3. Slides were washed for 3 min five times with PBS.
4. Samples were then blocked for 60 minutes at room temperature.
5. Slides were washed for 3 min five times with PBS.
6. Samples were incubated with primary antibodies in blocking solution at room temperature for 60 minutes.

   Slide 1 = CD31 (anti-mouse monoclonal) at a concentration of 1:100
   MHC II (anti-rabbit monoclonal) at a concentration of 1:1000
   Slide 2 = MHC I (anti-mouse monoclonal) at a concentration if 1:500
   Slide 3 = CD31 (anti-mouse monoclonal) at a concentration of 1:100
   Slide 4 = Blocking solution alone

7. Slides were washed for 3 min five times within PBS.
8. Slides 3 and 4 underwent a secondary primary antibody incubation at room temperature overnight and were wached for 3 min five times with PBS.

   Slide 3 = Ki-67 (anit-rabbit monoclonal) at a concentration of 1 in 250
   Slide 4 = Blocking solution alone

9. Samples were incubated with secondary antibodies in blocking solution in the dark at room temperature for 2 hours.

   Slide 1, 3 & 4 = Alexa Fluor 488 at a concentration of 1:500
   = Alexa Fluor 594 at a concentration of 1:500
   Slide 2 = Alexa Fluor 488 at a concentration of 1:500

10. Slides were washed for 3 min five times with PBS in the dark.
11. 1-2 drops of fluoromount aqueous mounting medium was applied to each slide and a cover slip was placed over the samples.

8.3 WESTERN BLOT PROTOCOL OPTIMISATIONS

Western blotting combines the process of gel electrophoresis with the specifity of antibody-antigen detection. This allows the quantification of the content of a specific protein in a sample. Within this thesis western blotting was used for the quantification of mitochondrial enzymes CS, COXII and COXIV and signalling proteins VEGF and PGC-1α

CS, COXII and COXIV

CS antibody: Rabbit monoclonal antibody (Abcam: ab129095)
COXII antibody: Mouse monoclonal antibody (Abcam: ab110258)
COX IV antibody: Mouse monoclonal antibody (Abcam: ab33985)

These proteins have regulary been measured in the literature. As a result an initial protocol based from previous publications was trialled. The initial trials of these proteins are displayed in Figure 8.10. 15ug and 30ug of protein loading was trialled. There was a strong blot when only 15ug of protein was loaded. With clear positive bands and lack of non-specific bands no further optimisation was undertaken upon these protocols.

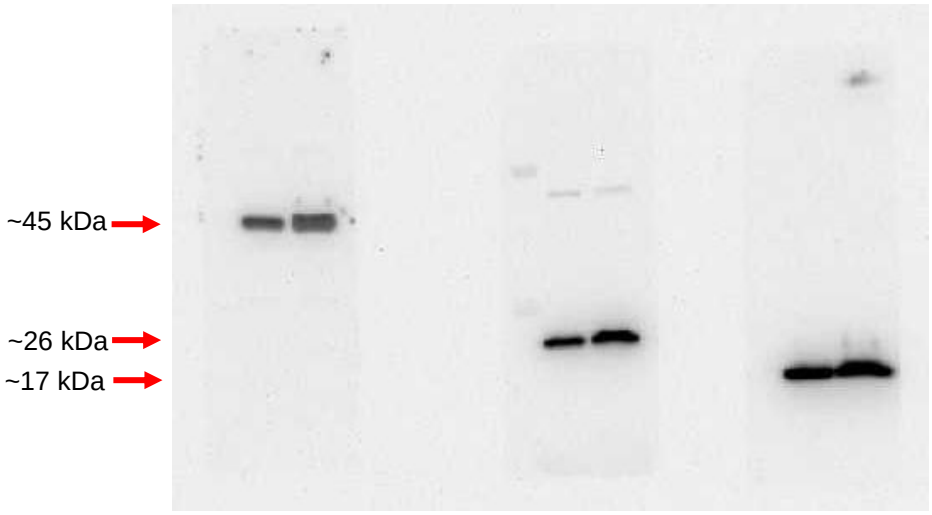

Figure 8.10. Western blots for (left to right) CS, COXII and COXIV with 15 ug loaded on the left-hand lanes and 30 ug loaded on the right-hand lanes.

VEGF

VEGF antibody: Rabbit monoclonal antibody (ab52917)

Initially two different protein loading concentrations: 30 µg and 60 µg and two blocking protocols: 5% milk and 5% BSA both in TBST were tested (Figure 8.11). Loading of 60 µg produced a clearer band near the correct molecular weight. BSA was an ineffective blocking solution so milk was used, but there was still non specific bands with this blocking protocol.

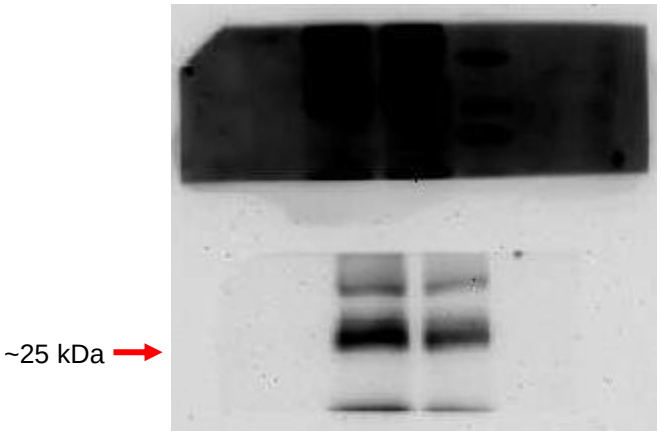

Figure 8.11. Western blots for VEGF top) blocked in 5% BSA, bottom) blocked in 5% milk. Left-hand lane 60 µg protein loaded and right-hand lane 30 µg protein loaded

Next lower primary antibody concentrations were trialled to try to reduce the non-specific bands. The concentrations trialled were 1 in 1000, 1 in 2000 and 1 in 4000 (Figure 8.12). This trial was run with an new vial of antibody. There was not a single clear positive band at any concentration. This experiment was repeated on different samples and produced the same result. As a result a new antibody was trialled.

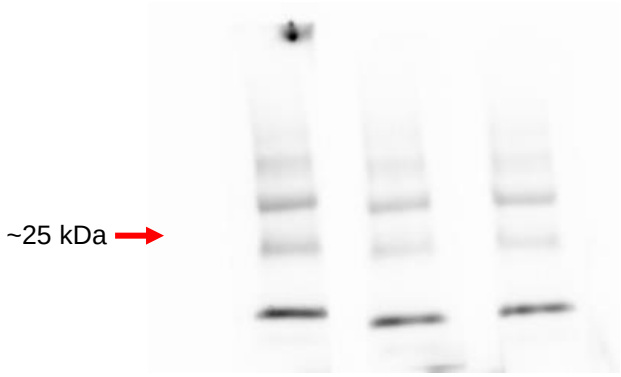

Figure 8.12. Western blots for VEGF at a primary antibody concentration of (left to right) 1 in 1000, 1 in 2000 and 1 in 4000.

Second VEGF antibody: Mouse monoclonal antibody (Santa-Cruz: sc-7269)

Initial protein loading centrations of 30 µg and 60 µg were triallled (Fig. 8.13A). There were two faint bands around the correct molecular weights for the VEGF monomer and dimer. Given the bands were faint the exposure time was increased in the next trial from initial exposure of 10 s for a total of 1200 s to an initial exposure time of 30 s for a total of 3600 s and a reduced concentration of milk was used in the blocking solution (2%). A higher primary antibody concentration was also trialled (1 in 500) (Fig.8.13B). The increase in exposure time resulted in a clear positive band which was not enhanced with a primary antibody concentration of 1 in 500.

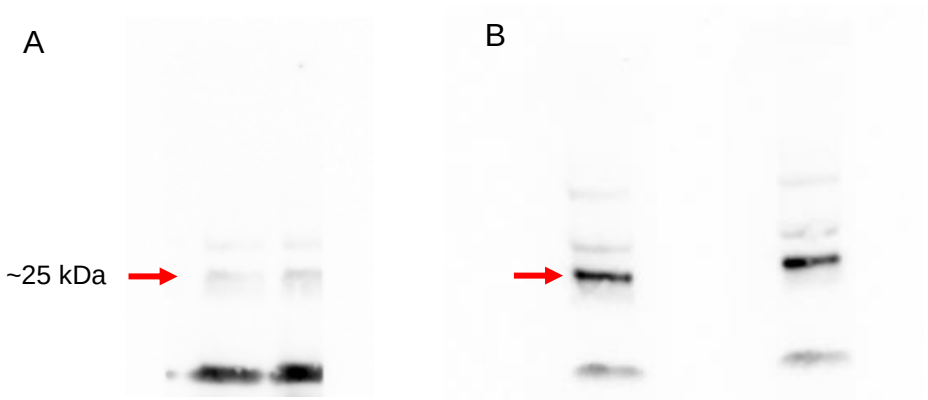

Figure 8.13. Western blots for VEGF. A) left hand lane 30 µg, right hand lane 60 µg. Exposure: initial time of 10 s for total of 12000 s. B) left-hand lane primary antibody concentration of 1 in 500 and right-hand lane 1 in 1000. Exposure: initial time 30 s for 3600 s.

PGC-1α

PGC-1α antibody: Mouse monoclonal antibody (Merck: 4C1.3)

Initially two different protein loading concentrations were trialled: 30 µg and 60 µg, with an initial primary antibody concentration of 1 in 1000 (Fig. 8.14A). A clear band at a high molecular weight was visible, although there was some background staining. A lower primary antibody concentration (1 in 2000) was trialled and both primary and secondary antibodies were diluted in 5% milk (increased from 3%) to try and reduce non-specific bands (Fig. 8.14B).

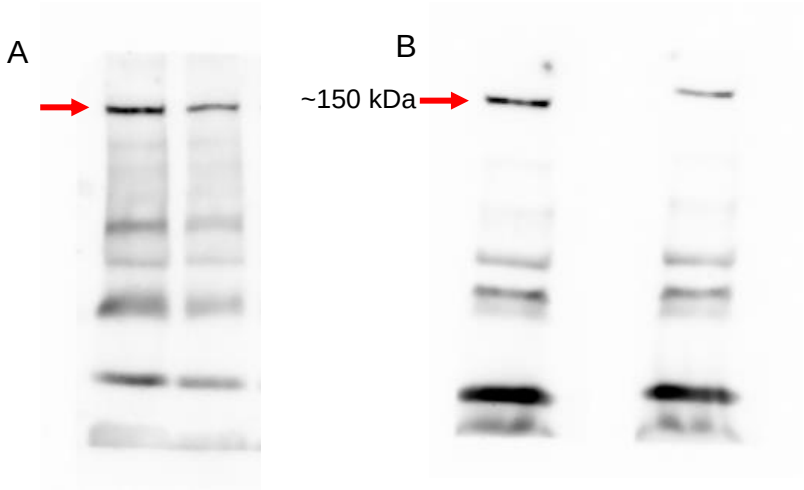

Figure 8.14. Western blots for PGC-1α A) left hand lane 60 µg, right hand lane 30 µg, with antibodies diluted at 1 in 1000 in 3% milk. B) left-hand lane primary antibody concentration of 1 in 1000 and right-hand lane 1 in 2000 with antibodies diluted in 5% milk.

A higher concentration of milk reduced the non-specific bands. The clear band produced was higher than the estimated molecular weight (~150 vs ~113 kDa), however, with no other bands near the estimated molecular weight it seemed possible that this could be a positive band. To confirm this a positive control was run alongside the sample to check if they occurred at the same molecular weight. As demonstarted in Figure 8.15A, however, the band for the muscle sample was occuring above that of the main band of the positive control, although there was still a weaker band of the control at a similar molecular weight. A further experiment was run against another PGC-1α antibody (Abcam) to identify if it was an issue with the antibody or positive control (Figure 8.15B).

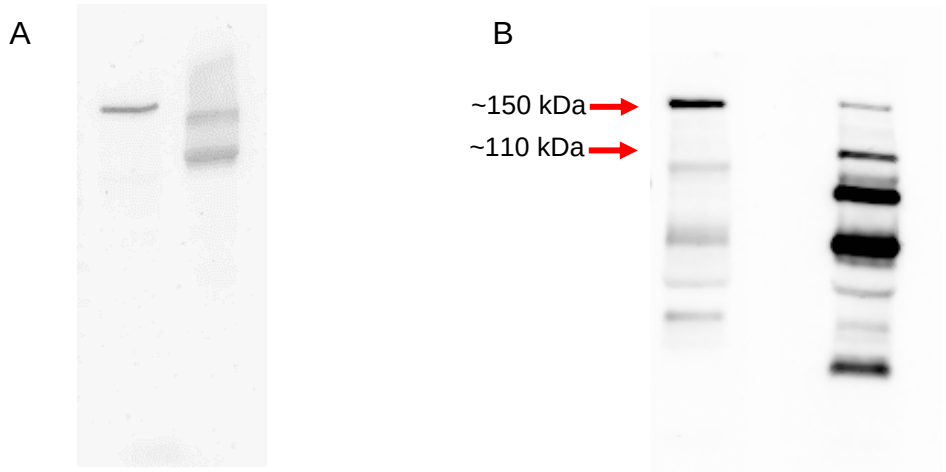

Figure 8.15. Western blot for PGC-1α A) left lane muscle sample, right lane positive control. B) left lane current Merck monoclonal antibody, right lane Abcam polyclonal antibody.

The abcam antibody produced multiple bands; a weak one was evident at the same molecular weight as that produced by the merck antibody, but there was a clearer band at the antibodies stated molecular weight (~100 kDa) and further larger bands at lower molecular weights, so the 'correct' band was also not clear for this antibody.

A further experiment was run on experimental samples using the Merck antibody to see if the changes were as would be expected (Fig. 8.16A), alongside another high molecular weight protein (mTOR ~289 kDa) to confirm if higher weight proteins in the muscle samples were running through the gels correctly (Fig 8.16B). mTOR came out at the correct molecular weight suggesting that there was no issue with the running protocol. The experimental samples also decreased across the training period which based upon previous literature (e.g. Perry *et al*. 2010) would be unexpected.

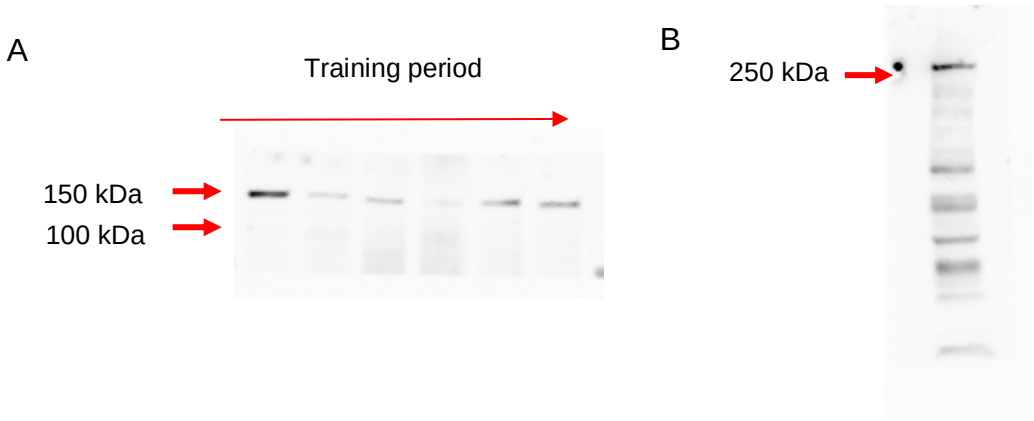

Figure 8.16. A) Western blot for PGC-1α on experimental samples across the training period from Chapter 6. B) Western blot for mTOR (~289 kDa).

A final experiement was run using muscle sampes which had not boiled prior to gel loading. This produced a similar single clear band again at a molecular weight of ~150 kDa.

Therefore, following this series of trials being unable to produce a valid positive band for PGC-1α protein at the correct molecular weight, the muscle samples from Chapter 6 were not analysed for PGC-1α protein.

8.4 WESTERN BLOT OPTIMISED PROTOCOL

Sample preperation

1. Muscle samples were weighed and 100 µl of lysis buffer was added per 10 mg of protein.
2. Samples were blitzed in a tissue lyser twice for 2 min at 20 Hz and were centrifuged at 12000 g for 10 min.
3. The supernatant was transferred to a fresh eppendorf tube.
4. Samples were diluted 1 in 10 in dH_2O and protein concentrations were determined by Pierce 660 protein assay.
5. Samples were prepared to the desired concentraion of protein with 4 x LDS sample buffer (2.5 µl per 10 µl), 0.1% β-mercaptoethanol (0.5 µl per 10 µl) and the remaining volume of dH_2O. Samples were then boiled for 5 minutes at 95°C and were frozen at -20°C until analysis.

Gel Running

1. Samples were loaded onto TGX gels. CS, COXII and COXIV = 15 µg and VEGF = 60 µg. 5 µl of pre-stained ladder was loaded at intervals across the gels.
2. Running buffer was added to the tank and the gels were run at a constant voltage of 100 v for 80 min.

Transfer

1. Gels were removed from casing and were incubated in transfer buffer for 5 min.
2. Transfer sandwiches were made with scoth pad, filter paper, TGX gel, PDVF membrane, filter paper and scoth pad. A roller was used to remove any air bubbles.
3. The remaining transfer buffer was added to the tank and the transfer was run at a constant voltage of 30 v for 90 min.

Immuno-Blot

1. Membranes were removed from the transfer sandwich and were washed for 5 min in TBST.
2. Membranes were blocked in 5% milk in TBST for 1 hour at room temperature on a rocker.
3. Membranes were washed for 5 min three times in TBST.

4. Membranes were cut at the appropriate molecular weight for the proteins of interest and incubated overnight at 4°C on a rocker at ~60 rpm at dilutions displayed in the table. GAPDH was used as a control protein.

Table 8.1. Molecular weights and primary antibody solutions for the proteins of interest.

Protein	Mol. weight	Primary antibody solution
CS	~45 kDa	1 in1000 in 3% Milk
COXII	~26 kDa	1 in 1000 in 3% Milk
COXIV	~17 kDa	1 in 1000 in 3% Milk
GAPDH	~38 kDa	1in 10 000 in 3% Milk
VEGF	~25 kDa	1 in 1000 in 2% Milk

5. Membranes were washed for 5 min three times in TBST.
6. Membranes were then incubated in the appropriate secondary antibody (anti-mouse horesradish peroxidase-conjugated secondary antiboy (Dako) or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad)) at a concentration of 1 in 10 000 for 60 min at room temperature.
7. Membranes were washed 5 min three times in TBST.
8. Membranes were then incubated in a 1:1 solution of enhanced chemiluminescence substrate (ClarityMax, Bio-rad) for 5 min in the dark.
9. Membranes were then visualised using image analysis with exposure times listed in the table below.

Table 8.2. Exposure times for the proteins of interest.

Protein	Exposure time
CS	10 s for total of 1200 s (total images = 60)
COXII	10 s for total of 1200 s (total images = 60)
COXIV	10 s for total of 1200 s (total images = 60)
GAPDH	10 s for total of 1200 s (total images = 60)
VEGF	30 s for total of 3600 s (total images = 60)

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