MECHANISTIC CONTROL OF THE COLD-INDUCED AUGMENTATION OF THE TRANSCRIPTIONAL CO-ACTIVATOR PGC-1α

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy.

June 2017
ABSTRACT

Cold water immersion is commonly used to alleviate the stress and damage that ensues following strenuous exercise. Alongside its purported performance and analgesic benefits recent literature highlights the positive impact it may have towards endurance adaptive responses, particularly on key markers of mitochondrial biogenesis. Despite these recent advances showing PGC-1α, the ‘master regulator’ of mitochondrial biogenesis, being augmented in its post-exercise response by cold water immersion, the precise controlling mechanisms remain to be determined. However, it has been suggested that local cooling effects on AMPK and p38 MAPK related signalling and/or increased systemic β-adrenergic stimulation are involved.

Study 1 (Chapter 4) examined whether post-exercise cold-water immersion induced augmentation of PGC-1α mRNA is mediated through local or systemic mechanisms. Participants completed acute cycling followed by seated-rest (CON) or single-leg cold-water immersion (CWI; 10 min, 8°C) with muscle biopsies obtained pre-, post- and 3 h post-exercise from a single limb in the CON condition but from both limbs in CWI (thereby providing tissue from a CWI and non-immersed limb, NOT). Muscle temperature decreased following CWI (-5°C), with lesser changes observed in CON and NOT (-3°C; P<0.05). A significant interaction effect was present for AMPK phosphorylation (P=0.031). Exercise (CON) increased gene expression of PGC-1α 3 h post-exercise (~5-fold; P<0.001). Post-exercise CWI augmented PGC-1α expression above CON in immersed (CWI; ~9-fold; P=0.003) and NOT limbs (~12-fold; P=0.001). Plasma Normetanephrine concentration was higher in CWI vs. CON post-immersion (860 vs. 665 pmol·L⁻¹; P=0.034). Data herein reports for the first time that local cooling of the immersed limb evokes transcriptional control of PGC1-α in the non-immersed limb, suggesting increased systemic β-adrenergic activation of AMPK may mediate, in part, post-exercise cold-induction of PGC-1α mRNA.

Study 2 (Chapter 5) assessed the impact of combining a post-exercise cooling stimulus with prior low glycogen as both stressors are shown to separately enhance the post-exercise PGC-1α response. A single-leg depletion protocol and bi-lateral muscle biopsies with and without post-exercise CWI were utilised to give the following conditions: High glycogen control (HI CON), Low glycogen control (LO CON), High glycogen CWI (HI CWI) and Low glycogen CWI (LO CWI). HI limbs began the experimental day ~190 mmol·kg⁻¹ dry
weight (dw) with low limbs at ~85 mmol·kg⁻¹·dw glycogen before undergoing the same relative exercise protocol as Chapter 4. PGC-1α mRNA was different between conditions ($P = 0.039$) with HI glycogen limbs showing greater expression than contralateral LO glycogen limbs ($P < 0.05$). PGC-1α mRNA increased to a greater extent in CWI HI vs. CON HI (ES 0.67 Large). Data herein supports previous research that shows post-exercise CWI is able to augment PGC-1α above the exercise response alone, however this response was not evident in heavily depleted limbs (~85 mmol·kg⁻¹·dw), suggesting a critical threshold may exist for the expected enhancement of PGC-1α to occur when exercise is commenced in a low glycogen state.

Chapter 6 sought to examine the contribution of CWI (Chapter 4, Experiment 1) and/or low muscle glycogen (Chapter 5, Experiment 2) in the activation of PGC-1α via either the canonical (Exon 1a) or the alternative promoter (Exon 1b) regions. Data was obtained using muscle biopsy samples collected from the previous chapters (Chapter 4 and 5). Exercise increased the expression of promoter specific PGC-1α, with greater fold changes seen in Exon 1b. Experiment 1 (Chapter 4) showed PGC-1α Exon 1b expression closely matched the pattern of expression seen for total-PGC-1α, with large, systemic cold-induced increases in the non-immersed (NOT, 2344 fold change from Pre, $P = 0.010$) and immersed (CWI, 1860 fold change from Pre, $P = 0.07$), compared with the control limb (CON, 579 fold change from Pre). Results from experiment 2 (Chapter 5) saw PGC-1α Exon 1a and 1b gene expression increase post-exercise, with the Exon 1b response showing lower fold-changes at 3h post-exercise compared to those from Experiment 1 (chapter 4), despite the same exercise protocol being utilised (~200 fold increases in experiment 2 vs. ~2000 fold increases in experiment 1). The data suggests that depletion exercise in the days prior to the experimental day may have raised basal RNA levels, which may have therefore contributed to dampened fold-changes seen post-exercise when relativized to pre-exercise values. The lack of a cold augmentation in promoter specific PGC-1α gene expression in experiment 2 suggests this response may be extremely acute, and may not occur when cooling is undertaken on the third day of exercise.

This thesis provides a novel insight into the influence of local, systemic and upstream activating mechanisms regulating the post-exercise, post-cooling and exercising in low glycogen states upon PGC-1α. These findings provide mechanistic application for future
study designs and practical application in the support for the use of CWI when the intended target is an upregulation of the gene PGC-1α.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere thanks and gratitude to Professor Warren Gregson for providing the opportunity to pursue a PhD. You instilled a thirst for research in me at undergraduate level that continued through Masters and into Post-Graduate level. Your unwavering confidence in me throughout my time as a student and laboratory technician at LJMU has helped shape my career path and led me to where I am today – and for that, I will be forever grateful. Your support has shown that with hard work, anything is possible, and any obstacle can be overcome. I only hope we can continue to conduct research and discuss science together going forward. I would also like to thank Dr James Morton and Dr Adam Sharples for their continued support and advice throughout my PhD. With a director of studies based overseas, you two were the “faces on the ground” and bore the brunt of my endless questions and problems. With your own students and heavy workload, not once did you turn me away, not have time for me, or complain. James, you have taught me so much about the world of science, academia and research from impromptu conversations and thorough feedback. You always manage to treat your post-graduate students on a level playing field with yourself, and this gives us confidence to pursue the excellence and rigour you quietly expect. Adam, you taught me biochemistry and molecular laboratory skills that have been critical to my research, and will be critical for any future research I complete. I cannot thank you enough for your patience, persistence and openness in developing me into a more complete scientist. Whether it was late night lab sessions, long email threads or persistent knocking on your door, you always found a way of turning things towards a more positive nature, often re-inspiring me in the process. Thank you all again, for your time, patience and support to get me this far. I feel so very fortunate to have learnt from some of the best there is in Sports Science.

Thank you to my parents, brother and sister. You raised me and taught me the values that have made me who I am. You have shown me that real wealth is based around love, family and joy; not how much money you have in the bank. You taught me critical values in life that are so transferrable to science you don’t realise; things such as being inquisitive, enjoying what you do, doing the basics well, and striving for improvement whilst maintaining integrity and pride. I hope I have managed to make you proud.
To my own little family, I dedicate this thesis. Everything I do is for you three! Danielle, you have been and still remain my rock. You put a hold on your career to take care of our boys and allow me to pursue mine. You are the one who saw all the late nights, long weekends, brilliant highs and terrible lows. You put up with all of the mood swings, stress and worry whilst keeping a smile on your face, going off to work yourself and seeing our boys never went without. I could not have completed this without you. To my boys, Jacob and Noah, you make all of the trials and tribulations worth it. Coming home to you two can make any troubles disappear. Although you are both still young, you have always been so mature in the way you accepted that daddy had to work at weekends; whether it was to bring in extra money, or to collect data, or to work on his thesis. I only hope that in seeing this it instils a behaviour in you both to actively pursue your dreams, and that nothing of substance is gained without a lot of hard work. I hope reading this at some point in the future you can be as proud of me as I am of the young men you are becoming.

To all my fellow PhD students and colleagues at LJMU past and present, there are too many of you to name. You have been an unbelievable source of motivation, cooperation, stimulation but most of all inspiration. Thank you to the Football Exchange at LJMU for funding this PhD. A special thanks to Chris Mawhinney and the technical staff Dean and Gemma, for all their assistance, sometimes at short notice. I have made so many friends in my time at LJMU across so many disciplines it really demonstrates the values and spirit of what RISES is. I will forever remember my time here and miss it dearly. Finally, to all of my participants that willingly gave their time, effort, blood, sweat, tears….and muscle! You guys are critical to the advancement of science. Your part is often undervalued, but I thank you all immensely.
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<tbody>
<tr>
<td>1RM</td>
<td>1-Repetition Max</td>
</tr>
<tr>
<td>12RM</td>
<td>12-Repetition Max</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate activated Protein Kinase</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating Transcription Factor 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>β2-AR</td>
<td>β2-Adrenergic Receptor</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>CON</td>
<td>Control condition</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome C Oxidase</td>
</tr>
<tr>
<td>COXIV</td>
<td>Cytochrome C Oxidase Subunit 4</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine Palmitoyltransferase 1</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element Binding Protein</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate Synthase</td>
</tr>
<tr>
<td>CWI</td>
<td>Cold Water Immersion</td>
</tr>
<tr>
<td>CWT</td>
<td>Contrast Water Therapy</td>
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<tr>
<td>DOMS</td>
<td>Delayed Onset Muscle Soreness</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIMD</td>
<td>Exercise Induced Muscle Damage</td>
</tr>
<tr>
<td>ELISA</td>
<td>Solid Phase Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERR-α</td>
<td>Estrogen-Related Receptor alpha</td>
</tr>
<tr>
<td>ES</td>
<td>Effect Size</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose Transporter Type 4</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>HIIT</td>
<td>High-Intensity Interval Training</td>
</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
</tr>
<tr>
<td>HSF-1</td>
<td>Heat Shock Factor-1</td>
</tr>
<tr>
<td>HWI</td>
<td>Hot Water Immersion</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>MA</td>
<td>Metanephrine</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte Enhancing Factor 2</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin Heavy Chain</td>
</tr>
<tr>
<td>MKK</td>
<td>MAP Kinase Kinases</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-Esterified Fatty Acids</td>
</tr>
<tr>
<td>NCAM⁺</td>
<td>Neural Adhesion Molecule Cells</td>
</tr>
<tr>
<td>NMA</td>
<td>Normetanephrine</td>
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</table>
NOT Non-Immersed limb condition
NRF1/2 Nuclear Respiratory Factor 1/2
p38-MAPK p38- Mitogen-Activated Protein Kinase
P53 Tumour-suppressor protein 53
P70S6K Ribosomal Protein S6 Kinase beta 1
Patm Atmospheric Pressure
PCR Polymerase Chain Reaction
PDK4 Pyruvate Dehydrogenase Kinase 4
PGC-1α Peroxisome proliferator-activated receptor γ coactivator-1α
PPO Peak Power Output
Phyd Hydrostatic Pressure
PKA Protein Kinase A
PPARγ Peroxisome Proliferator-Activated Receptor Gamma
RBE Repeated Bout Effect
RER Respiratory Exchange Ratio
RPE Rating of Perceived Exertion
RPL13a Ribosomal protein L13a
rt-qRT PCR Reverse transcriptase quantitative Real-Time Polymerase Chain
Reaction
Ser Serine
SDH Succinate Dehydrogenase
SIRT1 Sirtuin 1
SPE Solid Phase Extraction
Tcore Core Temperature
TFAM Mitochondrial Transcription Factor A
Thr Threonine
Tmus Skeletal Muscle Tissue Temperature
Trec Rectal Temperature
Tsk Skin Temperature
TWI Thermoneutral Water Immersion
TWP Thermoneutral Water Immersion Placebo
Tyr Tyrosine
UCP3 Uncoupling Protein 3
VEGF Vascular Endothelial Like Growth Factor
Vmax Maximum Velocity
\( \dot{V}O_{2\text{max}} \) Maximal Oxygen Uptake
\( \dot{V}O_{2\text{peak}} \) Peak Oxygen Uptake
WBC Whole-Body Cryotherapy
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1.1 BACKGROUND
It is well established that exercise that incorporates a novel eccentric component, or is of a particularly high-intensity or long duration, elicits muscle structure damage and disturbances in cellular and metabolic homeostasis, inflammation, and discomfort for the athlete (Friden & Lieber, 1992; Pournot et al., 2011). These alterations to metabolic homeostasis, damage to the muscle structure and ensuing inflammatory response are paralleled by other undesirable variables such as energy substrate depletion (Tee et al., 2007), hyperthermia (Tyler et al., 2015), and oxidative stress (Close et al., 2004). Collectively, this response can negatively affect an athlete through diminished quality of training and performance (Marcora & Bosio, 2007). This potential for decreased performance in training and competition, and need for ‘optimal recovery’, has ultimately led to significant levels of research optimising appropriate and effective recovery strategies.

Of particular interest is cold-water immersion (CWI). Since inflammation is fundamental in the aetiology of exercise stress, whole limb cooling via cold-water immersion is frequently applied immediately following exercise in an attempt to alleviate some of the physiological and functional deficits often associated with exercise stress and/or fatigue (Bailey et al., 2007). Historically cold application was used in the treatment of soft tissue injury (Swenson et al., 2011), more recently its popularity stems from an ability to reduce perceptions of DOMS often associated with intense exercise (Howatson & van Someren, 2008) and induce acute subsequent performance benefits (Leeder et al., 2012). The mechanisms of CWI are proposed to be related to temperature and pressure induced changes in blood flow and reduced muscle temperature (Gregson et al., 2011), subsequently reducing post-exercise inflammation (Howatson & van Someren, 2008; Leeder et al., 2012). Specifically, this reduction of inflammatory processes is said to be partially mediated
through temperature-induced reductions in micro-vascular blood flow around the injured tissue (Gregson et al., 2011). It is therefore proposed that whole-limb CWI is likely to be effective by virtue of its effect on deep muscle blood flow (Gregson et al., 2011; Mawhinney et al., 2013). As a proposed anti-inflammatory, CWI cannot repair the primary (structural) damage resulting from the exercise insult, but can act to protect against secondary damage caused by oedema, cytokines and oxidative stress (White & Wells, 2013). Recent data indicates the effects of CWI might not act by way of reducing markers of intra-muscular inflammation, but potentially through influencing other aspects of the inflammatory response; particularly analgesic perception and reduction of oedema (Peake et al., 2016; Allan & Mawhinney, 2017).

Whilst the acute recovery period is vital for restoration of energy stores and recovery from exercise induced damage, it is also the window for mediating adaptation via cell signalling and remodelling (Close et al., 2005; White & Wells, 2013). At a molecular level, adaptations to training can be described as repeated, transient increases in gene expression that ultimately lead to increased, steady state levels of specific proteins (Coffey & Hawley, 2007). Numerous signalling mechanisms induce changes in transcriptional activity that will independently, or together, activate phosphorylation networks. This will in turn activate transcription factors within the cell, causing them to bind to the promoter region of the gene of interest. In response to endurance based exercise protocols, transcription factor activation and messenger-RNA (mRNA) accumulation generally occurs within several hours after exercise (Pilegaard et al., 2005) which subsequently may induce translation of the specific proteins in the hours to days following (Perry et al., 2010). What is important for endurance performance is the training induced increase of skeletal muscle mitochondrial content, termed mitochondrial biogenesis. This is vital for mediating energy homeostasis and
resistance from fatigue (Holloszy, 1967; Coffey & Hawley, 2007) by improving the tissue’s capacity for oxygen consumption and ATP provision (Hood, 2009). The transcription factor co-activator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a critical regulator of mitochondrial biogenesis (Wenz, 2013) and therefore the oxidative adaptive process. Research has shown acute skeletal muscle contraction increases PGC-1α mRNA abundance (Pilegaard, et al., 2003), whilst exercise training is associated with increased PGC-1α protein content (Burgomaster et al., 2008).

In the initial discovery by Puigserver and colleagues (Puigserver, et al., 1998), PGC-1α was not detectable in resting muscle of mice; however, exposure to cold temperatures showed marked increases. Moreover, the application of a cold stimulus (e.g. CWI) may further enhance the expression of PGC-1α mRNA (Slivka et al., 2013; Ihnsan et al., 2014a; Joo et al., 2016), total protein content (Ihnsan et al., 2015), and therefore the adaptive process, in human skeletal muscle beyond that of exercise alone. Despite recent advances establishing that acute and regular cold application/exposure has the ability to accelerate PGC-1α mRNA expression and protein content above that of the exercise stimulus alone the precise mechanisms controlling this remain to be fully elucidated. Indeed, recent research surrounding isoform specific responses of PGC-1α to different modes and intensities of exercise offer an interesting concept to such an investigation (Popov et al., 2014; 2015a; 2015b). With evidence showing different PGC-1α promoters important for gene expression at rest and exercise it remains to be seen what other external stressors can influence promoter-specific gene expression.

Moreover, recent evidence suggests that completing exercise training under the influence of reduced carbohydrate (CHO) stores can enhance markers of mitochondrial biogenesis,
and hence oxidative adaptation, to a greater extent than when aerobic exercise is performed in a glycogen-replete state (Hansen et al., 2005; Psilander et al., 2013). The mechanisms behind a reduced-CHO augmentation of mitochondrial biogenesis appear to occur upstream of PGC-1α and be dependent upon AMP-activated Protein Kinase (AMPK) and p38-Mitogen-activated protein kinase (p38-MAPK) activation (Chan et al., 2004; Steinberg et al., 2006). With pre-exercise low glycogen concentration leading to gains in the post-training oxidative adaptive process this has become an important area of research with plenty of avenues to explore. As both methods have been shown to augment the adaptive process through the PGC-1α pathway, it is suggested that a combination of CWI and reduced CHO availability has the potential to accelerate the activity of PGC-1α, and other transcriptional regulators of mitochondrial biogenesis, above levels previously seen.
1.2 AIMS AND OBJECTIVES OF THESIS

The aim of this thesis is to establish the humoral and molecular mechanisms implicated in the cold augmentation of the transcriptional co-activator PGC-1α when CWI is applied following high intensity exercise. Having established potential controlling mechanisms a further aim is to assess whether a combination of stressful stimuli can enhance the post-exercise PGC-1α response to an even greater extent. As such, it is hoped that the studies contained within this thesis will help inform recovery prescription guidelines as well as scientific study designs.

The aims of this thesis will be achieved by the following objectives:

a) Examining whether the CWI induced increase in key regulators of mitochondrial biogenesis (PGC-1α) observed post-exercise is mediated through local or systemic mechanisms.

b) Examine whether exercise commenced with low muscle glycogen further stimulates the post-exercise and CWI-induced expression of PGC-1α.

c) Examining the influence of CWI and low muscle glycogen levels on the promoter specific regulation of the PGC-1alpha gene.
CHAPTER 2

LITERATURE REVIEW
2.1 EXERCISE RECOVERY

The stress of regular training and competition has the ability to impair performance, be it through reduced quality of training or impaired competitive performance. Inadequate recovery between training sessions or after competition may ultimately lead to fatigue; defined as the sensation of tiredness with associated decrements in muscle function and performance (Abbiss & Laursen, 2005). Ultimately, if fatigue is allowed to accumulate it may lead to overtraining/overreaching, increasing the likelihood of injury and reduced performance (Soligard et al., 2016).

Recovery can be defined as the process of restoring the body’s physiological and psychological function in order to return to a pre-fatigued level of performance (Versey et al., 2013). Therefore, accelerated recovery should allow the athlete improved subsequent performance and quality of training, thus maintaining a suitable or improved stimulus for adaptation. Consequently, the importance of recovery to the professional athlete has led to a wide variety of recovery strategies being investigated, with a preliminary focus on providing supporting evidence to practices currently being utilised in the field. Some of the most popular include active recovery, stretching (Barnett, 2006), compression garments (Pruscino et al., 2013), sleep (Fullagar & Bartlett, 2016), nutritional strategies, massage, cryotherapy and hydrotherapy (Reilly & Ekblom, 2005). Hydrotherapy can be further devolved into hot-water immersion (HWI) and contrast-water therapy (CWT) whilst cryotherapy methods include ice application and/or ingestion, cooling sprays and whole-body cryotherapy (WBC) (Meeusen & Lievens, 1986; Vaile et al., 2008a). Possibly the most prevalent recovery strategy spans both hydrotherapy and cryotherapy, and is termed CWI.
2.2 COLD WATER IMMERSION

The use of CWI for recovery from exercise stems from its use in athletic settings, where ice application in the immediate treatment of acute soft-tissue injuries and rehabilitation work was often combined with rest, compression and elevation (Meeusen & Lievens, 1986). Proposed benefits of CWI were initially focussed around the analgesic effects of cold temperatures. Cold-induced alterations in neurological processes, such as decreased nerve conduction velocity (Algaflly & George, 2007), allow for greater pain tolerance alongside decreased pain sensation (Galvan et al., 2006). Mechanisms by which CWI is purported to benefit recovery are hypothesised to be a combined result of temperature and pressure induced changes in blood flow and reduced muscle temperature (Gregson et al., 2011). Cold temperatures may facilitate recovery from exercise that is metabolically stressful by reducing intramuscular temperature and metabolism (Ihsan et al., 2013), whilst a cold-induced reduction in muscle blood flow (Gregson et al., 2011) may limit inflammatory signalling, oedema and thus any subsequent secondary damage/cell stress to the muscle fibres (Swenson et al., 1996; Puntel et al., 2011). Despite the recent surge in CWI research, the mechanisms of action are still not fully understood. Physiological mechanisms and effects of CWI on exercise recovery are discussed in more detail in Sections 2.3-2.4.

Despite its popularity in the elite setting CWI continues to lack constructive evidence based guidelines. Its use is largely dependent on numerous external factors that control its application such as mode of exercise, cost and logistics. Immersion is usually legs only or whole body depending on the predominant muscle mass used in the athletes chosen sport. Across the board CWI is adapted to suit logistical issues and independent budgets, however, one thing that remains consistent when applying CWI for recovery purposes is the lack of agreement in application guidelines, highlighted recently by McGorm and colleagues.
(2015). Through the use of a 15-question online survey 29 respondents from 17+ sports showed nearly 80% use temperatures between 5-15°C, with 73% using a cold pool or “makeshift bath” (wheelie bin). Furthermore, 83% of respondents immersed for durations of 10 minutes or less, 17% used water immersion outside the range of 5-20°C and nearly a third failed to monitor and/or regulate the temperature of the water. The final point alone may reduce the effectiveness of the strategy employed due to the transfer of heat from the body to the water.

2.2.1 Temperature.

As the mechanisms of action of CWI are largely thought to be derived from a reduction in muscle tissue temperature (Section 2.4.1) it is sensible to suggest that the temperature of water should be adequate to reduce tissue temperature in a time frame no longer than the duration of immersion. Few studies have attempted to determine the ideal immersion temperature (Yeargin et al., 2006; Vaile et al., 2008b; Almeida et al., 2016). Yeargin et al. (2006) reported CWI of 14°C (12 min, legs only) preferably improved subsequent 2-mile running race performance vs. 5°C and a control. Vaile et al. (2008b) compared intermittent immersions in 10, 15 and 20 °C water for 5 min and continuous immersion in 20 °C for 15 min following cycling. All CWI conditions supported maintenance of subsequent (1h later) cycling time-trial performance (10 °C: -0.6 %, 15 °C: 0.4 %, 20 °C: -1.0 %, 20 °C continuous: -0.6 %) compared with active recovery (-4.1 ± 1.8 %), with no significant differences between CWI conditions. More recently, Almeida et al. (2016) investigated the effect of differing immersion protocols on the recovery of heart rate variability post-exercise. Their findings indicated 15 min at 14°C was superior when compared to a control, 5 min at 9°C, 5 min at 14°C and 15 min at 9°C.
2.2.2 Duration.

As with temperature, a range of durations are currently applied in practice with durations as short as 30s utilised (McGorm et al., 2015). Ultimately such short durations will be unable to elicit sufficient reductions to either core or muscle temperatures and have any subsequent impact on physiological mechanisms such as fluid shifts as a result of hydrostatic pressure (Section 2.2.3); which have been shown to require at least 10 min (Hinghofer-Szalkay et al., 1987). Although it is likely that longer durations reduce tissue temperature to a greater extent, primarily through the laws of thermodynamics and heat transfer, they are often less likely to be applied. Longer durations may cause unnecessary discomfort to the athlete whilst increasing the likelihood of shivering thermogenesis, negatively impacting metabolic recovery through increased energy expenditure (Ihsan et al., 2015). Moreover, it has been shown that immersion durations of 30 min, irrelevant of temperature, may exacerbate the exercise-induced inflammatory response (White et al., 2014). Despite this, a recent review (Machado et al., 2016) suggests a dose-response relationship may occur for recovery with post-exercise CWI, particularly in the management of muscle soreness. Machado and colleagues (2016) stated that longer durations (10-15 min) are responsible for improved recovery, particularly immediately following exercise, and thus suggested 11-15 min as the optimal duration for CWI.

2.2.3 Depth.

The depth of immersion has a large impact on the hydrostatic pressure and fluid shifts that are proposed to benefit the athletes’ recovery (discussed in detail in Section 2.4.3). Indeed, Johansen et al. (1995) showed during neck deep immersion that central venous pressure rose by 12 mmHg and remained elevated, whilst waist deep immersion saw marginal increases (1.6 mmHg). However, many factors have a role to play in determining the ideal
CWI protocol, lending support to an individualised prescription dependent upon all of these factors. Only one study exists that assessed the impact of different depths of water immersion (seated vs. standing) upon athletic recovery (Leeder at al., 2015) with results indicating any slight differences in hydrostatic pressure caused by different depths lead to no differences between the two protocols. A paucity of data exists to support the importance of water immersion depth upon physiological mechanisms associated with benefits to recovery. Moreover, it is important to note the variable methods utilised by different groups and the impact this may have upon the physiological response to water immersion, with a particular focus on blood flow and fluid shifts.

During water immersion hydrostatic pressure acts on the body, with the magnitude having a linear relationship with both water density and immersion depth according to the equation:

\[ P_{\text{hyd}} = P_{\text{atm}} + g \times p \times h \]

where \( P_{\text{hyd}} \) = water pressure (Pa), \( P_{\text{atm}} \) = atmospheric pressure (sea level *1,013 Pa), \( g \) = gravity (9.81 m/s²), \( p \) = water density (1,000 kg/m³) and \( h \) = height of the water (m) (Wilcock et al., 2006). Therefore, hydrostatic pressure is influenced primarily by immersion depth, with a depth of 1m having a similar pressure to that of normal diastolic blood pressure (74mm Hg). Hydrostatic pressure upon the body is proposed to cause fluid shifts within the body, displacing gas and fluids to areas of lower pressure, thus a person standing in water will experience inwards and upwards compression on the body, displacing fluids from the lower extremities into the thoracic region (Wilcock et al., 2006). The importance of fluid shifts to the recovery process is highlighted by the fact that the human body is comprised of 50-60% fluid, located in intracellular, interstitial (between cells) or intravascular (blood plasma) space (Wilcock et al., 2006). CWI is said to benefit recovery through haemodilution: blood displacement from the interstitial to the intravascular spaces. In doing
so, fluids leaving the interstitial spaces are replaced by intercellular fluids, creating an intracellular-intravascular osmotic gradient said to allow greater metabolite removal implicated in the development of fatigue (Ihsan et al., 2016). One thing to note is that a change in temperature of the water may cause alterations in density (i.e. colder water has a greater density than thermoneutral water) and therefore alterations to pressure and the equation outlined above. Indeed, such changes in density and therefore pressure with temperature may impact the hydrostatic pressure upon the limb and therefore any fluid shifts thereafter; the extent of which is unknown.

2.3 CWI AND EXERCISE RECOVERY

CWI is regularly used to combat deleterious effects of exercise that is of a particularly intense or damaging nature, in order to reduce pain and improve subsequent training intensity and/or performance. A summary of this research is discussed below with interested readers directed to excellent reviews and meta-analyses on this topic for further information (Leeder et al., 2012; Versey et al., 2013).

2.3.1 Metabolic Stress

Over the last two decades, much research utilising post-exercise CWI as a method for promoting recovery has assessed recovery from high-intensity exercise. In this sense, high-intensity exercise refers to exercise that involves a high metabolic cost with an eccentric component (i.e. repeated sprints) (Leeder et al., 2012). Indeed, a plethora of research exists showing positive effects of post-exercise CWI upon specific performance variables. These performance improvements (versus a control or in comparison to pre-exercise values) have been reported across a range of exercise modalities. Particularly, ones that incorporate a
longer duration and/or an intermittent element, including, but not limited to, running (Bailey et al., 2007; Brophy-Williams et al., 2011), high-intensity interval training (Broatch et al., 2014), cycling (Lane & Wenger 2004; Vaile et al., 2008a; Vaile et al., 2011), BMX (Marquet et al., 2015), exhaustive anaerobic exercise (Pournot et al., 2011) and various match-play situations (Montgomery et al., 2008; Parouty et al., 2010; Rowsell et al., 2011).

Data also shows neutral (neither positive or negative) and negative effects of CWI on exercise of a shorter duration and/or greater intensity; including time trial and sprint performance (Peiffer et al., 2010a; Higgins et al., 2011), power output, sprint cycling, maximal voluntary contraction and sprint swimming performance (Schniepp et al., 2002; Crowe et al., 2007; Peiffer et al., 2009a; Parouty et al., 2010). Inconsistencies are clear and shall continue to remain due to differences in exercise stimuli, immersion protocols, subject training status, individual differences and performance measures assessed. Despite this, assessment of the current literature indicates that CWI following high-intensity, short-duration exercise is largely associated with neutral (and some negative) effects, whilst greater recovery tends to occur following exercise of a greater endurance component (longer duration, endurance and intermittent codes).

One explanation for the inconsistent impact of CWI upon recovery from short-duration, high intensity exercise and/or maintenance of performance is the likely reduction in skeletal muscle tissue temperature resulting from different immersion protocols applied in these studies. Ultimately, a reduction in tissue temperature will reduce nerve conduction velocity leading to associated decrements in skeletal muscle performance (Algaflow et al., 2007). Indeed, skeletal muscle function is greater when warmer (Faulkner et al., 1990). Shorter immersion durations have been investigated (Almeida et al., 2016), however CWI is
efficient by way of physiological impact and therefore protocols need to ensure they use the correct temperature, duration and depth of immersion in order to stimulate such physiological mechanisms purported to assist in recovery. Likewise, the greater impact of post-exercise CWI upon subsequent performance measures in exercise of longer durations may be associated with improved thermoregulatory control in the form of lower core body temperature observed following CWI (Peiffer et al., 2010b).

2.3.2 Mechanical Stress

Mechanical stress is often associated with exercise protocols consisting of large eccentric components, but relatively low metabolic cost (Leeder et al., 2012). To this extent, CWI has been shown to have no effect on the restoration of muscle strength, limb circumference/volume and isokinetic torque (Gulick et al., 1996; Paddon-Jones & Quigley, 1997; Leeder et al., 2012; Schimpchen et al., 2016; Argus et al., 2016), whilst having more success in recovering estimated muscle power (Fonseca et al., 2016), 30 s continuous jump test (Garcia et al., 2016), jump height (Webb et al., 2013), single- and 2-leg counter movement jumps (Abaidia et al., 2016) and submaximal muscle function (Ascensão et al., 2011; Roberts et al., 2014). Improvements in mechanical exercise performance also vary in duration, with some benefits reported to last for at least 3 days after the initial exercise insult. A good example of this is maximal voluntary contraction assessments, where improvements are regularly reported after post-exercise CWI at 24 (Skurvydas et al., 2006; Bailey et al., 2007; Pournot et al., 2011), 48 (Skurvydas et al., 2006; Bailey et al., 2007; Ingram et al., 2009) and 72 h post-immersion (Skurvydas et al., 2006; Vaile et al. 2008c).

The effects of CWI on recovery from intense exercise is highlighted by a recent systematic meta-analysis review (Leeder et al., 2012). The authors confirm CWI is effective at reducing
the experienced DOMS following damaging exercise, suggesting that inconsistencies in research outcomes could be related to different immersion protocols, measures of damage/soreness and/or the type of preceding exercise. Investigations with a focus on DOMS cover a variety of stimuli, ranging from high-intensity interval (Brophy-Williams et al., 2011), team sports (Elias et al., 2013), eccentric damaging exercise (Crystal et al., 2013) and power based protocols (Counter-movement jumps; Jakeman et al., 2009). In a meta-analysis, Hohenauer and colleagues (2015) and Bleakley et al. (2012) showed CWI was successful in significantly alleviating the symptoms of DOMS 24, 48, 72 and 96 h after application of the cooling stimulus.

Whilst most evidence supports the use of CWI to benefit post-exercise muscle soreness there is some evidence that displays CWI having no effect (Eston & Peters, 1999; Howatson et al., 2009; Sellwood et al., 2007), highlighting the modality’s equivocal nature. The largely perceptual nature of muscle soreness measures could be a reasonable explanation for this lack of agreement, with ongoing debate existing surrounding the potential of the placebo effect during the use of CWI (Section 2.4.2, Broatch et al., 2014). Despite this, sufficient evidence exists for the benefit of CWI upon subjective measures of soreness (Bailey et al., 2007; Cortis et al., 2010).

2.4 PHYSIOLOGICAL RESPONSES TO CWI FOLLOWING EXERCISE

The use of CWI as a method of recovery stems from its popularity in therapeutic and rehabilitation settings for the treatment of acute soft-tissue injuries (Meeusen & Lievens, 1986). Much of the attention CWI has received over the past decade has sought to
investigate the physiological mechanisms by which it is purported to enhance recovery. It is widely speculated that these mechanisms are related to changes in body temperature and blood flow (Leeder et al., 2012; Ihsan et al., 2016; Figure 2.1). This next section will focus individually on physiological mechanisms to give an overview of what effects CWI has upon human physiology.

![Figure 2.1: Possible mechanisms mediating the effects of CWI on EIMD (Reproduced from Ihsan et al., 2016, Sports Med, with permission of the publisher Springer).](image)

2.4.1 Thermoregulatory

The pattern of cooling post-exercise upon various temperature measures is shown in Figure 2.2. Generally, skin temperature decreases exponentially through cooling via heat extraction and conduction (Gregson et al., 2011), reaching its lowest point earliest and increasing exponentially through the post-cooling period. The magnitude of temperature decrease is dependent on the water temperature, with colder temperatures associated with greater

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2.4.1 Thermoregulatory

The pattern of cooling post-exercise upon various temperature measures is shown in Figure 2.2. Generally, skin temperature decreases exponentially through cooling via heat extraction and conduction (Gregson et al., 2011), reaching its lowest point earliest and increasing exponentially through the post-cooling period. The magnitude of temperature decrease is dependent on the water temperature, with colder temperatures associated with greater
reductions in skin temperature (Gregson et al., 2011). Skin temperature is reduced during CWI independent of being preceded by exercise or rest. Roberts et al. (2015) showed CWI at 10°C for 10 min decreased thigh skin temperature from a post-exercise value of ~35°C to a minimum of ~24°C at the end of immersion, whilst Gregson et al. (2011) showed CWI per se at 8°C for 10 min reduced thigh skin temperature from a resting pre-immersion value of ~30°C to a minimum of ~15°C.

![Figure 2.2](image)

**Figure 2.2:** Relative pattern of temperature change in different tissue layers during exercise, cooling and post-cooling period, from White & Wells (2013), Extreme Physiol Med. Permission for reproduction granted by BioMed Central. Tsk = Skin temperature, Tc-general=core temperature following cryotherapy applied to large mass, Tc-local = core temperature following cryotherapy applied to small mass, Tm@1 and Tm@3 = muscle tissue temperature at 1 and 3cm depth.

Core temperature decreases following CWI preceded by rest (Gregson et al., 2011; Joo et al., 2016) or exercise (Mawhinney et al., 2013), with the rate largely dependent on the
thermal gradient and peripheral blood flow (White & Wells, 2013). The reactivity of core temperature to the cooling stimulus is slower than more peripheral measures, whilst the magnitude of change is greater when cooling is applied following exercise (~1-2°C decrease from Pre-immersion values: Peiffer et al., 2009a; Joo et al., 2016) vs. rest (~0.2°C decrease from Pre-immersion values: Gregson et al., 2011; Joo et al., 2016); likely attributable to the large increases in core temperature that occur following exercise.

Skin cooling during CWI decreases muscle temperature via conductive heat exchange and convective cooling of the limb (Gregson et al., 2011). Thus, changes in deeper intramuscular temperature occur at a slower rate than more superficial depths. In this way, deep muscle temperature (3cm) continues to cool through the post-cooling phase as heat is transferred to warmer superficial tissues, with temperatures at 2 and 1cm depths re-warming and returning to baseline within 3h post-immersion (Gregson et al., 2013). Indeed, the greater the difference in temperature between the tissue and the water of immersion (i.e. greater thermal gradient), the faster the temperature will change (Taylor et al., 2008; Gregson et al., 2011; Mawhinney et al., 2013). Additionally, the change in intramuscular temperature is also related to the duration of cold exposure (Meeusen & Lievens, 1986), with very large decreases seen (~18°C) following 3h of CWI (Abramson et al., 1966). Surrounding adiposity will also effect the heat transfer gradient due to insulation. A difference of 4°C is noted (3cm and 1cm depths) between high and low adiposity of the calf (>20mm vs. <8mm skinfold thickness, respectively) 30 min following ice pack application (Myrer et al., 2001), whilst the time taken to decrease intramuscular temperature at 1cm depth, by 7°C increased with greater levels of adiposity (Otte et al., 2002).
2.4.2 Analgesic

The analgesic effect of reduced muscle and skin temperature represents a key mechanism by which CWI is purported to have positive effects on post-exercise recovery. Herrera et al. (2010; 2011) have shown cooling reduces the neural conductance velocity in sensory and motor neurons. Importantly, sensory neurons were influenced by more modest changes in temperature, suggesting an analgesic benefit occurs before cooling impairs contraction kinetics (White & Wells, 2013). Further cold-induced alterations in neurological processes, such as decreased nerve conduction velocity, allow for greater subjective interpretations of post-exercise recovery. Measures of pain tolerance and decreased pain sensation (Galvan et al., 2006; Algafly & George, 2007), measures often related to DOMS, have been shown to be improved following CWI.

Separately, it has been suggested that CWI has a large psychophysiological benefit and that any proposed analgesic benefit in recovery might be due to individual perception of recovery and a possible placebo effect (Broatch et al., 2014). In this sense, CWI may be effective by virtue of its analgesic properties upon perception of pain acting as a placebo. Indeed, many CWI studies have focussed on subjective measures of muscle soreness (Bailey et al., 2007; Ingram et al., 2009; Rowsell et al., 2011; Stanley et al., 2012) therefore it is plausible that positive subjective outcomes are a result of expectation rather than altered physiological mechanisms. Recently the impact of the placebo has been demonstrated by Broatch and colleagues (2014). In a matched groups design 30 males performed an acute high-intensity interval training (HIIT) session consisting of 4 x 30s sprints, immediately followed by 15min of either CWI (10°C, n=10), thermoneutral water immersion (TWI, 34.7°C, n=10) or a thermoneutral water immersion placebo (TWP, 34.7°C, n=10). In this respect, the placebo condition was achieved by misleading participants to believe a pH
balanced skin cleanser, added to the water in sight of the subject was beneficial for recovery from HIIT. Main findings showed TWP was superior to TWI for recovery of quadriceps strength after acute HIIT, whereas no difference was seen between TWP and CWI, despite a reduction in intramuscular temperature seen for CWI. Results were attributed to improved subjective ratings of pain and readiness for exercise in the CWI and TWP conditions.

2.4.3 Cardiovascular

Purported effects of CWI may be mediated through vasoconstrictive reductions in microvascular blood flow to the injured muscle, which would reduce oedema and the induction of inflammatory events (Gregson et al., 2011). Indeed, early use of cryotherapy is associated with significantly less swelling (oedema) between ruptured myofibers, inflammation and tissue necrosis, alongside accelerated early regeneration (Schaser et al., 2007; Järvinen et al., 2013). At rest, cold water immersion (8°C) is shown to distribute more blood flow to the skin, than in thermo-neutral conditions (22°C), whilst total limb blood flow remained unchanged between conditions (Gregson et al., 2011), indicating a possible reduction in skeletal muscle blood flow may be responsible for associated benefits of CWI upon exercise induced muscle damage. In contrast, post-exercise CWI was shown to reduce whole-limb blood flow (Vaile et al., 2011), however, the technique utilised in this study fails to distinguish between muscle and skin blood flows. Therefore, Mawhinney et al. (2013) examined the influence of CWI on limb (duplex ultrasound) and cutaneous blood flows (laser Doppler flowmetry) following exercise. Twelve participants completed a continuous cycle exercise protocol at 70% peak power output until a core temperature of 38°C was attained, followed by either CWI (10-min in 8°C or 22°C water) or passive rest. Results showed a greater decrease in femoral artery and cutaneous blood flows throughout the recovery period in the cooling conditions in comparison to passive rest, however no
difference was found between the immersion conditions. Despite greater reductions in muscle temperature occurring in colder water, it was deemed to be a magnitude not large enough to induce a significant difference in femoral artery blood flow between the cooling conditions. The fact that similar reductions in muscle blood flow are shown to occur, independent of water temperature, offers greater insight into the mechanistic background by which CWI may alleviate the stress associated with intense exercise.

In addition to recovery benefits arising from cold related reduction in tissue temperature, CWI is proposed to decrease microvascular blood flow (Lee et al., 2005). Near infrared spectroscopy (NIRS) has often been used to estimate changes in local muscle blood flow following post-exercise CWI, with muscle blood volume shown to decrease when CWI is applied after high intensity intermittent (Ihsan et al., 2013) and resistance (Roberts et al., 2015) exercise protocols. However, this technique of assessment has been criticised (Mawhinney et al., 2013) as it only provides indirect estimates of changes in blood volume within the microcirculation and not changes in blood flow per se. As NIRS uses the different absorption of near infrared light to determine concentration changes in oxygenated haemoglobin, deoxygenated haemoglobin and total haemoglobin it is liable to error following stress such as cooling as the signal cannot differentiate the contribution of myoglobin from haemoglobin (Davis et al., 2006).

Mechanistically, the hydrostatic pressure (see section 2.2.3) from water immersion may promote post-exercise recovery by way of reducing swelling, oedema and subsequent inflammatory events. Indeed, exercise causes blood plasma, fluid and inflammatory cell population movement into the muscles, the volume of which is relative to the intensity of exercise (Miles et al., 1983). As such, it is suggested this leads to a reduced oxygen delivery
via compression of localised capillaries and infiltration of inflammatory leukocytes and monocytes, both of which contribute to cellular damage (Pedersen & Toft, 2000). Limb girth is a simple way of assessing muscle oedema and swelling (Eston & Peters, 1999) however the effectiveness of this method is unconvincing (Goodall & Howatson, 2008).

2.4.4 Inflammation

Historically, cold, mainly in the form of icing, was originally used to treat soft tissue injury (Swenson et al., 2011). The term inflammation is often used loosely to encompass associated effects of exercise induced muscle damage, including oedema, swelling and DOMS. However, the inflammatory response is also characterised by physiological, cellular and molecular changes in the injured muscle (Peake et al., 2017a), often termed secondary damage. In response to stress and/or muscle damage induced by exercise the inflammatory response comprises the release of intracellular proteins such as cytokines, chemokines and myokines alongside satellite and inflammatory cell populations (such as neutrophils, macrophages and T cells) (Peake et al., 2017a). It is the dynamics of these cells’ interaction with each other within the extracellular matrix that determines the effectiveness and efficiency of recovery (Peake et al., 2017a).

A common assessment of post-exercise inflammation is the measure of systemic inflammatory markers in the blood, such as immune leukocytes and monocytes (Broatch et al., 2014, Gonzalez et al., 2014; Jajtner et al., 2014), inflammatory cytokines (Peake et al., 2008; Broatch et al., 2014; Stacey et al., 2010; Rowsell et al., 2014; Townsend et al., 2015), lymphocytes and neutrophils (Stacey et al., 2010; Broatch et al., 2014), and c-reactive protein (Brophy-Williams et al., 2011; Ascensão et al., 2011). The resultant impact of CWI is as equivocal as many other variables within the field, with results showing reductions
(Stacey et al., 2010; Ascensão et al., 2011; Gonzalez et al., 2014; Jajtner et al., 2014; Rowsell et al., 2014), no differences (Peake et al., 2008; Stacey et al., 2010; Rowsell et al., 2014; Townsend et al., 2015) and, in situations where the immersion duration is extended too long (30 min), potential elevation of the systemic inflammatory response (White et al., 2014).

Original observations of the benefit of cryotherapy techniques upon secondary damage to skeletal tissue were seen following crush injury in rodent models. Crush injuries in rats were treated with continuous cryotherapy, in the form of ice application, or no treatment at all (Merrick et al., 1999). Greater reductions in markers of oxidative and cellular damage (triphenylformazan) were seen in those tissues treated with 30min continuous ice application, indicating that cryotherapy reduces the magnitude of secondary injury.

A recent well-designed study by Peake and colleagues (2017b) incorporated several local (gene expression within the muscle) and systemic (blood plasma/serum concentration) inflammatory markers to investigate the effectiveness of CWI versus active recovery after a bout of resistance exercise. Results showed cytokines, neutrophils and heat shock proteins (HSP; help prevent aggregation of denatured proteins, Morton et al., 2009) did not differ significantly between CWI or active recovery. Moreover, whilst cold activated chaperone proteins (Cold shock proteins) can be found in humans (Lindquist et al., 2014) this remains an unexplored area of interest that may offer further explanation to the efficacy of CWI. Whilst the findings of Peake and colleagues (2017b) indicate that cold water immersion is no more effective than active recovery for reducing inflammation or cellular stress in muscle after a bout of resistance exercise, it remains to be determined if such measures are different when compared with no recovery protocol, or following other exercise modalities.
Indeed, the authors comment that the stress applied during the exercise protocol used was similar to that of a standard training session. Therefore, future research should focus on the impact of CWI on a heightened inflammatory response that occurs in eccentric, unaccustomed activity and/or ultra-endurance events. Despite this, the study does go some way in refuting the long revered hypothesis that CWI benefits recovery through reducing subsequent inflammatory events (Meeusen & Lievens, 1986).

To date, research surrounding the physiological effects of CWI remains largely contradictory. Whilst a reduction in tissue temperature may provide analgesic benefits because of reduced neural conduction velocity (Herrera et al., 2010) and reductions in muscle blood flow may serve to prevent or reduce formation of oedema and swelling (Gregson et al., 2011; Mawhinney et al., 2013), evidence for increased metabolite removal via a greater osmotic gradient as a result of hydrostatic fluid shifts is weak. Moreover, molecular and biochemical evidence is contrasting, with systemic markers of inflammation in the blood being seen to reduce after CWI (Ascensão et al., 2011; Jajtner et al., 2014), whilst local markers within skeletal muscle show no change compared with active recovery (Peake et al., 2017b). Whilst a lack of agreement between studies is clear, it is apparent that studies highlighting a negative effect of CWI upon physiological mechanisms of recovery are small in number, and generally associated with short-duration, high-intensity exercise; with CWI having a greater impact following exercise of longer durations.
2.5 MOLECULAR MECHANISMS REGULATING SKELETAL MUSCLE MITOCHONDRIAL BIOGENESIS

In any sport, particularly those that are endurance based, constant energy supply is important for proper tissue function. Mitochondria are often termed the “powerhouse of the cell” and serve a critical function in the maintenance of cellular energy homeostasis, improving the tissues capacity for oxygen consumption and adenosine triphosphate (ATP) provision (Hood, 2009) whilst also playing pivotal roles in thermogenesis, lipid metabolism, calcium signalling, metabolite synthesis and apoptosis (Kelly & Scarpulla, 2004; Ploumi et al., 2017).

Mitochondrial biogenesis is a tightly regulated process that depends on both the mitochondrial and nuclear genomes. Synchronised transcription and translation of nuclear and mitochondrial genes is needed for the generation of new organelles (Ploumi et al., 2017). Regulation of mitochondrial biogenesis occurs with the activation of sensor enzymes such as transcription factors, coactivators and regulators in response to various stimuli, such as nutrient availability, hormones, growth factors and temperature fluctuations. Importantly, the transcription factors bind to specific DNA sequences of target genes, controlling the rate of mRNA transcription and subsequent translation of functional proteins. The nuclear respiratory factors (NRF1 and NRF2), estrogen-related receptors (ERR-α) and PGC-1α are major modulators in mitochondrial proliferation (Palikaras & Tavernarakis, 2014). In particular, NRF-1/-2 have been implicated in the activation of mitochondrial transcription factor A (TFAM), a key component in the transcription of mitochondrial DNA (mtDNA) (Ekstrand et al., 2004; Wenz, 2013).
The importance of mitochondrial biogenesis became of greater relevance to exercise physiologists after pioneering work showed increases in mitochondria size, number and/or volume occurred after a physiological stimulus (Holloszy, 1967; Gollnick & King, 1969). The plasticity of the response was highlighted by the fact that mitochondrial content could be reduced by prolonged periods of muscle disuse, such as denervation (Wicks & Hood, 1991) or immobilization (Booth & Kelso, 1973). Since the publication of Holloszy’s (1967) pioneering work demonstrating that exercise training stimulates mitochondrial oxygen uptake and respiratory enzyme activity in animal skeletal muscle, a number of studies have also investigated the mitochondrial content and function in response to several weeks of exercise training in human muscle (Hoppeler et al., 1973, Tonkonogi et al., 2000, Nielsen et al., 2010). Exercise-induced mitochondrial biogenesis corresponds closely to the functional energy requirements necessary to improve oxidative phosphorylation, ATP provision and fatigue resistance (Hood, 2009). Improved substrate utilisation, increased glucose uptake and a greater reliance on fat metabolism are key components to this adaptive response (Player & Lewis, 2012). Moreover, the importance of exercise training for the control of mitochondrial biogenesis is highlighted by the beneficial effects of exercise on age-related mitochondrial dysfunction (Joseph et al., 2016) and other metabolic disorders such as type 2 diabetes (Mogensen et al., 2007) and obesity (Wells et al., 2008).

2.5.1 Peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC-1α)

PGC-1α has widely become known as the “master regulator” of mitochondrial biogenesis. Much attention has been given to this transcriptional coactivator since its discovery in 1998, where it was identified as a coactivator of the peroxisome proliferator-activated receptor gamma (PPARγ) in brown adipose tissue (Puigserver et al., 1998). Transcriptional co-activators respond to cellular signals to enhance target transcripts by binding with
transcription factors or nuclear receptors, but not to DNA directly (Hood et al., 2016). In this way, as a transcriptional coactivator, PGC-1α is unable to directly bind to DNA and exerts its influence by co-activating downstream transcription factors (such as NRF-1/2, EERα, TFAM) important for the regulation of the nuclear and mitochondrial genomes (Egan & Zierath, 2013).

PGC-1α is highly expressed in skeletal muscle, but is also present in other oxidative, mitochondria-rich tissues including the heart, kidney, liver, adipose tissue and brain (Finck & Kelly, 2006). Analysis of expression of PGC-1α within different tissues displayed significant correlation between high oxidative capacity and PGC-1α concentration (Ircher et al., 2003). Moreover, PGC-1α has a key role in driving the formation of slow twitch muscle fibres and increasing mitochondrial enzyme expression (Lin et al., 2002; Handschin et al., 2007), whilst selective deletion of PGC-1α in skeletal muscle attenuates the exercise induced increases in mitochondrial enzymes (Geng et al., 2010). Indeed, in its role as “master regulator” of mitochondrial biogenesis, and adaptive responses to an oxidative phenotype, substantial evidence exists demonstrating exercise-induced mitochondrial biogenesis is modulated through PGC-1α dependant mechanisms (Calvo et al., 2008; Leick et al., 2010; Little et al., 2011; Safdar et al., 2011; Uguccioni & Hood, 2011; Zhang et al., 2014). Studies have investigated the PGC-1α response to acute bouts of endurance exercise in animals (Baar et al., 2002) and in humans using cycling (Mathai et al., 2008; Gibala et al., 2009; Leick et al., 2010) and treadmill protocols (Bartlett et al., 2012; Joo et al., 2016); with ~3-10 fold-increases seen within the first few hours post-exercise, dependent on exercise intensity (Egan et al., 2010), training status and basal PGC-1α content (Gibala, 2009). Additionally, several weeks of endurance training increases PGC-1α mRNA and PGC-1α protein in human skeletal muscle with greater mitochondrial enzyme activities.
In contrast, evidence exists to suggest that PGC-1alpha gene expression is not necessary for the endurance adaptive response, with other mitochondrial stimulatory pathways suggested to be upregulated to maintain exercise induced mitochondrial biogenesis (Leick et al. 2008; Rowe et al., 2012; Ballman et al., 2016).

In a hallmark study by Perry et al. (2010) the time course of increases in PGC-1α mRNA and PGC-1α protein during exercise training was well documented. PGC-1α mRNA increased ~10-fold 4h after the first high-intensity training session and returned to rest levels by 24h. Subsequent high-intensity sessions increased expression again, but to a slightly lesser degree, before again returning to baseline. This ‘saw-tooth’ pattern continued (See Figure 2.3) with the magnitude of the 4h increase after each training session progressively decreasing; resulting in a ‘stair-case’ type response over seven training sessions. Increased PGC-1α protein expression (23%) was observed 24h after the 1st session. Unlike the reduced PGC-1α mRNA responses over seven training sessions, PGC-1α protein expression continued to increase, attaining a plateau by the 5-7th training session therefore showing repeated transient bursts of mRNA precede, and are required, for the transcriptional protein response.
Figure 2.3: Skeletal muscle PGC-1α mRNA (A) and protein content (C) throughout 2 weeks of high-intensity interval training. Adapted from Perry et al. (2010), The Journal of Physiology. Permission for reproduction granted by the publisher John Wiley and Sons. Values are means ± S.E.M. for 8–9 participants. *Significantly different from Pre, §significantly different from all 24 h time points, #significantly different from 1st-4 h, b significantly different from all 4 h time points, †significantly different from 1st-24 h (P < 0.05).

2.5.1.1 Promoter and isoform specific regulation of PGC-1α

For nearly twenty years, many studies have focused on PGC-1α function, the molecular mechanisms of its activation, and the regulation of PGC-1α gene expression. However, skeletal muscle expresses several PGC-1α isoforms via two different promoters offering additional avenues of mechanistic interest. The canonical (proximal) promoter contains two
major transcription initiation sites at 90 and 119 base pairs (bp) upstream of the initiation transcription codon ATG (Popov et al., 2015a). An alternative promoter is also located 14 kb upstream of the canonical promoter. The canonical promoter originates at the first exon (1a) of the canonical PGC-1α-a mRNA isoform (Martinez-Redondo et al., 2015). Because of alternative splicing, the alternative promoter directs the transcription of two different first exons (1b and 1c) (Popov et al., 2015a). The proximal (canonical) PGC-1α gene promoter has higher basal expression than the alternative promoter, however the alternative promoter is more responsive to stimulation (Martinez-Redondo et al., 2015). Indeed, several groups (Norrbom et al., 2011; Ydfors et al., 2013; Popov et al., 2014) have shown that in human skeletal muscle, acute exercise induces PGC-1α gene expression, mainly via the alternative promoter (Exon 1b).

Evidence in rodent skeletal muscle suggests activation of exercise-induced expression via the alternative promoter (Exon 1a) is regulated by the β2 adrenergic receptor- protein kinase A (PKA)- cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) signalling pathway (Chinsomboon et al., 2009; Tadaishi et al., 2011; Popov et al., 2015b) and/or AMPK pathways (Norrbom et al., 2011; Wen et al., 2014). Moreover, administration of clenbuterol to mice is shown to induce not only PGC-1α4 isoform expression in skeletal muscle (Ruas et al., 2012) but also PGC-1α2 and PGC-1α3 (Exon 1b derived), while reducing the levels of canonical promoter-derived PGC-1α1 (Exon 1a derived) (Martinez-Redondo et al., 2015). Despite this, evidence in humans is limited. Recently in humans, endurance exercise with blood flow restriction was shown to attenuate PGC-1α isoform expression (Conceição et al., 2016). However, the promoter specific response of PGC-1α to physiologically derived β2 adrenergic activity, rather than external and/or pharmaceutical activation, in humans remains to be determined.
Figure 2.4: (A) The schematic structure of the 5' region of the human PGC-1α gene (Miura et al. 2008). The proximal promoter drives the transcription of PGC-1α exon 1a-derived transcripts and the alternative promoter the transcription of PGC-1α exon 1b and 1b'-derived transcripts. (B) Detailed descriptions of primer pairs and their possible cDNA targets are presented. Reproduced from Silvennoinen et al. (2015), Physiol Rep, as per the Creative Commons Attribution License (CC BY).

Several mechanisms have been identified that lead the increased expression and activation of PGC-1α following an exercise stimulus. Metabolic sensors, AMPK and p38 MAPK are two key activators of PGC-1α (Gibala et al., 2009). Moreover, the suggested impact of β-adrenergic activity (Puigserver et al., 1998) also remains important. These and the
subsequent downstream impact of greater PGC-1α expression, activation and post-translational modification are discussed in greater detail in the following sections. It should be noted that activation of PGC-1α is also possible via acetylation and methylation, however these methods are not within the remit of this review and are not discussed further.

2.5.2 AMP-dependent protein kinase (AMPK)

AMPK is a serine/threonine protein kinase that responds to cellular energy status (Kemp et al., 1999). Any stress that results in a change of the adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratio will result in increased activation of AMPK. Activated AMPK stimulates energy generating processes such as glucose uptake, glucose transporter type 4 (GLUT4) translocation, fatty acid oxidation and translocation into the mitochondria (Hayashi et al., 1998; Winder, 2001; Jäger et al., 2007; Takaguri et al., 2016) whilst decreasing energy-consuming processes like protein and glycogen synthesis (Carling & Hardie, 1989; Richter et al., 2009). AMPK has further implications on metabolic health by enhancement of muscle insulin sensitivity (Kjøbsted et al., 2016) and promotion of mitochondrial fusion and function (Kang, 2016). As such, AMPK is regularly referred to as the ‘energy sensor’, playing a key role in the regulation of cellular metabolism.

Exercise is one of the most powerful physiological activators of AMPK. Activation of AMPK occurs in several manners outlined well by Jørgensen et al. (2006), however in skeletal muscle phosphorylation at Thr 172 of the α-subunit can enhance AMPK activity >100-fold (Hardie et al., 2003). Whilst phosphorylation of AMPK has been shown after multiple sporting modalities, one thing that is critical in controlling the response is the intensity of exercise; with greater intensity resulting in greater levels of activation (Wojtaszewski et al., 2000; Egan et al., 2010). However, this response has been disputed
with similar phosphorylation levels noted in matched work high-intensity vs. continuous exercise (Bartlett et al., 2012). Additionally, studies suggest a muscle fibre type-specific AMPK expression occurs (Kristensen et al., 2015), whilst trained individuals display greater protein content of AMPK (Nielsen et al., 2003), despite training reducing the phosphorylation response to a single-bout of exercise (McConnell et al., 2005).

The importance of AMPK upon PGC-1α driven mitochondrial biogenesis is highlighted by AMPK activation increasing PGC-1α gene expression (Irrcher et al., 2008; Zhang et al., 2014), including direct phosphorylation of PGC-1α (Jäger et al., 2007). Activity of PGC-1α can be regulated through inhibitory acetylation by GCN5 and stimulatory deacetylation through SIRT1 (Cantó & Auwerx, 2009). However, the importance of deacetylation of PGC-1α for exercise induced mitochondrial biogenesis has been questioned (Philp et al., 2011). Moreover, AMPK may also exert its effects in favour of mitochondrial biogenesis via tumour suppressor protein 53 (p53) (Bartlett et al., 2013). p53 regulates contractile-induced increases in mitochondrial content via modulating TFAM content and/or activity, as p53 knock-out (KO) mice display reduced skeletal muscle mitochondrial DNA, TFAM mRNA and protein levels (Saleem et al., 2009; Bartlett et al., 2013).

2.5.3 p38 mitogen-activated protein kinase (MAPK)

p38 MAPK is often referred to as a stress-activated protein kinase due to its frequent activation by a range of environmental stresses and inflammatory cytokines. The four p38 MAPK isoforms are all encoded by different genes and have differential tissue expression; p38α is ubiquitously expressed in most cell types with p38β preferentially expressed in the brain, p38δ the endocrine glands and p38γ skeletal muscle (Li et al., 1996; Cuadrado & Nebrada, 2010). p38 MAPK is inactive in the non-phosphorylated state and
activated by dual kinases termed MAP kinase kinases (MKK’s) phosphorylating on Threonine- Glycine- Tyrosine (Thr-Gly-Tyr) motifs. The contribution of MKK3 and MKK6 to p38γ activation strongly depends on the nature and strength of the stimulus; with both essential for activation induced by environmental stress and mainly MKK6 in response to the cytokine TNFα (Risco & Cuenda, 2012). Downstream p38MAPK phosphorylates the Serine/Threonine (Ser/Thr) residues of its substrates including kinases, transcription factors and cell cycle regulators playing important roles in skeletal muscle differentiation (Cuenda & Cohen, 1999), development (Foster et al., 2012) and adaptation through mitochondrial biogenesis and angiogenesis with its impact upon PGC-1α (Pogozelski et al., 2009).

Akimoto and colleagues (2005) demonstrated the importance of p38MAPK to the PGC-1α response, when PGC-1α promoter activity was enhanced in C2C12 cells following activation of p38MAPK, which could also be blocked by specific inhibitors. Moreover, p38MAPK stimulated activation of downstream transcription factors, that are also upstream regulators of PGC-1α has been shown, with activating transcription factor2 (ATF2) and myocyte enhancing factor2 (MEF2) of significant interest (Akimoto et al., 2005, Zarubin & Han, 2005).

Since the seminal work showing the MAPK pathway is stimulated by exercise in human skeletal muscle (Aronson et al., 1997) a plethora of studies has shown greater rates of phosphorylation following different exercise modalities. Phosphorylation of p38MAPK is independent of exercise intensity (Egan et al., 2010; Bartlett et al., 2012) and occurs systemically (Widegren et al., 1998), in this sense to the same extent in an exercised vs. non-exercised limb.
2.5.4 Downstream of PGC-1α

Activation of calcium signalling pathways during exercise may play a role in the stimulation of PGC-1α transcription through calcineurin and calcium-dependent protein kinases (Wu et al., 2002), ultimately leading to the activation of the transcription factors CREB, ATF2 and MEF2 (Lin et al., 2005). Importantly, activation of PGC-1α by CREB and MEF2 is controlled by an auto regulatory loop, whereby MEF2 binds to the PGC-1α promoter when co-activated by PGC-1α (Handschin et al., 2003).

The wider impact of PGC-1α on phenotypic changes within skeletal muscle is highlighted by its post-translational modifications and downstream interactions with multiple important transcription factors. Two proteins implicated in the coordination of mitochondrial biogenesis are the Nuclear respiratory factors 1 and 2 (NRF1 and 2) (Baar, 2004). NRF’s are important in the control of nuclear oxidative phosphorylation genes and the expression of nuclear encoded factors important to mitochondrial transcription, protein import machinery and assembly (Wenz, 2013). NRF-1 and 2 are targets of PGC-1α (Wu et al., 1999) and in this way, stimulate expression of TFAM; a mitochondrial matrix protein important in the replication and transcription of mtDNA (Virbasius & Scarpulla, 1994). Moreover, ERRα is also under the control of PGC-1α and plays its role in PGC-1α induced mitochondrial biogenesis through controlling vital mitochondrial processes such as oxidative metabolism, through a network of protein kinases and by regulating the expression of sirtuins (Ranhotra, 2014).

Of the aforementioned transcription factors (NRF1, NRF2, TFAM, ERRα) important to PGC-1α derived mitochondrial biogenesis, data exists to show the impact exercise may have upon their activity and function. Following an acute bout of swimming NRF-1 and -2 DNA-
binding activities increase at 12–18h (Baar et al., 2002), suggesting a rapid and coordinated activation following acute exercise. Additionally, Egan et al. (2013) showed increases in NRF2, TFAM and ERRα mRNA expression were present across a 14-day training period. Moreover, acute exercise induces translocation of p53 to the mitochondria, promoting interaction with TFAM, positively affecting mtDNA transcription (Saleem & Hood, 2013). This therefore represents an early exercise response which can promote long-term beneficial mitochondrial and metabolic adaptations in skeletal muscle.

2.5.5 Beta-adrenergic stimulation

The importance of β-adrenergic stimulation to muscle contraction is well described (Cairns & Borrani, 2015). Its relative importance to PGC-1α and mitochondrial biogenesis was alluded to in the seminal work by Puigserver and colleagues (1998), whereby PGC-1α was found to be induced in brown fat and myotube cultures following exposure to cold and the β-adrenergic agonist, isoproterenol. Indeed, prior to the discovery of PGC-1α earlier work in rats (Ji et al., 1986) and humans (Svedenhag et al., 1984) had suggested that blocking the exercise induced increase in β-adrenergic receptor activity, via treatment with β-antagonists/blockers propranolol and atenolol, dampened training induced mitochondrial biogenesis through reduced levels of mitochondrial enzymes (i.e. citrate synthase (CS), succinate dehydrogenase (SDH)). However, this was not without contrasting evidence, with a lack of change seen for CS and SDH following 6 weeks of daily adrenaline injections in rats (Fell et al., 1981). In a well-designed series of experiments, Miura and colleagues (2007) showed upregulation of PGC-1α expression in skeletal muscle by exercise is mediated, at least in part, by β2-adrenergic receptor activation. In this study mice injected with the β2-adrenergic agonist clenbuterol increased PGC-1α expression more than 30-fold in skeletal muscle, which could subsequently be inhibited with pre-treatment of the β2-
adrenergic blocker propranolol. Moreover, in ex-vivo experiments, exposure of rat muscle
to a β2-adrenergic agonist increased levels of PGC-1alpha mRNA, whilst a similar response
was not seen in beta-less knockout mice. In response to exercise (45 min treadmill running),
PGC-α mRNA increased 3-5-fold, and was inhibited ~70% by propranolol or the β2-
adrenergic receptor (AR)-specific inhibitor ICI 118,551. The exercise-induced increase in
PGC-1alpha mRNA in beta-less mice was also 36% lower than that in wild-type mice. A
year later the same group (Miura et al., 2008) suggested that the acute adrenergic response
to pharmacological activation, via injections of clenbuterol, in mice, was isoform specific
with PGC-1α -b and -c increasing (Exon 1b derived), but not PGC-1α –a (Exon 1a derived),
supported by data showing β-adrenergic receptor blocker (ICI 118551) inhibited PGC-1α -b
and –c (Exon 1b), but not -a (Exon 1a) isoform expression (Tadaishi et al., 2011). These
studies support the findings by Puigserver et al. (1998) and implicate adrenergic
mechanisms in the regulation of PGC-1α and mitochondrial biogenesis.

Despite this, recent work tends not to agree. In rats, β-antagonist/blocker propranolol
treatment failed to affect the training response of PGC-1α, TFAM, p-CREB and
Cytochrome C Oxidase Subunit 4 (COXIV) (Feng et al., 2013). In humans Robinson et al.
(2010) showed 1 hour of resting infusion with the β-AR agonist isoproterenol had no impact
on expression of PGC-1α, TFAM, NRF-1 and 2, Cytochrome C Oxidase (COX) or
NADHox expression. In addition, a year later Robinson et al. (2011) showed β-
antagonist/blocker propranolol did not blunt the PGC-1α mRNA response to 1 hour of
cycling. Reasons for these discrepancies are unclear but are suggested to be a combination
of experimental methods used (i.e. agonists vs. antagonists) and complications associated
with adrenergic blocker treatments, such as the possibility of incomplete adrenergic
blockade (Ihsan et al., 2014b). Moreover, it is plausible that other exercise-induced
pathways compensate for any blockade of PGC-1α induced of mitochondrial biogenesis (Ihsan et al., 2014b). Indeed, Kim et al. (2013) indicated that the reason for the lack of effect of β-adrenergic stimulation on PGC-1α expression in rat muscle might be that catecholamines do not activate p38MAPK and its subsequent increase in ATF2 phosphorylation.

One thing of importance is that much of the work conducted utilises pharmacological stimulation or inhibition either at rest, or prior to an exercise stimulus. To the author’s knowledge, only one study has investigated the impact of physiological increases of catecholamines and β-adrenergic activity on the PGC-1α response. Brandt et al. (2016) instructed participants complete four different exercise protocols where the workload of the legs was matched: 1) - cycling at 171 ± 6 W for 60 min (control); 2) - cycling at 171 ± 6 W for 60 min, with addition of intermittent arm exercise (98 ± 4 W). 3) - cycling at 171 ± 6 W interspersed by 30 sec sprints (513 ± 19 W) every 10 min (distributed sprints); and 4) - cycling at 171 ± 6 W for 40 min followed by 20 min of six 30 sec sprints (clustered sprints). Results showed no differences in PGC-1α mRNA expression despite elevated plasma adrenaline levels, and are also in contrast to those of Egan et al. (2013) showing PGC-1α mRNA expression is dependent on exercise intensity.

Cold is sensed in the central nervous system and results in increased sympathetic output to peripheral tissues, including skeletal muscle (Miura et al., 2008). Indeed, the magnitude of the increase in sympathetic discharge to skeletal muscle is influenced by both the size of the tissue area exposed to cooling (Seals, 1990) and the magnitude of the cooling stimulus (Kregel et al., 1992). It is suggested that catecholamine production in response to cold triggers the activation of β-adrenergic receptors, resulting in the elevation of intracellular
cAMP and subsequent binding of CREB to the promoter region of PGC-1α (Miura et al., 2008; Ihsan et al., 2014b). Indeed, the sympathetic response to cold stimulation has been consistently shown (Gregson et al., 2013; Joo et al., 2016; Ishida et al. 2016). However, the importance of cold-stimulated catecholamine release to the post-exercise PGC-1α response in humans remains to be determined.

2.6 THE INFLUENCE OF POST-EXERCISE COOLING ON TRAINING ADAPTATION

Whilst the impact of CWI on acute recovery of performance (Section 2.3) is extensive, data on the impact of regular CWI on training adaptations is less prevalent. Currently, a paradox exists on advocating the use of CWI for reducing the stress associated with intense or damaging exercise versus the negative impact it may have on the window of adaptation and necessary stress response required for molecular training adaptations (Howatson et al., 2009).

It has been suggested that the mechanisms of action of regular CWI may blunt the cell signalling responses to training, and thus if CWI were to be utilised on a regular basis, the permanent disruption of the mechanisms of fatigue and proposed reduction of inflammatory events may harm an athlete’s training progression (Versey et al., 2013). Much of the research to date shows a negative influence on performance characteristics when CWI is applied throughout a period of training on a regular basis. Yamane and colleagues (2006; 2015) noted detriments of regular CWI upon strength adaptations, among other measures, in a series of studies. Yamane et al. (2006) demonstrated an attenuation of endurance performance, maximal oxygen uptake, arterial diameter and muscular endurance following 4-6 weeks of lower leg (cycling) or arm flexor (handgrip dynamometer) training completed
with CWI (2 x 20 min at 5°C) or passive control. More recently, the same group (Yamane et al., 2015) used five sets of eight wrist-flexion exercises at a workload of 70-80% of the single repetition maximum, 3 times a week for 6 weeks in healthy subjects. Participants immersed their experimental forearms in cold water (20 min at 10°C) after wrist-flexion exercises, and another group served as controls (noncooled group). Wrist-flexor thicknesses of experimental arms increased after training in both groups, but to a lesser extent in the cooled group. Maximal muscle strength and brachial-artery diameter did not increase in the cooled group, while they increased in the noncooled group. Additionally, Frohlich and colleagues (2014) utilised single-leg 1 repetition max (1RM) and 12 repetition max (12RM) tests to assess a 5-week resistance-training programme. Directly after each training session, CWI was applied for one randomly assigned leg, consisting of three 4- minute intervals with a 30-s rest period; the contralateral leg was not cooled. Training significantly improved 1RM and 12RM, however these improvements tended to be larger in the control limb than the CWI limb (1.6-2.0%).

In contrast to the resistance adaptive response, Aguiar et al. (2016) recently showed CWI (10 °C for 15 min) after HIIT training (8–12 cycling sessions (90–110 % of peak power) for 60s followed by 75s of active recovery, 3x per week for 4 weeks) did not alter exercise performance in a 15km cycling time trial. Time trial performance, total mean power output, mean power per km all improved following training, with no differences evident between CWI or control (recovery at room temperature) groups. Vaile et al. (2008a) showed improved sprint and time trial performance was noticeable in cyclists, when exercise each day for 5 days was followed by CWI vs. passive recovery. Moreover, Halson and colleagues (2014) showed the regular use of CWI in elite cyclists showed no decrements to sprint performance. Competitive cyclists were randomized to CWI (15 min at 15°C) or
control (passive recovery) groups. The assessment period consisted of 7 d of baseline training, 21 d of intensified training, and an 11 d taper. Each week cyclists completed a high intensity interval-cycling test and two 4-min bouts separated by 30 min. CWI was performed four times per week for 15 min at 15°C. Between baseline and taper, cyclists in the CWI group had an unclear change in overall 4-min power relative to control (2.7%, ±5.7%), although mean power in the second 4-min effort relative to the first was likely higher for the CWI group relative to control (3.0%, ±3.8%). The change in 1-s maximum mean sprint power in the CWI group was likely beneficial compared to control (4.4%, ±4.2%). Differences between groups for a 10-min time trial were unclear (- 0.4%, ±4.3%). Thus, whilst some effects of CWI on performance were unclear, data did not support the speculation that CWI is detrimental to performance, particularly following increased training load in competitive cyclists.

For endurance adaptation, Ihsan and colleagues (2015) utilised a single-leg immersion protocol (10°C for 15 min) to assess the impact of regular CWI on an immersed vs. non-immersed limb, when cooling was applied following each training session (3x week, for 4 weeks). Training sessions included long (6 - 8 min), moderate (2 min) and short (30-151s) interval bouts performed at 80% - 110% Vmax. Whilst improvements were seen in participants’ peak oxygen uptake and running velocity by 5.9% and 6.2%, respectively, any performance benefits of training with or without CWI recovery cannot be determined due to the single-leg immersion technique adopted. Interpretation of single-leg immersion studies are difficult, as they do not take into account potential systemic effects of cooling.

Considering the advances of molecular biochemistry in recent decades, it has become far easier to investigate the mechanisms behind such physiological developments, in particular
the regulation of key genes and molecular pathways of adaptation to different stimuli. A few key papers have taken advantage of such advancements, showing CWI has the ability to augment the acute and chronic PGC-1α response when used post-exercise. Importantly, the increase in PGC-1α occurred to a greater extent in a cooled vs. control trial, suggesting that CWI may help towards a greater oxidative phenotypic adaptation. Slivka et al. (2013) showed PGC-1α mRNA increases 8-fold after 4 hours recovery in a cold environment (7°C) vs. 4-fold at room temperature (20°C), however doubts remained over an increase in metabolic rate; a possible effect of shivering thermogenesis that suggests increases in PGC-1α mRNA are a result of involuntary contraction rather than the cold stimulus per se. Ihsan Mohammed in 2014 (Ihsan et al., 2014a) used a single-leg immersion protocol to minimise the impact and possibility of shivering thermogenesis. Their group showed acute post-exercise CWI (10°C for 15 min) was able to increase PGC-1α mRNA ~4-fold; however, limitations are again present due to a lack of exercise induced PGC-1α mRNA seen, as would be expected after high-intensity exercise (Egan et al., 2013).

Ihsan and colleagues built on their acute data with a training study previously mentioned (Ihsan et al., 2015). Again, following each training session participants immersed a single leg into cold water (10°C for 15 min) with the non-immersed leg acting as a control. Repeated CWI resulted in higher total AMPK, phosphorylated AMPK (upstream effectors of PGC-1α), phosphorylated acetyl-CoA carboxylase and β-3-hydroxyacyl-CoA-dehydrogenase (important in fatty acid metabolism) and the protein subunits representative of complex-I and III (important in aerobic respiration). Large effect sizes (Cohen’s d > 0.8) were noted with changes in protein content of p38MAPK, PGC-1α and PPARα in the immersed vs. control leg. Ultimately, these results implicate CWI as stimulating several
important molecular markers for oxidative adaptation, benefiting athletes in sports dependent on oxidative metabolism.

Since this seminal work in the area more results are emerging with supportive data towards oxidative adaptation, showing regular CWI increases mRNA expression of heat shock factor 1 (HSF-1), TFAM and pyruvate dehydrogenase kinase 4 (PDK4) (all important in oxidative adaptation) after 4 weeks of HIIT (Aguiar et al., 2016). However, despite this the authors were unable to detect any cold-induced augmentation of PGC-1α mRNA to levels greater than the control group as shown in studies by Slivka et al. (2013, 2014) and Ihsan et al. (2014a). Our lab recently demonstrated post-exercise CWI augments the acute exercise-induced expression of PGC-1α mRNA (~6-fold) in human skeletal muscle compared to exercise per se (~3-4-fold). Additionally, we were the first group to show CWI per se (i.e. without prior exercise) mediates the activation of PGC-1α (1.4-fold) and vascular endothelial growth factor (VEGF) mRNA (2-fold) expression in human skeletal muscle (Joo et al., 2016); important in mitochondrial biogenesis and angiogenesis respectively.

Roberts et al. (2015) are the first to take advantage of such analytical advancements when looking at strength and resistance training stimuli followed by CWI. A chronic 12-week lower-body training regime was undertaken to investigate the influence of regular cold-water immersion on changes in muscle mass and strength. Participants completed two training sessions per week followed immediately by CWI (10°C for 10min) or active recovery (low-intensity cycling). Muscle mass increased for both groups but to a lesser extent in the CWI group (difference of 206g between groups; P<0.05) whereas cross-sectional area and number of myonuclei increased significantly in the active recovery group, but not CWI. Leg press and knee extension strength were also significantly improved in the
active recovery group vs. CWI (differences of 34kg and 18kg respectively). This was followed up with an investigation into the acute response of the hypertrophy signalling pathways and satellite cell activity; two key markers of the adaptive response to an acute bout of strength exercise. This time, using a randomised crossover design, participants completed a bout of single-leg strength exercise followed by either CWI or active recovery as before. Phosphorylation and total protein content of ribosomal protein S6 Kinase beta 1 (P70S6K), a key player in hypertrophy and anabolic signalling, was significantly greater at 2h and 24h post-exercise in the active recovery trial vs. CWI. Total protein content of P70S6K did not change in the CWI trial. The number of Pax7+ and neural cell adhesion molecule cells (NCAM+) cells increased by 48% and 10% respectively after the active recovery trial, whereas no increase was seen at any time points for the CWI trial. These investigations offer new insights into the impact CWI may have on acute signalling and chronic morphological and functional changes after resistance exercise training. Lindsay et al. (2016) offer support to the work of Roberts et al. (2015) by showing repetitive cryotherapy attenuates the in vitro and in vivo mononuclear cell activation response.

Thus, a paradox exists and the question as to whether CWI should be employed as a method of post-exercise recovery remains. However, the answer to the question has evolved to be dependent upon which exercise stimulus is being employed and thus which molecular adaptive pathway is being stimulated. Maybe the more pertinent question is which pathway is more important for the athlete? However, it would be naive for scientists and coaches alike to confidently answer such a question at this stage. The current state of research calls for further investigation into the correct periodization/individualisation of CWI towards the goals of the athlete, particularly as the impact of regular CWI upon concurrent training practices is unknown. Until further research clarifies, CWI can be useful within competition...
settings, particularly those requiring a short turn-around (such as tournament situations, athletic meets and cycling tours), if not for the benefits of improved subsequent performance then for the reduction in delayed onset muscle soreness, analgesic effects (Leeder et al., 2012) or potential placebo effect (Broatch et al., 2014). However, there is no justification to use CWI during a strength/power adaptive cycle as this potentially negates the hypertrophy response sought.

Importantly, recent research investigating the endurance adaptive mechanisms to post-exercise cooling have utilised a single leg immersion study design (Ihsan et al., 2014a, 2015). As previously mentioned, the interpretation of single-leg immersion studies is difficult, as they do not take into account potential systemic effects of cooling. Indeed, systemic control of important signalling kinases (p38MAPK) have previously been shown to respond in a systemic, rather than localised, manner (Widegren et al., 1998). Moreover, single-leg immersion designs that utilise a contra-lateral limb as a control (Ihsan et al., 2014a; Frohlich et al., 2014) may be liable to error by underestimating the actual response occurring in the immersed limb if relativised to a non-immersed limb instead of a resting control. Moreover, with several mechanisms thought to have a controlling role in the cold-induction of PGC-1α it is ever more important to determine whether the cause of such augmentation is systemic (β-adrenergic mechanisms) or local (as a result of reduced tissue temperature) in nature.

2.7 THE INFLUENCE OF NUTRITIONAL MODULATIONS ON TRAINING ADAPTATION

The importance of correct and sufficient nutrition for acute and chronic exercise is well described (Cermak & Van Loon, 2013). In addition to benefiting competition performance,
it is recommended that endurance athletes ensure high CHO availability before, during and after training sessions to support high daily training volumes and intensities and promote optimal recovery (Bartlett et al., 2014). Despite this, a growing body of evidence demonstrates potential benefits of restricted CHO availability (both endogenous and exogenous) in training-induced adaptations. Indeed, endurance based sessions commencing with low CHO availability are able to enhance the activation of cell signalling pathways (Bartlett et al., 2013), upregulate oxidative enzymes (Morton et al., 2009), increase lipid oxidation (Yeo et al., 2008) and improve exercise capacity (Hansen et al., 2005). Thus, the term “train low, compete high” was spawned.

More specifically to the PGC-1α response, Pilegaard et al. (2005) demonstrated that attenuating post-exercise muscle glycogen re-synthesis in the short-term recovery phase (24 h) further amplifies the transcription and mRNA expression of PDK4, uncoupling protein 3 (UCP3), lipoprotein lipase (LPL), and carnitine palmitoyltransferase 1 (CPT1) (all important metabolic regulators), but importantly also augmented the proposed master regulator of mitochondrial biogenesis, PGC-1α. A similar response is seen in PGC-1α mRNA expression after exercise is commenced with low glycogen (Psilander et al., 2013; Bartlett et al., 2013). Mechanisms controlling such a response are speculated to be a result of increased activity and nuclear abundance of AMPK (Steinberg et al., 2006) and p38MAPK (Chan et al., 2004) when exercise is commenced with low muscle glycogen. Despite this, and in contrast, other studies exist where low muscle glycogen was unable to stimulate greater post-exercise PGC-1α expression (Cluberton et al., 2005; Mathai et al., 2008; Cochran et al., 2010; Jensen et al., 2015; Impey et al., 2016). One explanation for the equivocal nature of the low glycogen – PGC-1α response may be the differences in exercise intensity. Indeed, PGC-1α gene expression increases in an intensity dependant manner.
(Egan et al., 2013), however it is suggested that if the duration and intensity-dependent activated PGC-1α increases to such an extent, subsequent dietary interventions may be incapable of further activating PGC-1α (Jensen et al., 2015).

The impact of low muscle glycogen upon promoter specific PGC-1α expression has not been identified in current literature. Indeed, activation (by 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)) and suppression of AMPK, an important energy sensing kinase that is upregulated in circumstances of low muscle glycogen (Wojtaszewski et al., 2003), was found not to be important for an increase in any PGC-1α isoform expression after high intensity exercise (Tadaishi et al., 2011). However, to the best of the author’s knowledge, no study has directly assessed the impact of reduced muscle glycogen on isoform/promoter specific PGC-1α regulation. Moreover, the combined impact of prior low glycogen and post-exercise CWI, two strategies reported to augment post-exercise PGC-1α mRNA expression, upon the total PGC-1α response remains to be determined.

2.8 SUMMARY

Post-exercise CWI remains one of the most popular recovery tools utilised in athletic recovery and investigated in scientific literature. Evidence supporting the use of post-exercise CWI in return to performance and on recovery from muscle soreness is strong, despite being heavily dependent on the variable measured, subject characteristics, mode of exercise and immersion protocol utilised. It is clear that for post-exercise CWI to be effective it must be administered correctly in order to activate the associated physiological and molecular mechanisms responsible for improved recovery. Despite this, there remains a lack of agreement on the correct and/or most efficient technique for implementing post-exercise CWI. Whilst equivocal data on the impact of CWI on the post-exercise
inflammatory response remains, alongside the paradox that CWI may stimulate and facilitate cell signalling related to mitochondrial biogenesis yet dampen the adaptive response to resistance training, further mechanistic research is required to determine how these mechanisms are controlled, and if they can be altered further.

Evidence suggesting different mechanisms of control of the “master regulator” of mitochondrial biogenesis PGC-1α (local tissue cooling vs. systemic adrenergic activation) requires further investigation, whilst it is unknown whether the influence of prior low glycogen availability and post-exercise CWI can further enhance cell signalling responses important in oxidative adaptation, to a greater extent when combined than individually.

With the knowledge that PGC-1α is not only augmented by post-exercise CWI, but is expressed via two different promoter regions that respond differently to low energy and heightened adrenergic environments, it is important to understand the promoter specific mechanisms to post-exercise CWI alone, or when combined with prior low glycogen availability.
CHAPTER 3

GENERAL METHODOLOGY
3.1 GENERAL METHODOLOGY

The present chapter describes the measurement techniques used within this thesis for the collection of thermoregulatory and metabolic measures and the biochemical and molecular analysis undertaken. It further provides a description of the exercise and CWI protocols used within the following experimental chapters.

3.1.1 Location of testing and ethical approval

All exercise testing was performed in the Exercise Physiology Research Labs of the Tom Reilly Building. Additional rooms were utilised for their specific purpose; such as the Muscle Biopsy Suite and Water Immersion Lab. Biochemical analysis of blood and muscle samples was undertaken in either the Tom Reilly Building or newly refurbished Life Sciences Building adjacent. Analysis of bloods for catecholamines was outsourced to Prof. William Fraser’s Lab at the University of East Anglia. Laboratory conditions were maintained through central university control at 21.2 ± 0.01°C. The use of an electronic fan was used to circulate air only, at no point was a fan pointed directly at the participant or located within 3m of the participant. Moreover, all measurements were taken at the same time of day in order to avoid circadian variation in internal body temperature (Reilly & Brooks, 1990). All study designs within this thesis and the corresponding protocols and procedures were approved by the ethical committee of Liverpool John Moores University.

3.1.2 Participants

All participants used herein were young, healthy and recreationally active volunteers recruited from within the School of Sport and Exercise Sciences or from various external contacts. All participants gave written informed consent to participate after details and procedures of the study had been fully explained and were fully aware of the right to
withdraw, without explanation, at any point (Appendix 1-4, 6). All participants were non-smokers with no history of neurological disease or musculoskeletal abnormality and none were under any pharmacological treatment during the course of the study. Participants were instructed to refrain from exercise, caffeine and alcohol consumption at least 48 hours prior to any laboratory visit. Where necessary participants diet and exercise was prescribed to adhere to the study design.

3.1.3 Anthropometry

Subject’s height was measured whilst standing in the Frankfort plane using a stadiometer (Seca, Birmingham, U.K.). Semi-nude body mass was recorded using precision calibrated weighing scales (Seca, Birmingham, U.K.). All assessments were undertaken on their first visit to the laboratory and re-assessed prior to each experimental test.

3.2 CARDIORESPIRATORY MEASUREMENTS

3.2.1 Heart Rate

Participants were fitted with a short-range radio telemetry system for the measurement of heart rate (HR) (Polar RS400, Kempele, Finland) during all water immersions and exercise experiments. The chest strap was worn directly below the pectoral muscles with the transmitter positioned centrally on the sternum. This device allowed for the recording of HR at 5-second intervals, with the data being extracted using Polar Pro Trainer (Polar, Kempele, Finland) software at the end of each visit.

3.2.2 Assessment of respiratory gas exchange

During all resting and CWI measures participants were fitted with a facemask for measurement of respiratory gas exchange. Expired fractions of oxygen and carbon dioxide
were analysed via breath-by-breath measurement using an on-line gas analysis system (Oxycon Pro, Jaeger, Wurzburg, Germany) after calibration with known reference gases. Any respiratory measures performed during exercise were undertaken using a mouthpiece and nose clip, as opposed to a facemask. Preliminary testing indicated a facemask was too claustrophobic when worn for a high intensity cycling protocol lasting 47 minutes and prevented participants from completing.

3.3 ASSESSMENT OF MAXIMAL OXYGEN UPTAKE (\(\dot{\text{V}}\text{O}_{2\text{max}}\)) AND PEAK POWER OUTPUT (PPO)

The maximal incremental cycling protocol used has previously been described in detail (Pedersen et al. 2008). Pedersen et al. (2008) commenced the test protocol at a rate of 3 W·Kg\(^{-1}\)Body Mass (BM) using endurance trained cyclists, however as participants in the present studies were not endurance trained cyclists covering >250km per week in training, and after preliminary testing, a starting point of 2 W·Kg\(^{-1}\)BM was utilised. This alteration is in line with research by Hawley & Noakes (1992), who reduced the workload to this measure for endurance-trained females. From here, the work rate was increased by 50 W after the first 150 seconds, and then by 25 W every 150 seconds thereafter, until exhaustion. Participants were encouraged to maintain a cycling cadence above 70 rev.min\(^{-1}\), with exhaustion being reached when participants could no longer maintain a cadence of 70 rev.min\(^{-1}\) for 15 seconds consecutively. All tests were completed using an electronically braked cycle ergometer (Lode Excalibur Sport, Lode, Netherlands). The \(\dot{\text{V}}\text{O}_{2\text{max}}\) was taken from an average of the highest two consecutive \(\dot{\text{V}}\text{O}_{2}\) values attained in any 10 s period and was stated as being achieved by the following end-point criteria: 1) heart rate within 10 b.min\(^{-1}\) of age-predicted maximum, 2) Respiratory Exchange Ratio (RER) > 1.1, and 3) plateau of oxygen consumption despite increasing workload. Expired fractions of oxygen
and carbon dioxide were analysed via an on-line gas analysis system (see section 3.2.2). HR was measured continuously using short-range radio telemetry (see section 3.2.1). PPO was calculated using the equation below (Pedersen et al., 2008); where CB is the wattage of the last complete bout, FB is the fraction of the final bout completed and 25 is the increment of 25W between each successive bout.

\[
PPO = CB + (FB \times 25)
\]

3.4 MEASUREMENT OF PSYCHO-PHYSIOLOGICAL VARIABLES

3.4.1 Rating of Perceived Exertion (RPE)

Participants’ reported ratings of perceived exertion during exercise were recorded using a 15-point Borg Scale (Borg, 1982). Participants were familiarised to the scale during preliminary laboratory visits to ensure full comprehension of the subjective and descriptive nature of such a measure. The category ratio scale that was used is displayed in Figure 3.1.
<table>
<thead>
<tr>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>No Exertion At All</td>
</tr>
<tr>
<td>7</td>
<td>Extremely Light</td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Very Light</td>
</tr>
<tr>
<td>10</td>
<td>Light</td>
</tr>
<tr>
<td>11</td>
<td>Somewhat Hard</td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Hard</td>
</tr>
<tr>
<td>14</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Very Hard</td>
</tr>
<tr>
<td>16</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Extremely Hard</td>
</tr>
<tr>
<td>18</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Maximal Exertion</td>
</tr>
</tbody>
</table>

**Figure 3.1**: Borg scale for reporting subjective RPE during exercise (Borg, 1982).
3.4.2 Subjective Shivering Scale

Participants reported ratings of perceived shivering during immersion, and in the post immersion resting periods, using a 4-point shivering scale (Wakabayashi et al., 2006). As with RPE, all participants were familiarised to the scale during preliminary laboratory visits to ensure full comprehension of the subjective and descriptive nature of such a measure. The category ratio scale that was used is displayed in Table 3.1.

**Table 3.1:** Subjective shivering scale used for ratings of perceived shivering during immersion and resting periods.

<table>
<thead>
<tr>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No Shivering</td>
</tr>
<tr>
<td>2</td>
<td>Slight Shivering</td>
</tr>
<tr>
<td>3</td>
<td>Moderate Shivering</td>
</tr>
<tr>
<td>4</td>
<td>Heavy Shivering</td>
</tr>
</tbody>
</table>
3.4.3 Thermal Comfort

At the same time points for assessment of Subjective Shivering, participants also reported subjective Thermal Comfort (Young et al. 1987) (Figure 3.2).

![Thermal Comfort Scale](Figure 3.2: Thermal comfort scale used for subjective rating of thermal strain.)
3.5 THERMOREGULATORY VARIABLES

3.5.1 Rectal, Skin and Muscle temperature

Muscle temperature was assessed using a needle thermistor (13050; Ellab, Rodovre, Denmark) inserted into the vastus lateralis. Thigh skinfold thickness was measured using Harpenden skinfold callipers (HSK BI; Baty International, West Sussex, UK) and divided by 2 to determine the thickness of the thigh subcutaneous fat layer over each participant’s vastus lateralis. Area of insertion was calculated as half the length of the femur, over the ‘belly’ of the vastus lateralis. The needle thermistor was then placed at a depth of 3cm, plus one-half of the skinfold measurement, for the determination of deep muscle temperature (3cm). After stabilisation of temperature, the thermistor was withdrawn at increments of 1cm for the determination of muscle temperature at 2cm and 1cm below the subcutaneous layer. Core temperature was assessed via a rectal thermistor (MRV-55044-A, Ellab, Roedovre, Denmark) self-inserted 10-15cm beyond the anal sphincter. Skin temperature probes were attached to the lateral upper thigh and medial calf (MHF-18050-A, Ellab) using adhesive surgical tape. Rectal temperature, skin temperature and muscle temperature were recorded at regular intervals throughout the experimental test day using an electronic measuring system (CTF 9004, ELLAB). Skin temperature probes and the rectal probe were attached and inserted, respectively, at the start of the day and at least 30 minutes prior to any resting measures.

3.6 HIGH INTENSITY INTERMITTENT CYCLING PROTOCOL

The High Intensity Intermittent cycling protocol used throughout the studies in this thesis has been used before, with a particular focus on the metabolic demands of such a protocol (Stepto et al., 2001). After a short warm up consisting of 2 min of cycling at 25%, 50% and
60% PPO each, at a self-selected cadence, participants rested for 2 minutes prior to commencing the protocol. The interval training protocol consisted of 8 x 5 minute bouts at 82.5% PPO (which has previously been shown to closely relate to ~85% peak oxygen uptake (\(\dot{V}O_2\) peak)) with 60 seconds of rest between each bout. Stepto and colleagues (2001) ensured that subjects continued cycling during the rest period at a rate <100W. However, they used highly trained athletes with a PPO >400W. After preliminary trials, it was decided that recreationally active participants would not pedal during the rest period. The cycle ergometer (Lode Excalibur Sport, Lode, Netherlands) was individually adapted to each participant’s specifications prior to arrival at the laboratory. It should be noted that a high-intensity intermittent cycling protocol was utilised in order to minimise muscle damage associated with mechanical stress (i.e. eccentric contractions) and to allow greater augmentation of the PGC-1\(\alpha\) response (Egan et al., 2010) and therefore superior analysis of any controlling mechanisms involved in such augmentation.

3.7 COLD WATER IMMERSION

3.7.1 Single-leg CWI

In the first study (See Chapter 4), a single-leg CWI design was utilised to allow for determination of the local and systemic effect of CWI on both lower limbs. During this, participants immersed a single leg in 8°C water for 10 minutes to the gluteal fold. Participants were strapped to an electronic hoist which allowed the researchers to lower them a suitable way into the cold bath (ECB, Cheltenham, UK). Stools were placed to the outside of the bath to support the subject whilst straddling the bath, always ensuring the non-immersed leg remained completely dry with the use of a towel. At the end of immersion, participants were raised using the electronic hoist and remained seated in a supine position whilst post-immersion variable measures were taken. An illustration of
single-leg CWI is shown in Figure 3.3. Despite other immersion temperatures and durations being suggested as superior for recovery from stressful exercise (Machado et al., 2016) the immersion temperature and duration herein was chosen to remain consistent with previous work from our labs (Gregson et al., 2013; Joo et al., 2016; Mawhinney et al., 2013), particularly as this has been shown to induce a significant adrenergic response in the post-exercise period (Gregson et al., 2013).

Figure 3.3: Illustration of single-leg CWI method.
3.7.2 Two-legged CWI

In the second study (Chapter 5), a two-legged immersion approach was taken as this follows general practice in field settings where CWI is used as a recovery method. Participants were lowered into the cold bath using the electronic hoist to their umbilicus for 10 minutes at 8°C (Figure 3.4). At the end of immersion, participants were raised from the water using the electronic hoist and remained seated in a semi-reclined position under the temperature-controlled laboratory.

Figure 3.4: Illustration of 2-legged CWI method.
3.8 PROCUREMENT AND STORAGE OF BLOOD SAMPLES

Venous blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using standard venepuncture techniques (BD Nexiva Closed IV Catheter 22G Blue, Becton Dickinson, Oxford, UK). Blood samples (~10ml) were collected into vacutainer tubes (Becton Dickinson, Oxford, UK) containing Ethylenediaminetetraacetic acid (EDTA) or serum separation and stored on ice or at room temperature (serum samples, ~1h) until centrifugation at 1500 rev.min⁻¹ for 15-min at 4°C. Following centrifugation, aliquots of plasma and serum were stored at -80°C for later analysis.

3.9 ANALYSIS OF BLOOD SAMPLES

Samples were analysed for serum glucose, lactate, non-esterified fatty acids (NEFA) and glycerol concentration using commercially available kits (Randox Laboratories, Antrim, UK). Serum samples were also analysed for insulin using a solid phase enzyme-linked immunosorbent assay (ELISA, KAQ1251, Life Technologies, UK). Plasma metanephrine and normetanephrine concentrations were measured using liquid chromatography–tandem mass spectrometry (Peaston et al., 2010). Normetanephrine (NMA), metanephrine (MA) and their deuterated internal standards are extracted from plasma using a polymeric weak cation exchange solid phase extraction (SPE). The extracts eluted from the SPE plate are then dried under nitrogen and reconstituted prior to injection. The internal standards used are d3-NMA and d3-MA. Analysis is carried out by high performance liquid chromatography coupled to a tandem mass spectrometer in electrospray ionization positive mode. Identification and quantification is based on multiple reaction monitoring of each specific target analyte transition.
3.10 MUSCLE BIOPSIES

Samples from the vastus lateralis (~30–50 mg wet wt) were obtained under local anaesthesia (0.5% Marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL) (Figure 3.5). When the biopsy needle is inserted through the muscle fascia, the biopsy gun operates with a feed-forward mechanism of 2.5–3.5 cm, dependant on the angle of insertion (Morton et al., 2009). Once obtained, samples were immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

Figure 3.5: Image of the biopsy gun technique utilised.
3.11 ANALYSIS OF SKELETAL MUSCLE mRNA USING QUANTITATIVE REAL-TIME PCR

3.11.1 Muscle sample homogenisation

Skeletal muscle samples (~30-50 mg) were transferred to 2ml lysing tubes containing 1.4mm ceramic beads (Lysing Matrix D, MP Biomedicals, UK) and 1ml ice-cooled TRIzol reagent and homogenised at 6 m/s for 3 x 40 seconds, separated by 5 minutes cooling on ice (MP Fastprep-24, MP Biomedicals, UK). Preliminary analyses using this method showed better mRNA concentrations were gained should RNA isolation continue immediately from this point, rather than centrifuge the homogenate. By principle, the ceramic beads contain much of the cell debris already and allow for removal of an aqueous phase immediately after homogenisation. Thus, the aqueous phase was transferred from the 2ml lysing tube into a labelled RNase-free Eppendorf before beginning phase separation. TRIZOL is a mono-phasic solution of phenol guanidine isothiocyanate that disrupts cells and dissolves all cell components other than RNA and DNA. All procedures below were carried out using RNA-free pipette tips and pipettes that were handled for RNA use only. A different tip was used for every sample to ensure no cross contamination. All work surfaces and equipment were sprayed with 75% ethanol and then RNase ZAP (Ambion-The RNA Company, Cheshire, UK) before commencing work with RNA.

3.11.2 RNA isolation and extraction

200µl of chloroform was added per 1ml TRIZol reagent used during homogenisation and shaken vigorously by hand for 15 seconds before being incubated at room temperature for 3 minutes. Samples were then centrifuged at 12000g for 15 minutes at 4°C. After centrifugation, the samples were separated into their red phenol, middle interphase and upper aqueous phase. The upper aqueous phase was carefully removed into a clean, labelled
Eppendorf, ensuring the middle interphase was not disturbed, and mixed with 500µl isopropanol (per 1ml TRIzol). After vortex for 15s, the sample was incubated at room temperature for 10 minutes before further centrifugation (12000g for 10 minutes at 4°C). The resulting supernatant was removed and the remaining RNA pellet washed in 1ml ice-cooled 75% ethanol (per 1ml TRIzol), vortexed briefly before centrifugation at 7500G for 8 minutes at 4°C. The ethanol was subsequently removed and the RNA pellet allowed to air dry before re-suspension in 30µl RNA storage solution (RNAlater, Invitrogen, UK). Samples were incubated in a block heater at 50°C for 10 minutes to assist with re-suspension before proceeding to measurement. RNA concentration and purity were assessed by UV spectroscopy at optical densities of 260 and 280nm with the use of a Nanodrop 2000 (Thermo Fisher Scientific, UK). A target of $A_{260} / A_{280}$ ratio was set at 2.0. 70ng RNA was used for each polymerase chain reaction (PCR) reaction.

3.11.3 Primer Design

Primer sequences (Table 3.2) were identified using Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Sequence homology searches ensured specificity; all primers had no potential unintended targets following a blast search. The primers were ideally designed to yield products spanning exon-exon boundaries to prevent any amplification of gDNA. Three or more GC bases in the last five bases at the 3’ end of the primer were avoided. Secondary structure interactions (hairpins, self-dimer and cross dimer) within the primer were avoided. All primers were between 16 and 25 bp, and amplified a product of between 67 – 212bp. Primers were purchased from Sigma (Suffolk, UK).
3.11.4 Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT PCR)

rt-qRT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotor-gene 3000Q (Qiagen, Crawley, UK) supported by rotor-gene software (Hercules, CA, USA). rt-qRT-PCR was performed as follows: hold 50°C for 10 min (reverse transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial denaturation step) and PCR steps of 40 cycles; 95°C for 10s (denaturation), 60°C for 30s (annealing and extension). Upon completion, dissociation/melting curve analysis were performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for each target gene suggesting amplification of a single product, Appendix 8). Following initial screening of suitable housekeeping genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed the most stable C<sub>t</sub> values across all RT-PCR runs, participants and regardless of experimental condition (23.54 ± 1.69 CT; 7% Co-efficient of variation; Chapter 4) and was selected as the reference gene in all RT-PCR assays. The relative gene expression levels were calculated using the comparative C<sub>t</sub> (ΔΔC<sub>t</sub>) equation (Schmittgen & Livak, 2008) where the relative expression was calculated as 2<sup>-ΔΔct</sup> and where C<sub>t</sub> represents the threshold cycle. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH; participants own reference, not pooled) within same subject and condition and to a calibrator of Pre-exercise. In Chapter 4, the calibrator of Pre-exercise for the immersed and non-immersed legs was the same sample.
**Table 3.2:** Primer sequences used during Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>AAGACCTTGGGCTGGGACTG</td>
<td>TGGCTCGGCTGCGAC</td>
</tr>
<tr>
<td>NM_002046.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RPL13a</strong></td>
<td>GGCTAAACAGGTACTGCTGGG</td>
<td>GAAAGCCAGGTACTTCAACTT</td>
</tr>
<tr>
<td><strong>Total PGC-1alpha</strong></td>
<td>TGCTAAACGACTCCGAGAA</td>
<td>TGCTAAACGACTCCGAGAA</td>
</tr>
<tr>
<td>NM_013261.3</td>
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<td></td>
</tr>
<tr>
<td><strong>PGC-1alpha alternative promoter (Exon 1b)</strong></td>
<td>CTATGGATTCAATTTTGAAATGTGC</td>
<td>CTGATTGGTCACTGCACCAC</td>
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<tr>
<td>XM_011513766.1</td>
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</tr>
<tr>
<td>XM_005248132.1</td>
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Glyceraldehyde 3-phosphate dehydrogenase – GAPDH; Ribosomal protein L13a - RPL13a; Peroxisome Proliferator-activated receptor gamma coactivator 1-alpha – PGC-1α; Tumour suppressor protein 53 - p53; Sirtuin 1 – SIRT1; Cytochrome C oxidase subunit 4 – COXIV; Citrate synthase – CS; Mitochondrial transcription factor A – TFAM; Nuclear respiratory factor 2 – NRF2; Estrogen-related receptor alpha – ERRα; Glucose transporter type 4 – GLUT4.
3.12 ANALYSIS OF SKELETAL MUSCLE SAMPLES USING SDS-PAGE AND WESTERN BLOTTING

3.12.1 Sample preparation

Approximately 30 mg of frozen muscle was homogenized using 2.4 mm ceramic beaded tubes (6 m/s for 3 x 40 seconds, separated by 5 minutes cooling on ice; MP Fastprep-24, MP Biomedicals, UK), in 500 µl of ice-cold lysis buffer [25 mM Tris-HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-pyrophosphatase, 1% Triton X-100] and supplemented with a protease phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor 186 Cocktail, Thermo Scientific, # 78442). The resulting homogenates were centrifuged at 14,000 g for 10 min at 4°C, and the supernatant was collected.

3.12.2 Determination of protein quantity using Bicinchoninic Acid (BCA kit) and SDS page

The protein content of the supernatant was determined using a bicinchoninic acid assay (Sigma, UK). Each sample was diluted with an equal volume of 2X Laemmli buffer (National Diagnostics) and boiled for 10 min at 90°C. 40 µg of protein from each sample was loaded (65 µg for phosphorylated analytes) and then separated in Tris-glycine running buffer (10x Tris/glycine, Geneflow, Staffordshire, UK) using self-cast 10% separating [33% Protogel; (30% w/v acrylamide: 0.8% (w/v) Bis-Acrylamide stock solution (37.5:1)), 25% Protogel resolving buffer (1.5M Tis-HCL, 0.4% SDS, pH 8.8), 41% ddH₂O, 100µl 10% APS, 20µl TEMED] and 4% stacking [13% Protogel, 25% Protogel Stacking buffer (0.5M Tris HCL, 0.4% SDS, pH 6.8), 61% ddH₂O, 100µl 10% APS, 20µl TEMED] gels (National Diagnostics, Geneflow, UK) (Figure 3.6).
3.12.3 Western Blotting

Gels were transferred semidry onto nitrocellulose membrane (Transblot Turbo, Bio-Rad) for 30 min at 25V and 1.0 mA in transfer buffer [10% TRIS/glycine (Sigma), 20% methanol, 70% ddH2O). After transfer, membranes were briefly washed in TBST (0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1% Tween-20] before being blocked for 1 h at room temperature in TBST with 1% BSA. The membranes were then washed for 3 x 5 min in TBST before being incubated overnight at 4°C with antibodies for anti-phospho-AMPK Thr172 (cat no: 2532), p38 MAPKThr180/Tyr182 (cat no: 9211) (Cell Signalling) as well as total protein content of AMPK (cat no: 2531), p38 MAPK (cat no: 9212) (Cell Signalling, UK), GAPDH (25778; Santa Cruz), and PGC-1α (Calbiochem, Merck Chemicals, UK) all at concentrations of 1:1000 in 1 x TBS. The following morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, UK) for 1h at room temperature. After a further 3 x 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL) for 5 min (30s for GAPDH). Membranes were visualized using a Bio-Rad Chemi-doc system, and band densities were determined using Image Lab image-analysis software (Bio Rad, UK). Samples from each subject for all exercise conditions were run on the same gel and statistical analysis conducted on raw densitometry data. Phosphorylated AMPK Thr172 and p38 MAPK Thr180/Tyr182 were relativized to their total counterpart, as these did not change significantly across blots or samples (P > 0.05). PGC-1α was relativized to GAPDH.
3.13 Muscle glycogen

For Chapter 5, to ensure all participants reported with similar levels of muscle glycogen they first underwent a two-legged glycogen depleting cycling protocol consisting of 2 min at 90% PPO, followed immediately by a 2-min recovery period at 50% PPO. They repeated this work to rest ratio until 2 min cycling at 90% PPO could not be maintained, determined as an inability to maintain a cadence of 70 rev.min\(^{-1}\). At this point, exercise intensity was lowered to 80% PPO, with the same work:rest ratio. When participants could no longer maintain this intensity, it was lowered to 70% and finally 60% PPO with the same work to rest ratio. When the participants were unable to cycle for 2 min at 60% PPO, the exercise protocol was terminated. This intermittent pattern of exercise has previously been shown to evoke glycogen depletion in both type I and type II fibres (Kuipers et al., 1987). After the completion of the two-legged glycogen depletion protocol, participants were provided with a high CHO diet for the next ~22 h (CHO 8 g·kg\(^{-1}\) body mass, PRO 1.4 g·kg\(^{-1}\) body mass
FAT 0.5 g·kg\(^{-1}\) body mass. Feeding began immediately after the cessation of exercise for 4x hourly intervals that evening. Participants were also provided with breakfast for the following morning and returned to the laboratory post-breakfast to collect food for the rest of the day (~8g CHO·kg\(^{-1}\)bw). The purpose of this initial glycogen depletion protocol with high CHO refeed was to ensure participants had a similar level of muscle glycogen prior to commencing the second evening depletion.

The protocol used to deplete a single leg of muscle glycogen in the day prior to the experimental day (Chapter 5) has been utilised before (Pilegaard et al., 2002). Approximately 15 h prior to the experimental trial, participants attended the laboratory for a single-leg glycogen depletion protocol, to deplete their dominant leg only. Glycogen depletion of the dominant leg was undertaken as to ensure similar muscle recruitment patterns and therefore glycogen depletion between trials. Single-leg glycogen depletion involved 20 min continuous single-leg cycling at 75% PPO, followed by intermittent cycling at a work:rest ratio of 90s:90s. Intermittent cycling began at 90% PPO decreasing in 5% decrements when such a workload cannot be maintained for 5s consecutively, to 55% PPO. Immediately following this participants’ completed an all-out one-legged cycling bout at 85% PPO before going on to 30 minutes of 2-arm cycling at 50W to decrease liver glycogen levels (Pilegaard et al., 2002). Participants then underwent an overnight fast before returning to the lab the next day.

Prior to commencing trials participants underwent at least two familiarization sessions for both the single- and 2-leg depletion protocols. Particular care was taken to ensure participants were comfortable with the technique required for single-leg cycling. Furthermore, during single-leg cycling the spare pedal was weighted to assist with rotation,
this method has previously been used in other single-leg cycling protocols (MacInnis et al., 2017). In both depletion protocols, participants were blinded to time elapsed, power output and number of stages completed and were only informed of whether they were completing a ‘high’ or ‘recovery’ interval. HR was measured continuously throughout exercise (Polar RS400, Kempele, Finland) and RPE (Borg, 1982) recorded with each reduction in PPO. Water intake was consumed ad libitum throughout exercise with the same total volume consumed in subsequent trials.

This method of single leg depletion has previously been successful in producing contralateral high and low glycogen limbs (High ~600 and Low ~300 mmol·kg\(^{-1}\)dw); Pilegaard et al., 2002). We further conducted pilot work on these depletion protocols to ensure we could match these previous results, with that pilot work showing slightly lower values (High ~400 and Low ~175 mmol·kg\(^{-1}\) dw).

Muscle glycogen concentration was determined according to the method described by Van Loon et al. (2000). Approximately 2-3 mg of freeze-dried sample was dissected free of all visible non-muscle tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3-4 hours at 100°C. After cooling to room temperature, samples were neutralized by the addition of 250 µl 0.12 mol.L\(^{-1}\) KOH saturated with KCl. Following centrifugation, 150 µl of the supernatant was analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HLK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol·kg\(^{-1}\)dw and intra assay coefficients of variation was <5%.
CHAPTER FOUR

Post-exercise cold-water immersion modulates skeletal muscle PGC-1α mRNA expression in immersed and non-immersed limbs: evidence of systemic regulation
This study was published in Journal of Applied Physiology. In doing so, permission to reproduce the work in this thesis is automatically granted.


*J Appl Physiol*, DOI: 10.1152/japplphysiol.00096.2017

4.1 INTRODUCTION

It is well established that the transcriptional co-activator PGC-1α, the proposed “master regulator” of skeletal muscle mitochondrial biogenesis, is sensitively controlled by acute and chronic exercise (Egan et al., 2010; Perry et al., 2010; Bartlett et al., 2012; 2013). Upstream control of PGC-1α includes phosphorylation by energy and stress sensing kinases AMPK (Jäger et al., 2007) and p38 MAPK (Akimoto et al., 2005). Consistent with its initial discovery as cold-inducible (Puigserver et al., 1998; Oliveira et al., 2004), recent studies have examined the potential of acute post-exercise cold exposure to also modulate PGC-1α expression. For example, in human tissue, both cold-ambient temperatures (Slivka et al., 2012; 2013) and post exercise cold-water immersion (Ihsan et al., 2014a; 2015) enhances the skeletal muscle PGC-1α gene transcription and protein translational response versus exercise alone. The precise mechanism(s) mediating cold-induced regulation of the PGC-1α transcriptional pathway have yet to be fully determined, though the cooling-induced
alterations in muscle blood flow (Gregson et al., 2011; Mawhinney et al., 2013) are unlikely to mediate these effects (Norr bom et al., 2004; Taylor et al., 2016). It is noteworthy, however, that chronic cold-induced changes in PGC-1α protein content arise in conjunction with increased activity of signalling kinases AMPK and p38 MAPK (Ihsan et al., 2015). These data suggest that cooling of the skeletal muscle tissue may play a role in mediating the post-exercise cold-induction of PGC-1α mRNA through activation of local signalling kinases.

Alternatively, systemic control via increased β-adrenergic activity is suggested to play a potent role in mediating the effects of cold exposure on PGC-1α expression via AMPK (Manfredi et al., 2013) and β2-adrenergic receptor (Miura et al., 2007) mechanisms. Indeed, plasma noradrenaline concentrations remain higher following high-intensity exercise after cold-water immersion versus control conditions (Gregson et al., 2013). Studies to date have utilised the non-immersed limb as the control condition without the use of a true control (no cooling) condition (Frohlich et al., 2014; Ihsan et al., 2015; Ihsan et al. 2014a). By assuming the response to cold-water immersion is mediated locally, such experimental designs do not permit examination of the role of systemic versus localised mechanisms in mediating cold-induced changes in PGC-1α. Indeed, Ihsan et al. (2014a) observed that PGC-1α gene transcription was not induced in a non-immersed control limb, despite the limb having previously been exercised, and exercise being a potent stimulus to induce PGC-1α expression. This would therefore suggest that an induction of PGC-1α expression in an immersed limb occurred by way of localised cold-induced mechanisms. In this regard it is suggested that the increased systemic β-adrenergic activity associated with post-exercise cooling of the immersed limb could also modulate PGC-1α expression in the non-immersed contralateral limb.
Therefore, the aim of the present study was to examine whether the cold-water induced increase in PGC-1α mRNA observed post exercise is mediated through local or systemic mechanisms. To this end, we employed a novel experimental design (in a repeated measures crossover design) where ten recreationally active males completed an acute cycling protocol (8x5 min at ~80% peak power output) followed by a seated-rest condition (CON) or single-leg cold-water immersion (CWI; 10 min, 8°C). Muscle biopsies were obtained pre-, post- and 3 h post-exercise from a single limb in the CON condition but from both limbs post-exercise in the CWI trial (thereby providing tissue from a CWI and non-immersed limb, NOT). In this way, our design allowed us to obtain tissue from true control conditions but yet, also sample tissue from an immersed and non-immersed limb that was subject to the same hormonal milieu.

4.2 METHODS

4.2.1 Participants
Ten recreationally active healthy males (age 26 ± 4 y; body mass 79.29 ± 6.73 kg; height 180 ± 5 cm; \( \dot{V}O_{2\text{peak}} \) 51.46 ± 9.07 mL·kg\(^{-1}\)·min\(^{-1}\) peak power output (PPO) 265.2 ± 38.33 W; mean ± SD) participated in this study. Participants were instructed to refrain from exercise, alcohol and caffeine 48 hours prior to the test day. All participants gave written informed consent to participate after details and procedures of the study had been fully explained. Participants had no history of neurological disease or musculoskeletal abnormality and none were under any pharmacological treatment during the course of the study. Each subject was medically screened by a practising GP prior to participation for their individual risk associated with high-intensity exercise, muscle biopsy procedures and immersion in cold water. All procedures performed in the study were approved by the Ethics
Committee of Liverpool John Moores University and in accordance with the 1964 Helsinki declaration and its later amendments.

4.2.2 Experimental Design.

In a repeated-measures crossover design, participants completed two high-intensity intermittent cycling protocols followed either by 10 min seated rest (CON) or single-leg CWI (10 min at 8°C). Muscle biopsies were obtained from the vastus lateralis immediately before, post- and 3 h post-exercise. During the CWI trial muscle biopsies in the post-exercise period were taken from both the immersed and non-immersed leg. This design allowed us to assess the impact of the cold stimulus locally (immersed leg) and systemically (non-immersed leg) against a relevant resting control. Both experimental trials were conducted in a counterbalanced, randomized order with at least 10 days between conditions.

4.2.3 Experimental Protocol.

Participants attended the laboratory on 3 separate occasions. On the first occasion, participants completed an incremental exercise test to fatigue for the determination of \( \dot{V}O_{2\text{max}} \) and PPO (Hawley & Noakes, 1992). Results from this test were used to determine the power output necessary for cycling at a proportion of PPO on subsequent test days (detailed below). Prior to the first test day, participants completed a 24-hour food diary to be replicated before the second trial. Upon arrival at the laboratory (0900h), participants were fitted with a heart-rate monitor (Polar RS400, Kempele, Finland), skin and rectal temperature probes (MHF-18050-A and MRV-55044-A, Ellab, Rodovre, Denmark). Legs were marked for subsequent insertion of muscle temperature needles; area of insertion was calculated as half the length of the femur, over the ‘belly’ of the vastus lateralis. The needle thermistor was then placed at a depth of 3cm, plus one-half of the skinfold measurement,
for the determination of deep muscle temperature (3cm). Following 10-min resting in a supine position, baseline measures of heart rate (HR), temperature and oxygen uptake (\(\dot{V}O_2\); Oxycon Pro, Jaeger, Wuerzberg, Germany) were assessed. Resting venous blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using venepuncture cannulation (BD Nexiva Closed IV Catheter 22G Blue, Becton Dickinson, Oxford, UK). Resting muscle temperature was assessed using a needle thermistor (13050; Ellab, Rodovre, Denmark) inserted into the vastus lateralis at 3cm depth as previously described (Mawhinney et al., 2013). Finally, resting muscle biopsy samples from the vastus lateralis (~30–50 mg wet wt) were obtained under local anaesthesia (0.5% Marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL). At rest only a single leg was biopsied in the cooling trial (CWI) in attempt to reduce the stress experienced by participants. This leg was randomised between the immersed and non-immersed limbs to exclude potential variation caused by leg dominance; all subsequent biopsies were completed in both legs, at each time point, 2cm proximal to the previous incision.

Following the resting biopsy, participants completed a high-intensity intermittent cycling protocol consisting of 8 × 5min bouts at 82.5% PPO separated by 1 min rest (adapted from Stepto et al., 2001) followed by either single-legged CWI (CWI: 10 min at 8°C; NOT: non-immersed leg i.e. 10 min at room temperature) or a control condition (CON; seated rest). CWI was conducted using a specialised seated mechanical hoist to lower the subject so that one leg was placed inside the cold bath, allowing the other (NOT) to remain outside, and dry. Participants then recovered in a semi-reclined position under normal laboratory temperatures (~21°C) until 3-hours post-exercise. Measures of heart rate, skin temperature (thigh and calf) and rectal temperature were recorded throughout the exercise and recovery
periods. Oxygen uptake was measured during the final minute of each high-intensity bout of exercise, during immersion, immediately post-immersion and again at 1, 2 and 3 h post-exercise. Ratings of perceived exertion (RPE) were assessed during the final minute of each exercise bout (Borg, 1982), whilst subjective measures of perceived shivering were assessed throughout water immersion and the 3 h recovery period using a visual analogue scale from 1 (No shivering) to 5 (Intense Shivering) (Joo et al., 2016). Laboratory temperatures remained stable throughout (21.18 ± 0.76°C) and at no point were participants allowed to rub themselves dry or shower (changing into dry shorts after immersion was allowed). Participants were advised to wear the same clothes between trials. Muscle temperature was assessed post-exercise, immediately post-immersion and at 1, 2 and 3 h post-exercise. Venous blood samples were also drawn at these times. Bi-lateral muscle biopsies occurred immediately after exercise and 3 h post-exercise.

Venous blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using standard venepuncture techniques (BD Nexiva Closed IV Catheter 22G Blue, Becton Dickinson, Oxford, UK). Blood samples (~10ml) were collected into vacutainer tubes (Becton Dickinson, Oxford, UK) containing EDTA and stored on ice until centrifugation at 1500 rev.min\(^{-1}\) for 15-min at 4°C. Following centrifugation, aliquots of plasma were stored at -80°C for later analysis. Plasma metanephrine and Normetanephrine concentrations were measured using liquid chromatography tandem mass spectrometry as previously described (Peaston et al., 2010). All samples were analysed in duplicate, with the mean value employed.

4.2.4 rt-qRT-PCR.
rt-qRT-PCR amplifications were performed as specified in Section 3.11 using specific Primer designs outlined in Table 4.1. Following initial screening of suitable reference genes, GAPDH showed the most stable C\textsubscript{t} values across all RT-PCR runs, participants and regardless of experimental condition (23.54 ± 1.69 C\textsubscript{t}; 7% Co-efficient of variation) and was selected as the reference gene in all RT-PCR assays. The average PCR efficiency was 90% and variation for all genes was less than 4.3%. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH; subject’s own samples reference) within same subject and condition and to a calibrator of Pre-exercise.

4.2.5 SDS-PAGE and Western Blotting.

SDS-PAGE and western blotting was performed as specified in Section 3.12. Samples from each subject for all exercise conditions were run on the same gel and statistical analysis conducted on raw densitometry data. Phosphorylated AMPK\textsuperscript{Thr172} and p38 MAPK\textsuperscript{Thr180/Tyr182} were normalised to their total protein, as these did not change significantly across blots or samples ($P > 0.05$). PGC-1\textalpha was normalised to GAPDH.
### Table 4.1: Primer sequences used for real-time PCR.

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Glyceraldehyde 3-phosphate dehydrogenase—GAPDH; Peroxisome Proliferator-activated receptor gamma coactivator 1-alpha—PGC-1α; Tumour suppressor protein 53—p53; Sirtuin 1—SIRT1; Cytochrome C oxidase subunit 4—COXIV; Citrate synthase—CS; Mitochondrial transcription factor A—TFAM; Nuclear respiratory factor 2—NRF2; Estrogen-related receptor alpha—ERRα.
4.2.6 Statistical analysis.

All data are presented as mean ± SD, unless otherwise stated. Baseline data, distance cycled, exercise HR and RPE were compared between conditions using a Paired Samples T-test. Blood, temperature (skin, rectal, muscle) and post-exercise HR, oxygen uptake and subjective ratings were analysed using a two-factor (two condition x time) within-participants general linear model. For muscle derived variables, a two-factor (three condition x time) within-participants general linear model was used to evaluate the effect of time (baseline vs. post exercise) with shared baseline data used for NOT and CWI (Statistical Package for the Social Sciences version 21.0; SPSS Inc., Chicago, IL). A two-factor (three condition x time) within-participants general linear model was subsequently used to evaluate the influence of the cooling intervention following exercise and the 3h post exercise period. The main effects for condition and time was followed up using planned LSD multiple contrasts. Where a significant condition by time interaction was observed, the post exercise to 3h post exercise change scores were calculated and compared across the 3-conditions using LSD multiple contrasts. The ES magnitude was classified as trivial (<0.2), small (0.2-0.6), moderate (0.6-1.2), large (1.2-2.0) and very large (>2.0-4.0) (Hopkins et al., 2009). The α level for evaluation of statistical significance was set at \( P < .05 \).

4.3 RESULTS

4.3.1 Exercise Response.

Distance cycled (CON 32.52 ± 4.21 km, CWI 32.33 ± 4.33 km; \( P = 0.629 \), ES 0.04 Trivial), heart rate (\( P = 0.309 \), ES 0.13 Trivial), \( \dot{V}O_2 \) (ml·kg\(^{-1}\)·min\(^{-1}\) \( P = 0.855 \), ES 0.02 Trivial) and RPE (\( P = 0.637 \), ES 0.08 Trivial) were similar between CON and CWI trials (data not shown). Mean HR during the final minute of exercise was 182 ± 8 beats.min\(^{-1}\) in CON and
183 ± 8 beats.min⁻¹ in CWI, equating to ~94% HR max. The RPE in the final exercise bout was 19 ± 1 AU and 19 ± 1 AU in the CON and CWI trials respectively.

4.3.2 Recovery Response.

Metabolic Responses. Heart rate (ES 0.90 Moderate) and VO₂, (ES 1.20 Moderate) was higher in CWI vs CON during the post-exercise recovery period (Table 4.2; \( P < 0.001 \)). The change in HR and VO₂, over time was also different between conditions (\( P <0.001 \)) with increases in HR and VO₂, occurring during the initial 2 minutes of immersion. Following immersion, HR and VO₂ dropped below pre-immersion values and remained lower throughout the 3h recovery period (HR, ES >0.92 Moderate; VO₂, ES >1.25 Large) (\( P < 0.05 \)).
Table 4.2: Heart rate and oxygen uptake during immersion and the post-immersion period (n=10, mean ± SD).

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<td>109±16</td>
<td>104±13</td>
<td>102±13</td>
<td>101±13</td>
<td>90±13</td>
<td>90±12</td>
<td>89±11</td>
<td>88±11</td>
<td>88±11</td>
<td>75±13</td>
</tr>
<tr>
<td>VO₂ (ml·kg⁻¹·min⁻¹)</td>
<td>CON</td>
<td>4.6±0.9</td>
<td>4.2±0.8</td>
<td>4.3±0.7</td>
<td>4.4±0.8</td>
<td>4.3±0.6</td>
<td>4.3±0.9</td>
<td>4.4±0.8</td>
<td>4.5±0.8</td>
<td>4.3±0.7</td>
<td>4.2±0.7</td>
<td>4.3±0.8</td>
<td>4.5±0.8</td>
</tr>
<tr>
<td></td>
<td>CWI</td>
<td>7.05±1.4</td>
<td>8.1±1.4</td>
<td>6.5±1.2</td>
<td>6.3±0.7</td>
<td>6.0±1.0</td>
<td>5.9±1.4</td>
<td>5.2±1.4</td>
<td>4.8±1.0</td>
<td>4.7±0.8</td>
<td>4.4±0.9</td>
<td>4.1±1.2</td>
<td>4.3±0.8</td>
</tr>
</tbody>
</table>

Values are mean ± SD. A main effect for condition and time along with a significant interaction between condition and time were found for HR and VO₂ (P < 0.05).

# Significant difference from pre-immersion (P < 0.05).
4.3.3 Thermoregulatory Responses.

Rectal temperature was similar between conditions throughout the post-exercise period (CON 37.52 ± 0.24°C, CWI 37.48 ± 0.05°C, ES 0.49 Small, \( P = 0.217 \)). The change in rectal temperature over time was different between conditions, with a small decline in rectal temperature occurring after 3 minutes of immersion until 3h post-exercise (\( P=0.034 \), ES 0.22 Small). Thigh skin temperature was generally lower throughout the post-exercise recovery period in CWI versus CON (ES 6.26 Very Large) and NOT (ES 6.46 Very Large) (Figure 4.1a, \( P < 0.001 \)). The change over time was also different between conditions, with thigh skin temperature continually decreasing in CWI and remaining lower than pre-immersion values until 1h post exercise (ES 3.0 Very Large, \( P < 0.001 \)). Values in CON and NOT limbs remained similar to pre-immersion throughout the 3h recovery period (\( P = 0.10 \), ES 0.57 Small).

Post-exercise muscle temperature (3 cm depth) was similar between CON (38.75°C), CWI (38.86°C) and NOT (38.54°C) (Figure 4.1b; \( P > 0.05 \)). During the 3 h recovery period muscle temperature (3cm) was lower in CWI versus CON (ES 1.60 Large) and NOT (ES 1.77 Large) (\( P < 0.001 \)). The change in muscle temperature over time was also different between conditions (\( P < 0.001 \)). Muscle temperature declined to a large extent immediately after immersion in the CWI limb, followed by a further gradual reduction during the remaining 3h post-exercise period (\( P < 0.001 \)). In CON and NOT conditions, muscle temperature was reduced to a lesser extent immediately following immersion followed by a further gradual reduction during the 3h post-exercise period (\( P = 0.246 \), ES 0.34 Small) (See Figure 4.1b).
Subjective shivering ratings were greater in CWI vs CON during the post-exercise recovery period (ES 1.20 Large, $P = 0.067$). The change in shivering over time also tended to be different between conditions ($P = 0.062$), with ‘slight’ shivering observed in the CWI condition during the first 2 minutes following immersion (ES >0.60 Moderate).
Figure 4.1: Temperature response post-exercise for thigh skin (a) and deep muscle (3cm depth) (b) with/without CWI. Values are mean ± SD. A main effect for time, condition and time*condition interaction was found in both thigh and muscle temperature measures ($P<0.05$). *Significantly different from pre-immersion (Skin) or post-exercise (muscle) ($P<0.05$). # Significantly different from CON and NOT ($P<0.05$). Post Ex = Post Exercise, Post Im= Post Immersion, +1h= 1h post- exercise, +2h= 2h post-exercise, +3h Post= 3h post-exercise.
4.3.4 AMPK and P38 MAPK activity and total abundance

Phosphorylation of AMPK\textsuperscript{Thr172} was not increased post-exercise ($P = 0.242$, ES 0.20 Small). At post-exercise and 3h post-exercise phosphorylation of AMPK\textsuperscript{Thr172} was similar between conditions ($P = 0.846$, ES 0.03 Trivial). However, the change in AMPK\textsuperscript{Thr172} between these time points was different between conditions ($P = 0.031$; Figure 4.2). AMPK\textsuperscript{Thr172} phosphorylation increased in CWI vs. CON ($P = 0.027$, ES 1.22 Large) with a moderate increase in AMPK\textsuperscript{Thr172} phosphorylation also observed in NOT vs. CON ($P = 0.145$, ES 0.70 Moderate). Representative Western blots are shown in Figure 4.5.

Exercise induced a small increase in phosphorylation of p38MAPK\textsuperscript{Thr180/Tyr182} ($P = 0.056$, ES 0.44 Small, Figure 4.3). At post exercise and 3h post-exercise phosphorylation of p38MAPK\textsuperscript{Thr180/Tyr182} was similar between conditions ($P = 0.672$; ES 0.03, Trivial). No differences in the change in phosphorylation between these time points was observed between conditions ($P = 0.268$, Figure 4.3). Representative Western blots are shown in Figure 4.5.

4.3.5 PGC-1alpha mRNA and protein abundance.

PGC-1\textalpha mRNA expression was moderately increased with exercise ($P = 0.066$, ES 0.92 Moderate, Figure 4.4a). At 3h post-exercise, expression was greater in CWI (ES 1.2 Moderate, $P = 0.003$) and NOT (ES 1.6 Large, $P = 0.001$) versus CON, but was similar between CWI and NOT (ES 0.6 Small, $P = 0.141$) (Figure 4.4a). This reflected the greater change in expression in CWI and NOT conditions between post exercise and 3h post exercise time points ($P = 0.001$, Figure 4.4a). PGC-1\textalpha protein content was not influenced by exercise ($P = 0.092$) or CWI ($P = 0.471$, Figure 4.4b). Representative Western blots are shown in Figure 4.5.
Figure 4.2: Phosphorylation of AMPK$^{Thr172}$ fold changes from Pre, expressed relative to total AMPK as total AMPK did not change significantly across blots or samples ($P > 0.05$). Values are mean ± SD. $\$ $ Significant time*condition interaction ($P = 0.031$). Post Ex = Post Exercise, 3h Post= 3h post-exercise.
Figure 4.3: Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ fold change from Pre, expressed relative to total p38MAPK as total p38 MAPK did not change significantly across blots or samples ($P > 0.05$). Values are mean ± SD. Post Ex = Post Exercise, 3h Post= 3h post-exercise.
Figure 4.4: PGC-1α mRNA (a) and protein content (b) $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD. A main effect for condition and time was found ($P < 0.001$). There was also a significant interaction between condition and time ($P = 0.001$) *Significantly different from baseline ($P < 0.05$). # Significantly different from CON ($P < 0.05$). Post Ex = Post Exercise, 3h Post= 3h post-exercise.
Figure 4.5: Representative Western Blots from the control (CON) non-immersed (NOT) and immersed (CWI) legs before (Pre), after (Post), and 3h after (3h) exercise. p-, phosphorylated; t-, total. Phosphorylated AMPK Thr172 and p38 MAPK Thr180/Tyr182 were relativized to their total counterpart, as these did not change significantly across blots or samples ($P > 0.05$). PGC-1α was relativized to GAPDH. kDa= Kilodaltons.
4.3.6 Additional gene expression

Exercise induced increases in SIRT1 ($P = 0.057$, ES 0.8 Moderate) and NRF2 ($P = 0.028$, ES 0.6 Moderate) mRNA (Figure 4.6). No changes were seen between conditions, or between conditions over time ($P > 0.05$). Gene expression analysis for p53, COXIV, CS, TFAM and ERRα mRNA was not influenced by exercise or CWI ($P > 0.05$).

4.3.7 Plasma Metanephrine and Normetanephrine.

Metanephrine concentrations were similar between conditions ($P = 0.159$, ES 0.15 Trivial). The change in metanephrine over time was also similar between conditions ($P = 0.299$). Metanephrine concentration was increased post-exercise (ES 2.46 Very Large) and post-immersion (ES 0.77 Moderate) vs. baseline ($P \leq 0.02$). Normetanephrine values were greater in CWI vs. CON ($P = 0.034$, ES 0.43 Small) with the largest difference seen post-immersion (860 vs. 665 pmol·L$^{-1}$, CWI vs. CON, respectively). The change in Normetanephrine over time was similar between conditions ($P = 0.821$). Normetanephrine concentrations increased with exercise (ES >4.70; $P < 0.001$) and remained above baseline post-immersion (ES 1.52 Large) and 1h post-exercise (ES 1.06 Moderate) ($P < 0.001$). Concentrations returned to baseline at 2h post-exercise (See Table 4.3).
Figure 4.6: mRNA $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD. A main effect for time was found for NRF2 ($P = 0.028$). #Significantly different from baseline ($P < 0.05$). * Different from baseline ($P = 0.057$).
Table 4.3: Catecholamine concentrations (pmol·L⁻¹) pre and post exercise, with and without CWI (n=10, mean ± SD).

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Baseline</th>
<th>Post-Exercise</th>
<th>Post-Immersion</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normetanephrine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>326.7±165.6</td>
<td>1711.9±411.10</td>
<td>665±291.8</td>
<td>615.7±368.3</td>
<td>489.3±407.0</td>
<td>426.9±260.0</td>
</tr>
<tr>
<td>CWI</td>
<td>478.2±189.2</td>
<td>1774.1±358.4</td>
<td>859.8±264.5</td>
<td>716.8±257.3</td>
<td>621.2±247.5</td>
<td>517.2±191.0</td>
</tr>
<tr>
<td>Metanephrine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>199.7±62.0</td>
<td>489.2±234.4</td>
<td>231.4±90.5</td>
<td>193.4±75.2</td>
<td>167.9±62.1</td>
<td>145.8±68.4</td>
</tr>
<tr>
<td>CWI</td>
<td>201.6±36.7</td>
<td>509.6±153.9</td>
<td>281.8±104.8</td>
<td>245.6±78.3</td>
<td>176.5±43.9</td>
<td>157.5±46.4</td>
</tr>
</tbody>
</table>

Values are mean ± SD. A main effect for condition was found for Normetanephrine (P = 0.034). A main effect for time was found for both Normetanephrine and Metanephrine (P < 0.001). # Significant difference from baseline (P < 0.05). * Significant difference versus the CON condition (P < 0.05).
4.4 DISCUSSION

The aim of the present study was to examine whether the post-exercise cold-water induced increase of PGC-1α mRNA is mediated through local or systemic mechanisms. Using a novel experimental design, data herein shows for the first time the appearance of systemic “cross-talk” between immersed and non-immersed limbs, as evidenced by the similar increase in PGC-1α mRNA in these limbs after single-legged CWI. Additionally, it is suggested that this effect could be mediated by β-adrenergic induced stimulation of AMPK. In addition to providing novel data on the potential mechanisms mediating post-exercise cold-induced enhancement of PGC-1α expression, data herein also has potential implications for research designs that utilise non-immersed limbs as control conditions.

Since its initial discovery (Puigserver et al., 1998) the importance of the post-exercise PGC-1α response to the oxidative adaptive process has been examined extensively, with mRNA increases ranging 5 to 10-fold commonly observed at 3-4 hours following exercise (Bartlett et al., 2012; 2013; Impey et al., 2016; Perry et al., 2010). More recently, cold-ambient temperatures (Slivka et al., 2012; 2013) and post exercise cold-water immersion (Ihsan et al., 2014a; 2015; Joo et al., 2016), have also been shown to enhance (~2- to 4-fold greater) the skeletal muscle PGC-1α gene transcription and protein translational response above exercise alone. In line with such observations, the high-intensity intermittent cycling protocol used in the present study elicited a ~5-fold increase in PGC-1alpha mRNA at 3 hours post-exercise. Furthermore, CWI enhanced this response to a greater extent than exercise alone (~9-fold increase vs. Pre in the CWI immersed limb).
There have been suggestions that reduced tissue temperature is responsible for the differences observed between cold-treated and control limbs. This stems from the initial discovery that PGC-1alpha was cold inducible in animals (Bruton et al., 2010; Eddy & Storey, 2003; Puigserver et al., 1998). In humans, recent data from Ihsan and colleagues (2014a; 2015) implicates a reduction in tissue temperature in the cold-induced increases of PGC-1α, as increases in mRNA (3h post-exercise) and protein content (after 4 weeks of training) were seen only in a cooled limb, and not in the contralateral non-immersed limb. Potential mechanisms underpinning such responses include activation of non-noxious thermoreceptors via reduced skin temperature (Hensel & Boman, 1960; Ishida et al., 2016). Within the present study, both the NOT and CWI limbs displayed similar acute PGC-1α mRNA expression (~12- and ~9-fold, respectively) in the 3 h recovery period, whilst skin and muscle temperature were significantly reduced in the CWI limb only. Indeed, the non-immersed limb (NOT) showed a similar temperature profile (skin and muscle temperature) to that of CON, where the magnitude of PGC-1α mRNA response was almost half (~5-fold) when compared with CWI and NOT. When taken together, these data suggest that alterations to local muscle temperature do not play a significant role in cold-induced regulation of PGC-1α expression.

As a result of increased local PGC-1α gene expression in the non-immersed exercised limb to a similar magnitude as the immersed limb, we sought to consolidate the role of the upstream kinases AMPK and p38-MAPK (Canto & Auwerx, 2010; Jäger et al., 2007; Wright et al., 2007) in their ability to regulate PGC-1α transcription. p38MAPK is a stress activated kinase that has been shown extensively to be phosphorylated after acute exercise, independent of intensity (Egan et al., 2010). Moreover, p38MAPK can exert its effect upon PGC-1α transcription via
upregulated ATF2 activity at the PGC-1α promoter (Akimoto et al., 2005). In the current study, exercise induced a small (ES 0.44, 1.5-fold) increase in phosphorylation with no further response to cooling. Moreover, our data supports previous data showing acute post-exercise phosphorylation of p38MAPK locally in skeletal muscle tissue occurs systemically (Widegren et al., 1998). Exercise-induced intensity dependant AMPK phosphorylation is a well reported phenomenon (Gibala et al., 2009; Cochran et al., 2010; Little et al., 2011) in rodent and human studies (Oliveira et al., 2004; Egan et al., 2010). Moreover, AMPK is implicated in PGC-1α activity via direct phosphorylation, initiating many of the important gene regulatory functions of PGC-1α in skeletal muscle (Jäger et al., 2007). The post-exercise increase in phosphorylation of AMPK in the present study was similar in magnitude to previous work (Bartlett et al., 2012) from our laboratory, albeit failing to achieve statistical significance. Notwithstanding this, large and moderate effect sizes were observed at 3 h post-exercise in the CWI and NOT limbs vs. CON, respectively. These greater increases from post-exercise to 3 h post-exercise in the immersion trial (CWI and NOT limbs), compared to a slight decline observed in CON (Figure 4.2) suggest the increases in phosphorylation of AMPK during the post-cooling period are controlled by a systemic mechanism, possibly adrenergic control via cold-augmented plasma Normetanephrine.

Adrenaline and noradrenaline are both dual α- and β- adrenergic agonists. Previous in vivo and in vitro incubation techniques utilising α- and β- adrenergic agonists have reported increased AMPK activation in rodent skeletal muscle (Minokoshi et al., 2002), adipose tissue (Koh et al., 2007) and cell cultures (Yin et al., 2003) implicating catecholamines as a potential AMPK activator. Despite this, support for the above hypothesis is currently conflicting, and is limited
by distinct differences in methodological design and species studied. In human muscle, acute infusion of adrenergic agonists/antagonists have previously shown to be ineffective at altering PGC-1α transcriptional activity (Robinson et al., 2010; 2011) and its upstream effector p38-MAPK (Kim et al., 2013). In contrast, treatment with β-adrenergic agonists/antagonists has been shown to induce and inhibit PGC-1α respectively (Miura et al., 2007), whilst incubation of rodent skeletal muscle with the adrenergic agonist phenylephrine increased the activity of the upstream regulator of PGC-1α, AMPK (Minokoshi et al., 2002). When results from these studies are taken together, it could be suggested that adrenergic activation of AMPK is a potential mechanism to explain the systemic increases in PGC-1α gene expression described herein.

In humans, one study has investigated the impact of higher catecholamine levels on AMPK phosphorylation in human skeletal muscle (Kristensen et al., 2007). These authors assessed muscle biopsies from an exercised and non-exercised limb in conditions of heightened catecholamine release. Results showed AMPK activity was restricted to contracting muscle only, with no systemic effects notable in the non-exercised limb despite the increased catecholamine levels. Importantly, in our study all limbs were exercised before undergoing cold exposure. It is therefore suggested that the cold induction of β-adrenergic pathways (via increased catecholamines) presented in this manuscript allows an additive response to exercise stimulated AMPK phosphorylation. Further studies are now required to verify this signalling response in related experimental conditions in human skeletal muscle.
Another pathway by which increased catecholamine’s may enhance PGC-1α transcription is via increased activation of β-adrenergic receptors. Activation of these receptors increases intracellular cAMP, which could ultimately activate CREB function on the PGC-1α promoter (Akimoto et al., 2008). However, evidence exists to show β-adrenergic stimulation does not activate a p38MAPK - ATF2 - CREB - PGC-1α signalling axis in skeletal muscle (Kim et al., 2013). These findings resonate with results from the present study as no changes in phosphorylation of p38MAPK were observed alongside increased plasma Normetanephrine concentrations. Further work is required to investigate the influence of the dual stress of exercise and cold temperature upon this signalling axis.

Downstream of PGC-1α, Slivka and colleagues (2013) noted that recovery in cold ambient temperatures (4h in 7°C ambient air) reduced the expression of the transcription factors NRF2 and ERRα, whilst having no effect on TFAM. Their importance to the adaptive response is highlighted by their roles in oxidative metabolism. Our data suggest the immersion protocol used herein was not sufficient to induce such changes. Indeed, 10 minutes of single-legged immersion offers a much smaller cooling stimulus than the 4 hours in cool ambient temperatures, as used by Slivka et al. (2013). In addition, an acute increase in PGC-1α gene expression was not followed by changes in PGC-1α total protein content, perhaps due to the acute time-frame of sampling applied in the present study. With this lack of change in total protein content it is however, unlikely that changes in gene expression of downstream genes such as NRF2, TFAM, COXIV, CS, ERRα presented here, would be affected by its upstream protein function as a transcription factor (PGC-1α). More research is required to understand the effect and dose response of a cold stimulus on downstream targets of PGC-1α.
It is difficult to explain the differences in PGC-1alpha mRNA results between the present study and Ihsan et al. (2014a), particularly the difference in non-immersed limbs. Moreover, reasons as to why Ihsan and colleagues failed to see the expected exercise-induced response in their non-immersed control limb, with minor changes from baseline at 3h post-exercise (1.5-fold increase vs. 3-5-fold increase usually seen), remain unclear. One possible explanation might include the differing muscle recruitment patterns occurring between the exercise protocols utilised (cycling vs. running). Ultimately, results from the present study have future implications on scientific study designs. Those wishing to investigate cold induced post-exercise responses in skeletal muscle must be aware of the evidence that implicates a systemic response of Normetanephrine, and a local response of p-AMPK in both immersed and non-immersed limbs. Further evidence is required to support the impact of systemic transcriptional responses in unilateral research designs, as such designs may be liable to error; potentially underestimating the actual response occurring in the immersed limb if relativized to a non-immersed limb instead of a resting control. Ultimately, the choice of scientific design lies with the question posed, as contralateral designs remain useful for understanding both local and systemic responses.

In summary, the present study characterises for the first time the mechanistic control of cold induced PGC-1α mRNA expression. Data herein indicate a reduction in tissue temperature (2-3°C) plays a limited role as similar levels of PGC-1α mRNA expression are observed in an immersed and non-immersed limb despite a reduction in tissue temperature in the CWI limb only. Moreover, a cold-induced systemic increase in plasma Normetanephrine may impact localised phosphorylation events of the signalling kinase AMPK\textsuperscript{Thr172}, with potential
downstream effects upon rates of PGC-1α mRNA expression. Future studies should investigate the role of β-adrenergic receptors in Normetanephrine induced AMPK phosphorylation and the signalling role of MEF2 and CRE/ATF2 sites to confirm a link between catecholamines and PGC-1α. Moreover, due to the acute nature of the present study more work is required to investigate whether the response seen herein is maintained over a more chronic term.
CHAPTER FIVE

The impact of combined pre-exercise low glycogen and post-exercise CWI on acute PGC-1α gene expression.
5.1 INTRODUCTION

The importance of the transcriptional co-activator PGC-1α to exercise induced oxidative adaptation is well reported (Perry et al., 2010; Bartlett et al., 2012; 2013). Indeed, this master regulator of mitochondrial biogenesis has been found to be up regulated by an exercise stimulus in an intensity dependent manner (Egan et al., 2010). As a transcriptional co-activator, PGC-1α is unable to directly bind to DNA and instead exerts its influence by co-activating a number of downstream transcription factors (such as NRF-1/2 EERα, TFAM) that are important for the regulation of the nuclear and mitochondrial genomes (Lin et al., 2005; Egan & Zierath, 2013), ultimately improving oxidative capacity (Bonafiglia et al., 2017).

Several mechanisms have been identified that regulate the increased expression and activation of PGC-1α following an exercise stimulus. Metabolic sensors, AMPK and p38 MAPK are two key activators of PGC-1α (Gibala et al., 2009; Ihsan et al., 2015). More recently, cold-ambient temperatures (Slivka et al., 2012; 2013) and post exercise cold-water immersion (Ihsan et al., 2014a; 2015; Joo et al., 2016), have also been shown to enhance the skeletal muscle PGC-1α gene transcription (~2- to 4-fold greater) and protein translational response versus exercise alone. This follows the initial discovery of PGC-1α expressed in rat skeletal muscle only after cold exposure (Puigserver et al., 1998). Mechanisms responsible are unknown, with β-adrenergic activation of AMPK (Allan et al., 2017; Ihsan et al., 2015) and β-AR activation of a cAMP-CREB-PGC-1α signalling axis (Akimoto et al., 2008) suggested to play a role. Furthermore, the findings from Chapter 4 (Allan et al., 2017) indicate that cold augmentation of PGC-1α mRNA is systemic in nature, occurring in the presence of heightened β-adrenergic activator normetanephrine, following greater activation of the signalling kinase AMPK and independent of reductions in muscle tissue temperature.
The importance of correct and sufficient nutrition for acute and chronic exercise is well described (Cermak & Van Loon, 2013). Despite this, a growing body of evidence demonstrates potential benefits of restricted CHO availability in exercise-induced adaptations, albeit at the expense of performance capacity (Impey et al., 2016). Indeed, endurance based exercise sessions commencing with low CHO availability can enhance the activation of cell signalling pathways (Bartlett et al., 2013), upregulate oxidative enzymes (Morton et al., 2009), increase lipid oxidation (Yeo et al., 2008) and therefore over time enable improvements in exercise capacity (Hansen et al., 2005). Importantly, Pilegaard and colleagues (2005) demonstrated that attenuating post-exercise muscle glycogen re-synthesis in the short-term recovery phase (24 h) further amplifies the gene expression or activity of PGC-1α, suggesting such a response occurs following a second consecutive exercise day. The augmented PGC-1α mRNA expression response following exercise with low glycogen availability has since been replicated in subsequent studies (Psilander et al., 2013; Bartlett et al., 2013), whilst disputed by others (Jensen et al., 2015). Mechanisms controlling such a response are speculated to be a result of increased activity and nuclear abundance of AMPK (Wojtaszewski et al., 2003; Steinberg et al., 2006) and p38MAPK (Chan et al., 2004) when exercise is commenced with low muscle glycogen.

To this end, investigations to date suggest that PGC-1α gene expression can be regulated by both internal (low glycogen) and external (cold stimuli) stress. Whilst available data demonstrates these stressors have the ability to augment post-exercise PGC-1α gene expression individually, no evidence exists investigating the potential further augmentation upon the PGC-1α response when both low glycogen and cold stimuli are combined. Therefore, the aim of the
Present study was to determine the relative influence of reduced muscle glycogen and post-exercise cold-water immersion in mediating post-exercise increases in skeletal muscle signalling (AMPK and p38MAPK activity) and PGC-1α transcription.

5.2 METHODS

5.2.1 Participants
Nine recreationally active healthy males (age 22 ± 3 yrs.; body mass 74.18 ± 7.88 kg; height 180.50 ± 6.60 cm; peak power output (PPO) 271.8 ± 25.8 W; mean ± SD) participated in this study. Participants were instructed to refrain from exercise, alcohol and caffeine 48 hours prior to the first depletion protocol, and not to stray from the prescribed meal plan or exercise within the 48 hours preceding the experimental day. All procedures performed in the study were approved by the Ethics Committee of Liverpool John Moores University and in accordance with the 1964 Helsinki declaration and its later amendments.

5.2.2. Preliminary testing: Prior to commencing the experimental trials the participants completed an incremental exercise test to fatigue for the determination of VO2peak and PPO (see Section 3). Results from this test were used determine the Watts necessary for cycling at a proportion of PPO on subsequent test days (See Section 3). Further preliminary visits encompassed familiarisation to the glycogen depletion protocols to be completed prior to the experimental day.
5.2.3 Experimental design

In a repeated-measures, randomized crossover design, with at least 14 d between trials, participants reported to the laboratory a total of nine times, where the first three were familiarisation sessions. In order to establish a research design that allowed the investigation of 4 separate conditions from 2 visits participants underwent a single-leg depletion protocol and bi-lateral muscle biopsies with and without post-exercise CWI to give the following conditions: High glycogen control (HI CON), Low glycogen control (LO CON), High glycogen CWI (HI CWI) and Low glycogen CWI (LO CWI).

Two-legged glycogen depletion, Visit 1: Participants arrived at the laboratory 40 h prior to the experimental trial at 1600 h and undertook a 5-min warm-up at 100 W. From here, participants performed an intermittent cycling protocol aimed to deplete both limbs of muscle glycogen, as described in Section 3.13. Participants were then fed high CHO diets to refuel both limbs to a similar extent (Appendix 5).

Single-leg glycogen depletion, Visit 2: Approximately 15 h prior to the experimental trial, participants attended the laboratory for a single-leg glycogen depletion protocol to deplete their dominant leg only, as described in Section 3.13. An overnight fast followed.

Experimental trial, Visit 3: Upon arrival at the laboratory participants were fitted with a heart-rate belt (Polar RS400, Kempele, Finland), skin and rectal temperature probes (MHF-18050-A and MRV-55044-A, Ellab, Rodovre, Denmark) and legs were marked for subsequent insertion of muscle temperature needles (See Section 3.5.1). Following 10-minutes in a supine position
baseline measures of HR, temperature and oxygen uptake (\(\dot{V}O_2\); Oxycon Pro, Jaeger, Wuerzberg, Germany) were assessed. Resting venous blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using venepuncture cannulation (BD Nexiva Closed IV Catheter 22G Blue, Becton Dickinson, Oxford, UK). Resting muscle temperature was assessed using a needle thermistor (13050; Ellab, Rodovre, Denmark) before resting bi-lateral muscle biopsies were sampled from the vastus lateralis (~30-50 mg wet wt). Immediately after the resting biopsy participants completed a high-intensity intermittent cycling protocol, consisting of 8 × 5 min bouts at 82.5% PPO separated by 1 min rest (See Section 3.6) followed by either two-legged CWI (CWI: 10 min at 7.96 ± 1.05°C) or a control condition (CON; seated rest). From here, participants recovered in a semi-reclined position under normal laboratory temperatures until 3-hours post-exercise. Measures of heart rate, skin temperature (thigh and calf) and rectal temperature were recorded throughout the exercise and recovery periods. Oxygen uptake was measured during the final minute of each high-intensity bout of exercise, during immersion and immediately post-immersion to assess for shivering thermogenesis and again at 1, 2 and 3h post-exercise. Laboratory temperatures remained stable throughout (~21°C) and at no point were participants allowed to rub themselves dry or shower (changing into dry clothes after immersion was allowed). Muscle temperature was assessed post-exercise, 1, 2 and 3h post-exercise, whilst venous blood samples were also drawn at these times. Further bi-lateral muscle biopsies occurred immediately after exercise and 3h post-exercise. Biopsies were obtained from both limbs at all time points to allow for comparison between high (HI) and low (LO) glycogen legs in both CON and CWI trials. All incisions were individually anaesthetised, separated by 2-3cm and included four passes of muscle tissue per biopsy.
5.2.4 Blood analysis.

All samples were analysed in duplicate. Samples were analysed for serum glucose, lactate, NEFA, and glycerol concentration using commercially available kits (Randox Laboratories, Antrim, UK). Plasma metanephrine and Normetanephrine concentrations were measured using liquid chromatography tandem mass spectrometry as previously described (Peaston et al., 2010). Serum samples were also analysed for insulin using a solid phase enzyme-linked immunosorbent assay (ELISA, KAQ1251, Life Technologies, UK), according to manufacturer’s instructions (See Section 3.9).

5.2.5 Muscle glycogen

Muscle glycogen concentration was determined according to the method described in Section 3.13.

5.2.6 rt-qRT-PCR.

rt-qRT-PCR amplifications were performed as specified in Section 3.11 using specific Primer designs outlined in Table 5.1. The average PCR efficiency was 91.25% and variation for all genes was less than 6.3%. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH; subject’s own samples reference) within the same subject and condition and to a calibrator of Pre-exercise.
Table 5.1: Primer sequences used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AAGACCTTGGGCTGGGACTG</td>
<td>TGGCTCGGCTGGCGAC</td>
<td>168</td>
</tr>
<tr>
<td>PGC-1alpha</td>
<td>TGCTAAACGACTCCGAGAA</td>
<td>TGCAAAGTTCCCTCTCTGCT</td>
<td>67</td>
</tr>
<tr>
<td>p53</td>
<td>ACCTATGGAAAACCTTTCTGAA</td>
<td>CTGGCATTCTGGGAGCTTC</td>
<td>141</td>
</tr>
<tr>
<td>SIRT1</td>
<td>CGGAACAATAACCTCCACCT</td>
<td>CATGAAACAGACACCCCC</td>
<td>186</td>
</tr>
<tr>
<td>COXIV</td>
<td>CGAGCAATTCCACCTGTT</td>
<td>GTCACGCGCATCCATATA</td>
<td>94</td>
</tr>
<tr>
<td>CS</td>
<td>CCTGCCATAATGACCCATGTT</td>
<td>CATATACTGGAGCAGCAGC</td>
<td>137</td>
</tr>
<tr>
<td>TFAM</td>
<td>TGGCAAGTTGTCCAAAGAAAC</td>
<td>GTTCCTCCAACGCTGGGC</td>
<td>135</td>
</tr>
<tr>
<td>NRF2</td>
<td>AAATTGAGATTGATGAAACAGA</td>
<td>TATGGCCTGGCTACACAT</td>
<td>95</td>
</tr>
<tr>
<td>ERRα</td>
<td>TGCCAAATTCACTCTGTC</td>
<td>CCAGCTTACCCCATAGAA</td>
<td>212</td>
</tr>
<tr>
<td>GLUT4</td>
<td>TCTCCAACCTGGACGACCAAC</td>
<td>CAGCAGGGAGCCGCAAAT</td>
<td>101</td>
</tr>
</tbody>
</table>

Glyceraldehyde 3-phosphate dehydrogenase – GAPDH; Ribosomal protein L13a- RPL13a; Peroxisome Proliferator-activated receptor gamma coactivator 1-alpha – PGC-1α; Tumour suppressor protein 53-p53; Siruin 1 – SIRT1; Cytochrome C oxidase subunit 4 – COXIV; Citrate synthase – CS; Mitochondrial transcription factor A – TFAM; Nuclear respiratory factor 2 – NRF2; Estrogen-related receptor alpha – ERRα; Glucose transporter type 4 – GLUT4.
5.2.7 SDS-PAGE and Western Blotting

SDS-PAGE and western blotting was performed as specified in Section 3.12. Samples from each subject for all exercise conditions were run on the same gel and statistical analysis conducted on raw densitometry data. Phosphorylated AMPK Thr172 and p38 MAPK Thr180/Tyr182 were relativized to their total counterpart, as these did not change significantly across blots or samples ($P > 0.05$).

5.2.8 Statistical analysis

All data are presented as mean ± SD, unless otherwise stated. Baseline data, distance cycled, exercise HR and RPE were compared between conditions using a Paired Samples T-test. A two-way (4 conditions x time) within participant’s general linear model for condition (CON HI, CWI HI, CON LO, CWI LO) and time was used to evaluate muscle glycogen, thigh skin temperature, muscle temperature, gene expression and protein activation responses. A two-way (2 conditions × time) within-participants general linear model was used to evaluate all blood measures (glucose, lactate, NEFA, glycerol, insulin, normetanephrine, metanephrine), rectal temperature, subjective and physiological responses (HR, RPE, shivering, $\dot{V}O_2$). The main effects for condition and time were followed up using planned LSD multiple comparisons. The ES magnitude was classified as trivial (<0.2), small (>0.2-0.6), moderate (>0.6-1.2), large (>1.2-2.0) and very large (>2.0-4.0) (Hopkins et al., 2009). The $\alpha$ level for evaluation of statistical significance was set at $P < .05$. 
5.3 RESULTS

5.3.1 Depletion response
In the two-legged depletion protocol no difference was present between conditions for distance cycled (CON 67.1 ± 18.1 km, CWI 67.3 ± 6.1 km, $P = 0.394$, ES 0.01 Trivial), time to depletion (CON 134 ± 30 min, CWI 130 ± 12 min, $P = 0.669$, ES 0.19 Small) or number of intervals completed (8 ± 2 per stage for both CON and CWI, $P = 0.669$, ES 0.20 Small). In the single-leg depletion protocol no significant difference was present between conditions for distance cycled in the steady-state period (CON 9.5 ± 1.2 km, CWI 8.5 ± 0.9 km, $P = 0.194$, ES 1.09 Large), number of high intensity bouts completed (CON 6.8 ± 2.0 per stage, CWI 6.4 ± 2.0 per stage, $P = 0.235$, 0.18 Trivial) and distance cycled during intervals (CON 47.5 ± 8.8 km, CWI 47.9 ± 7.3 km, $P = 0.587$, ES 0.06 Trivial). Moreover, no differences were found between conditions for time (CON 40.7 ± 9.0s, CWI 37.5 ± 8.4s) and distance (CON 0.6 ± 0.2 km, CWI 0.4 ± 0.1 km) completed in the all-out one-leg cycle ($P = 0.197$ ES 0.38 Small and 0.094 ES 0.94 Large, respectively).

5.3.2 Exercise response
Distance cycled in the high-intensity intermittent cycling protocol (CON 26.8 ± 2.8 km, CWI 26.7 ± 3.4 km; $P = 0.946$, ES 0.01 Trivial), heart rate ($P = 0.992$, ES 0.004 Trivial), $\dot{V}O_2$ (ml·kg$^{-1}$·min$^{-1}$; $P = 0.602$, ES 0.24 Small) and RPE ($P = 0.849$, ES 0.07 Trivial) were similar between CON and CWI trials. Mean HR during the final minute of exercise was 179 ± 7 beats.min$^{-1}$ in CON and 177 ± 9 beats.min$^{-1}$ in CWI ($P > 0.05$), equating to ~80% HR max. The RPE in the final exercise bout was 19.6 ± 0.7 AU and 19.3 ± 0.5 AU in the CON and CWI trials respectively.
5.3.3 Metabolic Response

Heart rate was similar between conditions \((P = 0.584, \text{ES} \ 0.18 \ \text{Trivial})\) during the immersion and recovery period. The change in HR over time was also similar between conditions \((P = 0.137)\), declining during the post-immersion period in both conditions. During the same period oxygen uptake was greater in CWI vs. CON \((P = 0.014, \text{ES} \ 1.83 \ \text{Large})\). The change in \(\dot{V}O_2\) over time was also different between conditions \((P = 0.045)\) with increases in \(\dot{V}O_2\) occurring during the initial 2 minutes of immersion. Following immersion, \(\dot{V}O_2\) dropped below pre-immersion values and remained lower throughout the 3h recovery period \((\text{ES} >0.58 \ \text{Small})\) \((P < 0.05)\). Subjective ratings of shivering were greater in CWI \((P = 0.052, \text{ES} \ 0.94 \ \text{Large})\). The change in subjective ratings of shivering over time also tended to be greater in CWI with values increasing during immersion and the 10-minute period immediately post-immersion \((P = 0.089)\) (Table 5.2).
Table 5.2: Heart rate ($n=9$) and oxygen uptake ($n=8$) during immersion and the post-immersion period (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Immersion</th>
<th>Post-Immersion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Im</td>
<td>2min</td>
</tr>
<tr>
<td>HR (beats.min$^{-1}$)</td>
<td>CON</td>
<td>84 ±5</td>
</tr>
<tr>
<td></td>
<td>CWI</td>
<td>95 ±19</td>
</tr>
<tr>
<td>VO$_2$ (ml·kg$^{-1}$·min$^{-1}$)</td>
<td>CON</td>
<td>5.4 ±0.8</td>
</tr>
<tr>
<td></td>
<td>CWI</td>
<td>8.5 ±2.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD. A main effect for condition and time along with a significant interaction between condition and time was found for VO$_2$ ($P < 0.05$).

*Significant difference from pre-immersion ($P < 0.05$). PreIm = Pre Immersion, AU = arbitrary units.
5.3.4 Blood plasma and muscle metabolites

Plasma glucose, lactate, NEFA, insulin and glycerol concentrations were similar between conditions ($P < 0.05$) (Figure 5.2). The change in these measures over time was also similar between conditions ($P < 0.05$). Significant differences were seen over time for glucose, lactate, glycerol and NEFA. Glucose increased immediately post-exercise ($P = 0.012$ vs. Pre), before decreasing to values below pre-exercise at 1h ($P = 0.011$), were it remained. Lactate increased post-exercise ($P = 0.001$ vs. Pre) and had returned to pre-exercise values by 1h post-exercise ($P = 0.055$). Glycerol increased post-exercise, remaining greater than pre-exercise values at 3h post-exercise ($P < 0.001$). NEFA increased from Pre at 1h post-exercise ($P < 0.001$), remaining at these levels by 3h post-exercise ($P < 0.001$).

Muscle glycogen concentrations were greater in the HI limbs compared to contralateral LO limbs throughout the experimental design (CON HI vs. CON LO: $P = 0.017$, ES 1.04 Moderate; CWI HI vs. CWI LO: $P = 0.001$, ES 1.18 Moderate; Figure 5.1). The concentration of muscle glycogen was similar between respective HI (CON HI vs. CWI HI: $P = 0.819$, ES 0.07 Trivial) and LO conditions (CON LO vs. CWI LO: $P = 0.751$, ES 0.14 Trivial; Figure 5.1). The change in muscle glycogen concentrations over time was similar between conditions ($P = 0.116$). Muscle glycogen was decreased immediately following exercise ($P = 0.001$, ES 1.11 Moderate) and 3h post-exercise ($P = 0.003$, ES 1.10 Moderate) compared to pre-exercise values.
Figure 5.1: Skeletal muscle glycogen content immediately pre- and post-exercise and after 3h of recovery. Biopsies were obtained from both limbs in each condition (CON or CWI) with limbs starting the day being high (HI) or low (LO) in glycogen stores. A main effect for time ($P = 0.001$) and condition ($P = 0.003$) was observed. No interaction effects were present ($P > 0.05$). * Significantly different from PRE. # Significantly different from contralateral LO limb (n = 9, mean ± SD). PRE= Pre exercise, POST= Post-exercise, +3hr= 3h post-exercise.
Figure 5.2: Plasma derived metabolic markers measured pre-exercise, post-exercise, 1h, 2h and 3h following the cessation of exercise (n=9, mean ± SD). * Significantly different from pre-exercise.
5.3.5 Thermoregulatory Response

Rectal temperature (Trec) was similar between conditions during exercise and post-exercise recovery ($P = 0.887$, ES 0.02 Trivial). The change in Trec over time was also similar between conditions ($P = 0.252$, Figure 5.3b). During the immersion and recovery period, Trec decreased from the fifth minute of immersion until 3h post-exercise ($P < 0.001$). Thigh skin temperature (Tthigh) was lower during immersion and the post-immersion period in CWI vs. CON conditions ($P < 0.001$, ES 2.48 Very Large). The change in Tthigh over time was also different between conditions, with Tthigh continually decreasing during cooling in CWI conditions, remaining lower than pre-immersion values at 3h post exercise ($P = 0.001$, Figure 5.3a). Muscle temperature (Tmus) was lower following immersion ($P < 0.001$) in the CWI limbs ($P < 0.001$, ES 0.90 Moderate). The change in Tmus over time was also different between conditions. Muscle temperature declined to a large extent immediately after immersion in the CWI limb, followed by a further gradual reduction during the remaining 3h post-exercise period ($P < 0.001$, ES 1.0 Moderate, Figure 5.4).
Figure 5.3: Thigh skin temperature (°C) (a) and rectal temperature (°C) (b) during immersion and the 3h post-exercise period. *Significantly different from pre-imm. #Significantly different from CON (n = 9 skin, n = 8 rectal; mean ± SD). Pre-Im = Pre Immersion.
Figure 5.4: Deep muscle temperature (3cm; °C). *Significantly different from PRE.
#Significantly different from CON (n = 8, mean ± SD).
5.3.6 Plasma Metanephrine and Normetanephrine

Metanephrine concentrations were similar between conditions \((P = 0.955, \text{ ES} \ 0.02 \ \text{Trivial, Figure 5.5})\). The change in metanephrine over time was also similar between conditions \((P = 0.438)\). Metanephrine concentration was increased post-exercise \((P < 0.001, \text{ ES} \ 2.10 \ \text{Very Large})\) and remained above baseline at 1h post-exercise \((P = 0.02, \text{ ES} \ 0.59 \ \text{Small})\).

Normetanephrine concentrations were similar between conditions \((P = 0.130, \text{ ES} \ 0.14 \ \text{Trivial, Figure 5.5})\). The change in normetanephrine over time was different between conditions, with normetanephrine concentrations decreasing to a greater extent in CON during the 3h post-exercise period \((P = 0.026, \text{ ES} \ 1.60 \ \text{Large})\). Normetanephrine concentration increased post-exercise \((P = 0.002, \text{ ES} \ 1.50 \ \text{Large})\) and remained above baseline in CWI conditions until 2h post-exercise \((P < 0.05; \ P = 0.058)\).
Figure 5.5: Plasma adrenergic catecholamines measured pre-exercise, post-exercise, 1h, 2h and 3h following the cessation of exercise ($n=9$, mean ± SD). A main interaction effect was present for normetanephrine ($P = 0.026$). *Significantly different from Pre-Exercise. Pre-Ex = Pre Exercise, Post-Ex= Post Exercise, +1h= 1h post-exercise, +2h= 2h post-exercise, +3h Post= 3h post-exercise.
5.3.7 AMPK and P38MAPK activity and total abundance

Phosphorylation of AMPK\textsuperscript{Thr172} was similar between conditions ($P = 0.443$), with no change over time ($P = 0.684$; Figure 5.6). Representative Western blots are shown in Figure 5.7.

Phosphorylation of p38MAPK\textsuperscript{Thr180/Tyr182} was similar between conditions ($P = 0.202$), with no change over time ($P = 0.283$; Figure 5.6). Representative Western blots are shown in Figure 5.7.
Figure 5.6: Phosphorylation of AMPK$^{\text{Thr}172}$ (a) and p38MAPK$^{\text{Thr}180/\text{Tyr}182}$ (b) fold changes from Pre, expressed relative to total AMPK ($P > 0.05$). Values are mean ± SD.
Figure 5.7: Representative Western Blots from CON HI, CON LO, CWI HI, CWI LO limbs before (Pre), after (Post), and 3h after (3h) exercise. Phosphorylated AMPK Thr172 and p38 MAPK Thr180/Tyr182 were relativized to their total counterpart ($P > 0.05$). kDa= Kilodaltons; P= phosphorylated; T= total.

5.3.8 Gene expression

PGC-1α mRNA was increased from baseline at 3h post-exercise ($P < 0.001$; ES 1.99 Large). At 3h post exercise, PGC-1α mRNA expression was greater in the HI vs. LO glycogen limbs (CON HI vs. CON LO: $P = 0.023$, ES 1.22 Large; CWI HI vs. CWI LO: $P = 0.019$, ES 1.43 Large; $P = 0.039$). Expression was also higher in CWI HI vs. CON HI limb ($P = 0.281$, ES 0.67 Large) (Figure 5.8). These differences reflected greater changes in expression between post exercise and 3h post exercise time points ($P < 0.001$).

Gene expression analysis for COXIV, CS, TFAM, SIRT1, NRF2 and GLUT4 mRNA (Figure 5.9) was not influenced by CWI or exercise ($P > 0.05$).
**Figure 5.8:** PGC-1α mRNA $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD. *Significantly greater than Pre and Post-Exercise ($P < 0.001$), # Significantly greater than LO counterpart ($P = 0.023$), + Greater than CON HI ($P = 0.281$, ES 0.67 Large).
**Figure 5.9:** mRNA $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD.
5.4 DISCUSSION

The aim of the present study was to investigate the potential further augmentation of post-exercise PGC-1α in response to combined low glycogen (internal) and cold (external) stimuli. A further aim was to investigate potential mechanisms in control of such a response. A key finding of the present study is that post-exercise CWI is able to augment the PGC-1α mRNA response to exercise vs. a non-immersed control condition, as has previously been shown (Chapter 4); however, this response was not evident in limbs where muscle glycogen is depleted to ~85 mmol·kg⁻¹·dw prior to exercise.

The impact of training with reduced glycogen levels are well documented, with low muscle glycogen availability resulting in greater activation of mitochondrial signalling enzymes compared to when the same exercise is commenced with normal glycogen values (Hawley & Morton, 2014). Indeed, Hansen et al. (2005) observed limbs that trained twice every second day (low glycogen; vs. limbs that trained once every day, high glycogen) showed improved time to exhaustion, greater resting muscle glycogen levels and greater activity of the mitochondrial enzymes 3-hydroxyacyl-CoA dehydrogenase and citrate synthase; all important markers of endurance adaptation and/or mitochondrial biogenesis. More recently, commencing exercise with muscles in a low glycogen state has been shown to augment the exercise induced expression of the transcriptional coactivator, and so-called ‘master regulator’ of mitochondrial biogenesis, PGC-1α (Psilander et al., 2013; Bartlett et al., 2013). Meanwhile, data from our lab and others has confirmed the role of post-exercise CWI in further augmenting the PGC-1α response to exercise (Ihsan et al., 2014a; Joo et al., 2016; Allan et al., 2017). The idea that both mechanisms could influence the acute post-exercise PGC-1α response to a greater extent than
individually has not previously been investigated. Presently, glycogen depletion of a single limb was successful in reducing muscle glycogen content versus the contralateral limb (~85mmol·kg\(^{-1}\)dw vs. ~200mmol·kg\(^{-1}\)dw, \(P < 0.05\), ES 1.11 Moderate). Muscle glycogen concentration was also similar between HI limbs and between LO limbs throughout the experimental design irrespective of condition (CON or CWI). This is in line with previous research that shows CWI does not negatively impact the acute (up to 4h) phase of post-exercise muscle glycogen resynthesis (Gregson et al., 2013). Moreover, HI limbs herein (~200mmol·kg\(^{-1}\)dw) failed to replete to the desired level and are better described as Low glycogen, whilst LO limbs herein (~85mmol·kg\(^{-1}\)dw) are better described as Very low glycogen. A lack of clarity on what constitutes high and low levels of muscle glycogen exists, however, low glycogen is often referred to as anything below 200-300 mmol·kg\(^{-1}\)dw (Psilander et al., 2013; Camera et al., 2015; Knuiman et al., 2015) with high glycogen being above ~550 mmol·kg\(^{-1}\)dw (Impey et al., 2016; Knuiman et al., 2015).

Limbs that commenced the high-intensity intermittent cycling protocol with ‘high’ glycogen concentrations (CON HI, CWI HI; ~200 mmol·kg\(^{-1}\)dw) showed large increases in PGC-1\(\alpha\) expression at 3h post-exercise, with post-exercise CWI augmenting the exercise-induced increase further (CWI HI ~8-fold vs. CON HI ~6-fold), as previously observed (Ihsan et al., 2013; Joo et al., 2016; Allan et al., 2017). However, when exercise was commenced with low glycogen concentrations (CON LO, CWI LO; ~85mmol·kg\(^{-1}\)dw) the expected augmentation of PGC-1\(\alpha\) versus a HI equivalent was not evident, with CWI having no additional benefit and showing the lowest fold change at 3h post-exercise versus all other conditions (CWI LO ~2.5-fold change from Pre). This is in agreement with other studies that show low muscle glycogen
is unable to stimulate greater post-exercise PGC-1α mRNA expression (Cluberton et al., 2005; Mathai et al., 2008; Cochran et al., 2010; Jensen et al., 2015; Impey et al., 2016) with differences in exercise intensity thought to be responsible (Egan et al., 2013).

It should be noted, previous literature using glycogen depletion protocols have failed to reach pre-exercise values as low as presently attained (~85 mmol·kg⁻¹·dw vs. ~184 mmol·kg⁻¹·dw Camera et al., 2015; ~166 mmol·kg⁻¹·dw Psilander et al., 2013; ~135 mmol·kg⁻¹·dw Taylor et al., 2013), ~103 mmol·kg⁻¹·dw Bartlett et al., 2013). Importantly, glycogen concentration in the present HI limbs are comparable to those of the low condition from previous research (Camera et al., 2015, Psilander et al., 2013) stimulating similar levels of PGC-1α gene expression 3h post-exercise (6-8 -fold increase in CON HI and CWI HI PGC-1α mRNA, Psilander et al., 2013, Taylor et al., 2013) irrespective of the mode of exercise completed. Taken together, it could be suggested a critical level of glycogen depletion exists for the benefits of low glycogen concentration upon the post-exercise PGC-1α mRNA response. Indeed, results in the present LO limbs surpassed such a critical threshold thus offering an explanation for the lack of cold and/or low glycogen induction in PGC-1α gene expression. Pilegaard et al. (2002) suggested such a threshold of glycogen might be evident for the expression of other genes (PDK4, UCP3). Further work is required to investigate whether a critical level of pre-exercise glycogen concentration exists before negating the post-exercise PGC-1α mRNA response.

Previous suggested mechanisms controlling the cold-induced (Ihsan et al., 2015) and low glycogen stimulated (Hawley & Morton, 2013) augmentation of PGC-1α are the upstream kinases AMPK and p38-MAPK. p38MAPK is a stress activated kinase that has been shown
extensively to be phosphorylated after acute exercise, independent of intensity (Egan et al., 2010). p38MAPK phosphorylates and activates PGC-1α (Puigserver et al., 2001; Knutti et al., 2001). Furthermore, Chan and colleagues (2004) demonstrated that reduced muscle glycogen availability increased the phosphorylation of nuclear p38MAPK versus the same exercise with normal glycogen stores. In the current study small (~1.5 fold, $P > 0.05$) increases in the phosphorylation of p38MAPK were present in the 3h post-exercise period, however no differences were evident in response to glycogen or immersion status. These results were to be expected given that the present study utilised contralateral HI and LO glycogen limbs and that p38MAPK has previously been shown to be activated systemically (Widegren et al., 1998; Allan et al., 2017). Exercise-induced intensity dependant AMPK phosphorylation is a well reported phenomenon (Gibala et al., 2009; Cochran et al., 2010; Little et al., 2011) in rodent and human studies (Oliveira et al., 2004, Egan et al., 2010). Moreover, AMPK is implicated in PGC-1α activity via direct phosphorylation, initiating many of the important gene regulatory functions of PGC-1α in skeletal muscle (Jäger et al., 2007). The phosphorylation of AMPK in the present study was incongruent to previous work from our laboratory (Bartlett et al., 2012, Allan et al., 2017), with control limbs (CON HI, CON LO) showing a reduction in phosphorylation at 3h post-exercise. Meanwhile, data from CWI limbs (CWI HI and CWI LO) showed post-exercise phosphorylation of AMPK$^{Thr172}$ was maintained at 3h irrespective of glycogen concentration.

Similar p-AMPK$^{Thr172}$ in contralateral limbs during the same experimental condition (similar CON HI and CON LO, similar CWI HI and CWI LO) provides stimulus to the suggestion that AMPK is systemically regulated (i.e. via catecholamines; Chapter 4), and induced by a cold
stimulus (Ihsan et al., 2015; Chapter 4). The hypothesis that catecholamines are implicated as a potential AMPK activator is conflicting (discussed in detail in Chapter 4). Briefly, only one study in humans has assessed the impact of higher catecholamine levels on AMPK phosphorylation in human skeletal muscle (Kristensen et al., 2007), with results showing AMPK activity restricted to contracting muscle only, with no systemic effects notable in the non-exercised limb despite the increased catecholamine levels. In the present study, two-legged CWI (8°C for 10 min) slowed the decrease in post-exercise normetanephrine concentration up to 2h post-exercise. It is suggested that this greater adrenergic response seen for CWI HI and CWI LO limbs may provide the difference between the maintenance of p-AMPK at 3h post-exercise, and the decrease noted in CON HI and CON LO limbs. Such an adrenergic response following CWI has been seen previously using a two-legged (Gregson et al., 2013) and single-legged (Chapter 4 – Allan et al., 2017) immersion design. The impact of β-adrenergic stimulation of p-AMPK<sub>Thr172</sub> requires further investigation, with a particular emphasis on β-AR function within this response.

It is difficult to explain the reasons for the lack of expected increases seen in post-exercise phosphorylation of AMPK<sup>Thr172</sup> and PGC-1α gene expression, particularly in LO glycogen limbs, however this could be related to cycling technique. It is well established that power output is depressed in glycogen-depleted limbs (Gejl et al., 2014). Indeed, Ørtenblad et al. (2011) demonstrated glycogen depletion at levels above (167 mmol·kg<sup>-1</sup>dw) those seen in the present LO limbs (~85mmol·kg<sup>-1</sup>dw) are associated with impaired sarcoplasmic reticulum function by diminished vesical Ca<sup>2+</sup> release, and thus reductions in peak power. Taken together it could be suggested participants herein preferentially turned the pedals with the HI glycogen
limb, unconsciously supporting the depleted LO limb and thus offering a greater exercise stimulus to the HI limb vs. LO limb; resulting in the lack of expected PGC-1α and p-AMPK response in the LO limbs. Indeed, previous research using the high-intensity intermittent cycling protocol employed here demonstrated a greater distance (~6km greater) covered by comparable participants (Chapter 4). The difference between that study and the present study is that the present study included 2 days of glycogen depletion protocols prior to the experimental protocol. Taken together with the fact that one limb was glycogen depleted during the experimental protocol, a critical level of glycogen being surpassed may have been a key factor in being unable to produce an intensity necessary to obtain expected levels of phosphorylation of AMPK and gene expression of PGC-1α in the LO limbs. Future investigations should look to assess any imbalance in power output between depleted and non-depleted limbs upon subsequent p-AMPK and PGC-1α gene expression.

In summary, the present study failed to see a benefit of combined low glycogen exercise with post-exercise CWI. Despite this, data herein characterises for the first time the potential existence of a critical threshold for the expected augmentation in post-exercise PGC-1α gene expression seen when exercise commences with low glycogen concentrations. Data herein suggest that whilst the cold induced increase in PGC-1α mRNA remains in limbs that begin exercise with glycogen concentrations >180mmol·kg⁻¹dw, the cold induced and low glycogen induced augmentation of post-exercise PGC-1α mRNA is abolished in limbs with glycogen concentrations ~85mmol·kg⁻¹dw, potentially due to reduced contraction kinetics. Moreover, this study offers support of previous work (Chapter 4 – Allan et al., 2017) that suggest cold-induced systemic control of AMPK phosphorylation potentially occurs via adrenergic
mechanisms. Further investigation is required to assess the role of β-adrenergic receptors in Normetanephrine induced AMPK phosphorylation and the signalling role of MEF2 and CRE/ATF2 sites to confirm a link between catecholamines and PGC-1α. Moreover, more work is required to confirm the existence of a critical glycogen threshold for controlling post-exercise PGC-1α gene expression.
CHAPTER SIX

Local and systemic impact of the cold and low glycogen on PGC-1α promoter specific gene expression.
6.1 INTRODUCTION

Since the discovery of the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) by Puigserver and colleagues (1998), it has been shown to be highly expressed in multiple human tissues, including the largest metabolically active tissue, skeletal muscle (Fick & Kelly, 2006). Here, it has been previously shown to play a key role in driving the formation of slow twitch muscle fibres (Lin et al., 2002) and is inextricably linked to improving oxidative capacity (Irrcher et al., 2003) via increasing mitochondrial biogenesis after exercise (Uguccioni & Hood, 2011). Indeed, ~3-10 fold-increases in PGC-1α transcription are often seen within the first few hours post-exercise (Bartlett et al., 2012; Joo et al., 2016; Allan et al., 2017), a response that is exercise intensity dependant (Egan et al., 2010), yet somewhat variable based on prior training status and/or basal PGC-1α content (Gibala, 2009).

More recently it has been shown that skeletal muscle expresses several different transcript variants that produce different protein isoforms of PGC-1α (reviewed in Popov et al. 2015a), highlighting a potential broad spectrum of alternative functions for this protein. To date, more than eight alternative protein isoforms have been described in human skeletal muscle, where the gene expression of these isoforms is thought occur via two alternate promoter regions on the same gene (Popov et al., 2015a; Martinez-Redondo et al., 2016). The proximal (canonical) PGC-1α gene promoter (Exon 1a) is constitutively expressed at rest compared to the alternative promoter (Exon 1b). The alternative promoter is more responsive post exercise stimulation (Martinez-Redondo et al., 2015; Popov et al., 2015b), where several transcript variants are expressed from the alternative gene promoter (Exon 1b & 1c) leading to the protein isoforms
of PGC-1α2, PGC-1α3 and PGC-1α4 (Martinez-Redondo et al., 2016). Although originating from the same promoter and upon identical stimuli, the different PGC-1α isoforms regulate altered transcriptional programs. Several groups have shown that in rodent and human skeletal muscle, acute exercise induces PGC-1α gene expression, mainly via the alternative promoter (Exon 1b) (Norrbom et al., 2011; Ydfors et al., 2013; Popov et al., 2014; Wen et al., 2014; Popov et al., 2015b). However, in humans, gene expression of exon 1c is not highly expressed post exercise with levels reported to be very low to negligible (Popov et al., 2015b).

Whilst evidence exists that supports exercise regulation of PGC-1α via a program of expression of distinct transcript variants, the contribution of post-exercise cold water (Chapter 4) or prior low muscle glycogen (Chapter 5) in the activation of PGC-1α via either the canonical (Exon 1a) or alternative (Exon 1b) promoter, and therefore the gene expression of Exon 1a vs. Exon 1b, has not been determined. Moreover, PGC-1α expression driven by exercise, cold and low glycogen stimuli is thought to be regulated by AMPK (Norrbom et al., 2011; Wen et al., 2014) or β-adrenergic receptor, protein kinase A (PKA), cAMP response element binding protein (CREB) signalling (Chinsomboon et al., 2009; Tadaishi et al., 2011). Therefore, of particular interest is whether the individual impact of exercise, cold stress and low glycogen would preferentially activate either the Exon 1a or Exon 1b PGC-1α promoter and whether the combined impact of these stimuli would further enhance PGC-1α gene expression. Indeed it has been suggested that Exon 1a may largely be AMPK activated, whilst Exon1b is more susceptible to β-adrenergic stimulation (Popov et al., 2015a). Thus, the aim of this study was to: 1) assess the impact of local and systemic cooling (using the samples collected from Chapter 4) on PGC-1α promoter specific mRNA expression, and 2) assess the impact of combined post-
exercise cooling and prior low glycogen content (using the samples collected from Chapter 5) within skeletal muscle on the post-exercise PGC-1α transcript variant response. It is hypothesised that cold-water immersion and prior low glycogen combined would increase the exercise induced PGC-1α alternative (Exon 1b) promoter to a greater extent than cold water or low glycogen alone.

6.2 METHODS

For full methodological details of experiment 1 (chapter 4) and experiment 2 (chapter 5) see section 4.2 and 5.2 respectively. Briefly, Chapter 5 investigated the local versus systemic response of post-exercise CWI by comparing an immersed limb, with a non-immersed limb and a complete (rested, no immersion) control. Results showed the cold-induction of PGC-1α transcription can be evoked systemically, with a similar augmentation in the non-immersed as well as the immersed limb versus the control. Chapter 6 built on this and investigated whether this cold induced augmentation can be further increased by commencing exercise with low glycogen. Results showed the potential existence for a critical threshold in low glycogen stimulated PGC-1α augmentation, however further research is required to fully clarify such a response. This chapter took muscle biopsies from Chapter 4 and Chapter 5 and used them to isolate mRNA (See Section 3.11.2) and assess promoter specific PGC-1α gene expression (exon 1a and 1b).

specificity and that all primers had no potential unintended targets. The primers were ideally
designed to yield products spanning exon-exon boundaries to prevent any amplification of
gDNA. Three or more GC bases in the last five bases at the 3’ end of the primer were avoided.
Secondary structure interactions (hairpins, self-dimer and cross dimer) within the primer were
avoided. All primers were between 20 and 25 bp, and amplified a product of between 127-153
bp. Primers were purchased from Sigma (Suffolk, UK).

rt-qRT-PCR amplifications were performed as specified in Section 3.11 using specific Primer
designs outlined in Table 6.1. Following initial screening of suitable reference genes, GAPDH
showed the most stable C_t values across all RT-PCR runs, participants and regardless of
experimental condition (23.54 ± 1.69 C_t; 7% Co-efficient of variation) and was selected as the
reference gene in all RT-PCR assays. The average PCR efficiency was 90% and variation of
efficiencies across all genes was low at 2.8%. mRNA expression for all target genes was
calculated relative to the reference gene (GAPDH; subject’s own samples reference) within
same subject and condition and to a calibrator of Pre-exercise.

6.2.1 Statistical analysis

Experiment 1- A two-way (3 condition × time) within-participants general linear model was
subsequently used to evaluate the influence of the cooling intervention following exercise and
the 3h post exercise period. The main effects for condition and time was followed up via the
use of planned LSD multiple contrasts. Where a significant condition by time interaction was
observed, the post exercise to 3h post exercise change scores were calculated and compared
across the 3-conditions using LSD multiple contrasts. The ES magnitude was classified as
trivial (<0.2), small (>0.2-0.6), moderate (>0.6-1.2), large (>1.2-2.0) and very large (>2.0-4.0) (Hopkins et al., 2009). The $\alpha$ level for evaluation of statistical significance was set at $P < .05$.

**Experiment 2**- A two-way (4 condition x time) within participant’s general linear model for condition (High glycogen control (HI CON), Low glycogen control (LO CON), High glycogen CWI (HI CWI)) and time (Pre, Post, 3h) was used to evaluate the influence of low glycogen and CWI following exercise and the 3h post exercise period. The main effects for condition and time were followed up using planned LSD multiple comparisons. The ES magnitude was classified as trivial (<0.2), small (>0.2-0.6), moderate (>0.6-1.2), large (>1.2-2.0) and very large (>2.0-4.0) (Hopkins et al., 2009). The $\alpha$ level for evaluation of statistical significance was set at $P < .05$.

Independent t-tests were used to assess the difference in mean baseline and 3h post-exercise gene expression (CT scores) between Exon 1a and Exon 1b promoter regions. Moreover, further independent t-tests were used to assess the difference in Pre-exercise and 3h post-exercise Exon 1b expression between HI and LO glycogen limbs.
<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Length (base pairs)</th>
<th>Location</th>
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</thead>
<tbody>
<tr>
<td><strong>Exon 1a</strong></td>
<td>ATGGAGTGACATCGAGTGTGCT</td>
<td>GAGTCCACCCAGAAAGCTGT</td>
<td>127</td>
<td>159-285</td>
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<td>NM_013261.4</td>
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<tr>
<td><strong>Exon 1b</strong></td>
<td>CTATGGATTCAATTCTTGAAATGTGC</td>
<td>CTGATTTGTCACTGCACCAC</td>
<td>153</td>
<td>77-229</td>
</tr>
<tr>
<td>XM_011513.766.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>XM_005248.132.1</td>
<td></td>
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*Exon 1a = PGC-1α Exon 1a; Exon 1b = PGC-1α Exon 1a.*
6.3 RESULTS

Experiment 1- Promoter-specific PGC-1α gene expression in immersed vs. non-immersed limbs

*Exon 1a-* Exercise significantly increased the expression of Exon1a mRNA ~2-4 fold from Pre (Pre – Post Exercise: $P < 0.001$, ES 2.50 Very Large, Figure 6.1). In the 3h post-exercise recovery period no change in level of expression was seen ($P = 0.202$, ES 0.27 Small) between conditions ($P = 0.146$, ES 0.74 Moderate). The change over time was significant ($P = 0.05$).

*Exon 1b-* Exercise significantly increased the expression of Exon 1b mRNA (Pre – Post Exercise: $P = 0.016$, ES 1.14 Large, Figure 6.1). In the 3h post-exercise recovery period Exon 1b expression increased significantly ($P = 0.007$, ES 1.15 Large) between conditions ($P = 0.011$). Post-hoc analysis showed NOT (2344-fold change from Pre, ES 1.12 Large) significantly increased Exon 1b gene expression compared to CON (579-fold change from Pre, $P = 0.010$) and CWI (1860-fold change from Pre, ES 0.61 Moderate) on average increased Exon 1b gene expression versus CON, an observation that approached statistical significance ($P = 0.07$). No significant change in Exon 1b gene expression between NOT and CWI ($P = 0.185$, ES 0.29 Small) was evident. No significant interaction effect was present ($P = 0.092$).
Figure 6.1: PGC-1α mRNA specific to Exon1a (a) and Exon1b (b) $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD. *Significantly different from baseline ($P < 0.05$). # Significantly different from CON ($P < 0.05$). $ Different from CON ($P=0.070$). Post= Post-exercise, +3h= 3h post-exercise.
**Experiment 2- Promoter-specific PGC-1α gene expression in cold water and low glycogen conditions**

Baseline/Pre-exercise expression was basally/constitutively higher in Exon1a than Exon1b, as would be expected based on previous literature (Martinez-Redondo et al., 2015; Popov et al., 2015b), demonstrated by later cycle threshold (Ct) values (lower basal expression) for Exon1b vs. Exon 1a (Ct 23.69 ± 2.52 vs. 21.99 ± 2.64 respectively, t = 0.006) at baseline. Therefore, when gene expression 3h post exercise increased for Exon 1b (low Ct value/higher expression of 20.41 ± 4.01 vs. a higher Ct/lower expression of 22.82 ± 4.13 (t = 0.014) for Exon 1a) this resulted in changes in the magnitude of 100-1000 fold for Exon 1b vs. fold changes in the 10’s for Exon 1a. Moreover, pre-exercise expression of Exon 1b gene expression was higher in LO glycogen limbs than HI (Ct 22.58 ± 2.70 vs. 24.81 ± 1.76, LO vs. HI limbs, t = 0.006), with gene expression increasing to a similar level in both HI and LO glycogen limbs (Ct 21.36 ± 3.96 vs. 19.46 ± 3.94, LO vs. HI limbs, t = 0.159).

**Exon 1a-** Exercise evoked an increase in the expression of Exon1a mRNA, peaking immediately post exercise (~1.5-2-fold change from Pre, ES 1.07 Moderate) before decreasing towards baseline at 3h post-exercise (~1-1.5-fold change from Pre, P = 0.065, ES 0.33 Small, Figure 6.2). No statistical significance was present between conditions or for the change over time (P = 0.086 and 0.492, respectively).

**Exon 1b-** Exercise evoked an increase in the expression of Exon1b mRNA, with the greatest increases seen at 3h post-exercise (CON HI and CWI HI ~159-fold change from Pre, CON LO ~ 58-fold change from Pre, CWI LO ~56-fold change from Pre, P = 0.057, ES 1.20 Moderate/Large). Significant differences were observed between conditions (P = 0.035, Figure 6.2) with both HI limbs having greater expression than their corresponding LO limb.
at 3h post-exercise (CON $P = 0.025$, ES 0.67 Moderate; CWI $P = 0.062$, ES 1.14 Large). No difference was present in both LO or both HI limbs when the CWI intervention was applied (LO $P = 0.398$, ES 0.26 Small; HI $P = 0.966$, ES 0.007 Trivial) suggesting a cold-induced response in exon 1b gene expression was not present. No significant interaction was seen ($P = 0.111$).
Figure 6.2: PGC-1α mRNA specific to Exon1a (a) and Exon1b (b) $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD. $^\dagger$ Trend towards increase from Pre ((a) $P=0.065$) (b) $P=0.057$. A main effect for condition and time was found ($P < 0.001$). *Different from corresponding LO limb ($P = 0.025$), +Different from corresponding LO limb ($P=0.062$). Post= Post-exercise, +3h= 3h post-exercise.
6.4 DISCUSSION

This is the first study to investigate the relative influence of localised and systemic cooling as well as the impact of prior reduced glycogen with and without post-exercise cooling on the promoter specific mRNA expression of the transcriptional coactivator PGC-1α. Data herein provides novel results to show PGC-1α mRNA expression from the alternative promoter (Exon 1b) mirrors the pattern of expression for total PGC-1α previously described in Chapter 5, thus the contribution to total PGC-1α mRNA expression post-exercise is likely a result of changes occurring from the alternative promoter (Exon 1b) (Figure 5.8). Moreover, a systemic response to CWI is seen from the alternative promoter (Exon 1b) with large (~2000) fold-changes from Pre-exercise in the immersed and contralateral non-immersed limbs, versus a non-immersed control (<1000 fold change from Pre). When levels of muscle glycogen are reduced below 85 mmol·kg⁻¹·dw this response is abolished, with LO glycogen limbs showing reduced mRNA expression via the alternative promoter (Exon 1b) versus the contralateral HI glycogen limb. Moreover, a cold stimulus had no influence upon promoter specific expression when glycogen concentration was low. Finally, data herein go some way to confirm previous reports that PGC-1α alternative promoter (Exon 1b) gene expression is responsible for the regularly observed exercise induced increase in total PGC-1α mRNA, in an intensity dependent manner.

The transcriptional coactivator PGC-1α has become a key target in many scientific studies focussing on skeletal muscle adaptation to different training stimuli because of its critical role in mediating mitochondrial biogenesis (Hood, 2009). The expression of this gene occurs via two different promoter regions, offering additional avenues of mechanistic interest. The proximal (canonical) PGC-1α gene promoter (Exon 1a) has higher basal expression; however, an alternative promoter (Exon 1b and 1c) is more responsive to
exercise stimulation (Martinez-Redondo et al. 2015). Indeed, several groups (Norrbom et al., 2011; Ydfors et al., 2013; Popov et al., 2014) have shown in human skeletal muscle that acute exercise induces PGC-1α gene expression mainly via the alternative promoter (Exon 1b). This occurs particularly via Exon 1b as gene expression of Exon 1c post-exercise in humans is very low to negligible (Popov et al. 2015b) and thus exon 1c was not measured in the present study.

In Experiment 1 of the present study, we sought to investigate the local vs. systemic effect of post-exercise cooling upon the promoter specific expression of PGC-1α mRNA. Expression from the canonical promoter (Exon1a) showed a significant increase post exercise in all limbs (2-4 fold change from Pre, $P < 0.05$). Large fold changes were present in expression from the alternative promoter (Exon1b), with values ranging ~1000-2000 fold increases from Pre-exercise values ($P < 0.05$). The sizeable difference in change of expression between the different promoter regions is likely to be due to the differential expression at baseline and response to an exercise stimulus. Indeed, Exon 1a is constitutively expressed at higher levels basally (at rest) to a greater extent than Exon 1b, whilst Exon 1b is constitutively expressed at lower basal levels, however, is more responsive to an exercise stimuli (Martinez-Redondo et al., 2015; Popov et al., 2015b). Data herein (Experiment 2) support previous data with lower Ct values (thus more gene expression) of Exon 1a at baseline vs. Exon 1b. Moreover, as the pattern of response to exercise from the alternative (Exon 1b) promoter closely mirrors that of total PGC-1α gene expression seen previously (Chapter 4) this data offers further support to previous evidence showing the response of total PGC-1α mRNA to acute exercise is largely driven by the alternative promoter (Exon 1b) (Norrbom et al., 2011; Ydorfs et al., 2013; Silvennoinen et al., 2015; Popov et al., 2015b). Additionally, data from our second experiment, to
investigate the impact of commencing exercise with low glycogen combined with post-exercise cooling on the promoter specific response, shows PGC-1α alternative promoter (Exon 1b) expression continued to rise at 3h post-exercise in all conditions, whilst expression from the canonical promoter (Exon 1a) decreases between the same time points (post-exercise – 3h post-exercise). This emphasises the role of the alternative promoter to the exercise induced augmentation in total PGC-1α gene expression.

Importantly, in Experiment 1, the local vs. systemic investigation, PGC-1α gene expression from the alternative promoter (Exon 1b) showed large increases in response to high-intensity intermittent cycling (CON, <1000 fold change from Pre), which was augmented even further in limbs exposed to systemic cold stress (NOT and CWI, ~2000 fold change from Pre, \(P = 0.07\) and \(<0.05\) vs. CON, respectively). Indeed, the fact that a similar pattern of response is seen in total PGC-1α mRNA (Chapter 4) suggests the contribution to cold induced total-PGC-1α gene expression is also driven by the alternative (Exon1b), and not the canonical (Exon1a) promoter region. This data is the first to show such a response to cold in either animal or human studies.

Previous evidence in rodent skeletal muscle has suggested that activation of exercise-induced expression via the alternative promoter (Exon 1b) is regulated by the β2 adrenergic receptor- protein kinase A (PKA)-cAMP response element-binding protein (CREB) signalling pathway (Chinsomboon et al., 2009; Tadaishi et al., 2011; Popov et al. 2015b). Ruas and colleagues (2012) showed administration of the β-adrenergic agonist clenbuterol in mice, induced expression of PGC-1a2 and 3 (Exon 1b derived) whilst levels of the canonical promoter derived PGC-1α1 (Exon 1a) were reduced. Moreover, Norrbom et al. (2011) showed AMPK activator AICAR stimulates transcription from both Exon 1a and 1b
promoter regions, whilst AICAR injected simultaneously with noradrenaline stimulated Exon 1b to a greater extent, thus suggesting noradrenaline had an additive effect on alternative (Exon 1b) promoter gene expression. We show a similar response occurs in humans whilst investigating Experiment 1; whereby higher levels of normetanephrine in the cooled condition occurs alongside continued increases in alternative promoter expression (Exon 1b) at 3h post-exercise. We have previously shown (Chapter 4 and Chapter 5) that the cooling conditions used in both experiments herein successfully increased and maintained plasma normetanephrine concentration, a β-adrenergic agonist. Thereby it would seem logical that the increased β-adrenergic activity that follows post-exercise CWI may play a sufficient role in the alternative promoter (Exon1b) driven cold-augmentation of total PGC-1α gene expression. Further work is required to confirm this response in humans.

The benefit of training with low glycogen levels upon the post-exercise PGC-1α mRNA response is well established (Psilander et al., 2013; Bartlett et al., 2013). We have shown previously (Chapter 5) that a potential critical limit of glycogen depletion may exist for such a benefit to occur, as limbs depleted below 85 mmol·kg\(^{-1}\)dw glycogen concentration failed to show an expected augmentation of total PGC-1α gene expression following high intensity intermittent cycling. It is suggested the very low levels of glycogen herein (Chapter 5) may have also prevented a cold induction of PGC-1α gene expression, with similar levels of expression in both LO glycogen limbs (CON LO and CWI LO), despite larger expression in the high glycogen cooled limb (CWI HI) versus its contralateral low glycogen cooled limb (CWI LO). Additionally, PGC-1α canonical (Exon 1a) promoter gene expression closely matched the pattern of response of total PGC-1α gene expression.
Mechanisms responsible for the lack of low glycogen stimulated augmentation of PGC-1α gene expression above the exercise induced response are unknown. However, it must be noted that exercise intensity may have confounding implications. More specifically, had participants struggled to produce a similar pedal force in glycogen depleted limbs, in comparison with the high glycogen contralateral limb, during the high-intensity intermittent cycling protocol, as would be expected due to impaired calcium release (Ørtenblad et al. 2011), it may be that cycling technique was altered to preserve total power output. With this said, participants could have subconsciously turned the pedals with the higher glycogen limb, sparing the already depleted (LO) limb. As a result, the higher glycogen limb will have been exposed to sufficiently greater exercise intensity and total work load, thus resulting in the discrepancy seen in total PGC-1α (Chapter 5) and Exon 1b (present Experiment 2) gene expression at 3h post-exercise from what would be expected.

As Exon 1a gene expression has previously been shown not to be dependent on exercise intensity (Popov et al., 2015b) it would suggest the lower expression from this promoter region in LO glycogen limbs (CON LO and CWI LO) at 3h post exercise is a result of the lower glycogen concentration. However, it should be noted that this suggestion is confounded by the reality that LO glycogen limbs underwent depleting exercise the night before the experimental day, whilst the HI limbs did not. This may therefore have led to higher absolute pre-exercise mRNA values in the LO vs. HI glycogen limbs, ultimately lowering the potential fold changes seen at 3h post exercise when compared with pre-values. Moreover, the smaller fold changes noted in the second experiment herein from the alternative promoter (Exon 1b) (~200 fold change from Pre), in comparison with Experiment 1 (~2000 fold change from Pre), despite both sets of participants undergoing the same relative exercise protocol, is perhaps a product of the same limitation. The prior
exercise completed in the second experiment (2 nights of depletion prior to the experimental day) may have altered the absolute basal levels of PGC-1α Exon 1a and 1b, limiting the fold changes seen at 3h post exercise; particularly as the 48 hours prior to experiment 1 included restricted levels of exercise and lower mean Ct values (therefore higher gene expression) were seen for LO glycogen limbs vs. HI glycogen limbs Pre-exercise. Indeed, maximum fold changes for total PGC-1α expression were lower in experiment 2 (Chapter 5, Figure 5.8) (max. ~8 fold change from Pre) vs. experiment 1 (Chapter 4, Figure 4.4) (max. ~12-fold change from Pre). This hypothesis is supported by Chris Perry and colleagues (2010) who showed the PGC-1α mRNA response to high intensity interval training was diminished almost 2-fold on the third day of exercise, compared to the first. Despite this, significant main effects over time suggest the greater fold changes seen at 3h post-exercise in HI glycogen limbs are also somewhat a result of exercise stimulation. Future studies should look to match the work and intensity done prior to the experimental day whilst altering the glycogen content of the muscles.

Despite the fact that differing exercise intensity may have played a role in blunting the response of exercising with low glycogen upon PGC-1α (Chapter 5), it is interesting that an expected cold-induction of PGC-1α mRNA was not seen in CWI limbs of the second experiment of this chapter, from either promoter region (Exon 1a and 1b). Indeed, we have previously shown that cold water immersion without prior exercise is able to evoke an increase in total PGC-1α gene expression (Joo et al., 2016). Thus, it is particularly confusing as to how a cold augmentation in total PGC-1α mRNA was seen in Chapter 5 between CON HI and CWI HI limbs, yet not for promoter specific expression (particularly Exon 1b) in the present chapter. Taken together, data from Experiment 1 established a close relationship between total and alternative promoter (Exon1b) derived PGC-1α mRNA in cooled
conditions that is not supported by the present data in this chapter from Experiment 2. Indeed, the fact that no cold-induction of promoter specific derived PGC-1α mRNA occurred in Experiment 2 could suggest alternative promoter (Exon 1b) expression is not as important in the overall cold induction of total PGC-1α gene expression as initially thought; with other, unidentified mechanisms yet to be discovered. Despite this, there are also other potential causes for such a response. Indeed, as previously mentioned, data herein are measured as fold-changes from pre exercise values. Prior exercise in the days leading up to Experiment 2 may have subsequently resulted in higher baseline absolute values and thus smaller fold changes at 3h post exercise, shown by the higher levels of pre-exercise expression in Exon 1a vs. Exon 1b, and LO vs. HI glycogen limbs for Exon 1b. If this hypothesis were true it begs the question that the cold-induced augmentation of PGC-1α is an acute response that occurs after a single exercise + cooling session, and is less likely to occur to a similar extent following several exercise + cooling sessions. Future work should look to follow a similar design as Perry and colleagues (2010) whereby the impact of CWI upon PGC-1α mRNA (total and promoter specific) is measured at regular acute intervals following exercise 4 times over the course of 7 days. This would allow determination of the time course changes in the PGC-1α response to several acute periods of cooling, and may explain why no cooling induced alterations in PGC-1α are noted in Experiment 2 herein, particularly as this experiment followed 2 days of prior depleting exercise.

In conclusion, this is the first study to show the systemic cold-induced augmentation of total PGC-1α as seen previously (Chapter 4) is largely a result of increased expression from the alternative promoter (Exon 1b), rather than canonical promoter (Exon 1a). Moreover, further research is required to confirm the suggestion that a critical level of glycogen is necessary to allow the expected low glycogen stimulated post-exercise augmentation in
PGC-1α mRNA. Evidence herein further supports the notion that PGC-1α gene expression from the alternative promoter (Exon 1b) is more reactive to an exercise and cold stimulus. Moreover, reduced gene expression fold changes in LO vs. HI glycogen limbs at 3h post-exercise (Exon 1b) likely occurs as a combined result of higher absolute basal expression pre-exercise and greater intensity/workload in the HI glycogen limb during exercise.
The aim of this chapter is to integrate and interpret the findings within this thesis. The initial section provides an overview of the findings in relation to the original aims and objectives of the thesis, set out in Chapter 1. A general discussion is then presented where specific attention is given to how the data herein has advanced the understanding of the transcriptional co-activator PGC-1α, its expression in the acute post-exercise period and the influence of the stress of low muscle glycogen and post-exercise CWI.

7.1 Realisation of aims and objectives

The aim of this thesis was to establish whether the cold induction of critical regulators in mitochondrial biogenesis are controlled systemically, or more locally through temperature induced changes in cell signalling pathways of the cooled muscle. Once established, subsequent aims served to investigate whether such a response could be enhanced when exercise was commenced with low glycogen stores. Additionally, this thesis sought to establish the impact of these stressors on the promoter specific response of the gene PGC-1α.

It was established in Study 1 (Chapter 4) that transcriptional regulation of the “key regulator” in mitochondrial biogenesis, PGC-1α responds in a systemic manner to local cooling. Similar increases in gene expression in a cooled and non-cooled contralateral limb, occurred to a greater extent than a control, uncooled limb post-exercise. Muscle temperature decreased in the cooled limb (CWI), not the uncooled limbs (NOT and CON), suggesting the gene expression response was not a result of local cooling of skeletal muscle tissue. Systemic increases in the β-adrenergic agonist normetanephrine during the post-exercise period of the cooling trial (CWI and NOT limbs) plays a role in this cold augmentation of PGC-1α gene expression via phosphorylation of the upstream signalling kinase AMPK.
Study 2 progressed upon the results from Study 1 to assess the impact of further stress upon the post-exercise and cooling response of PGC-1α, notably prior reduced glycogen concentration. In the higher glycogen limbs results were as expected, with CWI enhancing the post-exercise response compared with CON. No response of PGC-1α was noted in either low glycogen limb, with or without cooling. CWI produced expected local and systemic results by decreasing muscle temperature and increasing plasma normetanephrine. Data from Study 2 (Chapter 5) implicates a critical level of glycogen depletion exists for the augmentation of PGC-1α gene expression after exercise, and after exercise followed by cooling. In Study 3 (Chapter 6) the aim was to further assess the controlling mechanisms responsible for the regularly seen cold augmentation of post-exercise PGC-1α gene expression. In measuring the promoter specific response of PGC-1α data show for the first time the systemic cold-augmentation of PGC-1α largely occurs in a similar fashion to that of exercise induced PGC-1α, via the alternative promoter region (Exon 1b). Data show little impact of reduced glycogen on the promoter specific response, however, further data is required to confirm this response.

7.2 General discussion of findings

Whilst it has been apparent for some time that the transcriptional co-activator PGC-1α plays a critical role in mediating cell signalling pathways associated with mitochondrial biogenesis (Wenz, 2013), more recent work shows post-exercise CWI augments the exercise-induced PGC-1α response (Ihsan et al., 2014a; 2015; Joo et al., 2016). Despite these recent advances in the literature, the precise controlling mechanisms of the cold-induced augmentation in post-exercise PGC-1α gene expression remained to be determined. Upstream control of PGC-1α includes phosphorylation by energy and stress sensing kinases AMPK (Jäger et al., 2007) and p38 MAPK (Akimoto et al., 2005). Importantly, long-term
cold-induced changes in PGC-1α protein content arise in conjunction with increased activity of AMPK and p38 MAPK (Ihsan et al., 2015). Alternatively, β-adrenergic activity is suggested to play a potent role in mediating the effects of cold exposure on PGC-1α expression via AMPK (Manfredi et al., 2013). To date, studies investigating the impact of CWI upon post-exercise gene expression have utilised the non-immersed limb as the control condition without the use of a true control (no cooling) condition (Frohlich et al., 2014; Ihsan et al., 2014a; 2015). By assuming the response to cold-water immersion is mediated locally, such experimental designs do not permit examination of the role of systemic versus localised mechanisms in mediating cold-induced changes in PGC-1α.

Study 1 (Chapter 4) utilised a bi-lateral muscle biopsy design with single-leg CWI in order to establish the localised (cooled limb) and systemic (non-cooled limb) response to post-exercise CWI, versus a complete control. Similar acute PGC-1α gene expression in the immersed and non-immersed limbs, both greater than the control limb, led to the conclusion that the cold-augmentation of the post-exercise PGC-1α response is influenced systemically. Greater plasma normetanephrine concentration during the post-exercise period for the cooled conditions (immersed and non-immersed limbs) alongside an interaction effect noted for the phosphorylation of AMPK, provides support for the hypothesis that β-adrenergic activity plays a potent role in mediating the effects of cold exposure on PGC-1α expression via AMPK (Manfredi et al., 2013). Moreover, that muscle temperature was significantly lower in the immersed vs. non-immersed limb, despite similar augmentation of PGC-1α gene expression, indicates that the cold-induced regulation of this gene is not locally controlled by mechanisms of reduced tissue temperature, but influenced by systemic mechanisms, likely β-adrenergic activation. Importantly, recent work utilising a contralateral control limb design and local cooling of one limb (via ice application) has
concluded this method of cooling does not impact the expression of mitochondrial-related genes (including PGC-1α) (Shute et al., 2017) and the metabolome and transcriptome of human skeletal muscle (Sarver et al., 2017). This lack of difference noted between a cooled and non-cooled limb could well be a factor of the systemic regulation noted in our results herein (Chapter 4), highlighting our earlier point that such study designs do not allow for examination of both the localised and systemic responses with the comparative results being underrepresented where a true control condition is missing.

As a result of establishing that the cold-induced augmentation of the post-exercise PGC-1α gene expression response was systemic in nature investigations progressed to assess whether such a response could be increased to an even greater extent. In this sense, we sought to take advantage of the fact that previous research has shown exercise commencing with low glycogen concentrations enhances the post-exercise PGC-1α response (Psilander et al., 2013; Bartlett et al., 2013). Theoretically, with exercise alone enhancing post-exercise PGC-1α mRNA 3-4 fold as is regularly shown (Slivka et al., 2013; Ihsan et al., 2014a), and post-exercise CWI enhancing this response up to 6-10 fold (Ihsan et al., 2014a; Joo et al., 2016; Allan et al., 2017) we assessed whether post-exercise CWI of limbs that commence exercise with low glycogen concentrations would see a response greater than this.

In utilising a bilateral muscle biopsy design four conditions were established from two experimental visits. Depleting a single limb in the day prior to the experimental test day allowed measurement of high and low glycogen conditions, with and without post-exercise CWI. Whilst high glycogen limbs produced results as expected, with the high glycogen cooled limb producing greater expression of PGC-1α mRNA than the high glycogen control limb (~8 vs. ~6-fold increase from Pre exercise, respectively) the results were not matched
in the low glycogen limbs. Indeed, low glycogen prior to commencing exercise had no beneficial impact on the rate of PGC-1α gene expression, with or without CWI. Thus, data shows a critical level of glycogen depletion exists for the benefits of low glycogen concentration upon the post-exercise PGC-1α mRNA response.

Indeed, that results in the low glycogen limbs of Chapter 5 surpassed such a critical threshold might explain the lack of cold and/or low glycogen induction in PGC-1α gene expression. However, this Chapter failed in its aim to have a high vs. low glycogen limb as rates of repletion in the intended high glycogen limb did not occur as had done in pilot testing. Thus, the data from Chapter 5 are better described as low vs. very low glycogen, rather than low vs. high glycogen limbs. The identification of a critical threshold of glycogen depletion to maintain the associated augmentation of post-exercise PGC-1α after exercise is commenced with very low glycogen concentrations requires further investigation. Whilst it is not bold to suggest such an occurrence, considering this is not the first time such a threshold has been suggested to occur (Pilegaard et al., 2002), it is important to establish the mechanisms by which such a threshold would prevent the expected augmentation of PGC-1α. Alternatively, it is suggested that such low levels of muscle glycogen may influence the efficiency of muscle recruitment and therefore not provide a sufficient stimulus in which to augment PGC-1α gene expression.

One emerging area of research is that PGC-1α gene expression occurs by way of two separate promoter regions; a proximal (canonical, Exon 1a) and an alternative (Exon 1b/c) promoter, with this alternative promoter responsible for the exercise-induced increases of PGC-1α mRNA (Martinez-Redondo et al., 2015; Popov et al., 2015b). In Chapter 4, design of RT-PCR primers for PGC-1α demonstrated that these promoter regions differentially
express PGC-1α mRNA dependent upon the stress applied (i.e. baseline vs. post-exercise). Further analysis of the samples collected from Chapters 4 & 5 would allow determination of the influence of post-exercise CWI and prior low glycogen upon the differential expression from the two promoter regions; and may go some way to explaining the mechanisms involved in such responses. Data from Chapter 6 were able to show PGC-1α mRNA expression from the alternative promoter (Exon 1b) closely follows the pattern of expression for total-PGC-1α gene expression, with similar systemic increases in immersed and non-immersed limbs; suggesting a role for Exon 1b derived PGC-1α in the cold-augmented post-exercise response. Moreover, data confirmed previous reports that show the exercise-induced response is promoter specific, with PGC-1α gene expression occurring via the alternative (Exon 1b) promoter following high-intensity exercise (Martinez-Redondo et al., 2015; Popov et al., 2015b).

Mechanistic studies like those in this thesis often offer great implication for future research design. Researchers wanting to investigate the cold-induced post-exercise responses in skeletal muscle must be aware of the evidence of systemic regulation. In the case of the present thesis, this means the implication that a systemic response of normetanephrine may have upon the local response of p-AMPK and PGC-1α. Future work should look to establish further evidence to support the impact of systemic transcriptional responses in unilateral research designs, as these might be liable to error. As such, utilising a contralateral limb as a control limb may underestimate the actual response occurring in the intervention (immersed/cooled) limb. The choice of scientific design lies with the question posed, as contralateral designs remain useful for understanding both local and systemic responses. Ultimately, it is hoped that the results from Chapter 5 and Chapter 6 stimulate more questions than answers, and allow for further investigation into the mechanisms controlling
this cold-induced augmentation of PGC-1α. Results from Chapter 6 are the first to identify the promoter specific PGC-1α transcriptional response to CWI in either animals or humans. As this is a new and emerging area that can be easily investigated with the careful and correct design of suitable qRT-PCR primers it is suggested that all future work where PGC-1α plays a key role in determining answers to the research question should look to analyse the promoter specific response to different stimuli, in different populations in order to expand knowledge in the area.

7.3 Practical applications

In an applied sense, results from Chapters 4 and 5 offer further support to literature that shows PGC-1α gene expression is augmented to a greater extent than exercise alone, when the exercise is followed by CWI (Ihsan et al., 2013; Joo et al., 2016). Moreover, with much debate around the efficacy of CWI upon the adaptive response, stemming from the work of Roberts et al. (2014) showing post-exercise CWI dampens strength gains and adaptive processes (satellite cell activation) following a programme of resistance training, data from this thesis offers further support to the other side of the current paradox; that the oxidative adaptive response is augmented by post-exercise CWI.

The fact that a greater augmentation of the transcriptional co-activator PGC-1α and phosphorylation of AMPK were seen with exercise followed by CWI, in an immersed and contralateral non-immersed limb, suggests that single legged CWI is an appropriate technique to use for systemic cellular and molecular signal generation important in mitochondrial biogenesis and oxidative adaptation. However, coaches and sports scientists alike should be aware that in doing so they are preventing the athlete from gaining other benefits associated with CWI, such as hydrostatic pressure fluid shifts, reduced
swelling/oedema and reduced sensory neural conductance velocity associated with reduced perception of pain and fatigue.

With the apparent suggestion that a critical level of glycogen availability exists in order to produce an exercise intensity required to promote cellular and molecular activation of the pathways important for oxidative adaptation (AMPK, PGC-1α) coaches, athletes and sports scientists should be clear on the positive and negative implications associated with “train low” strategies. Indeed, if glycogen availability is allowed to reach levels seen in Chapter 5 (~85 mmol·kg⁻¹·dw) there is the potential for impaired contraction kinetics (Ørtenblad et al., 2011) to disrupt the quality of subsequent training sessions and/or performance. Following this it is difficult to suggest any benefits of an athlete combining low glycogen strategies with post-exercise CWI for the purpose of improving recovery and acute adaptive responses. Further work is required to determine critical levels of glycogen depletion that will allow continued exercise intensity and support a post-exercise adaptive signalling response.

Whilst data herein support the use of post-exercise CWI to promote oxidative cellular and molecular adaptive responses in the exercised muscle it is ever more important that applied practitioners understand the surrounding literature in order to successfully apply that knowledge to practice. The use of academic consultants to professional clubs is one way of addressing such issues. Whilst recent evidence shows there are no positive (or negative) implications of post-exercise CWI upon the inflammatory and cellular stress response, CWI may be useful in other ways. If not for the important signalling mechanisms involved in oxidative adaptation noted in this thesis, or to combat post-exercise inflammatory events (Peake et al. 2017b), CWI can still be important for greater functional recovery, improved
subsequent performance, reductions of DOMS and improved perception of recovery; be it via sensory or placebo mechanisms. The focus for any applied practitioner should now be how to suitably periodise and individualise recovery programmes to each athlete with particular attention being paid to their training and competition schedule, environmental conditions and personal preferences.

7.4 Limitations

*Subject characteristics.* The current thesis recruited participants who were physiologically active and free from current injury or illness. However, one thing that was not accounted for was the training status of individual participants that may undoubtedly differ due to the specific nature of their training history (i.e. running, cycling, team sports) and that provides altered adaptive stimuli (i.e. frequency and intensity). As this thesis contained large amount of cell-signalling analysis, with training history potentially impacting upon this (Coffey et al., 2006), future studies should pay particular attention to the inclusion criteria for subject recruitment so as to minimise the effect of training status upon cell signalling responses.

*Translation progression.* Whilst measuring mRNA content is an extremely useful tool in the acute response to exercise and/or external stimuli, increased mRNA content for a given protein may not necessarily translate into increased protein abundance (Baar et al., 2002; Hornberger et al., 2016). Thus, the need for further training studies highlighting the impact of regular use of CWI upon the cellular and molecular responses is required. This would allow for a better assessment of the impact of interventions on the PGC-1α protein, but analysis of other important factors (i.e. mitochondrial enzyme activity, mitochondrial quality and quantity, performance characteristics) would allow assessment upon the impact of adaptation towards a more oxidative phenotype.
Intensity dependency of PGC-1α. As was highlighted in Chapter 5, the lack of expected increases seen for post-exercise phosphorylation of AMPK\textsuperscript{Thr172} and PGC-1α gene expression, particularly in low glycogen limbs may have been a result of poor cycling technique. One explanation may be that the depleted limbs in the low glycogen conditions were unable to produce the same power output as their contralateral high glycogen counterparts, particularly as low glycogen concentrations are linked to diminished sarcoplasmic reticulum function and therefore decreased power output (Ørtenblad et al., 2011). Therefore, to maintain total power output during the cycling protocol utilised herein participants could have preferentially chosen to pedal more with the high-glycogen limb, thereby exposing that limb to a greater exercise intensity and total workload and creating an imbalance in the resulting expression of PGC-1α mRNA. Whilst no visible signs of preferential choice in limb used to turn the pedals were evident during testing procedures, future research should look to study designs that do not allow for this potential imbalance. Running protocols or cycle ergometers with individual power meters on each pedal are simple solutions to prevent such a problem.

Depletion protocols raising absolute baseline levels of mRNA. As discussed in Chapter 6, mRNA data herein are measured as fold-changes to Pre-exercise values. Prior exercise in the form of glycogen depleting protocols (Chapter 5) in the days leading up to the experimental day may have subsequently resulted in higher baseline absolute values and thus smaller fold changes at 3h post exercise. Indeed, this hypothesis is supported by Perry and colleagues (2010) who showed the PGC-1α mRNA response to high-intensity interval training was diminished almost 2-fold on the third day of exercise compared to the first. Ideally, bilateral muscle biopsy studies like the one utilised herein (Chapter 5) should look to match the work done in each limb in the days prior to experimental days. This however,
due to the nature of bilateral study designs, is difficult to ensure particularly where glycogen depletion of a single limb is involved. Future work of a similar design should look to complete four separate condition visits, rather than utilise a bilateral design, ensuring the days prior to the experimental day are matched for work and intensity and with the only difference occurring being that required to alter muscle glycogen concentration (i.e. diet alteration to promote/negate muscle glycogen resynthesis).

7.5 Recommendations for future research

The following points are recommendations for potential future work so as to gain a more complete insight into the controlling mechanisms of the cold-induced augmentation of PGC-1α, particularly with lessons learnt and ideas gained from the investigations in this thesis:

Recommendation 1: The role of β-adrenergic receptors in Normetanephrine induced AMPK phosphorylation.

Results from Chapter 4 suggest control of the post-exercise cooling response in PGC-1α are systemic in nature, potentially via an increase in the β-adrenergic agonist normetanephrine upon AMPK phosphorylation. Future work should look to confirm such a mechanism by assessing the role of β-adrenergic receptors and the downstream cell signalling that potentially leads to the augmentation of PGC-1α.

Recommendation 2: The signalling role of MEF2 and CRE/ATF2 sites to confirm a link between catecholamines and PGC-1α.

Considering that MEF2 and CRE/ATF2 sites are required for PGC-1α transcription (Akimoto et al., 2008) measurement of CREB/ATF2 phosphorylation would allow links to
be made between catecholamine response and PGC-1α transcription, especially considering β-adrenergic stimulation is mediated by activation of p38 MAPK-CREB- ATF2, signalling axis, mediating the increase in PGC-1α transcription (Kim et al., 2013).

Recommendation 3: Establish the impact of very low glycogen concentrations prior to commencing exercise upon the post-exercise and post-exercise plus cooling response. Chapter 5 in this thesis shows data to suggest a critical limit of glycogen depletion may exist for the augmentation benefits associated with commencing exercise with low glycogen to be seen. Future work should look to address this hypothesis particularly as this is not the first time a critical threshold of glycogen for gene expression has been suggested (Pilegaard et al., 2002).

Recommendation 4: The impact of regular acute CWI upon PGC-1α mRNA (total and promoter specific). Future work should look to follow a design similar to that of Perry and colleagues (2010) where gene expression and protein content are measured at regular acute intervals following exercise 4 times over the course of 7 days. This would allow determination of the time course changes in the PGC-1α response to several acute periods of cooling. Gene array analysis would also be useful here, followed up with rt-qRT-PCR, to establish important cell-signalling pathways involved in the longer-term adaptive processes controlled by the so-called master regulator, PGC-1α.

Recommendation 5: The impact of regular CWI on concurrent training adaptations. As previously mentioned, due to the sheer numbers of athletes that partake in training programmes that contain both endurance and resistance type exercises the influence of post-
exercise cooling upon concurrent training will be of great applied interest. Indeed, with the current hypothesis that concurrent training may lead to cell-signalling interference of the resistance side of the coin (Coffey & Hawley, 2017) it would be interesting to see the influence cold water immersion may have upon these cell signalling processes.

7.6 Conclusion

The investigation in Chapter 4 has major implications for future research designs. As discussed in Section 7.2, recent work highlighting a lack of impact of localised cooling on a non-cooled contralateral limb utilised as the control condition (Shute et al., 2017; Sarver et al., 2017) may be a result of systemic regulation producing similar responses in both limbs. Without the comparison with a true control it remains impossible to assess the impact of systemic regulators in the measured variables. These recent studies highlight the importance of findings from Chapter 4. Chapters 4 and 5 offer further support to the already established notion that the post-exercise PGC-1α response is augmented to a greater extent when exercise is followed by CWI. Moreover, Chapter 5 indicates a critical limit exists for glycogen concentration to influence PGC-1α gene expression. The mechanisms controlling this response are suggested to be molecular in nature or as a result of decreased efficiency in muscle fibre recruitment, and remain to be determined. Chapter 6 is the first investigation, in animals and humans, to show the post-exercise cooling response of promoter specific PGC-1α gene expression. Importantly data herein show the cold induced augmentation seen in recent literature (Ihsan et al., 2013; Joo et al., 2017; Allan et al., 2017) is largely a result of alternative (Exon 1b) promoter specific gene expression. Importantly it is suggested that any future investigations assessing the acute PGC-1α response also measures the promoter specific response due to the ease of measurement and impact such data would have on the understanding and controlling mechanisms behind the “master regulator” of mitochondrial
biogenesis, PGC-1α. The results from this thesis not only offer mechanistical insight for future research design, but go some way in supporting previous work that provides evidence of post-exercise CWI positively influencing the signalling pathways associated with mitochondrial biogenesis. Ultimately, this offers confidence for practitioners that CWI is an effective recovery strategy, not only by way of analgesic and pain relieving properties, but via molecular mechanisms linked to an improved oxidative phenotype. The emphasis now should be on the correct periodisation of CWI, and how it is aligned to the individual athlete.
Figure 7.1: Synthesis of findings in relation to physiological mechanisms by which post-exercise CWI is purported to promote recovery. Black text = physiological mechanisms of post-exercise CWI, Red text = molecular and signalling mechanisms influencing PGC-1α gene transcription (addition to knowledge provided by this thesis). Dark boxes = outcome, dashed boxes = key physiological response to cooling. CBV= Central Blood Volume, NCV= Neural Conductance Velocity, Tmus= Muscle temperature, Tskin= Skin temperature, O2= oxygen, AMPK= AMP-activated protein kinase, p38MAPK= p38 mitogen activated protein kinase, PGC-1α= Peroxisome proliferator activated-receptor gamma coactivator -1 alpha.
CHAPTER 8

REFERENCES


Ydfors, M., Fischer, H., Mascher, H., Blomstrand, E., Norrbom, J. & Gustafsson, T. 2013. The truncated splice variants, NT-PGC-1α and PGC-1 α4, increase with both endurance and resistance exercise in human skeletal muscle. *Physiol Rep, 1*(6), e00140.


APPENDIX 1
PARTICIPANT INFORMATION SHEET

Title of Project: The impact of glycogen manipulation on the mechanisms responsible for the benefits of post-exercise cold water immersion (CWI).

Research team: Mr Robert Allan, Dr James Morton, Dr Adam Sharples & Prof. Warren Gregson.
School of Sport and Exercise Sciences, Faculty of Science.

You are being invited to take part in a research study. Before you decide to take part, it is important that you understand why the research is being done and what it involves. Please take time to read the following information. If you are unsure of anything then do not hesitate to ask for more information. Take the time to decide if you want to take part or not.

1. What is the purpose of the study?
   Short and long-term training results in muscle damage, causing inflammation, athlete discomfort and reduced performance. Cold water immersion (CWI) promotes the recovery of soft tissue (i.e. muscle) injury by reducing the inflammatory response, swelling and pain in injured tissue. It has additionally been shown able to maintain, and even improve, performance short-term. Post-exercise CWI can also increase the endurance adaptive response to exercise to a greater extent than exercise alone. We are now interested in seeing whether this amplification of the adaptive response by CWI can be extended further by altering carbohydrate status.

2. Do I have to take part?
   No, it is voluntary and entirely your own choice. If you do decide to take part you will be given this information sheet and asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any stage throughout the testing period, for any reason, will not affect your rights/any future treatment/service you receive.

3. Am I eligible to take part?
   • To be eligible to take part in this study you must meet the following criteria:
   • Male, aged 18-40.
   • Physically active and free from injury or illness (engaging in some form of physical training).
   • Not suffering from / no history of Raynaud’s syndrome (excessively reduced blood flow in response to cold or emotional stress).
   • No serious food allergies e.g. to nuts

4. What will happen to me if I take part?
   You shall be required to attend the Tom Reilly Physiology laboratories 9 times in total. The first 3 visits shall be to complete a two-legged test to assess your maximum oxygen consumption and peak power output. The test shall commence at a power output of 2Watts per 1kg of your body mass, increasing by 50Watts after the first 150s and 25Watts every 150s thereafter. One of these 3 initial visits shall include familiarisation to the water immersion protocol by immersing you into 8°C water via an electronic hoist for 10 minutes. Additionally, these visits will provide you with a
chance to witness any unfamiliar invasive procedures (such as bloodletting, muscle temperature and rectal temperature measurement) and allow time for open discussions around anything to do with the project. The third visit includes a medical screen by a Doctor.

The next 6 visits shall take place on 3 consecutive days of two separate weeks (Control and Cold-Water Immersion conditions separated by a minimum of 10 days).

**Day 1 and 4:** You will arrive on Monday evening (for example purposes; days may change due to availability) and complete a two-legged cycling protocol with the aim of depleting glycogen stores in the legs. After a 5-min warm-up at 100 Watts, you shall cycle for 2 min at 90% of your predetermined peak power, followed by a 2-min recovery period at 50%. This work to rest ratio is maintained until you can no longer complete 2 min cycling at 90%. At this point the high-intensity bout will be lowered to 80% before being lowered further 70% and finally 60% as and when the target of 60 rev.min\(^{-1}\) cannot be met. When you are unable to cycle for 2 min at 60% the exercise protocol shall be terminated. That evening you shall receive a meal plan for a high CHO meal before returning to the lab the next morning after an overnight fast.

**Day 2 and 5:** The next morning you shall receive a high CHO meal plan (~8g·kg\(^{-1}\) body mass of CHO) for the day, before undergoing a single-leg depletion protocol that evening. Single-leg glycogen depletion involves 20 minutes continuous single-leg cycling at 75% of your predetermined ‘one-leg maximal oxygen consumption’, followed by intermittent cycling (90s at 90% : 90s rest) decreasing in 5% decrements to 55%. Immediately after this you shall complete an all-out one legged cycling bout at 85% one-leg maximal oxygen consumption before going on to 30 minutes of 2-arm cycling. You shall then undergo an overnight fast before returning to the lab the next day.

**Day 3 and 6; Test day:** you will complete a high-intensity endurance cycling protocol (8 x 5 minutes at 85% of your maximal endurance capacity, separated by 60s rest). During exercise your heart rate will be measured via a loose fitting band strapped around your chest, expired air shall be assessed via a small facemask at regular intervals. Following completion of the exercise protocol you will undergo two-legged cold water immersion (8°C) for 10 minutes (CWI condition) or sit on a chair at room temperature for 10min (Control condition). On both occasions you will then remain seated at room temperature until 3h post-exercise. Muscle temperature will be monitored immediately after exercise and CWI as well as at 1 and 3 hours post-exercise by inserting a needle to a depth of 3-4cm into the upper thigh. Rectal and skin temperature will also be monitored at various points throughout the day. Skin temperature will be assessed by taping small metal thermistors to different regions of the body (thigh and calf) prior to exercise. Blood samples will be drawn from the forearm pre-exercise, immediately post-exercise, post-CWI and at 1, 2, and 3 hours after completing the cycling protocol. Blood samples will be taken by a fully certified and experienced phlebotomist. A muscle biopsy will be obtained from both the thighs pre-, post-exercise and at 3 hours post-exercise. The total number of biopsies over the course of the study will be 12.

Please note that on completion of the research we may wish to keep both blood and muscle tissue for future research in the area of sport and exercise. This tissue will be treated in the strictest confidence with no association made between your identity and the data observed.

**Blood plasma shall be frozen and stored for later analysis of markers of muscle metabolism and catecholamines. Muscle samples are immediately frozen in liquid nitrogen for later analysis of**
markers of mitochondrial adaptation. For more information please feel free to contact Robert Allan on the details at the bottom of this form.

5. Are there any benefits / risks involved?
There are some invasive procedures involved that may cause slight discomfort, a brief outline of which is highlighted below:

**CWI;** upon immersion (to waist height) you may experience what is known as a “cold-shock” response. This involves short, sharp fast breathing whilst you experience numbness and a “pins and needles” sensation during acclimation to the temperature, normally lasting 2-3 minutes.

**Muscle Temperature;** you will experience a small amount of sharp pain that will disappear quickly, similar to that of a needle injection. Upon removal of the needle there may be a small amount of bleeding; this is normal and usually minimal.

**Rectal Temperature;** measured by inserting a rectal probe 15 cm beyond the external anal sphincter prior to the exercise insult to provide an indication of your internal body temperature throughout the day. This may initially result in you feeling some discomfort which will disappear as you progress with the test.

**Bloodletting;** Cannulation, a small plastic tube inserted into the vein of the arm to allow for multiple blood sampling and thereby reducing the need for repeated needle insertions, shall be performed at the start of the day and removed at the end. This is a routine procedure and should result in no more than a small amount of discomfort on insertion of the needle.

**Muscle Biopsies;** will be fully explained prior to participating in the study and will be conducted by a fully qualified member of staff. A clinical assessment by a medic shall be completed prior to commencing any biopsies. Further information on the procedure is included in the accompanying biopsy information sheet. Should you have any concerns after your biopsy we can arrange for assessment by a medic.

**Benefits**
The study offers no direct benefits for your participation. However, taking part in this trial means that you are contributing to important research. Without such volunteers, research such as this and many other important studies would not be possible. The researcher team is comprised of experienced exercise physiologists and sports nutritionists, who will be on hand to answer any questions and/or offer information regarding training and diet habits and an insight into the research setting if this is of interest to you.

6. Will my taking part in the study be kept confidential?
In the study, any data collected from you is fully anonymised. Data and tissue samples may be used in further investigations to develop current or future research with your consent. Any data and samples not needed following completion of the study will be destroyed. Your confidentiality is of utmost importance and thus will not be compromised at any point.

**This study has received ethical approval from LJMU's Research Ethics Committee**  
REC Reference Number  **15/SPS/030**  
Date: 31-07-2015

**Contact Details of Researcher-Principal Investigator**
Robert Allan
Football Exchange / Muscle Metabolism Research Group
Liverpool John Moores University
Contact Details of Research-Supervisor

Prof. Warren Gregson
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Tom Reilly Building, Byrom Street, L33 AF
W.Gregson@ljmu.ac.uk
APPENDIX 2

Participant Consent Form and PAR-Q

Title of project
The impact of carbohydrate (CHO) manipulation on the systemic response of post-exercise cold-water immersion (CWI) on markers of mitochondrial biogenesis.

Name of Researchers and School/Faculty
Mr. Robert Allan, Dr James Morton, Dr Adam Sharples, Dr Warren Gregson
The Research Institute for Sport and Exercise Sciences

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights.

3. I understand that any personal information collected during the study will be anonymised and remain confidential

4. I agree to take part in the above study

5. I consent to the removal and storage of tissue samples which will be collected during the study

6. I agree to the storage of my tissue samples for future research purposes

Name of Participant            Date            Signature

Name of Researcher            Date            Signature
Non-Validated Questionnaires (PARQ, Muscle Biopsy, Cryotherapy)

Physical Activity Readiness Questionnaire (PAR-Q)

For most people, physical activity should not pose any problem or hazard. The PAR-Q is designed to identify the small number of adults for whom physical activity might be inappropriate or those who should seek medical advice concerning the type of activity most suitable for them.

<table>
<thead>
<tr>
<th>Question</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Do you have a bone or joint problem such as arthritis, which has been aggravated by exercise or might be made worse with exercise?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>2. To your knowledge, do you have high blood pressure?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>3. To your knowledge, do you have low blood pressure?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>4. Do you have Diabetes mellitus or any other metabolic disorder?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>5. Has your doctor ever said that you have raised cholesterol (serum level above 6.2mmol/L)?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>6. Do you have or ever suffered a heart condition?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>7. Have you ever felt pain in your chest when you do physical exercise?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>8. Is your doctor currently prescribing you drugs or medication?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>9. Have you ever suffered from shortness of breath at rest or with mild exercise?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>10. Is there any history of Coronary Heart Disease within your family?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>11. Do you ever feel feint, have spells of dizziness or have ever lost consciousness?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>12. Do you currently drink more than the average amount of alcohol per week (21 units for men and 14 units for women (1 unit = ½ pint of beer/cider/larger or 1 small glass of wine))</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>13. Do you currently smoke?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>14. Do you NOT currently exercise regularly (at least 3 times per week) and/or work in a job that is physically demanding.</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>
15. Are you, or is there any possibility that you might be pregnant?  
   YES                                      NO

16. Do you know of any other reason why you should not participate in a programme of physical activity?  
   YES                                      NO

If you have answered YES to any of the above please give details:……………………......................................…………………………………………………
   …
   …
   …

…. If you answered YES to one or more questions:  
If you have not already done so, consult with your doctor by telephone or in person before increasing your physical activity and/ or taking a fitness appraisal. Inform your doctor of the questions that you answered ‘yes’ to on the PAR-Q or present your PAR-Q copy. After medical evaluation, seek advice from your doctor as to your suitability for:  
1. Unrestricted physical activity starting off easily and progressing gradually, and …  2. Restricted or supervised activity to meet your specific needs, at least on an initial basis.

If you answered NO to all questions:  
If you answered the PAR-Q honestly and accurately, you have reasonable assurance of your present suitability for:  

Assumption of Risk  
I hereby state that I have read, understood and answered honestly the questions above. I also state that I wish to participate in activities, which may include aerobic exercise, resistance exercise and stretching. I realise that my participation in these activities involve the risk of injury and even the possibility of death. I hereby confirm that I am voluntarily engaging in an acceptable level of exercise, which has been recommended to me.

<table>
<thead>
<tr>
<th>Name (Print):</th>
<th>Investigator’s Name:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject’s Signature:</td>
<td>Signature:</td>
</tr>
<tr>
<td>Date:</td>
<td>Date:</td>
</tr>
</tbody>
</table>
APPENDIX 3
Liverpool John Moores University Muscle Biopsy
Information Sheet

The muscle biopsy technique is a commonly employed technique within the exercise sciences. It is usually employed in studies that are examining the structure, metabolic and cellular state of a muscle in response to acute and chronic bouts of exercise. The following information is designed to inform all individuals interested in participating in a muscle biopsy study of the specific procedures and their associated risks and discomfort. Muscle biopsies are always carried out by fully qualified and experienced medical doctors.

The procedure of a muscle biopsy and possible associated discomfort

The muscle biopsy involves the removal of a small piece of muscle tissue from one of the muscles in your leg using a sterile hollow needle. The area over the outside of your lower thigh muscle (vastus lateralis muscle) and/or (calf) gastrocnemius muscle will be carefully cleaned. A small amount of local freezing (anesthetic) will be injected into and under the skin. You will likely experience a burning sensation while the freezing is injected, but this will only last momentarily. Then a small, 4 – 5 mm incision will be made in your skin in order to create an opening for the biopsy needle. There is often a small amount of bleeding from the incision, but this is usually minimal. The biopsy needle will then be inserted through the incision into the thigh/calf muscle and a small piece of muscle (20 – 50 mg) will be quickly removed and the needle taken out. During the time that the sample is being taken (about 5 seconds), you may feel the sensation of deep pressure in the muscle, and on some occasions this is moderately painful. However, the discomfort very quickly passes and you are quite capable of performing exercise and daily activities. There may be some minimal bleeding when the needle is removed which may require application of pressure for a few minutes. Following the biopsy, the incision will be closed with sterile tape (steri-strips), and wrapped with a tensor bandage. Once the freezing wears off, your leg may feel tight and often there is the sensation of a deep bruise.

What to do following a muscle biopsy

After the procedure, you may feel some mild discomfort and possibly see some bruising. This often feels like the sensation of a ‘dead leg’ and your leg may feel discomfort when walking down the stairs etc. This is perfectly normal and should not cause you any undue concern. The tightness in the muscle usually disappears within 2 days. Seven to ten days after the biopsy you will be asked to visit the doctor who performed the biopsy at the Research Institute for Sport and Exercise Sciences for a formal assessment of how the biopsy site is healing.

Potential risks associated with muscle biopsies

The local freezing will likely result in a burning feeling in the muscle at the time of the injection. This will last only 5 – 10 seconds. There is an extremely low risk of allergic reaction to the local injection (1 in 1 million). The chance of a local skin infection in less than 1 in 1000. Carefully cleaning the skin and keeping the area clean until the skin heals will minimize this. Most subjects experience local soreness and stiffness in the leg for two or three days after the biopsy similar to a deep bruise. There is a very low risk of internal bleeding at the biopsy site which can result in more prolonged pain and stiffness in the leg. On occasions, a small lump of scar tissue may form under the site of the incision, but this normally disappears within 2-3 months, or within a few weeks if massaged. A small visible scar often remains from the biopsy incision. There is the possibility of a small area of numbness (about the size of a two pence piece) around the biopsy site. This usually resolves over 5 – 6 months. There is a very low risk (estimated at less than 1/5000) of damage to a small nerve branch to the muscle. This would result in partial weakness of the muscle and would likely have no impact on day-to-day activities. Nerve injuries like this usually resolve in 8 – 12 months, but there is a theoretical risk of mild leg weakness.
Problems or concerns
Infection can be serious and if you therefore experience a lot of bleeding from the biopsy site, swelling or infection around the biopsy site, faintness, light headedness, heart pain, chest pain or increasing pain in your leg which is not relieved by Paracetamol, you must contact the doctor who did the biopsy right away. However, if for some reason, you are not able to contact this physician then you should contact your family doctor or go to the Accident and Emergency Department.

MUSCLE BIOPSY SUBJECT SCREENING FORM
Research Institute for Sport and Exercise Science Liverpool John Moores University.
Tom Reilly Building
Byrom Street Campus
L3 3AF

To help us ensure your safety and well-being please answer the following questions.
1. Have you ever had a negative or allergic reaction to local freezing (e.g. during dental procedures)?
   No □ Yes □

2. Do you have any tendency toward easy bleeding or bruising (e.g. with minor cuts or shaving)?
   No □ Yes □

3. Are you currently taking any medication
   No □ Yes □

4. Have you ever fainted or do you have a tendency to faint when undergoing or watching medical procedures?
   No □ Yes □

5. Will you contact the physician who did the biopsy directly if you have any concerns about the biopsy site including: excessive redness, swelling, infection, pain or stiffness of the leg?
   No □ Yes □

6. Are you willing to visit the physician who did the biopsy 7 – 10 days following the biopsy for an assessment of the biopsy site? No □ Yes □

7. Are you willing to allow samples collected from you to be used for analysis of mRNA abundance and protein content for the purpose of the research study outlined in the Subject Information sheet and future research of the same interest? No □ Yes □

Subject Name (print): ____________________________
Subject Signature: ________________________________
Date: ________________

Signature of Person Conducting Assessment: ________________________
CRYOTHERAPY SCREENING FORM
Tom Reilly Building
Liverpool John Moores University

To help us ensure your safety and well-being, please answer the following questions.

1. Have you ever suffered from any of the following cold related conditions?
   - Raynaud’s disease
   - Vasospastic diseases (blood vessels don’t dilate properly)
   - Cold hypersensitivity
   - Compromised local circulation
   
   No [ ] Yes [ ]

2. Have you ever had a negative or allergic reaction to cryotherapy (cold) exposure?
   
   No [ ] Yes [ ]

3. Has your doctor ever said you have a heart condition?
   
   No [ ] Yes [ ]

4. Do you currently have or ever been diagnosed with high blood pressure?
   
   No [ ] Yes [ ]

5. Have you been diagnosed with a rheumatoid condition?
   
   No [ ] Yes [ ]

Subject name (print): ______________________________
Subject signature: ______________________________
Date: ______________
Signature of person conducting assessment: ____________________
APPENDIX 4

Post biopsy treatment guidelines

After the procedure, you may feel some mild discomfort and possibly see some bruising. The biopsy site will often feel like the sensation of a ‘dead leg’ and your leg may feel discomfort when walking down the stairs etc. This is perfectly normal and should not cause you any undue concern. The tightness in the muscle will usually disappear within 2 days.

Please refer to the following advice in order to limit the possibility of infecting the wound and speeding up the repair process.

Key points:
- Try to keep the wound clean at all times. The most serious risk to you is infection.
- Do not take the sterile plaster off for 2 days (48h).
- If the plaster falls off in the first 48h please replace it with one of the spares we provided.
- After 48h do not re-apply a plaster to the site.
- Replace the steri-strips if they fall off until the wound has healed. If you are unsure about this please contact the research team and we can do this for you.
- DO NOT SWIM OR HAVE A BATH FOR AT LEAST 4 DAYS AFTER THE BIOPSY.
- You may shower but try to keep the plaster or steri-strip dry.
- Do not use soap or abrasive materials over the wounds.
- Do not exercise for 48h following the biopsy (unless the study protocol requires this)
- Regularly apply ice for short periods (20 minutes) for the first 48 hours after the biopsy.
- Bandaging of the leg is at your discretion. It will help reduce soreness but can be uncomfortable to wear.
- You can take paracetamol or another pain killer

Infection can be serious; therefore, if you experience a lot of bleeding from the biopsy site, swelling or infection around the biopsy site, faintness, light headedness, heart pain, chest pain or increasing pain in your leg which is not relieved by Paracetamol, you must contact the study team who will put you in touch with the study medic. However, if for some reason, you are not able to the study team then you should contact your GP.

Study team Contact Details: _____________
## APPENDIX 5

### DEPLETION/REPLETION MEAL PLAN

<table>
<thead>
<tr>
<th>Time</th>
<th>Meal</th>
<th>Description</th>
<th>CHO</th>
<th>PRO</th>
<th>FAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>6pm</td>
<td>PM MEAL</td>
<td>380 ml Lucozade original</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 x jaffa cakes</td>
<td>34.4</td>
<td>2.4</td>
<td>4</td>
</tr>
<tr>
<td>7pm</td>
<td></td>
<td>coco pops 100g</td>
<td>85</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200ml whole milk</td>
<td>9.4</td>
<td>6.5</td>
<td>7.2</td>
</tr>
<tr>
<td>8pm</td>
<td></td>
<td>Hartleys strawberry jam (15g) x2</td>
<td>18.2</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 white bread</td>
<td>83.2</td>
<td>18.8</td>
<td>3.6</td>
</tr>
<tr>
<td>9pm</td>
<td></td>
<td>GO Energy drink (500ml)</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>One banana</td>
<td>23</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>364.8</td>
<td>34.5</td>
<td>17.3</td>
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<table>
<thead>
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<th>Description</th>
<th>CHO</th>
<th>PRO</th>
<th>FAT</th>
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<tr>
<td>7am</td>
<td></td>
<td>coco pops 100g</td>
<td>85</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200ml whole milk</td>
<td>9.4</td>
<td>6.5</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 x white toast (warbutons white toastie) ~ 95g</td>
<td>83.2</td>
<td>18.8</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hartleys strawberry jam (15g) x2</td>
<td>18.2</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>8am</td>
<td></td>
<td>380ml lucozade original</td>
<td>64.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10am</td>
<td>Snack 1</td>
<td>Yeo valy yoghurt (150g)</td>
<td>10</td>
<td>7</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tin peaches drained (250g)</td>
<td>27.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO Energy drink (500ml)</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1pm</td>
<td>meal2</td>
<td>Tesco chicken breast 100g</td>
<td>0</td>
<td>23.5</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tilda white basmati (250g pack)</td>
<td>70.4</td>
<td>7.2</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tesco broccoli 50g</td>
<td>0.9</td>
<td>2.2</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO Energy drink (500ml)</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2pm</td>
<td>snack2</td>
<td>GO Energy drink (500ml)</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3pm</td>
<td>meal3</td>
<td>Tesco chicken breast 100g</td>
<td>0</td>
<td>23.5</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tilda white basmati (250g pack)</td>
<td>70.4</td>
<td>7.2</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tesco broccoli 50g</td>
<td>0.9</td>
<td>2.2</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO Energy drink (500ml)</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5/6pm</td>
<td>depletion</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
APPENDIX 6

PARTICIPANTS WANTED!
Are you a keen cyclist?
Are you interested in optimising your post-exercise recovery?
The Research Institute of Sport and Exercise Sciences are looking to investigate the mechanisms responsible for the benefit of Cold Water Immersion / Ice baths!
To be eligible for this study, you must meet the following criteria;
- Male, aged 18-40
- Physically fit and free from injury or illness
- Undertake regular exercise in the form of cycling

The research involves 3 visits to the Tom Reilly Building Laboratories, Liverpool, two of which will contain Cold Water Immersion (8°C) and muscle biopsies.

For more information on the study aims and procedures please do not hesitate to contact us on:
R.J.Allan@2013.ljmu.ac.uk  Robert Allan, PhD Student
The acute cold-induced increase in PGC-1α is mediated systemically through increased β-adrenergic stimulation.


INTRODUCTION

Post-exercise cold water immersion (CWI) increases the PGC-1α mRNA response to exercise (Ihsan et al. 2014). The molecular mechanisms mediating this response are yet to be fully elucidated but may involve upregulation of the local signalling kinases AMPK and p38 MAPK (Ihsan et al. 2015) and/or increased systemic β-adrenergic stimulation (Puigserver et al. 1998; Ihsan et al. 2014). Using a single leg immersion protocol our aim therefore was to examine whether the cold-induced post exercise increase in skeletal muscle PGC-1α mRNA is locally and/or systematically mediated.

METHODS

In a repeated measures cross-over design 10 active subjects completed high-intensity intermittent cycling (8 x 5 minute bouts separated by 60 sec rest, ~80% peak power output) followed by seated rest (CON) or single-leg cold water immersion (CWI; 10 min, 8°C). Muscle biopsies of the immersed (CWI) and CON legs were taken pre, post- and 3h post-exercise. In the non-immersed leg (NOT) biopsies were taken post- and 3h post-exercise. qRT-PCR and Western Blots were performed to assess gene expression (mRNA) and signalling kinase activity respectively. Plasma normetanephrine concentration was used as a measure of the systemic β-adrenergic response. Two factor (condition × time) within-subjects GLM with repeated measures determined treatment differences. LSD pairwise comparisons was used to locate the difference.

RESULTS

Exercise per se induced increases in PGC-1α mRNA 3h post-exercise (~5-fold; P < 0.001). These changes were further elevated following CWI (~9-fold; P < 0.001) and were similar in both CWI and NOT (~12-fold; P < 0.05). Plasma normetanephrine concentration was higher in CWI vs. CON (P = 0.034) (860 vs. 665 pmol·L^{-1} post-immersion).

DISCUSSION

We report for the first time that local cooling of the immersed limb evokes transcriptional control of PGC1-α in the non-immersed limb. This suggests that increased systemic β-adrenergic stimulation, and not the effects of localised cooling on skeletal muscle signalling kinases, mediates the post-exercise increases the PGC-1α mRNA. Results from the present study have important implications for research designs which adopt contralateral non-immersed limbs as a control condition, whilst presenting new directions for mechanistic research into cold-induced PGC-1α responses.

REFERENCES


CONTACT
R.J.Allan@2013.ljmu.ac.uk

2747 characters
APPENDIX 8

Melt curve analysis presented single reproducible peaks for each target gene suggesting amplification of a single product.

GAPDH

RPL13a

Total-PGC-1α
Alternative promoter EXON 1b

Canonical Promoter EXON 1a

p53
GLUT4