The Influence of Cold-Water Immersion on the Adaptive Response to High-Intensity Interval Training in Human Skeletal Muscle

James R. Broatch

Bachelor of Science (Health and Sports Science)

Bachelor of Applied Science (Honours) in Human Movement

College of Sport and Exercise Science

Institute of Sport, Exercise & Active Living (ISEAL)

Victoria University

Submitted in fulfilment of the requirements of the degree

Doctor of Philosophy

March 2015

Abstract

Despite a lack of understanding of the underlying mechanisms, cold-water immersion (CWI) is extensively used by athletes for recovery. Previous evidence demonstrates its effectiveness in reducing muscle soreness, with the effects on muscle function unclear (260). Given the subjective nature of soreness, the efficacy of post-exercise CWI may be confounded by a potential placebo effect. Debate also exists surrounding the merit of CWI in athletic training regimes. While better recovery may improve subsequent training quality and stimulus (490), there is suggestion that CWI may attenuate long-term skeletal muscle adaptations (523). Conversely, CWI may stimulate the expression of genes key to mitochondrial biogenesis (192). To fully understand the mechanisms underlying CWI, and its influence on athletic performance, it is crucial to investigate these issues further.

This thesis firstly aimed to investigate if the placebo effect is responsible for any short-term performance or psychological benefits following post-exercise CWI. To assess this, CWI was compared with a placebo and thermo-neutral control condition in the recovery from a single bout of high-intensity interval training (HIT). A recovery placebo was shown to be superior in the recovery of muscle strength over 48 h as compared with a control, and as effective as CWI, attributed to improved psychological ratings of well-being. This suggests that the placebo effect may account for some of the observed benefits following CWI, or alternately, that it is as strong as the commonly-hypothesised physiological benefits.

For the remaining studies, this thesis aimed to investigate the underlying molecular mechanisms by which CWI may alter cellular signalling and the long-term adaptive response to HIT in human skeletal muscle. It was demonstrated that CWI augments the post-exercise response of a number of signalling proteins and genes associated with mitochondrial adaptations. The oxidative stress imposed by CWI may serve to augment p53 activation post-exercise, leading to a greater up-regulation of its downstream targets. However, despite these alterations in cellular signalling, regular post-exercise CWI did not promote an improved adaptive response to HIT, as measured by markers of mitochondrial biogenesis and other aerobic adaptations.

Student Declaration

"I, James R. Broatch, declare that the PhD thesis entitled "The Influence of Cold-Water Immersion on the Adaptive Response to High-Intensity Interval Training in Human Skeletal Muscle" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature: Date:

Acknowledgements

I would firstly like to extend my sincere thanks and appreciation to my principal supervisor Prof. David Bishop. In spite of your many other commitments you have provided continual support, encouragement, insight and guidance throughout my research and in the completion of this thesis. Thanks must also go out to my co-supervisor Dr. Aaron Petersen for his continual support during the past four years. I cannot thank both of you more for your tireless efforts, and I look forward to working with you in the future.

To my amazing family, especially Mum, Caroline, Claire, and Tony. Words cannot express how much support and encouragement you have shown me throughout this journey. I am indebted to you all. Your belief and confidence in me has meant more than you will ever know, and makes all the hard work worthwhile.

Expressions of gratitude are extended to those who have read and reviewed this thesis, and to those who have assisted with the data analysis and project design. Your time, support and expertise have had a huge impact on my ability to complete this thesis on time, and without your critiques and assistance it would not be what it is. In particular, thanks are extended to Dr. Shona Halson, Dr. Xu Yan, Dr. Jujiao Kuang, Dr. Lauren Banting, Prof. Remco Polman, Dr. Chris Shaw and Dr Jon. Bartlett.

To my fellow VU PhD students and support staff at VU. Your contribution and assistance to my study and thesis was invaluable, and I only hope I was as of much assistance to you as you were all to me. In particular, I would like to thank Cian McGinley, Rodrigo Oliveira, Chris Hedges, Cesare Granata, Karen Hill, Jackson Fyfe, Brad Gatt and Jess Meilak. Your continual feedback and support contributed significantly the quality of my work, and my quality of life!

To the subjects who participated in this study, your time and participation has made this project possible. Finally, thanks must go to Exercise and Sports Science Australia (ESSA) for funding part of this project.

List of Publications, Awards and Grants:

Publications:

- **Broatch, J. R.,** Petersen, A., Bishop, D. J. (2014). Postexercise cold-water immersion benefits are not greater than the placebo effect. *Medicine and Science in Sports and Exercise.* 46(11), 2139-47.
- **Broatch, J. R.,** Petersen, A., Bishop, D. J. (2015). Post-exercise cold-water immersion activates PHF20 and p53 signalling in human skeletal muscle. *American Journal of Physiology* (to be submitted).
- **Broatch, J. R**., Petersen, A., Bishop, D. J. (2015). Regular cold-water immersion following high-intensity interval training does not hinder the adaptive response in human skeletal muscle. *Journal of Applied Physiology* (to be submitted).

Awards:

- Young Investigator Award Runner-up - *Exercise and Sports Science Australia Conference*, Adelaide, Australia 2014

Grants:

- Exercise and Sports Science Australia Research Grant (2011) - $10,000.

Conference Presentations:

- **Broatch, J** – Postexercise cold-water immersion benefits are not greater than the placebo effect. Oral presentation given at: *6th Exercise & Sports Science Australia Conference.* Apr 10th-12th, 2014. Adelaide, Australia.
- **Broatch, J** – Post-exercise cold-water immersion activates PHF20 and p53 signalling in human skeletal muscle. *Australian Physiological Society Conference.* Nov 30th – Dec 3rd, 2014. Brisbane, Australia.
- **Broatch, J** – Regular cold-water immersion following high-intensity interval training does not hinder the adaptive response in human skeletal muscle. *European College of Sports Science Conference.* Jun 24th-27th, 2015. Malmö, Sweden.

Table of Contents

Abstract 2

Student Declaration 3

Acknowledgements 4

List of Publications, Awards and Grants: 5

List of Figures 9

List of Tables 11

List of Abbreviations 12

CHAPTER ONE: Literature Review 15

1.1 Introduction 16

1.2 Cold-Water Immersion 17

1.2.1 Background 17

1.2.2 Proposed Mechanisms of Action 19

1.2.2.1 Hydrostatic Pressure 19

1.2.2.2 Cold Temperature Effects 20

1.2.3 Application and Current Guidelines 21

1.2.3.1 Temperature 22

1.2.3.2 Duration 23

1.2.3.3 Summary 24

1.2.4 CWI Effects on Recovery 24

1.2.4.1 Performance 25

1.2.4.2 Markers of Exercise-Induced Muscle Damage 27

1.2.4.3 Psychological/Placebo Effect 29

1.2.4.4 Summary of CWI and Recovery 30

1.2.5 CWI and Adaptations to Training 30

1.3 High-Intensity Interval Training 32

1.3.1 Physiological Remodelling after HIT 33

1.3.2 Mitochondrial Biogenesis 33

1.3.2.1 Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha 35

1.3.2.2 Adenosine Monophosphate-Dependent Protein Kinase 40

1.3.2.3 Reactive Oxygen Species 43
1.3.2.4 Mitogen-Activated Protein Kinases 45
1.3.2.5 Tumour Suppressor Protein p53 50
1.4 Potential Influence of Cold on Training Adaptations 54
1.4.1 Cold-Stimulated Thermogenesis and Mitochondrial Biogenesis 54
1.4.1.1 Shivering Thermogenesis 55
1.4.1.2 Non-Shivering (Adaptive) Thermogenesis 58
1.4.2 Thermal Shock Response 60
1.4.2.1 Cold Shock 61
1.4.2.2 Rewarming 62
1.4.2.3 Cell Cycle Arrest, DNA Damage and p53 64
1.4.3 Cold-Induced Oxidative Stress 66
1.5 Potential Role for Post-Exercise CWI 67
1.5.1 Cell Signalling 67
1.5.1.1 Adaptive Thermogenesis 67
1.5.1.2 Oxidative Stress 70
1.5.1.3 Thermal Shock 72
1.5.2 Training Adaptations 74
1.5.2.1 Training Quality 74
1.5.2.2 Molecular Markers 75
1.6 Summary 76
1.7 Overall Aims 77
CHAPTER TWO: Cold-Water Immersion and the Placebo Effect 78
2.1 Introduction 79
2.2 Methods 80
2.3 Results 87
2.4 Discussion 92
CHAPTER THREE: The Influence of Cold-Water Immersion on the Molecular Responses Following a Single Bout of High-Intensity Interval Training 98
3.1 Introduction 99
3.2 Methods 101
3.3 Results 106
3.4 Discussion 112

CHAPTER FOUR: The Effects of Regular Post-Exercise Cold-Water Immersion on the Adaptive Response to High-Intensity Interval Training 119

4.1 Introduction 120

4.2 Methods 122

4.3 Results 130

4.4 Discussion 136

CHAPTER FIVE: Thesis Summary 142

Summary of Key Findings 143

Practical Applications 148

Limitations and Considerations 149

Future Directions 149

CHATER SIX: References 153

APPENDICES 187

List of Figures

Chapter One:

Figure 1-1: Physiological response to training, recovery, and supercompensation... 18

Figure 1-2: Intramuscular temperature drop (pilot data, n = 5) comparing cold-water immersion temperatures of 10 and 14°C..... 23

Figure 1-3: Exercise factors involved in altering PGC-1α activity..... 37

Figure 1-4: Potential intracellular signalling mechanisms involved in high-intensity interval training-induced mitochondrial biogenesis..... 40

Figure 1-5: Potential signalling pathways by which exercise may influence p53 signalling..... 53

Figure 1-6: Potential molecular mechanisms by which post-exercise cold-water immersion may improve mitochondrial biogenesis..... 68

Figure 1-7: PGC-1α mRNA prior to exercise, following 15 min of cold-water immersion at 10°C and 3 h post-exercise..... 69

Chapter Two:

Figure 2-1: Schematic representation of the experimental design..... 81

Figure 2-2: Muscle temperature (4 cm into the *vastus lateralis* muscle) during the exercise and 15-min recovery protocols..... 88

Figure 2-3: Percent changes (from baseline) in maximal voluntary isometric contraction peak torque, mean torque, and rate of force development..... 90

Figure 2-4: Blood markers at baseline, post-exercise, post-recovery, and 1, 24 and 48 hours post-exercise..... 92

Chapter Three:

Figure 3-1: Representative western blots from muscle sampled pre-exercise (Pre), immediately post-recovery (Post), and 3 h post recovery (3 h) for control (CON) and cold-water immersion (CWI) conditions..... 107

Figure 3-2: Phosphorylation of p38 MAPK$^{Thr180/Tyr182}$ and AMPK^{Thr172} from muscle sampled pre-exercise, post-recovery, and 3 h post recovery for control and cold-water immersion conditions..... 107

Figure 3-3: Total PHF20 protein and phosphorylation of p53^{Ser15} from muscle sampled pre-exercise, post-recovery, and 3 h post recovery for control and cold-water immersion conditions..... 108

Figure 3-4: Gene expression (NRF1, NRF2, Tfam and PGC-1α) from muscle sampled 3 h post-recovery for control and cold-water immersion conditions. 109

Figure 3-5: Gene expression (Drp1, Mfn2, SCO2 and AIF) from muscle sampled 3 h post-recovery for control and cold-water immersion conditions. 110

Figure 3-6: Gene expression (GPX-1, MnSOD and catalase) from muscle sampled 3 h post recovery for control and cold-water immersion conditions. 111

Figure 3-7: Gene expression (CIRP and RBM3) from muscle sampled 3 h post-recovery for control and cold-water immersion conditions. 111

Chapter Four:

Figure 4-1: Schematic Diagram of the Training Study 123

Figure 4-2: Total HSP27 and HSP70 content before and after six weeks of high-intensity interval training for the control and cold-water immersion (CWI) conditions. 131

Figure 4-3: Total PGC-1α, PHF20 and p53 protein content before and after six weeks of high-intensity interval training for the control and cold-water immersion conditions.. 132

Figure 4-4: Representative western blots before and after six weeks of high-intensity interval training for the control and cold-water immersion conditions..... 132

Figure 4-5: Maximal activity of the mitochondrial enzyme citrate synthase before and after six weeks of high-intensity interval training for the control and cold-water immersion conditions. 133

Figure 4-6: Mitochondrial respiration measurements at different coupling control states.. 134

Figure 4-7: Peak power output achieved during the graded exercise test, and peak oxygen uptake achieved during the steady-state cycle to fatigue bout to fatigue, before and after six weeks of high-intensity interval training 135

Figure 4-8: 2km and 20km cycling time-trial performance before and after six weeks of high-intensity interval training 136

List of Tables

Chapter One:

Table 1-1: A summary of current evidence regarding short- and long-term effects of cold exposure on endurance-related skeletal muscle adaptations. 56

Chapter Two:

Table 2-1: Subjective ratings of perceived recovery effectiveness (belief effect)..... 88

Table 2-2: Subjective ratings of readiness for exercise, fatigue, vigour, sleepiness and pain. 89

Chapter Three:

Table 3-1: Primer sequences used for real-time PCR 105

List of Abbreviations

1-RM	1 repetition maximum
ACADM	Acyl-CoA dehydrogenase
ACC	Acetyl-CoA carboxylase
AIF	Apoptosis inducing factor
Akt	Protein kinase B
ALAS	D-Aminolevulinic acid synthase
AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
ATF	Activating transcription factor
β2M	Beta-2 microglobulin
CaMK	Calcium/calmodulin-dependent protein kinase
CIRP	Cold-inducible RNA-binding protein
CK	Creatine kinase
COX	Cytochrome *c* oxidase
CREB	cAMP response element-binding protein
CRP	C-reactive protein
CS	Citrate synthase
CSP	Cold-shock protein
CWI	Cold-water immersion
DDR	DNA damage response
DOMS	Delayed onset muscle soreness
Drp1	Dynamin-related protein 1
EIMD	Exercise-induced muscle damage
ERG	ETS-related gene
ERK	Extracellular signal-regulated kinase
ERR	Estrogen-related receptor
FABP	Fatty acid-binding protein

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN5	General control of amino acid synthesis protein 5
GPX-1	Glutathione peroxidase 1
GXT	Graded exercise test
H2AX	H2A histone family, member X
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
HIT	High-intensity interval training
HSE	Heat-shock element
HSF	Heat-shock factor
HSP	Heat-shock protein
IL	Interleukin
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
Mdm2	Mouse double minute 2 homolog
Mfn2	Mitofusin-2
miRNA	Micro RNA
MnSOD	Mitochondrial superoxide dismutase 2
mtDNA	Mitochondrial DNA
mtHSP70	Mitochondrial heat-shock protein 70
MVC	Maximal voluntary isometric contraction
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activate B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF	Nuclear respiratory factor

NUGEMP	Nuclear genes encoded for mitochondrial proteins
OXPHOS	Oxidative phosphorylation
p90rsk	p90 ribosomal s6 kinase
PGC-1α	*PPARγ* coactivator 1-alpha
PHF20	Plant homeodomain finger protein 20
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PRMT1	Protein arginine methyltransferase 1
RBM3	RNA-binding motif protein 3
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
SCAS	Succinyl-CoA synthetase
SCF	Skp, Cullin, F-box containing complex
SCO2	Synthesis of COX 2
SIRT1	Silent mating type information regulation 2 homolog 1
TBP	TATA-binding protein
Tfam	Mitochondrial transcription factor
T_m	Muscle temperature
TT	Time trial
USF	Upstream stimulatory factor
VO_{2max}	Maximal oxygen uptake
W_{max}	Maximal aerobic power

CHAPTER ONE

Literature Review

1.1 Introduction

To be successful in their chosen sport, athletes must achieve an adequate balance between training and recovery. However, recovery is often overlooked in an attempt to increase training overload, intensity and volume (87). Residual fatigue and inadequate recovery between training sessions can place great physiological strain on the musculoskeletal system, potentially causing injury and symptoms of overreaching, fatigue and reduced performance (405). Thus, hastening recovery between training bouts and competition is imperative to ensure athletes can train frequently without the associated risks.

A large body of research has focused on modalities designed to hasten recovery after exercise, with one of the most prevalent techniques being cold-water immersion (CWI). Although the specific mechanisms by which CWI hastens recovery remain to be elucidated, increased hydrostatic pressure and/or a reduction in muscle temperature may play a role in reducing exercise-induced inflammation, oedema and pain sensation (509, 29). However, despite its popularity, there is conjecture about the merit of CWI in the recovery from exercise.

One area of contention is the possible contribution of psychological factors to the purported benefits of CWI (165). To date, no study has controlled for a potential CWI placebo effect. Debate also exists concerning the effects of CWI on the adaptive response to exercise training. Improvements in markers of skeletal muscle recovery following CWI (26, 326, 486) may allow athletes to train at a higher quality and/or load in subsequent sessions, potentially providing a greater stimulus for adaptation (490). However, this may be counteracted by potential long-term detrimental effects on skeletal muscle adaptation (523, 176, 187). Given the widespread use and acceptance by athletes of CWI as a recovery modality, it is important these issues are investigated.

1.2 Cold-Water Immersion

1.2.1 Background

Cryotherapy refers to the local or general application of cold in order to lower tissue temperature, with the intention of a therapeutic outcome (315). A reduction in tissue temperature is believed to promote many physiological responses, including analgesia, reduced metabolism and enzyme activity, and a vasoconstrictive response to reduce blood flow to the muscle (188). As a result, cryotherapy may diminish the effects of soft tissue injury (161), via an attenuation of oedema, inflammation, pain, and haematoma formation (188).

In an athletic setting, ice application is the most commonly used method of cryotherapy, used in both the immediate treatment of injury and/or in rehabilitation (48). Immediately following acute soft-tissue injuries, like moderate articular ligament and myotendinous injuries (especially muscle strains and contusions), ice application is purported to reduce local metabolism and minimise tissue damage (290). During rehabilitation, ice is predominantly used to relieve pain, reduce inflammation, and reduce joint oedema, promoting an earlier return to exercise (122, 239). Cryotherapy, in the form of ice packs, is also often combined in clinical settings with elevation and compression, and even in post-surgical rehabilitation (315). However, support for the efficacy of cryotherapy to treat acute soft-tissue injuries and post-surgical rehabilitation is divided (48). Many reviews support its use in both settings (239, 313, 309, 410), with others questioning the validity of its physiological and clinical effects (292, 314, 315, 479). There is currently insufficient evidence to suggest cryotherapy improves clinical outcomes in the management of soft tissue injuries (92), and more quality trials are needed to provide evidence-based guidelines for its use (48, 190).

Apart from injury, cryotherapy is also heavily utilised in athletic situations not typically associated with overt injury. Thermoregulation is an important consideration in sport and exercise, with elevations in core body temperature being associated with a reduction in performance (153). Cooling before or during exercise with the use of vests, or the ingestion of cold drinks, is common practice for many athletes, particularly those exercising in the extreme heat and/or for long

durations (498). Cryotherapy is also a popular post-exercise recovery tool, implemented mainly as whole-body cryotherapy or CWI. In recovery, cryotherapy is believed to limit the extent of exercise-induced muscle damage (EIMD), and other fatigue-induced decrements in functional capacity and athletic performance (490, 509). Cryotherapy in recovery aims to hasten the restoration of the body's physiological processes, thus ensuring athletes can train frequently without the associated risks (Fig 1-1) (62). A hastened return of pre-exercise function may also serve to ultimately improve subsequent training and/or competition quality, or enhance the effect of a given training load (490, 29).

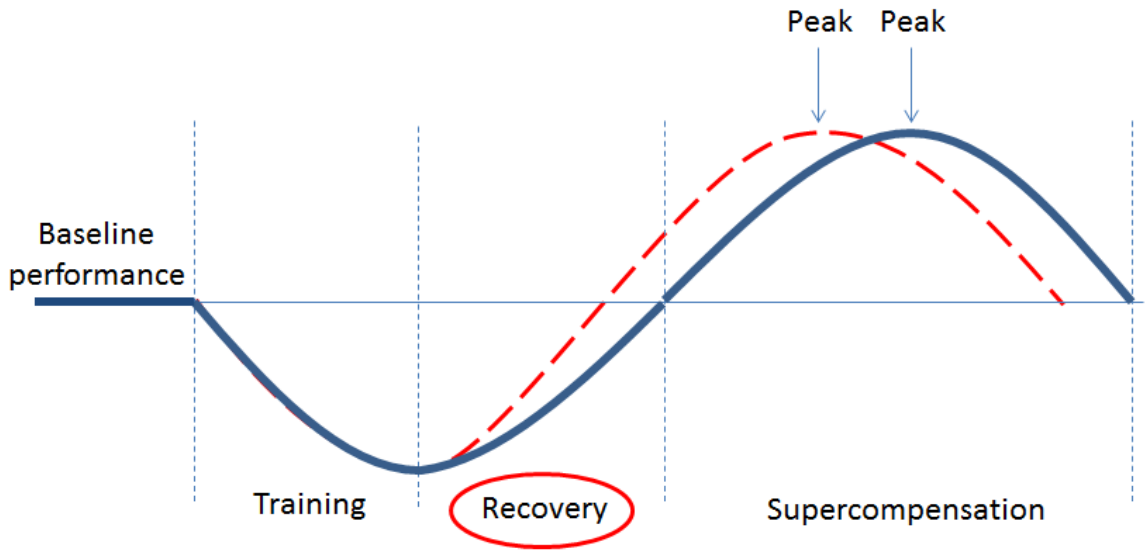


Figure 1-1: Physiological response to training, recovery, and supercompensation. Recovery modalities (e.g., cold-water immersion) aim to accelerate the recovery and supercompensation responses. ***Adapted from Hausswirth & Mujika (168).***

Receiving a lot of attention in recent literature, CWI has emerged as a popular cryotherapy tool that has been proposed to enhance athletic recovery following competition and training (509, 388). The supposed benefits in recovery from exercise from CWI are related to reductions in exercise-induced inflammation, oedema and pain sensation (29). Despite these physiological responses being heavily dependent on exercise intensity, duration and mode (472), CWI is utilised by athletes from a wide spectrum of different exercise modes (260). Although the specific mechanisms by which CWI hastens recovery remain to be elucidated, increased hydrostatic pressure and/or a reduction in muscle temperature may play a major role (509).

1.2.2 Proposed Mechanisms of Action

1.2.2.1 Hydrostatic Pressure

When immersed in water, a compressive force is exerted on the body called hydrostatic pressure (509). The degree of pressure exerted is dependent on immersion depth, and is calculated as follows:

$$P_{hyd} = P_{atm} + g * p * h$$

where P_{hyd} = hydrostatic water pressure (Pa), P_{atm} = atmospheric pressure (sea level ~ 1013 Pa), g = gravity (9.81 m/s^2), p = water density (1000 kg/m^3), and h = height of the water (m) (490, 509). Hydrostatic pressure will cause an upward and inward displacement of body fluid, aiding the return of fluid from the muscles to the blood (509). Central blood volume has been demonstrated to increase as a result (210, 12), which in turn may improve diffusion gradients via dilution of the blood (253). When used in the recovery from exercise, transportation of substrates and metabolism of waste products may improve, as suggested by faster blood lactate clearance following post-exercise water immersion (88, 338). These benefits may be particularly favourable following exercise that induces a pronounced depletion in muscle energy stores and an increase in metabolites, such as repeated high-effort bouts required in tournament situations (509). The shift of fluid from muscles to blood is also hypothesised to reduce exercise-induced muscle oedema, preventing the slowing of substrate transportation (227). A reduction in oedema can therefore assist nutrient delivery, maintaining muscle function and assisting muscle repair (509). It must also be noted that the benefits associated with hydrostatic pressure from water immersion transpire without a compensatory increase in heart rate and energy expenditure, as would be expected with low-intensity active recovery (427, 338).

Another potential benefit of an increased hydrostatic pressure during recovery is the reported feelings of buoyancy. When immersed in water, our bodies will experience an upward thrust as a result of the displaced water, proportional to the immersion depth (509). This net upward force makes the body buoyant, and may assist in the recovery from exercise via reductions in fatigue

perception. Immersion in water may conserve energy required by gravitational and postural muscles (509), reduce neuromuscular signal magnitudes (392), and modify peripheral processes associated with muscle contraction (242). Apart from the proposed benefits of immersion in water *per se*, immersion temperature and an associated reduction in tissue temperature is also believed to play a large role in aiding recovery from exercise.

1.2.2.2 Cold Temperature Effects

Core (139, 97), skin (139, 98, 302, 97), and intramuscular temperature (335, 56, 302, 97) have been shown to decrease following CWI, dependent on water temperature (302) and duration of immersion (373). The extent of tissue cooling from CWI is heavily determined by factors relating to thermal conductivity of the tissue, including contact area, absolute temperature differential, as well as tissue conductivity due to insulating adipose tissue (292). The physical properties of the cooling medium will also determine its capacity to cool, including the medium's thermal conductivity, specific heat capacity, and density (474, 400). For example, water has more than 3400 times the volume-specific heat capacity of air, meaning it has a much larger capacity to accept heat and that this transfer will also occur at a much faster rate (474). Cold water will also reduce muscle temperature more effectively than ice due to a greater surface area and larger transfer of heat (292), highlighting the effectiveness of CWI in cooling tissue as compared with ice application.

Cold-water immersion will also induce peripheral vasoconstriction (372), a response that is thought to play an integral role in improving post-exercise recovery. Decreased tissue temperature serves to excite cutaneous thermoreceptors, in turn increasing sympathetic nervous activity, norepinephrine release, and adrenergic stimulation to constrict local vasculature (78, 108, 230). The vasoconstrictive response following CWI will reduce capillary permeability and blood flow (315, 123, 488, 130, 191), and increase peripheral blood flow resistance to conserve core temperature (51, 365, 457). Perhaps the most advantageous outcome of vasoconstriction is its ability to decrease the swelling associated with exercise-induced muscle damage. Reduced permeability of capillary, cellular and lymphatic vessels following vasoconstriction will prevent

swelling from fluid diffusion (123), thereby limiting the acute inflammatory response following exercise-induced muscle damage (99, 58, 362). As such, CWI is often used in recovery as an anti-inflammatory intervention, which may reduce pain, swelling, and the loss of force generation and range of motion (99, 453).

Another important response to cold application is an alteration in neurological processes, such as decreased pain sensation and increased pain tolerance (73, 240). When tissue is cooled, nerve conduction velocity is reduced via a decrease in the production of acetylcholine (2), a neurotransmitter essential in the propagation of somatic stimuli. Pain and temperature sensory receptors located in the skin decrease their rate of firing after cooling, leading to a reduction in pain sensation (analgesia) (9, 315). Furthermore, reduced nerve conduction velocity following cold may also reduce pain caused by muscle spasm in the injured area (470). Cold-induced vasoconstriction may also improve subjective ratings of pain via a reduction in inflammation and the osmotic pressure of exudate, which in turn may reduce the pressure exerted on pain signalling nociceptors (260). In regards to CWI, a recent meta-analysis has endorsed its efficacy at reducing delayed onset of muscle soreness (DOMS), courtesy of an analgesic effect that may last up to 96 h post exercise (260). However, reduced nerve conduction velocity may also reduce muscle contractile speed and force-generating capacity up to 30 minutes post application (418, 189). For example, muscle strength and power output have been shown to be impaired immediately after muscle cooling (103, 429). As such, CWI that can adequately reduce tissue temperature may serve as an effective way to treat symptoms of exercise-induced muscle damage (e.g., muscle spasm and pain), but should not be used immediately prior to exercise that is dependent on performance outcomes.

1.2.3 Application and Current Guidelines

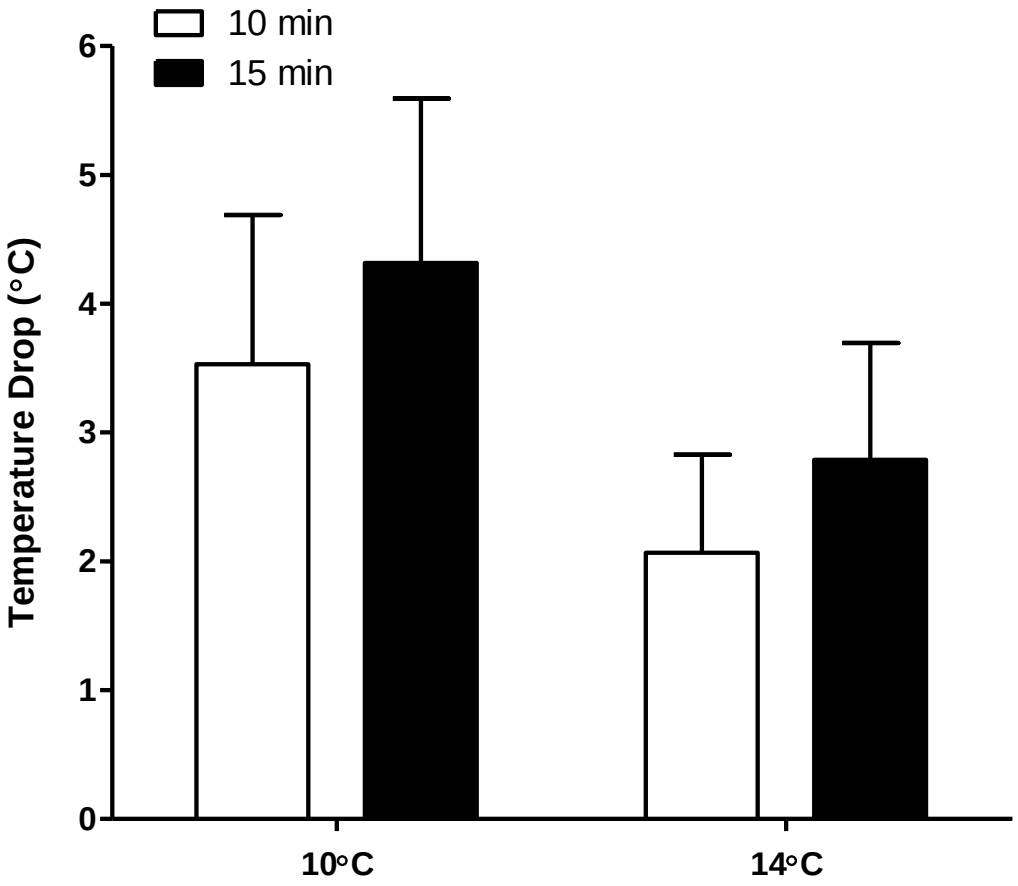
Despite its popularity, most athletes and sports practitioners follow anecdotal guidelines when prescribing CWI (49, 509). Due to a lack of empirically-based recommendations, there is limited evidence concerning optimal temperature, duration, timing and frequency. A number of considerations must be made when prescribing post-exercise CWI, including body composition,

periodization and timing of training/competition, exercise modality, general logistics (e.g., large sporting teams), ambient temperatures, and financial constraints. These considerations may potentially explain the varying CWI protocols used in both athletic and research settings. Although no standard CWI protocol exists, water cooler than 15°C and immersions of at least 10 min in duration are typically utilised (490).

1.2.3.1 Temperature

No clear recommendations exist regarding the optimal water temperature needed to achieve therapeutic reductions in muscle blood flow and tissue temperature following CWI. Reports of similar reductions in blood flow following cold (8°C) and temperate (22°C) water immersion for 10 minutes (160, 302) highlight that blood flow reductions aren't solely dependent on the extent of tissue cooling. The mechanism by which blood flow is reduced following temperate water immersion may be explained by the activation of non-noxious (independent of noxious cooling) thermoreceptors, which are known to operate at such temperatures (173). In regards to tissue temperature, greater reductions are seen with colder immersion temperatures (160, 302). From a thermodynamics perspective, the greater the temperature difference between a subject's tissue and the surrounding water, the faster its temperature will change (474). The extent of insulating adipose tissue surrounding the muscle will also determine the rate and degree of intramuscular cooling (336, 360), which should be taken into account when administering CWI to individuals of varying body composition (260). Some studies have utilised immersion temperature as cold as 5°C (437, 362, 523, 528) in an attempt to maximise cooling, but analysis of the literature reveals that most studies utilise more comfortable immersion temperatures of between 10-15°C (123, 155, 187, 449, 65, 26, 523, 414, 415, 326, 373, 375, 372, 374, 195, 246, 487, 174, 251, 485, 237, 61, 205, 461, 102, 367, 435, 57, 176, 388, 325). Cutaneous pain nerve endings begin to fire below a skin temperature of 15°C, a threshold at which the temperature stimulus becomes merged with pain (288). As such, cold-water immersion temperatures of between 10-15°C may provide an adequate balance between the maximal reduction in tissue temperature and comfort levels. Figure 1-2 reports pilot data demonstrating the extent of deep (~2.5 cm) intramuscular cooling during 10-15 min of CWI at either 10°C or 14°C.

As expected, 10°C was more effective in reducing muscle temperature over 10-15 min immersion durations.



<u>Figure 1-2:</u> Intramuscular temperature drop (pilot data, *n* = 5) comparing cold-water immersion temperatures of 10 and 14°C. Thermistors were inserted into the *vastus lateralis* to a depth of ~2.5 cm (excluding adipose tissue).

1.2.3.2 Duration

As shown in Figure 1-2, and reported previously (373), withdrawal of heat from tissue immersed in cold water is greater with longer immersion durations. Accordingly, most studies to date have varied their immersed duration with the immersion temperature, so as to minimise discomfort and maximise the desired physiological response. For example, more temperate immersions of ~15°C and upwards have typically been combined with durations of ~15-20 minutes (449, 123, 251), whereas shorter durations of ~one minute have been used with much colder immersion temperatures of ~5°C (437). Cold application in a clinical

setting may be as short as thirty seconds (509), but it is unlikely that immersions this short will elicit a significant drop in muscle temperature (229, 315). Similarly, short immersion durations may not allow adequate fluid shifts within the muscle (155), as increases in blood plasma fraction (movement of interstitial-intravascular fluid) during water immersion have been observed to take at least 10 minutes (210, 177). Although longer durations will result in larger reductions in muscle temperature, durations longer than 20-30 minutes may be detrimental to recovery. Participant comfort may be compromised with long durations, and the initiation of a shivering response may potentially counteract the recovery process by increasing energy expenditure (49). As such, durations of ~15-20 minutes seem to provide a good balance between the desired physiological effects (e.g., fluid shifts and decreased muscle temperature) and comfort.

1.2.3.3 Summary

Although an optimal prescription for post-exercise CWI does not currently exist, a temperature of ~10-15°C for 15-20 minutes seems the most appropriate to achieve the desired benefits. However, much of the ambiguity regarding prescription is related to the lack of empirical evidence surrounding its physiological benefit. Furthermore, it is currently unknown what physiological response needs to be achieved for CWI to have its maximum benefit. Until this is known it is not possible to prescribe an 'optimal' protocol. In addition, optimal CWI conditions may be specific to the type of exercise performed and individual body composition, making it difficult for coaches and sport scientists alike to advocate a universal prescription. If a reduction in blood flow is the most desirable physiological effect, then comfortable immersion temperatures may be as beneficial as considerably colder immersions. Furthermore, any additional physiological benefit arising from colder immersion temperatures are likely to be mediated by the purported benefits of reduced tissue temperature (e.g., analgesia) rather than any additional reduction in blood flow (160, 302).

1.2.4 CWI Effects on Recovery

For a detailed review of the original research performed to date examining the effects of CWI on the recovery from exercise, the reader is referred to an

excellent review on this topic (490). A summary of this research is discussed below, with a particular focus on CWI's effect on the recovery of exercise performance. Evidence investigating the effect of CWI on certain physiological markers, including muscle soreness and training adaptations, will also be discussed.

1.2.4.1 Performance

A number of sports, and in particular sporting competitions, may require an athlete to perform numerous high-quality exercise bouts within a short period of time. For example, international swimming competitions can require repeated maximal performances within the same day, with some recoveries between events as short as 30 minutes (367). Recovery is therefore of utmost importance for these athletes, as they attempt to hasten the return to baseline performance for subsequent efforts.

Evidence supporting the use of CWI to improve recovery on the same day is limited, but two studies have reported CWI to improve the recovery of strength/power (388) and 30-minute cycling (485) performance. Pournot et al. (388) reported a greater recovery of baseline maximal muscle strength and countermovement vertical jump height 1 h post-exercise when CWI (15 min at 10°C) was used for post-exercise recovery, as compared with contrast-water therapy, thermo-neutral water immersion, and a passive recovery. This research suggests that a reduction in tissue temperature may improve performance within one hour post-exercise, a finding pertinent for athletes required to perform multiple bouts on the same day. Cold-water immersions of varying temperatures (5, 10 and 15°C) have also been shown to maintain performance (total work) during two 30-minute cycling bouts (15 min at 75% peak power followed by a 15-min all-out time trial) separated by one hour, as compared with a 4.1% reduction with an active recovery (40% VO_{2max}) condition (485). These cycling bouts were performed in the heat however, so maintenance of cycling performance with the CWI conditions was attributed to a reduction in thermal strain. Given these are the only two studies (to the author's knowledge) reporting a beneficial effect, they

provide limited support for CWI's efficacy in improving the recovery of performance within one hour of exercise

In contrast, the majority of research investigating CWI's efficacy on same-day performance has demonstrated no beneficial effect. For example, treadmill running to exhaustion (88), all-out cycling time-trial performance (375, 61, 461), and isometric strength (26, 373) have all been demonstrated to be similar to a control condition following post-exercise CWI when measured within four hours of the initial exercise bout. In fact, some studies have even demonstrated a detrimental effect of post-exercise CWI on repeat anaerobic exercise bouts performed within 1 h, including maximal 30-s cycling (435, 102) and 100-m swimming (367) performance. Both negligible and detrimental effects of CWI on repeat performance within a short recovery period may be related to a cold-induced reduction in nerve conduction velocity and the associated reduction in muscle performance (9, 418, 189) - an effect that may also be further exacerbated with longer immersion durations (100). Furthermore, a cold-induced inhibition of heart rate (214, 44), and an increase in parasympathetic activity (61), may compromise the cardiac response (and thus oxygen delivery) at the start of exercise (367). Parouty et al. (367) state these detrimental effects can be overcome with shorter immersion times and an appropriate warm-up before subsequent high-intensity efforts, while retaining CWI's proposed recovery benefits. Despite this suggestion, the weight of evidence provides little support for a beneficial effect of CWI in recovery when the subsequent bout is within 24 h.

A lot of support for the use of CWI in the recovery from exercise is demonstrated between 24-72 h after an initial exercise bout. Performance improvements have been reported for muscle strength (449, 26, 388, 487, 195) and power (487), electrically-stimulated muscle function (449), cycling (486, 251, 374, 485), running (326, 528, 415, 57), climbing (174), vertical jump height (449, 326, 236) and range of motion/flexibility (123, 326, 246) tests following CWI. Furthermore, these performance improvements have been reported following a range of exercise modalities, including running (26, 57, 528), cycling (485, 486) (251, 375), basketball (326), soccer (415), simulated team-sport exercise (195, 236), exhaustive intermittent anaerobic exercise (388), and eccentric exercise

(449, 487, 123, 246), with the improvements reported to last for days after the initial exercise bout. For example, maximum voluntary contraction strength has been reported to be improved 24 h (449, 26, 388), 48 h (449, 26, 487, 195) and 72 h (449, 487) post-CWI, as compared with a passive control. However, there is a lack of consistency regarding the reported benefits of CWI in recovery. CWI has also been demonstrated to have no influence (123, 437, 362, 237, 205, 155, 236) or even a detrimental (372, 523) effect on recovery following exercise, further highlighting the equivocal nature of CWI research. Inconclusive results are evident in a number of measures, including muscle strength and function (123, 437, 155, 187, 362, 205), range of motion (155, 187, 437), vertical jump height (237, 205) and intermittent running performance (236). The lack of consistency with performance outcomes with CWI research is commonly attributed to methodological discrepancies between studies. For example, differences exist between a number of variables, including immersion temperature, immersion duration, exercise protocol employed, and performance measures recorded (195).

1.2.4.2 Markers of Exercise-Induced Muscle Damage

Apart from its effect on performance, the effectiveness of CWI in recovery is also commonly determined from a number of indirect physiological markers of EIMD. These markers include the appearance of intramuscular proteins in the blood following an initial damaging insult (e.g., eccentric muscle contractions), inflammatory markers in both the muscle and blood, and muscle soreness (82). CWI is believed to reduce the appearance of these markers, mainly via pressure-induced fluid shifts, and vasoconstrictive-induced reductions in blood vessel permeability and inflammation.

Following an initial mechanical insult, a number of myoproteins are passively leaked into the blood from disrupted skeletal muscle. The quantification of these proteins are a cost-effective and simple method by which to indirectly assess the extent of muscle damage (123). Creatine kinase (CK) is the most extensively used biochemical marker in the assessment of recovery from muscle damage (26, 123, 449, 437, 155, 28, 325), most likely due to the magnitude of increase following muscle damage as compared with other myoproteins (82). A

reduction in membrane permeability following CWI is believed to attenuate the efflux of CK, a notion supported in the literature (260, 449, 123). However, evidence also exists demonstrating no effect of CWI on the appearance of CK (437, 187, 26). This may be due to CWI having no effect on CK, or may conversely support suggestions that CK does not accurately reflect the extent of muscle damage and subsequent functional loss following strenuous exercise (495). CK released from the muscle is heavily dependent on individual variability (350), and blood levels of this protein represent both clearance from the bloodstream as well as release from the muscle, meaning the high variability in this measure may be due to changes in blood flow (260). A number of other myoproteins have also been utilised in CWI research, including myoglobin (26, 96, 17, 325), lactate dehydrogenase (LDH) (428, 260, 388), and fatty-acid binding protein (FABP) (325). However, evidence supporting the effectiveness of CWI in reducing these biomarkers is also conflicting.

Secondary to the initial trauma, CWI is also thought to benefit athletic recovery by suppressing the ensuing inflammatory response (99, 58, 362). This response is characterised by the infiltration of fluid, plasma proteins and inflammatory cell populations to the injured tissue (371, 453, 293), which collectively function to clear cellular debris from the injured area and prepare for regeneration (82). Common inflammatory markers measured in CWI research include immune and leukocyte cells (461, 369, 505, 388), inflammatory cytokines (461, 505, 369, 325), and c-reactive protein (CRP) (57, 256, 154). Similar to the response of myoproteins, the influence of CWI on the accumulation of inflammatory markers is unclear, with studies reporting a favourable alteration in some markers (461, 388, 369, 325), no difference at all (369, 325), and even an exacerbation of certain markers (505). Limb girth is also often measured as an indication of muscle oedema and swelling (123, 437), but evidence of CWI's effectiveness with this measure is largely inconclusive (155, 187, 437). The biological significance of these findings remains to be determined, and further research is needed to clarify the effects of cryotherapy on the immune and inflammatory responses after contraction-induced muscle injury.

CWI appears to be most effective in providing an analgesic effect and reducing pain associated with DOMS (260). Peaking around 24-48 h post-exercise (83, 342), DOMS may originate from swelling and pressure on the muscle itself, as well as noxious chemicals released upon muscle damage (352). Given its potential to hinder a number of muscular performance measures, including range of motion and force production, muscle soreness is a common measure in CWI research (362, 123, 449, 26, 437, 155, 195, 236). Most evidence supports CWI's ability to reduce muscular soreness (487, 26, 195); however, this is by no means indisputable. CWI has also been shown to have no effect on muscle soreness (187, 437, 123), again highlighting the equivocal nature of CWI's effectiveness to reduce a number of physiological markers of muscle damage. A potential explanation for this is that muscle soreness has both physiological and perceptual, and therefore subjective, origins. As such, debate exists as to whether the proposed benefits of CWI are due to the physiological responses it is designed to elicit, or to an alteration in pain and fatigue perceptions. Given the perceptual nature of DOMS, it is possible that the placebo effect may have an influence on this measure (260).

1.2.4.3 Psychological/Placebo Effect

A potential explanation for the variable response to CWI may be linked, at least in part, to psychological factors (461). Other recovery techniques, such as massage, have been reported to improve recovery via psycho-physiological mechanisms such as decreased sensation of pain and fatigue (497). CWI has been shown to enhance feelings of recovery (367), and reduce perceptions of fatigue (367, 464, 461, 415) and muscle soreness (26, 195, 464, 415), when compared with a control condition. Furthermore, recent reviews have concluded that CWI is effective in inducing an analgesic effect (78) and reducing subjective measures of muscle soreness (260), with its effects on muscle function unclear. Given the subjective nature of muscle soreness, and increasing popularity of CWI as a recovery strategy, it is plausible that athletes believe and expect CWI to improve recovery from exercise.

One major limitation with the CWI research performed to date is that no study has incorporated a placebo condition. This is a crucial omission given the placebo effect is a well-accepted phenomenon within medicine, and is even utilised as a therapeutic intervention (see review by Beedie & Foad (35)). The placebo effect also influences sport performance (35), and has a potentially long-lasting effect (38). For example, merely expecting an intervention to have a positive effect has been shown to improve an athlete's performance post-placebo administration (307). Pollo et al. (387) reported a 7.8% decrease in perceived fatigue and an 11.8% increase in leg extension strength when supplying a caffeine ergogenic placebo. Furthermore, Clark et al. (81) reported a 4% improvement in mean power over a 40-km cycling time-trial when administering a carbohydrate placebo. To fully understand the mechanisms underlying CWI, and its influence on athletic performance, it is crucial that future studies control for the placebo effect.

1.2.4.4 Summary of CWI and Recovery

In summary, evidence supporting the use of CWI to improve the recovery of skeletal muscle is equivocal. The lack of consistency in the observed effects of CWI is commonly attributed to methodological discrepancies between studies, such as the immersion and exercise protocols used and the performance outcomes measured (195). However, CWI appears to be most effective in providing an analgesic effect and reducing the pain associated with DOMS (260). As muscle soreness has both physiological and subjective origins, debate exists as to whether the proposed benefits of CWI are due to the physiological responses it is designed to elicit, or to an alteration in pain and fatigue perceptions. As such, another potential explanation for the variable response to CWI may be linked, at least in part, to psychological factors and a potential placebo effect (461). To fully understand the mechanisms underlying CWI, and its influence on athletic performance, it is crucial to control for the placebo effect.

1.2.5 CWI and Adaptations to Training

To date, most research has focused on the potential benefits of CWI to promote faster recovery from a training session or a match. As a consequence,

little is known about the effect of regular post-exercise CWI on adaptations to exercise training. It is currently unknown whether the physiological benefits that have been associated with post-exercise application of CWI translate into an improved adaptive response to exercise training. Indeed, a hastened restoration of the body's physiological processes may ultimately improve subsequent training load and quality, potentially providing a greater stimulus for adaptation. Conversely, a reduction of exercise-induced fatigue and inflammation may serve to decrease the stimulus for adaptation (490). Further clarification of this issue will provide evidence on the merit of post-exercise CWI as an integral component of athletic training regimes (29).

To the author's knowledge, only six studies have investigated the merit of regular CWI in training (523, 176, 134, 65, 187, 164). A study by Yamane et al. (523) first alluded to the potential detrimental effects of CWI in training. They demonstrated an attenuation of endurance performance, maximal oxygen uptake, arterial diameter and muscular endurance following four to six weeks of regular post-exercise CWI as compared with a passive control. Several methodological limitations have been raised with this study however, including participant training status (untrained), low participant numbers (n = 6), and an atypical immersion temperature (5°C) and duration (2 x 20 min) (490). Subsequent research investigating the effects of regular CWI on training adaptations is equivocal, with regular CWI shown to decrease repeat sprint performance (176) and attenuate improvements in leg strength (134), or to have no effect on muscle strength (187). Furthermore, regular CWI during three weeks of intensified cycling training was shown to have negligible or beneficial effects on cycling performance in competitive cycling (164), supporting the notion that CWI may assist recovery from individual training session and increase training quality (490). Consistent with the CWI data following a single training session or match, conclusive evidence either supporting or refuting the merit of CWI in exercise training is lacking, and further research is warranted. Of note, despite the increasing popularity of high-intensity interval training, there is very limited research investigating the effects of CWI on adaptations to high-intensity interval training.

1.3 High-Intensity Interval Training

Skeletal muscle is a highly malleable tissue, capable of altering muscle protein abundance in response to disruptions in cellular homeostasis. Exercise provides one such disruption to cellular homeostasis, and can be broadly grouped into aerobic/endurance or strength/resistance. Endurance exercise is predominantly associated with skeletal muscle phenotype alterations related to improved mitochondrial volume and aerobic capacity (21, 89, 119), whereas resistance exercise is more closely associated with improved muscle mass, fibre hypertrophy, strength, and anaerobic capacity (21, 89, 119). Accumulative disruptions to cellular homeostasis via repeated bouts of exercise, termed training, can drastically alter muscle phenotype, with the stimulus intensity and duration determining numerous morphological and metabolic muscular adaptations (21).

Low-volume, high-intensity interval training (HIT) has recently emerged as a popular mode of training. It is characterised by repeated, brief, intermittent bursts of vigorous exercise, interspersed with periods of rest or low-intensity exercise (143). 'Wingate' training remains the most common form of HIT, and may also be referred to as 'sprint'-interval training (SIT). This type of training typically consisting of 4-6 x 30-s 'all-out' efforts at a supra-maximal workload (~200% VO_{2max}), interspersed with ~4 min of recovery (143). Given the intense nature of Wingate training, more practical models of low-volume HIT are also routinely employed. These models incorporate lower work intensities (e.g., constant load at ~90% max heart rate) and longer rest durations (e.g., 60 s), but also more repetitions (e.g., 10) (280). Such short-duration, intense muscular work is typically associated with strength/resistance training, linking HIT with pathways that stimulate protein synthesis and muscle growth. However, HIT does not appear to induce marked fibre hypertrophy (412, 483, 146), but is conversely a potent stimulator of muscle phenotype adaptations comparable to that of traditional high-volume, long-duration endurance exercise (143, 399). Given the relatively low time commitment needed, HIT has been proposed as a time-efficient strategy to maximise endurance-related adaptations.

1.3.1 Physiological Remodelling after HIT

HIT protocols close to, or above maximal oxygen uptake (VO_{2max}) intensity, maximally stress the oxidative pathways and may therefore be an effective stimulus for enhancing aerobic-related adaptations (318). Similar to traditional high-volume, long-duration, endurance training, skeletal muscle metabolic adaptations induced by HIT are primarily associated with a shift towards an endurance phenotype. For example, endurance-like adaptations demonstrated with HIT include improved peripheral vascular structure and function (399), increased resting glycogen content (64, 144), reduced rate of glycogen and phosphocreatine utilisation (63), increased capacity for whole-body and skeletal muscle lipid oxidation (63), and improved endurance exercise performance (64, 144).

HIT has also been shown to improve key mitochondrial adaptations, including an increase in the maximal activity and content of mitochondrial enzymes (144, 64), improved ability to combat the accumulation of metabolites associated with fatigue (412), and increased mitochondrial respiration (157). Increases in maximal activity and content of mitochondrial enzymes, and improvements in mitochondrial function, are the result of a process termed mitochondrial biogenesis.

1.3.2 Mitochondrial Biogenesis

One of the most dramatic phenotype changes in skeletal muscle following endurance exercise is an increase in mitochondrial protein expression, commonly termed ‘mitochondrial biogenesis’ (185, 278, 524, 119). While mitochondrial biogenesis is sometimes used in reference to the formation of new mitochondria, it more accurately represents the remodelling of existing networks via a complex interplay of fission and fusion processes (419). New proteins are recruited into the mitochondrial reticulum, thereby generating new mitochondrial compartments (47). Given the complex nature of mitochondria, analysis of mitochondrial biogenesis should include a range of measurements assessing protein synthesis rate, as well as mitochondrial content (e.g., transmission electron microscopy or citrate synthase activity) and function (e.g., mitochondrial respiration) (47).

Improvements in muscle oxidative function are the result of hundreds of different nuclear and mitochondrial genes being transcribed to produce proteins and molecules needed for the assembly and expansion of the mitochondrial reticulum. Approximately 95% of mitochondrial proteins are expressed from nuclear genes, with the other 5% come from the mitochondria's own independent genome (149). As such, mitochondrial biogenesis relies heavily on the transport of nuclear-coded proteins into the organelle, with the assistance of cytosolic chaperones (e.g., heat shock protein 70 and mitochondrial import stimulating factor) and transport machinery (e.g., such as the inner and outer mitochondrial membrane translocases) (184, 196). Although tiny compared with the nuclear genome, mitochondrial specific DNA (mtDNA) encodes thirteen proteins involved in the electron transport chain (e.g., cytochrome c oxidase subunits), but is equally crucial for mitochondrial biogenesis and respiratory chain function (286).

Integral to the expression of proteins necessary for mitochondrial biogenesis is the expression and activation of numerous nuclear and mitochondrial transcription factors. Transcription factors bind to specific DNA sequences of target genes and control the rate of transcription of DNA into mRNA (255). As such, their activation represents the critical first step in gene expression and subsequent translation of a functional protein (254). Considering most mitochondrial proteins are nuclear-encoded, a number of transcription factors work on nuclear genes encoding mitochondrial proteins (NUGEMPs). Such transcription factors include (but are not limited to) nuclear respiratory factor-1 (NRF-1) and -2 (NRF-2), cAMP response element (CRE) binding protein (CREB), early growth response gene-1 (Erg-1), estrogen-related receptor (ERRα), upstream stimulatory factor-1 (USF-1), and activator protein 1 (AP-1) (431, 184, 286). NRF-1 and -2 have been widely implicated in the transcriptional activation of multiple genes associated with mitochondrial biogenesis, including mitochondrial transcription factor (Tfam), and dimethyladenosine transferase 1 (TFB1M) and dimethyladenosine transferase 2 (TFB2M) (431, 432, 148, 518), key components of mtDNA replication and transcription (224, 295). Importantly, many of these transcription factors have been shown to be induced following

single and repeated bouts of contractile activity (93, 198, 317, 521), as well as following endurance exercise (22, 444, 333).

Exercise-induced mitochondrial biogenesis corresponds closely to cellular energy requirements, and functions to improve oxidative phosphorylation, ATP yield, and fatigue resistance (184). Substrate utilisation is also improved, with more β-oxidation enzymes improving muscle fatty acid metabolism, increasing glycogen sparing and reducing accumulation of lactate (184). Long-term mitochondrial adaptations are dependent on the metabolic and molecular responses following a single bout of contractile activity, which are in turn dependent on exercise mode (e.g., running vs cycling), duration, frequency and intensity (60, 143, 185).

A number of stimuli have been implicated in the activation of cellular cascades altering muscle phenotype following contractile activity (e.g., endurance exercise), including mechanical strain, ATP turnover, calcium flux, redox balance, reactive oxygen species (ROS) production, and intracellular oxygen pressure (119, 185). Although the precise molecular mechanisms underlying skeletal muscle adaptation to HIT have not been fully elucidated, robust changes in intramuscular ATP and ROS concentrations are thought to be integral stimuli (143, 146, 278). These stimuli serve to activate certain signalling kinases and phosphatases, which in turn generate and/or activate transcription factors and co-regulator proteins for their corresponding role in the transactivation of target genes (185). Depending on the initial stimulus, this complex network exerts molecular control over contractile, metabolic and mitochondrial adaptations (119).

1.3.2.1 Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha

Commonly considered the "master-regulator" of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-alpha (PGC-1α) is a transcriptional co-activator shown to regulate skeletal muscle mitochondrial function and biogenesis (395, 396, 394, 286). Being a transcriptional co-activator, PGC-1α cannot bind to DNA directly. Instead, PGC-1α co-activates a number of transcription factors (e.g., NRF-1/2, ERRα, and Tfam) involved in the regulation of nuclear and mitochondrial genes (119, 286,

273). PGC-1α is highly expressed in oxidative tissues like skeletal muscle (396, 197), where it plays an integral role in adaption following exercise.

Given its vital role in mitochondrial biogenesis, PGC-1α transcription is similarly regulated by signalling pathways that initiate exercise-induced oxidative phenotype alterations (Fig 1-3). For example, repeated contractile activity will result in a transient increase in sarcoplasmic calcium concentrations, and a concomitant depletion in cellular energy stores that will increase the AMP:ATP ratio. These events are respectively implicated in the increase of PGC-1α mRNA expression via the activation of the calcium-sensitive calcineurin and calcium/calmodulin-dependent protein kinase (CaMK) (166, 312), and the energy sensitive AMP protein kinase (AMPK) (200, 213). Other exercise-induced signals implicated in PGC-1α transcription include the accumulation of ROS as a natural by-product of cellular respiration (447), muscle glycogen depletion (383), and PGC-1α itself via an auto-regulatory loop with myocyte enhancer factor 2 (MEF2) (166). The downstream implications of these signalling cascades will be discussed in detail later in this review.

As a fully functional protein, PGC-1α regulates mitochondrial biogenesis by undergoing specific post-translational modifications. Central to its regulatory role in mitochondrial biogenesis, phosphorylation of PGC-1α will induce its translocation into the nucleus, where it is subsequently stabilised to function as a transcriptional co-factor. Both adenosine monophosphate-dependent protein kinase (AMPK) (Thr^{177} and Ser^{538} residues) (204) and p38 mitogen activate protein kinase (MAPK) (Thr^{262}, Ser^{265} and Thr^{298}) (394) can phosphorylate PGC-1α directly, which in turn increases the protein's activity (356). Deacetylation of PGC-1α permits it to function as a coactivator of numerous transcription factors (27, 46, 84); however, conjecture exists as to how this occurs exactly. Both the removal of acetyl groups by sirtuin 1 (SIRT1) and/or the removal of histone acetyltransferase (GCN5) (126) have been implicated. The presence of SIRT1 has been suggested to be critical for PGC-1α activation (68) and nuclear abundance (279), but conversely a 2.5-fold increase in SIRT1 was shown to actually decrease whole muscle PGC-1α content (162). This contradiction may be explained by GCN5, whose translocation out of the nucleus (upon AMPK activation) is now

believed to deacetylate PGC-1α (381). Other post-translational modifications include methylation by the protein arginine methyl-transferase 1 (PRMT1) and ubiquitination by Skp/Cullin/F-box-cell division control 4 (SCF^{Cdc4}) (126). Methylation strongly enhances PGC-1α-mediated transcription (477), and ubiquitination targets PGC-1α for proteasomal degradation (358).

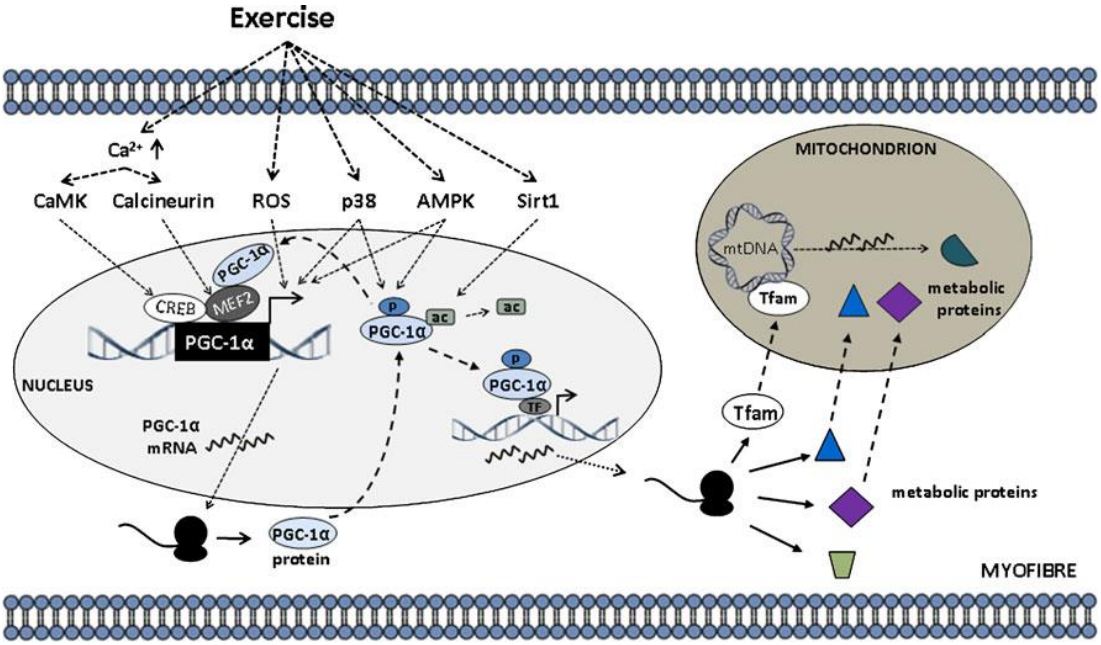


Figure 1-3: Exercise factors involved in altering PGC-1α activity. Ca^{2+}, calcium; CaMK, calcium/calmodulin-dependent protein kinase; ROS, reactive oxygen species; p38, p38 mitogen-activated protein kinase; AMPK, adenosine monophosphate-dependent protein kinase; Sirt1, Sirtuin 1; CREB, cAMP response element binding protein ; MEF2, myocyte enhancer factor 2; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TF, transcription factor; Tfam, mitochondrial transcription factor; mtDNA, mitochondrial DNA; p, phosphorylated; ac, acetylated. ***Olesen et al. (356)***

Increased PGC-1α protein content mediates numerous regulatory pathways associated with mitochondrial biogenesis. Overexpression of PGC-1α protein in wild-type mice has been shown to drive the conversion from fast to slow muscle fibres, increase mitochondrial enzyme expression, and improve resistance to electrically-stimulated fatigue (274), traits indicative of an endurance-trained, oxidative phenotype. Furthermore, other rodent studies have demonstrated improved insulin resistance (40), protection against sarcopenia (502), and improved exercise capacity (66) following PGC-1α overexpression, highlighting its importance in muscle metabolic health. It is important to note however, exercise-induced adaptive gene expression in skeletal muscle is not entirely

dependent on PGC-1α. Leick et al. (261) reported similar training-induced increases in cytochrome *c*, cytochrome *c* oxidase (COX), and aminolevulinate synthase (ALAS) 1 mRNA and protein levels in PGC-1α knock-out mice and wild-type mice, signifying that factors other than PGC-1α may also mediate training-induced mitochondrial adaptations.

Endurance exercise itself has routinely been implicated in the up-regulation of PGC-1α mRNA and/or protein (384, 515, 22, 416, 90, 382, 349, 377). A single bout of endurance exercise has been shown to increase PGC-1α transcriptional activity, resulting in elevated mRNA expression of both itself and a number of mitochondrial genes (384, 377). Resultant protein translation and increases in PGC-1α content have been suggested to enhance mitochondrial biogenesis and further adaptive phases in subsequent exercise bouts (377). PGC-1α protein content has been shown to increase rapidly in rat skeletal muscle, with an ~2.0 fold increase only 18 h after a single bout of swimming exercise (22, 515). In human skeletal muscle, a single bout of moderate-intensity cycling has been shown to both have no effect (91, 496) and increase (~23%) (298) PGC-1α protein content within 24 h after the exercise bout. However, this response may be regulated by training status and basal PGC-1α content (142), providing a potential explanation for these discrepancies. Six weeks of regular (3-5 d/wk), moderate-intensity (60-80% VO_{2max}), running (416) and cycling (63) have been demonstrated to increase PGC-1α content by ~ 1.5-2.0 fold in human skeletal muscle, consistent with data from endurance-trained (~8 wk treadmill running) rat skeletal muscle (473). Collectively, these data highlight that PCG-1α is a critical component of the adaptive response to endurance training (145).

Exercise-induced up-regulation of PGC-1α mRNA and protein content appears to be analogous between traditional long-duration endurance exercise and HIT. Sustained contractile activity for > 1 h was originally thought necessary for a significant increases in PGC-1α mRNA (417), with human studies demonstrating increases (~6-7 fold) in PGC-1α mRNA following 3 h of leg extension (384) and cycling (496) exercise. However, comparable increases (~3 fold) in PGC-1α mRNA are also evident following a single bout of low-volume HIT (146),

highlighting that HIT-mediated skeletal muscle remodelling may be regulated by signalling cascades normally associated with endurance training.

Given the potency of HIT to increase skeletal muscle oxidative capacity, recent research has investigated its influence on PGC-1α mRNA and protein levels. The first evidence that interval exercise was a potent stimulus for an increase in PGC-1α mRNA was demonstrated by De Filippis et al. (110), with large-volume HIT (4 x 10 min cycling at 70-90% HR_{max}) inducing an ~8 fold increase in lean individuals, 5 h post-exercise. Similar increases have also been reported with low volume, supra-maximal intensity cycling (4 x 30-s 'all-out' bouts) (278). Although studies have reported an increase in PGC-1α protein content following a single high-intensity interval cycling bout in humans (278), and a single high-intensity interval swimming bout in rodents (476), it is generally thought that more than one session of HIT is needed to increase PGC-1α protein content (146). For example, six weeks of 'Wingate' training (4-6 x 30-s 'all-out' bouts) increased PGC-1α protein by ~100% in human skeletal muscle, comparable to increases reported following traditional endurance training (63).

The potency of HIT to stimulate increases in PGC-1α mRNA comparable to (or even exceeding) that of long-duration endurance exercise is most likely due to the intensity of the exercise (118). For example, increasing exercise intensity coincides with greater bioenergetic process, calcium flux, and ROS production, stimuli that are all implicated in promoting PGC-1α transcription (118, 310, 312, 204). In particular, recent evidence has implicated both energy- (AMPK) and stress-dependent (p38 MAPK) kinase signalling as key regulators of PGC-1α mRNA expression and mitochondrial biogenesis following HIT (Fig 1-4).

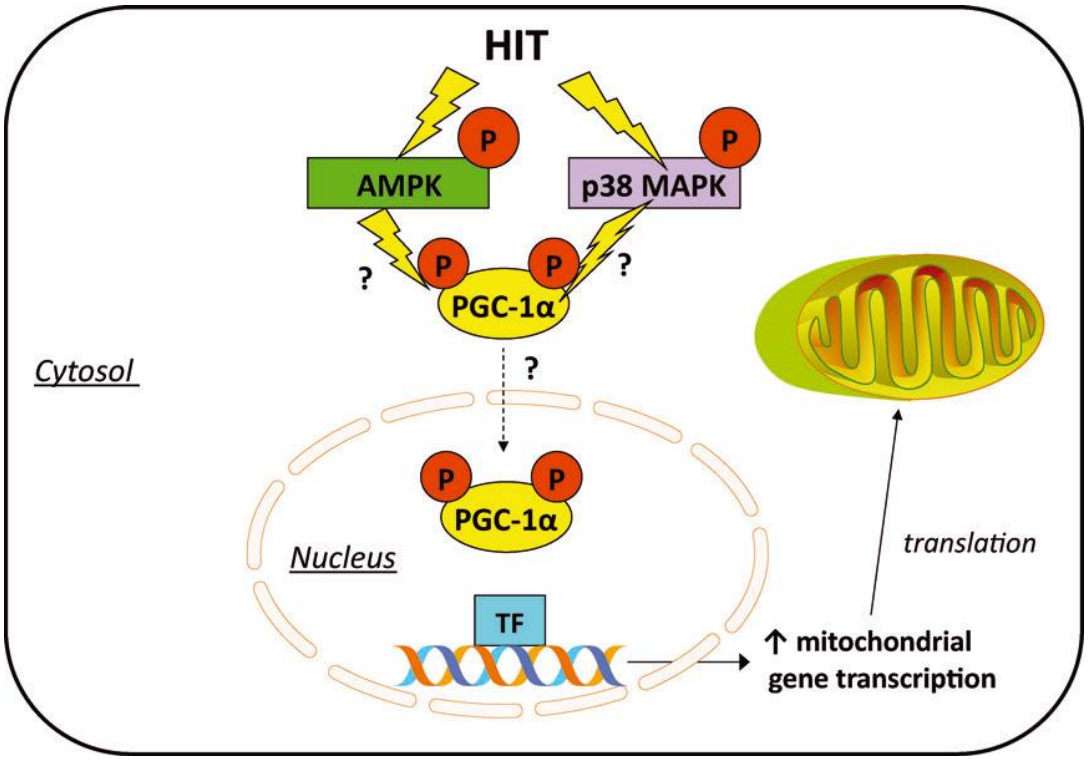


Figure 1-4: Potential intracellular signalling mechanisms involved in HIT-induced mitochondrial biogenesis. p38 MAPK, p38 mitogen-activated protein kinase; AMPK, adenosine monophosphate-dependent protein kinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TF, transcription factor; p, phosphorylated. Gibala et al. (143)

1.3.2.2 Adenosine Monophosphate-Dependent Protein Kinase

A potent stimulus for PGC-1α-mediated mitochondrial biogenesis in skeletal muscle is a deficit in cellular energy. Any cellular stress that depletes ATP stores and subsequently increases intracellular concentrations of AMP, most notably exercise, will activate the secondary messenger AMPK (167, 119, 135, 512). Activation via phosphorylation (Thr^{172}) has also been demonstrated in response to other cellular stresses that increase cellular AMP/ADP:ATP ratios (e.g., metabolic poisons, glucose deprivation or oxidative stress) (217), highlighting AMPK's role as an 'energy sensing' kinase (89).

Structurally, AMPK is a heterotrimeric protein formed by alpha (α), beta (β) and gamma (γ) subunits (465). Of the twelve different AMPK α/β/γ-heterotrimers, only three are present in human skeletal muscle and possess either an α1 or α2 catalytic subunit (46). Both the α1 and α2 subunits appear to be activated during high-intensity exercise in humans (146, 77); however, the

α2/β2/γ3 heterotrimer may be preferentially activated during short, intense exercise, and correlated with downstream phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) (46). Responsible for malonyl-CoA production, ACC inhibition promotes the oxidation of fat during exercise, and is a particularly sensitive measure of in-vivo AMPK signalling (77, 466). Knockout of the α2 subunit has been shown to reduce basal expression of some mitochondrial enzymes (CS, COX, *β*-HAD), with no change in training-induced increases in these markers; this has been attributed to a compensatory increase in AMPK-α1 activity (212).

Given the integral role of mitochondria in cellular energy homeostasis, AMPK's function as an energy sensing kinase is also closely interconnected with mitochondrial biogenesis. AMPK activation has been implicated in regulating numerous metabolic signalling cascades, serving to prevent anabolism, stimulate catabolism, and promote restoration of cellular energy stores (119). For example, AMPK activation inhibits protein (50, 19) and glycogen synthesis (71), whilst concomitantly stimulating insulin-independent glucose uptake (170, 339), fatty acid oxidation (259, 231), and the phosphorylation of metabolic enzymes (71). The importance of AMPK in skeletal muscle mitochondrial biogenesis is highlighted in rodent studies, whereby pharmacological activation of AMPK increases PGC-1α mRNA expression (511, 537, 469, 200). Once activated, AMPK appears to stimulate PGC-1α activity by removing histone deacetylase's (HDAC) repression of MEF2 (107), phosphorylating PGC-1α directly (204), and/or regulating post-translational acetylation of PGC-1α (268). As such, AMPK activation has been implicated in regulating mitochondrial biogenesis via PGC-1α-mediated gene transcription (204), and modulation of the DNA binding activity of transcription factors including NRF-1, MEF2 and HDAC (107, 310, 43).

Repeated contractile activity during exercise progressively depletes cellular energy stores, thus increasing the AMP:ATP ratio and activating AMPK (181). In rat skeletal muscle, 6 h of low-intensity swimming increased AMPK activation by up to 4-fold immediately post-exercise, with a concurrent increase in PGC-1α mRNA expression (475). Similar fold increases in AMPK activation have also been demonstrated immediately following thirty minutes of treadmill

running (511) and 3 h of low-intensity electrical stimulation designed to mimic endurance exercise (19), also in rats. Following activation, both the α1 and α2 isoforms enhance glucose transport and ACC phosphorylation in rodents (482), with the α2 isoform seemingly preferentially activated with higher intensity exercise (117, 482). These findings have also been replicated in humans (279, 31), suggesting that prolonged, low-intensity muscle contraction can activate AMPK.

Given the main stimulus for AMPK activation is cellular AMP levels, robust changes in intramuscular AMP/ADP:ATP ratios implicate intensity as an important modulator of AMPK activation, enzymatic activity, and subsequent activation of PGC-1α (512, 118, 142, 76, 459). Drastic reductions in ATP content and increases in AMP concentrations (to support ATP regeneration) play a pivotal role in AMPK activation, a response that is more profound with increasing exercise intensity (77). For example, 4 x 30-s Wingate efforts has been shown to decrease ATP content by ~40% (146), as compared with only ~8% reduction in ATP content following 1 h of low- to moderate-intensity (40-80% VO_{2peak}) cycling (77). Increased fast-twitch fibre recruitment, characteristic of short-duration high-intensity exercise, may also play a large role in ATP depletion and subsequent AMPK activation. For example, a single 25-s maximal isokinetic cycling effort has been shown to reduce ATP content by at least ~65% in type II muscle fibres, as compared with only ~25% in type I fibres (225). Activation of AMPK also seems to occur once a certain exercise-intensity threshold is reached. For example, low-intensity exercise at 50% of VO_{2max} has been reported not to activate either the α1 or α2 isoforms, but increasing exercise intensity to 70-75% VO_{2max} can induce a 3-4-fold increase in AMPK-α2 (135, 512). Recent evidence has also demonstrated concomitant activation of both AMPK and PGC-1α following supra-maximal (~200% VO_{2max}) cycling (146, 278), further implicating intensity as an important factor in AMPK activation.

The degree of AMPK activation following exercise is also dependent on an individual's training status and the adaptive status of the muscle. Trained individuals demonstrate a greater AMPK protein content (344), but both short- and long-term aerobic training has been demonstrated to reduce the amount of AMPK phosphorylation in response to a single bout of exercise undertaken at the

same absolute power (308, 532). For example, two weeks of cycling training, combining both moderate-intensity continuous exercise (~75% VO_{2max}) and HIT (90-100% VO_{2max}), significantly reduced both α1 and α2 AMPK activity during a work-matched (pre- vs post-training) cycling bout (308). However, adequate overload (e.g., HIT) has been shown to conserve AMPK activity in highly-trained individuals (80), most likely because of the extensive and rapid reduction in ATP content.

1.3.2.3 Reactive Oxygen Species

ROS refer to both oxygen-centred free radicals (e.g., superoxide – O_2^-) and non-radical but reactive derivatives of oxygen (e.g., hydrogen peroxide – H_2O_2). ROS are commonly formed during normal physiological processes by enzymatic or non-enzymatic sources (127). High levels of ROS have the potential to damage cellular components, and have been implicated in fatigue, disease and aging (249, 150). For example, ROS can cause DNA strand breaks and base repair damage (7, 493), alter enzyme function (397, 203, 207), reduce protein turnover (471, 406, 266), and compromise cellular integrity and function (127, 397). Conversely, low to moderate levels exert positive effects on immune system and essential metabolic functions (478), playing regulatory roles in the control of gene expression, cellular signalling pathways, and the production of force in skeletal muscle (454, 404, 403, 115).

Oxidative stress refers to the imbalance between ROS accumulation, and the body's ability to detoxify the reactive intermediates or repair the resulting damage via antioxidants. Although there is some conjecture to the exact definition of oxidative stress, it is generally accepted that if free radical and ROS production exceeds both endogenous and exogenous antioxidant content (and activity), an oxidative state is likely to exist (439, 390). Antioxidants function to both remove and inhibit the oxidative and damaging chain reactions caused by free radicals, and their efficiency depends heavily on exercise, training, and nutrition status (127, 111). The balance between ROS production and antioxidant levels is particularly pertinent in exercise physiology due to the significant increase in ROS production following exercise (127).

First demonstrated in 1978 by Dillard et al. (112), physical exercise is widely accepted as a potent producer of ROS (95, 492). Elevations in ROS are apparent following endurance (421, 25), isometric (8), and resistance exercise (305), whereby a shift in cellular homeostasis is thought to be the catalyst for its production. During exercise, ROS are a natural by-product of cellular respiration, generated by mitochondrial complexes 1 and 3, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (501, 390, 391). ROS levels can also be increased during intensified periods of training, particularly following sporadic and unaccustomed exercise, and are therefore implicated as a factor involved in overtraining syndrome (380, 311).

Recent evidence has also implicated transient increases in contraction-induced ROS as important stimuli for the signalling system driving mitochondrial biogenesis and oxidative phenotype alterations (115, 201). Potentially serving as a compensation for altered energy demands and respiratory chain defects (434, 3), low to moderate levels of ROS may have a beneficial, hormetic effect on the mitochondria (408). The apparent role of ROS in regulating muscular adaptation to endurance exercise is supported with antioxidant supplementation during exercise training shown to diminish markers of mitochondrial adaptations in rats (467), and either diminishing (409) or even completely abolishing (151) markers in humans (391). However, antioxidant supplementation has also been shown to have no effect on markers of mitochondrial biogenesis following a single exercise bout (151), or on mitochondrial electron transport chain protein content following exercise training (175), both in rat skeletal muscle. As such, investigations into the influence of ROS on both short- and long-term mitochondrial adaptive responses to exercise training are still inconclusive.

A number of recent studies have investigated the potential mechanisms by which ROS may act as a signalling molecule to induce mitochondrial biogenesis. For example, both nuclear factor-κB (NF-κB) and PGC-1α appear to be redox sensitive transcription factors, with ROS accumulation reported to activate their respective upstream kinases, IkB-α kinase (IKK) (216) and p38 MAPK (247, 398, 489, 481). Given the role of both NF-κB and PGC-1α in mitochondrial biogenesis (518, 27), these data establish a potential link between ROS and the induction or mitochondrial biogenesis. Recent research has also highlighted a complex

interaction between ROS and the tumour suppressor protein p53 (282). ROS has been implicated as both an upstream signal that triggers p53 activation, and a downstream factor that can be modulated by p53 (282). The role of p53 in mitochondrial biogenesis is discussed further in section 1.3.2.5.

Recent work in cultured cells and rodent skeletal muscle has demonstrated that ROS-mediated mitochondrial biogenesis is likely to occur via the up-regulation of PGC-1α mRNA (257, 460, 199, 447, 409), and subsequent increase in PGC-1α protein (222, 151). In support of this, research demonstrates a ROS-induced increase in the PGC-1α-targeted transcription factors NRF-1 and Tfam mRNA (257, 320, 151) and protein content (222). The ROS signals that regulate PGC-1α transcription are not fully understood, but ROS may directly increase PGC-1α mRNA via transcriptional activation of the PGC-1α promoter (199). Additionally, ROS may indirectly increase PGC-1α mRNA via ROS-induced ATP reductions and subsequent AMPK activation (199), or stress-induced p38 MAPK phosphorylation (222). Apart from ROS, reactive nitrogen species are also involved in the regulation of mitochondrial biogenesis (84, 347, 348). Nitric oxide (NO) is hypothesised to be an important regulator of PGC-1α, potentially via AMPK and p53-mediated pathways (11, 277). Taken together, these data demonstrate the potentially vital role of contraction-induced ROS as important signalling molecules driving mitochondrial biogenesis.

1.3.2.4 Mitogen-Activated Protein Kinases

Aptly named the 'stress-activated' kinases, MAPK signalling can be activated by a diverse array of stimuli, including inflammatory cytokines, osmotic stress, growth factors, and oxidative stress (119, 75, 287, 481). Increased contractile activity is one such stress, by which a number of biophysical and biochemical processes can activate three main MAPK subfamilies in human skeletal muscle; p38 MAPK, c-*jun* NH_2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) (524, 119). Dependent on type, duration, and intensity of exercise, MAPKs phosphorylate downstream target substrates throughout the cytoplasm and nucleus, including numerous transcription factors and coactivators (185). As such, MAPKs play an integral role in regulating

important cellular processes, including carbohydrate metabolism, inflammation, cellular growth or apoptosis, mitosis, differentiation and gene expression (287).

p38 MAPK:

Strongly activated by environmental stresses and inflammatory cytokines, p38 MAPKs have been implicated in the regulation of a wide spectrum of cellular processes (248, 376). There is also evidence that exercise-induced elevations in calcium concentrations (and CaMKII activation) (514), and ROS (247, 398, 489, 481), can up-regulate the p38 MAPK pathway. Upstream MAPK kinases (mainly MKK3 and MKK6) activate p38 MAPK in a stimulus-dependent manner, via dual phosphorylation of threonine and tyrosine residues on the activation loop (104). Downstream, p38 MAPK phosphorylates the serine and threonine residues of its substrates, including kinases, transcription factors and cell cycle regulators (376). p38 MAPK signal transduction is mediated by four isoforms (α, β, γ, δ), with the γ isoform being the most abundant (105), and expressed almost exclusively in skeletal muscle (104, 270). Several studies have demonstrated the importance of p38γ in various skeletal muscle processes and adaptive responses, including glucose uptake (178), muscle development (132) and mitochondrial biogenesis (386).

Recent evidence has implicated p38 MAPK as a fundamental regulator of PGC-1α-mediated mitochondrial biogenesis (243, 515). For instance, p38 plays a direct role in PGC-1α gene regulation by direct phosphorylation (5, 514, 501, 394). Phosphorylation by p38 MAPK enhances PGC-1α half-life, and disrupts p160 myb binding protein (p160MBP) from binding and repressing its function (125). As a result, p38 MAPK phosphorylation of PGC-1α increases its downstream co-activation of transcription factors (NRF-1/2) and regulation of NUGEMPs (125). This phosphorylation also occurs in parallel with PGC-1α nuclear translocation, and subsequent increases in mitochondrial enzyme mRNA (e.g., citrate synthase and cytochrome *c* oxidase), prior to any increase in mRNA or protein (514). As such, PGC-1α nuclear translocation occurring before the induction of PGC-1α protein expression may be mediated, at least in part, by p38-MAPK-dependent phosphorylation (525).

The integral role of p38 MAPK in mitochondrial biogenesis is further demonstrated in exercised skeletal muscle of transgenic mice. p38 MAPK activation following a single bout of endurance exercise induced a transient increase in PGC-1α mRNA (5), and targeted deletion of p38γ prevents training-induced increases in PGC-1α and other downstream mitochondrial biogenesis markers (386). Overexpression and activation of upstream kinases (MKK3/6) also initiate an increase in PGC-1α protein, as well as the downstream target cytochrome c oxidase (5), thereby demonstrating improved mitochondrial adaptation via the p38 MAPK signalling pathway. p38 MAPK's direct influence on PGC-1α was confirmed by Akimoto et al. (2005), whereby PGC-1α promoter activity in cultured C2C12 cells was increased following p38 MAPK activation, and decreased when specific p38 inhibitors were administered (5). p38 MAPK has also been shown to indirectly influence PGC-1α-mediated mitochondrial biogenesis, by activating numerous transcription factors and coactivators. For example, upstream transcription factors of the PGC-1α gene are shown to be stimulated by p38 in cultured cells, including activating transcription factor 2 ATF2 and MEF2 (5, 70, 534), whose activation coincides with an increase in PGC-1α expression (5, 515, 118). These findings highlight the importance of the p38-PGC-1α axis in mitochondrial biogenesis in skeletal muscle.

Phosphorylation of p38 MAPK in human skeletal muscle has been demonstrated following a variety of exercise modes, including cycling (146, 278, 506, 279, 85), marathon running (531, 54), running (118, 31), high-power resistance exercise (137), and unaccustomed resistance exercise in some (226, 91), but not all (510), studies. Phosphorylation also appears to be independent of exercise intensity, with similar increases reported between high-intensity and continuous running (31). This is consistent with previous reports that p38 phosphorylation is similar after low- and high-intensity cycling, despite large differences in intensity (40% vs 80% VO_{2max}) (118). Gibala et al. (2009) were the first to suggest that low-volume HIT stimulates PGC-1α signalling via p38 MAPK, but independent of the supposed upstream activator CaMKII (146). However, this temporal p38-PGC-1α association is inconsistent with cell culture work demonstrating that p38 MAPK inhibition can prevent the cytosolic calcium-induced increase in PGC-1α mRNA and protein content (514). These findings

have thus implicated other upstream signalling pathways in the activation of p38 MAPK, such as MKK3/6 (248) and AMPK (520). Activation of p38 MAPK following exercise is also a systemic response, as one-legged cycling at 70% VO_{2max} increased phosphorylation in both the exercised and contralateral unexercised leg (506).

Activation of p38 MAPK following exercise is not a forgone conclusion, as phosphorylation is heavily dependent on training status (513, 19). For example, greater phosphorylation has been demonstrated in untrained vs endurance-trained individuals following a single bout of high-intensity cycling, suggesting trained individuals may need a larger stimulus to invoke p38 MAPK activation (532). Training specificity is also a determining factor, as resistance-trained individuals performing an endurance exercise bout, and endurance-trained individuals performing a resistance exercise bout, both resulted in increased p38 phosphorylation, but not when they performed their specific exercise (91). In both instances, unaccustomed mechanical stress is likely to have caused an increase in phosphorylation. In summary, it is evident that p38 MAPK plays an integral role in the adaptive response to exercise, namely via the regulation of exercise-induced mitochondrial biogenesis.

c-Jun NH_2-Terminal Kinase:

Also belonging to the MAPK family, JNK responds to stress stimuli such as inflammatory cytokines, UV radiation, temperature, and osmotic shock. Activation of JNK occurs via the upstream kinases MKK4 and MKK7, and dual phosphorylation of threonine and tyrosine residues (491). Downstream, JNK regulates transcription by modification/activation of numerous transcription factors and target proteins, including c-Jun, ATF2, ETS domain-containing protein (ELK-1), SMAD4, p53, and heat-shock factor protein 1 (HSF1). Activation of JNK is also dependent on the accumulation of ROS, as demonstrated by a reduction in exercise-induced phosphorylation following administration of the anti-oxidant *N*-acetylcysteine (NAC) (379).

In regards to exercise, JNK phosphorylation appears to be dependent on intensity and/or levels of muscle damage, such that intense exercise and/or

exercise eliciting a large degree of muscle tension induces greater activation. Evidence of exercised-induced JNK phosphorylation was first demonstrated in exercised rats, with a 2-3 fold increase (similar to that of p38 MAPK) reported following ten to sixty minutes of treadmill running (20 $m.min^{-1}$ and 10% grade) (156). In a subsequent series of experiments, the authors set out to define JNK's role in adaptation to exercise in human skeletal muscle, and in particular the influence of exercise mode, intensity, and load. Marathon running and sixty minutes of cycling induced significant and transient increases in JNK phosphorylation (54, 14), and also increasing mRNA expression of its downstream target c-Jun following cycling (14). Exercise-induced increases in JNK activation were later determined to be significantly higher following eccentric contraction as compared with concentric contraction, demonstrating JNKs involvement in molecular and cellular adaptations related to muscle damage and injury-producing exercise (53). This finding was further supported in rat muscle, where static stretch produced a more profound activation of JNK than isometric contraction, confirming that the stretch component of muscle contraction (as typically seen in eccentric contractions) to be a major contributor to increases in JNK activity (55). Given that JNK phosphorylation increases linearly with escalating levels of muscular contraction force (296), these data represent a link between tension-dependent JNK activation and transcriptional responses in humans.

Extracellular Response Kinases

Abundantly expressed in skeletal muscle, ERK1/2 are rapidly activated in response to a wide variety of stimuli, including growth factors, receptor tyrosine kinases, and cellular stress (287, 337). As with the other MAPK isoforms, ERK1/2 are activated via phosphorylation by upstream kinases, namely MKK1 and MKK2. Several downstream substrates have been identified, most notably p90 ribosomal S6 kinase (p90rsk), a kinase known to regulate transcription factors like CREB, ETS domain-containing protein (ELK1), estrogen receptor-α (ERα), NF-κB, and c-Fos (133).

Exercise is a potent activator of ERK, with phosphorylation occurring rapidly and maintained throughout exercise (506, 287). Exercise-induced activation has been demonstrated in human skeletal muscle following a single bout of cycling above 70% VO_{2peak} (506, 532, 507), marathon running (531), and knee extensor resistance exercise (29 x ~70% 1-repetition maximum; 1-RM) (510), with further evidence implicating activation in an intensity-dependent manner (507). Similar to JNK, ERK activation originates locally in skeletal muscle rather than as a systemic response to exercise or contractile activity (513). In vitro studies have reported ERK1/2 activation following contraction of isolated rat muscle, suggesting that contraction *per se* may be sufficient in inducing ERK1/2 activation (287). ERK phosphorylation is also dependent on training status, with untrained subjects demonstrating a significantly larger activation as compared with endurance trained subjects following a single bout of high-intensity cycling (8 x 5 min, 60 s recovery, 85% VO_{2peak}) (532).

1.3.2.5 Tumour Suppressor Protein p53

Regarded as the 'Guardian of the Genome', tumour suppressor protein p53 plays an integral role in multicellular organisms, where it regulates cell cycle arrest, apoptosis, angiogenesis, genomic stability and cell deterioration (265). In response to genotoxic stress signals and associated DNA damage, p53 can activate DNA repair proteins, arrest cellular growth, or, in the case of irreparable damage, initiate programmed cell death (apoptosis). However, recent evidence has also implicated p53 activation in response to everyday stressors (e.g., metabolic stress from exercise), highlighting its role in pathways of energy metabolism such as exercise-induced mitochondrial biogenesis (30, 286).

Within the last decade, p53 has been heavily implicated as an important regulator of mitochondrial function, by which it exerts control over many different cellular pathways (424). As shown in a number of different cell and tissue types, p53 plays an important role in regulating mitochondrial content, oxidative capacity and exercise performance (301, 364, 422, 424, 425). The first reported evidence that p53 plays an integral role modulating mitochondrial function was demonstrated in mouse liver mitochondria and human isogenic colon cancer HCT116 cells (301). Targeted disruption of p53 in HCT116 cells was associated

with a significant reduction in oxygen consumption and an increase in lactate production. Furthermore, COX activity was decreased in p53 deficient cells, which the authors attributed to a reduction in synthesis of cytochrome *c* oxidase 2 (SCO2) content (a direct transcriptional target of p53 important for COX subunit 2 assembly).

Similar findings have also been reported in rodent skeletal muscle, with genetic deletion of p53 severely inhibiting mitochondrial function. p53 knockout mice exhibited reduced mitochondrial content (subsarcolemmal and intermyofibrillar), PGC-1α expression, and COX activity (422), reduced Tfam mRNA and protein content (364), and reduced exercise performance (swimming 'stress test') (301) as compared with wild-type animals. Saleem et al. (422) similarly demonstrated a reduction in mitochondrial respiration and exercise performance (rate of fatigue during electrical stimulation), and elevated ROS production (intermyofibrillar mitochondria), consistent with previous work (301). Furthermore, Park et al. (364) demonstrated that p53-ablated mice were unresponsive to endurance exercise training, with five weeks of treadmill exercise inducing no significant changes in VO_{2peak}, work capacity or respiratory exchange ratio (RER – marker of substrate oxidation) (364). Although current understanding of p53's role in skeletal muscle and exercise metabolism is still elementary, the above results implicate it as an important regulator of mitochondrial content, function and endurance exercise performance.

The early research in rodent and cancer cells has also shed some light on the potential upstream and downstream molecular signalling pathways involved in p53's influence on mitochondrial content and function, and the influence exercise has on this response. Contractile activity, in the form of electrical stimulation in rodents, induced a two-fold increase in p53 phosphorylation immediately post-exercise, with a possible associated increase in the activation of upstream kinases AMPK and p38 MAPK (422). This is consistent with previous work demonstrating that both AMPK (211) and p38 MAPK (441) phosphorylate p53 directly at the serine^{15} residue. More recently, Bartlett et al. (31) demonstrated comparable findings in human skeletal muscle, with a single bout of either high- or moderate-intensity exercise (work-matched) inducing parallel contraction-induced p53 phosphorylation, in a time course related to AMPK and p38 upstream

signalling. Reduced carbohydrate availability has also been demonstrated to elevate exercise-induced p53 phosphorylation (32), hypothesised to be mediated by an AMPK/p53 signalling axis (30). Taken together, it is currently unknown whether the established exercise-activated kinases AMPK and p38 MAPK are a means by which p53 initiates mitochondrial biogenesis in response to contractile activity (422), and future research in this area is warranted.

Subsequent to the phosphorylation of p53, research has documented the potential downstream pathways surrounding its role in exercise-induced mitochondrial biogenesis (Fig 1-5). Following ninety minutes of moderate-intensity exercise (15 $m.min^{-1}$) in mice, p53 has been shown to undergo post-translational modification and translocation into the mitochondria matrix, where it is involved in regulating the mitochondrial genome (425). Within the mitochondria, p53 interacts with Tfam to ensure genomic integrity and stability and to positively regulate mtDNA transcription and translation (425). Exercise may also cause p53 to translocate into the nucleus and regulate the expression of other proteins involved in mitochondrial biogenesis. For example, p53 nuclear translocation has been observed to regulate apoptosis inducing factor (AIF), dynamin-related protein 1 (Drp1), mitofusin-2 (Mfn2) and heat-shock protein 70 (HSP70) in cardiac and cancer cell lines (267, 462, 494). However, nuclear p53 has been shown to both increase (381) and decrease (425) following a single bout of exercise (60-90 min treadmill running at an average intensity of ~15 $m.min^{-1}$) in rodents, further warranting future research to confirm the role of p53 in mitochondrial biogenesis.

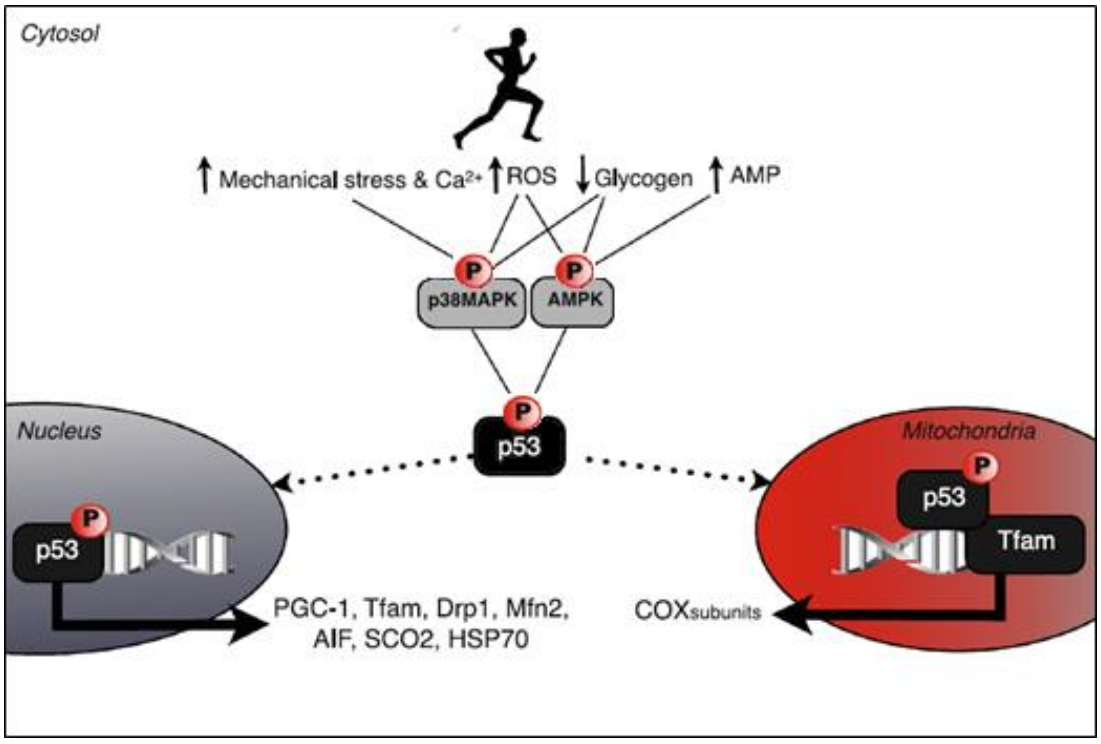


Figure 1-5: Potential signalling pathways by which exercise may influence p53 signalling. Ca^{2+}, calcium; ROS, reactive oxygen species; AMP, adenosine monophosphate; p38MAPK, p38 mitogen-activated protein kinase; AMPK, adenosine monophosphate-dependent protein kinase; p53, tumour suppressor protein p53; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Tfam, mitochondrial transcription factor; Drp1, Dynamin-related protein 1; Mfn2, Mitofusin 2; AIF, Apoptosis-inducing factor; SCO2, Synthesis of cytochrome c oxidase 2; HSP70, Heat-shock protein 70; COX, cytochrome *c* oxidase. Bartlett et al. (30).

In summary, exercise training is a powerful stimulus to increase mitochondrial biogenesis. While more research is required, the main molecular mechanisms underlying exercise-induced mitochondrial biogenesis are related to the complex interaction between metabolic stimuli (e.g., ATP turnover, calcium flux and redox balance), cellular signalling (e.g., AMPK, p38 MAPK, and ROS), regulatory proteins (e.g., PGC-1α and p53), and downstream targets (e.g., transcription factors and mitochondrial proteins).

1.4 Potential Influence of Cold on Training Adaptations

Despite its widespread use as a recovery modality, debate currently exists surrounding CWI's merit in promoting training adaptations (164). Most athletes and sports practitioners base treatment on anecdote rather than evidence-based practice and empirical evidence (509, 49), with insight into the underlying physiological mechanisms lacking. Research to date reports short-term benefits related to enhanced recovery of skeletal muscle performance (26, 147, 326, 449, 486, 487, 526); however, those benefits may be counteracted by possible long-term detrimental effects on adaptation (523). Post-exercise fatigue and/or inflammation are important stimuli for adaptation and performance improvements to occur (89, 82), and may be hindered by CWI (164). Conversely, CWI may expedite recovery and allow for greater training load or quality at a subsequent session (164), or even stimulate gene expression of key proteins involved in mitochondrial biogenesis (192). Given the widespread use and acceptance by athletes of CWI as a recovery modality, it is important that its effects on training adaptation be verified. Sections 1.4 and 1.5 will explore the potential molecular mechanisms by which; 1) the adaptive response in skeletal muscle may be altered following cold exposure, and 2) post-exercise CWI may affect human skeletal muscle adaptations following high-intensity interval training.

1.4.1 Cold-Stimulated Thermogenesis and Mitochondrial Biogenesis

Ultimately acting as a stress signal, variations in temperature influence a wide array of cellular responses. Hyperthermic and hypothermic conditions induce similar phenotype modifications, including 1) alterations to mRNA stability, transcription and translation, 2) denaturation and a generalised reduction in protein synthesis, 3) decreased metabolic rate, 4) initiation of cell cycle arrest/apoptotic pathways, and 5) disruptions to cell membrane permeability and cellular cytoskeletal structure (16, 218, 455, 385, 340). As such, temperature may influence certain adaptive mechanisms, including the regulation of mitochondrial biogenesis (452).

As seen in Table 1-1, cold exposure has been shown to be a powerful stimulus for mitochondrial phenotype alterations. Increased mitochondrial density and enzyme content are well-known characteristics of fish during acclimation to seasonal cold (34, 120). In cold-bodied fish, persistent cold stress compromises the ability to produce ATP, a potential mechanism that to stimulates aerobic metabolism and metabolic remodelling (351). Aerobic-related adaptations following cold exposure may be largely related to thermogenesis, whereby energy expenditure and metabolic demand are increased in an attempt to produce heat.

1.4.1.1 Shivering Thermogenesis

One possible mechanism by which cold exposure may promote mitochondrial biogenesis is via shivering thermogenesis. Shivering is the involuntary contraction of skeletal muscle in response to cold, by which a natural by-product is heat. Repeated muscular contractions during shivering may increase resting metabolic rate by up to five-fold (206) and oxygen consumption by more than two-fold (451, 452), which will be accompanied by a reduction in cellular energy stores (451). As a consequence of contraction-induced energy expenditure from shivering, free cytosolic Ca^{2+} and AMP levels will be increased. As a direct result, shivering-induced activation of CaMK and AMPK may be a means by which cold exposure may increase PGC-1α-mediated mitochondrial biogenesis (452).

In a series of experiments by Slivka et al. (451, 452), post-exercise cold exposure in humans was shown to increase PGC-1α mRNA, attributed to shivering thermogenesis. In their first experiment, participants were exposed to cold air (7°C) for 4 h following 1 h of cycling at 60% maximal aerobic power (W_{max}) (452). As compared with thermo-neutral (20°C; ~6.4 fold) and hot (33° C; ~4.8 fold) air, the cold condition induced significantly larger increases in PGC-1α mRNA (~8.5 fold), consistent with prior work in animal models (357, 436, 59, 194). Interestingly, this increase in PGC-1α mRNA did not translate into alterations in associated downstream markers of mitochondrial biogenesis, such as COX, uncoupling protein-3 (UCP3) and Mfn2 (452). This may be explained by sample timing however, as COX and Mfn2 mRNA have previously been shown to

Table 1-1: A summary of current evidence regarding short- and long-term effects of cold exposure on endurance-related skeletal muscle adaptations.

Study	Sample Size (sex)	Species	Exercise Protocol	Cold Intervention		Muscle Alterations (vs control)	
				Single	Repeated	mRNA	Protein
Ihsan et al. (192)	9 (M)	Human	Running 30 min (70% V_{max}) + HIT to exhaustion (100% V_{max})	CWI ~10°C for 15 min	-	↑ PGC-1α	-
Slivka et al. (451)	8 (M)	Human	Cycling 1 h (65% VO_{2max})	Cold air 7°C during exercise and 4 h of recovery	-	↑ PGC-1α ↓ ERR-α, NRF-2	-
Slivka et al. (452)	9 (M)	Human	Cycling 1 h (60% W_{max})	Cold air 7°C during exercise and 3 h of recovery	-	↑ PGC-1α	-
Kim et al. (235)	20 (M)	Rat	-	CWI 18°C for 1 h	CWI 18°C for 1 h 5 d/wk, 20 wk	Single: ↓VEGF Repeated: ↑ VEGF	Single: ↑VEGF Repeated: ↑ VEGF
Ijiri et al. (194)	12 (single) 18 (repeated)	Chickens	-	Cold air 4°C for 24 h	Cold air 4°C for 8 d	↑ PGC-1α (single) ↓ LDH and myostatin	↑ COX and LDH
Bae et al. (24)	10 (F)	Human	-	-	Habitual CWI divers	-	↑ IIx m. fibre % ↓ IIa m. fibre % ↓ m. fibre size ↑ capillary density
Young et al. (529)	18 (M)	Human	Cycling Training 1 h (60% VO_{2max}) 5 d/wk, 8 wk	-	CWI 20°C while training	-	↔ glycogen

Young et al. (530)	18 (M)	Human	Cycling Training 1 h (60% VO_{2max}) 5 d/wk, 8 wk	-	CWI 20°C while training	-	↔ CS activity
Bruton et al. (59)	20	Mice	-	-	Cold air 18°C for 4 wk + 4°C for 4-5 wk	↑ Tfam	↑ PGC-1α ↑ CS activity
Oliveira et al. (357)	-	Rats	-	-	Cold air 4°C for 4 d	↑ PGC-1α	↑ PGC-1α and UCP3
Seebacher et al. (436)	36 (M + F)	Rats	Running Training 30 min (60% W_{max}) 5 d/wk, 30 d		Cold air 12°C for 30 d	↑ NRF-1 and UCP1	-
Mitchell et al. (321)	32 (M)	Rats	Running Training 60 min (20 $m.min^{-1}$) 5 d/wk, 9 wk	-	Cold air 8°C and 4°C for 9 wk	-	↑ COX and mtHSP70
Mizunoya et al. (324)	12 (M)	Rats	-	-	Cold air 4°C for 4 wk	↑ PGC-1α and myoglobin	↑ PGC-1α
Stancic et al. (463)	42 (M)	Rats	-	-	Cold air 4°C for 1-45 d	-	↑ PGC-1α, Cyt c, ATP synthase, LDH, ACADM, SCAS

V_{max} = Maximal aerobic running velocity; HIT = High-intensity interval training; CWI = Cold-water immersion; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; VO_{2max} = Maximal oxygen uptake; ERR-α = Estrogen-related receptor alpha; NRF = Nuclear respiratory factor; W_{max} = Maximal aerobic power; VEGF = Vascular endothelial growth factor; CS = Citrate synthase; Tfam = Mitochondrial transcription factor; LDH = Lactate dehydrogenase; COX = Cytochrome *c* oxidase; UCP = Uncoupling protein; mtHSP70 = Mitochondrial heat-shock protein 70; Cyt c = cytochrome c protein; ACADM = Medium chain fatty acids acyl-CoA dehydrogenase; SCAS = succinyl-CoA synthetase.

peak closer to 24 h post exercise (72), and may need an increase in PGC-1α protein content to increase their transcription (456, 433). In a follow up study, participants exposed to cold air (7°C) for 3 h following 1 h of cycling at the power output associated with 65% VO_{2max} similarly demonstrated an increase in PGC-1α mRNA (~7.9 fold) as compared with a room temperature control (20°C; ~4.0 fold), and also no effect on the downstream transcription factors NRF-1 or Tfam (451). In fact, ERRα (inducer of Acetyl-CoA dehydrogenase) and NRF-2 (upstream of COXIV) were actually lower after recovery in the cold, raising the possibility that cold exposure alone or in combination with exercise may actually inhibit mitochondrial adaptations. It is likely that the increase in PGC-1α mRNA in these studies was the result of shivering-induced CaMK and/or AMPK activation, but whether this increase is likely to induce alterations in mitochondrial phenotype is unknown.

1.4.1.2 Non-Shivering (Adaptive) Thermogenesis

Mitochondrial biogenesis may also be promoted during cold exposure as a result of non-shivering, or adaptive, thermogenesis. Most adaptive thermogenesis in small mammals occurs in brown adipose tissue (395), but in mammals with relatively little brown fat skeletal muscle plays a much larger role (538). The adaptive thermogenesis response is characterised by the recruitment of uncoupling proteins (UCPs) to produce heat instead of shivering (59, 67). These proteins allow the dissipation of the proton gradient before it can be used to produce ATP, thus allowing the chemical energy stored within food to be dissipated as kinetic energy to produce heat (67). As such (and similar to shivering thermogenesis), adaptive thermogenesis in response to cold is accompanied by and related to a reduction in energy (ATP) stores (and an increase in the AMP/ATP ratio), increased oxygen consumption/metabolic rate, and increased mitochondrial-uncoupled respiration (272, 508).

Enhanced mitochondrial biogenesis is an important component of adaptive thermogenesis, a response that is potentially mediated by PGC-1α (395). Cold exposure has been shown to increase PGC-1α mRNA in brown adipose tissue (396), rodent (518) and chicken (194) skeletal muscle, and more recently in

human skeletal muscle following exercise (192). This response may be mediated via a cold-induced noradrenaline release (440) and subsequent stimulation of the sympathetic nervous system's β_3-adrenergic receptor and the downstream protein kinase A (PKA)/cAMP pathway (396, 271). In support of this, PGC-1α mRNA is increased in murine skeletal muscle following treatment with a β-adrenergic agonist, and abolished when treated with a β-adrenergic antagonist (322). Alternate mechanisms by which PGC-1α mRNA may increase as a result of adaptive thermogenesis include altered Ca^{2+} handling and potential CaMK/calcineurin activation (59), or increased AMP/ATP ratio as a result of uncoupled respiration and subsequent AMPK activation (357); however, evidence to support these theories is lacking. An increase in PGC-1α protein following nine weeks of cold acclimation (18°C for 4 wk + 4°C for 4-5 wk) has also been reported in rodents (59), as observed in skeletal muscle that does not participate in the shivering response (172). The essential role of PGC-1α in adaptive thermogenesis is supported by the observation that PGC-1α-KO mice were unable to withstand a cold stress (4°C) for longer than 6 h due to a continual decrease of core body temperature (263, 275). Given the well-established role of PGC-1α in the stimulation of mitochondrial biogenesis (356), cold-induced increases in mRNA and protein content may provide a plausible molecular basis for the connection between cold stimulus and mitochondrial biogenesis following increased thermogenic requirements (395).

Adaptive thermogenesis has also been demonstrated to improve a number of other markers of mitochondrial biogenesis or development. For example, lowering core temperature during nine weeks of endurance exercise (5 d/wk, 60 min at 6% grade at 20 $m.min^{-1}$) in rats increased COX activity and protein content, as well as mitochondrial heat-shock protein 70 (mtHSP70) content (321). The authors proposed that increased mitochondrial content and enzyme activity following regular cold exposure may serve to compensate for a cold-induced decrease in muscle compliance – i.e., reductions in oxygen diffusion (39) and enzyme catalytic activity (470). Furthermore, increased mtHSP70 content is also a sign of mitochondrial development, given its function as a chaperone for the mitochondrial protein transportation (HSP function is discussed further in 1.5.2) (328). Cold exposure has also been demonstrated to facilitate increases in slow- to

fast-twitch muscle fibre ratio, basal calcium levels (a method by which to increase PGC-1α mRNA via CaMK and calcineurin activation), and the activity of important mitochondrial enzymes like citrate synthase (CS), COX, LDH, acyl-CoA dehydrogenase (ACADM) and succinyl-CoA synthetase (SCAS) (194, 59, 463).

Skeletal muscle mitochondrial biogenesis following a cold stimulus may also be dependent on an exercise stimulus. Seebacher & Glanville (436) investigated a number of mitochondrial markers in rats that were exercised for five weeks at either 22 or 12°C, as compared with non-exercised rats kept at 22°C. Cold ambient temperatures additively increased exercise-induced increases in NRF-1 mRNA in skeletal muscle and *PPARδ* in brown adipose tissue, but only a combination of cold and exercise increased UCP-1 expression. Furthermore, cold stimulus alone had no influence on PGC-1α/δ, NRF-1, COX or CS activity. This is supported by Mitchell et al. (321), who demonstrated an increase in COX activity in the gastrocnemius muscle of rats exercised in cold temperatures, but not in the inactive muscle extensor digitorum longus, which received the cold stimulus but was not involved in contraction. These data demonstrate that a cold stimulus alone may be insufficient to induce changes in these markers of mitochondrial biogenesis, which are critically dependent on the presence of an exercise stimulus.

Further work is needed to determine if both shivering- and non-shivering-induced increases in PGC-1α mRNA and protein can result in increased mitochondrial protein content and function. As PGC-1α is a potent stimulator of transcriptional factors ERR-α, NRF-1/2 and Tfam, PGC-1α may mediate the adaptive response to cold application. Furthermore, this may provide a potential mechanism by which post-exercise CWI may improve skeletal muscle adaptations to training.

1.4.2 Thermal Shock Response

Thermal stress is one of the most common stresses experienced in a wide variety of organisms, including both prokaryotic and eukaryotic systems (455, 6). Whilst extensive research has defined the complex program of adaptive responses

to heat stress, little is known about the molecular mechanisms governing adaptations to cold stress (6). An innate ability to survive and adapt to thermal stress is dependent on the conserved and co-ordinated up-regulation or induction of both cold- (CSP) and heat-shock proteins (HSP), whose primary function is to protect and prepare for the cellular stresses imposed by thermal shock (276). Recent evidence, however, has implicated mild hypothermia and subsequent rewarming of cells inducing potentially damaging oxidative stress (340). This section will explore the current knowledge surrounding the mechanisms by which thermal shock serves to protect cells and/or stimulate adaptation, and more importantly its role in adaptations to exercise.

1.4.2.1 Cold Shock

Depending on the severity of the exposure, cold stress in mammalian cells can activate a stress response, potentially leading to cellular death by apoptosis or necrosis (455). Mild hypothermia (25-35°C) provides cells with a suitable environment to still proliferate and grow, with more extreme temperatures (0-10°C) likely to suspend growth completely (411). Activation of p38 MAPK and translocation of β-crystallin from the nucleus to the cytoplasm are known responses to mild hypothermia (94, 152), with apoptotic and necrotic responses reserved for more severe cold exposures (455, 158). Further to the exposure temperature and duration, cold-induced apoptosis is also dependent on other factors such as cell line, cell cycle phase and calcium levels (455). It is well accepted that a general response to cold stress is a suppression of transcription and translation, accompanied by a reduction in RNA degradation and alternate splicing of pre-mRNA (522, 16, 455). Furthermore, the translation and transcription of certain targets are actually improved with cold stress, courtesy of a select number of cold-shock proteins whose regulation is unaffected or even up-regulated (121, 114).

During cold exposure, the expression of a number of cold-shock proteins is rapidly and markedly increased, most notably cold-inducible RNA-binding protein (CIRP) and putative RNA-binding motif protein 3 (RBM3). In response to mild hypothermia (maximal expression around 32°C), both these proteins appear to play integral roles in adaptation to cold-stress, potentially by acting as RNA

chaperones during stress (114). In support of this role, CIRP has been demonstrated to translocate from the nucleus to the cytoplasm in response to UV irradiation, binding to the RNA of several stress-inducible molecules (527). Here, CIRP is thought to enhance translation of its target RNA species, at least in part through mRNA stabilisation (455). Furthermore, CIRP may also play an integral role in cell cycle control, either by supressing mitosis, assisting in maintaining differentiated states, or by playing a role in cell cycle arrest (136, 346).

Structurally similar to CIRP, RBM3 expression is also enhanced under cold stress. RBM3 is thought to facilitate global protein synthesis under cold stress, whereby specialised sequences in its mRNA 5' leader sequence allow translation at temperatures that protein synthesis is normally inhibited (114, 74, 455). In addition to this, but beyond the scope of this review, CIRP and RBM3 are also upregulated in response to hypoxia (500). In fact, the hypoxic signalling pathway is thought to share common components with cold-shock signalling pathways (152, 6). Oxygen is dissolved at higher concentrations as temperature drops (355), meaning that hypothermic stress may also induce oxygen stress (6). The above data demonstrate that via the up-regulation of CSPs, cold stress has the ability to alter gene expression, RNA processing and intracellular signalling (340).

1.4.2.2 Rewarming

Following an initial cold shock, gene expression is also altered upon return to normothermia whereby rewarming of the cell may induce numerous stress responses. As such, it is evident that cells somehow acclimatize to mild temperature and sense relative rather than absolute hyperthermia (281, 340, 250). By inducing a variety of cellular responses, such as stress-activated signalling and heat-shock protein expression, rewarming could potentially alter training-induced skeletal muscle adaptations.

Stress-Activated Signalling

Hypothermic conditions are believed to activate cellular stress responses, such as protein denaturation and stress-induced kinase activation (e.g., MAPK phosphorylation) (455). However, given that transcription and translation are reduced as a result of cold shock, stress-related signalling may not occur until

rewarming occurs. For example, p38 MAPK-dependent induction of interleukin-8 (IL-8) mRNA has been reported to occur upon rewarming human bronchial epithelial cells from 1°C to 37°C, despite p38 MAPK phosphorylation occurring when exposed to 1°C (152). Similarly, JNK phosphorylation is transiently increased upon severe hypothermia (5°C) in hepatoblastoma cells, but further increased upon the return to normothermia (355). As such, it appears that rewarming sustains or exacerbates the stress imposed by the initial cold shock (340).

Activation of these stress-induced kinases in response to thermal-shock may serve as stimuli for autophagy, or conversely to promote mitochondrial biogenesis. Autophagy is the process by which unnecessary cellular components are degraded, in this instance most likely as an adaptive response to cold stress to promote survival (244). For example, autophagy has been observed during the return to normothermia after cold-shock (289, 340), potentially mediated by JNK phosphorylation (244). Conversely, given that MAPK signalling is heavily implicated in PGC-1α activity (243, 514, 345), activation of these signalling kinases as a result of cold shock and subsequent rewarming may play a role in thermal-induced mitochondrial biogenesis. To date however, no research has investigated this response in human skeletal muscle.

Heat-Shock Response

Originally thought to be activated as a cell survival mechanism following heat shock (276), a return to normothermia following an initial cold shock may elicit phenotypical changes typically observed following heat stress. Non-lethal heat stress will alter the expression of certain genes to ultimately improve thermo-tolerance and the ability to survive subsequent heat stress (455). This heat-shock response is typically associated with the increased activation of heat-shock transcription factors (HSF), and the subsequent expression of HSPs.

The main function of heat-shock proteins is to act as protein chaperones, by which they assist in the refolding of denatured proteins, and also stabilise the confirmation of proteins important for a variety of different cellular regulatory processes (455, 368, 141). Further to this, HSPs also regulate cellular redox state and signal transduction (361), protein turnover (368), and inflammation (18).

Heat-induced activation of HSF-1 upregulates a number of heat-shock proteins, including HSP25, HSP70, HSP90, HSP105 and APG-1 (250, 281, 221, 179). HSF-1 is transported into the nucleus upon heat stress, where it binds to the heat shock element (HSE) in the promoter region of susceptible heat-shock protein genes (455). Oxidative stress, ischemia, depleted energy stores, viral infection and/or changes in pH can all also induce HSF activation and HSP expression, thereby highlighting their role in the cellular stress response (331, 327).

Heat-shock proteins are also heavily upregulated in response to a single bout or regular exercise training (232, 234, 331, 332, 304, 319, 284), implicating them in a number of skeletal muscle remodelling processes associated with exercise training (327). For example, HSPs act as chaperones for the importation of nuclear-encoded proteins destined for the mitochondria, and therefore play an essential role in mitochondrial biogenesis (184). HSPs are also implicated in regulating apoptosis (426), improving glucose tolerance/insulin sensitivity (79), and cytoprotective roles against exercise-induced cellular stress (331) and age-related reductions in muscle function (303). Basal HSP content appears to be higher and exercise-induced stress response lower, in trained as compared with untrained human skeletal muscle (331). Expression also appears to be dependent on exercise intensity and the associated increases in recruitment patterns and muscle load (284, 319), further highlighting the protective function HSPs play in response to cellular stress.

1.4.2.3 Cell Cycle Arrest, DNA Damage and p53

Given the evident stress imposed on a cell following cold-shock and subsequent heat-shock upon rewarming, thermal shock has also been implicated in activation of the p53 pathway. Genotoxic stresses, such as ultraviolet or ionising radiation, are well known activators of p53, inducing cell-cycle arrest for DNA repair or apoptosis. However, p53 has also been shown to be activated following ‘non-genotoxic’ physiological stresses such as thermal shock (300, 353), with suggestions that hypothermic and subsequent rewarming shocks may actually induce a DNA-damage response (340).

A well-accepted role of p53 in response to cold-shock is the regulation of cell cycle arrest. Both mild (300, 411) and severe (354) hypothermia are known to induce cell cycle arrest in rodents and cultured cells, most likely regulated by p53 and subsequent p21 activation. Also known as WAF1/CIP1, p21 is a cyclin-dependent kinase inhibitor that also regulates cell cycle progression (140). Upon rewarming, re-entry into cell cycle is likely mediated by increased levels of another stress protein, mtHSP70. mtHSP70 functions to prevent nuclear translation of p53 and subsequent p21 transcription (228), to alter the binding of p53 to cell centrosomes (291), and to promote monopolar spindle 1 (Mps1) protein kinase-mediated centrosome duplication (220). As such, these data implicate both p53 and mtHSP70 as important regulators of cell cycle arrest upon cold stress and rewarming.

A recent study by Neutelings et al. (340) sought to assess the effects of a mild cold shock on the cellular stress response and potential DNA damage response. Cultured cells exposed to 25°C for five consecutive days increased phosphorylation of p53 and H2A histone family member X (H2AX), a DNA structural protein whose phosphorylation is indicative of DNA double-strand breaks. Although the exact mechanisms leading to DNA damage remain elusive, the authors suggested that rewarming following a hypothermic shock may induce an acute and rapid oxidative burst, which in turn may initiate a DNA damage response and apoptosis (ROS are discussed further in Section 1.5.3) (340). This suggestion is supported in cultured rodent cells, where ROS production may play a key role in cold-induced apoptosis (402, 401). A low level of apoptosis was also reported following cold-stress, independent of MAPK stress signalling (340), potentially regulated by p53. p53-related genes, including p53-inducible gene 3 (PIG3) (343, 258), Bcl-2 associated X protein (443, 323), and mouse double minute 2 homolog (Mdm2) may all play a role in this response (340). These data heavily implicate p53 as an important regulator of cellular processes following hypothermic shock, and also the subsequent rewarming and associated oxidative stress.

1.4.3 Cold-Induced Oxidative Stress

Both adaptive and non-adaptive thermogenesis elevate metabolism and stimulate mitochondrial respiration, of which a natural by-product is reactive oxygen species (501). Shivering is an involuntary, repeated rhythmic muscle contraction, and has thus been associated with free-radical production (438). Non-shivering thermogenesis generates heat by allowing for rapid substrate oxidation with a low rate of ATP production, thereby increasing metabolism and potentially free-radical accumulation (49). ROS production may also be further exacerbated by the oxidation of certain molecules in the presence of oxygen, initiating a free-radical chain reaction (163). For example, catecholamine levels are increased following CWI, and are readily oxidised to superoxide anions with oxygen present (49). In support of this, baseline antioxidant levels are reportedly higher in individuals who habitually swim during winter, suggesting an adaptive response to repeated cold-induced oxidative stress (445, 446).

Another potential mechanism by which cold exposure induces oxidative stress is during rewarming. Upon return to normothermia after a hypothermic exposure, it is hypothesised that a small burst of ROS is induced, most likely the result of a sudden increase in mitochondrial electron transport (281). As discussed in section 1.4.2.3, this burst has the potential to induce DNA damage, trigger the DNA damage response (DDR) and provoke apoptosis/cell death (340). As such, an oxidative burst upon rewarming may serve as a potent stimulus for activation of the p53 pathway (340), potentially leading to mitochondrial biogenesis.

In summary, Section 1.4 has highlighted a number of molecular mechanisms by which the adaptive response in skeletal muscle may be altered following cold exposure. These include the stimulation of mitochondrial biogenesis as a result of thermogenesis, the stress-activated signalling and protein induction in response to thermal shock, and cold-induced elevations in ROS. With these mechanisms in mind, Section 1.5 will discuss the potential influence of post-exercise CWI on this response in human skeletal muscle, whilst also highlighting the research performed to date and directions for future investigation.

1.5 Potential Role for Post-Exercise CWI

Despite the popularity of post-exercise CWI in athletic training regimes, limited research has investigated its potential to improve the adaptive response to training. Should CWI application improve recovery between training sessions, it may serve to improve subsequent training load, thereby providing a greater stimulus for adaptation. Alternatively, given the mechanisms discussed in Section 1.4, CWI may stimulate a number of cellular responses to improve mitochondrial adaptations in human skeletal muscle. This section will focus on the potential influence of post-exercise CWI on the adaptive response to endurance-related training, with particular focus on the potential molecular pathways by which it may improve mitochondrial biogenesis.

1.5.1 Cell Signalling

As outlined in Figure 1-6, CWI may stimulate a number of cellular signalling pathways to improve mitochondrial adaptations in human skeletal muscle. Consistent with the mechanisms discussed in Section 1.4, CWI will significantly reduce muscle temperature (335, 56, 302, 97), following which a rewarming response will occur upon the return to normothermia. For example, CWI (10°C for 15 min) following exhaustive cycling exercise can reduce deep muscle temperature to ~33.6°C (56), and even below ~30°C at a depth of 3 cm (192). Furthermore, muscle temperature may continue to reduce up to ~1 h post-immersion (97, 159), the extent of which is dependent on water temperature (302) and duration of immersion (373). The mechanisms by which CWI may promote subsequent exercise-induced mitochondrial adaptations include adaptive thermogenesis, oxidative stress, and thermal shock.

1.5.1.1 Adaptive Thermogenesis

Only one study has been performed to date investigating the effects of post-exercise CWI on signalling responses associated with mitochondrial biogenesis (192). Following a single bout of high-intensity running (30 min continuous running at 70% VO_{2max} followed by intermittent running at 100% VO_{2max} until exhaustion), participants immersed one leg in 10°C water for 15 min, with the

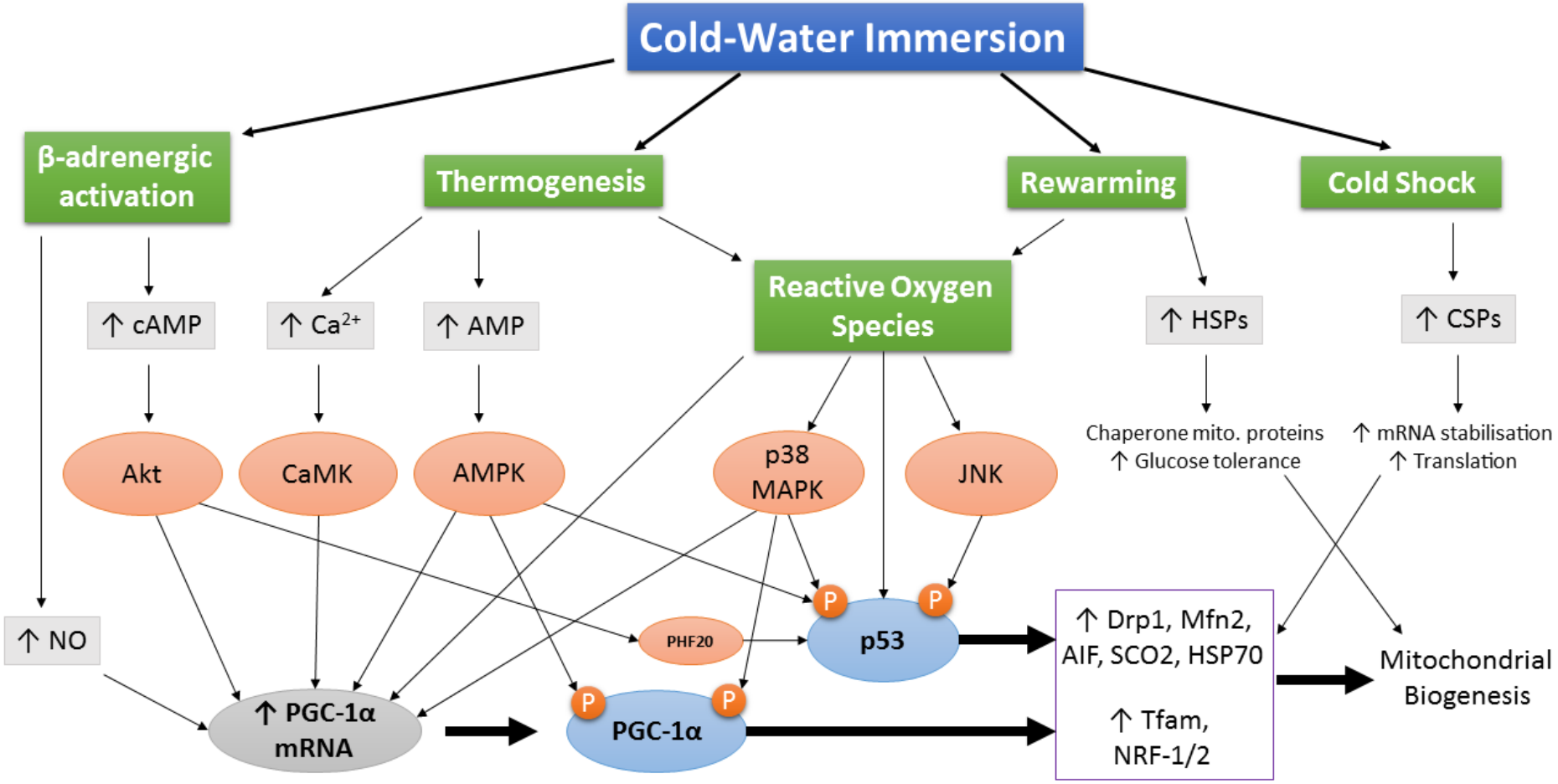
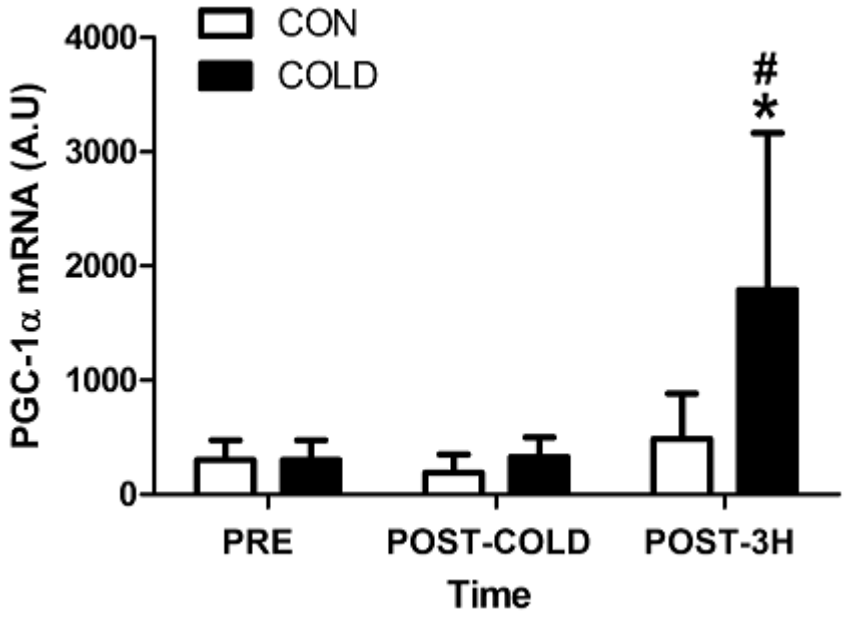


Figure 1-6: Potential molecular mechanisms by which post-exercise cold-water immersion (CWI) may improve mitochondrial biogenesis. cAMP, cyclic AMP; Ca^{2+}, calcium; AMP, adenosine monophosphate; ROS, reactive oxygen species; HSPs, heat-shock proteins; CSPs, cold-shock proteins; NO, nitric oxide; Akt, protein kinase B; CaMK, calcium/calmodulin-dependent kinase; p38MAPK, p38 mitogen-activated protein kinase; AMPK, adenosine monophosphate-dependent protein kinase; JNK, c-Jun N-terminal kinase; CREB, cAMP response element-binding protein; PHF20, plant homeodomain finger protein 20; p53, tumour suppressor protein p53; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Drp1, Dynamin-related protein 1; Mfn2, Mitofusin 2; AIF, Apoptosis-inducing factor; SCO2, Synthesis of cytochrome c oxidase 2; HSP70, Heat-shock protein 70; Tfam, mitochondrial transcription factor; NRF, nuclear respiratory factor.

other leg in ambient temperature air acting as the control (192). Importantly, the authors were able to prevent an increase in metabolic activity and microvascular perfusion with the CWI protocol, and therefore prevent a shivering thermogenesis response (193). As such, should thermogenesis occurring during a typical CWI protocol (e.g., 10°C for 15 min), it is likely to occur independent of shivering (i.e., adaptive). As seen in Fig 1-7, post-exercise CWI significantly increased (~6 fold) PGC-1α mRNA in the experimental leg (CWI) as compared with the control leg (~1.5 fold). However, a limitation with this study is the exercise-induced increase in PGC-1α mRNA reported in the control leg (~1.5 fold), which is considerably lower than what has been previously been reported (~3-4 fold minimum) 3 h after a single bout of high-intensity exercise (278, 146, 31). As such, the large increase in PGC-1α mRNA following CWI reported by Ihsan et al. (192) is likely to be confounded by the subdued response in the control condition, and may not truly reflect the potential of post-exercise CWI to augment PGC-1α-related mitochondrial biogenesis.



<u>Figure 1-7:</u> PGC-1α mRNA prior to exercise (PRE), following 15 min of cold-water immersion (CWI) at 10°C (POST-CWI) and 3 h post-exercise (POST-3H). *Significantly different from PRE in COLD ($p<0.01$). # Significantly different between CON and COLD ($p<0.05$) (192).

Despite this limitation, Ihsan et al. (192) do provide insight into the potential mechanisms of an increase in PGC-1α mRNA following post-exercise CWI. Given the apparent lack of shivering, this increase was attributed to adaptive

thermogenesis via a cold-induced β-adrenergic stimulation, and subsequent increase in NO synthesis. Although NO is important for mitochondrial biogenesis (348), and is known to cooperate with AMPK in regulating PGC-1α in skeletal muscle (277), the authors did not report a significant difference in nitric oxide synthase (NOS) markers between the cold and control leg. The lack of difference in NOS markers, despite an obvious difference in the downstream activator PGC-1α, may be explained by other upstream signals. For example, AMPK (107, 204, 268) and CaMK (166, 517, 516) are well-documented modulators of PGC-1α activity, and in turn are activated following cold exposure independent of shivering (321, 59). Cold exposure can also alter Ca^{2+} release (20) and re-uptake (15) within skeletal muscle not involved in shivering, in turn increasing free myoplasmic Ca^{2+} and inducing CaMK-induced PGC-1α activation (59). Alternatively, the increase in PGC-1α mRNA following post-exercise CWI may have been related to other pathways not discussed by Ihsan et al. (192), including a cold-induced increase in oxidative stress.

1.5.1.2 Oxidative Stress

As discussed in section 1.4.3, CWI may increase the production of ROS via thermogenesis and during the subsequent rewarming. However, the extent of free radical production following post-exercise CWI, and the effect this has on muscular recovery, is largely unknown. Despite evidence supporting the notion that CWI is associated with some degree of oxidative stress (445, 446, 116), no study has investigated its potential to promote ROS-induced mitochondrial biogenesis.

Previous research has implicated acute increases in contraction-induced ROS as important stimuli for the signalling system driving mitochondrial biogenesis and oxidative phenotype alterations (115, 201), in turn implicating CWI as a method to augment this response. This response is thought to be mediated by PGC-1α (3), potentially via phosphorylation of p38 MAPK (247) and/or protein kinase B (Akt) (37). Alternatively, cell culture models also implicate ROS-induced DNA damage as an activator of p53 following cold-stress (340), another protein key to mitochondrial biogenesis (424). In this instance, cold-induced oxidative stress burst from cold-stimulus may activate PGC-1α and

p53 to optimize respiration, and prevent future ROS generation. Although PGC-1α's role in promoting efficient oxidative phosphorylation (OXPHOS) is well established, it has also been speculated that p53 may be involved in the organisation of the OXPHOS complexes and favour efficient cooperation between them (42). These data implicate CWI-induced oxidative stress as a potentially potent stimulator of PGC-1α- and p53-mediated mitochondrial biogenesis, and provide an alternate explanation for the increase in PGC-1α mRNA reported by Ihsan et al. (192).

Although data supporting this is still inconclusive, low to moderate levels of ROS may have a beneficial effect on the mitochondria (408), potentially initiating mitochondrial biogenesis as a compensation for altered energy demands and respiratory chain defects (501, 434). ROS-induced mitochondrial biogenesis may serve as a hormetic stimulus to 'harden' and prepare an individual for subsequent ROS-related stress (446, 241), or, in this instance, future CWI. However, the degree of oxidative stress induced by CWI is currently unknown, and requires further investigation.

In the context of the recovery from exercise, CWI-induced ROS production/accumulation could conversely be detrimental to skeletal muscle and whole-body function. For example, high levels of ROS may damage cellular components compromising cellular integrity and function (127, 397), alter enzyme function (397, 203, 207), and reduce protein turnover (471, 406, 266). Careful consideration must be taken when prescribing CWI for athletes during intensive training programs, as CWI-induced elevations in ROS may exacerbate training-induced ROS production, and, more importantly, contribute to overtraining and/or apoptosis of skeletal muscle (311, 380). Given the potentially deleterious effect of ROS on skeletal muscle, this is of particular concern for the athletic populations. Implementing CWI in post-exercise recovery is designed to accelerate recovery, not exacerbate exercise-induced oxidative stress. Warmer immersion temperatures may serve to curtail these adverse responses, potentially attenuating ROS production during thermogenesis and rewarming, whilst concomitantly maximizing the purported physiological benefits of post-exercise CWI.

1.5.1.3 Thermal Shock

Another potential mechanism by which CWI may mediate mitochondrial adaptations is through the induction of CSPs during cooling, and HSPs during rewarming. As discussed in section 1.4.2, these proteins are important for a number of adaptive processes, including protection against cellular stress, mRNA stabilisation, protein translation rates, and the chaperoning of mitochondrial proteins (455). As such, CSP and HSP induction following thermal shock may play a role in the adaptive response to post-exercise CWI.

As a result of significant muscle cooling, post-exercise CWI may be a potent stimulus for the induction of CSPs. Given that muscle temperature is unlikely to drop below 25°C during a typical CWI protocol (e.g., 10°C for 15 min), cells are provided with an environment in which they can still proliferate and grow (411), as opposed to more extreme hypothermic conditions that may lead to cellular death by apoptosis or necrosis (455). As discussed in section 1.4.2.1, CSPs act as RNA chaperones during cold exposure, functioning to stabilise mRNA and improve global protein synthesis under cold stress. An up-regulation of CSPs following post-exercise CWI may serve to improve the transcription and translation of mitochondrial biogenic proteins up-regulated following the exercise and cold stimuli (e.g., PGC-1α and p53) (121, 114). This function may last up to at least 1 h post-CWI, at which time muscle temperature may still be below normothermia (97, 159). Despite their apparent role in adaptive pathways, research investigating the role of CSPs in exercise and CWI is currently lacking.

As discussed in Section 1.4.2.2, a return to normothermia following an initial cold shock may elicit phenotypical changes typically observed following heat stress. For example, data from cultured cells (340, 281, 250) have demonstrated a heat-shock-like response during rewarming, independent of an exercise stimulus. Heat-shock proteins are induced as a result of heat stress, functioning mainly to protect against cellular stress (Section 1.4.2.2). Exercise-induced activation of HSPs in human skeletal muscle is suggested to be mediated mainly by factors such as redox state (234, 129, 202), as opposed to elevated muscle temperature (330). As such, their induction may not be as evident

following recovery from hypothermia as compared with classical heat shock (411). However, whether or not rewarming following post-exercise CWI can induce a heat-shock response capable of altering mitochondrial gene expression in human skeletal muscle is currently unknown, and research is warranted to investigate this issue.

Thermal shock induced by CWI may also serve to active p38 MAPK and p53, both either directly or indirectly associated with mitochondrial biogenesis. In a similar 'hormetic' fashion as HSP proliferation, activation of p38 MAPK following the stress induced by rewarming, and/or p53 activation following DNA damage, may serve to facilitate mitochondrial biogenesis by better equipping the muscle for subsequent CWI-induced stress. MAPK signalling is heavily implicated in PGC-1α activity (243, 514, 345), and its activation following CWI may play a role in promoting cold-induced mitochondrial biogenesis. In regards to p53 activation, should post-exercise CWI induce cell cycle arrest and DNA damage upon rewarming, this is obviously deleterious to the adaptive response (340). However, it is currently unknown whether this finding translates to humans or to skeletal muscle. Conversely, should CWI increase p53 activation, it may serve as a potential method by which to increase the expression of genes associated with mitochondrial biogenesis, thereby improving mitochondrial function and exercise performance (33).

In summary, this section has identified a number of potential cellular signals by which post-exercise CWI may promote and/or assist mitochondrial adaptations. However, it must be noted that the rationale for the majority of these mechanisms are based on research performed in a variety of cell and tissue lines, Further research is warranted to determine if these responses are demonstrated in human skeletal muscle, thus contributing to data from the only study performed to date (192). Additional research is also needed to determine if these responses translate into improved training adaptations, including an increase in mitochondrial protein content, and improvements in mitochondrial function and exercise performance.

1.5.2 Training Adaptations

1.5.2.1 Training Quality

It has been suggested that the physiological benefits associated with post-exercise CWI may serve to improve subsequent training load and quality (29, 233). A hastened restoration of the body's physiological processes following a single bout of exercise may allow an athlete to train sooner and/or harder, potentially providing a greater stimulus for adaptation. In an attempt to quantify this response, research has investigated the effects of regular CWI during a training period on a number of performance and other non-invasive physiological measures of endurance adaptation. As discussed in Section 1.2.5, regular CWI during training has been shown to shown to have negligible or beneficial effects on cycling performance in competitive cyclists (164), supporting the notion that CWI may assist in the recovery from individual training sessions and increase subsequent training quality. However, data also exists to refute these suggestions, as demonstrated by an attenuation in endurance exercise performance, maximal oxygen uptake and arterial diameter following regular CWI (523). As such, conclusive evidence regarding the merit of CWI in improving training quality is lacking, and further investigation is warranted.

A potential limitation with assessing this hypothesis is the difficulty in quantifying the effects of post-exercise CWI on subsequent training quality and/or load. For example, training quality may be influenced by a number of factors independent of recovery, including nutrition, hydration, motivation, sleep quality, and training history (29). Furthermore, the performance and physiological markers that have previously been used (e.g., leg strength, maximal oxygen uptake, and endurance performance) provide little mechanistic insight on the effects of regular CWI on the adaptive response in skeletal muscle. As such, a more appropriate measure of the effects of CWI on training adaptations may be the quantification of adaptive molecular markers in skeletal muscle. For example, if regular CWI does indeed improve subsequent training quality, this should be represented in improvements in molecular markers such as mitochondrial protein content (e.g., PGC-1α, p53, CS) and mitochondrial function (e.g., mitochondrial respiration).

1.5.2.2 Molecular Markers

While research to date has investigated the effects of regular CWI on training-induced changes in performance, no study has investigated the effects of regular CWI on underlying skeletal muscle adaptations to aerobic training. Given the potential mechanisms discussed in section 1.5.1 and research performed to date, regular CWI may serve to increase the content of transcription factors (e.g., PGC-1α and p53) and HSPs key to mitochondrial biogenesis.

Regardless of the mechanisms by which CWI exposure may upregulate PGC-1α and p53, it represents a novel method by which alterations towards an aerobic phenotype can be obtained. It is currently unknown whether a single post-exercise CWI exposure can increase functional PGC-1α or p53 protein content, or whether a minimum number of repeated exposures are needed. Both PGC-1α (63) and p53 (157) have been reported to increase in content following HIT, but no study has investigated whether regular CWI can augment this response. Furthermore, there is limited research investigating the effect of a CWI on the associated downstream markers of mitochondrial biogenesis. Eight weeks of cycling (~60% VO_{2max}) in cold water (20°C) did not reduce CS activity in human skeletal muscle as compared with thermo-neutral water (35°C) (530), despite a reported correlation between PGC-1α and CS protein content (183). Future research is also warranted to investigate whether potential CWI-induced increases in PGC-1α and/or p53 allow for an increase in certain mitochondrial proteins in humans, as previously demonstrated in rodents exposed to regular cold air (59, 194, 357, 321). More importantly, it must be determined whether these adaptations will provide a basis for improved aerobic phenotype adaptations, including mitochondrial respiration, maximal oxygen consumption ($\dot{V}O_{2peak}$) and endurance exercise performance.

Elevated HSP content following regular CWI may share similar mechanisms to trained men displaying a selective up-regulation of basal HSP and antioxidant content (331). Repeated cold-induced stress, and a potential resultant increase in HSP expression, may lessen the stress response due to a conditioning response, functioning to better maintain homeostasis during the stress imposed by subsequent applications of CWI. For example, increased HSP70 content may also

protect against disruptions to the cytoskeleton/contractile machinery by maintaining redox balance and facilitating mitochondrial biogenesis (328), and also against heat-induced mitochondrial apoptosis (450). Whether an up-regulation of HSPs following CWI might occur as the result of cellular oxidative state, rewarming heat-stress, or a combination of the both, it is apparent this may serve to preserve mitochondrial function and even exercise performance. However, no study to date has investigated the potential of regular post-exercise CWI to improve HSP content.

1.6 Summary

Post-exercise CWI remains a popular cryotherapy tool that has been proposed to enhance athletic recovery following competition and training (509, 388). However, despite its popularity, a lack of empirically-based recommendations surrounding its physiological benefit means most athletes and sports practitioners follow anecdotal guidelines when prescribing CWI (49, 509). Evidence supporting the use of CWI to improve the recovery of certain markers of performance and muscle damage are equivocal, apart from its effectiveness in reducing muscle soreness. Given the perceptual nature of DOMS, it is possible that the placebo effect may have an influence on this measure (260). To date, no study investigating the effects of CWI on skeletal muscle recovery has controlled for the placebo effect, despite continual acceptance of its potential to confound research outcomes. To fully understand the mechanisms underlying CWI, and its influence on the recovery of athletic performance, it is crucial to control for the placebo effect.

Despite the suggestion of a potential placebo effect, research to date supports short-term benefits of CWI to enhance the recovery of skeletal muscle performance (26, 147, 326, 449, 486, 487, 526). However, these benefits may be counteracted by possible long-term detrimental effects on skeletal muscle adaptations to training (523). Post-exercise fatigue and/or inflammation are important stimuli for adaptation and performance improvements to occur, and may be hindered by CWI (523). Conversely, CWI may expedite recovery and allow for greater training load or quality at a subsequent session, thereby providing a greater stimulus for adaptation (490). Furthermore, evidence exists

that CWI may stimulate and facilitate cell signalling related to mitochondrial biogenesis (192), implicating it as a novel and practical method to further augment aerobic adaptations to exercise training. Given this conjecture, mechanistic research is required to determine the potential molecular mechanisms by which CWI may alter the signalling pathways and long-term adaptive response to training in human skeletal muscle.

1.7 Overall Aims

This thesis will firstly investigate if the placebo effect is responsible for any performance or psychological benefits following a single bout of HIT and CWI. It is hypothesised that the placebo effect would be, at least in part, responsible for any observed benefits of CWI. Second, this thesis will investigate the potential molecular mechanisms by which post-exercise CWI may affect the adaptive response to HIT in human skeletal muscle. The mechanisms by which this may occur include cold-induced alterations in mitochondrial biogenesis, cold- and heat-shock protein induction, and oxidative stress. In particular, the potential molecular pathways by which CWI may alter the mRNA and content of key proteins associated with mitochondrial biogenesis will be highlighted. Further clarification of these issues will provide insight into the merit of CWI following exercise as an integral component of athletic training regimes (29).

Specifically, the aims of each chapter of this thesis are listed below.

- Chapter Two: To examine the physiological merit of CWI for recovery from a single bout of HIT, by investigating if the placebo effect is responsible for any performance or psychological benefits.
- Chapter Three: To investigate the molecular mechanisms by which CWI may influence the signalling processes involved in mitochondrial biogenesis following a single bout of HIT.
- Chapter Four: To investigate the underlying molecular mechanisms by which CWI may alter the training adaptations associated with mitochondrial biogenesis following HIT.

CHAPTER TWO

Cold-Water Immersion and the Placebo Effect

This study is presented as the first in a series of related studies investigating the physiological and molecular merit of cold-water immersion in the recovery from and adaptation to high-intensity interval exercise. Its purpose was to determine whether the placebo effect would be, at least in part, responsible for any observed benefits of post-exercise cold-water immersion.

The reference for the published version of this study is as follows:

Broatch, J. R., Petersen, A., Bishop, D. J. (2014). Postexercise cold-water immersion benefits are not greater than the placebo effect. *Medicine and Science in Sports and Exercise.* 46(11), 2139-47. No citation to date, but this paper has already been tweeted 580 times to an upper-bound of 1,436,907 combined followers.

2.1 Introduction

To be successful in their chosen sport, athletes must achieve an adequate balance between training and recovery. Inadequate recovery between training sessions can place great physiological strain on an athlete, potentially leading to symptoms of overreaching, fatigue and reduced performance (405). Optimising recovery between training bouts and competition is frequently recommended to ensure that athletes can train frequently while reducing these associated risks. A large body of research has focused on modalities designed to hasten recovery after exercise, with one of the most prevalent techniques being CWI (29).

Cold-water immersion is believed to attenuate post-exercise reductions in functional capacity and athletic performance (509). Although the specific underlying mechanisms remain to be elucidated, hydrostatic pressure and a reduction in muscle temperature may assist in reducing oedema, pain and the accumulation of metabolites (49, 509). The physiological benefits of CWI reported to date include improved recovery of exercise performance (195, 26, 487, 488, 389), and reduced muscle soreness (414, 415, 17). However, many studies have reported CWI to have no influence on the recovery of exercise performance (415, 461, 437), or in reducing oedema and muscle soreness (437, 187).

An important unanswered question is whether the variable response to CWI can be attributed, at least in part, to psychological factors (461). Other recovery techniques, such as massage, have been reported to improve recovery via psycho-physiological mechanisms that include decreased sensation of pain and fatigue (497). CWI has been shown to enhance feelings of recovery (367), and reduce perceptions of fatigue (367, 464, 461, 415) and muscle soreness (26, 195, 464, 415), when compared with a control condition. Furthermore, a recent meta-analysis by Leeder (260) concluded that CWI is effective in reducing subjective measures of muscle soreness up to 96 h post exercise, with its effects on muscle function unclear. Given the subjective nature of muscle soreness, and increasing popularity of CWI as a recovery strategy, it is plausible that athletes believe and expect CWI to improve recovery from exercise.

One major weakness with the CWI research performed to date is that no study has incorporated a placebo condition. This is a crucial omission given that

the placebo effect is a well-accepted phenomenon within medicine, and is even utilised as a therapeutic intervention [see review by Beedie & Foad (35)]. The placebo effect also influences sport performance (35), and has a potentially long-lasting effect (38). Merely expecting an intervention to have a positive effect has been shown to improve an athlete's performance (307). Pollo et al. (387) reported a 7.8% decrease in perceived fatigue and an 11.8% increase in leg extension strength when supplying an ergogenic placebo. To fully understand the mechanisms underlying CWI, and its influence on athletic performance, it is therefore crucial to control for the placebo effect.

The aim of this study was to address this limitation and compare for the first time the effects of CWI to a placebo condition (thermo-neutral water immersion + placebo; TWP) that participants were informed was as effective as CWI. It was hypothesised that the placebo effect would be, at least in part, responsible for any observed benefits of CWI – i.e., TWP and CWI would elicit similar benefits in recovery, but superior benefits when compared with a thermo-neutral water immersion control condition (TWI). In addition, to assess any potential influence of reduced muscle temperature, independent of the effects of hydrostatic pressure on recovery, CWI was compared with TWI.

2.2 Methods

2.2.1 Participants

Thirty recreationally-active, healthy males (age 24 ± 5 y; body mass 78.7 ± 8.5 kg; height 179.3 ± 6.6 cm; VO_{2peak} 51.1 ± 7.0 $mL \cdot kg^{-1} \cdot min^{-1}$; mean ± SD) participated in this study. Informed consent was obtained prior to participation, and all participants were screened for immunological irregularities and cardiovascular risk factors associated with exercise. All procedures were approved by the Institution's Human Research Ethics Committee.

Each subject participated in five laboratory sessions over a two-week period (Fig 2-1). The study followed a parallel group design, in which participants were assigned to one of three recovery conditions in a randomised, counter-balanced fashion (ranked according to peak torque achieved during the MVC familiarisation). These conditions were cold-water immersion (CWI, n=10), a

thermo-neutral water immersion placebo (TWP, *n*=10), or thermo-neutral water immersion control (TWI, *n*=10) groups.

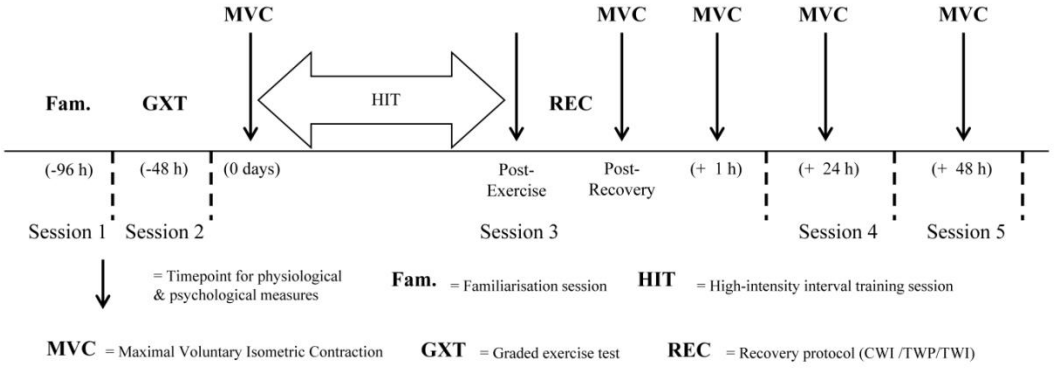


Figure 2-1: Schematic representation of the experimental design.

The initial testing session was a familiarisation session. Anthropometric measurements (height, mass and quadriceps skinfold thickness of the dominant leg) were taken, followed by familiarisation with the maximal voluntary isometric contraction (MVC), pain threshold/tolerance (algometer), thermistor insertion, HIT session, and recovery protocols. Two days after the familiarisation session, participants attended the lab (session two) to complete a graded exercise test (GXT) to determine peak oxygen uptake ($\dot{V}O_{2peak}$).

Following the preliminary sessions, participants completed session three, which was comprised of the single HIT session and designated recovery condition. Intra-muscular temperature (T_m) was documented during the exercise and recovery protocols of this session. An antecubital venous blood sample, girth and pain threshold/tolerance measures of the quadriceps of both limbs, and completion of a psychological questionnaire were performed before (baseline) and immediately after the HIT session (PE), immediately after recovery (PR), and 1 h, 24 h (session four) and 48 h (session five) after the HIT session. A MVC of the dominant leg knee extensors was also performed at these time-points, with the exception of PE (participants were unable to give a maximal effort whilst the thermistor was still inserted). Belief in the recovery effectiveness was assessed at the start (BE_{pre}) and end (BE_{post}) of the study, via a "belief questionnaire". Participants were asked to fast for 2 h, and refrain from exercise for 24 h preceding sessions three, four and five. Food diaries were recorded for the 24 h preceding session three, and participants were asked to replicate this diet for the

24 h preceding sessions four and five. Participants were allowed to consume water *ad libitum* during all sessions. All experimental sessions were conducted in a laboratory maintained between 19-22°C with ≥ 70% relative humidity.

2.2.2 Graded Exercise Test

The GXT was performed on an electronically-braked cycle ergometer (Lode, Excalibur Sport V2.0, The Netherlands). Following a 3-min warm-up at 75 W, the test increased by 30 W each minute thereafter until the participant reached volitional fatigue. Expired gases were analysed every 15 s using a metabolic cart (Moxus Metabolic System, AEI Technologies, USA), which was calibrated using known gas concentrations prior to each test (20.93% O_2, 0.04% CO_2 and 16.10% O_2, 4.17% CO_2; BOC Gases, Australia). Participants were instructed to maintain a pedalling cadence of 70 rpm and wore a heart rate monitor (RS800sd, Polar Electro Oy, Finland) during the test. The test was stopped when pedalling cadence dropped below 60 rpm. The $\dot{V}O_{2peak}$ as defined as the average of the two highest consecutive values reached during the test.

2.2.3 Intramuscular Thermistors

The site for thermistor insertion was determined as 5 cm lateral to the mid-point between the participant's anterior superior iliac spine and head of the patella, on the dominant leg. An 18 gauge needle (Optiva IV Catheter 18GX1.75", Smiths Medical, USA) was inserted at the marked site, after which it was subsequently removed whilst leaving the catheter in the quadriceps muscle. A needle thermistor probe (Model T-204A, Physitemp Instruments, USA) was inserted through the catheter, to a depth of 4 cm plus half the width of the thigh skinfold. The thermistor probe and catheter were securely covered and fastened to the leg, allowing for movement and continual measurement (4 Hz) of T_m. Complete temperature data sets were available for 23 participants (CWI, n=9; TWP, n=6; TWI n=8) as a result of the thermistors occasionally malfunctioning during high-intensity exercise.

2.2.4 High-Intensity Interval Training Session

The exercise protocol used in this study represents an increasingly popular form of high-intensity training (145). On an electronically-braked cycle ergometer (Lode, Excalibur Sport V2.0, The Netherlands), participants first completed a 5-min warm-up at 100 W. This was immediately followed by 4 x 30-s 'all-out' efforts at a constant resistance corresponding to 7.5% of body mass, separated by 4 min of rest. To eliminate individual variance with self-administered 'speeding up' of the flywheel, participants began each effort from a rolling start corresponding to 20 rpm lower than the peak rpm reached during familiarisation. The tester manually sped the flywheel to the desired rpm, before strapping the subject's feet to the pedals 15 s before each effort. During the effort, participants were given extensive verbal encouragement and asked to remain seated in the saddle.

2.2.5 Recovery Interventions

Five minutes after completing the HIT session, participants performed their assigned recovery intervention for 15 min. Seated (with legs fully extended) in an inflatable bath (iBody, iCool Sport, Australia), participants were immersed in water up to their umbilicus. Water temperature was maintained with a cooling/heating unit (Dual Temp Unit, iCool Sport, Australia), with validations taken every 3 min with a thermometer immediately adjacent to the thermistor site. TWI and TWP temperatures were maintained at 34.7 ± 0.1°C, and CWI temperatures at 10.3 ± 0.2°C. The CWI protocol was based on pilot work comparing the T_m drop elicited from a HIT bout and subsequent 15 min recovery, in 14°C (2.7 ± 1.7°C) or 10°C (4.7 ± 3.1°C) water. To facilitate the placebo effect, a de-identified, pH-balanced dissolvable skin cleanser (Cetaphil, Gentle Skin Cleanser, Australia) was added to the water for the TWP condition, in plain sight of the participant immediately prior to immersion. Investigators were not blinded to the experimental condition as this was revealed with different limb temperature when taking girth and algometer measures. Given its popularity in recovery (29), it was assumed that most participants had prior knowledge of CWI's purported benefits. To eliminate any potential bias, participants in the placebo condition were led to believe that a thermo-neutral water immersion (with the addition of

the skin cleanser) was beneficial in recovery from high-intensity exercise, which is considered will be more effective than convincing participants that CWI was detrimental.

2.2.6 Thermal Sensation and Comfort

During water immersion, thermal comfort (T_c) and sensation (T_s) were measured every 3 min using subjective visual scales (138). The scales ranged from 'very comfortable' to 'very uncomfortable' (white to black scale) for T_c, and from 'very cold' to 'very hot' (green to red scale) for T_s. Corresponding scores ranging from 0 to 20 were on the reverse side of each scale and only visible to the tester.

2.2.7 Recovery Information Sheets and Belief Questionnaires

At the beginning of the familiarisation session, participants were given an information sheet on the efficacy of their assigned recovery modality. CWI participants were shown peer-reviewed data on its effectiveness for repeat cycling performance (488). TWP participants were falsely led to believe they were using a newly-developed 'recovery oil', which was as effective as CWI in promoting recovery from high-intensity exercise. TWI participants were shown information on the benefits of water immersion alone, without an associated cold or 'recovery oil' influence (509). Apart from the addition of the skin cleanser, both the TWP and TWI conditions were identical. Following familiarisation of the MVC and algometer measures, and prior to familiarisation of the HIT and recovery protocols, subjects were asked to complete a 'belief' questionnaire, designed to measure the anticipated effectiveness of their assigned recovery technique (BE_{pre}). Participants were instructed to mark an 'X' on a 5-point likert scale between two extremes (0 indicating 'not effective at all' and 5 indicating 'extremely effective'). Approximately 5 min after the 48 h MVC (session five), participants were asked to complete a similar 'belief' questionnaire, designed to measure the perceived effectiveness of the completed recovery condition (BE_{post}).

2.2.8 Psychological Questionnaire

Participants were required to complete a psychological questionnaire documenting subjective ratings of readiness for exercise, fatigue, vigour, sleepiness, and muscular pain. Instructions were given to mark an 'X' on a 10 cm visual analogue scale (VAS) between two extremes (0 = 'least possible', 10 = 'most possible' for each rating), as described previously (437).

2.2.9 Maximal Voluntary Isometric Contraction

Muscle strength and power of the dominant leg knee extensors was measured using an isokinetic dynamometer (Cybex Isokinetic Dynamometer, Humac NORM, Canada). Participants were positioned to ensure the axis of rotation aligned with the femoral condyle. Following an initial warm-up of three sub-maximal efforts (50, 70 and 90% MVC), participants completed three maximal 5-s isometric contractions of the knee extensors, with 30 s of rest between each repetition. The knee extension angle was set at 60° as previously described (503, 504). The greatest peak (MVC_{max}) and mean (MVC_{avg}) torque achieved from all repetitions was recorded. This effort was also used to determine the rate of torque development (RTD). Absolute RTD was calculated from the average slope of the torque-time curve (Δ torque/Δ time) from contraction onset (torque > 7.5 N.m of baseline) to 200 ms (1).

2.2.10 Blood Analyses

A 20 gauge indwelling venous catheter (Optiva IV Catheter 20GX1.75", Smiths Medical, USA) was inserted into an antecubital vein 10 min prior to the first blood draw. Blood samples (~10 mL each) were collected into EDTA tubes (Greiner Bio-one, Germany), and analysed immediately for total leukocytes, neutrophils, lymphocytes (KX-21N, Sysmex, Japan). The remaining whole blood was centrifuged at 1,000 *g* and 4°C for 10 min. The acquired plasma was stored at -80°C for subsequent analysis. All samples were analysed for interleukin 6 (IL-6) by enzyme-linked immunosorbent assay (ELISA) with commercially-available multiplex kits (Fluorokine Multianalyte Profiling Kit, RND Systems, USA). All samples were analysed in duplicate. The IL-6 assay had an inter/intra-assay coefficient of variation (CV) of <9.4% across the range of 0.2-10.2 $pg.mL^{-1}$

2.2.11 Thigh Girth

Girth measurements were taken at the mid-point of the thigh on both limbs as an objective measure of exercise-induced oedema. With the participant standing, the thigh midpoint was determined as 4 cm distal to halfway between the greater trochanter and lateral epicondyle (238). Thigh circumference was measured around the thigh and over this mark, whilst the subject lay supine on a table, with the foot on the table surface and the knee at 90°. On each occasion investigators obtained three values which were averaged for data analysis.

2.2.12 Algometer

Objective measures of the pain threshold (P_{TH}) and pain tolerance (P_{TO}) were measured using a pressure algometer (FPX Algometer, Wagner Instruments, USA) applied to mid-belly sites on the quadriceps of both limbs. The assessment site was located at the point at which the girth circumference site met the line between the anterior superior iliac spine and head of the patella. With the participant instructed to relax the muscle, pressure was applied perpendicular to the long axis of the femur at a rate of 1 $kg \cdot cm^{-2} \cdot s^{-1}$ (128). The same instructor was used for all measurements. The first point at which discomfort and unbearable pain was reported corresponded to P_{TH} and P_{TO} respectively. The algometer had a 1-cm^2 application surface, and readings were displayed in kilograms of force. On each occasion investigators obtained three values which were averaged for data analysis.

2.2.13 Statistical Analyses

Data are reported in the text as means and standard deviations (mean ± SD), unless otherwise stated. For parametric data, comparisons between conditions were analysed using a multi-factorial mixed linear model (SPANOVA) with repeated measures for time. Fischer's LSD *post-hoc* test was performed when statistical significance was present. Data that violated Levene's test of homogeneity was log-transformed prior to analysis. Friedman's two-way ANOVA was used for non-parametric data not belonging to a particular distribution (psychological questionnaires). Pearson's correlation coefficient (*r*) was calculated (pooled data) to examine the relationship between recovery belief

(BE_{pre}) and exercise performance (recovery of MVC_{max} and MVC_{max} from baseline to 48 h). The level of significance for all data was set at $p < 0.05$. The above analyses were performed using IBM SPSS Statistics V20 (IBM Corporation, USA). In addition, effect sizes (ES) were calculated using Cohen's *d*, with magnitude-based inferences used to interpret the meaningfulness of results. Cohen's conventions for effect size (with 90% confidence intervals) were used for interpretation, where ES = 0.2, 0.5, and 0.8 are considered as small, medium, and large, respectively. The pooled SD was calculated when the SD were unequal.

2.3 Results

2.3.1 HIT Session

All groups performed similar volumes of total work during the single bout of HIT, 302.6 ± 14.8 kJ (CWI), 291.4 ± 42.2 kJ (TWP), and 324.0 ± 54.7 kJ (TWI) (p>0.05).

2.3.2 Muscle Temperature

For all conditions, T_m increased significantly from 36.0 ± 0.7°C to 37.6 ± 0.7°C as a result of exercise (p<0.05). After 15 min of immersion, CWI induced a 9.5 ± 10.3% reduction in T_m, significantly larger (p<0.05) than both the TWP (0.4 ± 0.8%) and TWI (0.5 ± 0.3%) (Fig 2-2).

2.3.3 Psychological Measures

T_c and T_s were the same for TWI and TWP during immersion. Participants perceived themselves to be significantly colder ($T_{s;}$ p<0.05) and more uncomfortable (T_c: p<0.05) in CWI as opposed to both TWI and TWP. BE_{pre} was significantly greater (p<0.05) for the TWP and CWI groups when compared with TWI (Table 2-1). There was no difference between the CWI and TWP conditions for BE_{pre}, or between any groups for BE_{post}.

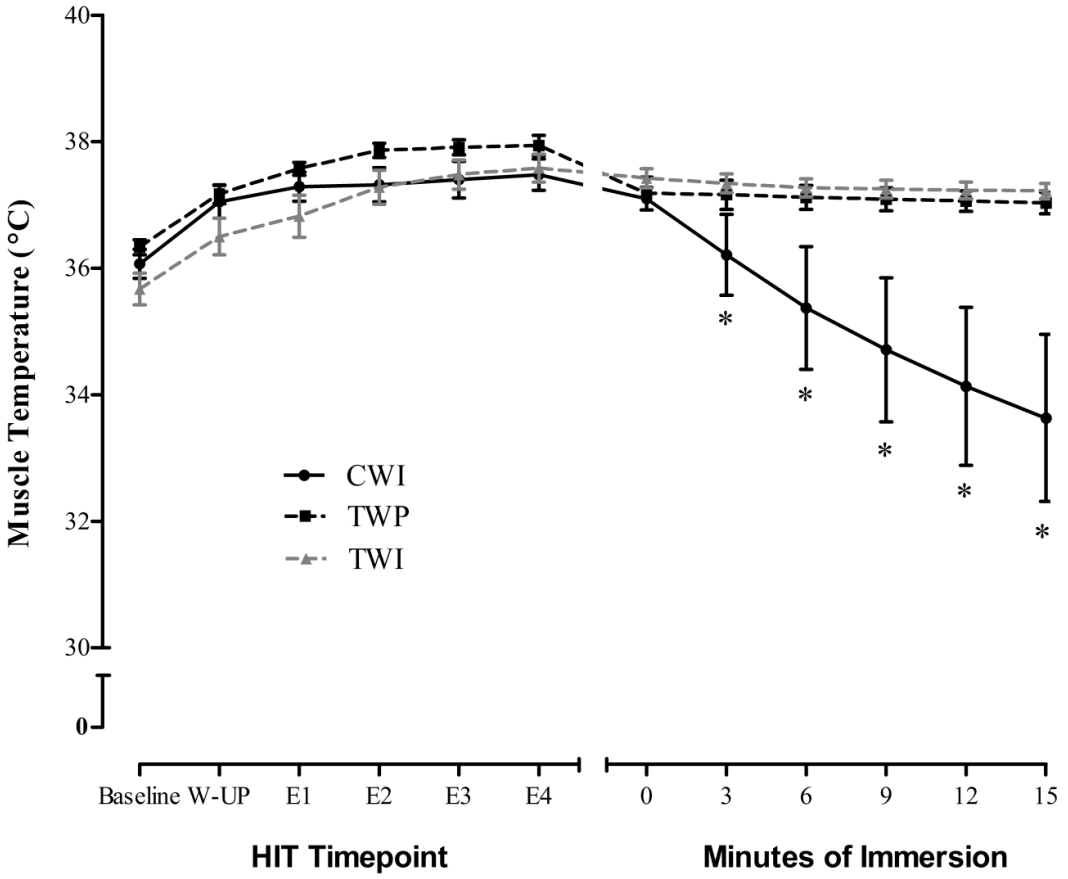


Figure 2-2: Muscle temperature (T_m; 4 cm into the *vastus lateralis* muscle) during the HIT and 15-min recovery protocols. W-UP = Warm-up, E1-4 = HIT effort 1, 2, 3 and 4. * Significantly different to both TWP and TWI (p<0.05). Values are mean ± SEM.

Readiness for exercise and vigour was significantly lower at PE (p<0.05) compared with baseline for all conditions (Table 2-2). Also PE, participants felt significantly more fatigued and in more muscular pain than baseline for all conditions (p<0.05). Mental readiness for exercise was higher for TWP participants compared with TWI at PR (p<0.05) and 48 h (ES = 0.96, p = 0.052).

Table 2-1: Subjective ratings of perceived recovery effectiveness (belief effect) before (Session 1; BE_{pre}) and after (Session 5; BE_{post}).

	BE_{pre}	BE_{post}
CWI	4.1 ± 0.8	4.2 ± 0.7
TWP	3.6 ± 0.7	4.1 ± 0.9
TWI	3.0 ± 0.9 *	3.6 ± 0.9

Values are mean (±SD), * denotes a significant difference to CWI and TWP (p<0.05)

Table 2-2: Subjective ratings of readiness for exercise, fatigue, vigour, sleepiness and pain. Time points are before exercise (baseline), immediately post-exercise (PE), post-recovery (PR), and 1 (1 h), 24 (24 h) and 48 (48 h) hours post-exercise.

	Time-point					
	Baseline	**PE**	**PR**	**1 h**	**24 h**	**48 h**
Phy. Ready						
CWI	7.7 ± 1.5	1.0 ± 1.5	4.8 ± 2.2	7.1 ± 1.8	8.4 ± 1.5	7.6 ± 1.9
TWP	7.1 ± 1.1	1.0 ± 0.7	3.7 ± 1.7	5.5 ± 2.1	6.8 ± 2.5 ^{c}	7.4 ± 1.8
TWI	7.4 ± 1.4	0.8 ± 0.7	3.1 ± 1.6 *	4.5 ± 1.5 *	6.7 ± 1.8 *	6.5 ± 2.3
Men. Ready						
CWI	7.2 ± 1.6	2.8 ± 2.5	5.5 ± 2.1	7.1 ± 1.9	7.9 ± 1.5	7.3 ± 1.9
TWP	6.7 ± 1.1	2.4 ± 1.9	4.5 ± 1.3	6.0 ± 1.9	7.0 ± 1.9	7.6 ± 1.2
TWI	7.3 ± 1.3	1.2 ± 0.9	2.7 ± 1.7$^{*, **}$	4.2 ± 2.2 *	6.5 ± 2.1	5.6 ± 2.6 ^{a}
Fatigued						
CWI	2.8 ± 1.9	9.1 ± 0.9	4.7 ± 2.0	3.2 ± 1.9	1.8 ± 1.5	3.0 ± 2.5
TWP	2.5 ± 1.6	9.0 ± 0.6	4.7 ± 1.5	4.2 ± 2.1	2.9 ± 1.9	2.7 ± 1.6
TWI	3.4 ± 1.8	8.4 ± 2.3	5.7 ± 2.1	3.9 ± 2.0	2.9 ± 2.0	3.7 ± 2.3
Vigorous						
CWI	6.7 ± 1.6	2.6 ± 2.4	5.3 ± 1.6	5.9 ± 1.7	6.7 ± 1.3	5.9 ± 1.9
TWP	6.0 ±1.6	2.9 ± 3.0	3.7 ± 1.8 *	4.6 ± 2.1	6.0 ± 2.4	6.2 ± 2.0
TWI	5.7 ± 1.6	0.7 ± 0.7 ^{*,a}	2.5 ± 1.4 *	3.6 ± 1.9 *	5.6 ± 2.1	5.0 ± 2.6
Sleep						
CWI	3.4 ± 2.2	3.4 ± 3.2	3.4 ± 3.0	3.2 ± 2.0	2.7 ± 1.5	3.7 ± 2.7
TWP	3.5 ± 1.8	3.7 ± 3.0	4.6 ± 2.3	4.2 ± 1.8	3.4 ± 1.6	4.0 ± 1.7
TWI	3.5 ± 2.0	4.0 ± 2.8	5.7 ± 2.6 ^{b}	5.0 ± 2.3	2.4 ± 1.3	3.9 ± 2.3
Pain						
CWI	1.5 ± 1.8	6.0 ± 1.5	2.5 ± 1.4	1.8 ± 1.0	1.4 ± 1.7	1.1 ± 1.3
TWP	1.9 ± 1.6	6.4 ± 2.4	3.4 ± 1.8	2.4 ± 1.4	2.1 ± 1.4	1.7 ± 0.9
TWI	1.5 ± 0.8	5.0 ± 1.9	2.0 ± 1.2 **	1.9 ± 1.3	1.3 ± 0.8	1.6 ± 1.1

Values are arbitrary units from 0-10 (0='least possible', 10='most possible' for each rating) on a VAS scale, presented as mean (± SD). * denotes a significant difference to CWI (p<0.05), ^{a} denotes a large effect compared with TWP (ES>0.8), ** denotes a significant difference to TWP (p<0.05), ^{b} denotes a large effect compared with CWI (ES>0.8), ^{c} denotes a moderate effect compared with CWI (ES=0.5-0.8). Phy. Ready = Physically Ready, Men. Ready = Mentally Ready.

Also compared with TWI, TWP participants were more vigorous at PE (ES = 0.93, $p = 0.063$) and in less pain at PR ($p<0.05$). In comparison with CWI, TWP participants reported a lower physical readiness for exercise at 24 h (ES = 0.74, $p = 0.075$), and a lower rating of vigour at PR ($p<0.05$). When compared with TWI, CWI participants felt more mentally ready for exercise at PR, 1 h and 24 h ($p<0.05$), more physically ready for exercise at PR and 1 h ($p<0.05$), and more vigorous at PE, PR, and 1 h ($p<0.05$).

2.3.4 MVC

There were no differences in MVC_{max} (CWI, 250.6 ± 48.7 $N.m^{-1}$; TWP, 234.4 ± 63.7 $N.m^{-1}$; and TWI, 240.4 ± 85.2 $N.m^{-1}$) and MVC_{avg} (CWI, 219.6 ± 43.3 $N.m^{-1}$; TWP, 207.0 ± 56.9 $N.m^{-1}$; and TWI, 211.6 ± 71.9 $N.m^{-1}$) at baseline for all groups. MVC performance declined as a result of the HIT session (Fig 2-3). At 1, 24 and 48 h, MVC_{max} was 13.1 ± 11.2% ($p<0.05$), 11.0 ± 9.0% ($p<0.05$) and 8.0 ± 7.0% ($p<0.05$) lower than baseline for TWI, respectively. Belief effect (BE_{pre}) was significantly correlated to the recovery of MVC_{max} ($r = 0.52$, $p<0.05$) and MVC_{avg} ($r = 0.41$, $p<0.05$) over the 48-h post-exercise period.

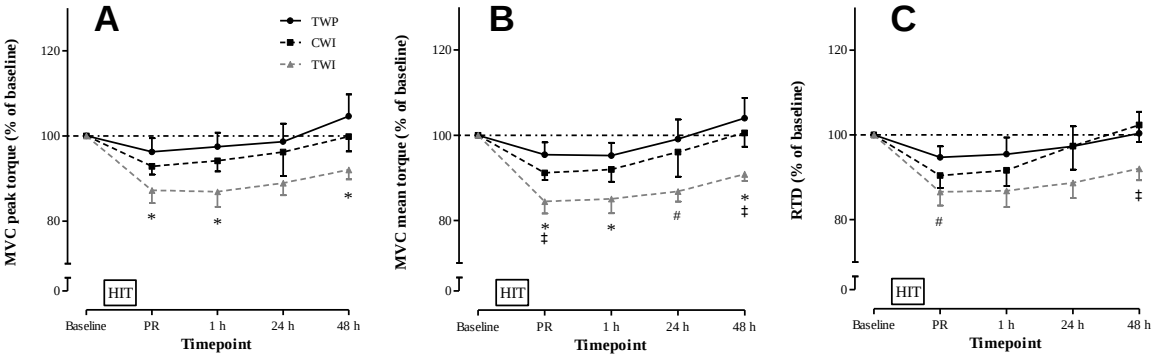


Figure 2-3: Percent changes (from baseline) in maximal voluntary isometric contraction (MVC) peak torque (A; MVC_{max}), mean torque (B; MVC_{avg}), and rate of force development (C; RTD), for cold-water immersion (CWI), thermo-neutral water immersion placebo (TWP) and thermo-neutral water immersion control (TWI) conditions. Time points are before exercise (baseline), post-recovery (PR), and 1 (1 h), 24 (24 h) and 48 (48 h) hours post-exercise. * Significantly different to TWP ($p<0.05$). ‡ a small effect compared with CWI (ES = 0.2-0.5). # a small effect compared with TWP (ES = 0.2-0.5). Values are mean ± SEM.

MVC_{max} was 9.0% (PR; $p<0.05$), 10.6% (1 h; $p<0.05$) and 12.6% (48 h; $p<0.05$) lower for TWI compared with TWP. MVC_{max} for CWI participants was not significantly different to either TWP or TWI conditions, at any time point. MVC_{avg} was 11.0% (PR; $p<0.05$), 10.2% (1 h; $p<0.05$), 12.3% (24 h; ES = 0.28, p = 0.06) and 13.2% (48 h; $p<0.05$) lower for TWI compared with TWP. There were no differences between CWI and TWP for MVC_{avg} at any time point. MVC_{avg} was 6.8% (PR; ES = 0.48, $p = 0.07$) and 9.7% (48 h; ES = 0.43, $p = 0.08$) lower for TWI compared with CWI.

RTD was lowest for TWI at every time point (Figure 3). Immediately PR, RTD was 8.1% lower for TWI compared with TWP (ES = 0.34, $p = 0.06$). There were no differences between CWI and TWP for RTD at any time point. At 48 h, RTD was 10.3% lower for TWI compared with CWI (ES = 0.48, $p = 0.08$).

2.3.5 Blood Plasma Variables

IL-6 concentration was significantly elevated from baseline at PE, PR and 1 h ($p<0.05$) for all conditions. Total leukocyte count was elevated PE for all conditions ($p<0.05$), and returned to baseline by PR. Lymphocyte count was higher PE than at baseline ($p<0.05$), whereas neutrophil count was significantly lower than at baseline ($p<0.05$), for all conditions. Of the time points measured, neutrophil count was highest at 1 h ($p<0.05$) for all conditions. There were no differences between groups for all blood markers (Fig 2-4).

2.3.6 Thigh Girth and Algometer

Thigh girth of both the dominant and non-dominant limbs were significantly elevated ($p<0.05$) PE for all conditions, and returned to baseline by PR. P_{TO} and P_{TH} did not differ from baseline for all conditions. There was also no interaction between groups for all thigh girth and algometer measures.

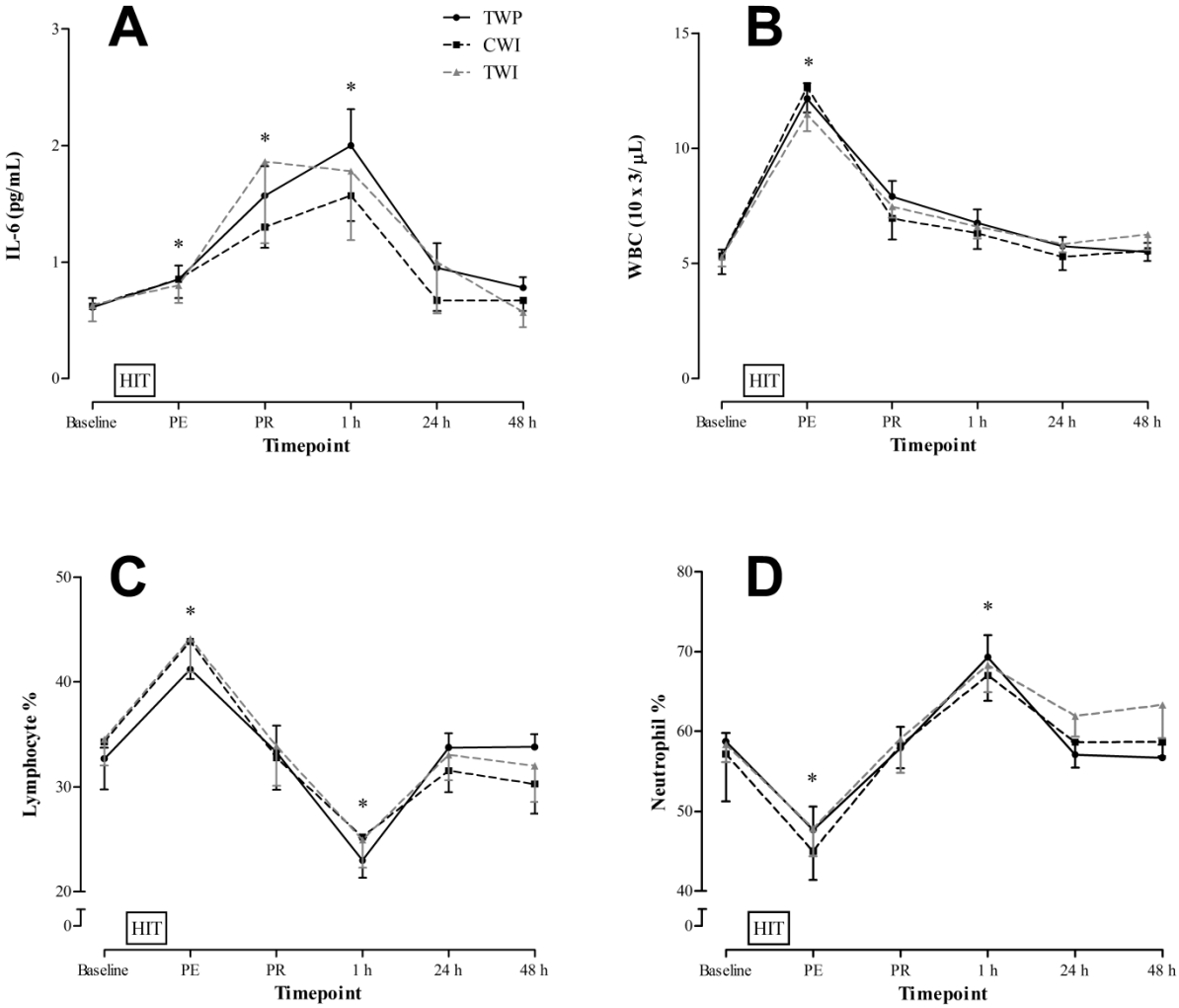


Figure 2-4: Blood markers at baseline, post-exercise (PE), post-recovery (PR), and 1 (1 h), 24 (24 h) and 48 (48 h) hours post-exercise. IL-6, Interleuken-6 (A); WBC, total white blood cell count (B); LYM, lymphocyte percentage in WBC (C); NEU, neutrophil percentage in WBC (D). * Significantly different to baseline for all conditions (p<0.05). Values are mean ± SEM

2.4 Discussion

The main finding of this study was that a thermo-neutral water immersion placebo is superior to a thermo-neutral water immersion control in assisting the recovery of quadriceps' muscle strength following a single HIT session. This coincided with improved subjective ratings of readiness for exercise and reduced subjective pain ratings in participants administered the TWP. CWI participants also displayed superior muscle strength and psychological measures when compared with TWI, but performed similarly to TWP despite a significant reduction in muscle temperature compared with the thermo-neutral conditions.

Furthermore, marked changes in immune markers were evident following a single bout of HIT, with no apparent difference between groups.

The results of this study demonstrate for the first time that a single bout of HIT, consisting of four 30-s 'all-out' cycling efforts, decreased peak quadriceps' strength by ~13% (1 h), ~11% (24 h) and ~8% (48 h) over a 48-h, post-exercise period for TWI. Despite the lack of eccentric load and subsequent muscle damage, a protocol of this nature can significantly hinder muscle strength up to 48 h post-exercise. Although many mechanisms may be responsible, a reduction in leg strength following high-intensity cycling may primarily be related to the inactivation of muscle cation pumps (264). In particular, sarcoplasmic reticulum Ca^{2+}-ATPase function has been shown to remain depressed for up to 36-48 hours post-exercise (484). A prolonged reduction in muscle strength following a single bout of HIT has the potential to hinder an athlete's capacity to repeat efforts requiring high speed, power and strength development in subsequent training sessions and/or competition.

A crucial component of this study was the effective deception of participants administered the placebo. After reading the information sheets, participants in the TWP condition rated the expected benefits of their assigned recovery protocol greater than participants in TWI. This was evident despite the two conditions being identical, apart from the addition of bath soap. The effectiveness of the deception is also supported by the psychological questionnaires, by which TWP participants reported greater mental readiness for exercise and lower pain levels immediately post-recovery compared with TWI. Similar results were observed for the CWI condition. Ratings of physical readiness for exercise, mental readiness for exercise, and vigour were also superior to TWI for up to 1 h post-exercise. Prior belief in the benefits of CWI, the provided information sheets, and the effects of the cold stimulus itself may all explain these differing perceptions of fatigue and recovery. Given the discomfort reported during CWI, as compared with both thermo-neutral conditions, participants may simply expect it to be beneficial. However, apart from CWI participants feeling more vigorous post-recovery as compared with TWP, subjective ratings of fatigue and recovery were superior for both these conditions

as compared with TWI. As such, participants in the TWP were effectively deceived and led to believe that a placebo was as effective as CWI in promoting recovery from high-intensity exercise, and superior to TWI.

As hypothesised, recovery of quadriceps muscle strength and RTD over the 48-h post-exercise period was superior with TWP as compared with TWI. Coinciding with the improved psychological ratings, MVC_{max} and MVC_{avg} were significantly higher for TWP compared with TWI post-recovery, and 1 h and 48 h post-exercise. Furthermore, there was a trend for an improved RTD (PR) and mean force (24 h) for TWP compared with TWI. Given that the only difference between the TWI and TWP conditions was the addition of bath soap, with water depth and T_m the same between the two conditions, this demonstrated that a placebo effect was achieved. In further support of this, BE_{pre} was also significantly correlated to a greater recovery of MVC, demonstrating that belief in the effectiveness of post-exercise recovery may be associated with subsequent performance. By deceiving subjects into thinking they are receiving a beneficial treatment, participants felt more recovered and a superior performance was witnessed.

Despite a large body of research on the placebo effect in medicine, there are few studies reporting the magnitude and extent of its effect in sports performance (35). Only recently has empirical evidence emerged in support of the placebo effect impacting on athletic performance, encompassing both endurance- (81, 36) and resistance-related (219, 387) exercise performance. After being informed that an ergogenic aid was likely to improve cycling time-trial performance, the placebo effect has been shown to account for a 3.8% improvement in mean power over a 40-km cycling time-trial (81), and a 2.2% increase in mean power over a 10-km cycling time trial (36) when given a carbohydrate and caffeine placebo, respectively. Alternately, both a caffeine and amino acid placebo, were reported to improve leg extension strength (total work performed during repeated repetitions until fatigue at 60% 1-RM) by 11.8% after 72 h (387), and 1-RM leg press by 21.1% after 48 h (219), respectively. The current study, in comparison, has demonstrated an improved maximal (12.6%) and mean torque (13.2%) during a maximal voluntary isometric contraction of the

quadriceps, 48 h after exercise, when administered TWP as compared with TWI. As such, this study has demonstrated that influencing one's belief in the efficacy of thermo-neutral water immersion as an ergogenic aid in recovery can account for a ~13% improvement in muscle strength 48 h after high-intensity exercise.

This is the first study to test the hypothesis that the short-term benefits of CWI may at least be partly placebo-related, and that altered perceptions of fatigue play an important role in the recovery from exercise. Consistent with past literature (449, 26, 487, 389), the CWI condition demonstrated a favourable return of muscle strength up to 48 h post-exercise, as compared with the TWI condition. Despite recent suggestions that the efficacy of CWI in recovery is limited to only subjective measures of pain and soreness (78, 260), this study has demonstrated that it is also beneficial for the recovery of quadriceps leg strength following HIT. However, the recovery of isometric leg strength and RTD during the 48-h post-exercise period followed the same pattern in the CWI and TWP conditions, with no significant differences between groups, and both conditions demonstrating a favourable return to baseline when compared with TWI. As such, when compared with TWP, a significantly colder water immersion and greater reduction in T_m (~3°C) in the CWI condition was not associated with greater recovery of muscle strength and RTD during a MVC. This is the first reported evidence that the manipulation of one's expectation of recovery, via a CWI placebo (i.e., TWP), can have a similar beneficial influence on the recovery of muscle strength as CWI itself. Previous research has alluded to the fact that the placebo effect may contribute to observed performance and physiological benefits following halftime cooling between two cycling efforts (186), and CWI following eccentric loading of the quadriceps (437). The findings of the current study have confirmed that the placebo effect may account for some of the observed benefits following CWI, or alternatively, that it is as strong as these physiological benefits.

The simultaneous assessment of objective physiological and performance variables with the subjective psychological and perceptual measures strengthens the suggestion of a placebo effect. Similar to previous research, a single bout of high-intensity anaerobic exercise promoted an increase in thigh girth (487), circulating leukocytes (461), and IL-6 concentration (461, 316), for all

conditions. Despite previous research demonstrating cold-induced alterations in these markers (461, 407), an ~3°C reduction in T_m did not influence these measures, suggesting CWI has no influence on these physiological markers. The acute immune response and elevation in the myokine IL-6 reported in the current study is consistent with that reported previously (316), but could be a result of elevated body temperature and/or metabolic status after high-intensity exercise (370). Whether this response is associated with a prolonged reduction in leg strength, and whether cold-water immersion could attenuate such a response, is unclear. Future research is warranted to investigate this issue, including comparisons to an exercise protocol likely to cause significant muscle damage, oedema and inflammation. Alternately, CWI (or the protocol used in this study) may not have induced the physiological response necessary to enhance recovery and improve performance. The apparent differences in MVC performance between groups, despite no difference in the physiological markers measured, reinforces the possibility of a CWI placebo effect. Altered perceptions of readiness for exercise and fatigue have the potential to influence performance, but will have little influence on physiological processes. These findings again highlight a potentially greater psychological role of CWI in recovery than the commonly-hypothesised physiological role.

2.4.1 Practical Applications/Implications:

Similar improvements in performance following CWI or a CWI placebo have highlighted the importance of belief in recovery. Regardless of any potential physiological role, it is important for coaches and sport scientists alike to educate their athletes on the benefits of recovery, and also encourage belief in the practice. This is particularly pertinent for athletes more responsive to the placebo effect, as it is well documented that some individuals show remarkable responses to placebo interventions, while others may not at all (41). A strong belief in CWI, combined with any potential physiological benefits, will maximise its worth in recovery from exercise. Future research in this area should investigate whether alternate exercise protocols (e.g., resistance training) would elicit a similar placebo effect. It is possible that CWI is more appropriate as a recovery modality following exercise or sport inducing a large degree of muscle damage or contusion injury. Furthermore, future research should investigate whether these results can be

replicated with well-trained athletes, and whether this placebo effect can influence athletic performance.

2.4.2 Conclusion

A HIT protocol of four 30-s maximal sprints can elicit a significant increase in T_m, accompanied by a significant reduction in muscle strength up to 48 h post-exercise. Despite being the same condition and eliciting the same physiological response, a CWI placebo administered after HIT was superior in the recovery of muscle strength as compared with a TWI control. A CWI placebo is also as effective as CWI itself in the recovery of muscle strength over 48 h. This can likely be attributed to improved subjective ratings of pain and readiness for exercise, suggesting that the hypothesised physiological benefits surrounding CWI may be at least partly placebo-related.

CHAPTER THREE

The Influence of Cold-Water Immersion on the Molecular Responses Following a Single Bout of High-Intensity Interval Training

The main finding from Chapter 2 was that a recovery placebo was superior to a thermo-neutral control condition in improving skeletal muscle recovery following a single bout of HIT. However, the CWI condition was reported to be equally as effective as the placebo condition, supporting previous suggestions that CWI is effective in improving skeletal muscle recovery. For example, leg strength decreased by ~10% 48 h after a single bout of HIT in the control condition, but returned to baseline levels in both the CWI and placebo conditions. As such, the placebo effect may account for some of the observed benefits following CWI, or alternatively, it may be as strong as the observed physiological benefits.

Chapter 3 aimed to investigate the underlying molecular mechanisms by which CWI may improve skeletal muscle recovery, and assess its potential to augment aerobic adaptations following a single bout of HIT. Considering CWI has previously been reported to augment the exercise-induced increase in PGC-1α mRNA (192), it was hypothesised that post-exercise CWI would serve as a stimulus to increase certain markers of exercise-induced mitochondrial biogenesis. Particular focus was given to the p53 and PGC-1α pathways.

The manuscript for this study is in the final stages of preparation:

Broatch, J. R., Petersen, A., Bishop, D. J. (2015). Post-exercise cold-water immersion activates PHF20 and p53 signalling in human skeletal muscle. *American Journal of Physiology* (in preparation).

3.1 Introduction

Cold exposure is a powerful stimulus for mitochondrial phenotype alterations, and increased mitochondrial density and enzyme content are well-known characteristics of fish during acclimation to seasonal cold (34, 120). In cold-bodied fish, persistent cold stress compromises the ability to produce ATP, a potential mechanism that stimulates aerobic metabolism and metabolic remodelling (351). These adaptive responses are thought to be mediated by the peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) family of transcriptional coactivators (262, 359), and associated downstream transcription factors such as nuclear respiratory factor 1 (NRF-1) (262, 306, 359). In humans, cold stimulation has also been demonstrated to augment the post-exercise gene expression of PGC-1α (451, 452, 192), an important regulator of skeletal muscle mitochondrial function, respiration and biogenesis (286).

Increases in PGC-1α mRNA following post-exercise muscle cooling have been attributed to both shivering and non-shivering (adaptive) thermogenesis (451, 452, 192). For example, 1 h of exercise followed by 3 h of recovery in a cold (7°C) environment induced significantly greater increases in PGC-1α mRNA in human skeletal muscle, compared with a control condition (452), consistent with increases in rodent skeletal muscle following cold exposure (4°C for 4 d) alone (357). This increase in PGC-1α mRNA was attributed to contraction-induced increases in calcium (Ca^{2+}) and adenosine monophosphate (AMP) as a result of shivering, which may serve to activate PGC-1α via the upstream kinases calcium/calmodulin-dependent protein kinase (CaMK) and AMP-activated protein kinase (AMPK) (452). However, PGC-1α mRNA has also been reported to increase in human skeletal muscle following post-exercise cold-water immersion (CWI) without shivering (192), consistent with work in adipose tissue and muscle cells (396, 518). The mechanisms responsible for this are unknown, but may be related to cold-induced increases in reactive oxygen species (ROS) and/or nitric oxide (NO), potentially regulating an adaptive thermogenesis response. These studies suggest that cold application may be a novel method to promote exercise-induced mitochondrial biogenesis in humans, but further research is required to identify additional underlying mechanisms.

Post-exercise CWI may also potentiate exercise-induced mitochondrial adaptations via activation of the PHF20-p53 pathway. Plant homeodomain finger protein 20 (PHF20) is a novel transcription factor previously demonstrated to transcriptionally up-regulate p53 (366), and promote stabilisation/activation of p53 in the nucleus via disruption of mouse double minute 2 homolog (Mdm2)-mediated degradation (106). Tumour suppressor protein p53 plays an integral role in the response to genotoxic stress signals and associated DNA damage, where it regulates cell cycle arrest, apoptosis, genomic stability and cell deterioration (265). Research has also implicated p53 activation in response to everyday stressors (e.g., metabolic stress from exercise), whereby activated p53 transcriptionally regulates genes and microRNAs (miRNAs) associated with energy metabolism (45). For example, exercise-induced ROS have been implicated as an activator of p53 (282), a mechanism by which p53 may regulate aerobic metabolism, PGC-1α expression and mitochondrial biogenesis (45, 30, 286). Cold-exposure may also induce oxidative stress (501), implicating post-exercise CWI as a stimulus to augment ROS production and mitochondrial biogenesis. Should CWI increase PHF20-p53 activity, it may serve as a potential method to increase the expression of genes associated with mitochondrial biogenesis, with possible benefits for improving mitochondrial content and function.

The aim of this study was to investigate the underlying molecular mechanisms by which CWI may alter the signalling pathways associated with mitochondrial biogenesis following a single bout of high-intensity interval training (HIT). In particular, this study investigated the response of the p53 pathway following a single exposure to post-exercise CWI. It was hypothesised that post-exercise CWI would augment p53 activation and PGC-1α gene expression, a response potentially induced by cold-induced oxidative stress. As such, this study aims to provide molecular insight into the merit of CWI following exercise, and, more importantly, assess its potential to augment aerobic adaptations.

3.2 Methods

3.2.1 Participants

Nineteen healthy males (age 24 ± 6 y; body mass 79.5 ± 10.8 kg; height 180.5 ± 9.9 cm; mean ± SD) participated in this study. Informed consent was obtained prior to participation, and all participants were screened for cardiovascular risk factors associated with exercise. Participants were recreationally-active several days a week, but not specifically trained to compete in any one sport. The study followed a parallel group design, in which participants were assigned to one of two recovery conditions in a randomised, counter-balanced fashion (ranked according to their VO_{2peak}). These conditions were CWI (*n*=9) or a passive control (CON, *n*=10). All procedures were approved by the Institution's Human Research Ethics Committee.

3.2.2 Pre-Experimental Procedures

Prior to the experimental protocol, participants made several familiarisation visits to the laboratory to become accustomed with the equipment and testing procedures, namely the single bout of HIT and their assigned recovery condition. During one of these visits participants performed a discontinuous graded exercise test (GXT) on an electronically-braked cycle ergometer (Lode, Groningen, The Netherlands), to determine maximal oxygen uptake ($\dot{V}O_{2peak}$; 46.5 ± 8.1 $mL \cdot kg^{-1} \cdot min^{-1}$). This protocol is described fully in Chapter 4. All exercise tests and recovery familiarisations were performed at least three days prior to the experimental protocol.

3.2.3 Experimental Protocol

Following an overnight fast and abstinence from exercise and alcohol for the preceding twenty-four hours, participants reported to the laboratory in the morning (0730) for the experimental trial. Upon arrival, the lateral aspect of the right thigh was anaesthetised (1% Xyolcaine), and three incisions were made through the skin and underlying fascia. The incisions were made approximately one-third of the distance from the knee to the hip, and spaced 1-2 cm apart. Using a 6-mm Bergström needle modified with suction (124), muscle biopsies were

taken from the belly of the *vastus lateralis*. A total of three muscle biopsies were taken during the experiment, with each biopsy taken from a separate incision site. The first biopsy was taken at rest, immediately before the HIT session (Pre). Participants then performed the single HIT bout and their designated recovery condition. Exactly two minutes post-recovery, a second muscle biopsy was taken (Post). Participants remained in the laboratory, fasted and at rest, until a third biopsy was taken 3 h post-recovery (3 h). The post and 3 h biopsy incision sites were covered with sterile gauze and a water-proof dressing (Tegaderm, 3M, North Ryde, Australia) during exercise and recovery. Following each biopsy, the corresponding incision site was closed with a suture. Samples were blotted on filter paper to remove blood and immediately snap-frozen in liquid nitrogen, then stored at -80°C until subsequent analyses. Participants were allowed to consume water *ad libitum* during the trial. All experimental sessions were conducted in a laboratory maintained between 19-22°C with ≥ 70% relative humidity.

3.2.4 High-Intensity Interval Training Session

On an electronically-braked cycle ergometer (Velotron, Racer-Mate, Seattle, WA), participants first completed a 5-min warm-up at 75 W. This was immediately followed by 4 x 30-s 'all-out' efforts at a constant resistance (7.5% of body mass), interspersed by 4 min of passive rest. To eliminate individual variance with self-administered 'speeding up' of the flywheel, participants began each effort from a rolling start corresponding to 120 rpm. During each effort, participants were given extensive verbal encouragement and asked to remain seated in the saddle.

3.2.5 Recovery Interventions

Exactly five minutes after completing the HIT session, participants performed their assigned recovery intervention for 15 min. Seated (with legs fully extended), participants were immersed in water up to their umbilicus (CWI: 10°C), or rested on a laboratory bed in the same position (CON: 23°C). The CWI condition was performed in an inflatable bath (iBody, iCool Sport, Australia), and water temperature was maintained with a cooling/heating unit (Dual Temp Unit, iCool Sport, Australia), as described previously (56).

3.2.6 Muscle Analyses

3.2.6.1 Preparation of Whole-Muscle Lysates for Western Blotting

A 10-15 mg piece of frozen muscle was added to ice-cold homogenising buffer (37.5 µL/mg tissue; 20 mM Tris, 137 mM NaCl, 1% Triton-X, 10% Glycerol, 2.7 mM KCl, 1 mM MgCl, 1 mM EDTA, 1 $\mu g.mL^{-1}$ aprotinin, 1 $\mu g.mL^{-1}$ leupeptin, 1 mM benzamedine, 1 mM Na_3VO_4, 1 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride – adjusted to pH 7.4), and homogenised on ice using a motorised pestle. The whole muscle lysate was used for Western blotting without centrifugation, (334). Protein concentration of the lysate was determined using a commercially-available colorimetric assay (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as the standard. All samples were diluted with distilled water to a standard concentration (1.5 µg. μL^{-1}), and further diluted with 2 x Laemmli buffer (125 $mmol.L^{-1}$ Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.015% bromophenol blue, 10% β-mercaptoethanol). The resultant lysate was split equally into two parts: one unboiled and the other boiled at 95°C for 5 min. Both lysate fractions were subsequently stored at -80°C for future analyses.

3.2.6.2 Western Blot Analysis

Muscle lysates were loaded equally (15 µg) and separated on 10% self-cast SDS-PAGE gels by means of electrophoresis, at 100 V for 1.5-2 h (Bio-Rad, Mississauga, ON, Canada). Unboiled muscle lysate was used for all proteins except p-$p53^{Ser15}$, in which samples were re-boiled at 95°C for 3 min prior to loading. Once resolved, gels were transferred wet onto polyvinylidene fluoride (PVDF) membranes in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.01% SDS) at 100 V for 90 min. Membranes were then blocked with 5% skim milk in Tris-buffered saline (TBST: 150mM NaCl, 20mM Tris, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. Following 5 x 5 min washes in TBST, the membranes were incubated overnight (4°C with gentle agitation) with monoclonal primary antibodies (1:1000 in 5% BSA or skim milk) against phosphorylated-$AMPK^{Thr172}$, p38 mitogen-activated protein kinase (p38 $MAPK^{Thr180/Tyr182}$), $p53^{Ser15}$, and total PHF20 (Cell Signalling, UK). The following morning, membranes were again washed 5 x 5-min in TBST, and incubated in the

appropriate species-specific horseradish peroxidise-conjugated secondary antibodies at room temperature for 90 min, diluted 1:10000 (1:5000 for p-AMPK only) in 5% skim milk (1% BSA for p-PHF20 only) and TBST. After a further 5 x 5 min wash in TBST, membranes were exposed to a chemiluminescent liquid (Clarity^{TM} Western ECL Substrate, Bio-Rad, Hercules, CA) and visualised using a VersaDoc 4000 MP imaging system (Bio-Rad, Hercules, CA). Band densities were determined using Image Lab 5.1 software (Bio-Rad, Hercules, CA). Membranes were then stained with 0.1% Coomassie R-350 (PhastGelTM Blue R, GE Healthcare) as previously described (499), thereby providing a loading and transfer control. Comparative samples for each participant were loaded into the same gel, and one lane for each gel was reserved for an internal standard to account for inter-gel variability. Raw blot density was normalised to the internal standard and coomassie stain prior to analysis.

3.2.6.3 Total RNA Isolation

Approximately 20-30 mg of frozen muscle was added to 1 g of zirconia/silica beads (1.0mm: Daintree Scientific, Tasmania, Australia) and 800 µL of commercially available TRIzol reagent (Invitrogen, Carlsbad, CA), and homogenised using an electronic homogeniser (FastPrep FP120 Homogenizer, Thermo Savant). Samples were centrifuged at 13000 rpm for 15 min (4°C) to pellet cell debris. The upper homogenate was then removed and pipetted into 250 µL chloroform (Sigma Aldrich, St Louis, MO), and again centrifuged at 13000 rpm for 15 min (4°C). Without disturbing the interphase, the top phase was pipetted into a fresh Eppendorf containing 400 µL of 2-isopropanol alcohol (Sigma-Aldrich, St Louis, MO) and 10 µL of 5 M NaCl, and stored at -20°C overnight to allow for RNA precipitation. The following morning the sample was centrifuged at 13000 rpm for 20 min (4°C), following which the majority of the isopropanol was aspirated. The remaining RNA pellet was washed once with 75% ethanol made with diethylpyrocarbonate-treated (DEPC) H_2O (Invitrogen Life Sciences), and centrifuged at 9000 RPM for 8 min (4°C). Ethanol was aspirated off, and the pellet was re-suspended in 5 µL of heated DEPC-treated H_2O. RNA concentration was quantified spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 (A_{260}) and 280 (A_{280}) nm, with an $A_{260/280}$ ratio of 1.78 ± 0.11. RNA was stored at -80°C until further analysis.

3.2.6.4 Real-Time RT-PCR

One microgram of RNA was reverse transcribed into cDNA using a thermal cycler (S1000™ Thermal Cycler, Bio-Rad, Hercules, CA) and a commercially available kit (iScript™ cDNA Synthesis Kit, Bio-Rad, Melbourne, Australia) with random hexamers and oligo dTs, according to the manufacturer's instructions. All samples and RT negative controls were run together to prevent technical variation. 'Real-time' PCR reactions (total volume 10 µL) were performed with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) as the fluorescent agent. The following cycling patterns were used: initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 60 s (Mastercycler® RealPlex^2, Eppendorf, Hamburg, Germany). Forward and reverse primers for target genes were designed using sequences obtained from GenBank (Table 3-1). All samples were run in duplicate with negative controls, using an automated pipetting system (epMotion 5070, Eppendorf, Hamburg, Germany). To account for the efficiency of RT and initial RNA concentration, the mRNA abundance of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and β_2 microglobulin (β2M) were quantified, and target genes were normalised to an average of all three. Relative changes in mRNA abundance were calculated as described previously (171).

Table 3-1: Primer sequences used for real-time PCR

Gene	Forward Primer	Reverse Primer
GAPDH	AAAGCCTGCCGGTGACTAAC	CGCCCAATACGACCAAATCAGA
TBP	CAGTGACCCAGCAGCATCACT	AGGCCAAGCCCTGAGCGTAA
β2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
PGC-1α	GGCAGAAGGCAATTGAAGAG	TCAAAACGGTCCCTCAGTTC
NRF-1	CTACTCGTGTGGGACAGCAA	AGCAGACTCCAGGTCTTCCA
NRF-2	AAGTGACAAGATGGGCTGCT	TGGACCACTGTATGGGATCA
Tfam	CCGAGGTGGTTTTCATCTGT	GCATCTGGGTTCTGAGCTTT
SOD2	CTGGACAAACCTCAGCCCTA	TGATGGCTTCCAGCAACTC
GPX1	CGCCACCGCGCTTATGACCG	GCAGCACTGCAACTGCCAAGCAG
Catalase	CTCAGGTGCGGGCATTCTAT	TCAGTGAAGTTCTTGACCGCT
CIRP	CCCGACTCAGTGGCCG	AGCGACTGCTCATTGGTGTC
RBM3	GGACGTTCTCGCTACGTACTC	ACATGGCAGTTCAAGTCCTGG

3.2.7 Statistical Analyses

Data are reported in the text as means and standard deviation (mean ± SD). Comparisons between conditions were analysed using a two-way general linear

model (ANOVA) with repeated measures for time, where the within-subject factor was time and the between-subject factor was condition (CON vs CWI). Independent t-tests were performed when statistical significance was present. Data that violated Levene's test of homogeneity were log-transformed prior to analysis. The level of significance for all data was set at $p < 0.05$. The above analyses were performed using IBM SPSS Statistics V20 (IBM Corporation, USA). In addition, effect sizes (ES) were calculated using Cohen's *d*, with magnitude-based inferences used to interpret the meaningfulness of results. Cohen's conventions for effect size (with 90% confidence intervals) were used for interpretation, where ES = 0.5, and 0.8 are considered as medium and large, respectively. Effect sizes are presented (± confidence intervals) for data demonstrating an effect size larger than 0.5. Raw Western blot densitometry data (correct to internal standard and coomassie stain) and changes in mRNA abundance (RT-PCR data) were log-transformed and used for statistical analyses. For graphical purposes, baseline Western blot and RT-PCT values were normalised to 1.0, such that post and 3 h values correspond to fold change from pre values.

3.3 Results

3.3.1 HIT Session

Both groups performed similar volumes of total work during the single bout of HIT, 260.0 ± 64.6 kJ (CON) and 261.6 ± 40.5 kJ (CWI) ($p > 0.05$).

3.3.2 Western Blots

Representative Western blots are shown in Figure 3-1. Significant main effects of time were observed for the phosphorylation (p-) of p38 MAPK$^{Thr180/182}$ ($p < 0.001$) and p-AMPK^{Thr172} ($p < 0.001$). Specifically, p-p38 MAPK$^{Thr180/182}$ was elevated post-recovery ($p = 0.001$, ES = 0.99 ± 0.49), but returned to basal levels by 3 h post-recovery ($p = 0.479$). p-AMPK^{Thr172} similarly increased post-recovery ($p < 0.001$, ES = 0.81 ± 0.40), and remained significantly elevated 3 h post-recovery ($p < 0.001$, ES = 0.92 ± 0.27). There were no main effects of condition for either p-p38 MAPK$^{Thr180/182}$ ($p = 0.858$) or p-AMPK^{Thr172} ($p = 0.945$) (Fig 3-2).

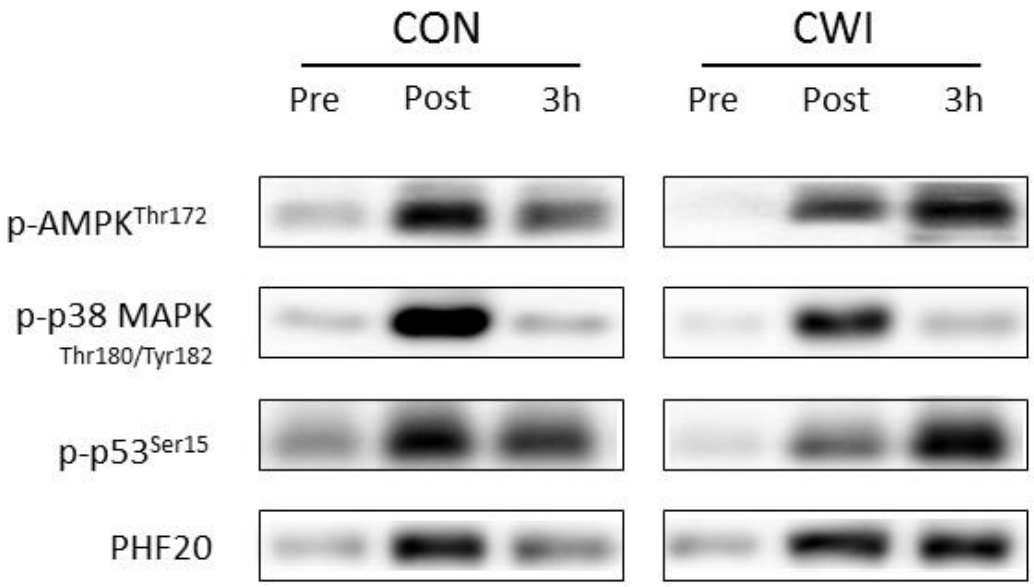


Figure 3-1: Representative western blots from muscle sampled pre-exercise (Pre), immediately post-recovery (Post), and 3 h post recovery (3 h) for control (CON) and cold-water immersion (CWI) conditions. p-, phosphorylated; AMPK, adenosine monophosphate-activated protein kinase; p38 MAPK, p38 mitogen-activated protein kinase; p53, tumour suppressor p53; PHF20, plant homeodomain finger protein 20; Thr, threonine; Tyr, tyrosine; Ser, serine.

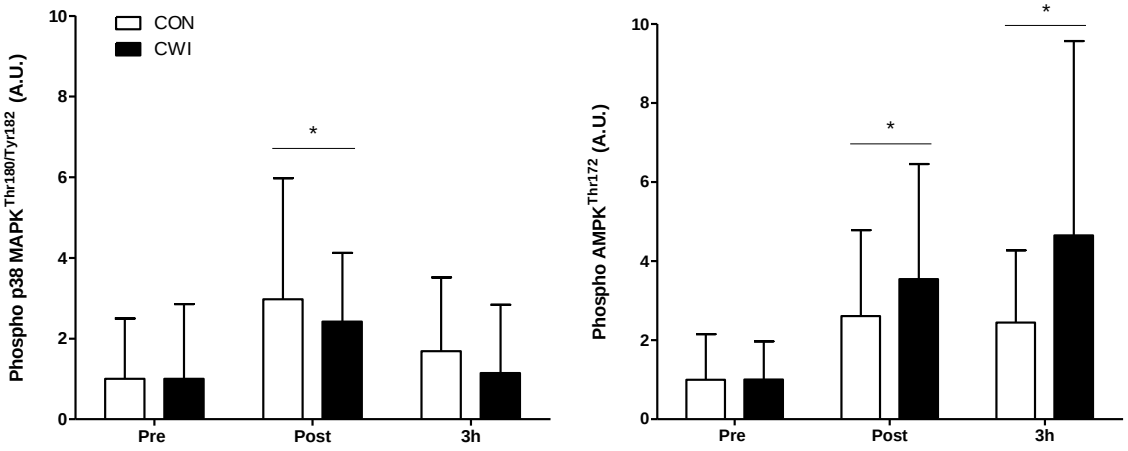


Figure 3-2: Phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK$^{Thr180/Tyr182}$) and adenosine monophosphate-activated protein kinase (AMPK^{Thr172}) from muscle sampled pre-exercise (Pre), post-recovery (Post), and 3 h post recovery (3 h) for control (CON) and cold-water immersion (CWI) conditions. Thr, threonine; Tyr, tyrosine. *Significant difference from pre (p < 0.05). Data are presented as mean ± S.D.

There were significant main effects of time for the content of PHF20 (p = 0.002) and p-p53^{Ser15} (p = 0.001). Specifically, total PHF20 protein content increased post-recovery (p = 0.007, ES 0.99 ± 0.72) and remained significantly elevated 3 h post-recovery (p = 0.003, ES 1.17 ± 0.65). Similarly, p-p53^{Ser15} was

elevated post-recovery (p = 0.001, ES 0.73 ± 0.43) and remained significantly elevated 3 h post-recovery (p = 0.002, ES 0.82 ± 0.45).

There were no significant main effects of condition for PHF20 content (p = 0.361) or p-p53^{Ser15} (p = 0.198). However, effect size calculations revealed that CWI resulted in larger (non-significant) increases in PHF20 content from pre-exercise to 3 h post-recovery (ES = 0.93 ± 1.12). Similarly, the increase in p-p53^{Ser15} content from baseline was larger for CWI post-recovery (ES = 0.55 ± 0.70) and 3 h post-recovery (ES = 0.71 ± 0.76) when compared with the CON condition (Fig 3-3).

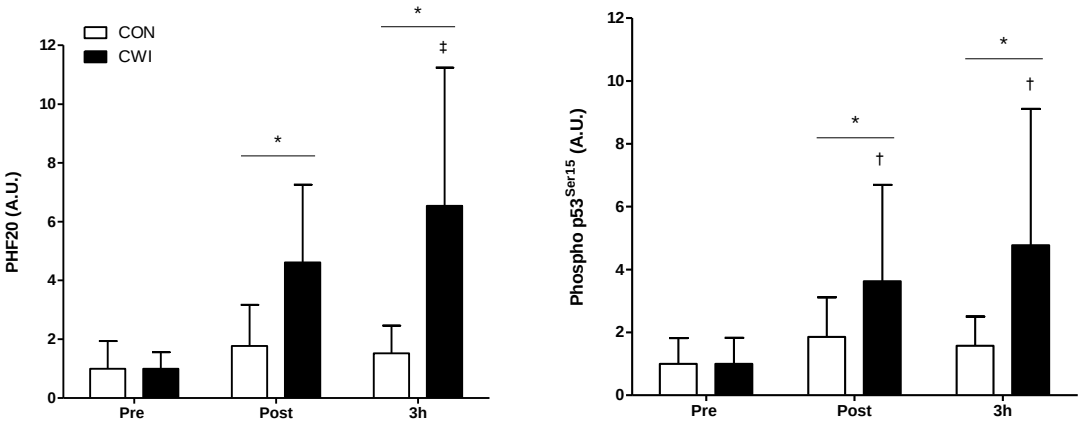


Figure 3-3: Total plant homeodomain finger protein 20 (PHF20) protein and phosphorylation of p53^{Ser15} from muscle sampled pre-exercise (Pre), post-recovery (Post), and 3 h post recovery (3 h) for control (CON) and cold-water immersion (CWI) conditions. *Significant difference from pre (p < 0.05). † Moderate effect (ES = 0.5-0.8) compared with CON. ‡ Large effect (ES > 0.8) compared with CON. Data are presented as mean ± S.D.

3.3.3 mRNA

All housekeeping genes (GAPDH, TBP and β2M) remained unchanged over time and as a result of condition (p > 0.05). A significant main effect of time (p < 0.001) was observed for PGC-1α mRNA, with post-analysis revealing it was significantly elevated 3 h post-recovery (p < 0.001, ES = 3.20 ± 0.37). However, no main effect of condition was observed for PGC-1α mRNA (p = 0.422). There were no main effects of time or condition for the mRNA content of nuclear respiratory factor-1 (NRF-1; p = 0.621 and 0.898, respectively), nuclear

respiratory factor-2 (NRF-2; p = 0.401 and 0.789, respectively) or mitochondrial transcription factor (Tfam; p = 0.321 and 0.882, respectively) (Fig 3-4).

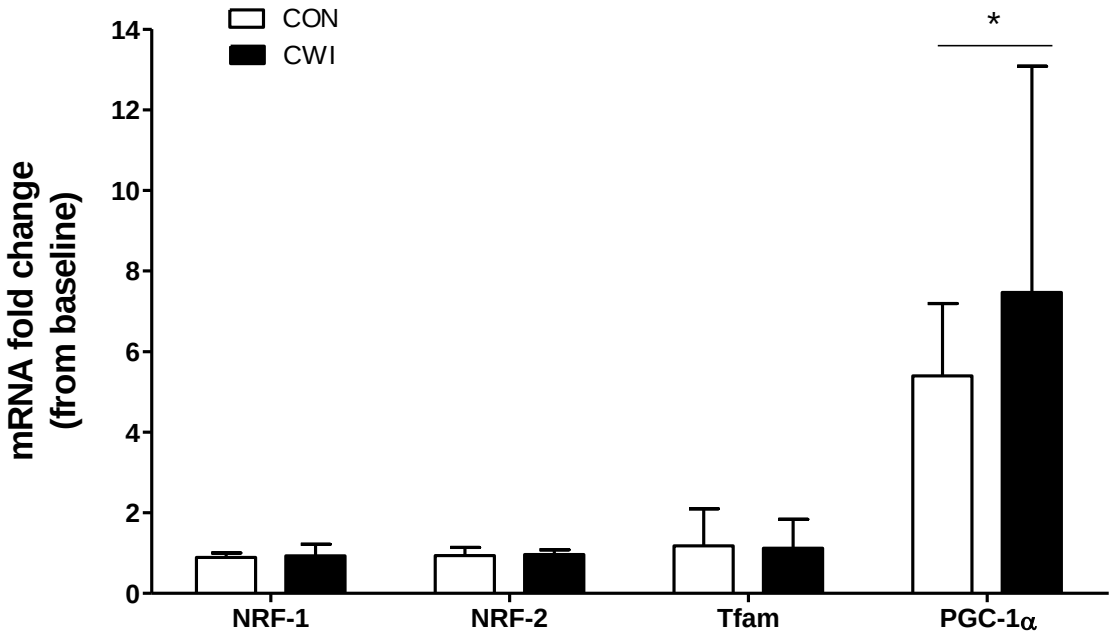


Figure 3-4: Gene expression from muscle sampled 3 h post-recovery for control (CON) and cold-water immersion (CWI) conditions. NRF-1/2, Nuclear respiratory factors 1/2; Tfam, Mitochondrial transcription factor; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-α. Corrected to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and β_2 microglobulin (β2M) housekeeping genes. *Significant difference from pre (p < 0.05). Data are presented as mean ± S.D.

There were no main effects of time for the mRNA content of p53 (p = 0.186), or the p53-downstream targets mitofusin-2 (Mfn2; p = 0.480), dynamin-related protein 1 (Drp1; p = 0.933), synthesis of cytochrome *c* oxidase (SCO2; p = 0.377) or apoptosis inducing factor (AIF; p = 0.339). A main effect of condition was demonstrated for Mfn2 (p = 0.050), but not p53 (p = 0.655), Drp1 (p = 0.586), SCO2 (p = 0.377) or AIF (p = 0.176). Specifically, Mfn2 mRNA was greater 3 h post-recovery for the CWI condition as compared with CON (p = 0.046, ES = 0.53 ± 0.45). At the 3 h post-recovery time-point, effect size calculations revealed greater mRNA content of AIF (ES = 0.72 ± 0.55) and SCO2 (ES = 0.72 ± 0.77) following CWI, as compared with the CON condition (Fig. 3-5).

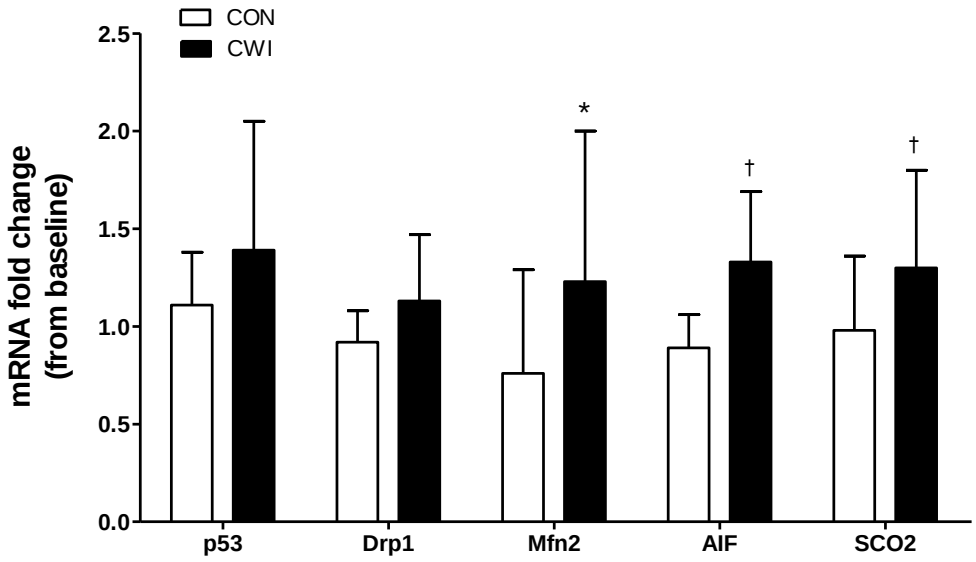


Figure 3-5: Gene expression from muscle sampled 3 h post-recovery for control (CON) and cold-water immersion (CWI) conditions. Drp1, dynamin-related protein 1; Mfn2, Mitofusin 2; AIF, apoptosis-inducing factor; SCO2, synthesis of cytochrome c oxidase 2. Corrected to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and β_2 microglobulin (β2M) housekeeping genes. *Significant difference from CON ($p < 0.05$). † Moderate effect (ES = 0.5-0.8) compared with CON. Data are presented as mean ± S.D.

There were no main effects of time or condition for the mRNA content of manganese superoxide dismutase (MnSOD; p = 0.165 and 0.534, respectively), glutathione peroxidase 1 (GPX-1; p = 0.155 and 0.180, respectively) or catalase (p = 0.086 and 0.311, respectively). However, effect size calculations revealed that CWI increased the mRNA content of catalase 3 h post-recovery (ES = 0.71 ± 0.65), as compared with the CON condition (Fig 3-6).

No main effects of time or condition were observed for the mRNA content of cold-inducible RNA-binding protein (CIRP; p = 0.456 and 0.326, respectively) or RNA-binding motif protein 3 (RBM3; p = 0.304 and 0.264, respectively). However, effect size calculations revealed that CWI induced a moderate increase in RBM3 (p = 0.154, ES = 0.50 ± 0.57) mRNA 3 h post-recovery, as compared with CON (Fig. 3-7).

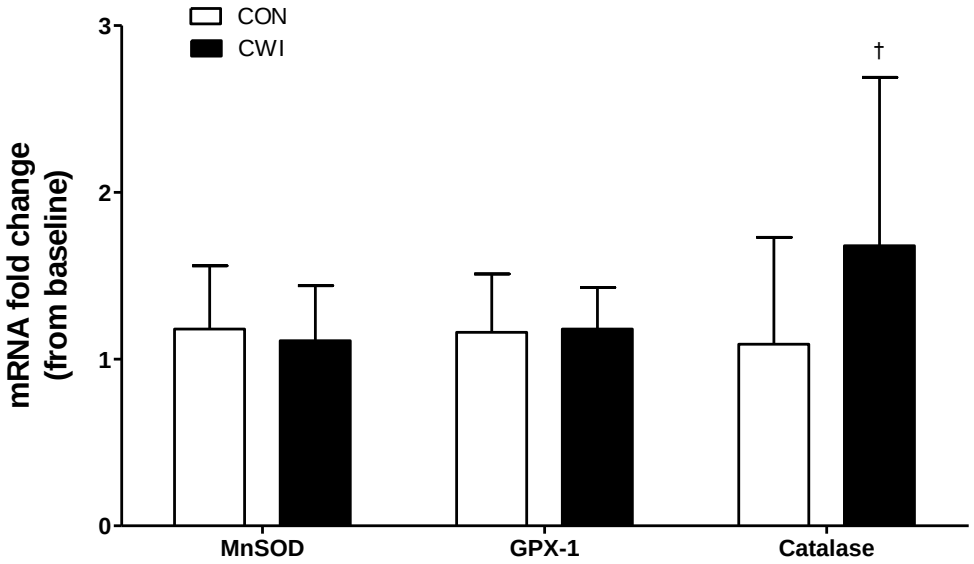


Figure 3-6: Gene expression from muscle sampled 3 h post-recovery for control (CON) and cold-water immersion (CWI) conditions. MnSOD, manganese superoxide dismutase; GPX-1, glutathione peroxidase 1. Corrected to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and β_2 microglobulin (β2M) housekeeping genes. † Moderate effect (ES = 0.5-0.8) compared with CON. Data are presented as mean ± S.D.

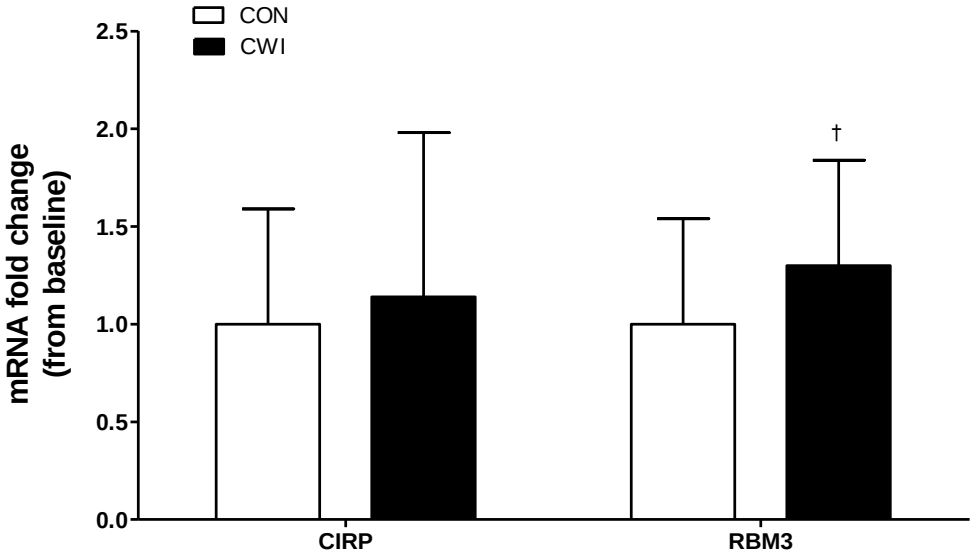


Figure 3-7: Gene expression from muscle sampled 3 h post-recovery for control (CON) and cold-water immersion (CWI) conditions. CIRP, cold-inducible RNA-binding protein; RBM3, putative RNA-binding protein 3. Corrected to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and β_2 microglobulin (β2M) housekeeping genes. † Moderate effect (ES = 0.5-0.8) compared with CON. Data are presented as mean ± S.D

3.4 Discussion

The main finding of this study was that post-exercise CWI, when compared with a passive control, altered some of the molecular signalling pathways associated with mitochondrial biogenesis following a single bout of high-intensity exercise. Despite no difference in PGC-1α mRNA, there was a greater post-exercise increase in both the total protein content of PHF20 and phosphorylation of p53 in participants administered CWI. The mRNA content of genes downstream of p53, including Mfn2, was also increased in CWI participants as compared with CON participants. The mechanisms by which post-exercise CWI augments the PHF20-p53 signalling pathway may be related to a cold-induced oxidative insult, as supported by increased gene expression of the antioxidant catalase.

This study provides the first evidence in human skeletal muscle that p53 phosphorylation is enhanced following post-exercise CWI. As shown in a number of different cell and tissue types, p53 plays an important role in regulating mitochondrial content and oxidative capacity (30). Consistent with previous work in human skeletal muscle (31), this study reports that $p53^{Ser15}$ phosphorylation was increased 3 h following a single bout of high-intensity exercise (4 x 30-s all-out cycling efforts) (Fig 3-3). In addition, post-exercise CWI enhanced this response in a manner similar to that reported in response to reduced carbohydrate availability (32). Thus, similar to other non-genotoxic stresses, such as fluctuations in oxygen or carbohydrate availability, the additional stress imposed by post-exercise CWI may have contributed to the observed greater increase in p53 activation (45).

Despite its role in the transcriptional regulation of p53 (366), this is the first study to demonstrate an increase in PHF20 protein content following a single bout of HIT. Furthermore, this response was augmented following post-exercise CWI. The mechanism by which this occurs is unknown, but may be related to cold-induced β-adrenergic stimulation and subsequent protein kinase B (Akt) phosphorylation (468, 366). The current study also observed a parallel increase in PHF20 content and $p53^{Ser15}$ phosphorylation, consistent with previous reports that PHF20 interacts with p53 protein inside the nucleus, promoting its

phosphorylation and stabilisation (106). As such, these findings raise the possibility that at least some of the CWI-induced increase in p53 phosphorylation may be mediated by an increase in PHF20 protein. Given the paucity of research examining the association of PHF20 and p53 following exercise, future research investigating these hypotheses is warranted.

Consistent with the upstream activation of p53, post-exercise CWI also increased the expression of a number of p53-target genes. Two proteins central to mitochondrial remodelling are the fusion protein Mfn2, and the fission protein Drp-1, both of which are activated by p53 (267, 494). Mfn2 is a mitochondrial GTPase that plays an important role in mitochondrial fusion, therefore contributing to the maintenance of the mitochondrial network (23). In contrast to the increase in Mfn2 mRNA following a single bout of continuous cycling (1 h at 60% W_{max}) (209, 452), there was no change reported in the control condition in the present study. However, Mfn2 mRNA was increased following CWI, and may be a result of upstream p53 phosphorylation. There was no change in Drp1 mRNA in either the CON or CWI in the present study, consistent with previous research demonstrating unaltered protein content following two to four weeks of HIT (377, 157). Further work is needed to verify these pathways in human skeletal muscle following exercise (32), and to investigate the influence post-exercise CWI has on the assembly, expansion and remodelling of the mitochondrial reticulum.

In conjunction with an inhibition of glycolysis, p53 has been shown to promote oxidative phosphorylation via translational activation of AIF and SCO2 (462, 301, 45). Typically known for its role in initiating apoptosis, positive transcriptional regulation of AIF by p53 under non-stressful conditions also promotes the proper assembly and function of mitochondrial complex I (462, 215). In the present study, AIF mRNA was unaltered in the CON condition. Studies demonstrating exercise-induced alterations in AIF are limited, with four to eight weeks of treadmill running causing no change in mRNA or protein content in animal soleus muscle (448, 297). Furthermore, four weeks of cycling training at varying intensities (ranging from ~60% to ~200% maximal aerobic power) had no effect on AIF protein content in human skeletal muscle (157). However, AIF protein has been reported to increase following repeated electrical stimulation (3

h/d for 7 d) in rat tibialis anterior muscle, suggesting its expression may be contraction- and/or fibre-type specific (4). Albeit insignificant, this is the first study to report a moderate increase in AIF mRNA (~33% at 3 h post-recovery) following post-exercise CWI. As such, CWI may serve as a stimulus to promote an increase in AIF gene expression, but further work is needed to determine if this will contribute to greater training-induced mitochondrial adaptations in human skeletal muscle.

SCO2 is required for the correct assembly of the mitochondrial DNA (mtDNA)-encoded COXII subunit (301, 536), and thus plays an important role in aerobic metabolism. Consistent with rodent studies, demonstrating unaltered skeletal muscle SCO2 mRNA in response to a single bout of endurance exercise (15 m/min for 90 min) (423), SCO2 mRNA was unchanged in the CON group in the current study. There was, however, a small and insignificant elevation in SCO2 mRNA content (~30% at 3 h post-recovery) in the CWI group, suggesting cold exposure may serve to increase its expression. Although also not significant, a similar response was demonstrated with reduced carbohydrate availability prior to a single bout of high-intensity interval exercise (6 x 3-min bouts at 90% VO_{2max}) in human skeletal muscle (32). A single bout of high-intensity exercise may have little effect on SCO2 mRNA expression 3 h post-exercise, but the additional stress imposed by reduced carbohydrate availability or CWI may serve as a stimulus to increase its expression. Consistent with the parallel increase in $p53^{Ser15}$ phosphorylation, these data indicate that CWI augments the mRNA content of three p53-target genes (Mfn2, AIF and SCO2).

It was hypothesised that the enhanced p53 activation observed in the current study may have been related to a cold-induced elevation in ROS. Recent research has highlighted the complex interaction between ROS and p53, whereby ROS has been implicated as both an upstream signal that triggers p53 activation, and a downstream factor that can be modulated by p53 (282). In the context of the current study, CWI may have induced ROS production via thermogenesis, where an elevated metabolism and stimulation of mitochondrial respiration will elevate ROS as a natural by-product (501). The return to normothermia following a hypothermic insult may also induce a small burst of ROS, most likely the result of

a sudden increase in mitochondrial electron transport (340). ROS production may be further exacerbated by the oxidation of certain molecules (e.g., cold-induced elevations in catecholamines) in the presence of oxygen, initiating a free-radical chain reaction (163). Regardless of the potential mechanism of ROS production during post-exercise CWI, the subsequent activation of p53 may serve as a hormetic stimulus to 'harden' and prepare an individual for subsequent ROS-related stress (445).

Following activation by ROS, p53 drives the expression of a range of antioxidant targets, including (but not limited to) GPX1, MnSOD, and catalase (363, 223). In the current study, there was a moderate increase in catalase mRNA content (~68% at 3 h post-recovery) following post-exercise CWI, compared with the passive control, further supporting CWI-induced p53 activation. Increased catalase expression and/or activity in response to CWI is most likely a result of p53-inducible ribonucleotide reductase (p53R2) activation, whereby a p53/p53R2-catalase pathway serves to protect against endogenous ROS under physiological conditions (223). In the present study, GPX-1 mRNA was unaltered in both the CON and CWI. Expression of GPX-1 mRNA has been reported to be mediated by p53 in cell culture models, but transcription levels were also shown to be tissue specific (420). Further research is needed to elucidate this potential link in human skeletal muscle. Although no changes in MnSOD mRNA were observed at any time point, the exact mechanisms surrounding the complex interactions between p53 and MnSOD are still not fully understood. For example, mitochondrial translocation of p53 has been shown to interact with MnSOD and inhibit its action (535), whereas either diminished or overexpressed MnSOD results in oxidative stress and damage (180, 10). CWI also increased AIF mRNA, another redox-sensitive p53 target shown to possess nicotinamide adenine dinucleotide (NADH) oxidase and antioxidant activity (286). In summary, these data suggest that the potential mild and subtle oxidative stress imposed by post-exercise CWI may activate p53 and augment an antioxidant response, potentially limiting oxidative stress and protecting cells from subsequent oxidative damage (45).

Contrary to expectations, the potential CWI-induced elevation in oxidative stress did not increase p38 MAPK activation, despite ROS being a well-accepted

upstream activator (208). It is possible that this response was masked by one of the many other known upstream signals for p38 MAPK activation, such as the unaccustomed mechanical stress induced by "all-out" cycling efforts. Alternatively, other stress/mitogen-activated protein kinases, such as c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), may respond more robustly to a hypothermic insult. For example, JNK has been implicated in both upstream activation by ROS (379) and downstream activation of p53 (442), and thus seems a potential candidate for CWI-induced p53 activation. Consistent with previous work, AMPK phosphorylation was increased as a result of high-intensity cycling (146, 278). Prolonged activation of AMPK until 3 h post-recovery was most likely due to glucose deprivation following an extended fast (~12 h by 3 h post-recovery) and completion of an intense exercise bout (69). There was however, no difference in AMPK phosphorylation between the CON and CWI conditions. These data indicate that differences in p38 MAPK or AMPK phosphorylation are not responsible for the greater p53 activation following CWI, further supporting the hypothesis that ROS are a potential upstream activator.

Both energy- (AMPK) and stress-dependent (p38 MAPK) kinase signalling (204, 5) have been implicated in PGC-1α activation following HIT (286), a response that was supported in the present study. Consistent with previous research (278), this study demonstrated an increase in PGC-1α mRNA following a single bout of high-intensity interval exercise (Fig 3-4). However, post-exercise CWI had no additional influence on p38 MAPK or AMPK activation, consistent with the lack of response of their downstream target PGC-1α. This contrasts with previous reports that post-exercise CWI (10°C for 15 min) (192) and cold ambient exposure (4°C for 3-4 h) (451, 452) augments PGC-1α mRNA levels, compared with a passive room temperature control. However, certain methodological differences can explain the inconsistencies between these studies and the current study. For example, it is unlikely that the 15-min CWI protocol utilised in the current study would have elicited a shivering response of the same magnitude as the 3-4 h cold exposure used in the two studies from Slivka (452, 451). In regards to the study by Ihsan (192), the large CWI-induced increase in PGC-1α mRNA reported was likely confounded by the subdued

response in the control condition. Ihsan (192) reported an ~1.5-fold increase in PGC-1α mRNA 3 h after a single bout of high-intensity exercise, which is considerably lower than the > 3-fold increase observed in both the current study and previous studies (278, 146). The lack of a CWI-induced increase in PGC-1α mRNA in the current study is also supported by the absence of an effect on the mRNA levels of downstream transcription factors NRF-1, NRF-2 and Tfam. Despite the absence of a cold-induced increase in PGC-1α mRNA, this study has for the first time shown that the p53 pathway is activated following CWI, a pathway also heavily implicated in promoting aerobic phenotype alterations. Therefore, this study provides an alternate and novel explanation by which CWI may enhance contraction-induced mitochondrial biogenesis.

Another novel aspect of this study was to investigate changes in the mRNA content of the cold-shock proteins (CSPs) CIRP and RBM3 following post-exercise CWI. It is well accepted that transcription and translation is suppressed in response to cold stress, accompanied by a reduction in RNA degradation and alternate splicing of pre-mRNA (522, 16, 455). An increase in RBM3 following CWI may serve improve the transcription and translation of a number of mitochondrial biogenesis markers up-regulated following exercise (121, 114), potentially by stabilising mRNA and improving global protein synthesis under cold stress (455). In contrast to the moderate (non-significant) increase in RBM3 mRNA, CIRP mRNA was unchanged 3h following post-exercise CWI. However, the expression of CIRP mRNA has been reported to peak between 6-24 h following mild (32°C) cold exposure in mouse fibroblasts (346), which may explain the lack of response 3 h after post-exercise CWI. Further research is required to clarify the role of CSPs in the adaptive response to exercise and cold-stress in human skeletal muscle.

This study provides novel data that p53 phosphorylation is enhanced with post-exercise CWI in human skeletal muscle. In support of this, CWI increased the mRNA content of the p53-inducbile genes Mfn2, AIF and SCO2, which is also the first evidence of an increase in these targets following exercise in humans. In a similar manner to other non-genotoxic stresses (e.g., fluctuations in oxygen or carbohydrate availability), the additional stress imposed by post-

exercise CWI may have contributed to an increase in p53 activation. This may be related to a cold-induced elevation in oxidative stress, where a thermogenic response may stimulate mitochondrial respiration and elevate ROS as a natural by-product. In support of this, CWI also increased the mRNA content of the antioxidant catalase. It is hypothesised that the oxidative stress imposed by post-exercise CWI may activate p53, potentially augmenting an antioxidant response to protect the cells from oxidative damage, as well as the expression of p53 downstream targets associated with mitochondrial biogenesis. Furthermore, CWI had no additional influence on p38 MAPK or AMPK activation, consistent with the lack of response of their downstream target PGC-1α. Therefore, this study provides an alternate and novel explanation by which CWI may enhance contraction-induced mitochondrial biogenesis.

CHAPTER FOUR

The Effects of Regular Post-Exercise Cold-Water Immersion on the Adaptive Response to High-Intensity Interval Training

The final study presented as part of this thesis aimed to investigate the adaptive response to CWI performed after every training session during six weeks of HIT. Given the documented upregulation of the PHF20-p53 signalling pathway following a single bout of HIT and subsequent CWI (Chapter 3), this study aimed to investigate whether this response would translate into long-term improvements in selected markers of mitochondrial biogenesis, aerobic adaptation and exercise performance.

The manuscript for this study is in the final stages of preparation:

Broatch, J. R., Petersen, A., Bishop, D. J. (2015). Regular cold-water immersion following high-intensity interval training does not hinder the adaptive response in human skeletal muscle. *Journal of Applied Physiology*. (in preparation).

4.1 Introduction

Many studies have reported better recovery of skeletal muscle performance by using cold-water immersion (CWI) (260). However, there is debate about whether regular use of CWI post exercise will hinder or promote training adaptations. Improvements in skeletal muscle recovery following CWI (26, 326, 486) may allow athletes to train with a higher quality and/or load in subsequent sessions, potentially providing a greater stimulus for adaptation (490). However, this may be counteracted by potential long-term detrimental effects on skeletal muscle adaptation (523, 176, 187). For example, cold exposure following exercise has been suggested to attenuate essential components of the muscle regeneration process, including heat-shock protein (HSP) expression, satellite cell proliferation, and inflammation (389, 523). While three studies to date have investigated the effects of regular CWI on training-induced changes in performance, no has investigated the effects of regular CWI on the underlying skeletal muscle adaptations to training.

Much of the rationale behind a possible CWI-induced attenuation in training adaptation is based on evidence that cold exposure reduces exercise-induced inflammation (389) - a response essential in adaptive processes such as muscle repair (82), cytokine-stimulated mitochondrial biogenesis (394), and capillary growth (113). Damaging exercise will promote post-exercise inflammation and satellite cell proliferation (82), a means to explain how a CWI-induced reduction in these processes may attenuate training adaptations such as muscle strength and hypertrophy (523). However, regular CWI has also been reported to reduce endurance-related adaptations following cycling training (523), despite a likely absence of muscle damage and inflammation with this type of exercise (82). Many methodological limitations have been raised (490) with the study by Yamane et. al (523), and subsequent research investigating CWI's effect on aerobic adaptations are equivocal. For example, regular CWI following training has been reported to attenuate improvements in repeated-sprint performance (176) or to augment (4.4% increase in 1-s maximum sprint power) improvements in cycling performance (164). Given the contrasting findings reported to date, clarification of the merit of CWI on adaptations to exercise training is warranted.

A limitation of the research to date is that no study has investigated the underlying skeletal muscle adaptations to training that may be influenced by the use of CWI following each training session. Nonetheless, studies that have investigated the effects of CWI on exercise-induced changes in skeletal muscle cell signalling may provide some insight to this response. Contrary to suggestions of a cold-induced attenuation in aerobic adaptations to exercise training, CWI has been demonstrated to augment the post-exercise gene expression of key proteins involved in mitochondrial biogenesis. A single bout of high-intensity exercise and subsequent CWI has been reported to augment the post-exercise increase of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA (192), the so called "master-regulator" of mitochondrial biogenesis. Although the exact mechanisms underlying a cold-induced increase in PGC-1α mRNA are largely unknown, the authors suggested that a β-adrenergic-induced adaptive thermogenesis response may have been responsible (192). However, no study has investigated whether these effects translate into increased PGC-1α content following regular post-exercise CWI. Furthermore, additional research is required to identify whether a potential CWI-induced increase in PGC-1α content serves to improve markers of mitochondrial function (mass-specific mitochondrial respiration) and content (citrate synthase activity).

Post-exercise CWI may also potentiate exercise-induced mitochondrial adaptations via activation of the p53 pathway. As shown in a number of different cell and tissue types, p53 plays an important role in regulating mitochondrial content, oxidative capacity and exercise performance (301, 364, 422, 424, 425) in response to numerous cellular stresses. In humans, a single bout of high-intensity exercise increases the activation of p53 via phosphorylation (31), a response that is augmented in the presence of additional cellular stressors, such as reduced carbohydrate availability (32) and post-exercise CWI (Chapter 3). Furthermore, post-exercise CWI also increased the protein content of plant homeodomain finger protein 20 (PHF20) (Chapter 3), a transcription factor that up-regulates p53 (364) and promotes its stabilisation/activation in the nucleus (106). Although one study has observed a significant increase in p53 and PHF20 protein content following four weeks of high-intensity interval training (HIT) (157), no study to date has investigated the effects of regular CWI on the content of these transcription

factors. Consistent with previous observations following a single bout of high-intensity exercise and subsequent CWI (Chapter 3), it was hypothesised that the accumulative oxidative stress imposed by regular CWI (281, 340, 501) may serve to increase PHF20 and p53 protein content, which in turn may improve training-induced mitochondrial adaptations.

Therefore, the aim of this study was to investigate the effects of regular post-exercise CWI on key markers of mitochondrial biogenesis. It was hypothesised that regular CWI would increase the protein content of key transcription factors, consistent with a upregulation of the PHF20-p53 signalling pathway reported following a single CWI session (Chapter 3). Given their integral role in promoting aerobic phenotype alterations, it was also hypothesised that an increase in these proteins would provide a basis for improved aerobic phenotype adaptations, including mitochondrial respiration, maximal oxygen consumption ($\dot{V}O_{2peak}$) and endurance exercise performance. Given the widespread use and acceptance by athletes of CWI as a recovery modality following training, this research will provide mechanistic insights into its effects on skeletal muscle adaptations to training.

4.2 Methods

4.2.1 Participants

Sixteen healthy males (age 25 ± 6 y; body mass 80.0 ± 11.7 kg; height 180.5 ± 9.9 cm; $\dot{V}O_{2peak}$ 44.7 ± 5.9 $mL \cdot kg^{-1} \cdot min^{-1}$; mean ± SD) participated in this study. Participants were recreationally-active several days a week, but not specifically trained to compete in any one sport. Informed consent was obtained prior to participation, and all participants were screened for cardiovascular risk factors associated with exercise. All procedures were approved by the Institution's Human Research Ethics Committee. The study followed a two-group parallel group design, in which participants were assigned, in a randomised, counter-balanced fashion (ranked according to their VO_{2peak}), to one of two recovery conditions. These conditions were CWI (*n*=8) or passive control (CON, *n*=8) groups.

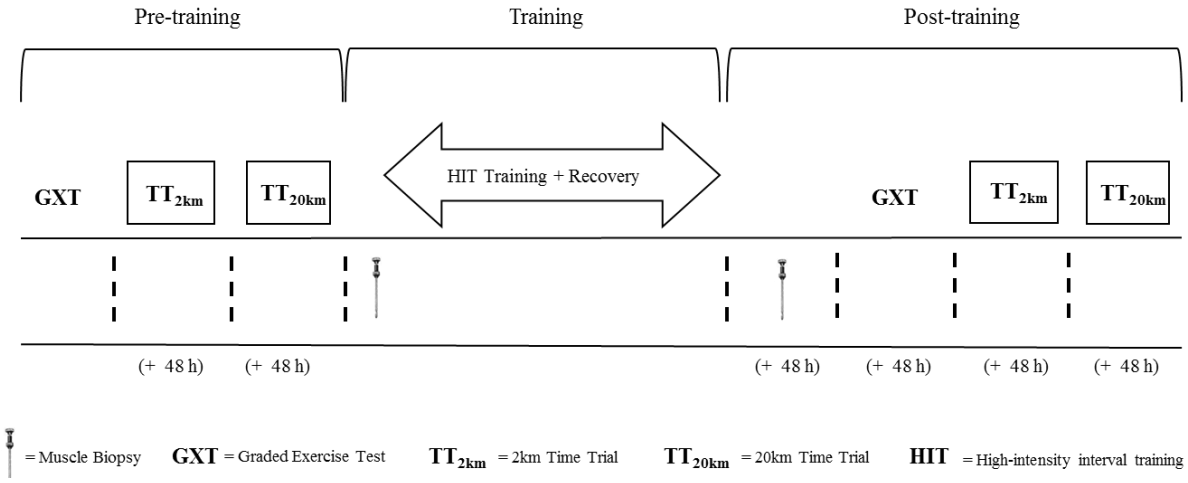


Figure 4-1: Schematic Diagram of the Training Study

4.2.2 Familiarisation

Prior to baseline testing, participants made several visits to the laboratory for familiarisation of the equipment and testing procedures. These sessions included a graded exercise test (GXT) to exhaustion, a 2-km cycling time trial (TT_2), and 20-km cycling time trial (TT_{20}), all performed on isolated days, and separated by at least 24 h. Participants also completed a familiarisation session of the HIT, and their assigned recovery condition.

4.2.3 Experimental Protocol

Briefly, the experimental protocol consisted of i) baseline testing; ii) a six-week training intervention, and iii) post-training testing (Fig. 4-1). All experimental sessions were conducted in a laboratory maintained between 19-22°C with ≥ 70% relative humidity.

4.2.3.1 Baseline Testing

Prior to the commencement of training, participants completed baseline trials of the TT_2, TT_{20}, and GXT protocols. All baseline trails were separated by at least 24 h, and performed in the morning (0700-1000) following an overnight fast. Participants were asked to refrain from exercise and alcohol consumption for 24 h prior to each trial. Immediately prior to the first training session, participants also underwent a resting needle biopsy procedure. The lateral aspect of the right thigh

was anaesthetised (1% Xylocaine), followed by a small incision through the skin and underlying fascia. Using a 6-mm Bergström needle modified with suction (124), the resting muscle biopsy was taken from the belly of the *vastus lateralis*, immediately before the first HIT session. Following the biopsy, the incision site was closed with a suture. A small piece (~15-20 mg) of fresh muscle was sectioned for immediate analysis of mitochondrial respiration. The remainder of the sample was blotted on filter paper to remove blood and immediately snap-frozen in liquid nitrogen, then stored at -80°C until subsequent analyses.

4.2.3.2 Training Intervention

Participants trained for a total of eighteen sessions over a six-week period. On an electronically-braked cycle ergometer (Velotron, Racer-Mate, Seattle, WA), participants first completed a 5-min warm-up at 75 W. This was immediately followed by 4-6 x 30-s 'all-out' efforts at a constant resistance corresponding to 7.5-9.5% of body mass, separated by 4 min of rest. To ensure a progressive training stimulus during the six-week training period, participants performed 4 repetitions in weeks 1-2, five in weeks 3-4, and six in weeks 5-6. Resistance ranged from 7.5-9.5% of body mass, so that the fatigue-induced reduction in power during the 30-s bout was at least 20 $W.sec^{-1}$. To eliminate individual variance with self-administered 'speeding up' of the flywheel, participants began each effort from a rolling start corresponding to 120 rpm. During each effort, participants were given extensive verbal encouragement and asked to remain seated in the saddle.

4.2.3.3 Post-Training Testing

A resting muscle biopsy was taken ~48-72 h after the final training session from the same leg used during the baseline testing. Participants also completed post-training trials for the TT_2, TT_{20}, and GXT protocols. The timing and nature of post-training testing was identical to baseline testing.

4.2.4 Exercise Performance Tests

4.2.4.1 Time Trials

On the same electronically-braked cycle ergometer used during training, participants were instructed to complete TT_2 and TT_{20} self-paced time-trials as quickly as possible. The only feedback given to participants during the trials was an update of distance covered: the half-way mark (1 km) during the TT_2 and every 2 km during the TT_{20}. Participants were allowed to control the gear ratio throughout the entire time trial, corresponding to three gearings on the chain-ring (28, 39 and 52) and ten on the rear sprocket (23, 21, 19, 17-11). Heart rate (RS800sd, Polar Electro Oy, Finland), exercise duration and average power were recorded during the trial.

4.2.4.2 Graded Exercise Test

Participants performed a discontinuous GXT on an electronically-braked cycle ergometer (Lode, Groningen, The Netherlands) to determine their lactate threshold (LT) and peak aerobic power (W_{peak}). An intermittent protocol was used, with 4-min exercise stages and 30-s rest stages. Following a 5-min steady state warm-up at 75 W, the workload was increased by 30 W every 4.5 min until the participant reached volitional fatigue. The starting workload varied between 60-120 W, ascertained from the familiarisation GXT and aiming to minimise the number of stages to a maximum of ten. Participants were instructed to maintain a pedalling cadence of 70 rpm and wore a heart rate monitor during the test. The test was stopped when pedalling cadence dropped below 60 rpm. Blood samples for the GXT were taken from a venous catheter (Optiva IV Catheter 20G x 1.75", Smiths Medical, USA) inserted into an antecubital vein 10 min prior to the first blood draw. The LT was determined as the workload at which venous blood lactate increased 1 mM above baseline (101), and was calculated using Lactate-E version 2.0 software (341). W_{peak} was calculated as previously reported (245, 169):

$W_{peak} = W_{final} + (\frac{t}{240} \cdot 30)$; where W_{final} was the power output of the last completed stage and *t* was the time in seconds of any final uncompleted stage.

4.2.4.3 Peak Oxygen Uptake

After volitional fatigue in the GXT, participants performed 5 minutes of passive rest before performing a $\dot{V}O_{2peak}$ test. This comprised of a steady-state cycle to fatigue at a supra-maximal power output corresponding to 105% of W_{peak}, previously reported to elicit $\dot{V}O_{2peak}$ values no different to that determined during a ramp incremental test performed 5 min previously (413). Participants were asked to maintain a pedalling cadence of 90-100 rpm until volitional fatigue, with the test terminated when cadence dropped below 80 rpm. Expired gases were analysed every 15 s using a custom-made metabolic cart. The gas analysers (S-3lA/II and CD-3A analysers, Ametek, PA, USA) were calibrated using known gas concentrations prior to each test (20.93% O_2, 0.04% CO_2 and 16.10% O_2, 4.17% CO_2; BOC Gases, Australia). $\dot{V}O_{2peak}$ was defined as the average of the two highest consecutive values reached during the test.

4.2.5 Recovery Interventions

Exactly five minutes after completing each training session, participants performed their assigned recovery intervention for 15 min. Seated (with legs fully extended), participants were immersed in water up to their umbilicus (CWI: 10°C), or rested on a laboratory bed (CON: 23°C). The CWI condition was performed in an inflatable bath (iBody, iCool Sport, Australia), and water temperature was maintained with a cooling/heating unit (Dual Temp Unit, iCool Sport, Australia) as previously described (56). The same CWI protocol has previously been reported to induce a 3.5 ± 3.7°C reduction in muscle temperature (T_m) following the same exercise protocol (Chapter 2).

4.2.6 Muscle Analyses

4.2.6.1 Preparation of Permeabilised Skeletal Muscle Fibres.

A small section (~15-20 mg) of fresh muscle was analysed for mitochondrial respiration pre- and post-training. The sample was immediately placed into a cold biopsy preservation solution (BIOPS; 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 50 mM K^+-4-morpholinoethanesulfonic acid (MES), 15 mM Na_2phosphocreatine, 20 mM

imidazole and 0.5 mM dithiothreitol (DTT) - pH 7.1 (378)), and subsequently mechanically separated using forceps. Fibres were permeabilised by gentle agitation (30 min at 4°C) in a solution of BIOPS containing 50 µg/mL of saponin, and washed (3 x 7 min at 4°C) by gentle agitation in the respiration medium solution (MiR05; 0.5mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO4, 20 mM HEPES, 110 mM sucrose and 1% bovine serum albumin (BSA) - pH 7.1 at 37°C (378)). This method selectively permeabilises the cellular membrane leaving the mitochondria intact, allowing for "*in-situ*" measurements of mitochondrial respiration.

4.2.6.2 High Resolution Respirometry

After washing, fibres were weighed (~3-4 mg wet weight) and assayed in duplicate in a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), with each chamber containing 2 mL of MiR05. Oxygen concentration (nmol/mL) and oxygen flux ($pmol \cdot s^{-1} \cdot mg^{-1}$) were measured at 37°C and recorded using DatLab software (Oroboros Instruments), corrected to instrumental background oxygen flux. Each chamber was continually re-oxygenated by direct syringe injection of O_2 into the chamber to allow for maintenance of O_2 concentration (275-450 nmol/mL) to avoid a potential oxygen diffusion limitation.

Mitochondrial respiration measurements were taking using a substrate-uncoupler-inhibitor titration (SUIT) protocol. Firstly, the substrates pyruvate (final chamber concentration; 2 mM) and malate (5 mM) were added in the absence of adenylates for measurement of leak respiration (L) with electron entry through Complex I (CI) (CI_L). Next, adenosine diphosphate (ADP) was added (5 mM) for measurement of maximal oxidative phosphorylation (OXPHOS) capacity (P) with electron input through CI (CI_P), followed by addition of succinate (10 mM) for measurement of P with electron supply through CI and Complex II (CII) combined ($CI+II_P$). This state provides convergent electron input to the Q-junction through CI (NADH provided by malate and pyruvate) and CII ($FADH_2$ provided by succinate) and supports maximal mitochondrial respiration by reconstruction of the citric acid cycle function. Cytochrome *c* (10 µM) was next added to assess outer mitochondrial membrane integrity – increases in O_2 flux > 6% after

cytochrome *c* addition indicated compromised membrane integrity, in which data was excluded. A series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (FCCP, 0.75-1.5 μM) were performed, to determine maximal uncoupled respiration and electron transport system (ETS) capacity (E), with convergent electron input through CI+II ($CI+II_E$). Rotenone (0.5 µM), an inhibitor of CI, was added next to determine E with electron input through CII alone ($CI+II_E$). Antimycin A (2.5 μM), an inhibitor of Complex III (CIII), was then added to measure residual oxygen consumption capacity (ROX); this was used to correct all respiration values. Mitochondrial respiration measures were expressed in both absolute oxygen flux (mass-specific mitochondrial respiration), and corrected to citrate synthase activity (mitochondrial-specific respiration). Complete mitochondrial respiration data sets were available for 12 participants (CWI, n=6; CON n=6) as a result of malfunctions with the respirometer and/or poor muscle sample quality.

4.2.6.3 Preparation of Whole Muscle Lysates

A 10-15 mg piece of frozen muscle was added to ice-cold homogenising buffer (37.5µL/mg of tissue; 20 mM Tris, 137 mM NaCl, 1% Triton-X, 10% Glycerol, 2.7 mM KCl, 1 mM MgCl, 1 mM EDTA, 1 $\mu g.mL^{-1}$ aprotinin, 1 $\mu g.mL^{-1}$ leupeptin, 1 mM benzamedine, 1 mM Na_3VO_4, 1 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride – pH 7.4), and homogenised on ice using a motorised pestle. The whole muscle lysate was used for enzyme activity assays and Western blotting, without centrifugation. Protein concentration of the lysate was determined using a commercially-available colorimetric assay (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as the standard. All samples were diluted with distilled water to a standard concentration (1.5 $\mu g.\mu L^{-1}$), and further diluted with 2x Laemmli buffer (125 $mmol.L^{-1}$ Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.015% bromophenol blue, 10% β-mercaptoethanol). The resultant lysate was split equally into two parts: one unboiled and the other boiled at 95°C for 5 min, and both subsequently stored at -80°C for future analyses.

4.2.6.4 Western Blot Analyses

Muscle lysates were loaded equally (15 µg) and separated on 10% self-cast SDS-PAGE gels by means of electrophoresis, at 100 V for 1.5-2 h (Bio-Rad, Mississauga, ON, Canada). Unboiled muscle lysate was used for all proteins except p53, in which samples were re-boiled at 95°C for 3 min prior to loading. Once resolved, gels were transferred wet onto polyvinylidene fluoride (PVDF) membranes in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.01% SDS) at 100 V for 90 min. Membranes were then blocked with 5% skim milk in Tris-buffered saline (TBST: 150mM NaCl, 20mM Tris, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. Following 5 x 5-min washes in TBST, the membranes were incubated overnight (4°C with gentle agitation) with monoclonal primary antibodies (1:500-5000 in 5% skim milk) against p53, PHF20, heat-shock protein 27 (HSP27), heat-shock protein 70 (HSP70) (Cell Signalling, UK), and PGC-1α (Calbiochem, ST1202). The following morning, membranes were again washed 5 x 5 min in TBST, and incubated in the appropriate species-specific horseradish peroxidise-conjugated secondary antibodies at room temperature for 90 min, diluted 1:7000-30000 in 5% skim milk and TBST. After 5 x 5-min further washes in TBST, membranes were exposed to a chemiluminescent liquid (ClarityTM Western ECL Substrate, Bio-Rad, Hercules, CA) and visualized using a VersaDoc 4000 MP imaging system (Bio-Rad, Hercules, CA). Band densities were determined using Image Lab 5.1 software (Bio-Rad, Hercules, CA). Membranes were then stained with 0.1% Coomassie R-350 (PhastGelTM Blue R, GE Healthcare) as previously described (499), thereby providing a loading and transfer control. Comparative samples for each participant were loaded into the same gel, and one lane for each gel was reserved for an internal standard to account for inter-gel variability. Raw blot density was normalised to the internal standard and coomassie stain prior to analysis.

4.2.6.5 Citrate Synthase Activity

The maximal enzyme activity of citrate synthase (CS) was determined using the lysate prepared for western blot analyses, and analysed on a spectrophotometer (Bio-Rad, Hercules, CA). Each well contained a 50-fold dilution of lysate (2mg/mL), 3mM Acetyl CoA, 1mM dystrobrevin beta (DTNB),

and 10 mM oxaloacetate (OAA), made to volume (250 µL) with Tris buffer (100 mM). Reactions were analysed over a 3 min period at 30°C, with absorbance (412 nM) recorded every 15 s. Samples were analysed in triplicate, with enzyme activity expressed as moles per hour per kilogram of protein ($\mu M.h^{-1}.kg^{-1}$ protein).

4.2.7 Statistical Analyses

Data are reported in the text as means and standard deviation (mean ± SD), unless otherwise stated. Comparisons between conditions were analysed using a two-way general linear model (ANOVA) with repeated measures for time, where the within-subject factor was time (training) and the between-subject factor was condition (CON vs CWI). Independent t-tests were performed when statistical significance was present. Data that violated Levene's test of homogeneity were log-transformed prior to analysis. The level of significance for all data was set at $p < 0.05$. The above analyses were performed using IBM SPSS Statistics V20 (IBM Corporation, USA). In addition, effect sizes (ES) were calculated using Cohen's *d*, with magnitude-based inferences used to interpret the meaningfulness of results. Cohen's conventions for effect size (with 90% confidence intervals) were used for interpretation, where ES = 0.2, 0.5, and 0.8 are considered as small, medium, and large, respectively. Effect sizes are presented (± confidence intervals) for data demonstrating an effect size larger than 0.2. Western blot densitometry data (after the internal standard and coomassie stain corrections) were log-transformed and used for statistical analyses. For graphical purposes, baseline Western blot values were normalised to 1.0, such that post values correspond to fold change from pre values.

4.3 Results

4.3.1 Training

Both groups performed similar volumes of training over the six weeks, 1518.7 ± 180.9 kJ and 1487.0 ± 242.4 kJ for CON and CWI respectively ($p > 0.05$). There was 98% adherence to the training programme, with 2 participants in the CON group and 3 participants in CWI group missing one training session each due to illness.

4.3.2 Western Blots

Representative western blots are shown in Fig 4-4. There was a main effect of time (p = 0.027) and condition (p = 0.040) for HSP70 content. Post-hoc analysis revealed a significant increase in HSP70 content following training in the CWI condition as compared with the CON condition (p = 0.018, ES = 0.71 ± 0.56). There was no main effect of time (p = 0.738) or condition (p = 0.686) for HSP27 content (Fig 4-2).

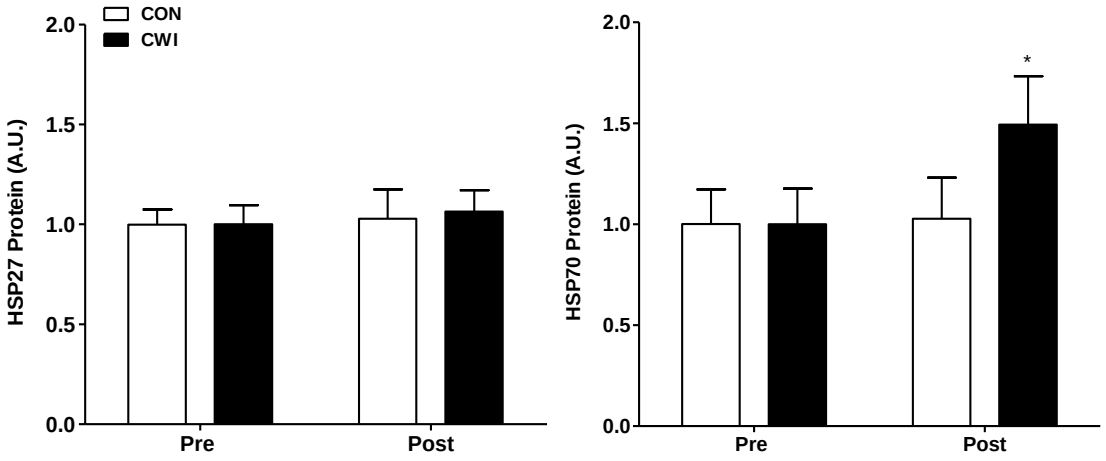


Figure 4-2: Total heat-shock protein 27 (HSP27) and 70 (HSP70) content before (Pre) and after (Post) six weeks of high-intensity interval training (HIT) for the control (CON) and cold-water immersion (CWI) conditions. Pre values have been normalised to 1.0 for graphical representation, such that post values correspond to fold change from pre values. * Significantly different to pre. Data are presented as mean ± S.E.M.

There were small, non-significant effects of time for PGC-1α (p = 0.437, ES = 0.21 ± 0.44), PHF20 (p = 0.138, ES = 0.32 ± 0.32) and p53 (p = 0.304, ES = 0.34 ± 0.55) content. Comparisons between the CON and CWI indicated no significant effects of condition for PGC-1α (p = 0.751), PHF20 (p = 0.310) or p53 (p = 0.446) protein content (Fig 4-3).

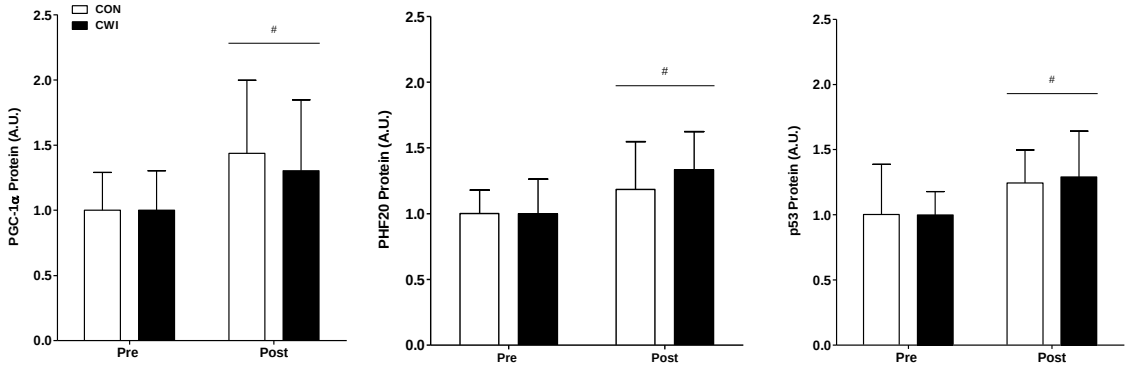


Figure 4-3: Total PGC-1α, PHF20 and p53 protein content before (Pre) and after (Post) six weeks of high-intensity interval training (HIT) for the control (CON) and cold-water immersion (CWI) conditions. Pre values have been normalised to 1.0 for graphical representation, such that post values correspond to fold change from pre values. # Small effect compared with pre. Data are presented as mean ± S.E.M.

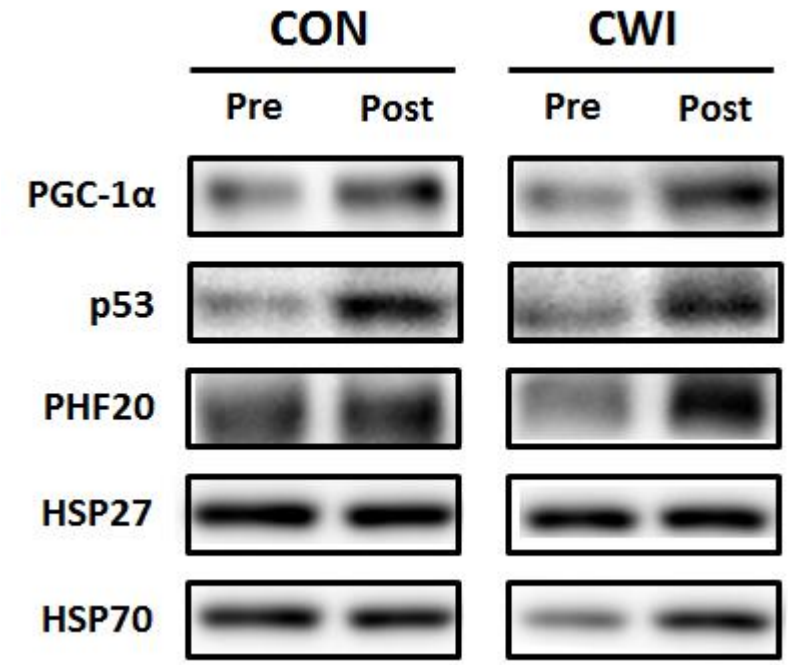


Figure 4-4: Representative western blots before (PRE) and after (POST) six weeks of high-intensity interval training (HIT) for the control (CON) and cold-water immersion (CWI) conditions.

4.3.3 CS Activity and Mitochondrial Respiration

There was a small, non-significant effect for time (p = 0.172, ES = 0.28 ± 0.35) in maximal citrate synthase activity, which increased 10.1 ± 23.8% following six weeks of HIT. However, there was no significant effect of condition (p = 0.656) (Fig 4-5).

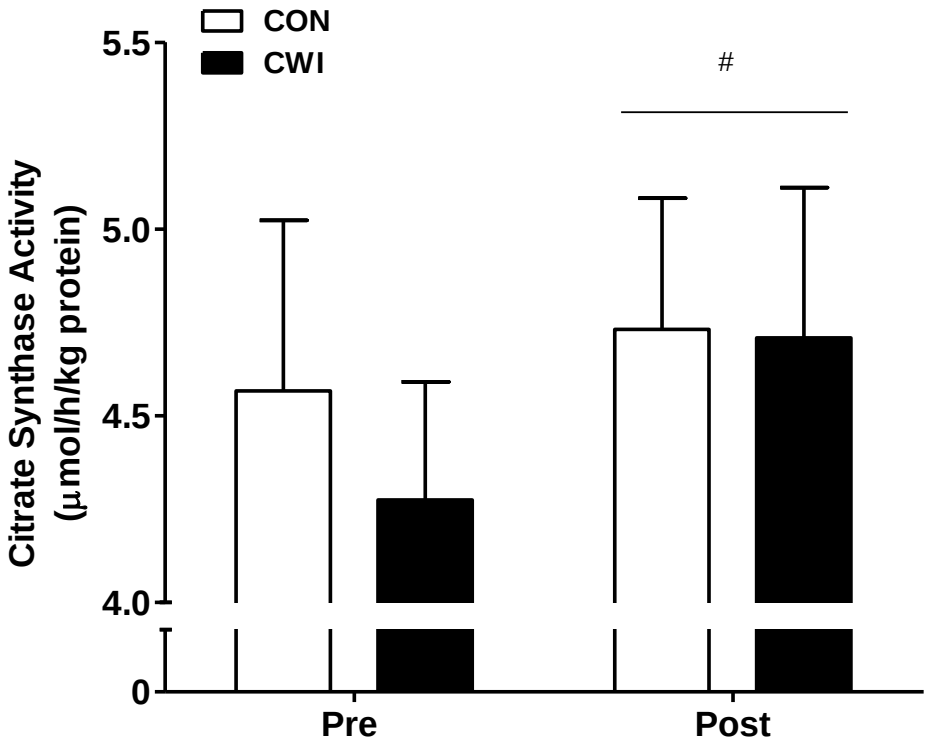


Figure 4-5: Maximal activity of the mitochondrial enzyme citrate synthase (CS) before (PRE) and after (POST) six weeks of high-intensity interval training (HIT) for the control (CON) and cold-water immersion (CWI) conditions. # Small effect compared with pre. Data are presented as mean ± S.E.M.

Respiration results are shown in Figure 4-6. There were no significant main effects of time (p = 0.102) or condition (p = 0.875) for mass-specific respiration. When CWI and CON conditions were pooled together, there were small increases in CI_P (p = 0.204, ES = 0.37 ± 0.49), $CI+II_P$ (p = 0.054, ES = 0.43 ± 0.35), $CI+II_E$ (p = 0.010, ES = 0.82 ±0.48) and CII_E (p = 0.063, ES = 0.61 ± 0.53) as a result of six weeks of HIT. When normalised to CS activity (mitochondrial-specific respiration), there were similarly no significant time (p = 0.231) or condition (p = 0.639) effects. Pooled data demonstrated small training-induced increases in $CI+II_E$ (p = 0.229, ES = 0.33 ± 0.47) and CII_E (p = 0.354, ES = 0.31 ± 0.58).

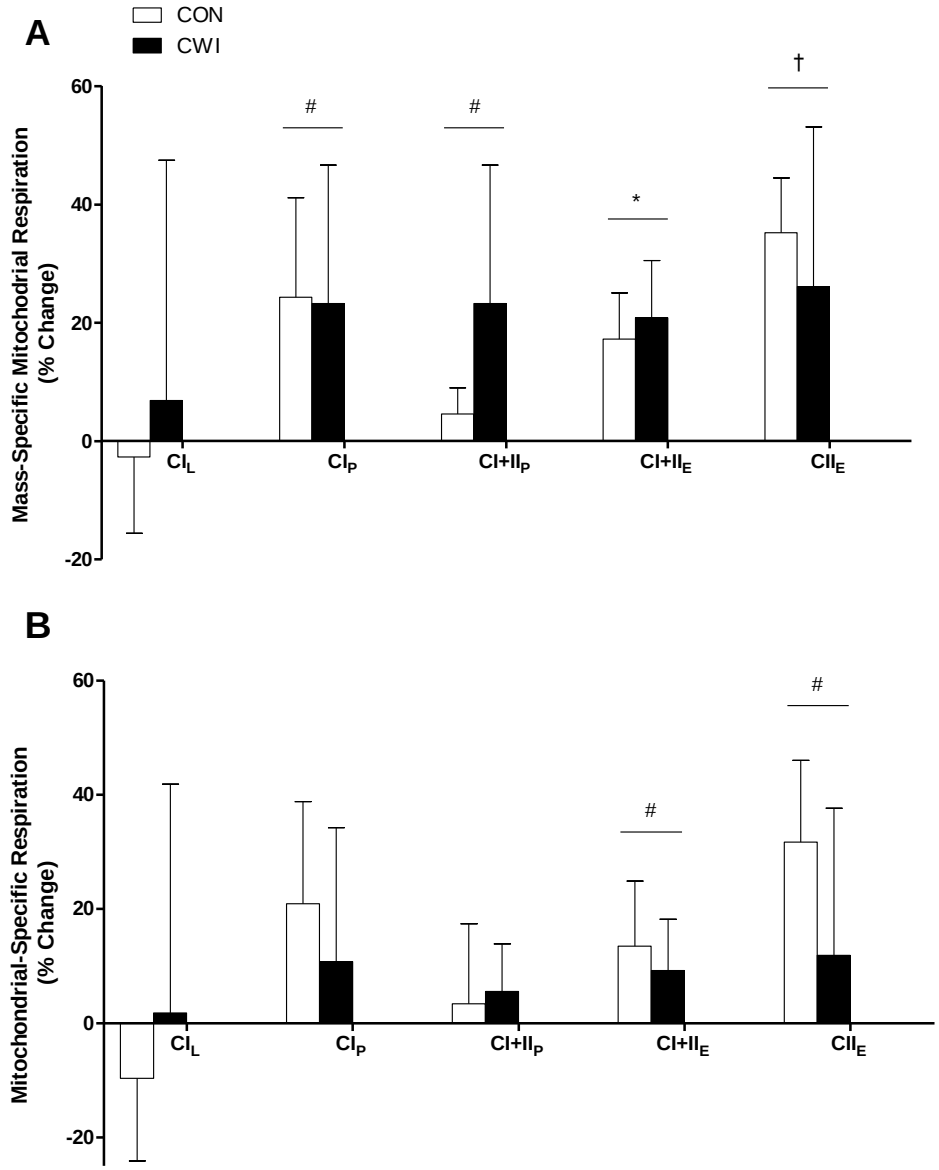


Figure 4-6: Mitochondrial respiration measurements at different coupling control states. A: mass-specific mitochondrial respiration, and B: mitochondrial-specific respiration (mass-specific respiration normalised to citrate synthase activity). Respiration was measured in permeabilised muscle fibres (*vastus lateralis)* obtained before (PRE) and after (POST) six weeks of high-intensity interval training (HIT) for control (CON, *n* = 6) and cold-water immersion (CWI, *n* = 6) conditions. CI_L: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P; maximal oxidative phosphorylation state (P) with saturating levels of ADP and limitation of flux by electron input through CI; $CI+II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI + Complex II (CII); $CI+II_E$: maximal electron transport system (ETS) capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI+II; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII. * Significant effect of training. # Small effect of training. † Moderate effect of training. Data are presented as mean ± S.E.M.

4.3.4 GXT and VO_{2peak}

Peak power output during the GXT increased by 5.8 ± 5.7 % and 6.1 ± 5.6% in the CON and CWI groups, respectively (main effect of time; p = 0.001, ES = 0.31 ± 0.12). Similarly, $\dot{V}O_{2peak}$ increased significantly as a result of training (main effect of time; p = 0.002, ES = 0.61 ± 0.28) - by 9.6 ± 6.7% in the CON group and 7.6 ± 11.0% in the CWI group. There were no main effects of condition for peak power output (p = 0.979) or $\dot{V}O_{2peak}$ (p = 0.633) (Fig 4-7).

As a result of training, the lactate threshold increased from 162.1 ± 26.7 W to 171.3 ± 36.6 W for the CON group, and from 155.8 ± 54.8 W to 160.2 ± 59.8 W for the CWI group, with no main effect of time (p = 0.074) or condition (p = 0.494).

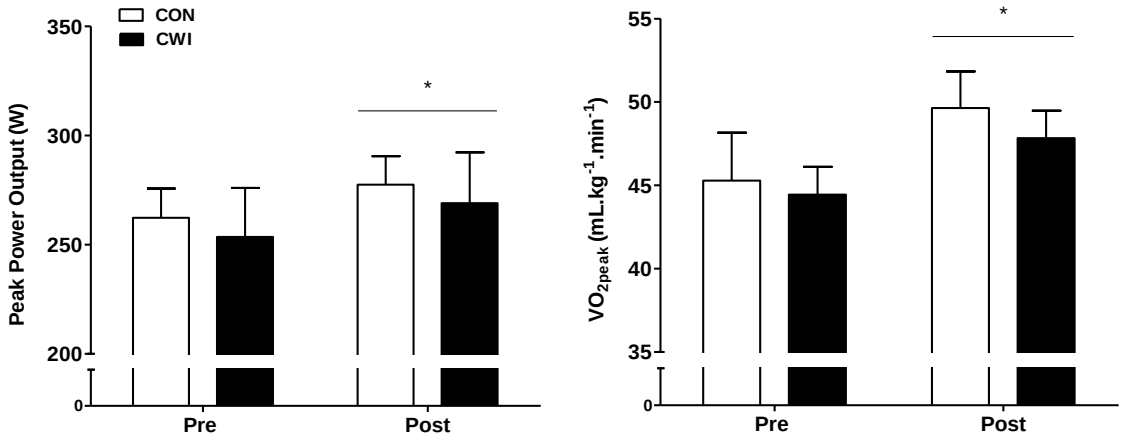


Figure 4-7: Peak power output (W) achieved during the graded exercise test (GXT), and peak oxygen uptake (VO_{2peak}; mL.kg^{-1}.min^{1}) achieved during the steady-state cycle to fatigue bout to fatigue, before (PRE) and after (POST) six weeks of high-intensity interval training (HIT). * Significantly different to pre-training (p<0.05). Data are presented as mean ± S.E.M.

4.3.5 Time Trials

The time required to finish the TT_2 decreased after training by 3.3 ± 4.5% and 2.4 ± 3.1% in the CON and CWI groups, respectively (main effect of time; p = 0.010, ES = 0.36 ± 0.21). TT_2 mean power also correspondingly increased from 261.3 ± 45.0 to 283.5 ± 46.1 W for the CON group, and from 248.1 ± 66.4 to 263.7 ± 59.2 W for the CWI group (main effect of time; p = 0.023, ES = 0.34 ± 0.21). There were no significant effects of time for TT_{20} duration (p = 0.224) or

mean power (p = 0.208). Furthermore, there were no main effects of condition for TT_2 duration (p = 0.699) and mean power (p = 0.666), or TT_{20} duration (p = 0.889) and mean power (p = 0.703) (Fig 4-8).

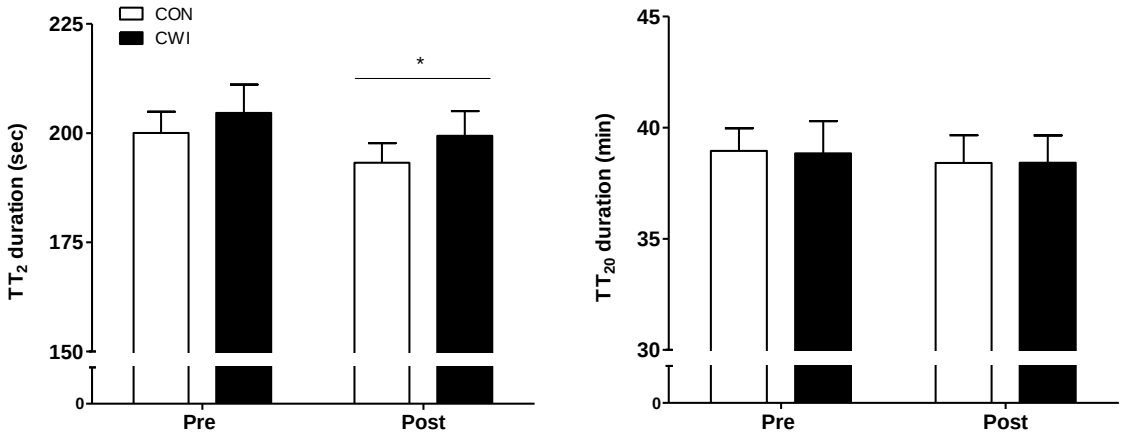


Figure 4-8: 2km (TT_2) and 20km (TT_{20}) cycling time-trial performance before (PRE) and after (POST) six weeks of high-intensity interval training (HIT). * Significantly different to pre-training (p<0.05). Data are presented as mean ± S.E.M.

4.4 Discussion

Following six weeks of HIT, there were small and non-significant increases in PGC-1α, p53 and PHF20 protein content, as well as citrate synthase activity and mitochondrial respiration. Additionally, HIT significantly increased peak aerobic power, $\dot{V}O_{2peak}$ and 2-km time-trial performance. Regular CWI performed after each training session had no effect on these markers, but served to increase the content of the mitochondrial chaperone protein HSP70. These observations demonstrate that CWI is not detrimental to endurance adaptations in response to six weeks of HIT.

This study demonstrates, for the first time, that six weeks of post-exercise CWI serves as a potent stimulus to increase training-induced HSP70 protein content. Heat-shock proteins are upregulated in response to both a single bout of exercise and to training (232, 234, 331, 332, 304, 319, 284), implicating them in a number of skeletal muscle remodelling processes associated with exercise training (327). For example, HSP70 acts as a chaperone for the importation of nuclear-encoded proteins destined for the mitochondria, and therefore plays an essential

role in mitochondrial biogenesis (184). Previous studies have suggested that cold exposure might remove the hyperthermic contribution to the adaptive response to training and potentially decrease HSP induction (523), particularly given that HSPs were originally identified to be induced following heat shock (276). However, the current study is consistent with reports that a return to normothermia following cold shock may elicit changes typically observed with heat stress, such as HSP expression (455). For example, cold stress increases HSP70 mRNA and/or protein induction in animal (269, 533, 321) and cell-culture models (250, 281, 340), most likely a result of heat-shock factor-1 (HSF-1) activation upon rewarming (455). This study therefore supports the hypothesis that cells may sense relative rather than absolute hyperthermia (281, 340, 250), which may explain an increase in HSP70 content following regular CWI. The lack of a training-induced increase in HSP70 for the CON condition, despite previous reports of an ~5 fold increase following four weeks high-intensity rowing (285), may be explained by the HIT protocol used. The minimum training dose needed to 'maintain' HSP levels is currently unknown, and the HIT protocol used in this study may not have been of sufficient volume and/or intensity to maintain HSP70 levels (284, 328). Nevertheless, this study demonstrates that regular CWI during HIT significantly increases HSP70 content, which may in turn facilitate training-induced mitochondrial adaptations (328).

HSPs serve to protect against oxidative stress, and their induction in human skeletal muscle is mediated by cellular redox state (129, 202). For example, targeted deletion of HSF-1 is associated with increased oxidative stress (109), and HSP70 regulates cellular redox state via the elimination of heat-induced ROS (458). In the current study, increased HSP70 content may have been in response to oxidative stress from exercise and/or CWI. Although exercise is a well-accepted inducer of oxidative stress (492), CWI may also induce oxidative stress via an elevation in metabolism/respiration (501), an acute burst of ROS upon rewarming (281, 340), and/or the oxidation of catecholamines (163). In support of this, a single bout of HIT and CWI resulted in increased mRNA of the antioxidants catalase and GPX-1 (Chapter 3). The apparent accumulation of exercise- and CWI-induced oxidative stress may challenge cellular integrity, implicating the induction of HSP70 to maintain redox balance and provide

important protective adaptive changes (533, 131, 129). However, six weeks of HIT and CWI had no effect on HSP27 content, despite its documented role in also protecting against oxidative stress (519). A potential explanation for this is that HSP27's primary function may be to aid in the repair of injured structures following contraction-induced muscle damage (329), a response unlikely to have occurred during six weeks of cycling (82).

To provide mechanistic insight into the effects of post-exercise CWI on the cellular mechanisms modulating adaptation to HIT, the protein content of several key regulators of mitochondrial biogenesis was measured (424, 518, 106). PGC-1α is a transcriptional coactivator critical in the regulation and coordination of skeletal muscle mitochondrial biogenesis (286). Total PGC-1α protein content has been reported to increase by up to ~2.0 fold following three to six weeks of supra-maximal (30-s work intervals at ~200% W_{peak}) HIT (430, 63, 157), consistent with the ~1.4 fold increase reported in the current study. However, no difference was observed between the CON and CWI conditions, despite previous reports of a greater exercise-induced increase in PGC-1α mRNA following a single bout of high-intensity cycling and post-exercise CWI (192). This may be explained by the non-significant increase in post-exercise PGC-1α mRNA content for the control condition, which is in contrast to most of the published research (278, 146, 31). The absence of any significant difference exercise-induced in PGC-1α mRNA expression between the CON and CWI conditions (Chapter 3) further supports the similar increase in PGC-1α protein content reported in the current study. In contrast to this finding, PGC-1α protein has been reported to increase (~50%) following cold-air exposure (4°C for as little as 4 d) in rodent skeletal muscle (357, 59). These increases were likely the result of shivering thermogenesis (357) and extended cold-exposure (i.e., 4°C, 24 h/d for 4-5 wk) (59) - which were not characteristic of the CWI protocol used in the current study. The present data therefore suggest that CWI has no effect on HIT-induced PGC-1α protein accumulation.

Despite its clear role in the regulation of exercise-induced mitochondrial biogenesis (424), only one study has investigated the effects of a period of exercise training on p53 content (157). This study observed an ~1.9 fold increase

following four weeks of HIT (157), comparable to the ~1.3 fold increase reported in the current study. Also consistent with the results of Granata et al. (157), there were similar increases (~1.3 fold vs ~1.8 fold) in PHF20 protein content following six weeks of HIT in the present study, further supporting suggestions of a co-ordinated up-regulation of the PHF20-p53 pathway following HIT (157). These findings therefore contribute to the emerging understanding of training-induced alterations of p53 and PHF20 content in human skeletal muscle.

Given the reported up-regulation in $p53^{Ser15}$ phosphorylation and PHF20 protein content following a single bout of HIT and CWI (Chapter 3), the current study also investigated whether regular CWI would augment the training-induced increase in the content of these transcription factors. Contrary to the hypothesis, p53 and PHF20 protein content was unaltered as a result of regular CWI, despite the augmented response observed following a single bout (Chapter 3). The discrepancy between these responses may be explained by the observation that training-induced adaptations are not necessarily predictable from the signalling response induced by a single exercise bout (86), or, in this instance, the accompanying post-exercise CWI. For example, many other signalling proteins, apart from those measured in Chapter 3, are likely to play a significant role in the adaptive response to exercise training (86). Furthermore, p53 regulates a number of different cellular processes, including the initiation of cell cycle arrest (to maintain cellular homeostasis) in response to cold shock (300, 411, 354). It is possible that p53 activation following a single bout of post-exercise CWI (Chapter 3) was a transient protective mechanism as opposed to a stimulus to promote mitochondrial biogenesis. In a similar manner as demonstrated with PGC-1α, and contrary to the hypothesis, these findings suggest that training-induced alterations in p53 and PHF20 content are not influenced by the addition of CWI following individual training sessions.

A novel aspect of this study was to directly assess the effect of regular post-exercise CWI on training-induced improvements in mitochondrial respiration in permeabilised muscle fibres. Despite a smaller than expected increase for the CON group in the current study there was an ~13% increase in maximal mass-specific respiration ($CI+II_P$) when participants were pooled together. This is

comparable to previous reports (~25% increase following 4 wk of HIT) in participants of a similar training status (157). A potential explanation for the smaller improvements reported in the current study is the greater number of 'all-out' bouts performed in the study by Granata et al. (157). However, consistent with the similar response for markers of mitochondrial adaptation (i.e., PGC-1α, p53 and PHF20 protein content), regular CWI had no influence on training-induced changes in mass-specific mitochondrial respiration.

To determine if the increases in mass-specific respiration were qualitative or quantitative, mass-specific respiration was corrected to CS activity (mitochondrial-specific respiration). Located exclusively in the mitochondria (480), CS is an enzyme commonly-used in exercise training studies as a biomarker of mitochondrial content (252, 47) that has been reported to increase following six weeks of HIT (63). When corrected to CS, training-induced increases in mitochondrial respiration were generally smaller, suggesting that the increases in mass-specific respiration may be due to increases in mitochondrial content (47), as supported by the increase in citrate synthase activity. Similar to the observations for mass-specific respiration, and consistent with similar training-induced increases PGC-1α, p53 and PHF20 protein content, CWI had no effect on changes in mitochondrial-specific respiration. As such, and contrary to the hypothesis, these data suggest that CWI does not serve to augment training-induced increases in mitochondrial respiration. Further research is warranted to investigate whether CWI can increase thermogenesis and augment mitochondrial respiration, and to investigate the effects of combining CWI with a larger training volume that will induce more robust increases in CS activity/mitochondrial content.

An important applied aspect of this research was to determine if regular CWI had any effect on exercise performance. Although it has previously been suggested that CWI may be detrimental to training-induced changes in exercise performance (523), this study provides evidence that regular CWI following high-intensity cycling has no detrimental (or advantageous) influence on certain physiological and performance markers of endurance adaptations, including time-trial performance, the lactate threshold, VO_{2peak}, and peak aerobic power. These

findings support the absence of a significant difference between conditions for the measured markers of mitochondrial biogenesis (i.e., p53, PGC-1α, CS etc.). Furthermore, this performance data supports the only other study performed to date investigating the effects of regular CWI following cycling training, whereby CWI performed four times per week during three weeks of cycling training did not impair performance adaptations in competitive cyclists (164). Given that the participants in the current study were considerably less trained in comparison, this suggests that training status does not appear to influence these effects. Finally, this data, along with that of Halson et al. (164) refutes concerns of CWI being detrimental to long-term adaptations to training (523, 176, 187).

In summary, the most important finding from the present study was that regular CWI following high-intensity cycling has no detrimental (or advantageous) influence on markers of endurance-related training adaptations and cycling performance. Despite the observed improvements in skeletal muscle recovery following a single bout of post-exercise CWI (Chapter 2), and in opposition to recent suggestions (490), CWI used regularly during a six-week training period did not improve training quality and/or load. Furthermore, and contrary to the hypothesis, a number of molecular markers associated with skeletal muscle metabolic adaptations and mitochondrial biogenesis were unchanged with CWI as compared with a passive control. Greater upregulation of the PHF20-p53 pathway following a single bout of post-exercise CWI (Chapter 3) did not translate into an increase in PHF20 or p53 content following regular CWI during HIT, suggesting that these signalling responses do not accurately predict training responses. Nonetheless, these findings do refute previous suggestions that exercise-induced training benefits may be counteracted by possible CWI-induced long-term detrimental effects on muscular adaptation. In contrast, regular post-exercise CWI may serve as a stimulus to augment training-induced improvements in HSP70 expression, which may in turn provide protection against oxidative stress.

CHAPTER FIVE

Thesis Summary

The overall aim of this thesis was to provide mechanistic insight into the merit of cold-water immersion (CWI) in the recovery from, and adaptation to, high-intensity interval training (HIT). Study 1 aimed to address the evident lack of placebo controls in research investigating the effects of CWI on skeletal muscle recovery. Studies 2 and 3 investigated the molecular mechanisms by which CWI may alter the adaptive response to HIT. This section will summarise the key findings and practical applications from the studies conducted, and recommend potential directions for future research.

Summary of Key Findings

- A recovery placebo administered after a single bout of HIT was superior to a control condition, and as effective as CWI. This suggests, for the first time, that the placebo effect may account for some of the observed benefits following CWI. Alternatively, given that CWI was demonstrated to be effective for skeletal muscle recovery, the placebo effect may be as strong as these physiological benefits.

- Further to the physiological benefits reported in Chapter 2, Chapter 3 demonstrates that post-exercise CWI also augments a number of signalling proteins and genes associated with mitochondrial adaptations. It is hypothesised that the oxidative stress imposed by a hypothermic shock and subsequent rewarming may serve to augment p53 activation post-exercise, leading to a greater up-regulation of some of its downstream targets.

- Despite the differences observed following a single bout of exercise (Chapter 3), repeated CWI following every training session did not augment (or hinder) the adaptive response to HIT, as measured by markers of mitochondrial biogenesis and other endurance-related adaptations. However, regular post-exercise CWI may serve as a stimulus to augment training-induced improvements in HSP70 expression.

The benefits of post-exercise CWI are not greater than the placebo effect:

Chapter 2 investigated the physiological merit of CWI for recovery from a single bout of HIT, by investigating if the placebo effect is responsible for any performance or psychological benefits. One major limitation with the CWI research performed to date is that no study has incorporated a placebo condition; this is a crucial omission considering its well-documented influence on athletic performance (35, 165). A lack of placebo control trials performed to date is not surprising given the difficulties in implementing a true placebo, such as the inability to alter an individual's perception of cold. By effectively deceiving the participants administered the placebo, a means to control for the placebo effect was provided. As such, this study significantly contributes to the understanding of the mechanisms underlying CWI, and its influence on athletic performance.

The main finding of the first study was that a CWI placebo is superior to a thermo-neutral water immersion control in assisting the recovery of quadriceps' muscle strength following a single bout of HIT. As these were essentially the same condition, and elicited the same physiological response, this can likely be attributed to improved subjective ratings of pain and readiness for exercise. By deceiving subjects into thinking they were receiving a beneficial treatment, participants felt more recovered and a superior performance was witnessed.

This is the first study to test the hypothesis that the commonly-hypothesised physiological benefits of CWI may at least be partly placebo-related, and that altered perceptions of fatigue play an important role in the recovery from exercise. As such, manipulation of one's expectation of recovery, via a CWI placebo, can have a similar beneficial influence on the recovery of muscle strength as CWI itself. Furthermore, these similarities occurred despite a significantly larger reduction in muscle temperature for the CWI condition. The findings from Chapter 2 have confirmed that the placebo effect may account for some of the observed benefits following CWI, or alternatively, that it is as strong as these physiological benefits.

Post-exercise CWI activates PHF20 and p53 signalling in human skeletal muscle:

Despite the benefits observed following a post-exercise CWI placebo, Chapter 2 also demonstrated that CWI was superior to a control condition in improving skeletal muscle recovery following a single bout of HIT. Chapter 3 aimed to investigate the underlying molecular mechanisms by which CWI may improve skeletal muscle recovery, and assess its potential to augment aerobic adaptations to a single bout of HIT. Given a previous report of CWI augmenting the exercise-induced increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA (192), it was hypothesised that post-exercise CWI would serve as a stimulus to increase exercise-induced mitochondrial biogenesis. Particular focus was given to the p53 and PGC-1α mitochondrial biogenic pathways.

The main finding of this study was that p53 phosphorylation was increased following post-exercise CWI in human skeletal muscle. In support of this, plant homeodomain finger protein 20 (PHF20) protein content was also increased following CWI, which may function to increase p53 transcription (366), and promote its stabilisation/activation in the nucleus (106). Consistent with the upstream activation of p53, post-exercise CWI also increased the transcription of a number of p53-target genes associated with aerobic metabolism, including apoptosis inducing factor (AIF) and synthesis of cytochrome *c* oxidase subunit-2 (SCO2). Also downstream of p53, CWI increased the mRNA content of mitofusin-2 (Mfn2), a protein crucial for mitochondrial fusion and biogenesis. These data provide the first evidence that post-exercise CWI increases p53 activation, and therefore may serve as a novel stimulus by which to enhance contraction-induced mitochondrial biogenesis.

It was hypothesised that the enhanced p53 activation following post-exercise CWI was the result of a cold-induced elevation in reactive oxygen species (ROS). Regardless of the potential mechanism of ROS production during post-exercise CWI, the subsequent activation of p53 may serve as a hormetic stimulus to ‘harden’ and prepare an individual for subsequent ROS-related stress (445). In support of this hypothesis, there was an increase in the mRNA of the antioxidant enzymes glutathione peroxidase 1 (GPX-1) and catalase. Both GPX-1

and catalase have the same action upon the reactive species hydrogen peroxide (H_2O_2), helping to reduce the severity of oxidative stress (127). These data support the hypothesis that the potential oxidative stress imposed by post-exercise CWI may activate p53 and augment an antioxidant response, which may in turn increase the efficiency of the antioxidant system during subsequent exercise bouts.

In further support that ROS are a likely upstream activator of p53 following post-exercise CWI, similar increases in p38 mitogen-activated protein kinase (MAPK) and adenosine monophosphate-activated protein kinase (AMPK) phosphorylation were reported between the CWI and control (CON) conditions. As these kinases have been linked to p53 phosphorylation following exercise (211, 441), they may not be responsible for CWI-induced p53 activation. Consistent with this, CWI did not augment an exercise-induced increase in PGC-1α mRNA, a well-documented downstream response following AMPK and/or p38 MAPK phosphorylation (200, 5). As such, this study reports for the first time that post-exercise CWI augments p53 signalling, a response heavily implicated in promoting aerobic phenotype alterations (422). Therefore, this study provides an alternate and novel explanation by which CWI may enhance contraction-induced mitochondrial biogenesis.

Regular CWI following HIT does not hinder the adaptive response in human skeletal muscle:

The last component of this thesis was to investigate the adaptive response to CWI performed after every training session during six weeks of HIT. Given the documented upregulation of the p53 signalling pathway in Chapter 3, it was hypothesised this would improve markers of mitochondrial biogenesis following training. Furthermore, it was also hypothesised this response would translate into an improvement in exercise performance and other markers of aerobic adaptations.

Regular CWI following six weeks of HIT served as a potent stimulus to increase training-induced heat-shock protein 70 (HSP70) content. The functional significance of increased HSP70 content following regular CWI may be closely related to its role in protection against oxidative stress (283), as HSP induction in

human skeletal muscle is mediated by cellular redox state (129, 202). An accumulation of exercise- and CWI-induced oxidative stress, as supported by an increase in GPX-1 and catalase mRNA reported in Chapter 3 following a single bout of HIT and CWI, may challenge cellular integrity, implicating the induction of HSP70 in providing important protective adaptive changes (533, 131, 129). Increased HSP content following CWI and HIT may serve as a conditioning response, functioning to better maintain homeostasis during the oxidative stress imposed from subsequent exercise and thermal stress. Furthermore, in support of work in cultured cells (179, 221, 250, 281), this study demonstrated a heat-shock response following CWI in human skeletal muscle, which would have most likely occurred during rewarming.

To provide mechanistic insight into the effects of post-exercise CWI on the cellular mechanisms modulating adaptations to HIT, the protein content of key regulators of mitochondrial biogenesis (424, 518, 106) was measured. Total content of PGC-1α, p53, and PHF20 all increased as a result of HIT, but increased similarly between the CWI and CON groups. As expected, regular CWI had no influence on total PGC-1α content, consistent with the mRNA response observed in Chapter 3. Similarly, CWI did not augment other markers of mitochondrial adaptations, including CS activity and mitochondrial respiration. In regards to PHF20 and p53, the discrepancy between the responses in Chapters 3 and 4 can be explained by the fact that training-induced adaptations are not necessarily predictable from those induced by a single exercise bout (86), or, in this instance, the accompanying post-exercise CWI. The influence of exercise training on p53 and PHF20 content are largely unknown, but these findings contribute to previous work demonstrating a co-ordinated up-regulation of the PHF20-p53 pathway following HIT (157). Furthermore, despite HSP70's documented role in mitochondrial biogenesis (184), an increase in this protein following CWI had no influence on mitochondrial adaptations.

Finally, the influence of regular CWI following HIT on exercise performance and other physiological markers of endurance adaptations was investigated. As expected, maximal oxygen uptake, peak aerobic power and 2-km time-trial performance all improved similarly in both groups as a result of training. However, regular CWI had no influence on these markers, consistent

with the lack of change in markers of mitochondrial biogenesis as compared with the CON condition. Although CWI-induced upregulation of the PHF20-p53 pathway following a single bout of HIT (Chapter 3) had no influence on the adaptive response to CWI during training, this data refutes concerns of CWI being detrimental to long-term adaptations to training (523, 176, 187).

Practical Applications

As identified in this thesis, there is a paucity of research investigating the mechanisms underlying the effects of post-exercise CWI. Whilst it is acknowledged that further research is needed to further elucidate the merit of CWI in athletic training regimes, the following recommendations are provided.

- Similar improvements in performance following CWI or a CWI placebo have highlighted the importance of belief in recovery. Regardless of any potential physiological role, it is important for coaches and sport scientists alike to educate their athletes on the benefits of recovery, and to also encourage belief in the practice. A strong belief in CWI, combined with any potential physiological benefits, will maximise its worth in the recovery from exercise.

- Despite recent suggestions that CWI may attenuate training-induced adaptations, this research provides data that, in contrast, it can up-regulate certain markers of mitochondrial biogenesis following a single bout of HIT. Up-regulation of the PHF20-p53 following CWI may serve as a practical method to modulate mitochondrial adaptations, potentially improving athletic performance.

- Recent identification of p53 as a molecular regulator of the mitochondrial and/or nuclear genome implicates CWI as a potential intervention to improve various indices of health. For example, reduced p53 is associated with tumour development (294), insulin resistance (13) and reduced longevity (299). As such, CWI may represent a novel approach to achieve

the therapeutic benefits of p53 signalling, with applications to both clinical and athletic populations.

- Regular CWI following HIT appears to have no detrimental (or advantageous) influence on exercise performance and other markers of endurance-related training adaptations. As such, this data does not support recent speculation that regular post-exercise CWI may impair endurance adaptations and associated markers of endurance exercise performance.

- Regular post-exercise CWI may conversely serve as a stimulus to augment training-induced improvements in HSP expression, potentially serving to protect against oxidative stress.

Limitations and Considerations

In conjunction with the practical applications mentioned above, it is also important to acknowledge any potential shortcomings and limitations of this research; these limitations are discussed below.

- There are some inherent limitations in the parallel-group design chosen, namely inter-subject variability. As participants did not act as their own control (cross-over design), an imbalance in certain covariates (e.g., mRNA and protein expression) may theoretically be present, thereby limiting the strength of our conclusions. However, a parallel-group design was chosen to avoid a possible order effect in Chapter 2 (i.e., belief in recovery effectiveness) and a 'carry-over' effect (i.e., residual training adaptations) in Chapters 3 and 4. Future research implementing a cross-over design will further complement the findings from this thesis.

- Caution is advised with the interpretation that CWI may serve as an intervention to improve certain indices of health and performance associated with p53 signalling. Although we demonstrated an increase in PHF20 protein and p53 phosphorylation following a single bout of HIT and CWI, this response was not conserved following 6 weeks of HIT and

CWI. This lack of change in p53 and PHF20 may simply represent an adaptation to regular CWI, which may in turn eradicate any potential CWI-induced health and/or performance benefits.

- While it is important that CWI did not hinder adaptations, there were also a number of non-statistically significant training-induced changes in the molecular markers measured. As such, despite the moderate to large effect sizes reported in these variables, it may be argued that CWI may have been more likely to impair the response to training that provoked greater molecular adaptations.

- Given the limited number of oxidative stress markers measured, it may be difficult to argue the presence of CWI-induced oxidative stress. Methods to assess oxidative stress and/or antioxidant status are complex, and interpretation of the values obtained from antioxidant mRNA (i.e., MnSOD, GPX-1 and catalase) alone could be a source of error (393). As such, a battery of measurements (i.e., including total antioxidant capacity and lipid/protein/DNA damage) may serve as a more reliable method to assess oxidative stress in future studies.

Future Directions

This thesis has demonstrated a number of novel mechanisms underlying the merit of CWI in training regimes. However, the findings from the present series of studies have identified a number of considerations for future research.

- Given the well-documented influence of the placebo effect in athletic performance, future CWI research should incorporate a placebo condition where possible, particularly when performance measures are the primary outcome.

- Future research should investigate whether exercise protocols likely to induce muscle damage and/or contusion injury (e.g., resistance training, marathon running, rugby union etc.) would elicit a similar placebo effect.

It is possible that CWI is more appropriate as a recovery modality following such exercise protocols, in which the desired physiological effect may be greater than the placebo effect.

- Given the relatively low training status of participants utilised in this thesis, future research should investigate whether the observed results are replicated in well-trained athletes, from a variety of different sports.

- Further work is needed to determine the role of p53 and its downstream targets in association with mitochondrial adaptions and aerobic metabolism, including both immediate signalling/mRNA alterations and long-term training-induced adaptations. This is particularly pertinent for human skeletal muscle, as much of the current knowledge of p53 and associated targets is derived from various cell lines and tissues (30). Furthermore, elucidating the optimal prescription of post-exercise CWI (e.g., duration, temperature, timing and dose) to promote p53 activation is needed.

- One of the main conclusions from this thesis is that oxidative stress and ROS accumulation may play a large role in the regulation of aerobic adaptations following post-exercise CWI. Future research should aim to collect a battery of ROS measurements, including direct free radical detection (e.g., electron spin resonance), total antioxidant capacity, isolated antioxidant activity assays, and markers of free-radical damage on lipids, protein and DNA. These measures will allow for a better quantification and understanding of the effect of CWI on ROS production.

- Given time and financial constraints, a comprehensive examination of all of the pathways investigated could not be provided. Future research could contribute to the current understanding by analysing a number of related upstream activators and downstream targets. For example nuclear factor kappa-light-chain-enhancer of activate B cells (NF-κβ) and c-Jun N-terminal kinase (JNK) are both redox sensitive, and both influence mitochondrial biogenic pathways. Other target include (but are not limited

to) protein kinase A (PKA), calcium/calmodulin-dependent protein kinase (CaMK), nitric oxide (NO) and H2A histone family, member X (H2AX).

- Heavily related to aerobic adaptations, but out of the scope of this thesis, future research should investigate the effects of post-exercise CWI on angiogenesis. Increased capillary density contributes to increases in maximal aerobic capacity of skeletal muscle (182, 52), and therefore may improve endurance exercise performance. Furthermore, vascular endothelial growth factor (VEGF) mRNA and protein content have been shown to increase following twenty weeks of CWI (18°C) in rat skeletal muscle (235), implicating post-exercise CWI as a potential method to improve vascularisation-induced improvements in aerobic capacity.

- Finally, a number of alterations can be implemented to the protocols utilised in the current series of studies. For example, future work should investigate the effects of CWI vs hot-water immersion, varying temperatures/durations of CWI (to alter the extent of muscle cooling), CWI alone vs in combination with exercise, and resistance vs endurance training. These alterations will compliment and further develop the mechanistic understanding of CWI on the adaptive response to exercise.

Chapter Six

References

1. AAGAARD, P., SIMONSEN, E. B., ANDERSEN, J. L., MAGNUSSON, P. & DYHRE-POULSEN, P. 2002. Increased rate of force development and neural drive of human skeletal muscle following resistance training. *J Appl Physiol,* 93**,** 1318-26.
2. ABRAMSON, D. I., CHU, L. S., TUCK, S., JR., LEE, S. W., RICHARDSON, G. & LEVIN, M. 1966. Effect of tissue temperatures and blood flow on motor nerve conduction velocity. *Jama,* 198**,** 1082-8.
3. ACIN-PEREZ, R., SALAZAR, E., BROSEL, S., YANG, H., SCHON, E. A. & MANFREDI, G. 2009. Modulation of mitochondrial protein phosphorylation by soluble adenylyl cyclase ameliorates cytochrome oxidase defects. *EMBO Mol Med,* 1**,** 392-406.
4. ADHIHETTY, P. J., LJUBICIC, V. & HOOD, D. A. 2007. Effect of chronic contractile activity on SS and IMF mitochondrial apoptotic susceptibility in skeletal muscle. *Am J Physiol-Endoc M,* 292**,** E748-E755.
5. AKIMOTO, T., POHNERT, S. C., LI, P., ZHANG, M., GUMBS, C., ROSENBERG, P. B., WILLIAMS, R. S. & YAN, Z. 2005. Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem,* 280**,** 19587-93.
6. AL-FAGEEH, M. B. & SMALES, C. M. 2006. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem J,* 397**,** 247-59.
7. ALESSIO, H. M. 1993. Exercise-induced oxidative stress. *Med Sci Sports Exerc,* 25**,** 218-24.
8. ALESSIO, H. M., HAGERMAN, A. E., FULKERSON, B. K., AMBROSE, J., RICE, R. E. & WILEY, R. L. 2000. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Med Sci Sports Exerc,* 32**,** 1576-81.
9. ALGAFLY, A. A. & GEORGE, K. P. 2007. The effect of cryotherapy on nerve conduction velocity, pain threshold and pain tolerance. *Br J Sports Med,* 41**,** 365-9; discussion 369.
10. ANSENBERGER-FRICANO, K., GANINI, D., MAO, M., CHATTERJEE, S., DALLAS, S., MASON, R. P., STADLER, K., SANTOS, J. H. & BONINI, M. G. 2013. The peroxidase activity of mitochondrial superoxide dismutase. *Free Radical Bio Med,* 54**,** 116-24.
11. AQUILANO, K., BALDELLI, S., PAGLIEI, B., CANNATA, S. M., ROTILIO, G. & CIRIOLO, M. R. 2013. p53 orchestrates the PGC-1alpha-mediated antioxidant response upon mild redox and metabolic imbalance. *Antioxid Redox Signal,* 18**,** 386-99.
12. ARBORELIUS, M., JR., BALLIDIN, U. I., LILJA, B. & LUNDGREN, C. E. 1972. Hemodynamic changes in man during immersion with the head above water. *Aerosp Med,* 43**,** 592-8.
13. ARMATA, H. L., GOLEBIOWSKI, D., JUNG, D. Y., KO, H. J., KIM, J. K. & SLUSS, H. K. 2010. Requirement of the ATM/p53 tumor suppressor pathway for glucose homeostasis. *Mol Cell Biol,* 30**,** 5787-94.
14. ARONSON, D., BOPPART, M. D., DUFRESNE, S. D., FIELDING, R. A. & GOODYEAR, L. J. 1998. Exercise stimulates c-Jun NH2 kinase activity and c-Jun transcriptional activity in human skeletal muscle. *Biochem Bioph Res Co,* 251**,** 106-10.
15. ARRUDA, A. P., KETZER, L. A., NIGRO, M., GALINA, A., CARVALHO, D. P. & DE MEIS, L. 2008. Cold tolerance in hypothyroid rabbits: role of skeletal muscle mitochondria and sarcoplasmic reticulum Ca2+ ATPase isoform 1 heat production. *Endocrinology,* 149**,** 6262-71.

16. ARS, E., SERRA, E., DE LA LUNA, S., ESTIVILL, X. & LAZARO, C. 2000. Cold shock induces the insertion of a cryptic exon in the neurofibromatosis type 1 (NF1) mRNA. *Nucleic Acids Res,* 28**,** 1307-12.
17. ASCENSAO, A., LEITE, M., REBELO, A. N., MAGALHAES, S. & MAGALHAES, J. 2011. Effects of cold water immersion on the recovery of physical performance and muscle damage following a one-off soccer match. *J Sport Sci,* 29**,** 217-225.
18. ASEA, A., KRAEFT, S. K., KURT-JONES, E. A., STEVENSON, M. A., CHEN, L. B., FINBERG, R. W., KOO, G. C. & CALDERWOOD, S. K. 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med,* 6**,** 435-42.
19. ATHERTON, P. J., BABRAJ, J., SMITH, K., SINGH, J., RENNIE, M. J. & WACKERHAGE, H. 2005. Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J,* 19**,** 786-8.
20. AYDIN, J., SHABALINA, I. G., PLACE, N., REIKEN, S., ZHANG, S. J., BELLINGER, A. M., NEDERGAARD, J., CANNON, B., MARKS, A. R., BRUTON, J. D. & WESTERBLAD, H. 2008. Nonshivering thermogenesis protects against defective calcium handling in muscle. *FASEB J,* 22**,** 3919-24.
21. BAAR, K. 2006. Training for endurance and strength: lessons from cell signaling. *Med Sci Sports Exerc,* 38**,** 1939-44.
22. BAAR, K., WENDE, A. R., JONES, T. E., MARISON, M., NOLTE, L. A., CHEN, M., KELLY, D. P. & HOLLOSZY, J. O. 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J,* 16**,** 1879-86.
23. BACH, D., PICH, S., SORIANO, F. X., VEGA, N., BAUMGARTNER, B., ORIOLA, J., DAUGAARD, J. R., LLOBERAS, J., CAMPS, M., ZIERATH, J. R., RABASA-LHORET, R., WALLBERG-HENRIKSSON, H., LAVILLE, M., PALACIN, M., VIDAL, H., RIVERA, F., BRAND, M. & ZORZANO, A. 2003. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. *J Biol Chem,* 278**,** 17190-7.
24. BAE, K. A., AN, N. Y., KWON, Y. W., KIM, C., YOON, C. S., PARK, S. C. & KIM, C. K. 2003. Muscle fibre size and capillarity in Korean diving women. *Acta Physiol Scand,* 179**,** 167-72.
25. BAILEY, D. M., DAVIES, B., YOUNG, I. S., JACKSON, M. J., DAVISON, G. W., ISAACSON, R. & RICHARDSON, R. S. 2003. EPR spectroscopic detection of free radical outflow from an isolated muscle bed in exercising humans. *J Appl Physiol,* 94**,** 1714-8.
26. BAILEY, D. M., ERITH, S. J., GRIFFIN, P. J., DOWSON, A., BREWER, D. S., GANT, N. & WILLIAMS, C. 2007. Influence of cold-water immersion on indices of muscle damage following prolonged intermittent shuttle running. *J Sport Sci,* 25**,** 1163-1170.
27. BAKKAR, N., WANG, J., LADNER, K. J., WANG, H., DAHLMAN, J. M., CARATHERS, M., ACHARYYA, S., RUDNICKI, M. A., HOLLENBACH, A. D. & GUTTRIDGE, D. C. 2013. IKK/NF-kB regulates skeletal myogenesis via a signaling switch to inhibit differentiation and promote mitochondrial biogenesis. *J Cell Biol,* 202**,** 825.
28. BANFI, G., MELEGATI, G. & VALENTINI, P. 2007. Effects of cold-water immersion of legs after training session on serum creatine kinase concentrations in rugby players. *Br J Sports Med,* 41**,** 339.
29. BARNETT, A. 2006. Using recovery modalities between training sessions in elite athletes: does it help? *Sports Med,* 36**,** 781-96.
30. BARTLETT, J. D., CLOSE, G. L., DRUST, B. & MORTON, J. P. 2014. The emerging role of p53 in exercise metabolism. *Sports Med,* 44**,** 303-9.

31. BARTLETT, J. D., HWA JOO, C., JEONG, T. S., LOUHELAINEN, J., COCHRAN, A. J., GIBALA, M. J., GREGSON, W., CLOSE, G. L., DRUST, B. & MORTON, J. P. 2012. Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol,* 112**,** 1135-43.
32. BARTLETT, J. D., LOUHELAINEN, J., IQBAL, Z., COCHRAN, A. J., GIBALA, M. J., GREGSON, W., CLOSE, G. L., DRUST, B. & MORTON, J. P. 2013. Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. *Am J Physiol-Reg I,* 304**,** R450-8.
33. BARTLETT, J. D. & MORTON, J. P. 2013. p53: the tumour suppressor turns mitochondrial regulator. *J Physiol,* 591**,** 3455-6.
34. BATTERSBY, B. J. & MOYES, C. D. 1998. Influence of acclimation temperature on mitochondrial DNA, RNA, and enzymes in skeletal muscle. *Am J Physiol,* 275**,** R905-12.
35. BEEDIE, C. J. & FOAD, A. J. 2009. The placebo effect in sports performance: a brief review. *Sports Med,* 39**,** 313-29.
36. BEEDIE, C. J., STUART, E. M., COLEMAN, D. A. & FOAD, A. J. 2006. Placebo effects of caffeine on cycling performance. *Med Sci Sports Exerc,* 38**,** 2159-64.
37. BEESON, C. C., BEESON, G. C., BUFF, H., ELDRIDGE, J., ZHANG, A., SETH, A., DEMCHEVA, M., VOURNAKIS, J. N. & MUISE-HELMERICKS, R. C. 2012. Integrin-dependent Akt1 activation regulates PGC-1 expression and fatty acid oxidation. *J Vasc Res,* 49**,** 89-100.
38. BENEDETTI, F., POLLO, A. & COLLOCA, L. 2007. Opioid-mediated placebo responses boost pain endurance and physical performance: is it doping in sport competitions? *J Neurosci,* 27**,** 11934-9.
39. BENTLEY, T. B. & PITTMAN, R. N. 1997. Influence of temperature on oxygen diffusion in hamster retractor muscle. *Am J Physiol,* 272**,** H1106-12.
40. BENTON, C. R., WRIGHT, D. C. & BONEN, A. 2008. PGC-1alpha-mediated regulation of gene expression and metabolism: implications for nutrition and exercise prescriptions. *Appl Physiol Nutr Me,* 33**,** 843-62.
41. BÉRDI, M., KÖTELES, F., SZABÓ, A. & BÁRDOS, G. 2011. Placebo effects in sport and exercise: A meta-analysis. *Eur J Men Health,* 6**,** 196-212.
42. BERGEAUD, M., MATHIEU, L., GUILLAUME, A., MOLL, U. M., MIGNOTTE, B., LE FLOCH, N., VAYSSIERE, J. L. & RINCHEVAL, V. 2013. Mitochondrial p53 mediates a transcription-independent regulation of cell respiration and interacts with the mitochondrial F(1)F0-ATP synthase. *Cell cycle,* 12**,** 2781-93.
43. BERGERON, R., REN, J. M., CADMAN, K. S., MOORE, I. K., PERRET, P., PYPAERT, M., YOUNG, L. H., SEMENKOVICH, C. F. & SHULMAN, G. I. 2001. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am J Physiol-Endoc M,* 281**,** E1340-6.
44. BERGH, U. & EKBLOM, B. 1979. Physical performance and peak aerobic power at different body temperatures. *J Appl Physiol,* 46**,** 885-9.
45. BERKERS, C. R., MADDOCKS, O. D., CHEUNG, E. C., MOR, I. & VOUSDEN, K. H. 2013. Metabolic regulation by p53 family members. *Cell Metab,* 18**,** 617-33.
46. BIRK, J. B. & WOJTASZEWSKI, J. F. 2006. Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol,* 577**,** 1021-32.
47. BISHOP, D. J., GRANATA, C. & EYNON, N. 2014. Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content? *BBM-Gen Subjects,* 1840**,** 1266-1275.

48. BLEAKLEY, C., MCDONOUGH, S. & MACAULEY, D. 2004. The use of ice in the treatment of acute soft-tissue injury - A systematic review of randomized controlled trials. *Am J Sport Med,* 32**,** 251-261.
49. BLEAKLEY, C. M. & DAVISON, G. W. 2010. What is the biochemical and physiological rationale for using cold-water immersion in sports recovery? A systematic review. *Br J Sports Med,* 44**,** 179-87.
50. BOLSTER, D. R., KUBICA, N., CROZIER, S. J., WILLIAMSON, D. L., FARRELL, P. A., KIMBALL, S. R. & JEFFERSON, L. S. 2003. Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle. *J Physiol,* 553**,** 213-20.
51. BONDEPETERSEN, F., SCHULTZPEDERSEN, L. & DRAGSTED, N. 1992. Peripheral and Central Blood-Flow in Man during Cold, Thermoneutral, and Hot Water Immersion. *Aviat Space Envir Md,* 63**,** 346-350.
52. BOOTH, F. W. & THOMASON, D. B. 1991. Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. *Physiol Rev,* 71**,** 541-85.
53. BOPPART, M. D., ARONSON, D., GIBSON, L., ROUBENOFF, R., ABAD, L. W., BEAN, J., GOODYEAR, L. J. & FIELDING, R. A. 1999. Eccentric exercise markedly increases c-Jun NH(2)-terminal kinase activity in human skeletal muscle. *J Appl Physiol,* 87**,** 1668-73.
54. BOPPART, M. D., ASP, S., WOJTASZEWSKI, J. F., FIELDING, R. A., MOHR, T. & GOODYEAR, L. J. 2000. Marathon running transiently increases c-Jun NH2-terminal kinase and p38 activities in human skeletal muscle. *J Physiol,* 526 Pt 3**,** 663-9.
55. BOPPART, M. D., HIRSHMAN, M. F., SAKAMOTO, K., FIELDING, R. A. & GOODYEAR, L. J. 2001. Static stretch increases c-Jun NH2-terminal kinase activity and p38 phosphorylation in rat skeletal muscle. *Am J Physiol-Cell Ph,* 280**,** C352-8.
56. BROATCH, J. R., PETERSEN, A. & BISHOP, D. J. 2014. Postexercise cold water immersion benefits are not greater than the placebo effect. *Med Sci Sports Exerc,* 46**,** 2139-47.
57. BROPHY-WILLIAMS, N., LANDERS, G. & WALLMAN, K. 2011. Effect of immediate and delayed cold water immersion after a high intensity exercise session on subsequent run performance. *J Sport Sci Med,* 10**,** 665-70.
58. BRUKNER, P. & KHAN, K. 1993. *Clinical Sports Medicine,* Sydney, McGraw-Hill Book Company Australia Pty Ltd.
59. BRUTON, J. D., AYDIN, J., YAMADA, T., SHABALINA, I. G., IVARSSON, N., ZHANG, S. J., WADA, M., TAVI, P., NEDERGAARD, J., KATZ, A. & WESTERBLAD, H. 2010. Increased fatigue resistance linked to Ca2+-stimulated mitochondrial biogenesis in muscle fibres of cold-acclimated mice. *J Physiol,* 588**,** 4275-88.
60. BUCHHEIT, M. & LAURSEN, P. B. 2013. High-intensity interval training, solutions to the programming puzzle. Part II: anaerobic energy, neuromuscular load and practical applications. *Sports Med,* 43**,** 927-54.
61. BUCHHEIT, M., PEIFFER, J. J., ABBISS, C. R. & LAURSEN, P. B. 2009. Effect of cold water immersion on postexercise parasympathetic reactivation. *Am J Physiol-Heart C,* 296**,** H421-7.
62. BURGESS, T. L. & LAMBERT, M. I. 2010. The efficacy of cryotherapy on recovery following exercise-induced muscle damage. *Int SportMed J,* 11.
63. BURGOMASTER, K. A., HOWARTH, K. R., PHILLIPS, S. M., RAKOBOWCHUK, M., MACDONALD, M. J., MCGEE, S. L. & GIBALA, M. J. 2008. Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol,* 586**,** 151-60.

64. BURGOMASTER, K. A., HUGHES, S. C., HEIGENHAUSER, G. J., BRADWELL, S. N. & GIBALA, M. J. 2005. Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol,* 98**,** 1985-90.
65. BURKE, D. G., MACNEIL, S. A., HOLT, L. E., MACKINNON, N. C. & RASMUSSEN, R. L. 2000. The effect of hot or cold water immersion on isometric strength training. *J Strength Cond Res,* 14**,** 21-25.
66. CALVO, J. A., DANIELS, T. G., WANG, X., PAUL, A., LIN, J., SPIEGELMAN, B. M., STEVENSON, S. C. & RANGWALA, S. M. 2008. Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *J Appl Physiol,* 104**,** 1304-12.
67. CANNON, B. & NEDERGAARD, J. 2004. Brown adipose tissue: function and physiological significance. *Physiol Rev,* 84**,** 277-359.
68. CANTO, C., GERHART-HINES, Z., FEIGE, J. N., LAGOUGE, M., NORIEGA, L., MILNE, J. C., ELLIOTT, P. J., PUIGSERVER, P. & AUWERX, J. 2009. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature,* 458**,** 1056-60.
69. CANTO, C., JIANG, L. Q., DESHMUKH, A. S., MATAKI, C., COSTE, A., LAGOUGE, M., ZIERATH, J. R. & AUWERX, J. 2010. Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. *Cell Metab,* 11**,** 213-9.
70. CAO, W., DANIEL, K. W., ROBIDOUX, J., PUIGSERVER, P., MEDVEDEV, A. V., BAI, X., FLOERING, L. M., SPIEGELMAN, B. M. & COLLINS, S. 2004. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. *Mol Cell Biol,* 24**,** 3057-67.
71. CARLING, D. & HARDIE, D. G. 1989. The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta,* 1012**,** 81-6.
72. CARTONI, R., LEGER, B., HOCK, M. B., PRAZ, M., CRETTENAND, A., PICH, S., ZILTENER, J. L., LUTHI, F., DERIAZ, O., ZORZANO, A., GOBELET, C., KRALLI, A. & RUSSELL, A. P. 2005. Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. *J Physiol,* 567**,** 349-58.
73. CHAPMAN, C. E. 1991. Can the use of physical modalities for pain control be rationalized by the research evidence? *Can J Physiol Pharmacol,* 69**,** 704-12.
74. CHAPPELL, S. A., OWENS, G. C. & MAURO, V. P. 2001. A 5' Leader of Rbm3, a Cold Stress-induced mRNA, Mediates Internal Initiation of Translation with Increased Efficiency under Conditions of Mild Hypothermia. *J Biol Chem,* 276**,** 36917-22.
75. CHEN, Z., GIBSON, T. B., ROBINSON, F., SILVESTRO, L., PEARSON, G., XU, B., WRIGHT, A., VANDERBILT, C. & COBB, M. H. 2001. MAP kinases. *Chem Rev,* 101**,** 2449-76.
76. CHEN, Z. P., MCCONELL, G. K., MICHELL, B. J., SNOW, R. J., CANNY, B. J. & KEMP, B. E. 2000. AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *Am J Physiol-Endoc M,* 279**,** E1202-6.
77. CHEN, Z. P., STEPHENS, T. J., MURTHY, S., CANNY, B. J., HARGREAVES, M., WITTERS, L. A., KEMP, B. E. & MCCONELL, G. K. 2003. Effect of exercise intensity on skeletal muscle AMPK signaling in humans. *Diabetes,* 52**,** 2205-12.
78. CHEUNG, K., HUME, P. & MAXWELL, L. 2003. Delayed onset muscle soreness : treatment strategies and performance factors. *Sports Med,* 33**,** 145-64.

79. CHUNG, J., NGUYEN, A. K., HENSTRIDGE, D. C., HOLMES, A. G., CHAN, M. H., MESA, J. L., LANCASTER, G. I., SOUTHGATE, R. J., BRUCE, C. R., DUFFY, S. J., HORVATH, I., MESTRIL, R., WATT, M. J., HOOPER, P. L., KINGWELL, B. A., VIGH, L., HEVENER, A. & FEBBRAIO, M. A. 2008. HSP72 protects against obesity-induced insulin resistance. *Proc Natl Acad Sci USA,* 105**,** 1739-44.
80. CLARK, S. A., CHEN, Z. P., MURPHY, K. T., AUGHEY, R. J., MCKENNA, M. J., KEMP, B. E. & HAWLEY, J. A. 2004. Intensified exercise training does not alter AMPK signaling in human skeletal muscle. *Am J Physiol-Endoc M,* 286**,** E737-43.
81. CLARK, V. R., HOPKINS, T. G., HAWLEY, J. A. & BURKE, L. M. 2000. Placebo effect of carbohydrate feedings during a 40-km cycling time trial. *Med Sci Sports Exerc,* 32**,** 1642-1647.
82. CLARKSON, P. M. & HUBAL, M. J. 2002. Exercise-induced muscle damage in humans. *Am J Phys Med Rehabil,* 81**,** S52-69.
83. CLARKSON, P. M., NOSAKA, K. & BRAUN, B. 1992. Muscle function after exercise-induced muscle damage and rapid adaptation. *Med Sci Sports Exerc,* 24**,** 512-20.
84. CLEMENTI, E. & NISOLI, E. 2005. Nitric oxide and mitochondrial biogenesis: a key to long-term regulation of cellular metabolism. *Comp Biochem Physiol A,* 142**,** 102-10.
85. COCHRAN, A. J., LITTLE, J. P., TARNOPOLSKY, M. A. & GIBALA, M. J. 2010. Carbohydrate feeding during recovery alters the skeletal muscle metabolic response to repeated sessions of high-intensity interval exercise in humans. *J Appl Physiol,* 108**,** 628-36.
86. COCHRAN, A. J., PERCIVAL, M. E., TRICARICO, S., LITTLE, J. P., CERMAK, N., GILLEN, J. B., TARNOPOLSKY, M. A. & GIBALA, M. J. 2014. Intermittent and continuous high-intensity exercise training induce similar acute but different chronic muscle adaptations. *Exp Physiol,* 99**,** 782-91.
87. COCHRANE, D. J. 2004. Alternating hot and cold water immersion for athlete recovery: a review. *Phys Ther Sport,* 5**,** 26-32.
88. COFFEY, V., LEVERITT, M. & GILL, N. 2004. Effect of recovery modality on 4-hour repeated treadmill running performance and changes in physiological variables. *J Sci Med Sport,* 7**,** 1-10.
89. COFFEY, V. G. & HAWLEY, J. A. 2007. The molecular bases of training adaptation. *Sports Med,* 37**,** 737-63.
90. COFFEY, V. G., SHIELD, A., CANNY, B. J., CAREY, K. A., CAMERON-SMITH, D. & HAWLEY, J. A. 2006. Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. *Am J Physiol-Endoc M,* 290**,** E849-55.
91. COFFEY, V. G., ZHONG, Z., SHIELD, A., CANNY, B. J., CHIBALIN, A. V., ZIERATH, J. R. & HAWLEY, J. A. 2006. Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans. *FASEB J,* 20**,** 190-2.
92. COLLINS, N. C. 2008. Is ice right? Does cryotherapy improve outcome for acute soft tissue injury? *Emerg Med J,* 25**,** 65-68.
93. CONNOR, M. K., IRRCHER, I. & HOOD, D. A. 2001. Contractile activity-induced transcriptional activation of cytochrome C involves Sp1 and is proportional to mitochondrial ATP synthesis in C2C12 muscle cells. *J Biol Chem,* 276**,** 15898-904.
94. COOP, A., WIESMANN, K. E. & CRABBE, M. J. 1998. Translocation of beta crystallin in neural cells in response to stress. *FEBS Lett,* 431**,** 319-21.
95. COOPER, C. E., VOLLAARD, N. B., CHOUEIRI, T. & WILSON, M. T. 2002. Exercise, free radicals and oxidative stress. *Biochem Soc T,* 30**,** 280-5.
96. CORBETT, J., BARWOOD, M. J., LUNT, H. C., MILNER, A. & TIPTON, M. J. 2012. Water immersion as a recovery aid from intermittent shuttle running exercise. *Eur J Sport Sci,* 12**,** 509-514.

97. COSTELLO, J. T., CULLIGAN, K., SELFE, J. & DONNELLY, A. E. 2012. Muscle, skin and core temperature after -110 degrees c cold air and 8 degrees c water treatment. *PloS one,* 7**,** e48190.
98. COSTELLO, J. T., DONNELLY, A. E., KARKI, A. & SELFE, J. 2014. Effects of whole body cryotherapy and cold water immersion on knee skin temperature. *Int J Sports Med,* 35**,** 35-40.
99. COTE, D. J., PRENTICE, W. E., JR., HOOKER, D. N. & SHIELDS, E. W. 1988. Comparison of three treatment procedures for minimizing ankle sprain swelling. *Phys Ther,* 68**,** 1072-6.
100. COULANGE, M., HUG, F., KIPSON, N., ROBINET, C., DESRUELLE, A. V., MELIN, B., JIMENEZ, C., GALLAND, F. & JAMMES, Y. 2006. Consequences of prolonged total body immersion in cold water on muscle performance and EMG activity. *Pflugers Arch,* 452**,** 91-101.
101. COYLE, E. F., MARTIN, W. H., EHSANI, A. A., HAGBERG, J. M., BLOOMFIELD, S. A., SINACORE, D. R. & HOLLOSZY, J. O. 1983. Blood Lactate Threshold in Some Well-Trained Ischemic-Heart-Disease Patients. *J Appl Physiol,* 54**,** 18-23.
102. CROWE, M. J., O'CONNOR, D. & RUDD, D. 2007. Cold water recovery reduces anaerobic performance. *Int J Sports Med,* 28**,** 994-8.
103. CROWLEY, G. C., GARG, A., LOHN, M. S., VAN SOMEREN, N. & WADE, A. J. 1991. Effects of cooling the legs on performance in a standard Wingate anaerobic power test. *Br J Sports Med,* 25**,** 200-3.
104. CUADRADO, A. & NEBREDA, A. R. 2010. Mechanisms and functions of p38 MAPK signalling. *Biochem J,* 429**,** 403-17.
105. CUENDA, A. & ROUSSEAU, S. 2007. p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta,* 1773**,** 1358-75.
106. CUI, G., PARK, S., BADEAUX, A. I., KIM, D., LEE, J., THOMPSON, J. R., YAN, F., KANEKO, S., YUAN, Z., BOTUYAN, M. V., BEDFORD, M. T., CHENG, J. Q. & MER, G. 2012. PHF20 is an effector protein of p53 double lysine methylation that stabilizes and activates p53. *Nat Struct Mol Biol,* 19**,** 916-24.
107. CZUBRYT, M. P., MCANALLY, J., FISHMAN, G. I. & OLSON, E. N. 2003. Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. *Proc Natl Acad Sci USA,* 100**,** 1711-6.
108. DATTA, A. & TIPTON, M. 2006. Respiratory responses to cold water immersion: neural pathways, interactions, and clinical consequences awake and asleep. *J Appl Physiol,* 100**,** 2057-64.
109. DAYALAN NAIDU, S., KOSTOV, R. V. & DINKOVA-KOSTOVA, A. T. 2015. Transcription factors Hsf1 and Nrf2 engage in crosstalk for cytoprotection. *Trends Pharmacol Sci,* 36**,** 6-14.
110. DE FILIPPIS, E., ALVAREZ, G., BERRIA, R., CUSI, K., EVERMAN, S., MEYER, C. & MANDARINO, L. J. 2008. Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise. *Am J Physiol-Endoc M,* 294**,** E607-14.
111. DEKKERS, J. C., VAN DOORNEN, L. J. & KEMPER, H. C. 1996. The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. *Sports Med,* 21**,** 213-38.
112. DILLARD, C. J., LITOV, R. E., SAVIN, W. M., DUMELIN, E. E. & TAPPEL, A. L. 1978. Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J Appl Physiol,* 45**,** 927-32.
113. DISTLER, J. H., HIRTH, A., KUROWSKA-STOLARSKA, M., GAY, R. E., GAY, S. & DISTLER, O. 2003. Angiogenic and angiostatic factors in the molecular control of angiogenesis. *Q J Nucl Med,* 47**,** 149-61.

114. DRESIOS, J., ASCHRAFI, A., OWENS, G. C., VANDERKLISH, P. W., EDELMAN, G. M. & MAURO, V. P. 2005. Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc Natl Acad Sci USA,* 102**,** 1865-70.
115. DROGE, W. 2002. Free radicals in the physiological control of cell function. *Physiol Rev,* 82**,** 47-95.
116. DUGUE, B., SMOLANDER, J., WESTERLUND, T., OKSA, J., NIEMINEN, R., MOILANEN, E. & MIKKELSSON, M. 2005. Acute and long-term effects of winter swimming and whole-body cryotherapy on plasma antioxidative capacity in healthy women. *Scand J Clin Lab Invest,* 65**,** 395-402.
117. DURANTE, P. E., MUSTARD, K. J., PARK, S. H., WINDER, W. W. & HARDIE, D. G. 2002. Effects of endurance training on activity and expression of AMP-activated protein kinase isoforms in rat muscles. *Am J Physiol-Endoc M,* 283**,** E178-86.
118. EGAN, B., CARSON, B. P., GARCIA-ROVES, P. M., CHIBALIN, A. V., SARSFIELD, F. M., BARRON, N., MCCAFFREY, N., MOYNA, N. M., ZIERATH, J. R. & O'GORMAN, D. J. 2010. Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. *J Physiol,* 588**,** 1779-90.
119. EGAN, B. & ZIERATH, J. R. 2013. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab,* 17**,** 162-84.
120. EGGINTON, S. & SIDELL, B. D. 1989. Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am J Physiol,* 256**,** R1-9.
121. ERMOLENKO, D. N. & MAKHATADZE, G. I. 2002. Bacterial cold-shock proteins. *Cell Mol Life Sci,* 59**,** 1902-13.
122. ERNST, E. & FIALKA, V. 1994. Ice freezes pain? A review of the clinical effectiveness of analgesic cold therapy. *J Pain Symtom Manag,* 9**,** 56-9.
123. ESTON, R. & PETERS, D. 1999. Effects of cold water immersion on the symptoms of exercise-induced muscle damage. *J Sport Sci,* 17**,** 231-8.
124. EVANS, W. J., PHINNEY, S. D. & YOUNG, V. R. 1982. Suction applied to a muscle biopsy maximizes sample size. *Med Sci Sports Exerc,* 14**,** 101-2.
125. FAN, M., RHEE, J., ST-PIERRE, J., HANDSCHIN, C., PUIGSERVER, P., LIN, J., JAEGER, S., ERDJUMENT-BROMAGE, H., TEMPST, P. & SPIEGELMAN, B. M. 2004. Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1alpha: modulation by p38 MAPK. *Genes Dev,* 18**,** 278-89.
126. FERNANDEZ-MARCOS, P. J. & AUWERX, J. 2011. Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. *Am J Clin Nutr,* 93**,** 884S-90.
127. FINAUD, J., LAC, G. & FILAIRE, E. 2006. Oxidative stress : relationship with exercise and training. *Sports Med,* 36**,** 327-58.
128. FISCHER, A. A. 1988. Documentation of myofascial trigger points. *Arch Phys Med Rehabil,* 69**,** 286-91.
129. FISCHER, C. P., HISCOCK, N. J., BASU, S., VESSBY, B., KALLNER, A., SJOBERG, L. B., FEBBRAIO, M. A. & PEDERSEN, B. K. 2006. Vitamin E isoform-specific inhibition of the exercise-induced heat shock protein 72 expression in humans. *J Appl Physiol,* 100**,** 1679-87.
130. FISCUS, K. A., KAMINSKI, T. W. & POWERS, M. E. 2005. Changes in lower-leg blood flow during warm-, cold-, and contrast-water therapy. *Arch Phys Med Rehabil,* 86**,** 1404-10.
131. FITTIPALDI, S., DIMAURO, I., MERCATELLI, N. & CAPOROSSI, D. 2014. Role of exercise-induced reactive oxygen species in the modulation of heat shock protein response. *Free Radic Res,* 48**,** 52-70.

132. FOSTER, W. H., TIDBALL, J. G. & WANG, Y. 2012. p38gamma activity is required for maintenance of slow skeletal muscle size. *Muscle Nerve,* 45**,** 266-73.
133. FRODIN, M. & GAMMELTOFT, S. 1999. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol,* 151**,** 65-77.
134. FROHLICH, M., FAUDE, O., KLEIN, M., PIETER, A., EMRICH, E. & MEYER, T. 2014. Strength training adaptations after cold-water immersion. *J Strength Cond Res,* 28**,** 2628-33.
135. FUJII, N., HAYASHI, T., HIRSHMAN, M. F., SMITH, J. T., HABINOWSKI, S. A., KAIJSER, L., MU, J., LJUNGQVIST, O., BIRNBAUM, M. J., WITTERS, L. A., THORELL, A. & GOODYEAR, L. J. 2000. Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle. *Biochem Bioph Res Co,* 273**,** 1150-5.
136. FUJITA, J. 1999. Cold shock response in mammalian cells. *J Mol Microbiol Biotechnol,* 1**,** 243-55.
137. GALPIN, A. J., FRY, A. C., CHIU, L. Z., THOMASON, D. B. & SCHILLING, B. K. 2012. High-power resistance exercise induces MAPK phosphorylation in weightlifting trained men. *Appl Physiol Nutr Me,* 37**,** 80-7.
138. GAOUA, N., GRANTHAM, J., RACINAIS, S. & EL MASSIOUI, F. 2012. Sensory displeasure reduces complex cognitive performance in the heat. *J Environ Psych,* 32**,** 158-163.
139. GARCÃA-MANSO, J. M., RODRÃGUEZ-MATOSO, D., RODRÃGUEZ-RUIZ, D., SARMIENTO, S., DE SAA, Y. & CALDERÃ³N, J. 2011. Effect of cold-water immersion on skeletal muscle contractile properties in soccer players. *Am J Phys Med Rehab,* 90**,** 356-363.
140. GARTEL, A. L. & RADHAKRISHNAN, S. K. 2005. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res,* 65**,** 3980-3985.
141. GEORGOPOULOS, C. & WELCH, W. J. 1993. Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol,* 9**,** 601-34.
142. GIBALA, M. 2009. Molecular responses to high-intensity interval exercise. *Appl Physiol Nutr Me,* 34**,** 428-32.
143. GIBALA, M. J., LITTLE, J. P., MACDONALD, M. J. & HAWLEY, J. A. 2012. Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol,* 590**,** 1077-84.
144. GIBALA, M. J., LITTLE, J. P., VAN ESSEN, M., WILKIN, G. P., BURGOMASTER, K. A., SAFDAR, A., RAHA, S. & TARNOPOLSKY, M. A. 2006. Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *J Physiol,* 575**,** 901-11.
145. GIBALA, M. J. & MCGEE, S. L. 2008. Metabolic adaptations to short-term high-intensity interval training: a little pain for a lot of gain? *Exerc Sport Sci Rev,* 36**,** 58-63.
146. GIBALA, M. J., MCGEE, S. L., GARNHAM, A. P., HOWLETT, K. F., SNOW, R. J. & HARGREAVES, M. 2009. Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. *J Appl Physiol,* 106**,** 929-34.
147. GILL, N. D., BEAVEN, C. M. & COOK, C. 2006. Effectiveness of post-match recovery strategies in rugby players. *Br J Sports Med,* 40**,** 260-263.
148. GLEYZER, N., VERCAUTEREN, K. & SCARPULLA, R. C. 2005. Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. *Mol Cell Biol,* 25**,** 1354-66.
149. GOFFART, S. & WIESNER, R. J. 2003. Regulation and co-ordination of nuclear gene expression during mitochondrial biogenesis. *Exp Physiol,* 88**,** 33-40.

150. GOLDEN, T. R., HINERFELD, D. A. & MELOV, S. 2002. Oxidative stress and aging: beyond correlation. *Aging cell,* 1**,** 117-23.
151. GOMEZ-CABRERA, M. C., DOMENECH, E., ROMAGNOLI, M., ARDUINI, A., BORRAS, C., PALLARDO, F. V., SASTRE, J. & VINA, J. 2008. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr,* 87**,** 142-149.
152. GON, Y., HASHIMOTO, S., MATSUMOTO, K., NAKAYAMA, T., TAKESHITA, I. & HORIE, T. 1998. Cooling and rewarming-induced IL-8 expression in human bronchial epithelial cells through p38 MAP kinase-dependent pathway. *Biochem Bioph Res Co,* 249**,** 156-60.
153. GONZALEZ-ALONSO, J., TELLER, C., ANDERSEN, S. L., JENSEN, F. B., HYLDIG, T. & NIELSEN, B. 1999. Influence of body temperature on the development of fatigue during prolonged exercise in the heat. *J Appl Physiol,* 86**,** 1032-9.
154. GONZALEZ, A. M., STOUT, J. R., JAJTNER, A. R., TOWNSEND, J. R., WELLS, A. J., BEYER, K. S., BOONE, C. H., PRUNA, G. J., MANGINE, G. T., SCANLON, T. M., BOHNER, J. D., OLIVEIRA, L. P., FRAGALA, M. S. & HOFFMAN, J. R. 2014. Effects of β-hydroxy-β-methylbutyrate free acid and cold water immersion on post-exercise markers of muscle damage. *Amino Acids,* 46**,** 1501-1511.
155. GOODALL, S. & HOWATSON, G. 2008. The effects of multiple cold water immersions on indices of muscle damage. *J Sport Sci Med,* 7**,** 235-241.
156. GOODYEAR, L. J., CHANG, P. Y., SHERWOOD, D. J., DUFRESNE, S. D. & MOLLER, D. E. 1996. Effects of exercise and insulin on mitogen-activated protein kinase signaling pathways in rat skeletal muscle. *Am J Physiol,* 271**,** E403-8.
157. GRANATA, C., DA SILVA FERMINO DE OLIVEIRA, R., LITTLE, J. P., RENNER, K. & BISHOP, D. J. 2015. Sprint interval, but not lower-intensity training increases mitochondrial respiration, and PGC-1a, p53, and PHF20 protein content in human skeletal muscle. *unpublished research*.
158. GREGORY, C. D. & MILNER, A. E. 1994. Regulation of cell survival in Burkitt lymphoma: implications from studies of apoptosis following cold-shock treatment. *Int J Cancer,* 57**,** 419-26.
159. GREGSON, W., ALLAN, R., HOLDEN, S., PHIBBS, P., DORAN, D., CAMPBELL, I., WALDRON, S., JOO, C. H. & MORTON, J. P. 2013. Postexercise cold-water immersion does not attenuate muscle glycogen resynthesis. *Med Sci Sports Exerc,* 45**,** 1174-81.
160. GREGSON, W., BLACK, M. A., JONES, H., MILSON, J., MORTON, J., DAWSON, B., ATKINSON, G. & GREEN, D. J. 2011. Influence of cold water immersion on limb and cutaneous blood flow at rest. *Am J Sport Med,* 39**,** 1316-1323.
161. GULICK, D. T., KIMURA, I. F., SITLER, M., PAOLONE, A. & KELLY, J. D. 1996. Various treatment techniques on signs and symptoms of delayed onset muscle soreness. *J Athl Training,* 31**,** 145-52.
162. GURD, B. J., YOSHIDA, Y., LALLY, J., HOLLOWAY, G. P. & BONEN, A. 2009. The deacetylase enzyme SIRT1 is not associated with oxidative capacity in rat heart and skeletal muscle and its overexpression reduces mitochondrial biogenesis. *J Physiol,* 587**,** 1817-28.
163. HALLIWELL, B. 1996. Mechanisms involved in the generation of free radicals. *Pathol Biol,* 44**,** 6-13.
164. HALSON, S. L., BARTRAM, J., WEST, N., STEPHENS, J., ARGUS, C. K., DRILLER, M. W., SARGENT, C., LASTELLA, M., HOPKINS, W. G. & MARTIN, D. T. 2014. Does hydrotherapy help or hinder adaptation to training in competitive cyclists? *Med Sci Sports Exerc,* 46**,** 1631-9.
165. HALSON, S. L. & MARTIN, D. T. 2013. Lying to win-placebos and sport science. *Int J Sports Physiol Perform,* 8**,** 597-9.

166. HANDSCHIN, C., RHEE, J., LIN, J., TARR, P. T. & SPIEGELMAN, B. M. 2003. An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proc Natl Acad Sci USA,* 100**,** 7111-6.
167. HARDIE, D. G. & SAKAMOTO, K. 2006. AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology,* 21**,** 48-60.
168. HAUSSWIRTH, C., MUJIKA, I. I. & INSTITUT NATIONAL DU SPORT ET DE L'ÉDUCATION PHYSIQUE (FRANCE) 2013. *Recovery for performance in sport,* Champaign, IL, Human Kinetics.
169. HAWLEY, J. A. & NOAKES, T. D. 1992. Peak power output predicts maximal oxygen uptake and performance time in trained cyclists. *Eur J Appl Physiol Occup Physiol,* 65**,** 79-83.
170. HAYASHI, T., HIRSHMAN, M. F., KURTH, E. J., WINDER, W. W. & GOODYEAR, L. J. 1998. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes,* 47**,** 1369-73.
171. HELLEMANS, J., MORTIER, G., DE PAEPE, A., SPELEMAN, F. & VANDESOMPELE, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol,* 8**,** R19.
172. HEMINGWAY, A. 1963. Shivering. *Physiol Rev,* 43**,** 397-422.
173. HENSEL, H. & BOMAN, K. K. 1960. Afferent impulses in cutaneous sensory nerves in human subjects. *J Neurophysiol,* 23**,** 564-78.
174. HEYMAN, E., B, D. E. G., MERTENS, I. & MEEUSEN, R. 2009. Effects of four recovery methods on repeated maximal rock climbing performance. *Med Sci Sports Exerc,* 41**,** 1303-10.
175. HIGASHIDA, K., KIM, S. H., HIGUCHI, M., HOLLOSZY, J. O. & HAN, D. H. 2011. Normal adaptations to exercise despite protection against oxidative stress. *Am J Physiol-Endoc M,* 301**,** E779-84.
176. HIGGINS, T. R., HEAZLEWOOD, I. T. & CLIMSTEIN, M. 2011. A random control trial of contrast baths and ice baths for recovery during competition in U/20 rugby union. *J Strength Cond Res,* 25**,** 1046-51.
177. HINGHOFER-SZALKAY, H., HARRISON, M. H. & GREENLEAF, J. E. 1987. Early fluid and protein shifts in men during water immersion. *Eur J Appl Physiol O,* 56**,** 673-8.
178. HO, R. C., ALCAZAR, O., FUJII, N., HIRSHMAN, M. F. & GOODYEAR, L. J. 2004. p38gamma MAPK regulation of glucose transporter expression and glucose uptake in L6 myotubes and mouse skeletal muscle. *Am J Physiol-Reg I,* 286**,** R342-9.
179. HOLLAND, D. B., ROBERTS, S. G., WOOD, E. J. & CUNLIFFE, W. J. 1993. Cold shock induces the synthesis of stress proteins in human keratinocytes. *J Invest Dermatol,* 101**,** 196-9.
180. HOLLEY, A. K., DHAR, S. K. & ST CLAIR, D. K. 2010. Manganese superoxide dismutase vs. p53: regulation of mitochondrial ROS. *Mitochondrion,* 10**,** 649-61.
181. HOLLOSZY, J. O. 2005. Exercise-induced increase in muscle insulin sensitivity. *J Appl Physiol,* 99**,** 338-43.
182. HOLLOSZY, J. O. & COYLE, E. F. 1984. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol,* 56**,** 831-8.
183. HOLLOWAY, G. P., PERRY, C. G., THRUSH, A. B., HEIGENHAUSER, G. J., DYCK, D. J., BONEN, A. & SPRIET, L. L. 2008. PGC-1alpha's relationship with skeletal muscle palmitate oxidation is not present with obesity despite maintained PGC-1alpha and PGC-1beta protein. *Am J Physiol-Endoc M,* 294**,** E1060-9.
184. HOOD, D. A., TAKAHASHI, M., CONNOR, M. K. & FREYSSENET, D. 2000. Assembly of the cellular powerhouse: current issues in muscle mitochondrial biogenesis. *Exerc Sport Sci Rev,* 28**,** 68-73.

185. HOOD, D. A., UGUCCIONI, G., VAINSHTEIN, A. & D'SOUZA, D. 2011. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle: implications for health and disease. *Compr Physiol,* 1**,** 1119-34.
186. HORNERY, D. J., PAPALIA, S., MUJIKA, I. & HAHN, A. 2005. Physiological and performance benefits of halftime cooling. *J Sci Med Sport,* 8**,** 15-25.
187. HOWATSON, G., GOODALL, S. & VAN SOMEREN, K. A. 2009. The influence of cold water immersions on adaptation following a single bout of damaging exercise. *Eur J Appl Physiol,* 105**,** 615-21.
188. HOWATSON, G. & VAN SOMEREN, K. A. 2008. The prevention and treatment of exercise-induced muscle damage. *Sports Med,* 38**,** 483-503.
189. HUBBARD, T. J., ARONSON, S. L. & DENEGAR, C. R. 2004. Does Cryotherapy Hasten Return to Participation? A Systematic Review. *J Athl Training,* 39**,** 88-94.
190. HUBBARD, T. J. & DENEGAR, C. R. 2004. Does Cryotherapy Improve Outcomes With Soft Tissue Injury? *J Athl Training,* 39**,** 278-279.
191. HUTTUNEN, P., RINTAMAKI, H. & HIRVONEN, J. 2001. Effect of regular winter swimming on the activity of the sympathoadrenal system before and after a single cold water immersion. *Int J Circumpol Heal,* 60**,** 400-6.
192. IHSAN, M., WATSON, G., CHOO, H. C., LEWANDOWSKI, P., PAPAZZO, A., CAMERON-SMITH, D. & ABBISS, C. R. 2014. Postexercise Muscle Cooling Enhances Gene Expression of PGC-1alpha. *Med Sci Sports Exerc,* 46**,** 1900-7.
193. IHSAN, M., WATSON, G., LIPSKI, M. & ABBISS, C. R. 2013. Influence of postexercise cooling on muscle oxygenation and blood volume changes. *Med Sci Sports Exerc,* 45**,** 876-82.
194. IJIRI, D., KANAI, Y. & HIRABAYASHI, M. 2009. Possible roles of myostatin and PGC-1alpha in the increase of skeletal muscle and transformation of fiber type in cold-exposed chicks: expression of myostatin and PGC-1alpha in chicks exposed to cold. *Domest Anim Endocrinol,* 37**,** 12-22.
195. INGRAM, J., DAWSON, B., GOODMAN, C., WALLMAN, K. & BEILBY, J. 2009. Effect of water immersion methods on post-exercise recovery from simulated team sport exercise. *J Sci Med Sport,* 12**,** 417-21.
196. IRRCHER, I., ADHIHETTY, P. J., JOSEPH, A. M., LJUBICIC, V. & HOOD, D. A. 2003. Regulation of mitochondrial biogenesis in muscle by endurance exercise. *Sports Med,* 33**,** 783-93.
197. IRRCHER, I., ADHIHETTY, P. J., SHEEHAN, T., JOSEPH, A. M. & HOOD, D. A. 2003. PPARgamma coactivator-1alpha expression during thyroid hormone- and contractile activity-induced mitochondrial adaptations. *Am J Physiol-Cell Ph,* 284**,** C1669-77.
198. IRRCHER, I. & HOOD, D. A. 2004. Regulation of Egr-1, SRF, and Sp1 mRNA expression in contracting skeletal muscle cells. *J Appl Physiol,* 97**,** 2207-13.
199. IRRCHER, I., LJUBICIC, V. & HOOD, D. A. 2009. Interactions between ROS and AMP kinase activity in the regulation of PGC-1alpha transcription in skeletal muscle cells. *Am J Physiol-Cell Ph,* 296**,** C116-23.
200. IRRCHER, I., LJUBICIC, V., KIRWAN, A. F. & HOOD, D. A. 2008. AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. *PloS one,* 3**,** e3614.
201. JACKSON, M. J. 2008. Free radicals generated by contracting muscle: by-products of metabolism or key regulators of muscle function? *Free Radical Bio Med,* 44**,** 132-41.
202. JACKSON, M. J., KHASSAF, M., VASILAKI, A., MCARDLE, F. & MCARDLE, A. 2004. Vitamin E and the oxidative stress of exercise. *Ann NY Acad Sci,* 1031**,** 158-68.
203. JACKSON, M. J. & O'FARRELL, S. 1993. Free radicals and muscle damage. *Br Med Bull,* 49**,** 630-41.

204. JAGER, S., HANDSCHIN, C., ST-PIERRE, J. & SPIEGELMAN, B. M. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc Natl Acad Sci USA,* 104**,** 12017-22.
205. JAKEMAN, J. R., MACRAE, R. & ESTON, R. 2009. A single 10-min bout of cold-water immersion therapy after strenuous plyometric exercise has no beneficial effect on recovery from the symptoms of exercise-induced muscle damage. *Ergonomics,* 52**,** 456-60.
206. JANSKY, L. 1998. Shivering. *In:* BLATTHEIS, C. M. (ed.) *Physiology and Pathophysiology of Temperature Regulation.* World Scientific.
207. JENKINS, R. R. 1988. Free radical chemistry. Relationship to exercise. *Sports Med,* 5**,** 156-70.
208. JI, L. L. 2007. Antioxidant signaling in skeletal muscle: A brief review. *Exp Gerontol,* 42**,** 582-593.
209. JIANG, H. K., WANG, Y. H., SUN, L., HE, X., ZHAO, M., FENG, Z. H., YU, X. J. & ZANG, W. J. 2014. Aerobic interval training attenuates mitochondrial dysfunction in rats post-myocardial infarction: roles of mitochondrial network dynamics. *Int J Mol Sci,* 15**,** 5304-22.
210. JOHANSEN, L. B., JENSEN, T. U., PUMP, B. & NORSK, P. 1997. Contribution of abdomen and legs to central blood volume expansion in humans during immersion. *J Appl Physiol,* 83**,** 695-9.
211. JONES, R. G., PLAS, D. R., KUBEK, S., BUZZAI, M., MU, J., XU, Y., BIRNBAUM, M. J. & THOMPSON, C. B. 2005. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell,* 18**,** 283-93.
212. JORGENSEN, S. B., TREEBAK, J. T., VIOLLET, B., SCHJERLING, P., VAULONT, S., WOJTASZEWSKI, J. F. & RICHTER, E. A. 2007. Role of AMPKalpha2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle. *Am J Physiol-Endoc M,* 292**,** E331-9.
213. JORGENSEN, S. B., WOJTASZEWSKI, J. F., VIOLLET, B., ANDREELLI, F., BIRK, J. B., HELLSTEN, Y., SCHJERLING, P., VAULONT, S., NEUFER, P. D., RICHTER, E. A. & PILEGAARD, H. 2005. Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. *FASEB J,* 19**,** 1146-8.
214. JOSE, A. D., STITT, F. & COLLISON, D. 1970. The effects of exercise and changes in body temperature on the intrinsic heart rate in man. *Am Heart J,* 79**,** 488-98.
215. JOZA, N., OUDIT, G. Y., BROWN, D., BENIT, P., KASSIRI, Z., VAHSEN, N., BENOIT, L., PATEL, M. M., NOWIKOVSKY, K., VASSAULT, A., BACKX, P. H., WADA, T., KROEMER, G., RUSTIN, P. & PENNINGER, J. M. 2005. Muscle-specific loss of apoptosis-inducing factor leads to mitochondrial dysfunction, skeletal muscle atrophy, and dilated cardiomyopathy. *Mol Cell Biol,* 25**,** 10261-72.
216. KABE, Y., ANDO, K., HIRAO, S., YOSHIDA, M. & HANDA, H. 2005. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal,* 7**,** 395-403.
217. KAHN, B. B., ALQUIER, T., CARLING, D. & HARDIE, D. G. 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab,* 1**,** 15-25.
218. KAITSUKA, T., TOMIZAWA, K. & MATSUSHITA, M. 2011. Transformation of eEF1Bdelta into heat-shock response transcription factor by alternative splicing. *EMBO Rep,* 12**,** 673-81.
219. KALASOUNTAS, V., REED, J. & FITZPATRICK, J. 2007. The effect of placebo-induced changes in expectancies on maximal force production in college students. *J Appl Sport Psych,* 19**,** 116-124.

220. KANAI, M., MA, Z., IZUMI, H., KIM, S. H., MATTISON, C. P., WINEY, M. & FUKASAWA, K. 2007. Physical and functional interaction between mortalin and Mps1 kinase. *Genes Cells,* 12**,** 797-810.
221. KANEKO, Y., NISHIYAMA, H., NONOGUCHI, K., HIGASHITSUJI, H., KISHISHITA, M. & FUJITA, J. 1997. A novel hsp110-related gene, apg-1, that is abundantly expressed in the testis responds to a low temperature heat shock rather than the traditional elevated temperatures. *J Biol Chem,* 272**,** 2640-5.
222. KANG, C., O'MOORE, K. M., DICKMAN, J. R. & JI, L. L. 2009. Exercise activation of muscle peroxisome proliferator-activated receptor-gamma coactivator-1alpha signaling is redox sensitive. *Free Radical Bio Med,* 47**,** 1394-400.
223. KANG, M. Y., KIM, H. B., PIAO, C., LEE, K. H., HYUN, J. W., CHANG, I. Y. & YOU, H. J. 2013. The critical role of catalase in prooxidant and antioxidant function of p53. *Cell Death Differ,* 20**,** 117-129.
224. KANKI, T., OHGAKI, K., GASPARI, M., GUSTAFSSON, C. M., FUKUOH, A., SASAKI, N., HAMASAKI, N. & KANG, D. 2004. Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA. *Mol Cell Biol,* 24**,** 9823-34.
225. KARATZAFERI, C., DE HAAN, A., FERGUSON, R. A., VAN MECHELEN, W. & SARGEANT, A. J. 2001. Phosphocreatine and ATP content in human single muscle fibres before and after maximum dynamic exercise. *Pflugers Arch,* 442**,** 467-74.
226. KARLSSON, H. K., NILSSON, P. A., NILSSON, J., CHIBALIN, A. V., ZIERATH, J. R. & BLOMSTRAND, E. 2004. Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. *Am J Physiol-Endoc M,* 287**,** E1-7.
227. KAUL, M. P. & HERRING, S. A. 1994. Superficial heat and cold: how to maximise the benefits. *Physician Sportsmed,* 22**,** 65-72.
228. KAUL, S. C., AIDA, S., YAGUCHI, T., KAUR, K. & WADHWA, R. 2005. Activation of wild type p53 function by its mortalin-binding, cytoplasmically localizing carboxyl terminus peptides. *J Biol Chem,* 280**,** 39373-9.
229. KAUPPINEN, K. 1989. Sauna, shower, and ice water immersion. Physiological responses to brief exposures to heat, cool, and cold. Part III. Body temperatures. *Arctic Med Res,* 48**,** 75-86.
230. KAUPPINEN, K., PAJARI-BACKAS, M., VOLIN, P. & VAKKURI, O. 1989. Some endocrine responses to sauna, shower and ice water immersion. *Arctic Med Res,* 48**,** 131-9.
231. KAUSHIK, V. K., YOUNG, M. E., DEAN, D. J., KUROWSKI, T. G., SAHA, A. K. & RUDERMAN, N. B. 2001. Regulation of fatty acid oxidation and glucose metabolism in rat soleus muscle: effects of AICAR. *Am J Physiol-Endoc M,* 281**,** E335-40.
232. KAYANI, A. C., CLOSE, G. L., JACKSON, M. J. & MCARDLE, A. 2008. Prolonged treadmill training increases HSP70 in skeletal muscle but does not affect age-related functional deficits. *Am J Physiol-Reg I,* 294**,** R568-76.
233. KELLMANN, M. 2010. Preventing overtraining in athletes in high-intensity sports and stress/recovery monitoring. *Scand J Med Sci Spor,* 20 Suppl 2**,** 95-102.
234. KHASSAF, M., CHILD, R. B., MCARDLE, A., BRODIE, D. A., ESANU, C. & JACKSON, M. J. 2001. Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. *J App Physiol,* 90**,** 1031-1035.
235. KIM, J. C., YI, H. K., HWANG, P. H., YOON, J. S., KIM, H. J., KAWANO, F., OHIRA, Y. & KIM, C. K. 2005. Effects of cold-water immersion on VEGF mRNA and protein expression in heart and skeletal muscles of rats. *Acta Physiol Scand,* 183**,** 389-97.

236. KING, M. & DUFFIELD, R. O. B. 2009. The effects of recovery interventions on consecutive days of intermittent sprint exercise. *J Strength Cond Res,* 23**,** 1795-1802.
237. KINUGASA, T. & KILDING, A. E. 2009. A comparison of post-match recovery strategies in youth soccer players. *J Strength Cond Res,* 23**,** 1402-1407.
238. KNAPIK, J. J., STAAB, J. S. & HARMAN, E. A. 1996. Validity of an anthropometric estimate of thigh muscle cross-sectional area. *Med Sci Sports Exerc,* 28**,** 1523-30.
239. KNIGHT, K. L., BRUCKER, J. B., STONEMAN, P. D. & RUBLEY, M. D. 2000. Muscle injury management with cryotherapy. *Athlet Ther Today,* 5**,** 26-30.
240. KNIGHT, K. L., RUBLEY, M. D., INGERSOLL, C. D. & BRUCKER, J. B. 2000. Pain perception is greater during ankle ice immersion than during ice pack application. *J Athl Training,* 35**,** S-45.
241. KOLETTIS, T. M. & KOLETTIS, M. T. 2003. Winter swimming: healthy or hazardous?. Evidence and hypotheses. *Med Hypotheses,* 61**,** 654-6.
242. KORYAK, Y. A. 2002. Surface action potential and contractile properties of the human triceps surae muscle: effect of "dry" water immersion. *Exp Physiol,* 87**,** 101-11.
243. KRAMER, H. F. & GOODYEAR, L. J. 2007. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. *J Appl Physiol,* 103**,** 388-95.
244. KROEMER, G., MARINO, G. & LEVINE, B. 2010. Autophagy and the integrated stress response. *Mol Cell,* 40**,** 280-93.
245. KUIPERS, H., VERSTAPPEN, F. T., KEIZER, H. A., GEURTEN, P. & VAN KRANENBURG, G. 1985. Variability of aerobic performance in the laboratory and its physiologic correlates. *Int J Sports Med,* 6**,** 197-201.
246. KULIGOWSKI, L. A., LEPHART, S. M., GIANNANTONIO, F. P. & BLANC, R. O. 1998. Effect of whirlpool therapy on the signs and symptoms of delayed-onset muscle soreness. *J Athl Training,* 33**,** 222-8.
247. KULISZ, A., CHEN, N., CHANDEL, N. S., SHAO, Z. & SCHUMACKER, P. T. 2002. Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. *Am J Physiol-Lung C,* 282**,** L1324-9.
248. KYRIAKIS, J. M. & AVRUCH, J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev,* 81**,** 807-69.
249. LACHANCE, P. A., NAKAT, Z. & JEONG, W. S. 2001. Antioxidants: an integrative approach. *Nutrition,* 17**,** 835-8.
250. LAIOS, E., REBEYKA, I. M. & PRODY, C. A. 1997. Characterization of cold-induced heat shock protein expression in neonatal rat cardiomyocytes. *Mol Cell Biochem,* 173**,** 153-9.
251. LANE, K. N. & WENGER, H. A. 2004. Effect of selected recovery conditions on performance of repeated bouts of intermittent cycling separated by 24 hours. *J Strength Cond Res,* 18**,** 855-60.
252. LARSEN, S., NIELSEN, J., HANSEN, C. N., NIELSEN, L. B., WIBRAND, F., STRIDE, N., SCHRODER, H. D., BOUSHEL, R., HELGE, J. W., DELA, F. & HEY-MOGENSEN, M. 2012. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol,* 590**,** 3349-60.
253. LASSITER, W. E. & GOTTACHALK, C. W. 1980. Volume and components of the body fluids. *In:* MOUNTCASTLE, V. B. (ed.) *Med Physiol.* 14th ed. St Louis (MO): CV Mosby.
254. LATCHMAN, D. S. 1993. Transcription factors: an overview. *Int J Exp Pathol,* 74**,** 417-22.
255. LATCHMAN, D. S. 1997. Transcription factors: an overview. *Int J Biochem Cell B,* 29**,** 1305-12.

256. LEAL JR, E. C., DE GODOI, V., MANCALOSSI, J. L., ROSSI, R. P., DE MARCHI, T., PARENTE, M., GROSSELLI, D., GENEROSI, R. A., BASSO, M., FRIGO, L., TOMAZONI, S. S., BJORDAL, J. M. & LOPES-MARTINS, R. A. B. 2011. Comparison between cold water immersion therapy (CWIT) and light emitting diode therapy (LEDT) in short-term skeletal muscle recovery after high - Intensity exercise in athletes-preliminary results. *Laser Med Sci,* 26**,** 493-501.

257. LEE, H. C., YIN, P. H., CHI, C. W. & WEI, Y. H. 2002. Increase in mitochondrial mass in human fibroblasts under oxidative stress and during replicative cell senescence. *J Biomed Sci,* 9**,** 517-26.

258. LEE, J. H., KANG, Y., KHARE, V., JIN, Z. Y., KANG, M. Y., YOON, Y., HYUN, J. W., CHUNG, M. H., CHO, S. I., JUN, J. Y., CHANG, I. Y. & YOU, H. J. 2010. The p53-inducible gene 3 (PIG3) contributes to early cellular response to DNA damage. *Oncogene,* 29**,** 1431-50.

259. LEE, W. J., KIM, M., PARK, H. S., KIM, H. S., JEON, M. J., OH, K. S., KOH, E. H., WON, J. C., KIM, M. S., OH, G. T., YOON, M., LEE, K. U. & PARK, J. Y. 2006. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. *Biochem Bioph Res Co,* 340**,** 291-5.

260. LEEDER, J., GISSANE, C., VAN SOMEREN, K., GREGSON, W. & HOWATSON, G. 2011. Cold water immersion and recovery from strenuous exercise: a meta-analysis. *Br J Sports Med,* 46**,** 233-240.

261. LEICK, L., WOJTASZEWSKI, J. F., JOHANSEN, S. T., KIILERICH, K., COMES, G., HELLSTEN, Y., HIDALGO, J. & PILEGAARD, H. 2008. PGC-1alpha is not mandatory for exercise- and training-induced adaptive gene responses in mouse skeletal muscle. *Am J Physiol-Endoc M,* 294**,** E463-74.

262. LEMOINE, C. M., GENGE, C. E. & MOYES, C. D. 2008. Role of the PGC-1 family in the metabolic adaptation of goldfish to diet and temperature. *J Exp Biol,* 211**,** 1448-55.

263. LEONE, T. C., LEHMAN, J. J., FINCK, B. N., SCHAEFFER, P. J., WENDE, A. R., BOUDINA, S., COURTOIS, M., WOZNIAK, D. F., SAMBANDAM, N., BERNAL-MIZRACHI, C., CHEN, Z., HOLLOSZY, J. O., MEDEIROS, D. M., SCHMIDT, R. E., SAFFITZ, J. E., ABEL, E. D., SEMENKOVICH, C. F. & KELLY, D. P. 2005. PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol,* 3**,** e101.

264. LEPPIK, J. A., AUGHEY, R. J., MEDVED, I., FAIRWEATHER, I., CAREY, M. F. & MCKENNA, M. J. 2004. Prolonged exercise to fatigue in humans impairs skeletal muscle Na+-K+-ATPase activity, sarcoplasmic reticulum Ca2+ release, and Ca2+ uptake. *J Appl Physiol,* 97**,** 1414-23.

265. LEVINE, A. J., HU, W. & FENG, Z. 2006. The P53 pathway: what questions remain to be explored? *Cell Death Differ,* 13**,** 1027-36.

266. LEVINE, R. L. 2002. Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radical Bio Med,* 32**,** 790-6.

267. LI, J., DONATH, S., LI, Y., QIN, D., PRABHAKAR, B. S. & LI, P. 2010. miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. *PLoS Genet,* 6**,** e1000795.

268. LI, L., PAN, R., LI, R., NIEMANN, B., AURICH, A. C., CHEN, Y. & ROHRBACH, S. 2011. Mitochondrial biogenesis and peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation by physical activity: intact adipocytokine signaling is required. *Diabetes,* 60**,** 157-67.

269. LI, S., REN, B., YANG, H., YANG, Y., JI, H. & NI, J. 2006. Effect of cold stress with different intensities on HSP70 expression in Wistar rat muscle, spleen and liver. *Chinese J App Enviro B,* 12**,** 235-238.

270. LI, Z., JIANG, Y., ULEVITCH, R. J. & HAN, J. 1996. The primary structure of p38 gamma: a new member of p38 group of MAP kinases. *Biochem Bioph Res Co,* 228**,** 334-40.
271. LIANG, H. & WARD, W. F. 2006. PGC-1alpha: a key regulator of energy metabolism. *Adv Physiol Educ,* 30**,** 145-51.
272. LICHTENBELT, W. V. M., KINGMA, B., VAN DER LANS, A. & SCHELLEN, L. 2014. Cold exposure – an approach to increasing energy expenditure in humans. *Trends Endocrin Met,* 25**,** 165-167.
273. LIN, J., HANDSCHIN, C. & SPIEGELMAN, B. M. 2005. Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab,* 1**,** 361-70.
274. LIN, J., WU, H., TARR, P. T., ZHANG, C. Y., WU, Z., BOSS, O., MICHAEL, L. F., PUIGSERVER, P., ISOTANI, E., OLSON, E. N., LOWELL, B. B., BASSEL-DUBY, R. & SPIEGELMAN, B. M. 2002. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature,* 418**,** 797-801.
275. LIN, J., WU, P. H., TARR, P. T., LINDENBERG, K. S., ST-PIERRE, J., ZHANG, C. Y., MOOTHA, V. K., JAGER, S., VIANNA, C. R., REZNICK, R. M., CUI, L., MANIERI, M., DONOVAN, M. X., WU, Z., COOPER, M. P., FAN, M. C., ROHAS, L. M., ZAVACKI, A. M., CINTI, S., SHULMAN, G. I., LOWELL, B. B., KRAINC, D. & SPIEGELMAN, B. M. 2004. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell,* 119**,** 121-35.
276. LINDQUIST, S. 1986. The heat-shock response. *Annu Rev Biochem,* 55**,** 1151-91.
277. LIRA, V. A., BROWN, D. L., LIRA, A. K., KAVAZIS, A. N., SOLTOW, Q. A., ZEANAH, E. H. & CRISWELL, D. S. 2010. Nitric oxide and AMPK cooperatively regulate PGC-1 in skeletal muscle cells. *J Physiol,* 588**,** 3551-66.
278. LITTLE, J. P., SAFDAR, A., BISHOP, D., TARNOPOLSKY, M. A. & GIBALA, M. J. 2011. An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1{alpha} and activates mitochondrial biogenesis in human skeletal muscle. *Am J Physiol-Reg I,* 300**,** R1303-10.
279. LITTLE, J. P., SAFDAR, A., CERMAK, N., TARNOPOLSKY, M. A. & GIBALA, M. J. 2010. Acute endurance exercise increases the nuclear abundance of PGC-1alpha in trained human skeletal muscle. *Am J Physiol-Reg I,* 298**,** R912-7.
280. LITTLE, J. P., SAFDAR, A., WILKIN, G. P., TARNOPOLSKY, M. A. & GIBALA, M. J. 2010. A practical model of low-volume high-intensity interval training induces mitochondrial biogenesis in human skeletal muscle: potential mechanisms. *J Physiol,* 588**,** 1011-22.
281. LIU, A. Y., BIAN, H., HUANG, L. E. & LEE, Y. K. 1994. Transient cold shock induces the heat shock response upon recovery at 37 degrees C in human cells. *J Biol Chem,* 269**,** 14768-75.
282. LIU, B., CHEN, Y. & ST CLAIR, D. K. 2008. ROS and p53: a versatile partnership. *Free Radical Bio Med,* 44**,** 1529-35.
283. LIU, Y., GAMPERT, L., NETHING, K. & STEINACKER, J. M. 2006. Response and function of skeletal muscle heat shock protein 70. *Front Biosci,* 11**,** 2802-27.
284. LIU, Y., LORMES, W., BAUR, C., OPITZ-GRESS, A., ALTENBURG, D., LEHMANN, M. & STEINACKER, J. M. 2000. Human skeletal muscle HSP70 response to physical training depends on exercise intensity. *Int J Sports Med,* 21**,** 351-5.
285. LIU, Y., MAYR, S., OPITZ-GRESS, A., ZELLER, C., LORMES, W., BAUR, S., LEHMANN, M. & STEINACKER, J. M. 1999. Human skeletal muscle HSP70 response to training in highly trained rowers. *J Appl Physiol,* 86**,** 101-4.
286. LJUBICIC, V., JOSEPH, A. M., SALEEM, A., UGUCCIONI, G., COLLU-MARCHESE, M., LAI, R. Y., NGUYEN, L. M. & HOOD, D. A. 2010. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. *Biochim Biophys Acta,* 1800**,** 223-34.

287. LONG, Y. C., WIDEGREN, U. & ZIERATH, J. R. 2004. Exercise-induced mitogen-activated protein kinase signalling in skeletal muscle. *Proc Nutr Soc,* 63**,** 227-32.
288. LOW, J. & REED, A. 1994. *Electrotherapy explained: principles and practices,* Oxford, Butterworth Heinemann.
289. LU, Z., DONO, K., GOTOH, K., SHIBATA, M., KOIKE, M., MARUBASHI, S., MIYAMOTO, A., TAKEDA, Y., NAGANO, H., UMESHITA, K., UCHIYAMA, Y. & MONDEN, M. 2005. Participation of autophagy in the degeneration process of rat hepatocytes after transplantation following prolonged cold preservation. *Arch Histol Cytol,* 68**,** 71-80.
290. LYNCH, S. A. & RENSTROM, P. A. F. H. 1999. Treatment of acute lateral ankle ligament rupture in the athlete - Conservative versus surgical treatment. *Sports Med,* 27**,** 61-71.
291. MA, Z., IZUMI, H., KANAI, M., KABUYAMA, Y., AHN, N. G. & FUKASAWA, K. 2006. Mortalin controls centrosome duplication via modulating centrosomal localization of p53. *Oncogene,* 25**,** 5377-90.
292. MAC AULEY, D. C. 2001. Ice therapy: how good is the evidence? *Int J Sports Med,* 22**,** 379-84.
293. MACINTYRE, D. L., REID, W. D. & MCKENZIE, D. C. 1995. Delayed muscle soreness. The inflammatory response to muscle injury and its clinical implications. *Sports Med,* 20**,** 24-40.
294. MADAN, E., GOGNA, R., BHATT, M., PATI, U., KUPPUSAMY, P. & MAHDI, A. A. 2011. Regulation of glucose metabolism by p53: emerging new roles for the tumor suppressor. *Oncotarget,* 2**,** 948-57.
295. MANIURA-WEBER, K., GOFFART, S., GARSTKA, H. L., MONTOYA, J. & WIESNER, R. J. 2004. Transient overexpression of mitochondrial transcription factor A (TFAM) is sufficient to stimulate mitochondrial DNA transcription, but not sufficient to increase mtDNA copy number in cultured cells. *Nucleic Acids Res,* 32**,** 6015-27.
296. MARTINEAU, L. C. & GARDINER, P. F. 2001. Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. *J Appl Physiol,* 91**,** 693-702.
297. MARZETTI, E., GROBAN, L., WOHLGEMUTH, S. E., LEES, H. A., LIN, M., JOBE, H., GIOVANNINI, S., LEEUWENBURGH, C. & CARTER, C. S. 2008. Effects of short-term GH supplementation and treadmill exercise training on physical performance and skeletal muscle apoptosis in old rats. *Am J Physiol-Reg I,* 294**,** R558-67.
298. MATHAI, A. S., BONEN, A., BENTON, C. R., ROBINSON, D. L. & GRAHAM, T. E. 2008. Rapid exercise-induced changes in PGC-1alpha mRNA and protein in human skeletal muscle. *J Appl Physiol,* 105**,** 1098-105.
299. MATHEU, A., MARAVER, A., KLATT, P., FLORES, I., GARCIA-CAO, I., BORRAS, C., FLORES, J. M., VINA, J., BLASCO, M. A. & SERRANO, M. 2007. Delayed ageing through damage protection by the Arf/p53 pathway. *Nature,* 448**,** 375-9.
300. MATIJASEVIC, Z., SNYDER, J. E. & LUDLUM, D. B. 1998. Hypothermia causes a reversible, p53-mediated cell cycle arrest in cultured fibroblasts. *Oncol Res,* 10**,** 605-10.
301. MATOBA, S., KANG, J. G., PATINO, W. D., WRAGG, A., BOEHM, M., GAVRILOVA, O., HURLEY, P. J., BUNZ, F. & HWANG, P. M. 2006. p53 regulates mitochondrial respiration. *Science,* 312**,** 1650-3.
302. MAWHINNEY, C., JONES, H., JOO, C. H., LOW, D. A., GREEN, D. J. & GREGSON, W. 2013. Influence of cold-water immersion on limb and cutaneous blood flow after exercise. *Med Sci Sports Exerc,* 45**,** 2277-85.
303. MCARDLE, A., DILLMANN, W. H., MESTRIL, R., FAULKNER, J. A. & JACKSON, M. J. 2004. Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction. *FASEB J,* 18**,** 355-7.

304. MCARDLE, A., PATTWELL, D., VASILAKI, A., GRIFFITHS, R. D. & JACKSON, M. J. 2001. Contractile activity-induced oxidative stress: cellular origin and adaptive responses. *Am J Physiol-Cell Ph,* 280**,** C621-7.
305. MCBRIDE, J. M., KRAEMER, W. J., TRIPLETT-MCBRIDE, T. & SEBASTIANELLI, W. 1998. Effect of resistance exercise on free radical production. *Med Sci Sports Exerc,* 30**,** 67-72.
306. MCCLELLAND, G. B., CRAIG, P. M., DHEKNEY, K. & DIPARDO, S. 2006. Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (Danio rerio). *J Physiol,* 577**,** 739-51.
307. MCCLUNG, M. & COLLINS, D. 2007. "Because i know it will!": Placebo effects of an ergogenic aid on athletic performance. *J Sport Ex Psy,* 29**,** 382-394.
308. MCCONELL, G. K., LEE-YOUNG, R. S., CHEN, Z. P., STEPTO, N. K., HUYNH, N. N., STEPHENS, T. J., CANNY, B. J. & KEMP, B. E. 2005. Short-term exercise training in humans reduces AMPK signalling during prolonged exercise independent of muscle glycogen. *J Physiol,* 568**,** 665-76.
309. MCDOWELL, J. H., MCFARLAND, E. G. & NALLI, B. J. 1994. Use of cryotherapy for orthopaedic patients. *Orthop Nurs,* 13**,** 21-30.
310. MCGEE, S. L., VAN DENDEREN, B. J., HOWLETT, K. F., MOLLICA, J., SCHERTZER, J. D., KEMP, B. E. & HARGREAVES, M. 2008. AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. *Diabetes,* 57**,** 860-7.
311. MCKENZIE, D. C. 1999. Markers of excessive exercise. *Can J Appl Physiol,* 24**,** 66-73.
312. MCKINSEY, T. A., ZHANG, C. L., LU, J. & OLSON, E. N. 2000. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature,* 408**,** 106-11.
313. MCLEAN, D. A. 1989. The use of cold and superficial heat in the treatment of soft tissue injuries. *Br J Sports Med,* 23**,** 53-4.
314. MCMASTER, W. C. 1977. A literary review on ice therapy in injuries. *Am J Sports Med,* 5**,** 124-6.
315. MEEUSEN, R. & LIEVENS, P. 1986. The use of cryotherapy in sports injuries. *Sports Med,* 3**,** 398-414.
316. MEYER, T., GABRIEL, H. H., RATZ, M., MULLER, H. J. & KINDERMANN, W. 2001. Anaerobic exercise induces moderate acute phase response. *Med Sci Sports Exerc,* 33**,** 549-55.
317. MICHEL, J. B., ORDWAY, G. A., RICHARDSON, J. A. & WILLIAMS, R. S. 1994. Biphasic induction of immediate early gene expression accompanies activity-dependent angiogenesis and myofiber remodeling of rabbit skeletal muscle. *J Clin Invest,* 94**,** 277-85.
318. MIDGLEY, A. W. & MC NAUGHTON, L. R. 2006. Time at or near VO2max during continuous and intermittent running. A review with special reference to considerations for the optimisation of training protocols to elicit the longest time at or near VO2max. *J Sport Med Phys Fit,* 46**,** 1-14.
319. MILNE, K. J. & NOBLE, E. G. 2002. Exercise-induced elevation of HSP70 is intensity dependent. *J Appl Physiol,* 93**,** 561-8.
320. MIRANDA, S., FONCEA, R., GUERRERO, J. & LEIGHTON, F. 1999. Oxidative stress and upregulation of mitochondrial biogenesis genes in mitochondrial DNA-depleted HeLa cells. *Biochem Bioph Res Co,* 258**,** 44-9.
321. MITCHELL, C. R., HARRIS, M. B., CORDARO, A. R. & STARNES, J. W. 2002. Effect of body temperature during exercise on skeletal muscle cytochrome c oxidase content. *J Appl Physiol,* 93**,** 526-30.

322. MIURA, S., KAWANAKA, K., KAI, Y., TAMURA, M., GOTO, M., SHIUCHI, T., MINOKOSHI, Y. & EZAKI, O. 2007. An increase in murine skeletal muscle peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) mRNA in response to exercise is mediated by beta-adrenergic receptor activation. *Endocrinology,* 148**,** 3441-8.
323. MIYASHITA, T. & REED, J. C. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell,* 80**,** 293-9.
324. MIZUNOYA, W., IWAMOTO, Y., SATO, Y., TATSUMI, R. & IKEUCHI, Y. 2014. Cold exposure increases slow-type myosin heavy chain 1 (MyHC1) composition of soleus muscle in rats. *Anim Sci J,* 85**,** 293-304.
325. MONTGOMERY, P. G., PYNE, D. B., COX, A. J., HOPKINS, W. G., MINAHAN, C. L. & HUNT, P. H. 2008. Muscle damage, inflammation, and recovery interventions during a 3-day basketball tournament. *Eur J Sport Sci,* 8**,** 241-250.
326. MONTGOMERY, P. G., PYNE, D. B., HOPKINS, W. G., DORMAN, J. C., COOK, K. & MINAHAN, C. L. 2008. The effect of recovery strategies on physical performance and cumulative fatigue in competitive basketball. *J Sport Sci,* 26**,** 1135-45.
327. MORTON, J. P., CROFT, L., BARTLETT, J. D., MACLAREN, D. P., REILLY, T., EVANS, L., MCARDLE, A. & DRUST, B. 2009. Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulate oxidative enzyme activity in human skeletal muscle. *J Appl Physiol,* 106**,** 1513-21.
328. MORTON, J. P., HOLLOWAY, K., WOODS, P., CABLE, N. T., BURNISTON, J., EVANS, L., KAYANI, A. C. & MCARDLE, A. 2009. Exercise training-induced gender-specific heat shock protein adaptations in human skeletal muscle. *Muscle Nerve,* 39**,** 230-3.
329. MORTON, J. P., KAYANI, A. C., MCARDLE, A. & DRUST, B. 2009. The exercise-induced stress response of skeletal muscle, with specific emphasis on humans. *Sports Med,* 39**,** 643-62.
330. MORTON, J. P., MACLAREN, D. P., CABLE, N. T., CAMPBELL, I. T., EVANS, L., BONGERS, T., GRIFFITHS, R. D., KAYANI, A. C., MCARDLE, A. & DRUST, B. 2007. Elevated core and muscle temperature to levels comparable to exercise do not increase heat shock protein content of skeletal muscle of physically active men. *Acta Physiol,* 190**,** 319-27.
331. MORTON, J. P., MACLAREN, D. P., CABLE, N. T., CAMPBELL, I. T., EVANS, L., KAYANI, A. C., MCARDLE, A. & DRUST, B. 2008. Trained men display increased basal heat shock protein content of skeletal muscle. *Med Sci Sports Exerc,* 40**,** 1255-62.
332. MORTON, J. P., MACLAREN, D. P. M., CABLE, N. T., BONGERS, T., GRIFFITHS, R. D., CAMPBELL, I. T., EVANS, L., KAYANI, A., MCARDLE, A. & DRUST, B. 2006. Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. *J App Physiol,* 101**,** 176-182.
333. MURAKAMI, T., SHIMOMURA, Y., YOSHIMURA, A., SOKABE, M. & FUJITSUKA, N. 1998. Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. *Biochim Biophys Acta,* 1381**,** 113-22.
334. MURPHY, R. M. & LAMB, G. D. 2013. Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. *J Physiol,* 591**,** 5823-31.
335. MYRER, J. W., MEASOM, G. & FELLINGHAM, G. W. 1998. Temperature changes in the human leg during and after two methods of cryotherapy. *J Athl Training,* 33**,** 25-9.

336. MYRER, W. J., MYRER, K. A., MEASOM, G. J., FELLINGHAM, G. W. & EVERS, S. L. 2001. Muscle Temperature Is Affected by Overlying Adipose When Cryotherapy Is Administered. *J Athl Training,* 36**,** 32-36.
337. NADER, G. A. & ESSER, K. A. 2001. Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol,* 90**,** 1936-42.
338. NAKAMURA, K., TAKAHASHI, H., SHIMAI, S. & TANAKA, M. 1996. Effects of immersion in tepid bath water on recovery from fatigue after submaximal exercise in man. *Ergonomics,* 39**,** 257-66.
339. NAKANO, M., HAMADA, T., HAYASHI, T., YONEMITSU, S., MIYAMOTO, L., TOYODA, T., TANAKA, S., MASUZAKI, H., EBIHARA, K., OGAWA, Y., HOSODA, K., INOUE, G., YOSHIMASA, Y., OTAKA, A., FUSHIKI, T. & NAKAO, K. 2006. alpha2 isoform-specific activation of 5'adenosine monophosphate-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle. *Metabolis,* 55**,** 300-8.
340. NEUTELINGS, T., LAMBERT, C. A., NUSGENS, B. V. & COLIGE, A. C. 2013. Effects of mild cold shock (25 degrees C) followed by warming up at 37 degrees C on the cellular stress response. *PloS one,* 8**,** e69687.
341. NEWELL, J., HIGGINS, D., MADDEN, N., CRUICKSHANK, J., EINBECK, J., MCMILLAN, K. & MCDONALD, R. 2007. Software for calculating blood lactate endurance markers. *J Sport Sci,* 25**,** 1403-9.
342. NEWHAM, D. J., MILLS, K. R., QUIGLEY, B. M. & EDWARDS, R. H. 1983. Pain and fatigue after concentric and eccentric muscle contractions. *Clin Sci,* 64**,** 55-62.
343. NICHOLLS, C. D., SHIELDS, M. A., LEE, P. W., ROBBINS, S. M. & BEATTIE, T. L. 2004. UV-dependent alternative splicing uncouples p53 activity and PIG3 gene function through rapid proteolytic degradation. *J Biol Chem,* 279**,** 24171-8.
344. NIELSEN, J. N., MUSTARD, K. J., GRAHAM, D. A., YU, H., MACDONALD, C. S., PILEGAARD, H., GOODYEAR, L. J., HARDIE, D. G., RICHTER, E. A. & WOJTASZEWSKI, J. F. 2003. 5'-AMP-activated protein kinase activity and subunit expression in exercise-trained human skeletal muscle. *J Appl Physiol,* 94**,** 631-41.
345. NISHINA, H., WADA, T. & KATADA, T. 2004. Physiological roles of SAPK/JNK signaling pathway. *J Biochem,* 136**,** 123-6.
346. NISHIYAMA, H., ITOH, K., KANEKO, Y., KISHISHITA, M., YOSHIDA, O. & FUJITA, J. 1997. A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. *J Cell Biol,* 137**,** 899-908.
347. NISOLI, E. & CARRUBA, M. O. 2006. Nitric oxide and mitochondrial biogenesis. *J Cell Sci,* 119**,** 2855-62.
348. NISOLI, E., CLEMENTI, E., PAOLUCCI, C., COZZI, V., TONELLO, C., SCIORATI, C., BRACALE, R., VALERIO, A., FRANCOLINI, M., MONCADA, S. & CARRUBA, M. O. 2003. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science,* 299**,** 896-9.
349. NORRBOM, J., SUNDBERG, C. J., AMELN, H., KRAUS, W. E., JANSSON, E. & GUSTAFSSON, T. 2004. PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol,* 96**,** 189-94.
350. NOSAKA, K. & CLARKSON, P. M. 1996. Variability in serum creatine kinase response after eccentric exercise of the elbow flexors. *Int J Sports Med,* 17**,** 120-7.
351. O'BRIEN, K. M. 2011. Mitochondrial biogenesis in cold-bodied fishes. *J Exp Biol,* 214**,** 275-285.
352. O'CONNOR, P. J. & COOK, D. B. 1999. Exercise and pain: the neurobiology, measurement, and laboratory study of pain in relation to exercise in humans. *Exerc Sport Sci Rev,* 27**,** 119-66.

353. OHNISHI, K. & OHNISHI, T. 2001. Heat-induced p53-dependent signal transduction and its role in hyperthermic cancer therapy. *Int J Hyperthermia,* 17**,** 415-27.
354. OHNISHI, T., WANG, X., OHNISHI, K. & TAKAHASHI, A. 1998. p53-dependent induction of WAF1 by cold shock in human glioblastoma cells. *Oncogene,* 16**,** 1507-11.
355. OHSAKA, Y., OHGIYA, S., HOSHINO, T. & ISHIZAKI, K. 2002. Phosphorylation of c-Jun N-terminal kinase in human hepatoblastoma cells is transiently increased by cold exposure and further enhanced by subsequent warm incubation of the cells. *Cell Physiol Biochem,* 12**,** 111-8.
356. OLESEN, J., KIILERICH, K. & PILEGAARD, H. 2010. PGC-1alpha-mediated adaptations in skeletal muscle. *Pflugers Arch,* 460**,** 153-62.
357. OLIVEIRA, R. L., UENO, M., DE SOUZA, C. T., PEREIRA-DA-SILVA, M., GASPARETTI, A. L., BEZZERA, R. M., ALBERICI, L. C., VERCESI, A. E., SAAD, M. J. & VELLOSO, L. A. 2004. Cold-induced PGC-1alpha expression modulates muscle glucose uptake through an insulin receptor/Akt-independent, AMPK-dependent pathway. *Am J Physiol-Endoc M,* 287**,** E686-95.
358. OLSON, B. L., HOCK, M. B., EKHOLM-REED, S., WOHLSCHLEGEL, J. A., DEV, K. K., KRALLI, A. & REED, S. I. 2008. SCFCdc4 acts antagonistically to the PGC-1alpha transcriptional coactivator by targeting it for ubiquitin-mediated proteolysis. *Genes Dev,* 22**,** 252-64.
359. ORCZEWSKA, J. I., HARTLEBEN, G. & O'BRIEN, K. M. 2010. The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *Am J Physiol-Reg I,* 299**,** R352-64.
360. OTTE, J. W., MERRICK, M. A., INGERSOLL, C. D. & CORDOVA, M. L. 2002. Subcutaneous adipose tissue thickness alters cooling time during cryotherapy. *Arch Phys Med Rehabil,* 83**,** 1501-5.
361. OTTERBEIN, L. E. & CHOI, A. M. 2000. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol-Lung C,* 279**,** L1029-37.
362. PADDON-JONES, D. J. & QUIGLEY, B. M. 1997. Effect of cryotherapy on muscle soreness and strength following eccentric exercise. *Int J Sports Med,* 18**,** 588-93.
363. PANI, G. & GALEOTTI, T. 2011. Role of MnSOD and p66shc in mitochondrial response to p53. *Antioxid Redox Signal,* 15**,** 1715-27.
364. PARK, J. Y., WANG, P. Y., MATSUMOTO, T., SUNG, H. J., MA, W., CHOI, J. W., ANDERSON, S. A., LEARY, S. C., BALABAN, R. S., KANG, J. G. & HWANG, P. M. 2009. p53 improves aerobic exercise capacity and augments skeletal muscle mitochondrial DNA content. *Circ Res,* 105**,** 705-12, 11 p following 712.
365. PARK, K. S., CHOI, J. K. & PARK, Y. S. 1999. Cardiovascular regulation during water immersion. *Appl Human Sci,* 18**,** 233-41.
366. PARK, S., KIM, D., DAN, H. C., CHEN, H., TESTA, J. R. & CHENG, J. Q. 2012. Identification of Akt interaction protein PHF20/TZP that transcriptionally regulates p53. *J Biol Chem,* 287**,** 11151-63.
367. PAROUTY, J., AL HADDAD, H., QUOD, M., LEPRETRE, P. M., AHMAIDI, S. & BUCHHEIT, M. 2010. Effect of cold water immersion on 100-m sprint performance in well-trained swimmers. *Eur J Appl Physiol,* 109**,** 483-90.
368. PARSELL, D. A. & LINDQUIST, S. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu Rev Genet,* 27**,** 437-96.
369. PEAKE, J., PEIFFER, J. J., ABBISS, C. R., NOSAKA, K., OKUTSU, M., LAURSEN, P. B. & SUZUKI, K. 2008. Body temperature and its effect on leukocyte mobilization,

cytokines and markers of neutrophil activation during and after exercise. *Eur J Appl Physiol,* 102**,** 391-401.

370. PEDERSEN, B. K. 2009. Edward F. Adolph distinguished lecture: muscle as an endocrine organ: IL-6 and other myokines. *J Appl Physiol,* 107**,** 1006-14.
371. PEDERSEN, B. K. & TOFT, A. D. 2000. Effects of exercise on lymphocytes and cytokines. *Br J Sports Med,* 34**,** 246-51.
372. PEIFFER, J. J., ABBISS, C. R., NOSAKA, K., PEAKE, J. M. & LAURSEN, P. B. 2009. Effect of cold water immersion after exercise in the heat on muscle function, body temperatures, and vessel diameter. *J Sci Med Sport,* 12**,** 91-6.
373. PEIFFER, J. J., ABBISS, C. R., WATSON, G., NOSAKA, K. & LAURSEN, P. B. 2009. Effect of cold-water immersion duration on body temperature and muscle function. *J Sport Sci,* 27**,** 987-993.
374. PEIFFER, J. J., ABBISS, C. R., WATSON, G., NOSAKA, K. & LAURSEN, P. B. 2010. Effect of a 5-min cold-water immersion recovery on exercise performance in the heat. *Br J Sports Med,* 44**,** 461-5.
375. PEIFFER, J. J., ABBISS, C. R., WATSON, G., NOSAKA, K. & LAURSEN, P. B. 2010. Effect of cold water immersion on repeated 1-km cycling performance in the heat. *J Sci Med Sport,* 13**,** 112-116.
376. PERDIGUERO, E. & MUÑOZ-CÁNOVES, P. 2008. Transcriptional regulation by the p38 MAPK signaling pathway in mammalian cells. *Topics in Current Genetics.*
377. PERRY, C. G., LALLY, J., HOLLOWAY, G. P., HEIGENHAUSER, G. J., BONEN, A. & SPRIET, L. L. 2010. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol,* 588**,** 4795-810.
378. PESTA, D. & GNAIGER, E. 2012. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *In:* PALMEIRA, C. M. & MORENO, A. J. (eds.) *Mitochondrial Bioenergetics: Methods and Protocols.* Springer Science+Business Media.
379. PETERSEN, A. C., MCKENNA, M. J., MEDVED, I., MURPHY, K. T., BROWN, M. J., DELLA GATTA, P. & CAMERON-SMITH, D. 2012. Infusion with the antioxidant N-acetylcysteine attenuates early adaptive responses to exercise in human skeletal muscle. *Acta Physiol,* 204**,** 382-92.
380. PETIBOIS, C., CAZORLA, G., POORTMANS, J. R. & DELERIS, G. 2002. Biochemical aspects of overtraining in endurance sports: a review. *Sports Med,* 32**,** 867-78.
381. PHILP, A., CHEN, A., LAN, D., MEYER, G. A., MURPHY, A. N., KNAPP, A. E., OLFERT, I. M., MCCURDY, C. E., MARCOTTE, G. R., HOGAN, M. C., BAAR, K. & SCHENK, S. 2011. Sirtuin 1 (SIRT1) deacetylase activity is not required for mitochondrial biogenesis or peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation following endurance exercise. *J Biol Chem,* 286**,** 30561-70.
382. PILEGAARD, H., ORDWAY, G. A., SALTIN, B. & NEUFER, P. D. 2000. Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol-Endoc M,* 279**,** E806-14.
383. PILEGAARD, H., OSADA, T., ANDERSEN, L. T., HELGE, J. W., SALTIN, B. & NEUFER, P. D. 2005. Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolis,* 54**,** 1048-55.
384. PILEGAARD, H., SALTIN, B. & NEUFER, P. D. 2003. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol,* 546**,** 851-8.
385. PLESOFSKY, N. & BRAMBL, R. 1999. Glucose metabolism in Neurospora is altered by heat shock and by disruption of HSP30. *Biochim Biophys Acta,* 1449**,** 73-82.

386. POGOZELSKI, A. R., GENG, T., LI, P., YIN, X., LIRA, V. A., ZHANG, M., CHI, J. T. & YAN, Z. 2009. p38gamma mitogen-activated protein kinase is a key regulator in skeletal muscle metabolic adaptation in mice. *PloS one,* 4**,** e7934.
387. POLLO, A., CARLINO, E. & BENEDETTI, F. 2008. The top-down influence of ergogenic placebos on muscle work and fatigue. *Eur J Neurosc,* 28**,** 379-388.
388. POURNOT, H., BIEUZEN, F., DUFFIELD, R., LEPRETRE, P. M., COZZOLINO, C. & HAUSSWIRTH, C. 2011. Short term effects of various water immersions on recovery from exhaustive intermittent exercise. *Eur J Appl Physiol,* 111**,** 1287-95.
389. POURNOT, H., BIEUZEN, F., LOUIS, J., MOUNIER, R., FILLARD, J. R., BARBICHE, E. & HAUSSWIRTH, C. 2011. Time-course of changes in inflammatory response after whole-body cryotherapy multi exposures following severe exercise. *PloS one,* 6**,** e22748.
390. POWERS, S. K. & JACKSON, M. J. 2008. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev,* 88**,** 1243-76.
391. POWERS, S. K., TALBERT, E. E. & ADHIHETTY, P. J. 2011. Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. *J Physiol,* 589**,** 2129-38.
392. POYHONEN, T. & AVELA, J. 2002. Effect of head-out water immersion on neuromuscular function of the plantarflexor muscles. *Aviat Space Envir Md,* 73**,** 1215-8.
393. PRIOR, R. L. & CAO, G. 1999. In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Bio Med,* 27**,** 1173-81.
394. PUIGSERVER, P., RHEE, J., LIN, J., WU, Z., YOON, J. C., ZHANG, C. Y., KRAUSS, S., MOOTHA, V. K., LOWELL, B. B. & SPIEGELMAN, B. M. 2001. Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. *Mol Cell,* 8**,** 971-82.
395. PUIGSERVER, P. & SPIEGELMAN, B. M. 2003. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev,* 24**,** 78-90.
396. PUIGSERVER, P., WU, Z., PARK, C. W., GRAVES, R., WRIGHT, M. & SPIEGELMAN, B. M. 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell,* 92**,** 829-39.
397. RADAK, Z., KANEKO, T., TAHARA, S., NAKAMOTO, H., OHNO, H., SASVARI, M., NYAKAS, C. & GOTO, S. 1999. The effect of exercise training on oxidative damage of lipids, proteins, and DNA in rat skeletal muscle: evidence for beneficial outcomes. *Free Radical Bio Med,* 27**,** 69-74.
398. RAINGEAUD, J., GUPTA, S., ROGERS, J. S., DICKENS, M., HAN, J., ULEVITCH, R. J. & DAVIS, R. J. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem,* 270**,** 7420-6.
399. RAKOBOWCHUK, M., TANGUAY, S., BURGOMASTER, K. A., HOWARTH, K. R., GIBALA, M. J. & MACDONALD, M. J. 2008. Sprint interval and traditional endurance training induce similar improvements in peripheral arterial stiffness and flow-mediated dilation in healthy humans. *Am J Physiol-Reg I,* 295**,** R236-42.
400. RAMIRES, M. L. V., NIETO DE CASTRO, C. A., NAGASAKA, Y., NAGASHIMA, A., ASSAEL, M. J. & WAKEHAM, W. A. 1994. Standard reference data for the thermal conductivity of water. *American Institute of Physics and American Chemical Society.***,** 1377-1381.
401. RAUEN, U., PETRAT, F., LI, T. & DE GROOT, H. 2000. Hypothermia injury/cold-induced apoptosis--evidence of an increase in chelatable iron causing oxidative injury in spite of low O2-/H2O2 formation. *FASEB J,* 14**,** 1953-64.

402. RAUEN, U., POLZAR, B., STEPHAN, H., MANNHERZ, H. G. & DE GROOT, H. 1999. Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *FASEB J,* 13**,** 155-68.
403. REID, M. B. 2001. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. *J Appl Physiol,* 90**,** 724-31.
404. REID, M. B. 2001. Nitric oxide, reactive oxygen species, and skeletal muscle contraction. *Med Sci Sports Exerc,* 33**,** 371-6.
405. REILLY, T. & EKBLOM, B. 2005. The use of recovery methods post-exercise. *J Sport Sci,* 23**,** 619-27.
406. RENKE, J., POPADIUK, S., KORZON, M., BUGAJCZYK, B. & WOZNIAK, M. 2000. Protein carbonyl groups' content as a useful clinical marker of antioxidant barrier impairment in plasma of children with juvenile chronic arthritis. *Free Radical Bio Med,* 29**,** 101-4.
407. RHIND, S. G., CASTELLANI, J. W., BRENNER, I. K., SHEPHARD, R. J., ZAMECNIK, J., MONTAIN, S. J., YOUNG, A. J. & SHEK, P. N. 2001. Intracellular monocyte and serum cytokine expression is modulated by exhausting exercise and cold exposure. *Am J Physiol-Reg I,* 281**,** R66-75.
408. RISTOW, M. & ZARSE, K. 2010. How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). *Exp Gerontol,* 45**,** 410-8.
409. RISTOW, M., ZARSE, K., OBERBACH, A., KLOTING, N., BIRRINGER, M., KIEHNTOPF, M., STUMVOLL, M., KAHN, C. R. & BLUHER, M. 2009. Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci USA,* 106**,** 8665-70.
410. RIVENBURGH, D. W. 1992. Physical modalities in the treatment of tendon injuries. *Clin Sports Med,* 11**,** 645-59.
411. ROOBOL, A., CARDEN, M. J., NEWSAM, R. J. & SMALES, C. M. 2009. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. *FEBS J,* 276**,** 286-302.
412. ROSS, A. & LEVERITT, M. 2001. Long-term metabolic and skeletal muscle adaptations to short-sprint training: implications for sprint training and tapering. *Sports Med,* 31**,** 1063-82.
413. ROSSITER, H. B., KOWALCHUK, J. M. & WHIPP, B. J. 2006. A test to establish maximum O2 uptake despite no plateau in the O2 uptake response to ramp incremental exercise. *J Appl Physiol,* 100**,** 764-70.
414. ROWSELL, G. J., COUTTS, A. J., REABURN, P. & HILL-HAAS, S. 2009. Effects of cold-water immersion on physical performance between successive matches in high-performance junior male soccer players. *J Sport Sci,* 27**,** 565-573.
415. ROWSELL, G. J., COUTTS, A. J., REABURN, P. & HILL-HAAS, S. 2011. Effect of post-match cold-water immersion on subsequent match running performance in junior soccer players during tournament play. *J Sport Sci,* 29**,** 1-6.
416. RUSSELL, A. P., FEILCHENFELDT, J., SCHREIBER, S., PRAZ, M., CRETTENAND, A., GOBELET, C., MEIER, C. A., BELL, D. R., KRALLI, A., GIACOBINO, J. P. & DERIAZ, O. 2003. Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. *Diabetes,* 52**,** 2874-81.
417. RUSSELL, A. P., HESSELINK, M. K., LO, S. K. & SCHRAUWEN, P. 2005. Regulation of metabolic transcriptional co-activators and transcription factors with acute exercise. *FASEB J,* 19**,** 986-8.
418. RUTKOVE, S. B. 2001. Effects of temperature on neuromuscular electrophysiology. *Muscle Nerve,* 24**,** 867-82.

419. RYAN, M. T. & HOOGENRAAD, N. J. 2007. Mitochondrial-nuclear communications. *Annu Rev Biochem,* 76**,** 701-22.
420. SABLINA, A. A., BUDANOV, A. V., ILYINSKAYA, G. V., AGAPOVA, L. S., KRAVCHENKO, J. E. & CHUMAKOV, P. M. 2005. The antioxidant function of the p53 tumor suppressor. *Nat Med,* 11**,** 1306-13.
421. SAHLIN, K., SHABALINA, I. G., MATTSSON, C. M., BAKKMAN, L., FERNSTROM, M., ROZHDESTVENSKAYA, Z., ENQVIST, J. K., NEDERGAARD, J., EKBLOM, B. & TONKONOGI, M. 2010. Ultraendurance exercise increases the production of reactive oxygen species in isolated mitochondria from human skeletal muscle. *J Appl Physiol,* 108**,** 780-7.
422. SALEEM, A., ADHIHETTY, P. J. & HOOD, D. A. 2009. Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. *Physiol Genomics,* 37**,** 58-66.
423. SALEEM, A., CARTER, H. N. & HOOD, D. A. 2014. p53 is necessary for the adaptive changes in cellular milieu subsequent to an acute bout of endurance exercise. *Am J Physiol-Cell Ph,* 306**,** C241-9.
424. SALEEM, A., CARTER, H. N., IQBAL, S. & HOOD, D. A. 2011. Role of p53 within the regulatory network controlling muscle mitochondrial biogenesis. *Exerc Sport Sci Rev,* 39**,** 199-205.
425. SALEEM, A. & HOOD, D. A. 2013. Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle. *J Physiol,* 591**,** 3625-36.
426. SAMALI, A. & ORRENIUS, S. 1998. Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress Chaperon,* 3**,** 228-36.
427. SANDERS, J. 1996. *Effect of contrast-temperature immersion on recovery from short-duration intense exercise.* Unpublished Thesis, University of Canberra.
428. SANTOS, W. O. C., BRITO, C. J., PINHO JR, E. A., VALIDO, C. N., MENDES, E. L., NUNES, M. A. P. & FRANCHINI, E. 2012. Cryotherapy post-training reduces muscle damage markers in jiu-jitsu fighters. *J Hum Sport Exer,* 7**,** 629-638.
429. SARGEANT, A. J. 1987. Effect of muscle temperature on leg extension force and short-term power output in humans. *Eur J Appl Physiol O,* 56**,** 693-8.
430. SCALZO, R. L., PELTONEN, G. L., BINNS, S. E., SHANKARAN, M., GIORDANO, G. R., HARTLEY, D. A., KLOCHAK, A. L., LONAC, M. C., PARIS, H. L., SZALLAR, S. E., WOOD, L. M., PEELOR, F. F., 3RD, HOLMES, W. E., HELLERSTEIN, M. K., BELL, C., HAMILTON, K. L. & MILLER, B. F. 2014. Greater muscle protein synthesis and mitochondrial biogenesis in males compared with females during sprint interval training. *FASEB J,* 28**,** 2705-14.
431. SCARPULLA, R. C. 1997. Nuclear control of respiratory chain expression in mammalian cells. *J Bioenerg Biomembr,* 29**,** 109-19.
432. SCARPULLA, R. C. 2008. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev,* 88**,** 611-38.
433. SCARPULLA, R. C., VEGA, R. B. & KELLY, D. P. 2012. Transcriptional integration of mitochondrial biogenesis. *Trends Endocrinol Metab,* 23**,** 459-66.
434. SCHEELE, C., NIELSEN, S. & PEDERSEN, B. K. 2009. ROS and myokines promote muscle adaptation to exercise. *Trends Endocrinol Metab,* 20**,** 95-9.
435. SCHNIEPP, J., CAMPBELL, T. S., POWELL, K. L. & PINCIVERO, D. M. 2002. The effects of cold-water immersion on power output and heart rate in elite cyclists. *J Strength Cond Res,* 16**,** 561-6.
436. SEEBACHER, F. & GLANVILLE, E. J. 2010. Low levels of physical activity increase metabolic responsiveness to cold in a rat (Rattus fuscipes). *PloS one,* 5**,** e13022.
437. SELLWOOD, K. L., BRUKNER, P., WILLIAMS, D., NICOL, A. & HINMAN, R. 2007. Ice-water immersion and delayed-onset muscle soreness: a randomised controlled trial. *Br J Sports Med,* 41**,** 392-7.

438. SELMAN, C., GRUNE, T., STOLZING, A., JAKSTADT, M., MCLAREN, J. S. & SPEAKMAN, J. R. 2002. The consequences of acute cold exposure on protein oxidation and proteasome activity in short-tailed field voles, microtus agrestis. *Free Radical Bio Med,* 33**,** 259-65.
439. SEN, C. K. 2001. Antioxidant and redox regulation of cellular signaling: introduction. *Med Sci Sports Exerc,* 33**,** 368-70.
440. SENDOWSKI, I., SAVOUREY, G., LAUNAY, J. C., BESNARD, Y., COTTET-EMARD, J. M., PEQUIGNOT, J. M. & BITTEL, J. 2000. Sympathetic stimulation induced by hand cooling alters cold-induced vasodilatation in humans. *Eur J Appl Physiol,* 81**,** 303-9.
441. SHE, Q. B., BODE, A. M., MA, W. Y., CHEN, N. Y. & DONG, Z. 2001. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res,* 61**,** 1604-10.
442. SHI, Y., NIKULENKOV, F., ZAWACKA-PANKAU, J., LI, H., GABDOULLINE, R., XU, J., ERIKSSON, S., HEDSTRÖM, E., ISSAEVA, N., KEL, A., ARNÉR, E. S. J. & SELIVANOVA, G. 2014. ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis. *Cell Death Differ,* 21**,** 612-623.
443. SHKRETA, L., FROEHLICH, U., PAQUET, E. R., TOUTANT, J., ELELA, S. A. & CHABOT, B. 2008. Anticancer drugs affect the alternative splicing of Bcl-x and other human apoptotic genes. *Mol Cancer Ther,* 7**,** 1398-409.
444. SHORT, K. R., VITTONE, J. L., BIGELOW, M. L., PROCTOR, D. N., RIZZA, R. A., COENEN-SCHIMKE, J. M. & NAIR, K. S. 2003. Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes,* 52**,** 1888-96.
445. SIEMS, W. G., BRENKE, R., SOMMERBURG, O. & GRUNE, T. 1999. Improved antioxidative protection in winter swimmers. *QJM,* 92**,** 193-8.
446. SIEMS, W. G., VAN KUIJK, F. J., MAASS, R. & BRENKE, R. 1994. Uric acid and glutathione levels during short-term whole body cold exposure. *Free Radical Bio Med,* 16**,** 299-305.
447. SILVEIRA, L. R., PILEGAARD, H., KUSUHARA, K., CURI, R. & HELLSTEN, Y. 2006. The contraction induced increase in gene expression of peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1alpha (PGC-1alpha), mitochondrial uncoupling protein 3 (UCP3) and hexokinase II (HKII) in primary rat skeletal muscle cells is dependent on reactive oxygen species. *Biochim Biophys Acta,* 1763**,** 969-76.
448. SIU, P. M., BRYNER, R. W., MARTYN, J. K. & ALWAY, S. E. 2004. Apoptotic adaptations from exercise training in skeletal and cardiac muscles. *FASEB J,* 18**,** 1150-2.
449. SKURVYDAS, A., SIPAVICIENE, S., KRUTULYTE, G., GAILIUNIENE, A., STASIULIS, A., MARNKUS, G. & STANISLOVAITIS, A. 2006. Cooling leg muscles affects dynamics of indirect indicators of skeletal muscle damage. *J Back Musculoskel,* 19**,** 141-151.
450. SLIMEN, I. B., NAJAR, T., GHRAM, A., DABBEBI, H., BEN MRAD, M. & ABDRABBAH, M. 2014. Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. *Int J Hyperthermia,* 30**,** 513-23.
451. SLIVKA, D., HEESCH, M., DUMKE, C., CUDDY, J., HAILES, W. & RUBY, B. 2013. Effects of post-exercise recovery in a cold environment on muscle glycogen, PGC-1alpha, and downstream transcription factors. *Cryobiology,* 66**,** 250-5.
452. SLIVKA, D. R., DUMKE, C. L., TUCKER, T. J., CUDDY, J. S. & RUBY, B. 2012. Human mRNA response to exercise and temperature. *Int J Sports Med,* 33**,** 94-100.

453. SMITH, L. L. 1991. Acute-Inflammation - the Underlying Mechanism in Delayed Onset Muscle Soreness. *Med Sci Sports Exerc,* 23**,** 542-551.
454. SMITH, M. A. & REID, M. B. 2006. Redox modulation of contractile function in respiratory and limb skeletal muscle. *Respir Physiol Neurobiol,* 151**,** 229-41.
455. SONNA, L. A., FUJITA, J., GAFFIN, S. L. & LILLY, C. M. 2002. Invited review: Effects of heat and cold stress on mammalian gene expression. *J Appl Physiol,* 92**,** 1725-42.
456. SORIANO, F. X., LIESA, M., BACH, D., CHAN, D. C., PALACIN, M. & ZORZANO, A. 2006. Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2. *Diabetes,* 55**,** 1783-91.
457. SRAMEK, P., SIMECKOVA, M., JANSKY, L., SAVLIKOVA, J. & VYBIRAL, S. 2000. Human physiological responses to immersion into water of different temperatures. *Eur J Appl Physiol,* 81**,** 436-42.
458. SREEDHAR, A. S., PARDHASARADHI, B. V., KHAR, A. & SRINIVAS, U. K. 2002. A cross talk between cellular signalling and cellular redox state during heat-induced apoptosis in a rat histiocytoma. *Free Radical Bio Med,* 32**,** 221-7.
459. SRIWIJITKAMOL, A., COLETTA, D. K., WAJCBERG, E., BALBONTIN, G. B., REYNA, S. M., BARRIENTES, J., EAGAN, P. A., JENKINSON, C. P., CERSOSIMO, E., DEFRONZO, R. A., SAKAMOTO, K. & MUSI, N. 2007. Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. *Diabetes,* 56**,** 836-48.
460. ST-PIERRE, J., DRORI, S., ULDRY, M., SILVAGGI, J. M., RHEE, J., JAGER, S., HANDSCHIN, C., ZHENG, K., LIN, J., YANG, W., SIMON, D. K., BACHOO, R. & SPIEGELMAN, B. M. 2006. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell,* 127**,** 397-408.
461. STACEY, D. L., GIBALA, M. J., MARTIN GINIS, K. A. & TIMMONS, B. W. 2010. Effects of recovery method after exercise on performance, immune changes, and psychological outcomes. *J Orthop Sports Phys Ther,* 40**,** 656-65.
462. STAMBOLSKY, P., WEISZ, L., SHATS, I., KLEIN, Y., GOLDFINGER, N., OREN, M. & ROTTER, V. 2006. Regulation of AIF expression by p53. *Cell Death Differ,* 13**,** 2140-9.
463. STANCIC, A., BUZADZIC, B., KORAC, A., OTASEVIC, V., JANKOVIC, A., VUCETIC, M., MARKELIC, M., VELICKOVIC, K., GOLIC, I. & KORAC, B. 2013. Regulatory role of PGC-1α/PPAR signaling in skeletal muscle metabolic recruitment during cold acclimation. *J Exp Biol,* 216**,** 4233-4241.
464. STANLEY, J., BUCHHEIT, M. & PEAKE, J. M. 2011. The effect of post-exercise hydrotherapy on subsequent exercise performance and heart rate variability. *Eur J Appl Physiol,* 112**,** 951-961.
465. STAPLETON, D., WOOLLATT, E., MITCHELHILL, K. I., NICHOLL, J. K., FERNANDEZ, C. S., MICHELL, B. J., WITTERS, L. A., POWER, D. A., SUTHERLAND, G. R. & KEMP, B. E. 1997. AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. *FEBS Lett,* 409**,** 452-6.
466. STEPHENS, T. J., CHEN, Z. P., CANNY, B. J., MICHELL, B. J., KEMP, B. E. & MCCONELL, G. K. 2002. Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. *Am J Physiol-Endoc M,* 282**,** E688-94.
467. STROBEL, N. A., PEAKE, J. M., MATSUMOTO, A., MARSH, S. A., COOMBES, J. S. & WADLEY, G. D. 2011. Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. *Med Sci Sports Exerc,* 43**,** 1017-24.
468. STUENAES, J. T., BOLLING, A., INGVALDSEN, A., ROMMUNDSTAD, C., SUDAR, E., LIN, F. C., LAI, Y. C. & JENSEN, J. 2010. Beta-adrenoceptor stimulation

potentiates insulin-stimulated PKB phosphorylation in rat cardiomyocytes via cAMP and PKA. *British journal of pharmacology,* 160**,** 116-29.

469. SUWA, M., EGASHIRA, T., NAKANO, H., SASAKI, H. & KUMAGAI, S. 2006. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. *J Appl Physiol,* 101**,** 1685-92.
470. SWENSON, C., SWÃ¤RD, L. & KARLSSON, J. 1996. Cryotherapy in sports medicine. *Scand J Med Sci Spor,* 6**,** 193-200.
471. SZWEDA, P. A., FRIGUET, B. & SZWEDA, L. I. 2002. Proteolysis, free radicals, and aging. *Free Radical Bio Med,* 33**,** 29-36.
472. TAKARADA, Y. 2003. Evaluation of muscle damage after a rugby match with special reference to tackle plays. *Br J Sports Med,* 37**,** 416-9.
473. TAYLOR, E. B., LAMB, J. D., HURST, R. W., CHESSER, D. G., ELLINGSON, W. J., GREENWOOD, L. J., PORTER, B. B., HERWAY, S. T. & WINDER, W. W. 2005. Endurance training increases skeletal muscle LKB1 and PGC-1alpha protein abundance: effects of time and intensity. *Am J Physiol-Endoc M,* 289**,** E960-8.
474. TAYLOR, N. A. S., CALDWELL, J. N., HEUVEL, A. M. J. V. D. & PATTERSON, M. J. 2008. To cool, but not too cool: That is the question-immersion cooling for hyperthermia. *Med Sci Sports Exerc,* 40**,** 1962-1969.
475. TERADA, S., GOTO, M., KATO, M., KAWANAKA, K., SHIMOKAWA, T. & TABATA, I. 2002. Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. *Biochem Bioph Res Co,* 296**,** 350-4.
476. TERADA, S., KAWANAKA, K., GOTO, M., SHIMOKAWA, T. & TABATA, I. 2005. Effects of high-intensity intermittent swimming on PGC-1alpha protein expression in rat skeletal muscle. *Acta Physiol Scand,* 184**,** 59-65.
477. TEYSSIER, C., MA, H., EMTER, R., KRALLI, A. & STALLCUP, M. R. 2005. Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation. *Genes Dev,* 19**,** 1466-73.
478. THOMAS, M. J. 2000. The role of free radicals and antioxidants. *Nutrition,* 16**,** 716-8.
479. THORSSON, O. 2001. [Cold therapy of athletic injuries. Current literature review]. *Lakartidningen,* 98**,** 1512-3.
480. TONKONOGI, M. & SAHLIN, K. 1997. Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. *Acta Physiol Scand,* 161**,** 345-53.
481. TORRES, M. & FORMAN, H. J. 2003. Redox signaling and the MAP kinase pathways. *BioFactors,* 17**,** 287-96.
482. TOYODA, T., TANAKA, S., EBIHARA, K., MASUZAKI, H., HOSODA, K., SATO, K., FUSHIKI, T., NAKAO, K. & HAYASHI, T. 2006. Low-intensity contraction activates the alpha1-isoform of 5'-AMP-activated protein kinase in rat skeletal muscle. *Am J Physiol-Endoc M,* 290**,** E583-90.
483. TREEBAK, J. T., BIRK, J. B., ROSE, A. J., KIENS, B., RICHTER, E. A. & WOJTASZEWSKI, J. F. 2007. AS160 phosphorylation is associated with activation of alpha2beta2gamma1- but not alpha2beta2gamma3-AMPK trimeric complex in skeletal muscle during exercise in humans. *Am J Physiol-Endoc M,* 292**,** E715-22.
484. TUPLING, A. R., GREEN, H. J., ROY, B. D., GRANT, S. & OUYANG, J. 2003. Paradoxical effects of prior activity on human sarcoplasmic reticulum Ca2+-ATPase response to exercise. *J Appl Physiol,* 95**,** 138-44.
485. VAILE, J., HALSON, S., GILL, N. & DAWSON, B. 2008. Effect of cold water immersion on repeat cycling performance and thermoregulation in the heat. *J Sport Sci,* 26**,** 431-40.

486. VAILE, J., HALSON, S., GILL, N. & DAWSON, B. 2008. Effect of hydrotherapy on recovery from fatigue. *Int J Sports Med,* 29**,** 539-44.
487. VAILE, J., HALSON, S., GILL, N. & DAWSON, B. 2008. Effect of hydrotherapy on the signs and symptoms of delayed onset muscle soreness. *Eur J Appl Physiol,* 102**,** 447-55.
488. VAILE, J., O'HAGAN, C., STEFANOVIC, B., WALKER, M., GILL, N. & ASKEW, C. D. 2011. Effect of cold water immersion on repeated cycling performance and limb blood flow. *Br J Sports Med,* 45**,** 825-829.
489. VEAL, E. A., FINDLAY, V. J., DAY, A. M., BOZONET, S. M., EVANS, J. M., QUINN, J. & MORGAN, B. A. 2004. A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. *Mol Cell,* 15**,** 129-39.
490. VERSEY, N. G., HALSON, S. L. & DAWSON, B. T. 2013. Water immersion recovery for athletes: effect on exercise performance and practical recommendations. *Sports Med,* 43**,** 1101-30.
491. VLAHOPOULOS, S. & ZOUMPOURLIS, V. C. 2004. JNK: a key modulator of intracellular signaling. *Biochemistry-Moscow+,* 69**,** 844-54.
492. VOLLAARD, N. B., SHEARMAN, J. P. & COOPER, C. E. 2005. Exercise-induced oxidative stress:myths, realities and physiological relevance. *Sports Med,* 35**,** 1045-62.
493. WALLACE, S. S. 2002. Biological consequences of free radical-damaged DNA bases. *Free Radical Bio Med,* 33**,** 1-14.
494. WANG, W., CHENG, X., LU, J., WEI, J., FU, G., ZHU, F., JIA, C., ZHOU, L., XIE, H. & ZHENG, S. 2010. Mitofusin-2 is a novel direct target of p53. *Biochem Bioph Res Co,* 400**,** 587-92.
495. WARREN, G. L., LOWE, D. A. & ARMSTRONG, R. B. 1999. Measurement tools used in the study of eccentric contraction-induced injury. *Sports Med,* 27**,** 43-59.
496. WATT, M. J., SOUTHGATE, R. J., HOLMES, A. G. & FEBBRAIO, M. A. 2004. Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. *J Mol Endocrinol,* 33**,** 533-44.
497. WEERAPONG, P., HUME, P. A. & KOLT, G. S. 2005. The mechanisms of massage and effects on performance, muscle recovery and injury prevention. *Sports Med,* 35**,** 235-56.
498. WEGMANN, M., FAUDE, O., POPPENDIECK, W., HECKSTEDEN, A., FROHLICH, M. & MEYER, T. 2012. Pre-cooling and sports performance: a meta-analytical review. *Sports Med,* 42**,** 545-64.
499. WELINDER, C. & EKBLAD, L. 2011. Coomassie staining as loading control in Western blot analysis. *J Proteome Res,* 10**,** 1416-9.
500. WELLMANN, S., BUHRER, C., MODEREGGER, E., ZELMER, A., KIRSCHNER, R., KOEHNE, P., FUJITA, J. & SEEGER, K. 2004. Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. *J Cell Sci,* 117**,** 1785-94.
501. WENZ, T. 2013. Regulation of mitochondrial biogenesis and PGC-1alpha under cellular stress. *Mitochondrion,* 13**,** 134-42.
502. WENZ, T., ROSSI, S. G., ROTUNDO, R. L., SPIEGELMAN, B. M. & MORAES, C. T. 2009. Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging. *Proc Natl Acad Sci USA,* 106**,** 20405-10.
503. WESTING, S. H. & SEGER, J. Y. 1989. Eccentric and concentric torque-velocity characteristics, torque output comparisons, and gravity effect torque corrections for the quadriceps and hamstring muscles in females. *Int J Sports Med,* 10**,** 175-80.

504. WESTING, S. H., SEGER, J. Y., KARLSON, E. & EKBLOM, B. 1988. Eccentric and concentric torque-velocity characteristics of the quadriceps femoris in man. *Eur J Appl Physiol O,* 58**,** 100-4.
505. WHITE, G. E., RHIND, S. G. & WELLS, G. D. 2014. The effect of various cold-water immersion protocols on exercise-induced inflammatory response and functional recovery from high-intensity sprint exercise. *Eur J Appl Physiol,* 114**,** 2353-2367.
506. WIDEGREN, U., JIANG, X. J., KROOK, A., CHIBALIN, A. V., BJORNHOLM, M., TALLY, M., ROTH, R. A., HENRIKSSON, J., WALLBERG-HENRIKSSON, H. & ZIERATH, J. R. 1998. Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. *FASEB J,* 12**,** 1379-89.
507. WIDEGREN, U., WRETMAN, C., LIONIKAS, A., HEDIN, G. & HENRIKSSON, J. 2000. Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. *Pflugers Arch,* 441**,** 317-22.
508. WIJERS, S. L., SCHRAUWEN, P., SARIS, W. H. & VAN MARKEN LICHTENBELT, W. D. 2008. Human skeletal muscle mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. *PloS one,* 3**,** e1777.
509. WILCOCK, I. M., CRONIN, J. B. & HING, W. A. 2006. Physiological response to water immersion: a method for sport recovery? *Sports Med,* 36**,** 747-65.
510. WILLIAMSON, D., GALLAGHER, P., HARBER, M., HOLLON, C. & TRAPPE, S. 2003. Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. *J Physiol,* 547**,** 977-87.
511. WINDER, W. W. & HARDIE, D. G. 1996. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol,* 270**,** E299-304.
512. WOJTASZEWSKI, J. F., NIELSEN, P., HANSEN, B. F., RICHTER, E. A. & KIENS, B. 2000. Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol,* 528 Pt 1**,** 221-6.
513. WRETMAN, C., LIONIKAS, A., WIDEGREN, U., LANNERGREN, J., WESTERBLAD, H. & HENRIKSSON, J. 2001. Effects of concentric and eccentric contractions on phosphorylation of MAPK(erk1/2) and MAPK(p38) in isolated rat skeletal muscle. *J Physiol,* 535**,** 155-64.
514. WRIGHT, D. C., GEIGER, P. C., HAN, D. H., JONES, T. E. & HOLLOSZY, J. O. 2007. Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. *J Biol Chem,* 282**,** 18793-9.
515. WRIGHT, D. C., HAN, D. H., GARCIA-ROVES, P. M., GEIGER, P. C., JONES, T. E. & HOLLOSZY, J. O. 2007. Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. *J Biol Chem,* 282**,** 194-9.
516. WU, H., KANATOUS, S. B., THURMOND, F. A., GALLARDO, T., ISOTANI, E., BASSEL-DUBY, R. & WILLIAMS, R. S. 2002. Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science,* 296**,** 349-52.
517. WU, Z., HUANG, X., FENG, Y., HANDSCHIN, C., FENG, Y., GULLICKSEN, P. S., BARE, O., LABOW, M., SPIEGELMAN, B. & STEVENSON, S. C. 2006. Transducer of regulated CREB-binding proteins (TORCs) induce PGC-1alpha transcription and mitochondrial biogenesis in muscle cells. *Proc Natl Acad Sci USA,* 103**,** 14379-84.
518. WU, Z., PUIGSERVER, P., ANDERSSON, U., ZHANG, C., ADELMANT, G., MOOTHA, V., TROY, A., CINTI, S., LOWELL, B., SCARPULLA, R. C. & SPIEGELMAN, B. M. 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell,* 98**,** 115-24.
519. WYTTENBACH, A., SAUVAGEOT, O., CARMICHAEL, J., DIAZ-LATOUD, C., ARRIGO, A. P. & RUBINSZTEIN, D. C. 2002. Heat shock protein 27 prevents cellular

polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Human molecular genetics,* 11**,** 1137-51.

520. XI, X., HAN, J. & ZHANG, J. Z. 2001. Stimulation of glucose transport by AMP-activated protein kinase via activation of p38 mitogen-activated protein kinase. *J Biol Chem,* 276**,** 41029-34.
521. XIA, Y., BUJA, L. M., SCARPULLA, R. C. & MCMILLIN, J. B. 1997. Electrical stimulation of neonatal cardiomyocytes results in the sequential activation of nuclear genes governing mitochondrial proliferation and differentiation. *Proc Natl Acad Sci USA,* 94**,** 11399-404.
522. YAMANAKA, K. 1999. Cold shock response in Escherichia coli. *J Mol Microb Biotech,* 1**,** 193-202.
523. YAMANE, M., TERUYA, H., NAKANO, M., OGAI, R., OHNISHI, N. & KOSAKA, M. 2006. Post-exercise leg and forearm flexor muscle cooling in humans attenuates endurance and resistance training effects on muscle performance and on circulatory adaptation. *Eur J Appl Physiol,* 96**,** 572-80.
524. YAN, Z. 2009. Exercise, PGC-1α, and metabolic adaptation in skeletal muscle. *Appl Physiol Nutr Me,* 34**,** 424-427.
525. YAN, Z., OKUTSU, M., AKHTAR, Y. N. & LIRA, V. A. 2011. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. *J Appl Physiol,* 110**,** 264-74.
526. YANAGISAWA, O., MIYANAGA, Y., SHIRAKI, H., SHIMOJO, H., MUKAI, N., NIITSU, M. & ITAI, Y. 2003. The effects of various therapeutic measures on shoulder strength and muscle soreness after baseball pitching. *J Sports Med Phys Fit,* 43**,** 189-201.
527. YANG, C. & CARRIER, F. 2001. The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. *J Biol Chem,* 276**,** 47277-84.
528. YEARGIN, S. W., CASA, D. J., MCCLUNG, J. M., KNIGHT, J. C., HEALEY, J. C., GOSS, P. J., HARVARD, W. R. & HIPP, G. R. 2006. Body cooling between two bouts of exercise in the heat enhances subsequent performance. *J Strength Cond Res,* 20**,** 383-9.
529. YOUNG, A. J., SAWKA, M. N., LEVINE, L., BURGOON, P. W., LATZKA, W. A., GONZALEZ, R. R. & PANDOLF, K. B. 1995. Metabolic and thermal adaptations from endurance training in hot or cold water. *J Appl Physiol,* 78**,** 793-801.
530. YOUNG, A. J., SAWKA, M. N., QUIGLEY, M. D., CADARETTE, B. S., NEUFER, P. D., DENNIS, R. C. & VALERI, C. R. 1993. Role of thermal factors on aerobic capacity improvements with endurance training. *J Appl Physiol,* 75**,** 49-54.
531. YU, M., BLOMSTRAND, E., CHIBALIN, A. V., KROOK, A. & ZIERATH, J. R. 2001. Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. *J Physiol,* 536**,** 273-82.
532. YU, M., STEPTO, N. K., CHIBALIN, A. V., FRYER, L. G., CARLING, D., KROOK, A., HAWLEY, J. A. & ZIERATH, J. R. 2003. Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. *J Physiol,* 546**,** 327-35.
533. ZHAO, F. Q., ZHANG, Z. W., WANG, C., ZHANG, B., YAO, H. D., LI, S. & XU, S. W. 2013. The role of heat shock proteins in inflammatory injury induced by cold stress in chicken hearts. *Cell Stress Chaperon,* 18**,** 773-783.
534. ZHAO, M., NEW, L., KRAVCHENKO, V. V., KATO, Y., GRAM, H., DI PADOVA, F., OLSON, E. N., ULEVITCH, R. J. & HAN, J. 1999. Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol,* 19**,** 21-30.
535. ZHAO, Y., CHAISWING, L., VELEZ, J. M., BATINIC-HABERLE, I., COLBURN, N. H., OBERLEY, T. D. & ST CLAIR, D. K. 2005. p53 translocation to mitochondria

precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase. *Cancer Res,* 65**,** 3745-50.

536. ZHOU, S., KACHHAP, S. & SINGH, K. K. 2003. Mitochondrial impairment in p53-deficient human cancer cells. *Mutagenesis,* 18**,** 287-92.
537. ZONG, H., REN, J. M., YOUNG, L. H., PYPAERT, M., MU, J., BIRNBAUM, M. J. & SHULMAN, G. I. 2002. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci USA,* 99**,** 15983-7.
538. ZURLO, F., NEMETH, P. M., CHOKSI, R. M., SESODIA, S. & RAVUSSIN, E. 1994. Whole-body energy metabolism and skeletal muscle biochemical characteristics. *Metabolis,* 43**,** 481-6.

Appendices

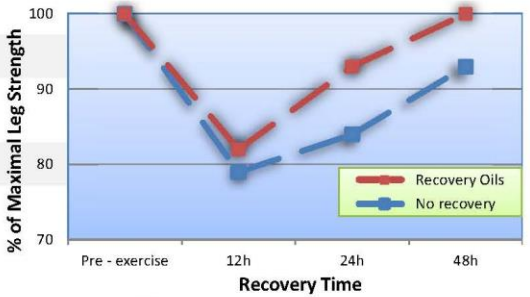
Recovery Information Sheets – Placebo Trial

"Bathing in recovery oils is a safe and easy way to promote recovery from exercise-induced muscle damage."
– Dr. L Nakamura- [redacted] *2010*

RECOVERY BATH OILS
WHAT DO THEY DO?

By facilitating muscle blood flow, waste product removal and a reduction in swelling after exercise, using recovery oils in a bath can greatly increase the rate of recovery after a hard training session. As a bonus, they reduce muscle and joint pains associated with post-exercise inflammation by sedating the nervous system, and therefore relaxing your muscles.

REDUCE:	IMPROVE:	PREVENT:
✓ INFLAMMATION	✓ PERFORMANCE	✓ MUSCLE DAMAGE
✓ MUSCLE ACHES	✓ BLOOD FLOW	✓ STRESS
✓ JOINT STIFFNESS	✓ MUSCLE FUNCTION	✓ HEALTH ISSUES



DISCOVERY

Dr Nakamura calls her discovery the "secret science of recovery". On one of her recent research trips, Dr Nakamura discovered this simple and effective way to recover from joint and muscle soreness normally associated with injury. Soaking in a bath of recovery oils will help relieve muscle aches after a heavy workout.

RECOVERY OIL AND SPORT

Water oil therapy is the combination of bath oils and water, and is quickly becoming the basis of many powerful therapeutic treatments used in sports therapy clinics around the world. Oil baths will help relax your muscles after a hard training session, and decrease the inflammatory response associated with reduced performance.

PERFORMANCE GUARANTEE

Rapid return of performance within 48 hours when using recovery oils as compared to no recovery at all!!
Following a bout of high-intensity interval exercise, preliminary research has shown a staggering **11%** improvement in leg strength 24h post-exercise, and up to an **8%** improvement 48h post-exercise.

SPORTS SCIENCE • MEDICINE• INFORMATION• INTELLIGENCE

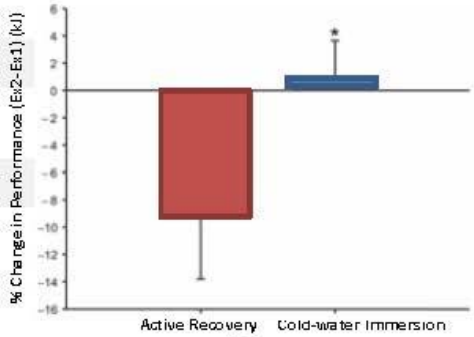
COLD-WATER IMMERSION

WHAT DOES IT DO?

"Cold-water immersion is a safe and easy way to promote recovery from exercise-induced muscle damage."
– Dr. L Nakamura- [redacted] *-2010*

By facilitating waste product removal and a reduction in exercise-induced inflammation and oedema, cold-water immersion can greatly increase the rate of recovery after a hard training session. As a bonus, it will reduce muscle and joint pains associated with post-exercise inflammation by sedating the nervous system, and therefore relaxing your body.

REDUCE:	IMPROVE:	PREVENT:
✓ INFLAMMATION	✓ PERFORMANCE	✓ MUSCLE DAMAGE
✓ MUSCLE ACHES	✓ MUSCLE FUNCTION	✓ STRESS
✓ SWELLING	✓ BLOOD FLOW	✓ FATIGUE



Vaile et. al. (2011) – Br J Sports Med; ***45****: 825-829.*

HISTORY

Traditionally used to treat acute traumatic injury, cryotherapy has become a popular tool in post-exercise recovery. Cold-water immersion as a method of cryotherapy is used to promote a reduction in inflammation, oedema and muscle soreness, physiological responses evident with exercise-induced muscle damage.

COLD-WATER AND SPORT

Cold-water immersion is quickly becoming the basis of recovery methods practiced in elite athletic populations around the world. It has the potential to attenuate reductions in functional capacity associated with reduced performance, such as force production/muscle strength and range of motion.

PERFORMANCE GUARANTEE

Extensive literature supports improved performance with cold-water immersion as a recovery technique. A recent study has shown total work during two high-intensity cycling bouts (separated by one hour) was unchanged with cold-water immersion, but reduced by **10%** with an active recovery protocol of very light cycling.

Information on your Immersion Protocol:

The thermo-neutral water immersion protocol used in this study is designed to act as a control condition, by which the results of the other two conditions will be compared.

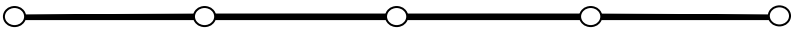
Immersion in water will elicit hydrostatic pressure on the body, aiding in the return of fluid from muscle to blood, the removal of waste products and a reduction in swelling. This effect is common across all conditions used in this study. The control condition is not, however designed to alter muscle temperature.

Thermo-neutral water temperature is ~34.5°C, designed to maintain your body's natural baseline temperature (37°C). This temperature will allow the natural dispersion of heat, thereby not changing the muscle's temperature.

Belief Effect Forms

Directions: Indicate on the line how you feel RIGHT NOW in response to each question.

1. How often do you perform recovery after exercise or competition?

Never  *Always*

1 2 3 4 5

What recovery techniques have you used (if any)?

_____

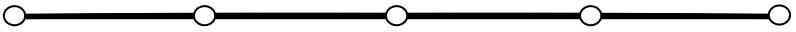
_____

2. How effective do you think post-exercise recovery techniques are in general?

Not effective at all  *Extremely effective*

1 2 3 4 5

3. How effective do you think the recovery technique you have been assigned will be?

Not effective at all  *Extremely effective*

1 2 3 4 5

4. Do you think the recovery technique that you have been assigned will be more effective than no recovery?

Not at all  *Most definitely*

1 2 3 4 5

Directions: Indicate on the line how you feel RIGHT NOW in response to each question

1. How effective do you think post-exercise recovery techniques are in general?

Not effective at all — 1 — 2 — 3 — 4 — 5 — *Extremely effective*

2. How effective was the recovery technique you used?

Not effective at all — 1 — 2 — 3 — 4 — 5 — *Extremely effective*

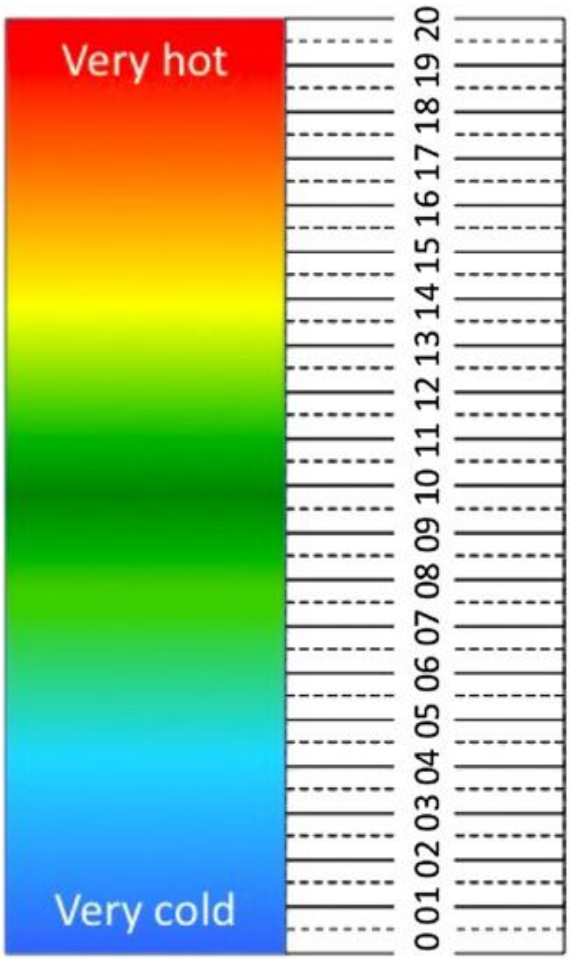
3. How effective do you think this recovery technique would have been as compared with no recovery technique at all?

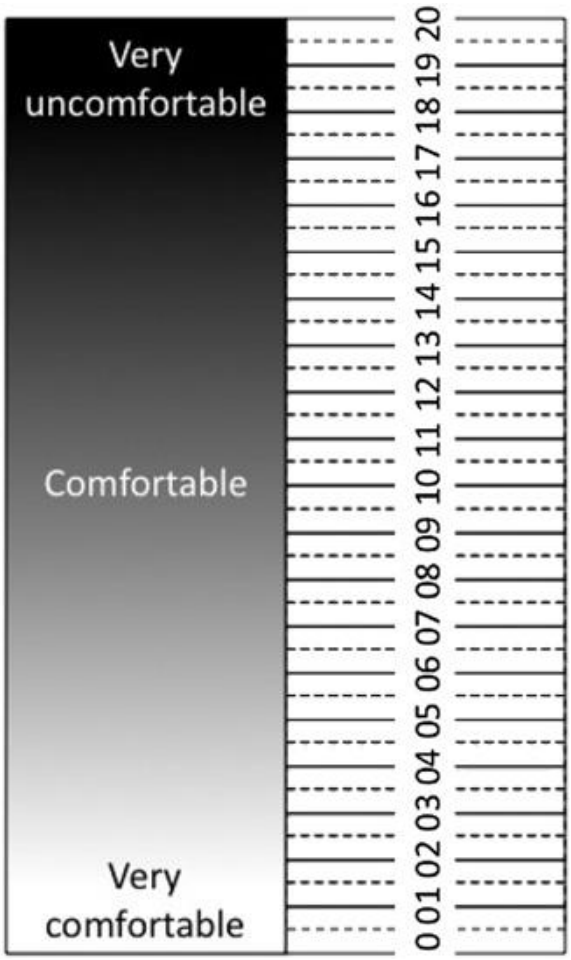
Not effective at all — 1 — 2 — 3 — 4 — 5 — *Extremely effective*

4. Would you consider using this recovery technique in the future?

Never again — 1 — 2 — 3 — 4 — 5 — *Most definitely*

Thermal Sensation and Comfort Scales





Psychological Questionnaire

Directions: Indicate on the line how you feel RIGHT NOW in response to each question

How **PHYSICALLY READY** are you right now to engage in strenuous exercise:

Not Ready At all ——— *Totally Ready*

How **MENTALLY READY** are you right now to engage in strenuous exercise:

Not Ready At all ——— *Totally Ready*

How would you rate your general feelings of **FATIGUE** right now:

Not Fatigued At all ——— *Extremely Fatigued*

How **VIGOROUS** do you feel right now:

Not vigorous at all ——— *Extremely vigorous*

How **SLEEPY** do you feel right now:

Not Sleepy At all ——— *Extremely Sleepy*

In how much muscular **PAIN** are you in right now:

Least possible pain ——— *Worst possible pain*

Risk Factor Questionnaire

EXERCISE PHYSIOLOGY UNIT

SCHOOL OF SPORT AND EXERCISE SCIENCE
PO BOX 14428
MELBOURNE MC, VIC 8001
TELEPHONE: (03) 9919 4129

Please return this form to:

School of School of Sport and Exercise Science
Victoria University of Technology
P.O. Box 14428
MELBOURNE MC8001

Telephone: (03) 9919 4593
Mobile:
Fax: (03) 9919 4891
Email:
Website:

NAME: _____ **DATE** _____

ADDRESS: _____ **SEX** **M / F**

_____**Postcode:** _____ **AGE** _____ **YRS**

TELEPHONE: Work: _____ **WEIGHT** _____ **KG**

TELEPHONE: Home: _____ **HEIGHT** _____ **M**

EMAIL: _____

MEDICAL HISTORY:
In the past have you ever had (tick No or Yes)

Medical Condition	NO	YES	Medical Condition	NO	YES
Heart Attack	❑	❑	Congenital Heart Disease	❑	❑
Chest Pain (angina)	❑	❑	Disease of Arteries/Veins	❑	❑
Heart Murmur	❑	❑	Asthma	❑	❑
Heart Rhythm Disturbance	❑	❑	Lung Disease (eg. emphysema)	❑	❑
Heart Valve Disease	❑	❑	Epilepsy	❑	❑
Stroke	❑	❑	Injuries to back, knees, ankles	❑	❑

List any prescribed medications being taken

_____

_____

List any surgical procedures that you have had (write the year in brackets):
example: appendix (1979)

_____

List any injuries in your past medical history

_____

_____

ALLERGIES: Do you have any allergies **NO** ❑ **YES** ❑

If yes, give details: _____

_____

SYMPTOMS DURING OR AFTER EXERCISE

As a result of exercise, have you ever experienced any of the following:

Symptom during exercise	**NO**	**YES**	**Symptom during exercise**	**NO**	**YES**
Pain or discomfort in the chest, back, arm, or jaw	❑	❑	Palpitations (heart rhythm disturbance)	❑	❑
Severe shortness of breath or problems with breathing during mild exertion	❑	❑	Pain in the legs during mild exertion	❑	❑
Dizziness, nausea or fainting	❑	❑	Severe heat exhaustion	❑	❑

CARDIOVASCULAR RISK FACTORS:

Do you have (tick NO, YES or circle ? for DON'T KNOW)

Cardiovascular Risk Factors	**NO**	**YES**	**DON'T KNOW**	
High Blood Pressure	❑	❑	**?**	
High Blood Cholesterol/Triglycerides	❑	❑	**?**	
Diabetes	❑	❑	**?**	
Current Smoker	❑	❑	Average/day =	
Ex-smoker	❑	❑	Average/day =	
Do you drink alcohol regularly?	❑	❑	Average/day =	drinks

FAMILY MEDICAL HISTORY:

Have members of your immediate family ever had any of the following conditions: (tick NO, YES or circle ? for DON'T KNOW). If you answer Yes or ?, write beside this the member of the family affected (F=father, M=mother, B=brother, S=sister, GM=grandmother, GF=grandfather).

FAMILY MEDICAL HISTORY	NO	YES	?	FAMILY MEMBER	AGE (Years)	ALIVE NOW?)
Heart Attack	❑	❑	?	_____	_____	_____
Chest Pain (Angina)	❑	❑	?	_____	_____	_____
Stroke	❑	❑	?	_____	_____	_____
High Blood Pressure	❑	❑	?	_____	_____	_____
High Blood Cholesterol/Triglycerides	❑	❑	?	_____	_____	_____
Diabetes	❑	❑	?	_____	_____	_____

PERSONAL LIFESTYLE:

A. Exercise

List the sports, exercise or physically active hobbies (eg. gardening or playing with the kids) that you are **currently** engaged in:

Sport/Activity	Day(s) of week Sa-Su-Mo-Tu-We-Th-Fr	Time of the day eg. 6 p.m.	Approximate duration eg. 30 minutes
		TOTAL	

B. Nutrition

List a typical day's eating pattern.

Breakfast	Lunch	Dinner	Snacks	Drinks

C. Rest/Recreation

How many hours sleep do you usually have? _____ hours/night

On average how much time do you spend each day on passive hobbies or just relaxing?

_____ minutes/hours per day.

Do you feel that you usually get enough restful sleep and time to relax? **Yes/No**

Client Declaration	**OFFICE USE ONLY** **CLEARANCE TO UNDERGO AN EXERCISE TEST**
I declare that the above information is To my knowledge true and correct, and that I have not omitted any information that is requested on this form. ***Signed:*** _____ ***Parent Signature: (for those under 18)*** _____ ***Date:*** _____/_____/_____	This person has been cleared to undergo a Fitness test: ❑ Without medical supervision ❑ With medical supervision ❑ A fitness test is not advisable at this time **Signed: Dr/Mr/Mrs/Ms** _____ **(Circle appropriate title: Physician/exercise physiologist)**

Please turn over and provide the information requested overleaf.

Venous Cannulation Questionnaire

EXERCISE PHYSIOLOGY UNIT

SCHOOL OF SPORT AND EXERCISE SCIENCE
PO BOX 14428
MELBOURNE MC, VIC 8001
TELEPHONE: (03) 9919 4129

In order to be eligible to participate in the experiment investigating:

"TAKING THE PLUNGE: DOES HYDROTHERAPY HELP OR HINDER ADAPTATION TO TRAINING."

you are required to complete the following questionnaire which is designed to assess the risk of you having an adverse event during venous blood sampling.

NAME: _____

DATE: _____ **AGE:** _____ **years**

1. Have you or your family suffered from any tendency to bleed excessively? (e.g., Haemophilia) or bruise very easily? Yes No Don't Know

If yes, please elaborate

_____

2. Are you allergic to local anaesthetic? Yes No Don't Know

If yes, please elaborate

_____

3. Do you have any skin allergies? Yes No Don't Know

If yes, please elaborate

_____

4. Have you any other allergies? Yes No Don't Know

If yes, please elaborate

_____

5. Are you currently on any medication? Yes No

If yes, what is the medication?

_____

6. Do you have any other medical problems? Yes No

If yes, please elaborate

_____

7. Have you ever fainted when you had an injection or blood sample taken? Yes No Don't know

If yes, please elaborate

_____

8. Have you previously had heparin infused or injected? Yes No Don't know

If yes, please elaborate

_____

9. Do you or other members of your family have Raynauds disease, or suffer from very poor circulation in the fingers, leading to painful fingers that turn white/blue? Yes No Don't know

If yes, please elaborate

_____

10. Do you suffer from thromboembolic disordesr e.g., DVT, PE, AMI? Yes No Don't know

If yes, please elaborate

_____

To the best of my knowledge, the above questionnaire has been completely accurately and truthfully.

Signature: _____

Date: _____

Participant Instruction Sheet – Placebo Trial

Thank-you for agreeing to participate in this research project entitled:

"The effects of water immersion therapies on post-exercise recovery."

Before you participate in this study, we kindly ask you read through this short instruction sheet designed to ensure you are properly prepared for the study. Furthermore, the accuracy of results we obtain can be influenced by factors such as nutrition and exercise, so we kindly ask you adhere to the following information throughout the study.

Exercise:

Previous exercise has the potential to hinder results obtained during the study, so we kindly ask that you refrain from **strenuous** exercise during the three days of the trial, i.e., sessions three to five.

Nutrition:

Prior to session three, we will ask that you fill in a food diary for preceding 24 hours.
As nutrition has the potential to affect the results we obtain, we kindly ask that you replicate this diet for 24 hours prior to the fifth and last session. Please also abstain from alcohol between sessions three-five, and avoid caffeine or caffeine products in the 2 hours prior to these sessions.

General Instructions:

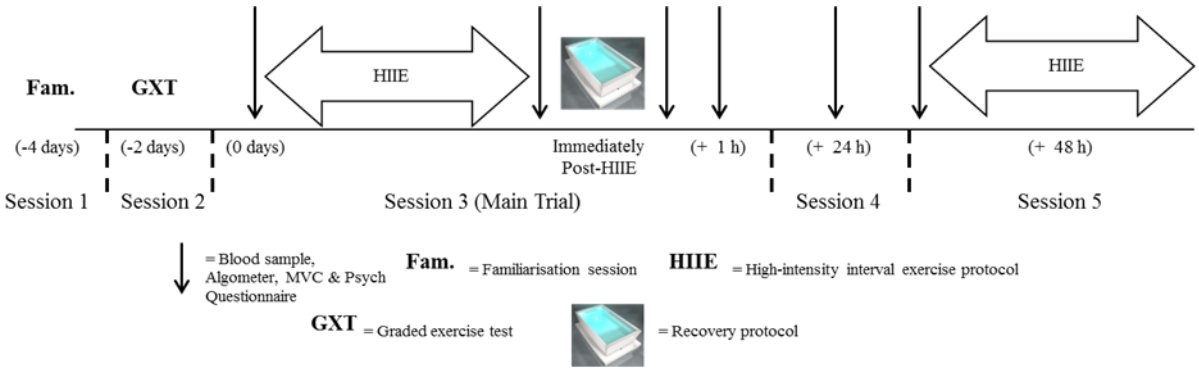
Please bring a towel and swimwear (loose fitting shorts and/or swimmers) for the trial day (session three). You will be required to complete your assigned recovery water immersion so will need a towel to dry yourself.

Drinking water will be provided throughout the entire trial.

Information on your assigned immersion protocol has been attached to this sheet for your reference.

Thank you once again for agreeing to participate in this study, and I hope that you find you participation rewarding. Should you have any questions in regards to this information, or should you not be able to follow the instructions for any reason at all, please contact the researcher on the following details:

Mr. James Broatch
Mobile: 0422 050 361
E-mail: james.broatch@live.vu.edu.au


Fam.
GXT
HIIE
HIIE
(-4 days)
(-2 days)
(0 days)
Immediately Post-HIIE
(+ 1 h)
(+ 24 h)
(+ 48 h)
Session 1
Session 2
Session 3 (Main Trial)
Session 4
Session 5
= Blood sample, Algometer, MVC & Psych Questionnaire
Fam. = Familiarisation session
HIIE = High-intensity interval exercise protocol
GXT = Graded exercise test
= Recovery protocol

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

EXERCISE PHYSIOLOGY UNIT

School of Sport and Exercise Science
PO Box 14428
Melbourne MC, VIC 8001
TELEPHONE: (03) 9919 4129

INFORMED CONSENT FOR UNDERTAKING AN EXERCISE TEST FOR VOLUNTEERS UNDER THE AGE OF 40 YEARS

1. EXPLANATION OF THE GRADED EXERCISE TEST

A VO_2max measures the maximal ability of the body to utilise oxygen during exercise. The test begins at a low intensity and progressively increases in intensity until the point at which the participant can no longer continue.

You will perform a graded exercise test on the bicycle ergometer or a motor-driven treadmill. The exercise intensities will begin at a level you can easily accomplish and will be advanced in stages, depending on your functional capacity. We may stop the test at any time if signs or symptoms occur or you may stop whenever you wish to because of personal feelings of fatigue or discomfort. We do not wish you to exercise at a level which is abnormally uncomfortable for you; for maximum benefit from the test, exercise as long as is comfortable.

2. RISK AND DISCOMFORTS

There exists the possibility of certain changes occurring during the test. They include abnormal blood pressure, fainting, disorders of heart beat, and in very rare instances, heart attack, stroke or death. Every effort will be made to prevent these by preliminary screening and careful monitoring during the test. Should you feel any symptoms if discomfort of any kind, indicate this to us and we will terminate the test immediately. Please note that body hair covering the sites needed to record an electrocardiograph (ECG) may need to be shaved. This will involve shaving up to 10 areas on the chest, each the size of a 50 cent piece.

3. RESPONSIBILITIES OF THE PARTICIPANT

Information you possess about your health status or previous experiences of unusual feelings with physical effort may affect the safety and value of your exercise test. You are responsible to fully disclose such information on the accompanying sheets or when requested by the testing staff. Furthermore you are expected to disclose any feelings of discomfort during the exercise test. The staff will take all reasonable precautions to ensure the safety and value of your exercise test but we can not be held responsible in the event that you fail to disclose important information to us.

4. BENEFITS TO BE EXPECTED

The results obtained from the exercise test assist in the evaluation of the types of physical activities you might engage in with no or low hazards.

5. INQUIRIES

Any questions about the procedures used in the graded exercise test or in the estimation of functional capacity are encouraged. If you have any doubts or questions, please ask us for further explanations.

6. MEDICAL SUPERVISION

Normally it is not necessary for someone under the age of 40 to need a doctor to be present for an exercise test, but we will arrange for a medically supervised test if you prefer. Note: if your cardiovascular risk factor and medical history indicate the need for medical coverage, we MUST arrange for a doctor to be present.

7. FREEDOM OF CONSENT

Your permission to perform this graded exercise test is voluntary. You are free to *deny consent now or withdraw consent at any time* (including during the exercise test) if you so desire.

I have read this form and I understand the test procedures and the conditions under which this test will be conducted. I consent to participate in this fitness test without medical supervision.

VOLUNTEER'S CONSENT

I have read this form and I understand the procedures involved and the conditions under which the tests will be conducted. I am under the age of 18 and consent to participate in this test **WITHOUT** medical supervision.

_____	_____	_____/_____/_____
Name of Volunteer	**Signature of Volunteer**	**Date**
_____	_____	_____/_____/_____
Name of Witness	**Signature of Witness**	**Date**

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled:

"The effects of water immersion therapies on post-exercise recovery."

This project is being conducted by a student researcher James Broatch as part of a PhD at Victoria University under the supervision of Prof. David Bishop [School of Sport and Exercise Science and ISEAL].

Project explanation

The project aims to investigate the effects of different recovery modalities implemented immediately following high-intensity interval exercise on performance, post-exercise inflammation and pain tolerance. The results of this research will have a significant and direct application to current sporting practice, and will be relevant to all athletes who perform high-intensity intermittent exercise. Furthermore, the results have the potential to drastically alter current sporting practice, and may provide the rationale for modifying current post-exercise recovery techniques.

What will I be asked to do?

We will first ask you to fill in several short questionnaires about your family medical history and your exercise habits to determine your ability to participate in this study. This study is divided in five sessions:
Participants will be required to attend 1x 2 hr familiarisation session, a 30 min pre-testing session, a 3 hr testing session, and two post-testing sessions of 30 and 60 min in duration. In session one you will be asked to perform a familiarisation session which will consist of familiarisation with the high-intensity interval exercise (HIIE), maximal voluntary contraction, algometer, and your assigned recovery protocol. An intramuscular temperature probe will also be inserted into your quadriceps muscle so as to familiarise you with how this feels during exercise. Session two pre-testing will consist of a graded exercise test (GXT) in which your VO_{2max} will be determined. Session three marks the start of the main trial, in which you will perform the HIIE followed immediately by your assigned recovery protocol. Muscle temperature will be monitored throughout this session via insertion of an intramuscular temperature probe into your quadriceps muscle. Blood samples and ratings for muscle soreness, pain tolerance/threshold, muscle strength and psychological mood will be taken/measured immediately before and after the HIIE test, and at 1 hour post-exercise. These measures will be taken again at 24 hours post-exercise (session four) and again at 48 hours post-exercise (session five). At the fifth and final session you will again be required to complete the HIIE protocol.

What will I gain from participating?

From participating in this study you can expect to increase your understanding of fitness tests used by sport scientists, which will be performed in a state-of-the-art purpose-built training facility. You will also gain the experience of having participated in an exercise science experiment designed to increase knowledge about the effectiveness of recovery protocols used after training and competition.

How will the information I give be used?

Your samples will be stored under alphanumeric codes (i.e., without your name or personal details) and only the researchers will be able to connect the samples to you. All of the blood samples collected will be used to

measure inflammatory markers present in the post-exercise recovery response. In the event of any fluid remaining it will be disposed of in a de-identified container (i.e., no coding present) via incineration using Victoria University's waste disposal contractor. The data that will be collected during the study will be used/published in peer-reviewed journals and conference presentations. No personal details will be revealed without your written consent.

What are the potential risks of participating in this project?

The HIIE, MVC and GXT all involve risks of fainting, muscle soreness and stiffness, and sudden death due to myocardial infarction. The risk of such events is in reality very low. Risks associated with venous catheterisation include discomfort, bruising and infection (for example puss, tenderness and/or redness). Risks associated with blood sampling include slight discomfort, with the possibility of bruising and infection. The muscle temperature probe technique is likely to cause some discomfort. The feeling has been described as being given a very localised "dead leg" that lasts approximately 1 to 2 days; although each individual will respond differently, with some scarcely feeling it. In all cases there is a very minimal risk of infection at muscle temperature sites.

Furthermore, the training protocol you will perform (high-intensity exercise with minimal recovery), can be accompanied in rare cases by the possibility of mood changes such as irritability and depression. These changes are however short term and can be reversed with a maximum of two to three weeks of recovery. To minimise the risk of any psychological stress, your results will remain confidential. Should you experience any adverse or unusual psychological changes, you will be immediately removed from the study and "debriefed" by speaking with the psychologist associated with this study, Dr Harriet Speed (Victoria University Psychologist). Dr Harriet Speed can be contacted on (03) 9919 5412. With this safeguard in place, you will have access to counselling, free of charge in the case of any psychological event.

How will this project be conducted?

All testing will be conducted in accordance with current guidelines for testing in the Exercise Physiology Laboratory, School of Sport and Exercise Science, Victoria University. Participants will initially be screened for cardiovascular risk factors and any health issues of relevance to the study. If you are deemed healthy and at low risk of any adverse events, you will be ask to complete five sessions of testing over 2 weeks. The main testing session (session three) will take approximately 2 hours to complete, while all other sessions will last only 30 minutes each.

Visit 1 (~ 2 h): Familiarisation session

During this session you will be asked to complete the algometer, maximum voluntary contraction, and high-intensity interval exercise protocols, followed by your assigned recovery protocol. A muscle temperature probe will also be inserted into your quadriceps muscle prior to these protocols, and will be left in your leg for the entire session. These will be performed so you are accustomed to the procedures when you perform the actual testing trial. Further information for these tests can be seen below.

Visit 2 (~30 min): Graded exercise test (GXT)

During this session you will undertake a maximal incremental exercise test to exhaustion for baseline determination of peak oxygen uptake (VO_{2Peak}). The test will be performed on a mechanically-braked bicycle ergometer and will consist of a 5 min warm up phase (75W), a one minute recovery phase, and a gradual incremental phase until fatigue. The incremental phase will start at 90 W and increase by 1 W every 2 seconds until you reach volitional fatigue. Pedalling cadence will be fixed at 70 revolutions per minute (RPM), and during this test you will be required to wear a heart rate monitor. Expired gases will also be analysed during the test with the use of a metabolic cart (Moxus Metabolic System). This test is routinely performed in our laboratory.

Visit 3 (~3 h): Main Trial

You will be asked to refrain from vigorous exercise in the 24 h preceding this trial, and to fast for at least 2 hours prior to the trial. During this session you will be required to perform the HIIE test followed immediately by 10 min of your designated recovery protocol. Some further measures will also be taken as outlined below.

High-intensity interval exercise (HIIE) test:
The HIIE protocol consists of 4 x 30-s 'all-out' efforts on a cycle ergometer with 4 min of rest between bouts. Before this test, you will warm up for 5 min on the cycle ergometer (100W), followed by 2 min of rest prior to commencing the protocol. You will be then instructed to pedal as fast as possible from the start of exercise and will be verbally encouraged throughout each 30-s bout to maintain maximal pedalling speed. During these bouts, we will be measuring your power output and rate of fatigue. This protocol will be completed again 48 hours later (session five) as detailed below.

Recovery protocol:
Immediately after the HIIE protocol, you will be required to perform one of three recovery protocols; a cold-water immersion (CWI) recovery protocol, in which you will be immersed in 10°C water up to the umbilicus for 10 min, a recovery-oil water immersion (RWI) protocol, in which you will be immersed in thermo-neutral water (~34.5°C), with the addition of recovery oils, to the umbilicus for 10min, and a control thermo-neutral water immersion, in which you will be immersed in thermo-neutral water (~34.5°C). Your assigned immersion will be performed in a specifically purchased recovery pools (iCool Sport, Australia), in which the water temperature will be constantly monitored and controlled to within ±1°C of the desired temperature. Immersions will be performed in the environmental chamber at Victoria University, and the ambient temperature will be set to a constant 23°C.

Temperature Probe:
During the HIIE and CWI protocols, intramuscular temperature will be monitored via a needle thermistor probe. This probe will be inserted into both your left and right vastus lateralis muscles (prior to the HIIE protocol), 4 cm below the skin. Insertion is performed via the use of a needle and cannula, with the needle being removed once the probe is inserted and in place. The probe will also be held in place with a plastic adhesive cover. After the cessation of the recovery protocol, the probe will be removed from your leg. This is a well-established, accepted and well-practiced technique used to document intra-muscular temperature. Mr. James Broatch is qualified to perform this procedure, and will do so under the supervision of Prof. David Bishop. Prof. Bishop has performed this procedure many times in previous studies.

Maximal Voluntary Contraction:
Immediately before and after exercise, and at 1 h post exercise, you will be required to complete 3 x 5 s maximal contractions of the quadriceps muscle, using an iso-kinetic dynamometer (Cybex). This protocol is designed to measure muscle strength of the knee extensors and is a well-established and accepted protocol. During the 5-s contractions, we will be encouraging you to push to your maximum.

Psychological Questionnaire:
Immediately before and after exercise, and at 1 h post exercise, you will be required to complete a one-page psychological questionnaire designed to document subjective ratings of pain, general feelings of fatigue, vigour, and sleep patterns. The questionnaire will take approximately 20 seconds to complete.

Algometer:
Immediately before and after exercise, and at 1 h post exercise, you will complete a pain threshold and pain tolerance test. This will require the use of a pressure algometer, which involves the application of $1cm^2$ flat surface to the skin at an approximate rate of 1 $kg.cm^{-2}.s^{-1}$. The first point at which discomfort is reported will correspond to your pain threshold, and the point at which pain becomes unbearable corresponded to pain tolerance.

Venous Blood Sampling:
For the main trial day, an indwelling venous catheter will be inserted into your brachial vein 10 min prior to the first blood draw. Six blood samples (10 mL) will be taken in total, four of which will be in this third session (prior to and immediately after exercise, immediately after the recovery protocol, and 1 h post-exercise). Two

further samples will be taken (by a venepuncture technique) in the following days as described below. Samples will be analysed for blood markers associated with the post-exercise inflammatory response. All techniques are established and available in the laboratories at Victoria University, and will be conducted by Mr. James Broatch, who is qualified and competent with these procedures. All techniques will be conducted in accordance with current guidelines for blood sampling exercise testing in the Exercise Physiology Laboratory, School of Sport and Exercise Science, Victoria University.

Visit 4 (~ 30 min): Blood sampling and psychological measures:

During this session, you will report to the laboratory for four measures. A venous blood sample will be taken, and you will again be required to complete the algometer, maximal voluntary contraction and the psychological questionnaire protocols.

Visit 5 (~ 60 min): Repeat Testing

During this fifth and final session, you will be asked to re-perform the HIIE test. You will again be asked to refrain from vigorous exercise in the 24 h preceding the trial, and to fast for at least 2 hours prior to the trial. A further venous blood sample will be taken, and you will again be required to complete the algometer, maximal voluntary contraction and the psychological questionnaire protocols.

Who is conducting the study?

The study is conducted by the School of Sport and Exercise Science, Victoria University, Footscray Park Campus. The main investigators are:

Prof. David Bishop, Telephone number: 9919 9471, Mobile: 0435 962 364, email: david.bishop@vu.edu.au
Dr. Aaron Petersen, Telephone number: 9919 9452, email: aaron.petersen@vu.edu.au
Mr. James Broatch, Mobile: 0422 050 361, email: james.broatch@live.vu.edu.au

Any queries about your participation in this project may be directed to the Principal Researcher listed above. If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study entitled:
"The effects of water immersion therapies on post-exercise recovery."

AIMS OF THE STUDY:
The major aim of this project is to compare the effects of two promising recovery techniques, following very intense exercise, on performance, post-exercise inflammation and pain tolerance in healthy young adults.

PROCEDURES INVOLVED AND NATURE OF THE PROJECT:

Participants will be requested to attend the Exercise Physiology Laboratory at Victoria University, Footscray Park Campus (School of Sport and Exercise Science, ISEAL, building P). During the study, you will be required to visit the labs on five separate occasions for exercise testing trials. Whilst each testing session may be tiring, you will recover very quickly.

Exercise testing procedure: You will be asked to undertake three high-intensity tests. This testing involves cycling exercises on a stationary cycle over two visits, separated by 48 hours, and also a graded exercise test to exhaustion.

Recovery Modality: You will be asked to perform one of three recovery techniques (for 10 minutes) immediately following the first high-intensity exercise test. You will be randomly allocated into one of the three recovery modality groups; a passive resting group, a therapeutic oil bath group, and a cold-water immersion group. 48 hours following the recovery modality, you will be required to re-perform the high-intensity exercise bout to assess the effectiveness of the assigned recovery modality.

Temperature Probe: You will be asked to have an intra-muscular temperature probe inserted into both your quadriceps muscles (left and right legs) for the session involving the first exercise test and recovery modality. This temperature probe is inserted with a needle and catheter. The needle will be removed after insertion, leaving the catheter in your leg as housing for the small and flexible probe, which is fed through the catheter until reaching the desired landmark. Both the catheter and probe will be implanted in the leg for the testing session.

Psychological Questionnaire: Immediately before and after the first exercise session, and at three further time points until the second exercise session, you will be required to complete a one-page psychological questionnaire designed to document subjective ratings of pain, general feelings of fatigue, vigour, and amount of sleep. The questionnaire will require a visual analogue score (VAS), in which you will mark between two extremes (e.g., 0 indicating 'no pain', 10 indicating 'extreme pain'), and will take approximately 20 seconds to complete.

Maximal Voluntary Contraction: Immediately before and after the first exercise session, and at three further time points until the second exercise session, you will be required to complete a maximal voluntary contraction muscle strength test.

Algometer: Immediately before and after the first exercise session, and at three further time points until the second exercise session, you will be required to complete a pain threshold and pain tolerance test with the use of a pressure algometer.

Blood samples: Blood samples will be taken before and after the first high-intensity exercise test from the antecubital vein. Three further samples will be taken within the 48 hours until the second high-intensity exercise test.

CERTIFICATION BY SUBJECT

I, _____(Phone No:)_____

of _____

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study:
"The effects of water immersion therapies on post-exercise recovery."
being conducted at Victoria University by: **Prof. David Bishop.**

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

Mr James Broatch

and that I freely consent to participation involving the below mentioned procedures:

- Pre-test screening
- VO_{2Peak} test (GXT)
- High-intensity interval exercise tests (HIIE)
- Recovery modality
- Temperature probe insertion
- Maximal voluntary contraction test (MVC)
- Psychological questionnaire
- Blood samples (venous and capillary samples)
- Pain Threshold Test (Algometer)

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed: _____

Date: _____

Any queries about your participation in this project may be directed to the researcher
Professor David Bishop
Telephone number: 03 9919 9471
Mobile number: 0435 962 364
Email: david.bishop@vu.edu.au

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate

You are invited to participate in a research project entitled:

"TAKING THE PLUNGE: DOES HYDROTHERAPY HELP OR HINDER ADAPTATION TO TRAINING?"

This project is being conducted by a student researcher James Broatch as part of a PhD at Victoria University under the supervision of Dr. Aaron Petersen and Prof. David Bishop [College of Sport and Exercise Science and ISEAL]

Project explanation

The project aims to investigate the efficacy of chronic exposure to hydrotherapy following exercise, and its effects on performance, fatigue and adaptations to training. The results of this research will have a significant and direct application to current sporting practice, and will be relevant to all athletes utilising post-exercise recovery techniques. Furthermore, the results have the potential to alter current sporting practice, and may provide the rationale for modifying current post-exercise recovery techniques.

What will I be asked to do?

If you give your consent to participate in this project, you will be asked to visit the Exercise Physiology Laboratory at Victoria University Footscray campus to take part in the following testing and training sessions:

- 2-3 x 30 min pre-testing sessions
- 1 x 4 h acute exercise trial session
- 1 x 6-7 week exercise training program
- 2-3 x 30 min post-training testing sessions

We will first ask you to fill in several short questionnaires about your family medical history and your exercise habits to determine your ability to participate in this study. Prior to beginning the testing sessions, you will be randomly allocated to either a cold-water immersion (CWI) or a passive control (CON) group, and either a high-intensity interval training (HIIT) or resistance training (RT) group. You will therefore be allocated to one of four groups; RT-CWI, RT-CON, HIIT-CWI, or HIIT-CON. The study is divided into four phases as listed above.

Phase 1 – Pre-Testing:
The first pre-testing session will consist of a dual-energy X-ray absorptiometry scan (DXA) to measure whole body muscle and fat mass (RT groups only) or a graded exercise test (GXT) to measure your VO_{2max} and lactate threshold (HIIT groups only). 48 h later, the second pre-testing session will consist of a baseline performance measure – a 50kJ (2km) cycling time trial (HIIT groups), and one repetition maximum (1RM) strength testing (RT groups). The high-intensity group will also be required to perform a third pre-testing session – a 750kJ (30km) cycling time trial 48 h after the 50kJ time trial.

Phase 2 – Acute Exercise Trial:
One week after baseline testing you will be asked to complete the acute exercise trial. This will consist of one bout of your assigned exercise protocol, followed immediately by your assigned recovery condition, with muscle

biopsy sampling, blood sampling, psychological measures and muscle temperature measurements taken throughout.

Phase 3 – Training Intervention:
The acute exercise trial will also double as the first session of a 6-week HIIT or 7-week RT program. During the training intervention you will be required to attend the laboratory multiple times during the week. Each session will comprise of an exercise bout followed immediately by your assigned recovery condition. Blood samples, psychological measures and performance measures will be taken at the end of each week (Fri session) to monitor your progress during the training intervention.

Phase 4 – Post-Testing:
Finally, a resting muscle biopsy and DXA scan (RT groups) or GXT (HIIT groups) will be performed 48 h after your last training bout at the post-training testing session. The HIIT groups will have an additional two post-training testing sessions to repeat the 50kJ and 750kJ time trials. These will be completed a further 48 h and 96 h after the first post-training testing session, respectively.

How will this project be conducted?

Pre-experiment subject screening:

All testing will be conducted in accordance with current guidelines for testing in the Exercise Physiology Laboratory, College of Sport and Exercise Science, Victoria University. You will initially be screened for cardiovascular risk factors and any health issues of relevance to the study via a questionnaire. Participants with the following conditions will be excluded from the research as it may be unsafe for you to conduct the exercise intervention: diabetes (Type 1 or 2), chronic heart disease, severe hypertension (systolic 160-179 mmHg systolic, diastolic 100-109 mmHg), severely overweight/obese (BMI> 30), if you have had uncontrolled metabolic (such as uncontrolled diabetes) and/or cardiovascular disease, any recent significant injury that will impede your ability to perform exercise during the study or any other contraindications that will impede your ability/safety during exercise. If you are deemed healthy and at low risk of any adverse events, we will then request you to complete the five phases involved in this study.

Pre-testing:

Duel energy x-ray absorptiometry (DXA) scan – (RT groups)
Your whole body muscle and fat mass will be assessed by dual-energy X-ray absorptiometry scan (DXA). This machine is located at Victoria University Footscray Park campus. You will only be scanned once throughout the entire study, which provides a dose which is only approximately 10% of one day's natural radiation exposure from the atmosphere. The DXA machine has been assessed for compliance and meets the requirements of the Victorian DHHS Radiation Safety section.

1RM testing – (RT groups)

Baseline performance for the RT groups will comprise of two maximal strength tests (bench press and leg press exercises). You will be required to perform three sets (50%, 70%, 90% effort, two-six repetitions) of sub-maximal bench press and leg press exercises, followed by one set to failure of one to four repetitions. Each set to failure will used to predict your one repetition maximum (1RM). For the bench press, you will use a self-selected hand position, and will be required to lower the bar to 90° angle at the elbows and then press the bar in a vertical movement so that the arms are fully extended. During the leg press, you will use a self-selected foot position, and will be required to lower the weights to a position where the knee is at a 90 degree angle.

Graded exercise test (GXT) – (HIIT groups)

During this session you will undertake a maximal incremental exercise test to exhaustion for baseline determination of peak oxygen uptake (VO_{2Peak}) and lactate threshold (LT). The test will be performed on an electronically-braked bicycle ergometer and will consist of 4-min incremental steps with 1-min rest between each

step. The test will start at 90 W and increase by 30 W for each subsequent step until you reach volitional fatigue. Pedalling cadence will be fixed at 70 revolutions per minute (RPM), and during this test you will be required to wear a heart rate monitor. Expired gases will also be analysed during the test with the use of a metabolic cart (Moxus Metabolic System). This test is routinely performed in our laboratory.

Time Trials – (HIIT groups)

Baseline performance measures for the HIIT groups will comprise of a 50 kJ time trial followed 2 days later by a 750 kJ time trial. You will perform the time trials on a cycle ergometer as quickly as possible. The only feedback to be provided will be work completed, presented as 'distance covered' (i.e., 50kJ is equated to 2km, and 750kJ equated to 30km). These performance trials will be completed again in the post-testing phase, and a pre-post comparison will represent the effect the training intervention had on your cycling time trial performance.

Acute Exercise Trial – (RT and HIIT groups):

You will be asked to refrain from exercise in the 24 h preceding this trial, and report to the lab following consumption of a standardised meal the night before, and a subsequent overnight fast. During this session you will be required to perform one bout of your assigned exercise protocol followed immediately by 15 min of your designated recovery condition. Details of the exercise protocols are seen below in the training intervention section - the acute exercise trial will also double as the first session of the exercise training interventions. Details of additional measures to be taken during the acute exercise trial are given below.

Recovery Protocol

You will be randomly allocated into a cold-water immersion (CWI) or a passive control (CON) group. CWI will consist of immersion in 10°C water for 15 min, whereas CON will be sitting passively in the testing laboratory (23°C) for the same duration. Your assigned recovery protocol will be performed in a specifically purchased recovery pool (iCool Sport, Australia), in which the water temperature will be constantly monitored and controlled to within ±1°C of the desired temperature. Immersions will be performed at Victoria University, and the ambient temperature will be set to a constant 23°C.

Temperature Probe

During the exercise and recovery protocols, intramuscular temperature will be monitored via a needle thermistor probe. This probe will be inserted into the quadriceps muscle of your dominant leg (prior to exercise), 4 cm below the skin. Insertion is performed via the use of a needle and cannula, with the needle being removed once the probe is inserted and in place. The probe will also be held in place with a plastic adhesive cover. After the cessation of the recovery protocol, the probe will be removed from your leg. This is a well-established, accepted and well-practiced technique used to document intra-muscular temperature. Dr. Aaron Petersen and Mr. James Broatch are qualified to perform this procedure, and have performed this procedure many times in previous studies.

Muscle biopsy

Upon arrival for the acute exercise trial you will have a resting muscle biopsy taken. For the RT groups, a second biopsy will be taken 1 h after your recovery protocol and a third biopsy 48 h after recovery. This last biopsy will be performed at rest prior to the second training session in the RT program (detailed below). For the HIIT groups, a second biopsy will be taken immediately after your recovery protocol, after which you will then rest in ambient room air until the third and final biopsy at 3 h post-recovery.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue which can be used for analysis of proteins, genes and muscle function. The muscle biopsy will be performed by an experienced medical doctor under sterile conditions, and will consist of an injection of a local anaesthetic in the skin overlying the thigh muscle, and then a

small incision (approx. 0.6 cm long) will be made in the skin. The biopsy needle will be then inserted into your muscle and a small piece of tissue will be removed. During this part of the procedure you will feel pressure and this will be quite uncomfortable and you may also experience some pain, but it will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. The size of muscle removed by the biopsy needle is similar to 3-4 grains of rice. This poses no long-term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months. Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days. You should avoid heavy knocks for 24 hours after biopsy. It is common for participants to experience some soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore soreness. In some rare cases mild haematomas have been reported, but these symptoms disappear within a week. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.

Psychological measures

During the acute exercise trial you will be required to fill out two questionnaires; the 6-item Profile of Mood Sate (POMS) questionnaire to assess fatigue, vigour, depression, anxiety, confusion and anger, and the preference for and tolerance of the intensity of exercise questionnaire. The tolerance questionnaire will be administered once only prior to the acute exercise trial. The POMS will also be administered before, during and after the acute exercise trial at pre-exercise, immediately post-exercise, and immediately post-recovery intervention. The questionnaires will take approximately 20 seconds each to complete.

Venous blood sampling

An indwelling venous catheter will be inserted into your forearm vein before the first muscle biopsy. Blood samples will be collected by a trained phlebotomist during the acute exercise trial at the same time points as the muscle biopsies. Samples will be analysed for hormones associated with the exercise and fatigue, such as cortisol, testosterone, ACTH, growth hormone and prolactin concentrations. All techniques are established and available in the laboratories at Victoria University, and will be conducted by people who are qualified and competent with these procedures. All techniques will be conducted in accordance with current guidelines for blood sampling exercise testing in the Exercise Physiology Laboratory, College of Sport and Exercise Science, Victoria University.

<u>Training Intervention:</u>

High-intensity interval training (HIIT) protocol

The training protocol will require you to train 3 days per week for 6 weeks. Training will consist of "all-out" 30-s efforts separated by 4 min of light cycling (0.5 W/kg). Before each training bout you will warm up for 5 min on the cycle ergometer (100W), followed by 1 min of rest prior to commencing the protocol. You will be then instructed to pedal as fast as possible from the start of exercise and will be verbally encouraged throughout each 30-s bout to maintain maximal pedalling speed. During these bouts, we will be measuring your power output and rate of fatigue. You will complete 4 all-out bouts per training session in weeks 1-2, 5 in weeks 3-4, and 6 per session in weeks 5-6. After every training session, you will perform your assigned recovery session as described earlier.

Resistance training (RT) protocol

The RT protocol will be a training program consisting of a 14-day baseline training period followed by a 21-day intensified training period and a 14-day taper (7 weeks in total). The baseline and taper phases will require you to train for three session per week (Tue, Thu and Fri), whilst the intensified training phase will consist five

consecutive days of training (Mon-Fri). After every training session, you will perform your assigned recovery session as described earlier.

Training measures

A number of performance, physiological and psychological measures will be taken throughout the training intervention. For the RT groups, you will be assessed for strength (bench press, leg press) and power measures (bench throw, vertical jump) on Fridays prior to, and each week throughout the intensified and tapering phases. For the HIIT groups, performance will be monitored as your power output and ability to resist fatigue during the 30-s efforts. Blood samples will also be collected on the same days as performance measures (at the end of each week), collected pre-exercise, post-exercise and post-recovery. You will finally be asked to complete the modified Sport Inventory for Pain (SIP-15), and the modified (one week time frame) Pittsburgh Sleep Quality Index (PSQI) questionnaires, which will administered on the same days as the performance assessments.

Post-training testing:

Two days after your last training bout a resting muscle biopsy, DXA scan (RT groups), and GXT (HIIT groups) will be performed at the post-training testing session. The HIIT groups will have an additional two post-training testing sessions to repeat the 50kJ and 750kJ time trials. These will be completed a further 48 h and 96 h after the first post-training testing session, respectively.

Important things to understand and what are the potential risks of participating in this project?

Before you volunteer to this study, make sure you read carefully the items below:

1. You are free to withdrawn from this study at any time without any consequences or need for explanation.
2. All exercise activity carries a risk of injury and risks of suffering a heart attack or stroke. It is important that you tell us if you have any medical condition.
3. For any medical emergencies a call to 000 will be made. The researchers will also commence appropriate resuscitation methods while waiting for an emergency team to arrive.
4. All exercise sessions involve risks of fainting, soft-tissue injury, muscle soreness and stiffness, and sudden death due to myocardial infarction. The risk of such events is in reality very low.
5. Risks associated with venous catheterisation and blood sampling include discomfort, bruising and infection (for example puss, tenderness and/or redness). All these events are very unlikely.
6. The muscle temperature probe technique is likely to cause some discomfort. The feeling has been described as being given a very localised “dead leg” that lasts approximately 1 to 2 days; although each individual will respond differently, with some scarcely feeling it. In all cases there is a very minimal risk of infection at muscle temperature sites.
7. Muscle biopsy risks include possibility of infection and localized altered sensation of skin and/or muscle. However significant adverse effects are rare and if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.
8. Due to the DXA scan, this research study involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisieverts (mSv) each year. The effective dose from this study is less than 0.02mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. This risk is believed to be minimal.
9. Furthermore, the training protocol you will perform (high-intensity or resistance exercise) can be accompanied in rare cases by the possibility of mood changes such as irritability and depression. These

changes are however short term and can be reversed with a maximum of two to three weeks of recovery. To minimise the risk of any psychological stress, your results will remain confidential. Should you experience any adverse or unusual psychological changes, you will be immediately removed from the study and "debriefed" by speaking with the psychologist associated with this study, Dr Harriet Speed (Victoria University Psychologist). Dr Harriet Speed can be contacted on (03) 9919 5412. With this safeguard in place, you will have access to counselling, free of charge in the case of any psychological event.

What will I gain from participating?

From participating in this study you can expect to increase your understanding of fitness tests used by sport scientists, which will be performed in a state-of-the-art purpose-built training facility. You will also gain the experience of having participated in an exercise science experiment designed to increase knowledge about the effectiveness of recovery protocols used after training and competition. Furthermore, due to the nature and duration of the training intervention, you may or may not see improvements in muscle size and strength (RT groups) or cardio-respiratory fitness (HIIT groups).

How will the information I give be used?

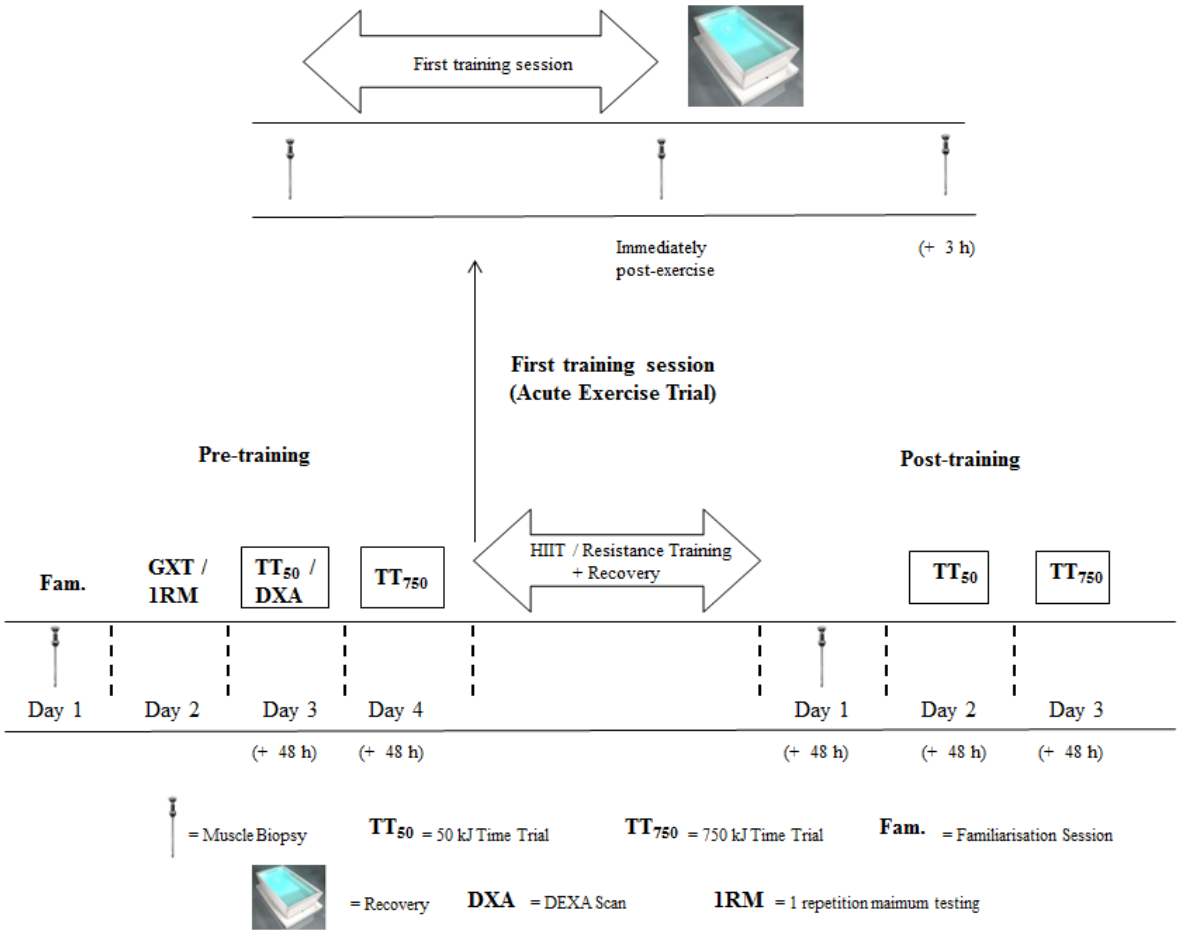
Your samples will be stored under alphanumeric codes (i.e., without your name or personal details) and only the researchers will be able to connect the samples to you. All of the muscle samples collected will be used to measure proteins involved in mitochondrial biogenesis and muscle growth following acute exercise and training. All blood samples will be used to measure hormones and inflammatory markers present in the post-exercise recovery response (cortisol, testosterone, ACTH, growth hormone and prolactin concentrations). The data that will be collected during the study will be used/published in peer-reviewed journals and conference presentations. No personal details will be revealed without your written consent.

Who is conducting the study?

The study is conducted by the College of Sport and Exercise Science, Victoria University, Footscray Park Campus. The main investigators are:

Dr. Aaron Petersen, Telephone number: 9919 9452, email: aaron.petersen@vu.edu.au
Mr. James Broatch, Mobile: 0422 050 361, email: james.broatch@live.vu.edu.au

Any queries about your participation in this project may be directed to the Principal Researcher listed above. If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.


First training session
Immediately post-exercise
(+ 3 h)
First training session (Acute Exercise Trial)
Pre-training
Post-training
Fam.
GXT / 1RM
TT50 / DXA
TT750
HIIT / Resistance Training + Recovery
TT50
TT750
Day 1
Day 2
Day 3
Day 4
Day 1
Day 2
Day 3
(+ 48 h)
(+ 48 h)
(+ 48 h)
(+ 48 h)
(+ 48 h)
= Muscle Biopsy
TT50 = 50 kJ Time Trial
TT750 = 750 kJ Time Trial
Fam. = Familiarisation Session
= Recovery
DXA = DEXA Scan
1RM = 1 repetition maimum testing

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study entitled:

"TAKING THE PLUNGE: DOES HYDROTHERAPY HELP OR HINDER ADAPTATION TO TRAINING"

AIMS OF THE STUDY:

The major aim of this project is to investigate the efficacy of chronic exposure to hydrotherapy following exercise, and its effects on performance, fatigue and adaptations to training. You will be allocated to one of two training interventions; a resistance training (RT) group or a high-intensity interval training (HIIT) group.

PROCEDURES INVOLVED AND NATURE OF THE PROJECT:

You will be randomly allocated into a resistance training (RT) or high-intensity interval training (HIIT) group. Pre-testing will consist of tests for bench press and leg press strength (RT group only), or a graded exercise test (GXT; HIIT group only). You will then be allocated to either a cold-water immersion (CWI) or thermo-neutral water immersion (TWI) group. Further pre-testing will also consist of a dual-energy X-ray absorptiometry (DXA) scan (RT only), or two time trials (50kJ and 750kJ; HIIT only).

One week after pre-testing you will perform an acute exercise trial, comprised of a single bout from the assigned training protocol followed immediately by the designated recovery condition. You will be asked to refrain from exercise in the 24 h preceding this trial, and report to the lab following a standardised meal the night before and an overnight fast. Muscle biopsy sampling, blood sampling, psychological measures and muscle temperature measurements will be taken throughout the acute exercise trial. This trial will also double as the first session of a 6-week HIIT or 7-week RT program. Blood samples, psychological measures and performance measures will be taken at the end of each training week (Fri) to monitor progress. Finally, a resting muscle biopsy, DXA scan (RT only), and repeat GXT and time trials (HIIT only) will be performed 48 h after the last training bout for post-training testing.

All procedures will be performed at the Sport, Exercise and Active Living precinct at Victoria University.

CERTIFICATION BY SUBJECT

I, _____(Phone No:)_____

of _____

certify that I am at least 18 years old and that I am voluntarily giving my consent to participate in the study:

"TAKING THE PLUNGE: DOES HYDROTHERAPY HELP OR HINDER ADAPTATION TO TRAINING"

being conducted at Victoria University by: **Dr. Aaron Petersen, Prof. David Bishop, Prof. Remco Polman and Mr. James Broatch.**

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

Dr. Aaron Petersen or Mr James Broatch

and that I freely consent to participation involving the below mentioned procedures:

- Pre-test screening
- Performance measures – 1-RM (RT) or GXT and time trials (HIIT)
- Resistance training (RT) or high-intensity interval exercise training (HIIT)
- DXA scan (RT group)
- Recovery modality
- Temperature probe insertion
- Muscle Biopsies
- Psychological questionnaires
- Blood samples (venous and capillary samples)

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed: _____

Date: _____

Any queries about your participation in this project may be directed to the principal researcher
Dr. Aaron Petersen
Telephone number: 03 9919 9452
Fax number: 03 9919 4891
Email: aaron.petersen@vu.edu.au

If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148