DOCTOR OF PHILOSOPHY

Environmental extremes and the immune response to exercise

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ENVIRONMENTAL EXTREMES AND THE IMMUNE RESPONSE TO EXERCISE

by

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Summary

Exercising in hot and cold conditions poses one of the greatest challenges to human homeostasis. Popular belief is that cold exposure increases susceptibility to upper respiratory tract infections, however, evidence to support a link between cold exposure, impaired immune function and increased incidence of infection is not well defined. On the other hand, if heat production during exercise is not effectively dissipated, core body temperature can rise to dangerous levels, thereby placing the individual at risk of developing exertional heat-illness (EHI), or the more serious, and potentially fatal, condition of exertional heat stroke (EHS). In addition to exercising in hot and humid environments, a number of risk factors for EHI/EHS have been identified. Other potential risk factors, which are less well supported, include the circulating inflammatory response that follows a muscle-damaging exercise bout. All together, these conditions might potentially affect athletes and military personnel, which are expected to perform arduous physical activity, often in extreme environments.

Hypothermia is common in trauma victims and is associated with an increase in mortality. Its causes are still not well understood. We found (Chapter 4) that after mild-hypothermia (Body rectal temperature (T_{re}) 35.17 ± 0.33 °C) vaccine-stimulated IFN-γ production significantly decreased by 44% suggesting temporary immune suppression. Moreover, despite rewarming and feeding, vaccine-stimulated IFN-γ production did not return to control values within 3-hour, suggesting more prolonged immune suppression, specifically, impaired antimicrobial capacity and increased risk of infection. This might partially explain the increased mortality reported after mild-hypothermia.

During exercise heat stress (HS) (Chapter 5), ΔT_{re} was significantly greater following EIMD than in CON (0.52 ºC). Therefore, HS was increased during endurance exercise in the heat conducted 30min trial after, and to a much lesser extent, 24h after muscle-damaging exercise. These data indicate that EIMD is a likely risk factor for EHI particularly during exercise-heat stress. After a repeated bout of muscle-damaging exercise (EIMD trial 2) (Chapter 6), final T_{re} during HS was lower (39.25 ± 0.47 ºC) than in EIMD trial 1 (39.59 ± 0.49 ºC), whilst no differences between repeat trials were observed in control trials (CON). Thus, incorporating a muscle-damaging bout into training is a strategy to reduce the risk of EHI in individuals undertaking heavy exercise with an eccentric heat component.

Further research is required clarify the role of thermo-genic activity on innate immune markers during cold exposure needs clarification. In addition, future studies would need to examine the supposed contribution of pyrogenic pathways after muscle damage upon exercise heat strain in more depth.

Declaration

This work has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Statement One

This thesis is the product of my own investigations, except where otherwise stated. Other sources are acknowledged giving explicit references.

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Statement Two

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Publications

I have been involved in all aspects of protocol design, data collection, data analyses and preparation of manuscripts for publication of the thesis chapters that follow.

However, I also gratefully acknowledge input from the co-authors of each publication.

The findings of some of the material presented in this thesis have been published as follows:

Full papers

Repeated muscle damage blunts the increase in heat strain during subsequent exercise heat stress.

Dolci A, Fortes MB, Walker FS, Haq A, Riddle T, Walsh NP.

Eur J Appl Physiol. **2015** Jul; 115(7):1577-88.

I was involved with study design, data collection, data analysis and manuscript writing.

Portable prehospital methods to treat shivering and non-shivering cold casualties.

Oliver S, Brierley J, Raymond-Barker P, Dolci A, Walsh NP.

Wilderness & Environ Med. **2015** Nov 11:012.

This publication is associated to the present thesis as part of a larger study (Chapter 4) in which I was a component. I was involved in data collection, data analysis and manuscript writing.

Muscle-damaging exercise increases heat strain during subsequent exercise heat stress.

Fortes MB, Di Felice U, Dolci A, Junglee NA, Crockford MJ, West L, Hillier-Smith R, Macdonald JH, Walsh NP.

Med Sci Sports Exerc. **2013** Oct; 45(10):1915-24.

I was involved with study design, data collection, data analysis and manuscript writing.

Exercising in a hot environment with muscle damage: effects on acute kidney injury biomarkers and kidney function.

Junglee NA, Di Felice U, Dolci A, Fortes MB, Jibani MM, Lemmey AB, Walsh NP, Macdonald JH.

Am J Physiol Renal Physiol. **2013** Sep 15; 305(6).

This publication is associated to the present thesis as part of a larger study (Chapter 5) in which I was a component. I was involved in data collection and data analysis.

Impaired immune function after mild hypothermia and prolonged cold exposure.

Dolci A, Oliver SJ, Brierley JL, Walsh N, Gleeson M.

(In preparation, 2016).

I am involved with study design, data collection, data analysis and manuscript writing.

Abstracts

The repeated bout effect blunts the increase in heat strain during exercise-heat-stress after exercise-induced muscle damage.

A. Dolci, M. B. Fortes, A. Haq, F. S. Walker, T. Riddle & N. P. Walsh FACSM

Invited speaker at the highlighted symposium of the American College of Sport Medicine (ACSM), Annual meeting.

May 2014, Orlando (USA).

Impaired immune function after mild hypothermia and prolonged cold exposure.

Dolci A, Oliver SJ, Brierley JL, Walsh N, Gleeson M.

Mini-talk presentation at European College of Sport Sciences (ECSS).

June 2013, Barcelona (Spain).

Short-term increase in plasma IL-6 after downhill running is associated with increased core temperature during subsequent exercise heat-stress.

M. B. Fortes, U. DiFelice, A. Dolci and N. P. Walsh FACSM

The Physiological Society: The Biomedical Basis of Elite Performance.

Presented by: Fortes MB.

March 2012, London (United Kingdom).

An Experimental Study of Practical Field Methods for Cold Casualty Protection.

Brierley JL, Oliver SJ, Dolci A, Walsh N, Gleeson M.

College of Sport Medicine (ACSM), Annual meeting.

Presented by: Brierley J.

May 2012, S. Diego (USA).

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List of Abbreviations

AB	Antibody
ACSM	American College of Sports Medicine
ACTH	Adrenocorticopic hormone
ALI	*Ad libitum* fluid intake
AMPS	Antimicrobial peptides
ANOVA	Analysis of variance
BBB	Blood brain barrier
BM	Body mass
BML	Body mass loss
°C	Degrees Celsius
$CD14^{+\,+}$	Cluster of differentiation 14
$CD16^{+}$	Cluster of differentiation 16
$CD4^{+}$	Cluster of differentiation 4
CK	Creatine Kinase
cm	Centimetre
CNS	Central Nervous System
CON	Control group
CV	Co-efficient of variation
Da	Dalton
DEXA	Dual Energy X-ray Absorptiometry
DOMS	Delayed Onset Muscle Soreness
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
EHI	Exertional Heat Illness
EHS	Exertional Heat Stroke
eHSP	Extracellular Heat Shock Protein
eHsp72	Extracellular heat shock protein 72
EIMD	Exercise-Induced Muscle Damage
ELISA	Enzyme-linked immunosorbent assay
ER	Energy Restriction
FFA	Free fatty acid
FI	Fluid Intake
Fig	Figure
FR	Fluid Restriction
g	Gram
h	Hour
HPA	Hypothalamic-pituitary-adrenal axis sympathoadrenal medullary
HR	Heart Rate
HR_{max}	Maximal heart rate
HS	Exercise-heat-stress test
HSE	Heat Shock Element
HSP	Heat Shock Protein
Hsp	Heat shock protein
Ig	Immunoglobulin

IL	Interleukin
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
°K	Degrees Kelvin
Kg	Kilogram
Km	Kilometre
L	Litre
LPS	Lipopolysaccharide
$\dot{M}$	Metabolic energy expenditure
m	Metre
MET	Metabolic Equivalent of Task
Mg	Milligram
Min	Minute
mL	Millilitre
Mmol	Millimole
mOsmol	Milliosmole
MRI	Magnetic Resonance Imaging
NBM	Nude Body Mass
NK	Natural Killer cells
NKCA	Natural Killer Cells Activation
NTS	Nucleus Tractus Solitarii
PGE2	Prostaglandin E2
r	Pearson's correlation coefficient
RA	Receptor agonist
RER	Respiratory Exchange Ratio
RH	Relative Humidity
RMR	Resting Metabolic Rate
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
SAM	Sympathoadrenal medullary
SD	Standard deviation
SEM	Standard Error of the Mean
SIg	Saliva Immunoglobulin
s-IgA	Saliva immunoglobulin-A
SIRS	Systemic Inflammatory Response Syndrome
SNS	Sympathetic Nervous System
SWOP	Second Window Of Protection
TBW	Total Body Water
Tc1/ Tc2	Type-1/Type-2
Th1	Type-1
Th2	Type-2
TLR	Toll like receptors
TNF	Tumour necrosis factor

T_{re}	Rectal temperature
T_{sk}	Skin temperature
TT	Time Trial
TTE	Time To Exhaustion
URI	Upper Respiratory tract Infection
URTI	Upper Respiratory Tract Infection
USG	Urine Specific Gravity
VO_{2max}	Maximal oxygen uptake
W	Watts
WURSS	Wisconsin Upper Respiratory Symptom Survey
yrs	Years

Thesis Format

A literature review (**Chapter 2**) provides a brief background and proposes the broad aims of the research presented in the thesis. A general methods chapter follows that outlines the common procedures and analyses performed in the subsequent three independent experimental studies (**Chapter 3**). The first study principally investigates the effects of mild-hypothermia on immune system function (**Chapter 4**). The second experimental study is divided into two chapters: the first investigates whether exercise-induced muscle damage (EIMD) increases heat strain during subsequent exercise heat stress, which in turn may increase the risk of exertional heat illness (**Chapter 5**). The second examines the influence of exercise-induced-muscle–damage repeated bout effect upon heat strain during subsequent exercise in the heat (**Chapter 6**). A general discussion (**Chapter 7**) contains a summary and critical analysis of the main findings of the research programme, highlighting limitations and potential areas for future research. Since all chapters are linked, at times there is necessary overlap between them. Throughout the thesis, abbreviations are defined at first use and for clarity a list of abbreviations, tables and figures appears prior in the **Frontispiece**.

Bold type is used to refer to sections elsewhere within the thesis.

CHAPTER ONE

General Introduction

Extreme temperature exposure or single-day, multiple strenuous exercise bouts (singly or in any combination) are common in active populations (e.g. military personnel, athletes, outdoor enthusiasts). As result, these individuals can have an increased susceptibility to Upper Respiratory Tract Infections (URTI) (**Chapter 4**) or be predisposed to exertional heat illness (EHI) and exertional heat stroke (EHS) (**Chapters 5 and 6**).

Popular belief points towards cold exposure increasing one's susceptibility to infections. Evidence to support a link between cold exposure and an increased infection incidence is lacking in human studies (Castellani and Rhind, 2002). The few human studies to investigate the effect of cold exposure on immune function report conflicting information that is dependent on the degree of hypothermia, the health of the individual, and the immune parameters studied (Lackovic et al., 1988; Brenner et al., 1999; Wenisch et al., 1996; Costa et al., 2010). Collectively, these studies suggest that lowering core temperature (core body temperature decrease > 1 °C) can disrupt circulating immune function, which may partly explain the increased URTI reported after cold exposure. Limitations of previous research are that they included unhealthy population (e.g. patients undergoing surgery) and they did not perform a thermoneutral control trial (Wenisch et al., 1996; Beilin et al., 1998). Therefore it is unclear whether surgery, anaesthesia, existing comorbidity and diurnal variation have independent effects for the altered immune function reported. Thus it remains to be shown in healthy humans whether immune function is impaired when core temperature is reduced (e.g. core temperature decrease of 1–2 °C).

Oppositely, active populations such as athletes and soldiers taking part in heavy training are also expected to perform multiple bouts of strenuous exercise in hot environments, which may predispose them to EHI or the more severe form, and often incurable EHS. Conventionally classified risk factors for EHI and EHS include hot and humid environmental conditions, unsuitable clothing, sleep disruption, absence of heat acclimation, high exercise intensity, poor physical fitness levels (or obesity) and

underlying undiagnosed medical conditions (Armstrong et al., 2007; Epstein et al., 1999; Rav-Acha et al., 2004). These conventional risk factors do not however explain all EHI events, this begs the question: "Which lesser known risk factors or pathways might play important roles in many cases of EHI?" (Sawka et al., 2011) (**Chapter 4**).

One further pathway was suggested for EHI progression; this relates to a systemic inflammatory reaction with an incremental increase in pyrogenic cytokines [e.g., interleukin (IL)-1β, IL-6, and tumour necrosis factor-α] (Bouchama, 1995; Lambert et al., 2001; Shephard and Shek, 1999). This may result in quicker progression toward EHI and/or EHS (Walsh et al., 2011). If firm evidence of a pyrogenic cytokine role in the development of EHI is uncovered, it would be of great interest to investigate, and develop strategies of avoiding or managing EHI. Reducing the amount of muscle damage and inflammation would plausibly decrease heat strain. Indeed, evidence showed that over 80% of all EHI casualties reported during Marine recruits training occurred in the first 3 days of unaccustomed exercise in the heat (Kark et al., 1996). This is also supported by data collected during Operation TELIC in the summer of 2003 where a heat illness rate of 50/1000 were recorded during the first 10-14 days of deployment among troops that were performing arduous physical activity in Afghanistan's tactical scenarios (Bolton et al., 2006). One obvious, but as yet un-investigated, avenue is the well-known repeated bout effect in muscle-damaging exercise, where a single session of eccentric exercise causes adaptations which in-turn result in decreased damage from subsequent training (Clarkson et al. 1992; Clarkson and Hubal 2002; McHugh et al. 1999) (**Chapters 5 and 6**).

Consequently, the aims of this thesis were to investigate:

1. The unknown effect of mild hypothermia and prolonged cold exposure on immune function in healthy humans.
2. If exercise-induced muscle damage (EIMD) increases heat strain during subsequent exercise-heat-stress, which in-turn may increase the risk of exertional heat illness.

3. If the repeated-bout of EIMD blunts the increase in rectal temperature (T_{re}) during subsequent endurance exercise in the heat.

CHAPTER TWO

Review of Literature

Introduction

In the 1950's a Canadian physician, Hans Selye pioneered the scientific study of stress. He first defined the word "stressor" (Selye, 1956) as influences that put the human body out of homeostatic balance. This definition came from his research on the physiological responses to exhaustive exercise, prolonged exposure to extreme environments, and other damaging stimuli.

Indeed, the human body tries to adapt to stressors by counteracting and re-establishing homeostasis (Elliot and Eisdorfer, 1982; Mausch and Rulkowska, 1993; Weiner, 1992); therefore, an adaptation represents improved body function (Baker, 1974; Cannon, 1929; Mrosovsky, 1990; Sherrington, 1941). This adaptation is also defined by its duration; short-term adaptations are classed as accommodations; intermediate-term adaptations as acclimation or acclimatization; and long-term adaptations as genetic adaptation (Armstrong, 2000; Fregly and Blatteis, 1996).

The nervous, endocrine and immune systems have been classically seen as independent responders to stressors. However, recently findings has led to the idea that for some purposes these systems interact as a large single, system serving an integrated function rather than as three distinct systems (Berne and Levy, 1996; DeSouza and Appel, 1991; Dunn, 1989; Dunn and Berridge, 1990; Guyton and Hall, 1996; Mason, 1972) (**Figure 1.**).

Therefore, the next chapter will focus on the immune responses to external stressors, such as physical exercise (e.g. moderate and intense) and extreme environments (e.g. cold and heat).

Each section will describe in detail the factors mentioned, providing a "picture" of the processes that the immune system uses to counteract stressors.

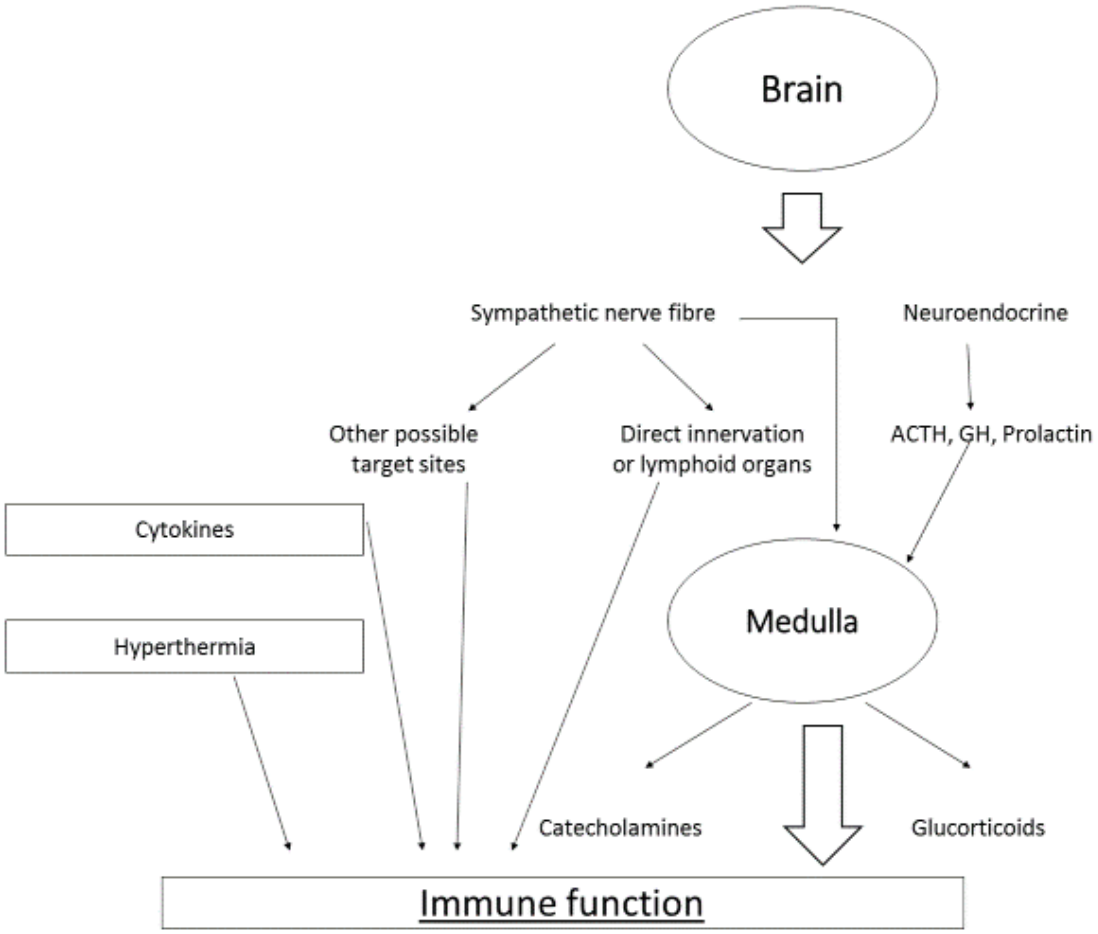

Fig. 1. The two pathways by which the CNS may communicate with the periphery include neuroendocrine outflow, via the hypothalamic–pituitary–target organ axis, and the automatic nervous system, through direct nerve fibre connections with cells or the organs of the immune system. ACTH, Adrenocorticotropic hormone; GH, Growth hormone (Adapted from Jonsdottir et al., 2000).

Immune function and exercise

From the early 20th century, exercise has been suggested by physicians as a cause of a decreased resistance to bacterial diseases (Bosch et al., 2002; Shephard, 1997; Shephard, 2010; Shephard and Shek, 1996; Walsh et al., 2011). These concerns were legitimised by an increased number of URTI reported after endurance competitions (Nieman et al., 1990; Peters, 1997; Peters and Bateman, 1983).

2.1. Effect of exercise on innate immune function

Presently, research in understanding if exercise-induced changes in innate immune function alter infectious disease susceptibility or outcome is at an early stage. It remains unclear if the exercise-induced effects on innate immune cells, due to regular exercise, mediate any anti-inflammatory effect. Innate immunity, following skin, represents our second line of defence against pathogens and unlike adaptive immune responses it does not strengthen after exposure. Moreover, its ability to recognise pathogens is less specific.

The innate immune system is based principally on cells and soluble factors. Soluble factors are a heterogeneous group of proteins, which include interferons that restrict viral infection, anti-microbial peptides which help to prevent bacterial growth, and complement proteins which control phagocytosis, inflammation and interact with antibodies (Woods et al., 1998). Taken together, Soluble factors help to activate other immune cells, such as macrophages.

Indeed, the main contributor of the innate immune response is the macrophage, followed by neutrophils, dendritic cells and monocytes. These versatile cells plays numerous roles but mainly perform phagocytic, antigen presentation, and regulation functions.

It is really important to note that innate and adaptive immune responses, thanks to antigen presentation and regulation functions are inextricably linked together (Delves et al., 2011; Glaser and Kiecolt-Glaser, 2005; Janeway and Medzhitov, 2002; Kindt et al., 2006; Murphy, 2011; Parham, 2009; Playfair and Bancroft, 2004; Shetty, 2010; Smith et al., 2012). Dendritic cells are the main envoys between the innate and adaptive immune system but dialogue between the innate and adaptive immune systems is not one sided. Elements of the adaptive response also support the function of the innate immune system. For example, antibodies secreted by B cells bind to macrophages and other phagocytes by interaction with Fc receptors on these cells. Phagocytes can

efficiently recognize, internalize, and destroy pathogens using these borrowed antibodies (Clark and Kupper, 2005).

2.1.1. Macrophages

Macrophage function appears to be dependent on exercise intensity, where moderate exercise enhances many macrophages functions (adherence, chemotaxis, phagocytosis and microbicidal activity), while acute exercise to exhaustion appears to have no effect on macrophage functions (Ortega, 2003). Stress hormones, such as cortisol seem to be involved in the regulation of this process (Baybutt and Holshner, 1990).

Usually, macrophages, the tissue resident differentiation of monocytes, serve three main functions in the immune system. These are phagocytosis, antigen presentation, and cytokine production. Acute exercise reduces IL-6, IL-1β and TNF-α production from stimulated monocytes, perhaps due to a reduction in the number of Toll-like receptors (TLRs) (Lancaster et al., 2005). Due to maturation status and exercise-induced changes in monocyte function for example, this may not reflect actual tissue macrophage function, which is far more important.

Moderate and intense physical activity affects the tissue macrophage, which has stimulatory effects on chemotaxis (Okutsu et al., 2008; Ortega et al., 1997), phagocytosis (Ortega et al., 1996) and production of reactive oxygen species (ROS) (Woods et al., 1993; Woods et al., 1994), reduced macrophage MHC class II expression (Woods et al., 1997) and antigen presentation (Ceddia et al., 1999; Ceddia and Woods, 1999) especially by endurance exercise. It appears that the volume and intensity of exercise decreases tissue macrophage and disease outcome in animal studies; however, there is no evidence, at present, to expand this effect to humans.

2.1.2. Neutrophils

Neutrophils contribute about 50-60% of the circulating blood leukocyte cells. They are vital in innate immune responses as they are attracted to areas of infection (chemotaxis) and they ingest microbes (phagocytosis). This process is followed by enzymatic attack

and digestion within intracellular vacuoles, using granular hydrolytic enzymes and ROS in the oxidative burst; this process is called degranulation.

Neutrophils exert their main function outside blood, via a process called diapedesis. Their ability to do this is based upon the ability of neutrophils to emigrate from the blood vessels into surrounding tissue. Acute or exhausting exercise does not seem to affect their ability to adhere to endothelium (Lewicki et al., 1987; Ortega et al., 1993). Typically, the pathogen is overwhelmed by the neutrophils (phagocytosis) by extending pseudopodia. When these extensions are fused together the pathogens is trapped within intracellular vacuoles and then the neutrophils begin their attack. This neutrophil ability has been used to assess neutrophil function *in vitro*. Most of the reports show that there is an increase in phagocytic activity of neutrophils during intense physical activity (Hack et al., 1992; Lewicki et al., 1987; Ortega et al., 1993; Rodriguez et al., 1991), however, this evidence is still under debate (Blannin et al., 1996; Gabriel et al., 1994; Nieman et al., 1998; Rodriguez et al., 1991).

Even if neutrophil phagocytic activity is just one process that contributes to immunological integrity, it has been shown that long periods of heavy training may lead to a decrease in this activity at rest (Blannin et al., 1996), leading to an increased risk of getting opportunistic infections. Certainly, disorders of neutrophil function and neutropenia are associated with recurrent infections (Yang and Hill, 1991) and there is now convincing evidence to show that highly trained athletes are more susceptible to infection than their relatively sedentary counterparts (Brenner et al., 1994; Shephard et al., 1991). Indeed, exercise seems to induce degranulation by increasing the plasma levels of elastase and myeloperoxidase (Blannin et al., 1996; Robson et al., 1999; Suzuki et al., 2003), although this might merely reflect the simultaneous neutrophilia often observed (Suzuki et al., 1999). Unfortunately, during subsequent exercise, neutrophils can enter into a refractory activation period after exercise that may lead to a lower degranulation response to subsequent bacterial exposure (Bishop et al., 2002; Bishop et al., 2003; Blannin et al., 1997), supported by the observation that no changes

in total elastase content have been reported after physical exercise, which could mean there was no a depletion of neutrophil granules.

An attenuated ROS production has been reported after high intensity exercise (Hack et al., 1992; Pyne, 1994). Indeed, it has been demonstrated that the generation of hydrogen peroxide (H_2O_2) by stimulated neutrophils is reduced during acute exercise by plasma borne inhibitors, such as nicotinamide adenine dinucleotide phosphate-oxidase inhibitors. Although other researchers have shown the converse, an increased production of hydrogen peroxide and hydrochlorous acid (HClO) after moderate intensity exercise(Smith et al., 1990). This ambiguity may be a consequence of a shift in neutrophil function that could be dependent on exercise intensity (Dziedziak, 1990; Pyne, 1994; Suzuki et al., 2003). Furthermore, a decrease in respiratory burst has been reported in the hours (h) following a bout of intense physical activity (Pyne, 1994). In this case, the exercise-intensity seems to be the most important factor; shown in the decreased neutrophil oxidative burst during a marathon race (Suzuki et al., 2003). This process appears to be mediated by catecholamines, especially adrenaline, which decreases neutrophil respiratory burst *in vitro* elevating cycling adenosine monophosphate (Tintiger et al., 2001). Furthermore, pro-inflammatory cytokines, such as IL-6, appear to be involved in regulating this process, which would also be in-line with the different result found at moderate and high exercise intensities (Peake et al., 2005).

Thus, the combined uses of the neutrophilic arsenal, creates a hostile environment for any foreign bodies, leading to their destruction. The ability of neutrophils to kill has been shown to be unaffected or enhanced by acute exercise, irrespectively of intensity (Lewicki et al., 1987; Ortega et al., 1993; Rodriguez et al., 1991). Resting neutrophil bactericidal activity was similar in trained and sedentary subjects (Lewicki et al., 1987). The contradictions in the effects of acute and chronic exercise on neutrophil functions are attributable to the differences in age, gender and initial fitness levels of the subject (Wolach et al., 2000).

2.1.3. Natural killer (NK) cells

NK cells may serve as a "front line of defence" before a specific response can be mounted by the adaptive immune response, and do not require recognition of an antigen-MHC class II combination. The effects of intense exercise on NK-cell function appear to be biphasic, with an initial increase followed by delayed suppression (Kappel et al., 1991; Nieman et al., 1993; Pedersen, 1991).

Exercise induces an increase in the absolute number and percentage of blood NK cells, which also results in an increased cytolytic activity (Gannon et al., 1995; Pedersen et al., 1988; Roberts et al., 2004). The delayed suppression of NK cell function seems to be mediated via prostaglandins released from the relatively numerous monocytes observed after intense exercise (Kappel et al., 1991; Pedersen et al., 1988).

2.1.4. Other contributors to innate immune function

The stress hormones adrenaline and cortisol are involved in many of the changes in innate immunity outlined above. Indeed, they regulate innate immune cell function and the production of the leukocytosis by bone marrow release and demargination (Ortega, 2003). Cortisol release and clearance is regulated by exercise intensity; moderately intense exercise, which is often associated with enhanced immune cell function, decreases cortisol levels. On the other hand, high intensity exercise, which can depress innate immune cell function, increases cortisol secretion. The similarities between acute exhaustive exercise and acute inflammatory response lead to the conclusion that other immunological mediators are involved (Camus et al., 1992). For example, IL-6 increases during endurance exercise and is released by exercising muscle (Steensberg et al., 2000). A consequence of this event accounts for increases in cortisol secretion, and IL-1ra and IL-10 expression (Steensberg, 2003).

2.2. Effects of exercise on acquired immune function

Acquired immunity combats infections by not allowing colonisation of pathogens and by destroying invading microorganisms. It is initiated, in most cases, by antigen presentation to T helper lymphocytes, within the peptide-binding groove of major histocompatibility complex class II molecules on antigen-presenting cells. The subsequent cell-mediated immune response is mediated by inflammatory monocytes (CD4+) T-cells.

According to the cytokines they produce, helper T-cell clones can be divided into two main phenotypes: Type-1 (Th1) and Type-2 (Th2). Th1 cells play an important role in the defence against intracellular pathogens and Th2 cells are involved in the protection against extracellular parasites and stimulation of B-cell antibody (AB) production. Cytotoxic T cells can also be classified into Type-1 (Th1) and Type-2 (Th2) cells according to their cytokine profiles.

2.2.1. T-cells

As outlined above, T cells play a fundamental role in the organization and regulation of the cell-mediated immune response to pathogens. To cope with the enormous variability of environmental antigens, T lymphocytes comprise a collection of cell clones, each featuring the ability to specifically recognize a given antigen (Von Boehmer, 1997). Specificity of recognition, memory of previous antigen encounter and lack of self-reactivity are the physiological hallmarks of T lymphocytes. These features are acquired during the maturation of T cells, which takes place in the thymus and later on in secondary lymphoid organs (Von Boehmer, 1997). Antigen recognition leads to the clonal amplification of antigen-specific T cells, which will eventually acquire effector functions ranging from the ability to kill infected cells to the production of soluble paracrine factors required for cooperation among immune cells (Lanzavecchia and Sallusto, 2000). A defect of their function, such as inherited or acquired immunodeficiency syndromes, might lead to an increased incidence of infections (Fabbri

et al., 2003), so it is reasonable to think that the increased incidence of URTI recorded between sports enthusiasts may be due to exercise-induced decreases in T-cell function.

The effect of acute exercise on cell-mediated immune function has been most commonly assessed measuring cytokine production and cell proliferation (Gleeson et al., 2013). It has been reported that exercise had little or no effect on the portion of circulating Th2 cells, where conversely it had a decreasing effect on Th1 cell numbers. This might be due to changes in the distribution of the cell between circulation and tissues ,cell death, or that the increase in plasma epinephrine during exercise contributes to the suppression of IL-2-producing T cells and that high plasma IL-6 helps to maintain the IL-4-producing T cells in the circulation. Thus epinephrine and IL-6 may participate in the exercise-induced shift toward a relative type 2 T-cell dominance. However, other physiological changes, such as local inflammation, may also play a role. In addition, the present study supports the idea that an exercise-induced increase in plasma IL-6 induces elevated levels of plasma cortisol (Steensberg et al., 2001).

Athletes are prone to viral infections. Some researchers have argued this is due to an impaired Th1 cellular response, leading to a reduced host-defence reaction against intracellular pathogens. Indeed, there are no differences between sedentary people and athletes in circulating lymphocyte numbers during rest (Nieman, 2000). In contrast, decreases in circulating numbers of Type-1 T cells, reduced T cell proliferative responses, and falls in stimulated B cell immunoglobulin synthesis have been reported in athletes after long periods of intensive training, suggesting a decrease in T-cell functionality (Baj et al., 1994; Lancaster et al., 2004; Verde et al., 1992). However, there are many other processes that lead to lymphocyte proliferation and cytotoxicity, including cytokine production and pathways that require other co-stimulatory signals, including specific antigen encounters.

Numerous studies report that lymphocyte proliferation decreases during and after exercise (Walsh et al., 2011). This exercise-induced decline could be, in part, due to

changes in B cell responses to mitogen stimulation. However, given the small number of circulating B cells, this contribution appears to be small.

2.2.2. Monocytes (CD14, CD16)

Monocytes can be divided in two populations, classical ($CD14^{++}$) and inflammatory ($CD14^{+}$ and $CD16^{+}$) cells, with their difference residing in how they express cell-surface tool-receptor 4 (TLR4), with inflammatory monocytes having twice the number of receptors as classical monocytes (Skinner et al., 2005). Even if they represent only ten per cent of total monocyte population, they are a significant inflammatory potential of the monocyte pool as a whole (Belge et al., 2002).

There is evidence of a temporary increase of the inflammatory monocyte percentage after a single bout of intense exercise, with a quick return to baseline levels during recovery. Cortisol may be involved in reducing CD14+, CD16+ monocyte numbers with exercise training (Fingerle-Rowson et al., 1998). Studies have shown that high-intensity training results in increases in regulatory T-cell numbers and activation was also associated with reduced pro-inflammatory and increased anti-inflammatory cytokine expression (Wang et al., 2012).

2.2.3. Immunoglobulin contribution to immune function

An AB, also known as an immunoglobulin (Ig), is a protein produced by plasma cells that is used by the immune system to identify and neutralize pathogens (Kenneth, 2005). The largest immunoglobulin pool in the blood is Immunoglobulin (Ig)G, with a smaller quantity of Immunoglobulin (Ig)A and Immunoglobulin (Ig)M. The amount of Immunoglobulin (Ig)D and Immunoglobulin (Ig)E is almost insignificant. Therefore, most studies have focused on exercise-induced changes in serum IgA, IgG and IgM.

Serum immunoglobulin concentrations appear to remain either unchanged or slightly increased in response to moderate physical activity. When athletes run 45 to 75 km at high intensities, serum immunoglobulin levels have been reported to be depressed for up to 2 days. Thus intense ultra marathon running may lead to greater and longer lasting

decreases in serum immunoglobulin levels than following exercise of shorter duration. IgA and IgG, immunoglobulins commonly found in airway and alveolar space secretions, may have diffused from the serum during recovery from prolonged endurance exercise non-specifically and/or in response to microbial agents and antigens introduced into the airways during the exercise bout (Nehlsen-Cannarella et al., 1991; Nieman et al., 1989; Potteiger et al., 2001).

2.3. Cytokines contribution to immune function

Cytokines are known as an important family of intercellular molecules that influence inflammatory and immune responses. Cytokines can be categorised as being pro- or anti-inflammatory. Pro-inflammatory cytokines include IL-6, IL-1α, and -1β, and TNF-α. Examples of anti-inflammatory cytokines include IL-10, the IL-1 receptor agonist (RA) as well as soluble TNF-receptor (sTNFr) 1 and 2 (Heled et al., 2013). Both pro- and anti-inflammatory cytokines counter-regulate one another, in sequence. This maintains their levels in homeostasis (Pedersen and Hoffman-Goetz, 2000; Suzuki et al., 2002). Cytokine IL-6 (an inflammatory) causes pyrogenesis, together with IL-1 and TNFα. Both neutrophils and monocytes produce inflammatory cytokines in local inflammation responses and during healing.

2.3.1. Interleukin (IL)-6: release and functions

Studies have demonstrated that the release of Interleukin (IL)-6 is induced by exercise and that this release almost exclusively accounts for the elevated systemic IL-6 concentration found (Steensberg et al., 2000). Indeed, by obtaining arterial-femoral venous differences from an exercising leg and a resting leg, it has been found that exercising muscles released the total amount of circulating IL-6 (Pedersen et al., 2001). This demonstrates that only muscle contraction induces IL-6 and that secreted factors (e.g. adrenaline) do not play an important role in the exercise-induced increase in the systemic IL-6 concentration. In addition, even if strong evidence has been provided to

support IL-6 induction by exercise, it has not been mentioned that other cellular sources within the muscle (e.g. macrophages, the endothelium of the muscle capillary bed, adipose tissue or bone) may be the source of the increased IL-6 release from the muscle during exercise.

There is also evidence that the kinetics of IL-6 concentration/release during exercise is closely related to the duration of the exercise bout. A plausible explanation of this process could be that the depletion of muscle glycogen during prolonged endurance exercise increases the release of IL-6 in response to an energy crisis (Steensberg et al., 2000). This augmented concentration of IL-6 may signal the liver to increase its glucose output to prevent a drastic fall in the blood glucose concentration the muscles are now using as their main energy source. Supporting this hypothesis, studies have showed that carbohydrate ingestion during exercise, which provides an exogenous energy source, attenuates the systemic IL-6 concentration (Nehlsen-Cannarella et al., 1997; Starkie et al., 2001). More evidence has been provided to confirm that the IL-6 response to exercise is mainly dependent on exercise duration with a minor role played by exercise intensity (Fischer, 2006).

This is supported by recently presented data which revealed that the inflammatory cytokine IL-6 increases significantly in the plasma after a marathon run even after adjustment for leukocyte (Bernecker et al., 2013) and more modest increases are reported with shorter duration or intermittent exercise protocols or when carbohydrates are provided during exercise (Fischer, 2006; Meckel et al., 2009). Exercise-induced IL-6 increase is temporary, returning to basal levels within one-hour post exercise (Steensberg et al., 2000). The major stimulus of IL-6 synthesis and release seems to be a decrease in muscle glycogen, which results in an exponential increase of IL-6 concentration (Keller et al., 2005; Pedersen and Fischer, 2007). Indeed, muscle-derived IL-6 is released into the circulation in large amounts and is likely to work in a hormone-like fashion, exerting its effect on the liver and adipose tissue, thereby contributing to the maintenance of glucose homeostasis during exercise.

In addition, it is likely that the great increase in IL-6 in response to long-duration exercise is independent of muscle damage, while muscle damage itself is followed by repair mechanisms including invasion of macrophages into the muscle leading to IL-6 production. IL-6 production in relation to muscle damage occurs later and is of smaller magnitude than IL-6 production related to muscle contractions. Increases in intracellular calcium and ROS production could also activate transcription factors, such as NF-IL-6 and p38/RK known to regulate IL-6 synthesis (Fischer, 2006). Using arterio-venous difference techniques or muscle biopsies does not provide definitive information on the actual cellular source of the exercise-induced increase in systemic IL-6 concentration. To determine the cellular source of the exercise-induced increase in IL-6, researchers obtained muscle biopsies at rest and following 2 h of cycling (Hiscock et al., 2004). The data provided from this study showed that IL-6 protein was mostly expressed by myocytes in the periphery of individual myofibres.

Even if the physiological process that leads to an increased release of IL-6 is now better understood, the biological role of this increased concentration has been, until very recently, unknown. The hypothesis that IL-6 is released from the exercising muscles to signal to the liver the need for a increased glucose production has been recently tested. Evidences show that increased exercise-induced levels of plasma IL-6, act in hormone-like manner to regulate fat metabolism increasing substrate mobilisation (Pedersen et al., 2001). Indeed, it is well know that strenuous exercise increases plasma concentrations of stress hormones and catecholamines (Steensberg, 2003). Therefore, it should be considered that IL-6 may affect the hypothalamus, increasing the release of adrenocorticopic hormone (ACTH) from the anterior pituitary gland or directly acting on the adrenal gland itself, to stimulate cortisol synthesis; evidence has been provided for both mechanisms but further research is required to confirm these processes. Therefore, an acute increase in IL-6 could be important to mobilize substrates in times of need, for example during exercise, through different processes (Glund and Krook, 2008) (**Chapters 5 and 6**).

In addition, increased synthesis of IL-6 induction preceded a rise of two anti-inflammatory cytokines, IL-1ra and IL-10 (Steensberg, 2003). It has been argued that if IL-6 was the initiator of this response, then IL-6 and IL-4 would stimulate monocytes and macrophages to produce IL-1ra, which inhibits the effect of IL-1. Monocytes, lymphocytes and B cells are known to have an important role in producing IL-10, which together with IL-4 can inhibit Type-1 T-cell cytokine production. Evidence for this has already been provided by a study which showed that strenuous exercise causes a decrease in the number of type-1 T-cells, whereas the population of Type-2 T-cells is unchanged. Both cortisol and adrenaline are shown to decrease production of Type-1 T-cell cytokines, whereas Type-2 T cell cytokine production is stimulated by IL-6 (Steensberg et al., 2001).

The exercise-induced IL-6 release may decrease the production Type-1 T cells, which consequently may lead to lower virus protection in the host as Type-1 T cells drive the immune system reaction against intracellular pathogens. This process might ultimately end with an increased incidence of URTI for serious exercisers. Interestingly, tissues other than skeletal muscles appear to be involved in the exercise-induced increase in systemic IL-6 concentrations. Data has shown that IL-6 is also released by the brain during exercise (Nybo et al., 2002) and IL-6 gene expression is increased within adipose tissue in response to exercise (Keller et al., 2003).

In conclusion, the stimulus for the increased IL-6 synthesis from tissue other than skeletal muscle is unclear, although glycogen depletion may be one possible explanation as it has been showed that low muscle glycogen is a stimulus for muscle-derived IL-6 production. This effect may be of interest for athletes or outdoor enthusiasts who appear to be more susceptible to exercise-acquired URTI.

2.3.2. Interleukin (IL)-4: release and functions

IL-4, a multifunctional pleiotropic cytokine discovered in the mid-1980s, remains a focus of attention and continues to spur vigorous research efforts. This mediator is produced mainly by activated T cells but also by mast cells, basophils, and eosinophils (Nelms et al., 1999). A typical cytokine structurally, with molecular weight varying between 12 and 20 kDa as a result of variable natural glycosylation, IL-4 shares sequence homology, cell surface receptors, intracellular signaling, and partial functional effects on cells with IL-13 (Kelly Welch et al, 2005; Murata et al., 1998). Functionally, IL-4 is best known for defining the so-called Th2 phenotype of lymphocytes and for regulating cell proliferation, apoptosis, and expression of numerous genes in various cell types, including lymphocytes, macrophages, and fibroblasts, as well as epithelial and endothelial cells LaPorte et al., 2008; Chomarat et al., 1998). Considering the already staggering and still rapidly growing volume of information available about IL-4, it is hardly possible to provide a systematic review of this pivotal regulator alone, even without considering important relevant aspects, for example, recent advances concerning functionally related Th2 mediators, such as IL-10, IFN-ϒ and, IL-13 As the field of IL-4 research has expanded, it has become obvious that the initial broad-strokes picture of IL-4 function created in the 1980s and 1990s needs to be adjusted to reflect the numerous intricacies related to IL-4 generation, receptor use, and the effects on cells. Specifically, it has emerged that instead of linear scenarios, the field of IL-4 biology is full of alternative pathways. In addition to the well-appreciated—now described at the textbook level—role of IL-4 in determining the alternative fates of Th lymphocytes (Zhu et al., 2008), particularly in promoting Th2 and inhibiting Th1 (Constant et al., 1997) differentiation, new knowledge about other IL-4-related, alternative branchings has emerged.

The effect of IL-4 following exercise is presented in more details in the following paragraph.

2.3.3. Effect of exercise on cytokines

Exercise and environmental stressors induce an increase in the systemic concentration and muscle levels of numerous cytokines. It is interesting to note that the function of many of these cytokines is not completely understood. Additional research is needed to augment our knowledge about the biological role of exercise and environmental induced release of cytokines.

The mRNA expression of a number of cytokines in response to prolonged exercise has been studied to understand if their exercise-induced patterns are similar to IL-6 (Chan et al., 2004; Nieman et al., 2003). The mRNA of many cytokines (e.g. IL-1β, IL-6, IL-8, IL-15, TNF-α, IL-12p35 and IFN-ϒ) is detectable at rest in muscle. On the other hand, other cytokines, such as IL-1α, IL-2, IL-4, IL-5, IL-10 and IL-12p40 are not detectable. There is evidence of an increase of mRNA expression for IL-6 and IL-8 during prolonged exercise (Nieman et al., 2006); one study also reported an increase in IL-1β (Lysiak et al., 2004), but that observation could be attributable to differences in exercise protocols. For example, a study that did not cause any muscle damage did not induce IL-1β mRNA expression (Chan et al., 2004). There was no increase in mRNA expression for any other cytokine following exercise.

A metabolic role for IL-6 and IL-8 is supported by the fact that exogenous carbohydrate ingestion decreases their mRNA expression. In contrast, no effect of carbohydrate ingestion was observed on IL-1β mRNA expression. Consequently, this evidence provides support for the notion that IL-8 may have a similar biological pattern to IL-6. However, it is important to mention that while an increase in the expression of both IL-6 and IL-8 mRNA within the contracting muscle after exercise has been recorded, only IL-6 is synthetized and released.

IL-1ra concentrations are increased in response to endurance exercise with a peak in magnitude that is similar to that of IL-6. Interestingly, the increase in IL-1ra concentration occurs later than that of IL-6. Knowing that IL-6 could induce IL-1ra, it is

plausible that the release of IL-6 from the contracting muscle during exercise elicits the release of IL-1ra from blood mononuclear cells. As IL-1ra is an anti-inflammatory cytokine, it seems reasonable that the increase in the IL-1ra concentration after a bout of exercise acts as a negative feedback mechanism, helping control the magnitude and duration of IL-6 mediated effects (Ostrowski et al., 1998).

In addition, exercise induces an increase of many cytokines in the systemic circulation and muscles, but there is little understanding of their function. Two main mechanisms appear to be involved in the anti-inflammatory chronic effects of exercise. The first is an increased synthesis and release of anti-inflammatory cytokines from contracting skeletal muscle (Mathur and Pedersen, 2008). The second is attributable to a reduced expression of TLRs on monocytes and macrophages (Flynn and McFarlin, 2006), which inhibits the downstream responses, such as pro-inflammatory cytokine production, antigen presentation and co-stimulatory molecule expression (Gleeson et al., 2006). However, these two processes do not completely account for the anti-inflammatory effects of exercise.

Other effects of exercise have been demonstrated, such as the inhibition of monocyte/macrophage infiltration into adipose tissue (Kawanishi et al., 2010), a reduction of circulating numbers of pro-inflammatory monocytes (Timmerman et al., 2008) and an increase the circulating numbers of IL-10 secreting regulatory T-cells (Wang et al., 2012).

As stated above, IL-6 appears responsible for the subsequent rise in circulating levels of other anti-inflammatory cytokines (IL-10 and IL-1ra) and also stimulates the release of cortisol from the adrenal glands (Steensberg, 2003). IL-1ra, which is mainly secreted by monocytes and macrophages, decreases the pro-inflammatory actions of IL-1β. Regulatory T cells, monocytes, Th2 cells, macrophages, CD8+ T cells and Th1 cells are primarily involved in IL-10 production.

The main role of IL-10 is counteracting the inflammatory responses in order to down-regulate adaptive immune effector responses and to minimise tissue damage in response to microbial challenges. This has been showed by a study conducted during Copenhagen marathon in May 1997 where they found that a dramatic increase in the inflammation responsive cytokine IL-6 was balanced by the release of the anti-inflammatory cytokine IL-10 (Ostrowski et al., 1999). In addition, several pro-inflammatory cytokines and other soluble mediators are inhibited by IL-10. Moreover, as insulin sensitivity is increased by IL-10, a protective effect against obesity, induced by macrophage infiltration in skeletal muscle, has been observed (Hong et al., 2009).

TLRs together with the IL-1 receptors form a receptor family, and they are generally expressed by macrophages and dendritic cells. They mainly detect and recognise microbial pathogens. They are transmembrane proteins and can be activated by endogenous signals of tissue damage, such as heat shock proteins (Kaisho and Akira, 2006). TLRs mainly produce pro-inflammatory cytokines after signalling of the antigen-presenting cell, and thus TLR pathways are fundamental in mediating whole body inflammation (Takeda et al., 2003). Physically active people have shown lower inflammatory response by their blood monocytes to endotoxin stimulation *in vitro* and lower TLR4 expression (Gleeson et al., 2006). The physiological stimulus that triggers an exercise-induced decrease in cell surface TLR expression is not fully understood. However, anti-inflammatory cytokines, stress hormones and heat shock proteins have been implicated (Gleeson et al., 2006).

2.4. Influence of exercise on URTI incidence

Exercise has been suggested by physicians as the cause of a decreased resistance to bacterial diseases (Bosch et al., 2002; Shephard, 1997; Shephard, 2010; Shephard and Shek, 1996; Walsh et al., 2011). As support to this, an increased number of URTI reported after endurance competitions has been showed (Nieman et al., 1990; Peters, 1997; Peters and Bateman, 1983).

2.4.1. Influence of exhaustive exercise on URTI incidence

A temporary fall in the circulating number of natural killer (NK) cell number has been observed after an exhaustive bout of exercise (Shinkai et al., 1992). This observation provides a partial explanation for the recorded increase in URTI, as a drop of NK cells offers a period when infecting microorganisms have easier access. This transient period is called an "open window". There is still debate around the length of this "open window"; some studies report that it could last for more than seven days (Shek et al., 1994), but most studies describe this reduction lasting just for a few hours. Also, doubts have been raised as to whether the "window" is open sufficiently long enough to lead to increased susceptibility to infections. In addition, it has also been observed that NK cells do not get damaged by exercise, instead they are temporarily relocated to blood vessel walls because of the exercise-induced secretion of catecholamines (Shephard et al., 2000). A more plausible explanation could be a depression of front-line defences resulting in a subsequent lower secretory rate of antimicrobial peptides (AMPS) from the nasal and salivary glands (Mackinnon, 1997; McDowell et al., 1991; Tharp and Barnes, 1990).

2.4.2. Influence of moderate exercise on URTI incidence

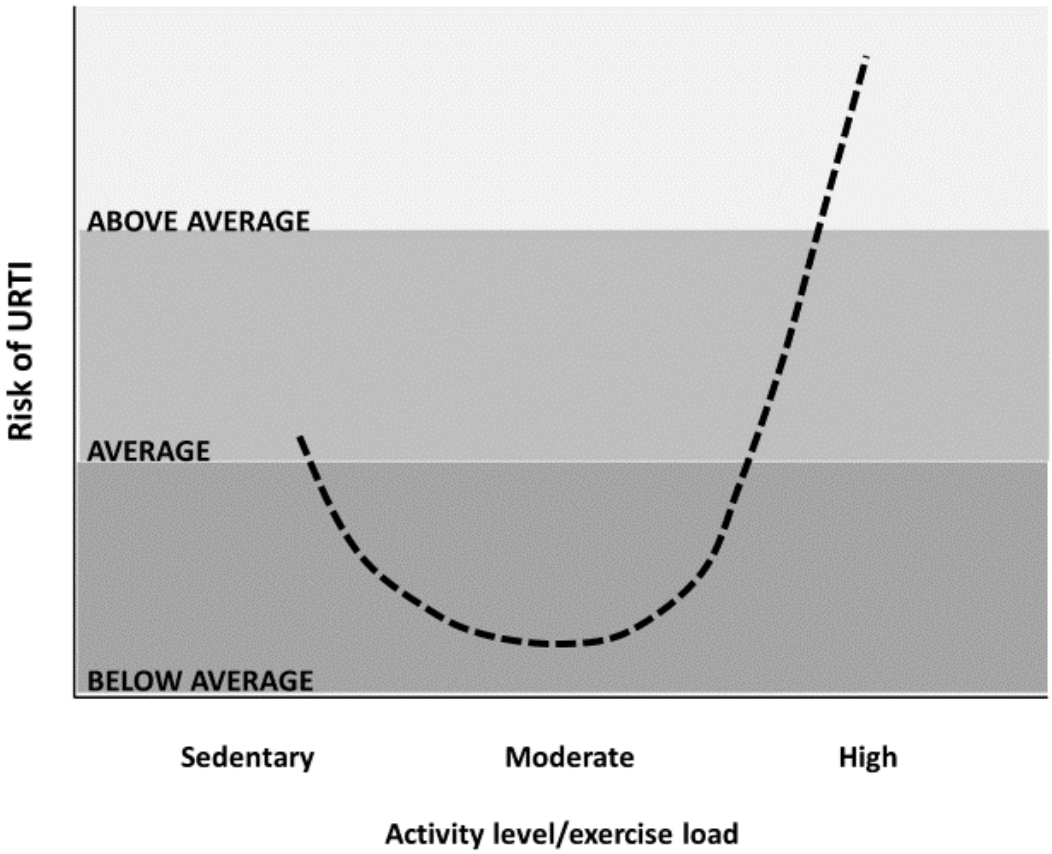

Fig. 2. The J-shaped model of the relationship between risk of Upper Respiratory Tract Infection (URTI) and exercise volume (Nieman et al., 1994).

It has been also observed that regular moderate sessions of exercise have a beneficial effect on health and that excessive volumes of training have negative consequences. Thus suggesting a J shape relationship between physical activity and resistance to diseases (**Figure 2.**; Nieman et al., 1994); however, this observation is based on a limited amount of data (Nieman et al., 1990; Shephard et al., 1999). In the case of the immune system it would seem that a disproportionate inflammatory reaction leads to tissue damage after exagerated training (Shek and Shephard, 1998).

Most of the studies on this topic were survey-based epidemiological studies and presented limitations on data reliability and study design, particularly on inclusion criteria, food intake, memory recall effect limitations, and population specificity for symptoms of Upper Respiratory Tract Infection (URI) (Cramer et al., 1991; Hemila et al., 2003; Kostka et al., 2000; Matthews et al., 2002; Nieman et al., 1990; Shephard and Shek, 1999). However, these results suggest that moderate physical activity was associated with a 20-30% reduction in annual risk of URTI when compared with sedentary subjects. In conclusion, the evidence from available studies show a decreased incidence, severity and duration of URTI in consequence of acute moderate (lasting ~30 min) physical activity (Nieman et al., 1990; Weidner et al., 1998).

The evidence mentioned above suggests that the human body can increase its ability to respond to bacterial challenges leading to the concept of "immunosurveillance", thereby providing a plausible explanation for the apparent relationship between moderate exercise and incidence of infections (Nieman, 2000). Conversely, physical activity showed little effect on the immune system and there is a lack of evidence to support this theory.

2.4.3. Influence of heavy exercise on URTI incidence

Little research has been conducted in the area of intense physical activity and risk of URTI. Some elegant studies have looked at the incidence of URTI after marathon and ultra-marathon competitions and there is evidence of an increased risk of URTI during the 7-14 days following the race (Nieman et al., 1990; Peters and Bateman, 1983; Peters et al., 2010). Even stronger evidence supports the idea that there is a linear relationship between exercise intensity and post-race diseases (Nieman, 1994), suggesting that the more acute the exercise stress the higher the susceptibility to URTI.

Scientific studies based on endurance competition and heavy volume training have investigated the relationship between high-intensity exercise and the immune system (Bury et al., 1998; Foster, 1998; Gleeson et al., 1999). Evidence suggests that

performing both a session of intense prolonged exercise and higher volumes of training is associated with increase susceptibility to URTI, thus supporting the J-shaped model. It is now well established that a depression of immune function can last for 72 hours following a bout of high intensity physical activity (Nieman, 2000), and it seems reasonable to relate this to the increased incidence of URTI, even if it has not been demonstrated definitively.

In conclusion, a suppression of immune function, following high intensity exercise could lead to an "open window" during which bacteria and viruses may invade the body, and thus augment the risk of infections (Nieman, 2000). However, a direct link between impaired immune function *in vivo* and increased incidence of URTI has not yet been demonstrated.

2.4.4. Effect of extreme environments on immune responses to exercise

The hypothesis that extreme environments could affect immune function while exercising was firstly proposed in the late 90's. Considering that exercise and environmental stress both affect the immune function it is easy to comprehend why Dr Shephard hypothesised that linking them together would cause greater disruption to immune function and host defence (Shephard, 1998).

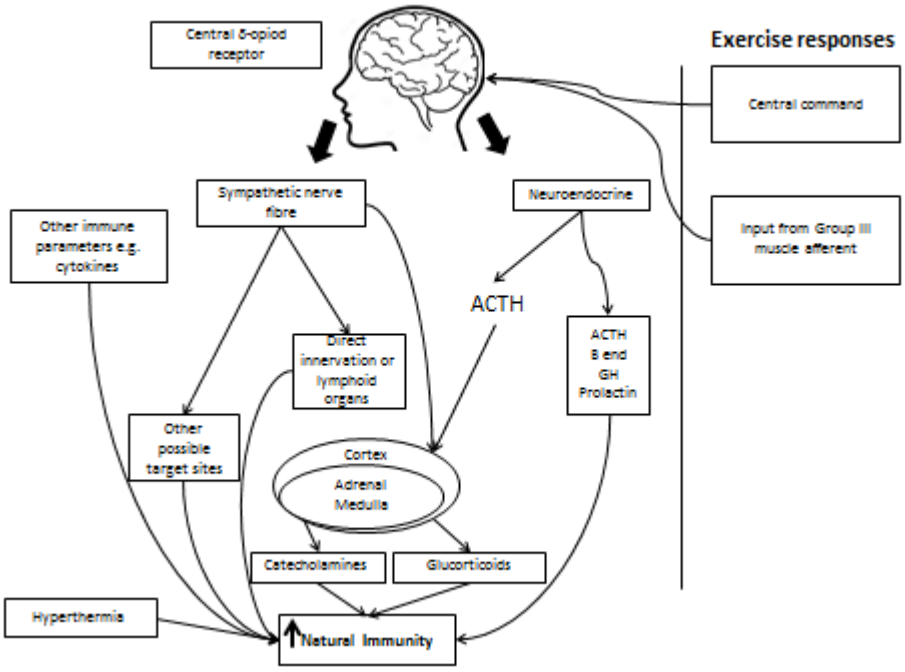

Fig. 3. The co-ordinated hormonal responses, supervised by the central nervous system to contrast stressors. ACTH, Adrenocorticotropic hormone; GH, Growth hormone (Jonsdottir, 2000).

Stressors are contrasted by co-ordinated hormonal responses supervised by the central nervous system (**Figure 3.**; Jonsdottir, 2000). The hypothalamic-pituitary-adrenal (HPA) axis and sympatho-adrenal medullary (SAM) axis provide the signals by which the brain modulates immune function to oppose stressors. Cortisol is produced by the HPA axis, and SAM axis regulates the production of catecholamines. Evidence supports an interaction between neuroendocrine responses and immune responses to exercise (Hoffman-Goetz and Pedersen, 1994).

To provide an example, during fever, the body's temperature is increased by endogenous pyrogens through an increase in the hypothalamic temperature set point. On the other hand, the hypothalamic temperature set point does not change during vigorous physical activity (Byrne et al., 2006; Pugh et al., 1967). However, decreased

heat dissipation during exercise causes the body's temperature to rise, often exceeding levels associated with fever and hyperthermia.

Exercise in hot conditions is often associated with an increase of catecholamines and cytokines, which amplify immune modifications, stress hormones, higher heart rate, and a decrease of endurance performance when compared with thermo-neutral conditions (Febbraio, 2001; Galloway and Maughan, 1997). Evidence shows that exercising in the heat poses a greater threat to immune function compared with thermo-neutral conditions (Rhind et al., 2004; Walsh et al., 2011; Walsh et al., 2011; Walsh and Whitham, 2006) (**Chapters 5 and 6**).

2.4.5. Hyperthermia and heat stroke: a role for cytokines?

Studies have reported an acute inflammatory response to heat strain, which involved hyperthermia-related pyrogens (IL-6, IL-1, and TNFα), of which IL-6 corresponded to the severity of the EHS, as well as their antagonist receptors (IL-6R, IL-1RA, and TNFr I&II), after being exposed to a mix of exertional components and heat. An uncontrolled anti-inflammatory response was related to poor prognosis in recovering patients (Bouchama et al., 1993; Hammami et al., 1997; Hashim et al., 1997).

A more distinct effect of environmental heat has been observed in neutrophil activation, which serves as a link between the inflammation and coagulation responses (Heled et al., 2013). Involvement of pyrogenic cytokines (IL-6, IL-8 and IL-1 RA) was also reported (Duque et al., 2014). Animal models reported similar results alongside increases in anti-inflammatory cytokines, such as IL-10 and IL-1 RA (Zhang et al., 2007). Again the elevation in plasma IL-6 levels was related to the severity of the heat stroke. A dysregulation of the inflammatory response in non-surviving patients leads to the hypothesis that it may contribute to fatal tissue injury. Nevertheless, the anti-inflammatory component is also heavily involved in heat stroke (Bouchama et al., 2005 *a*; Bouchama et al., 2005 *b*). In EHS, a major role for the endotoxemic component is reflected by cytokines, especially by the IL-1 cytokine family (IL-1α, IL-1β, and IL-1 RA).

Cytokines are involved from the early stages, pointing to multi-factorial damage due to excessive heat and EIMD. Once again, IL-6 is correlated with the severity of the heat stroke (Chang, 1993; Lu et al., 2004).

Following a bout of physically demanding exercise, plasma levels of both pro- and anti-inflammatory cytokines increase, and that this increase is enhanced by previous illness or inflammation (Bouchama and Knochel, 2002; Lim and Mackinnon, 2006; Suzuki et al., 2000). For example, performing physical activity in the heat contributes to and increases the level of complexity of the physiological processes mentioned above, providing a combination of stressors (Akimoto et al., 2000; Cox et al., 2007; Rhind et al., 2004; Walsh and Whitham, 2006). Indeed, submaximal exercise in a hot environment has been found to enhance the amount of pyrogenic cytokines following lipopolysaccharide (LPS) stimulation (Starkie et al., 2005). In addition, evidence demonstrates that skeletal muscle responds to heat stress with a distinct "stress-induced immune response," characterized by an early upregulation of IL-6, IL-10, and TLR-4 and suppression of IL-1β and TNF-α mRNA, a pattern discrete from classic innate immune cytokine responses (Leon et al., 2015; Welch et al, 1985). This effect is due to an increased amount of secreting cells such as like macrophages, B lymphocytes, and T lymphocytes (Janeway et al., 2001) (**Chapters 5 and 6**).

Following exercise, an inhibition of cytokine production was expected due to the release of stress hormones, but this has not been confirmed in studies that showed cytokines plasma levels to rise in the combined stress situation. It has also been shown that acclimation has considerable influence on the ability to perform exercise bouts in the heat. Indeed, heat acclimation was found to lower the increase in pro- and anti-inflammatory cytokines during exercise in the heat (Heled et al., 2013). Furthermore, thermoregulatory preconditioning has a considerable influence on the ability to perform exercise in the heat. In particular, heat acclimation was found to diminish the increase in IL-6 and IL-10 during exposure to exercise in the heat, while TNF-α remained unchanged. Supplementation with quercetin, a heat shock response inhibitor, abolished this effect,

suggesting that the mechanisms controlling the extent of the pro- and anti-inflammatory cytokine reaction to heat stress are enhanced by heat acclimation (Kuennen et al., 2013).

Cytokines have been shown to contribute to thermoregulation and heat stroke development via fever, leucocytosis, leukocyte activation, and stimulation of the HPA axis (Heled et al., 2013; Rav-Acha et al., 2004). However, the role of cytokines in the pathophysiology of heat illnesses is complex (**Figure 4.**). Increases in the production of both anti- and pro-inflammatory cytokines and the activation of several pathways belonging to physiological and biochemical processes have been highlighted in hyperthermia (Heled, Fleischmann, and Epstein, 2013). Current knowledge is summarised in **Fig. 4**.

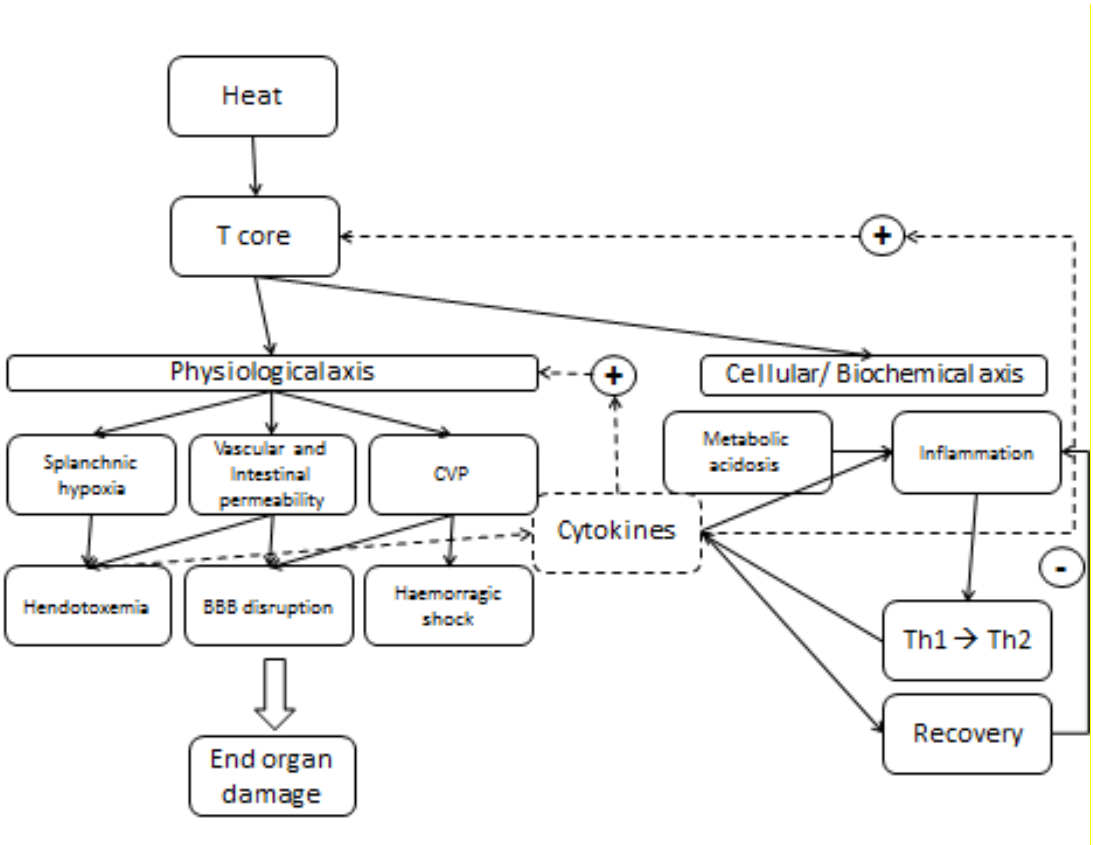

Fig. 4. Proposed roles of cytokines in hyperthermia and heat stroke, (Heled et al., 2013). T core, core body temperature; BBB, Blood-brain-barrier; CVP, Central venous pressure.

2.4.6. Considerations on the effect of exercise on innate and adaptive immune function

With regard to exercise-induced changes in innate immune function and how this relates with EHI and EHS, there is evidence to show how febrile response to LPS injection was nullified in test-rodents previously dosed with anti-IL-6 antibodies (Rummel et al., 2006). Furthermore rats injected with an IL-1 receptor antagonist had an increase rate of survival following experimentally induced heatstroke (Chiu et al., 1996). Another study clearly shows how exercise has a pyrogenic effect of similar magnitude to fever. For example, human plasma taken from subjects post-exercise induced fever in rodents, in contrast to pre-exercise plasma which did not. This indicates an exercise response, and not an allergic or immune response (Cannon and Kluger, 1983). A later study showed that doping human subjects with the cyclooxygenase (COX) inhibitor rofecoxib over a six day period, curtailed body temperature increase during prolonged exercise, which indicates that the prostaglandin-mediated inflammatory processes might exacerbate exercise-induced caused heat strain (Bradford et al., 2007). This prompted the proposition of an additional pathway in the development of EHI. This would involve a systemic inflammatory response (Bouchama, 1995; Lim and Mackinnon, 2006), with increased pyrogenic cytokines (e.g. IL-1β, IL-6 and TNF-α), which might in turn push the individual towards EHI and/or EHS (Walsh et al., 2011 *a*). Hence, it remains unclear whether EIMD increases heat strain during subsequent exercise heat stress, which in turn may increase the risk of EHI. As mentioned, it is well known that exercising muscle releases the inflammatory cytokine IL-6, and it has been demonstrated that EIMD elicits a greater circulating IL-6 response than non muscle-damaging concentric exercise. Therefore, it is plausible that EIMD and associated inflammation (increase in circulating pyrogenic cytokines) might increase heat strain at least in the short-term during subsequent exercise heat stress.

2.5. Heat Shock Proteins

In consequence to exposure to physiological stressors a challenge to homeostasis at the cellular level is posed, and this stimulates an increase in the expression of Heat Shock Proteins (Hsp). Although many studies have focused on the function of intra-cellular Hsp72 after exposure to acute stressors, such as exercise or extreme environments, little is known about the potential function of endogenous extra-cellular Hsp72 (eHsp72). What is known is that although eHsp72 might be a not widely recognized feature of the stress response, it may function as an endogenous "danger signal" for the immune system. Therefore, this allows a reaction and an adaptation to the new challenge the body is facing.

These molecules have a cytoprotective effect, indeed to maintain cellular homeostasis they act as molecular chaperones. In addition, they are also involved in the immune response modulating the inflammatory response following a stress (Horowitz et al. 1996). There is also evidence that Hsp72 is a marker of heatstroke risk (Pavlik et al., 2003; Westman and Sharma, 1998; Yang et al., 1998). Therefore it is of interest of the present thesis to investigate its role during exercise-heat stress and the subsequent effect on thermoregulation. Indeed, it could be suggested that Hsp might act as a link between the peripheral inflammatory response following eccentric-exercise and the CNS that regulate core body temperature. Hsps are characteristically divided into super-families based on their function, structure and molecular weight (Feige and Polla, 1994; Park et al., 2000; Westman and Sharma, 1998).

2.5.1. Heat Shock Protein 72 (Hsp 72) (**Chapter 6**)

Heat shock protein 72 is an inducible isoform of 70kDa of molecular weight.
Indeed, Hsp72 release depends on the Heat Shock Element (HSE) in the promoter region of the Hsp gene binding heat shock transcription factor-1 (Pirkkala et al., 2001). After Hsp72 mRNA transcription, Hsp72 protein synthesis starts (Kregel and Moseley, 1996). Expression of intracellular Hsp72 is the result of many signals such as ACTH (Blake et

al., 1991; Blake et al., 1994), corticosterone (Cvoro and Matic, 2002; Valen et al., 2000), catecholamines (Heneka et al., 2003; Matz et al., 1996; Matz et al., 1996), glycogen depletion (Febbraio et *al.*, 2002; Santoro, 2000), oxidative stress (Calabrese et al., 2001; Wallen et al., 1997) and heat/hyperthermia (Cvoro and Matic, 2002; King et al., 2002; Kregel and Moseley, 1996; Redaelli et al., 2001; Thomas et al., 2002) .

Studies have shown that stressors of exercising muscle (e.g. acidosis, increased temperature, free radical formation, ischaemia and glucose deprivation) induce an increase in intracellular Hsp72 (Salo et al., 1991; Skidmore et al., 1995; Zhou et al., 1997; Omatsky et al., 1995; Vesely et al., 1998; Martinez et al., 1999). Furthermore, exercise itself increases Hsp72 in many central and peripheral tissues (Walsh et al., 2001). This evidence suggests that exercise might affect the extracellular expression of Hsp72 (Febbraio et al., 2002; Fleshner and Johnson, 2005; Walsh et al., 2001; Yamada et al., 2008), because Hsp72 had been demonstrated *in vitro* to have potential immuno-stimulatory capabilities (Asea et al., 2000; Prohaszka et al., 2002; Wang et al., 2005).

Hsp72 has been detected in the circulation of humans (Pockley et al., 1998). It has been hypothesized that an acute bout of exercise would increase Hsp72 protein expression in contracting skeletal muscle, which would then be released into the extracellular environment, increasing the concentration of serum Hsp72. A study by Walsh et al. (2001) showed that after an acute-exercise bout serum eHsp72 was elevated, but the increase preceded the increase in mRNA and protein Hsp72 synthesis within the muscle tissue. This led to the conclusion that muscle cells were not responsible for the extracellular release of eHsp72. Further studies have corroborated this using different protocols (Marshall et al., 2006; Johnson et al., 2006). Studies have demonstrated that eHsp72 responses were dependent on the intensity and duration of exercise (Fehrenbach et al., 2005); therefore, demonstrating that exercise results in an increase in extracellular Hsp72 in humans.

2.5.2. Mechanism, releasing signal and origin of extracellular Hsp72

Many questions, however, have been raised about the possible mechanism, releasing signal and origin of extracellular Hsp72 release, and the biological significance of Hsp72 in the extracellular environment. Given that eHsp72 has been measured in interstitial space and in body periphery, something (e.g. cell, organ) must release it. Two mechanisms might, potentially, be involved; (1) Hsp72 is passively released from an intra-cellular pool following cellular lysis, or death, and (2) a receptor-mediated exocytotic pathway actively releases Hsp72 (Asea, 2007; Fleshner and Johnson, 2005). A further plausible explanation for Hsp72 presence is that it is co-ordinating further molecules, which the cell excreted/secreted (Prohaszka and Fust, 2004). Other studies suggest that Hsps are only released under pathological circumstances (Gallucci et al., 1999). Either way, this results in necrotic death rather than controlled apoptotic cell death (Kroemer et al., 2005). Some *in vivo* evidence links elevated eHsp72 with muscle damage markers.

One study on runners showed that heavy/repeated footfall damages erythrocytes (Gromov and Celis, 1991), and this might help towards elevating eHsp72. Indeed, exercise duration and the consequent induced muscle-damage, seems a principle elevator of eHsp72 (Fehrenbach et al., 2005). However, a direct correlation between eHsp72 levels and markers of muscle damage has not been shown in literature. Moreover, downhill running and running on a level gradient reported similar Hsp72 levels (Peake et al., 2005).

The fact that Hsp72 is released into the peripheral circulation within 10-30 minutes of stressor onset suggests that necrosis is not the only factor involved in such a big increase (Fleshner and Johnson, 2005). Indeed, 30 minutes is not enough time for the protein induction pathway, and moreover it is unlikely that such a large number of cells would simultaneously die through necrosis. Whilst a passive release through necrosis may be an important mechanism in pathological circumstances and trauma (da Rocha et al., 2005; Pittet et al., 2002), the fact that psychological stressors, such as predatory

fear and threat of electric shock, evoke a stress-induced eHsp72 release adds further evidence to the suggestion that a pathway other than necrosis is evident (Fleshner et al., 2004; Fleshner et al., 2003; Johnson et al., 2005). In particular, an active release mechanism for Hsps in the absence of lysis was demonstrated by stimulated cultured rat embryo cells that released a number of heat shock proteins. More recently, glia-like cells have been shown to actively release Hsp70 upon heat shock *in vitro*, prompting recent investigations into the mechanism of this selective exocytic release. It has been demonstrated that the presence of Hsp72 within plasma membrane detergent-resistant micro domains of epithelial cells increased significantly when exposed to heat shock. Recently, much interest has revolved around the role of exosomes in the selective release of Hsp72 (Clayton et al., 2001). Much of this interest is based on findings that exosomes contain many immuno-stimulatory molecules, such as major histocompatibility complex (MHC) I and II, co-stimulating molecules and adhesion molecules, suggesting that exosomes could provide an exocytic pathway for other potential immune-stimulatory molecules, such as Hsp72 (Johnson et al., 2006), and are small membrane-bound vesicles that are secreted by a number of eukaryocytes as the consequence of a fusion of multivesicular bodies with the plasma membrane (Denzer et al., 2000). Exosomes are released by many haemopoietic cells following changes in intracellular calcium levels (Savina et al., 2003). Hsp72 has been detected within exosomes in a variety of cell types, although it appears that cellular specificity exists with regard to exosomal Hsp72release/expression (Clayton et al., 2005). Whilst the supporting evidence to date suggests that the majority of Hsp72 is actively released via an exocytic pathway into the circulation from cells under times of stress, it is likely that exosomes/lysosomes are involved, although further research is needed to clarify the factors involved in Hsp72 release.

Exercise is a stress that tends to disrupt homeostasis inducing a multitude of physiological changes. In addition, these physiological changes are known to increase the concentration of intracellular Hsp72, providing a plausible answer to "What triggers

the specific release of eHsp72?". The term heat shock protein clearly elicits heat as the main stimulator of the Hsp response. Indeed, using an isolated working heart model at different temperatures (Staib et al., 2007), it has been implied that elevated temperature was a requirement for myocardial Hsp72 expression and might be concluded that HSF-1, which regulate Hsp72 synthesis, is activated by temperature (Ruell et al., 2006). However, it is still unclear if heat stress increases Hsp72 into the extracellular environment.

Whitham and Fortes, using an elegant model, which involved a thermal clamp protocol have studied the independent and combined effects of exercise and increased core temperature on eHsp72 (2008). The greatest eHsp72 increase was the result of combined exercise and increases in core temperature, although it must be mentioned that passive heating alone induced significant eHsp72 responses (Ruell et al., 2006). Therefore, the increases in eHsp72 during exercise are not just induced by increases in core temperature.

2.5.3. Other factors involved in the eHsp72 response

The eHsp72 response is clearly complex. Prolonged exercise decreases muscle glycogen and blood glucose. Inadequate refuelling directly leads to an increase in glycogenolysis within the liver and skeletal muscle which maintains blood glucose levels (Coyle et al., 1986). The liver releases a great deal of acute phase proteins, and it was therefore postulated that Hsps may originate there. Evidence supporting the described process stems from studies which used arterial-venous difference cannulation techniques (Febbraio et al., 2002). These evidenced significant Hsp72 release during cycling, from the hepatosplanchic viscera (Febbraio et al., 2002). Furthermore, this release was diminished following recent glucose ingestion therefore, researchers concluded that glucose ingestion reduces hepatic production (Febbraio et al., 2004; Jeukendrup et al., 1999). Further evidence that blood glucose may regulate eHsp72 production abounds in the literature (Krause et al., 2015; Keane et al., 2015).

Exercise challenges cellular integrity by releasing increased levels of ROS and reactive nitrogen species (RNS). ROS have been shown to induce Hsp expression in lymphocytes (Marini et al., 1996). The findings showed that heat, glucose availability and oxidative stress play significant roles in generating an eHsp72 exercise response (Fischer et al., 2006). These studies failed, however, to demonstrate a clear cause and effect relationship. Any association might, therefore, be mediated by a different signal, particularly should an active exocytic pathway be the release mechanism from the cell.

Campisi et al. (2003) suggested that eHsp72 could form part of the normal stress response. Hence it would seem plausible for eHsp72 release to be mediated by a signal associated with the HPA or the SAM axis, particularly since many stress-induced signals originate in the anterior pituitary. Also, adrenal glands were shown to increase the intra-cellular concentration of Hsp72 (Matz et al., 1996). Studies that utilised adrenalectomy, hypophysectomy and adrenergic receptor blockade to induce eHsp72 in animal subjects, suggested a probable catecholamine connection.

One hypothesis states that under times of stress, calcium flux within the cell is precipitated by increased noradrenaline acting on α-adrenergic receptors and, subsequently, Hsp72 is released in exosomes, since intra-cellular calcium affects them (Johnson and Fleshner, 2006). Furthermore, one mouse study measured the release of eHsp72 by noradrenaline stimulation of α-adrenergic receptors, but there is relatively little human research to date (Fleshner et al., 2003). Since catecholamines possibly stimulates eHsp72 release, it is also possible that any sympathetic nervous system stimulation could stimulate eHsp72. This could be shown by psychologically stimulating a subject during exercise to induce a greater cardiovascular response. Psychological stress has been proven to stimulate eHsp72 in animals (Fleshner et al., 2004), which would suggest that heightened mental stress during exercise might have an additive effect in inducing a greater eHsp72 response.

Studies on post-operative anaesthesia and curarization, which essentially block or reduce peripheral tissue afferent feedback, suggest a central command contribution in

sympathetic activity (Kjaer et al., 1989; Pawelczyk et al., 1997). This would provide a potential mechanism by which central drive could stimulate eHsp72, independent of afferent feedback. Further evidence shows that immune and epithelial cells can release Hsp72 into extracellular environments (Broquet et al., 2003). It would also be worth investigating whether there are any other tissue sources of eHsp72 during exercise. One concept skeletal muscle is highly active during exercise, and exercise-induced intracellular Hsp72 response has been widely demonstrated, which appears to present skeletal muscle cells as an additional generator of eHsp72. While this could be true, the appearance of eHsp72 preceded the increase in skeletal muscle Hsp72 mRNA and protein concentration in exercising humans, as already mentioned (Walsh et al., 2001). Further to this, eHsp72 was not detected after 4-5 hours of knee extensor exercise at 40% maximal leg power output in a muscle glycogen depleted state (Febbraio et al., 2002).

In conclusion, it might be said that Hsp72 is partly released by muscle damage. Additionally, cannulation studies revealed a possible Hsp72 release in the brain after 180 minutes of cycling at 60% VO_{2max}, albeit the response demonstrated significant subject specificity. Considering the involvement of sympathetic activation in Hsp72 expression (Johnson et al., 2005), it can be plausibly suggested that Hsp72 could be released by any tissue served by the sympathetic nervous system.

2.5.4. Potential functions of Hsp 72

A deeper understanding of the signalling mechanisms behind intracellular and extracellular Hsp72 expression becomes more urgent when one considers the potential roles played by Hsp on immune system function, and vice versa (Srivastava et al., 1986). Initiating an intracellular Hsp leads to stress tolerance and protection against otherwise potentially lethal stressors. It has been previously suggested that Hsp72 has a role to play in the protection of immune cells operating in stressful inflammation environments (Campisi et al., 2003; Breloer et al., 1999).

It has been also shown that Hsp72 demonstrates higher responses to heat shock in phagocytic leukocytes than in other immune cell types (Oehler et al., 2001), and this increase was attributed to the observation that phagocytes mainly produce reactive oxygen species. Pursuant to this, the resting and post exercise concentrations of eHsp72 initially decrease during heat acclimation (Marshall et al., 2006) and that leads to the conclusion that there is an increased cellular uptake of eHsp72 which improves stress tolerance to any subsequent heat exposure. So Hsp72 may, in trying times, protect cells from external stressors and also from cytotoxicity of the immune system itself. It would be fascinating to understand whether eHsp72 could be taken up by distant cells that might be undergoing stress and/or are incapable of inducing Hsp72 by themselves. To present one idea; motor neurons contain basal Hsp72, but are incapable of synthesizing it following heat shock (Whitham and Fortes, 2008). One study administered an exogenous application of Hsp72 to a motor neuron cell culture and this enhanced post heat shock motor neuron survival (Robinson et al., 2005). Glial cells also release Hsp72, and since they are in close proximity to motor neurones their production could bolster the stress tolerance of their neighbours (Guzhova et al., 2001). This attribute of Hsp72 could lead to innovative treatment for exertional heat illness, particularly heat stroke.

Whilst many cell types are known to release Hsp72, eHsp72 must bind to a receptor, thereby precipitating an event cascade resulting in the initiation of an immune response. For example, leukocytes and NK cells have been shown to bind with eHsp72 (Asea et al., 2000; Asea, 2006; Sondermann et al., 2000) and certain pattern recognition receptors (PRR's) have been shown to exhibit cytokine-like eHsp72 of actions (Asea, 2006). Some argue that since Hsp72 could bind with the plasma membrane of human monocytes that this could cause intracellular calcium flux, that concordantly results in up-regulation of the inflammatory cytokines TNF-α, IL-1β, and IL-6. It has also been shown that Hsp72 activates the complement cascade, independent of antibodies (Markievski et al., 2007). One molecule, the Cluster of Differentiation 14 (CD14), is a co-receptor for LPS detection expressed by macrophages, and has been shown to induce pro-inflammatory cytokines

via eHsp72. Consequentely, a bacterial activation of CD14 might lead to an eHsp72-initiated up-regulation of innate immunity (Asea et al., 2000). Since Hsp72 binds to and then stimulates CD40+ cells (e.g., macrophages and B cells) this could be through a co-stimulatory pathway which would consist of an interaction between CD40 on PAC and CD40L on T cells (Caux et al., 1994). Accordingly, Hsp72, it is postulated, could have a contributory role in the interphase between innate and adaptive immunity.

2.6. The pathophysiology of heat stroke (**Chapters 5 and 6**)

Humans maintain their body core temperature within a narrow range. Consequently, when body temperature becomes higher, clinical failure may quickly develop (Hales et al., 1996; Horowitz and Hales, 1998). Heat illness represents a family of disorders ranging in severity from temporary and mild to fatal, depending on a variety of factors and the presence of hyperthermia (Sawka et al., 2011). Together these factors affect the magnitude and rate in the rise of body temperature, by interfering with the homeostatic control systems that regulate temperature (Hales et al., 1996). Nevertheless, the cascade of pathophysiological consequences is similar in all cases, with the critical factor in the development of heat stroke being the inability to sustain circulatory function (Hales et al., 1996; Horowitz and Hales, 1998). Indeed, cardiovascular activity transports heat to the body surface, while perfusing all tissues at a rate adequate to meet their metabolic demand. For example, during mild heat stress, body surface blood flow is elevated, without impairing the blood supply to other tissues. As the heat load increases, skin blood flow is further augmented and profuse sweating occurs. This increases peripheral blood volume, concurrently, the enhanced evaporation decreases total blood volume and causes a depletion of central blood volume. In turn, this reduces central venous pressure, which diminishes skin blood flow and volume, as well as evaporative cooling via low-pressure baroreceptors. These factors in conjunction, may lead to hyperthermia as blood maintenance becomes difficult, as the thermoregulatory

demands conflict with blood pressure regulation requirements. This conflict may result in physiological breakdown and heat stroke syndrome development.

Body temperature control is considered secondary compared to cardiovascular need. Severe body hyperthermia is supplemented by cellular/molecular changes resulting in a potentially fatal cascade of events, including cerebral hypoxia, neural dysfunction and hypotension (Hales et al., 1996; Horowitz and Hales, 1998). Many studies have focused on experimental animal models, as well as case reports of humans suffering from heat stroke or post-mortem examinations. Substantiated causal relationships between elevated circulating plasma cytokines (Leon et al., 2006), brain cytokines and monoamines (Kao et al., 1994; Lin, 1999) have all been suggested in the pathophysiology of this syndrome (Bouchama, 2006; Bouchama and Knochel, 2002).

2.6.1. Hsp 72 as marker for Heat Stroke risk (**Chapter 6**)

The assertion that Hsp72 is a marker of heatstroke risk is based on studies demonstrating that a severe diffuse loss of Purkinje cells is associated with Hsp70 expression in heat stroke victims (Bazille et al., 2005), and that a marked elevation in inducible Hsp70 is found in CNS tissues in response to heat stress and hyperthermia (Pavlik et al., 2003; Westman and Sharma, 1998; Yang et al., 1998). Animal studies have also shown increased Hsp70 in extracellular space during heat stroke (Dehbi et al. 2010).

This protein protects nascent and unfolded proteins and further protects against tissue damage (Hausenloy and Yellon, 2006; Reshef et al., 1996), therefore, this protein is considered to be a reliable biomarker for stress. Indeed, many studies evidence that thermal preconditioning (leading to the overexpression of Hsp70) delays heat stroke induced by cerebral ischaemia and hypotension (Yang et al., 1998). The neuroprotective role of Hsp70 has been tested in many stress conditions (Welch, 2001; Yenari, 2002). However, whether HSPs have additional functions beyond their chaperoning abilities or if they mediate physiological processes through cellular safeguarding is an important

unanswered research question. It is based on the observation that thermal preconditioning, in contrast to ischaemic preconditioning, induces the "Second Window of Protection" (SWOP) in cardio protection, suggesting that SWOP and heat stress have distinct protection mechanisms and that they might not be exclusive to Hsp72 expression (Qian et al., 1999).

There have been suggestions that Hsp70 potentiates the baroreflex response by acting on the *Nucleus Tractus Solitarii (*NTS*)* (Li et al., 2001*)*, i.e. the baroreceptor afferent fibres. Li and colleagues (2001), demonstrated the Hsp70 levels in the NTS further enhanced the sensitivity of both the sympathetic and parasympathetic arms of the autonomic nervous system, and this mollified heat stroke induced cerebral ischaemia and hypotension. The opposite effect was seen when microinjections of anti-Hsp antibodies were applied to the NTS before thermal preconditioning. The same authors observed a threshold level of Hsp expression in the NTS had to be overcome to activate the baroreflex response.

These observations support the concept that thermal preconditioning and increased Hsp72 are fundamental to preserving synaptic transmission during thermal stress (Kelty et al., 2002). Since Hsp is absorbed by neurons it can control transmitter release by interacting with a variety of intracellular proteins (Jiang et al., 2000; Ohtsuka and Suzuki, 2000). One might conclude that Hsp72 protects the organism by multiple functions and direct interaction with autonomic neuronal networks. The interaction is not completely understood, but molecular pathways associated with synaptic transmission have been mooted. It has been also hypothesized that cell integrity is important to this process (Chamber et al., 2013).

2.6.2. Immune system and Heat stroke: Is there any link?

Recently, the debate on whether or not the immune system is involved in the aetiology of EHS has gathered pace (Muldoon et al., 2004; Muldoon et al., 2008). Unlike milder EHI, EHS is a life-threatening acute heat illness characterised by hyperthermia and

neurological abnormalities that can develop after exposure to high ambient temperature and humidity (**Figure 5.**).

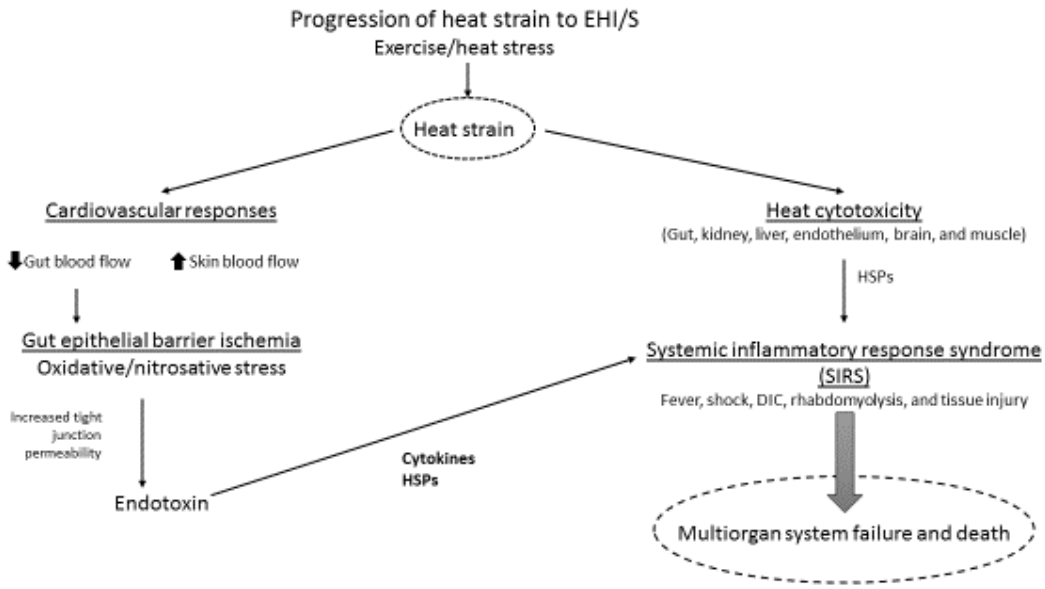

Fig. 5. Summary of Exertional Heat Stroke (EHS) pathophysiological responses ending in multi-organ system failure. In exercise-induced heat stress, an increase is observed in cutaneous blood flow and decrease in splanchnic blood flow. Gut epithelial membrane ischemia induces oxidative and nitrosative stress and this increases tight junction permeability and allows endotoxins to leak into the systemic and portal circulation. Toll-like receptors (e.g., TLR4) meet pattern-associated molecular patterns (PAMPs) on the cell membrane and stimulate pro- and anti-inflammatory cytokine production. Heat is toxic to many organs and this stimulates heat shock proteins (HSPs) secretion. These interact with cytokines and other proteins to lessen the systemic inflammatory response syndrome. A shift in the cytokine populations from an anti-inflammatory (Th2) to a pro-inflammatory (Th1) balance (i.e. anergy) is thought to mediate heat stroke symptoms that lead to multi-organ system failure and death (Sawka et al., 2011).

There is evidence of the involvement of immune system in the aetiology of EHS (Sawka et al., 2011; Lim and Mackinnon, 2006; Shephard and Shek, 1999) (**Figure 5.**). Indeed, exercise heat stress can cause a redistribution of blood flow which can lead to gastrointestinal ischaemia. That in turn results in damage to the intestinal mucosa and leads to the unwelcome LPS in the portal circulation (**Figure 5.**). The LPS is neutralised by the liver and then by monocytes and macrophages. However, these may be insufficient, resulting in increased concentrations of LPS in peripheral circulation. This

process may be exacerbated by heavy training where immune function is impaired (Bosenberg et al., 1988; Camus et al., 1997).

Many events are associated with LPS binding to protein; the transfer of LPS to its receptor complex (Toll-like receptor 4), subsequent nuclear factor-kappa B activation, and the translation and production of inflammatory mediators, including interleukins IL-1β, TNF-α, IL-6 and inducible nitric oxide synthase (Selkirk et al., 2008). These events can lead to systemic inflammatory response syndrome, intravascular coagulation and eventually multi-organ failure. This is an attractive model, particularly for cases of EHS that are otherwise difficult to explain, because the pyrogenic cytokines can alter thermoregulation and cause cardiovascular instability resulting in collapse of the athlete or soldier (Bosenberg et al., 1988; Bouchama et al., 1991; Camus et al., 1997; Walsh and Whitham, 2006).

It is likely that the more traditional predisposing factors for EHS, such as high heat load and underlying illness, alongside a possible muscle defect causing excessive endogenous heat production, play a more prominent role in EHS aetiology than immune dysregulation (Rav-Acha et al., 2004). Immune dysregulation might have a role to play in the aetiology of a small number of EHS cases that remain to be explained by more traditional predisposing factors (Rae et al., 2008). These considerations provide part of the rationale of the present thesis. Indeed, the effect of EIMD upon heat strain during subsequent exercise in the heat has been studied to clarify the role of inflammation on thermoregulation.

2.7. Cold stress and Immune function (**Chapter 4**)

It is of interest for exercise immunologists to have a better understanding of the effects of cold exposure on immune function and URTI incidence (Castellani et al., 2006). There is still a debate on whether cold exposure with or without exercise increases susceptibility to URTI and leads to immune function depression.

In a cold environment, any attempt by the body to maintain core temperature may be insufficient, consequently shivering thermogenesis and peripheral vasoconstriction begin (Castellani and Rhind, 2002). SAM and HPA axis lead these responses, which also have effects on the immune system; therefore, it conceivable to think that the immune system may also be affected. Evidence shows that peak infection and illness typically occur in the coldest times of the year and last longer. However, it must be noted that most of these epidemiological reports did not establish that cold exposure was the cause of URTI, as many other stressors were likely to have been present (Makinen et al., 2009; Shephard and Shek, 1998; Whitham et al., 2006).

Makinen and colleagues (2009), has recently provided evidence for a link between low ambient temperature and increased URTI. Indeed, a study on 892 Finnish military conscripts indicates that URTI, including the common cold, were preceded by three days of declining ambient temperature and humidity (Makinen et al. 2009). More compelling evidence indicates that patients who become cold during surgery suffer greater surgical wound infection post operation (Kurz et al., 1996). Further, URTI symptoms were more than doubled in the 4-5 days after people had their feet immersed in cold water for 20 minutes compared with non-chilled controls (Johnson and Eccles 2005). Consequently, decreased immune function with repeated cold exposure could be a mechanism which may partially explain for the increase in infection reported by athletes and soldiers during winter months.

Hypothermia is defined as core body temperature of less than 35 °C, and it is uncommon in athletes due to the metabolic heat produced while exercising, nonetheless

it has been reported in cold-water swimmers or hill walkers (Pugh et al., 1967). Indeed, hypothermia is more frequent in some active occupational populations exposed to cold air and cold water during recreational or professional activities (e.g. military personnel, hikers and outdoor enthusiasts) (Beeley et al., 1993; Brannigan et al., 2009; Giesbrecht, 2000; Keatinge et al., 2001; Nuckton et al., 2000).

A limited number of investigations (Brenner et al., 1999; Costa et al., 2010) have studied the effect of modest core temperature reduction (0.5-1 °C) on leukocyte cell trafficking and function in humans, and findings showed leukocytosis that lasted for two hours after cooling. In particular, an increase in neutrophils and a simultaneous decrease in lymphocytes were reported. This modest rise in neutrophils may be attributed to demargination of leukocytes from the blood vessel walls as a result of increased cardiac output and catecholamine action. Moreover, these studies reported that natural killer cell activity (NKCA) remained unaltered, but saliva secretory Immunoglobulin-A (SIgA) secretion rate, lymphocyte function and neutrophil function were all reduced (Beilin et al., 1998; Brenner et al., 1999; Costa et al., 2010; Wenisch et al., 1996). Furthermore, it has been suggested that mild hypothermia can disrupt circulating immune function (LaVoy et al., 2011), which may partly explain the increased infection in cold exposed athletes and soldiers. A limitation of this research is that the studies were focused on patients undergoing surgery and might not be applicable to a healthy population, additionally a thermo-neutral control trial was not performed and therefore any effect of diurnal variation cannot be excluded.

Because cold exposure causes an increased energy requirement, cold-induced diuresis and a blunted thirst response (Pugh et al., 1967), then a negative energy balance and dehydration, are more likely to affect those exercising and training in the cold. Physical activity in cold air versus thermo-neutral conditions has been shown to have minimal effect on mucosal immunity (Walsh et al., 2002). A limitation of this study, in the present context, is that data provided by Walsh and colleagues (2001) were collected after cold air exposure but core body temperature was not measured and it was also

unlikely to be decreased due to the metabolic heat produced whle exercising. However, there is limited evidence to support that exercising in cold air compared with thermo-neutral conditions decreases mucosal immune function. Another plausible explanation for the increased URTI, is the decreased epithelial barrier function caused by upper airway drying from breathing cold air during exercise (Giesbrecht, 1995). Indeed, cold exposure leads to decreases in nasal respiratory epithelium temperature and reduces mucocilliary clearance of pathogens. Further evidence was provided by Tomasi and colleagues (1985) who studied secretory IgA levels in nationally ranked Nordic skiers before and after the national cross-country races held. Comparing the skiers with age-matched controls, there was significantly lower level of salivary IgA before the race. Concentrations of IgA decreased further following the competition to very low levels (Tomasi et al., 1985). Furthermore, low skin temperature and subsequent cold-induced peripheral vasoconstriction may impair whole body peripheral immune cell trafficking and function (Webb, 1992). This could be even greater with an existing open wound. Indeed, wound healing is slower and infection higher in patients that become hypothermic during surgical operations (Kurz et al., 1996).

Another important factor that must be taken in account is the increased time spent in close contact with others during winter months that can increase pathogen exposure, and may therefore explain the increased reported URTI. This factor has been highlighted as a cause for increased infection for more than 80 years (Hill and Cobbold, 1929). Whether crowding is really greater in winter compared with summer has not been demonstrated yet. In summary, cold exposure is associated with a greater incidence of URTI in active populations. The mechanism responsible for the increased URTI, however, remains elusive and may not be related to a reduced immune function (Eccles, 2002).
While the clinical significance of cold/exposure induced immune function impairment is yet to be clarified in healthy individuals, there are indications that decreases in neutrophil function, saliva IgA and other antimicrobial proteins have been have been linked to an increased incidence of infection in otherwise healthy and active subject, in a

wealth of clinical trials (Smitherman and Peacock, 1995; Fahlman and Engels, 2005; Gleeson et al., 1999; Gleeson et al., 2013; Gleeson et al., 2011). On numerous occasions the individual contributions of these agents have been conferred a high level of clinical relevance (Albers et al., 2005; Walsh et al., 2011). Associations between URTI and other blood and salivary immune agents are yet to be extensively examined. Additionally, dysregulation of interferon-gamma (IFN-γ) production (a cytokine which has important anti-viral and immunomodulatory roles) was found for illness prone athletes, with normal function in regularly robust athletes (Gleeson et al., 2011). These 'first line of defence' chemicals form an innate part of immune system and a disruption by cold exposure could result in increased URTI. More research is required as to whether a decrease in innate immune function results from hypothermia or whether this is a consequence of extreme metabolic responses to the adverse/cold environment. These responses (or similar ones) could be attributed to moderate physical exercise.

To date it is unknown whether the high incidence of infection cases in the studies above stem from a cold-induced immune-function suppression. As already mentioned, cold exposure responses (e.g. shivering thermogenesis or peripheral vasoconstriction) are regulated by the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis, which are also instrumental in modulating the immune system (Costa et al., 2012). It would therefore follow that cold exposure might result in impaired immune function due to increased stress hormones (cortisol and catecholamines) which are released by the SNS and HPA axis during a thermoregulatory feedback process.

2.8. Exercise-Induced Muscle Damage (EIMD): mechanism, measures and consequences (**Chapters 5 and 6**)

In 1902, Hough first suggested that delayed onset muscle soreness (DOMS) was a consequence of micro-lesions in the muscle (Hough, 1902). For the next 9 decades, the study of muscle damage and muscle soreness received little attention, with only a few studies published. However, the interest in exercise-induced muscle damage (EIMD) has risen, with more than 100 studies conducted on humans. Most of the studies used eccentric actions of isolated muscle groups or exercises that are biased toward eccentric action, because it is well documented that eccentric actions result in muscle damage.

An additional EHI development pathway has been proposed which involves a systemic inflammatory response, with increased pyrogenic cytokines (e.g. IL-1β, IL-6 and TNF-α) that might accelerate progression towards EHI and/or EHS. It has been proven that exercise has a pyrogenic effect similar to fever.

It remains unclear whether EIMD increases the heat strain experienced during subsequent exercise-heat-stress, which may in-turn increase the risk of EHI.

2.8.1. Assessment of muscle damage

Muscle biopsies or magnetic resonance imaging (MRI) are the only ways to directly assess muscle damage in human skeletal muscle. These techniques both present limitations, for example muscle biopsy uses a small sample to estimate damage in an entire muscle, and imaging techniques present images that are not stationary. Due to these limitations indirect measures to assess damage have been developed. The three most commonly used indirect damage markers on humans are; (1) a subjective determination of muscle soreness (63% of studies), (2) blood protein assessment (52% of studies), and (3) maximal voluntary contraction force (50% of studies) (Warren et al., 1999).

In 1981, some of the first evidence of muscle fibre damage in humans after exercise was provided using biopsies (Friden et al., 1981), and this was later confirmed by a follow-up

study (Friden et al., 1983). The percentages of damaged muscle corresponded to 1.6%, 2.4%, and 0.6% of the fibre area, with disturbances predominantly occurring in Type II fibres. The greater damaged was observed immediately after exercise, compared with samples taken 24-48 hours after exercise cessation (Friden et al., 1983; Newham et al., 1983). In contrast, the effect of multiple biopsies taken over a period of seven days in control subjects and subjects who performed eccentric exercise was measured, showed both conditions resulted in similar changes in infiltrating neutrophils and macrophages (Malm et al., 2000; Roth et al., 2000). Consequently, the biopsy procedure itself can produce some changes. There is evidence from human trials that the initial bump of the exercise creates a jolt to fibres, which results in damage to the extracellular matrix and, possibly, capillaries. This activates an inflammatory response, which may be part of the repair and regeneration process.

Between indirect assessment methods, prolonged strength loss after eccentric exercise is considered to be one of the most valid and reliable measures of muscle damage in humans (Warren et al., 1999). Metabolic and neural fatigue strength losses (Edwards et al., 1977) immediately after exercise are restored quickly, with strength returning to baseline within hours after exercise (Jones et al., 1989; Newham et al., 1983).

2.8.2. Differences between exercise protocols

The main differences between concentric protocols and eccentric-biased downhill running protocols are a longer recovery period (up to 24 h post-exercise for eccentric exercise) with a comparable 10-30% force loss immediately after exercise (Eston et al., 1996; Eston et al., 2000; Mizrahi et al., 2001). Longer recovery time and higher degrees of strength loss are associated with high-force eccentric exercise (Clarkson and Newham, 1995) that can often generate up to 50-65% loss of force-generating capacity (Newham et al., 1987; Nosaka et al., 1991; Saxton et al., 1995). Prolonged force loss in the days after eccentric exercise can typically last between 1 and 2 weeks (Armstrong, 1990; Lieber and Friden, 1993), as a result of the initial damage and additional damage during the regeneration process.

Mechanical muscle strain has been shown to be a principle causes of EIMD. Mechanical muscle strain is when a muscle is lengthened beyond 140% of its optimum length during eccentric contractions. This both decreases its force production capacity post exercise and increases subsequent muscle injury (Faulkner et al., 1993). Human studies have reported that greater produced force losses after the subject exercised with muscles initially set at a longer length (Child et al., 1998; Newham et al., 1988; Nosaka and Sakamoto, 2001; Saxton and Donnelly, 1996). These observations indicate that exercise at longer initial muscle lengths creates a larger amount of strain on muscle fibres. This should coincide with a linear deformation of sarcomeres within muscles that generate lower strength peak, which ultimately increases EIMD.

The mechanism by which force is lost after eccentric exercise is yet to be established. One theory, the popping-sarcomere hypothesis, states that sarcomeres lengthen in a non-uniform manner during lengthening contractions (Morgan and Allen, 1999). If the muscle is lengthened slowly, sarcomere length uniformly stretches with increasing tension. If instead, sudden tension or of high force is applied, a non-uniform stretching takes place in the sarcomere resulting in excess stretching, generating cellular damage. The proposition: that when a muscle is lengthened, weak sarcomeres stretch to such a length that they are supported purely by passive elements; colloquially 'popped' (Morgan and Allen, 1999).

2.8.3. Indirect methods of muscle damage assessment

It has been often reported that the appearance of muscle proteins in the bloodstream following eccentric exercise provides an indirect indication of muscle damage. Assessments have been performed on the following muscle enzymes: lactate dehydrogenase, aspartate aminotransferase, carbonic anhydrase isoenzyme II, and creatine kinase (CK) (Sorichter et al., 1999). While all of these are now known to increase after damage-inducing exercise, CK has received the most attention. This is probably since it exhibits a high magnitude increase and the assay is relatively inexpensive. To date there has not been a systematic study of all of these markers in

combination. Another drawback is that the two types of exercise predominantly used in muscle damage studies (downhill running and high force muscular contractions) show different CK responses. It is problematic to use any of these muscle proteins from blood samples as muscle damage markers because blood concentration is a function of two individual processes: what the muscle produces, and what is cleared from the blood. Concentric exercise performed over several days after an eccentric exercise can result in increased levels of CK in the blood, but no evidence of damage has been found using direct measures. Therefore, it has been suggested that the increased CK levels was released from damaged muscle due a massaging action (Sorichter et al., 1995). However, exercising lightly in the days after a bout of high-force eccentric exercise resulted in lower increases in serum CK activity than performance of the eccentric exercise alone (Saxton and Donnelly, 1995). This effect could be due to a decrease in release from damaged muscle, but also faster clearance of CK from the blood.

A second bout of eccentric exercise resulted in a lower CK increase; probably because blood CK levels were elevated from previous exercise and this had already activated clearance (Nosaka and Clarkson, 1994). CK is released by the muscle, enters the lymphatic system and finally is diffused into the blood stream (Havas et al., 1997; Sayers et al., 2000). One of the major drawbacks when using CK as a muscle damage marker is related to broad range of inter-subject response variability (236 to 25,244 UI/ Litre (Nosaka and Clarkson, 1996). MRI assessments show that individuals with the highest blood CK activity have generally undergone the greatest damage, but this relationship is not perfect. The great variability in CK response to exercise is not understood the roles of gender, muscle mass or subject activity level are not fully understood. The source of this variability remains to be determined (Gunst et al., 1998). The same study showed that serum CK activity might be related to serum glutathione activity, which ought to act as a CK preserving agent when in the circulation (Gunst et al., 1998). To summarise, blood CK activity clearly provides an indirect qualitative

marker of muscle damage and that it may be influenced by several factors besides exercise-induced muscle damage.

2.8.4. Muscle soreness as a marker of muscle damage

Muscle soreness peaks 24-48 hours after the performance of the damage-inducing exercise bout (Clarkson et al., 1992; Ebbeling and Clarkson, 1989; Newham et al., 1983). The degree of soreness differs with mode and intensity of the exercise. The assessment of the amount of muscle damage is by a scale of 1 (no soreness) to 10 (very sore) and this correlates well with the increases in blood CK activity and force loss (Baird at el., 2012).

Different exercise protocols have different degrees of soreness, but they are similar in time-course (Clarkson et al., 2002). Muscle soreness could be the result of swelling and augmented pressure in the muscle where, as consequence of the damage, muscle area increases proportionally (Friden et al., 1988). The larger the damage/area, the longer it takes for the tissue fluid pressure to return to normal. It is possible that swelling in the muscle fibres activates free nerve ending in muscles, contributing to the sensation of soreness (Shellock et al., 1991). In addition, through the synthesis and release of inflammatory mediators and interactions with neurotransmitters and their receptors, the immune cells, glia and neurons form an integrated network that coordinates immune responses and modulates the excitability of pain pathways (Ke Ren et al., 2010). The immune system also reduces sensitization by producing immune-derived analgesic and anti-inflammatory or pro resolution agents (Ke Ren et al., 2010).

Chemicals (e.g. prostaglandins) are also implicated in producing the sensation of soreness (Ricciotti et al., 2012). Indeed, they activate Type III and Type IV nerve afferents, which send pain signals to the central nervous system (Buckworth et al., 2013). There is evidence that the presence of chemical mediators is reflected by the time-course of soreness, but the physical stimulus for the sensation of pain should be given by the increased pressure (Howell et al., 1993).

Muscle damage is caused more by eccentric contractions than by concentric contractions due the mechanical strain from extreme muscle fibre lengthening (Teague and Schwane, 1995). Eccentric contractions increase tension and a higher load is distributed across the same number of fibres (Enoka, 1996). This results in a higher ratio of load to fibre (Howell, 1995). However, since soreness peaks 24-48 hours after exercise and swelling is greatest several days after exercise, this suggests that damage is somehow exacerbated in the days following exercise. The inflammation after the initial insult is the most probable cause for continued damage and might contribute to the regeneration process. Enoka showed that reperfusion injury increases CK plasma activity and a biopsy showed histologic changes similar to those found after exercise-induced damage (Enoka, 1996). Reperfusion injury was due to restricted blood flow and then an increase in blood flow. It has been suggested that muscle damage associated with reperfusion injury might stem from capillary damage (Jones and Round, 1997).

Scientists have proposed that mechanical insult to muscle from eccentric contractions would most likely result in damage to the capillary endothelium. This is based on the similarities between reperfusion damage and exercise-induced muscle damage. Consequently, these burst capillaries could contribute to edema and compromised blood flow, causing further fibre damage (Jones and Round, 1997). Importantly, inflammation after muscle damage clears debris from the injury site to prepare for regeneration (MacIntyre et al., 1995). This is thought to be triggered by the initial mechanical insult and it is characterized by an infiltration of fluid and plasma proteins into the injured tissue and increased inflammatory cell populations (MacIntyre et al., 1995; Pedersen and Toft, 2000; Smith, 1991). This inflammatory cell increase is thought to amplify initial muscle injury by increased production of reactive oxygen species and an activation of phospholipases and proteases in injured tissue (MacIntyre et al., 1995). The development and duration of the inflammation can vary due to several factors, such as mode, intensity and duration of exercise (Brentano and Kruel, 2011; MacIntyre et al., 1995; Pedersen and Toft, 2000; Smith, 1991).

2.8.5. The repeated bout effect (**Chapter 6**)

A single bout of muscle-damaging eccentric produces an adaptation following there less damage is reported when the exercise bout is repeated after a week, or even up to 6 months following the initial damage (Clarkson et al., 1992; McHugh et al., 1999). Significantly, CK activity is dramatically increased after the first bout but the second bout results in virtually no change (Balnave and Thompson, 1993; Clarkson and Tremblay, 1988). When the second bout of muscle-damaging exercise occurred 2 to 6 days after the first bout, recovery time from the first bout did not differ and this did not result in a recovery setback (Nosaka and Clarkson, 1995; Paddon-Jones et al., 2000) .

The process behind the repeated bout effect is not understood. One can assume that the first bout of damage produces an adaptation i.e. the muscle is less vulnerable to subsequent exercise damage (Clarkson and Tremblay, 1988). Moreover, this did not depend on the degree of damage in the first bout, which might suggest neural factor involvement (Kuhl et al., 2010). One plausible explanation is greater recruitment pattern efficiency during the second bout (Golden and Dudley, 1992; Nosaka and Clarkson, 1995). For example, it was suggested that soreness was caused by muscle fibre rupture due to discrepancies in stimulating neurons activation (Hough, 1902; Hortobagyi et al., 1998). Other reached the conclusion that neural factors were at least partly involved in the adaptation process. The limitations of neural factor models came originated in animal models where the repeated bout effect was found in electrically-stimulated muscular contractions, but fibre recruitment was not found to be different (McHugh et al., 1999). Armstrong (1990) first suggested that muscle damage observed after eccentric exercise could stem from irreversible damage to stress-susceptible fibres. One study (Foley et al., 1999) may be taken to mean that the repeated bout effect is due to fragile fibre loss during the first bout of exercise. These findings suggest that stress-susceptible fibres develop over time quite naturally and are susceptible to eccentric exercise strain when they are irreversibly damaged or lost. When an exercise is repeated there follows further fibre damage, and these fibres would also be lost. If this continued a significant decline

in muscle mass would occur, which seems implausible. A contrary explanation is that damaged fragile fibres are repaired over time and that these repaired fibres are enhanced and less susceptible to damage. Evidence, suggests a 6 months development period for fragile fibre areas due to muscle disuse (Clarkson et al., 2002).

Immune responses to exercise-induced muscle damage are, it has been argued, diminished after repeated bouts of exercise. Evidence has shown decreases in the number of circulating neutrophils and lower levels of neutrophil and monocyte activation 3 weeks after the first bout of exercise (Pizza et al., 1996). Neutrophils and monocytes, it has been suggested, induce more damage as they enter the fibre, therefore, their lessened activity could result in less damage sustained. Cytokines (e.g., IL-6) are released, it is believed, from exercise-damaged muscle due to acute inflammation. Plasma IL-6 increases were shown to show similar patterns following each bout of eccentric exercise (Croisier et al., 1999). It has therefore been argued that IL-6 is either not related to acute inflammation or that inflammation is not reduced by the repeated bout effect (Margaritelis et al., 2015).

Animal studies show that the first bout of eccentric exercise could promote increased sarcomeres (Lynn and Morgan, 1994). Meanwhile human studies have shown sarcomere length may correlate to muscle injury (Clarkson et al., 2002). It has been further suggested that eccentric exercise produces an altered optimal angle for force production and that it was significantly greater in the leg that had been trained. This resulted in an increased muscle damage susceptibility (Whitehead et al., 1998). That in turn would suggest that concentric training reduces sarcomeres and leads to increased eccentric exercise-induced damage vulnerability. This evidence suggests that increasing sarcomeres may protect against injury (Ploutz-Snyder et al., 1998), which has particular implications for the repeated bout effect, and how it is taken under consideration in training plans.

2.9. Thesis objectives

In conclusion, the aim in the present thesis was to assess the effect of mild hypothermia and prolonged cold exposure on relevant measures of immune function in healthy humans. As cold exposure is known to activate the SNS and HPA axes that have immunosuppressive effects, we hypothesised that mild hypothermia would impair immune function, as assessed by neutrophil degranulation, and whole-blood culture cytokine release (IFN-γ and IL4).

In addition, it is of interest to investigate if prior EIMD increases heat strain during exercise-heat-stress conducted after muscle damage thus supporting the work of Montain et al. (2000). They showed that performing eccentric exercise might be an additional and novel risk factor for EHI due to the increased heat strain while exercising in the heat. Limitation of this study was to show a very small increase in core body temperature (0.2-0.3 °C) during exercise heat stress after muscle injury compared with control at 2 h and 7 h after injury. Evidently, it is of interest to investigate, and adopt actions that may reduce the risk of EHI. It is logical that a reduction of the degree of muscle damage experienced will also lessen the amount of heat strain met. One coherent, but unexplored pathway of research is the well-known repeated bout effect of muscle-damaging exercise.

As stated above, there are numerous factors that could influence the immune response to exercise and extreme environments.

With this in mind, the broad objectives of the following experiments were to investigate the influence of:

1. Mild hypothermia and prolonged cold exposure on measures of immune function in healthy humans.

2. Exercise-Induced Muscle Damage (EIMD) upon heat strain during subsequent exercise in heat.

3. If the repeated-bout effect of EIMD lower the increase in rectal temperature (T_{re}) experienced during subsequent endurance exercise under determined heat conditions.

CHAPTER THREE

General Methods

3.1. Ethical approval

Approval for all studies was obtained from the local Ethics Committee (School of Sport, Health and Exercise Sciences, Bangor University, Bangor).

The nature and purpose of each study was fully explained verbally and in writing to each volunteer (**Appendix A**). Each participant was made fully aware that they were free to withdraw from the study at any time and completed an informed consent form (**Appendix B**). To further confirm that participants were free from upper-respiratory tract infections, on each of the 14 days preceding both main trials, participants completed an illness-specific questionnaire (WURSS-44) (Barnett et al., 2002).

Following personal meetings we could confirm that all participants were non–heat or cold acclimated, nor accustomed to downhill running or regular eccentric exercise. In addition, participants were non-smokers and free from any known immune, cardiovascular, or metabolic diseases and not taking any medication (e.g., anti-inflammatories). Participants were also asked to refrain from exercise 72 h before and from alcohol and caffeine 24 h before all exercise bouts. Prior to every trial, participants were provided fluids to consume (40 $mL \cdot kg^{-1} \cdot NBM \cdot d^{-1}$) in the prior 24-hour period to ensure they began exercise bouts euhydrated.

3.2. Anthropometry

Prior to commencement of the first experimental trial, height was recorded using a wall stadio-meter (Bodycare Ltd, Warwickshire, UK) and Nude Body Mass (NBM) by digital platform scales accurate to the nearest 50 g (Model 705, Seca, Hamburg, Germany). To monitor NBM changes during exercise-induced dehydration (**Chapter 4**) or cold exposure participants removed clothing and were towel dried prior to being weighed.

3.3. Maximal oxygen uptake (VO_{2max})

For the determination of VO_{2max} and trial workloads, participants performed a continuous incremental exercise test to volitional exhaustion on a treadmill (**Chapters 5, 6**), (HP Cosmos Mercury 4.0, Nussdorf-Traunstein, Germany).

Treadmill tests: on completion of a 5 min warm-up period at 9 $km \cdot h^{-1}$ participants began running at 10 $km \cdot h^{-1}$ at a 1.0% gradient with 2 km·h-1 increments every 3 min until 14 km·h-1 was reached, after which the gradient was increased by 2.5% every 3 min increment until fatigue. During all VO_{2max} tests expired gas was analysed continuously using an on-line breath-by-breath system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany) to determine the volumes of expired gas, oxygen and carbon dioxide. Criteria for attaining VO_{2max} included the participant reaching volitional exhaustion, a HR within 10 $beats \cdot min^{-1}$ of age predicted HR max and a respiratory exchange ratio greater than or equal to 1.15 (Bird and Davison, 1997).

3.4. Blood collection, handling and analysis

Prior to commencement of every experimental trial, participants' fasted condition was verified by a finger-prick blood sample analysed for glucose and ensuring that the reading was <6 $mg \cdot dl^{-1}$ (ACCU-CHECK Aviva, Roche, Basel, Switzerland).

During experimental trials (**Chapters 4, 5** and **6**), blood samples were collected, without venestasis, by venepuncture from an antecubital vein, into separate vacutainers tubes (Becton Dickinson, Oxford, UK), containing the anticoagulants ethylenediaminetetraacetic acid (EDTA) and lithium heparin. Part of the blood collected was then immediately used to determine haematocrit. Haematocrit (heparinised blood) was determined in triplicate using the capillary method in accordance with Watson and Maughan (2014), and plasma volume changes were estimated (Dill and Costill, 1974). Blood in the EDTA tube and blood in the lithium heparin tube was immediately

centrifuged (1500 g for 10 min at 5 °C: Hettich Rotina 35R, Bach, Germany), the plasma was aspirated and stored at -80 °C for further analysis.

3.5. Urine collection, handling and analysis

During experimental trials (**Chapters 4, 5** and **6**), all urine passed was collected into 2 L plastic containers. A 3 mL sample of each urine sample at collection times was aspirated into two eppendorfs and frozen at -80ºC for further analysis.

Euhydration was verified by checking that urine-specific gravity (Atago Uricon-Ne refractometer, Atago Co., Ltd. Tokyo, Japan.) upon arrival at the laboratory was less than 1.028 (Armstrong et al., 2010). Urine colour was determined by a urine colour scale (Armstrong et al., 1994).

3.6. Body temperatures

Rectal core temperature was assessed using a standard flexible, sterile, disposable thermistor (Henleys Medical Supplies Ltd, Herts, UK) inserted 12 cm beyond the anal sphincter, with temperature recorded using a data logger (YSI model 4000A, YSI, Dayton, USA) (**Chapters 4, 5** and **6**). In both repeated trials, participants used the same thermistor which was sterilized after every trial. For skin temperature measurements ($T_{sk)}$, insulated thermistors (Grant EUS-U, Cambridge, UK) were attached to the skin via surgical tape which conducted heat and allowed evaporation (Hypafix, BSN medical GmbH, Hamburg, Germany). The amount of tape used to attach the thermistors was standardised both between and within participants. Temperature data collected were registered using a portable data logger (Grant SQ2020, Cambridge, UK), then mean T_{sk} was calculated using a weighted equation (Ramanathan, 1964).

3.7. Statistical Analysis

Prior to commencement of each study, a sample size calculation was performed (G*Power, 3.1.9.2) using mean and SD data from previous studies (Costa et al., 2011; Montain et al., 2000; Fortes et al., 2013). For a two-tailed test with alpha level set at 0.05, and power set at 0.8.

All data were checked for normality and sphericity and analyzed using either paired t-tests or fully repeated-measures ANOVA with the Greenhouse–Geisser correction applied to the degrees of freedom if necessary. All F values reported are for the time – trial interaction unless otherwise stated. Tukey's HSD or Bonferroni-adjusted paired t-test post hoc procedures were used to determine withinsubject differences where appropriate. All data were analyzed using SPSS version 14 software (IBM, NY) and Prism 6 (GraphPad Software Inc, La Jolla, CA, USA). Data are reported as mean (SD).

Statistical significance was accepted as $P < 0.05$.

CHAPTER FOUR

Influence of mild hypothermia and cold exposure on immune function as assessed by neutrophil degranulation and antigen-stimulated cytokine production in whole blood culture

4.1. Abstract

The aim was to assess the effect of mild hypothermia and prolonged cold exposure on relevant measures of immune function in healthy humans. As cold exposure is known to activate the SNS and HPA axes that have immunosuppressive effects, we hypothesised that mild hypothermia would impair immune function, as assessed by neutrophil degranulation, and whole-blood culture cytokine release (IFN-γ and IL4). In random order, nineteen men completed a thermal-neutral control trial and a cold trial. During the cold trial they were immersed in 13 °C water until rectal core temperature reached 36 °C. Afterwards they completed a seated and semi-nude cold air exposure (0 °C) for up to 3 hours or until rectal core temperature reached 35 °C. Rectal core temperature was lowest (21 (31) (mean (SD)) minutes into the cold air exposure (35.17 (0.33) °C). Immediately after the lowest rectal core temperature total leukocytes were increased (30% $P < 0.001$), neutrophil degranulation and IL4 were unchanged, and IFN-γ production was decreased (44%, $P = 0.04$). In the fifteen participants that completed the 3-hour cold-air exposure, and were not removed from the chamber due to a decrease in rectal core temperature below 35 °C, immune function was similarly altered immediately afterwards. Despite rewarming and feeding, leukocytes (+30% $P < 0.001$) and IFN-γ production after cold-air exposure did not return to control values within the 3-hour recovery period. This is the first study that reports that other stressors than exercise (e.g. cold) might affect interferon production even after recovery from hypothermic state. In conclusion, this study shows that mild hypothermia causes immune suppression in healthy young men that might, at least in part, explain the increased incidence of upper-respiratory infection reported after cold exposure. Further research is required to elucidate if the decrease in immune function observed is due to a lower core body temperature or to the metabolic responses to the adverse environment.

4.2. Introduction

Cold exposure and mild hypothermia are commonplace in many active and clinical populations (e.g. military personnel, open-water swimmers, patients undergoing surgery). Popular belief is that cold exposure increases susceptibility to infection, however, evidence to support a link between cold exposure and increased incidence of infection is not well defined in humans (Castellani and Rhind, 2002). Indeed, early research using viral inoculation before, or immediately after, cold exposure showed no increase in the frequency of colds in the six days after cold exposure (Dowling et al., 1958). A recent study of 892 Finnish military conscripts indicates that URTI, including the common cold, were preceded by three days of declining ambient temperature and humidity (Makinen et al., 2009). More compelling evidence indicates that patients who become cold during surgery suffer greater surgical wound infection post operation (Kurz et al., 1996). Further, URTI symptoms were more than doubled in the 4-5 days after people that had their feet immersed in cold water for 20 minutes compared with non-chilled controls (Johnson and Eccles, 2005). Whether the increased infection in these studies is related to a cold-induced suppression of immune function is not clear from these studies. Cold exposure responses, including peripheral vasoconstriction and shivering thermogenesis, are initiated by the SNS and HPA axis that are also potent modulators of the immune system (Costa et al., 2010). Therefore, it is plausible that cold exposure may lead to impaired immune function because of increases in stress hormones (cortisol and catecholamines) released by the SNS and HPA axis during thermoregulatory responses.

The few human studies to investigate the effect of cold exposure on immune function report conflicting information that is dependent on the degree of hypothermia, the health of the individual, and the immune parameter studied. A very mild decrease in core temperature (<0.5 °C) has no effect, or may even be immune-stimulatory, increasing circulating leukocytes and natural killer cell activity (Lackovic et al., 1988; Brenner et al.,

1999). In contrast, lymphocyte and neutrophil function are reduced when core temperature has been decreased by more than 1°C (Wenisch et al., 1996; Costa et al., 2010; Beilin et al., 1998). Collectively, these studies suggest lowering core temperature (e.g. core temperature decrease > 1 °C) can disrupt circulating immune function, which may partly explain the increased URTI reported after cold exposure. Limitations of previous research is that they included patients undergoing surgery, which have questionable relevance to healthy persons, as surgery, anaesthesia and existing comorbidity have independent effects on inflammation and immune responses (Wenisch et al., 1996; Beilin et al., 1998). Moreover, the study with healthy participants did not include a thermoneutral control trial and therefore it is unclear whether diurnal variation is responsible for the altered immune function reported. Thus, it remains to be shown in healthy humans whether immune function is impaired when core temperature is reduced (e.g. core temperature decrease of 1–2 °C).

In consequence, the purpose of this investigation was to determine, in healthy humans, the effect of cold exposure and mild hypothermia (rectal core temperature ~35 °C) on immune function. This was achieved by immersing participants in cold water until rectal core temperature reached 36 °C after which they were exposed to cold air for up to 3 hours.

Water immersion was chosen as water has many physical characteristics that differentiate it from air. It is reported that water has a thermal conductivity of 630.5 mW/m^2 per °K, whereas for air it is 26.2 mW/m^2 per °K. This 24-fold greater capacity for thermal conductivity translates into a much greater capacity of heat transfer (Casa et al., 2007) Generally, the immersed body portion is nearly 100% in direct contact with water that surrounds it, therefore the conductive potential is far superior to air. This results in volume-specific heat capacity of water nearly 3500 times greater than that of air (Toner et al., 1996; Golden et al., 2002). Thus, a person cools four times faster in water than in air of the same temperature. Also, a water temperature of 13 °C during experimental trials was chosen to reflect typical water average temperatures in British

Isles during summer (Tipton et al., 1989). In addition, 3-hours air cold exposure was previously choosen to reflect the average time to rescue in practical context (MacGregor, 1988; Grant et al., 2002). Indeed, even if the time for rescues varies greatly depending on weather conditions and location of the casualty it has been reported that the time taken for rescues is likely to be at least 2.25 hours, and non-helicopter evacuations could be in the region of 3.5 hours (MacGregor, 1988; Grant et al., 2002). To control for time of day, results of immune function were obtained serially during a cold exposure trial and were compared to measures collected at the same time of day on a thermoneutral control trial. Immune function was assessed by neutrophil function, and antigen-stimulated IFN-γ and IL4 cytokine production, measures are considered relevant as they are part of the innate immune system and therefore the 'first line of defence' against pathogens, and thus disruption by cold exposure of any of these parameters could lead to increased URTI. Indeed, decreases in neutrophil function, and dysregulation of interferon-gamma (IFN-γ) production, a cytokine that has important anti-viral and immunomodulatory roles, is associated with increased infection in illness prone athletes compared with healthy counterparts (Clancy et al., 2006; Fox et al., 2003; Gleeson et al., 2012; Yang and Gordon, 2002; Fahlman and Engels, 2005). As cold exposure is known to activate the sympathetic nervous system and HPA axis, we hypothesised that cold exposure leading to mild hypothermia would impair immune function.

4.3. Methods

4.3.1. Participants

Nineteen healthy, recreationally active males volunteered to participate in the study (mean (SD): age 20.7 (2.2) years, height 178 (7) cm, nude body mass 68.7 (6.2) kg, body fat 11 (4) %). Participants gave written informed consent before the study, which received local ethics committee approval. Participants were free from medication or supplements and had not reported an infection on the daily health questionnaire in the two weeks before the first experimental trial (Barrett et al. 2005).

4.3.2. Preliminary testing

To ensure habituation to the laboratory, cold-water immersion and blood collections, participants visited the laboratory before their first experimental trial and performed each procedure. For the 24 hours before the first trial participants recorded their food intake. They were asked to repeat the same diet before each trial. The day before each trial participants consumed water equal to 35 $ml \cdot kg^{-1}$ of body mass throughout the day (Oliver et al., 2007). Participants were also asked to abstain from strenuous exercise and alcohol during this 24-hour period.

4.3.3. *Experimental trials*

In random order separated by one week participants completed a control trial and a cold trial. Participants awoke at 07:00 hours after an overnight fast and were transported to the laboratory. On arrival participants were requested to empty their bladder and bowels and a urine sample was collected to determine hydration status from the measurement of urine specific gravity (Atago Uricon-Ne, NSG Precision cells, New York, USA). After voiding, anthropometric measures of height and body mass were obtained. To measure rectal core temperature participants inserted a thermocouple 12 cm beyond the external anal sphincter (Grant REC soft insertion probe thermocouple; Grant 2020 Squirrel data logger, Shepreth, UK). Energy expenditure and metabolic heat production were

determined indirectly by analysis of expired gasses (Feurer and Mullen, 1986; ISO 8996, 1989).

Participants began each trial at 08:00 hours resting and clothed on a steel framed plastic laboratory chair in a 20.2 ± 1.7 °C environment. After 30-minutes of being seated, baseline blood samples were collected (08:30 hours). On the cold trial, whilst wearing swimming shorts only, participants were immersed up to the axilla in 13.0 (0.1) °C stirred water until rectal core temperature reached 36 °C. Once removed from the water participants were lightly towel dried and dressed in dry shorts, socks and gloves before entering an environmental chamber at approximately 09:00 hours. (0°C, 40% relative humidity and 0.2 $m \cdot s^{-1}$ wind velocity, Delta Environmental Systems, Chester, UK). During the cold-air exposure participants remained seated on a steel framed plastic laboratory chair in a polythene survival bag for 3 hours or until rectal core temperature reached 35 °C. Once seated, at 1 and 2 hours participants consumed water equal to 6 $ml \cdot kg^{-1}$ body mass (412 (37) ml each serving (total 1236 (117) ml). The water was flavoured but had negligible nutritional value and was served at 36 °C in an insulated mug. 60-s expired gas samples were collected by Douglas bag method every 10 minutes and analysed for VO_2, VCO_2 and VE. No food was consumed during the cold air exposure. After the cold air exposure participants were immersed up to the axilla in 40 °C water until rectal core temperature reached 36 °C or for up to 1 hour. After participants were towelled dry, 1-hour recovery blood samples were obtained (~13:00 hours) and participants consumed a meal (5146 kJ; carbohydrate 58%, fat 28%; protein 14%). After a further 2 hours of seated rest, final blood samples were obtained (~15:00 hours). On the control trial, participants remained seated in the thermoneutral environment (20.2 ± 1.7 °C) and were asked to add and remove clothes to maintain thermal comfort (Hollies and Goldman, 1977). Participants consumed identical fluid and food as on the cold trial. An overview of the study design is depicted in **Figure 6**.

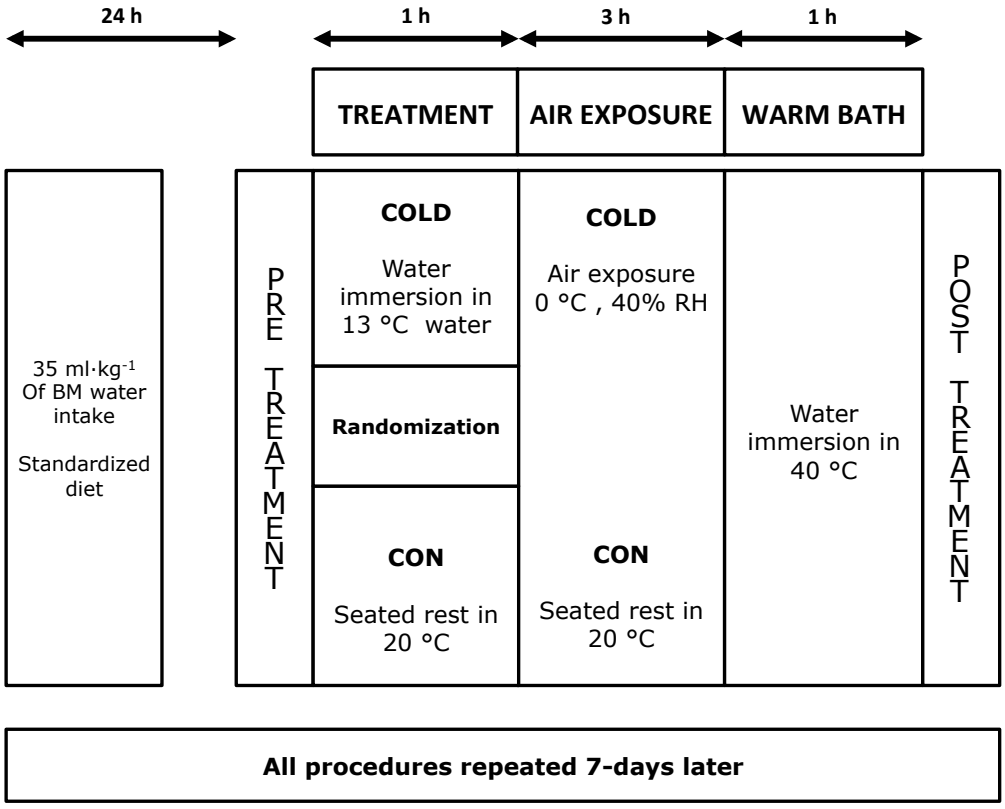

Fig. 6. Schematic of experimental procedures. Participants were removed from the environmental chamber if their rectal core temperature reached 35 °C. Participants were left in the Warm Bath until their rectal core temperature reached 36 °C or up to 1 hour**.** COLD, Cold trial; CON, thermo-neutral control trial; RH, relative humidity.

4.3.4. Sample collection and analytical methods

For both trials blood samples were collected at 08:30 hours (baseline), ~12:00 hours (immediately after the cold air exposure), ~13:00 hours (1 hour after the cold air exposure) and ~15:00 hours (3 hours after the cold exposure). On the cold trial only an additional blood sample was collected immediately after the lowest rectal temperature was recorded (~09:30 hours). Samples were obtained at 12:00, 13:00 and 15:00 hours after a baseline sample at 08:30 hours. These times of day were determined from average cold-water immersion times of pilot investigations.

4.3.5. Blood collection and analysis

Blood samples were collected, by venepuncture from an antecubital vein, into two K_3EDTA and two lithium heparin vacutainer tubes (Becton Dickenson, Oxford, UK). Haematocrit was determined by capillary method in triplicate using lithium heparin blood and a micro-haematocrit reader (Hawksley & Sons Ltd., Lancing, UK). One K_3EDTA vacutainer tube was placed on a roller and stored at room temperature before haematological analysis that included haemoglobin concentration, total and differential leukocyte counts (Gen-S, Beckman Coulter, High Wycombe, UK). All blood-borne parameters were corrected for changes in plasma volume, which was calculated from haematocrit and haemoglobin (Dill and Costill, 1974).

4.3.6. Antigen stimulated whole blood culture IFN-γ and IL-4 production

Stimulated whole blood culture production of IFN-*γ* and IL-4 was determined as previously described (Gleeson et al., 2011). Within 10 minutes of collection, and after mixing, 0.25 mL of lithium heparin whole blood was added into a culture well with 0.75 mL of medium (RPMI 1640, Sigma, Poole, UK) and 0.25 µL of vaccine stimulant (Pediacel Vaccine, Sanofi Pasteur, UK). The stimulant was a multi-antigen vaccine that contained diphtheria, tetanus, acellular pertussis, poliomyelitis and Haemophilus influenzae type b antigens, which was diluted in RPMI medium to a final concentration of 1:100. The culture well was then placed for 24 hours in an incubator (5% CO_2, 37 °C). After centrifugation at 5000 g for 4 minutes, supernatants were collected and stored frozen at -80 °C. After thawing ELISA was used to determine the cytokines IFN-γ (Cat. No. ab46045, Cambridge, United Kingdom) and IL-4 (Cat. No. ab46058 Abcam). Values that were below the detectable range on the normal sensitivity ELISA were re-assayed using high sensitivity ELISA (Cat. No. ab46063 (IFN-γ) and ab46050 (IL4), Abcam). The intra-assay coefficient of variation for IFN-γ and IL-4 concentration was 3.4 and 6.6% respectively.

4.3.7. Bacterially stimulated neutrophil degranulation

Neutrophil degranulation was determined as previously described (Laing et al., 2008). Briefly, 1 mL of lithium heparin blood was added to a micro-centrifuge tube containing 50 µL of bacterial stimulant solution (Sigma, Poole, UK). After a gentle vortex mix, this was incubated in a water bath at 37 °C for 1 hour. Samples were then centrifuged at 5000 g for 2 minutes, and the supernatant was immediately aspirated into an eppendorf and stored at -80 °C for further analysis. Plasma elastase concentration, a marker of neutrophil degranulation, was measured in unstimulated and bacterially stimulated lithium heparin plasma by ELISA (Cat No. RM191021100 Biovendor, Heidelberg, Germany). The intra-assay coefficient of variation for elastase concentration was 2.8%.

The remaining blood from lithium heparin and K_3EDTA samples was centrifuged at 1500 g for 10 minutes at 4 °C within 15 minutes of sample collection. The plasma was aspirated into eppendorfs and stored for further analysis. ELISA was used to determine cortisol concentration from lithium heparin plasma (Cat No. EIA1887, DRG Diagnostics, Marburg, Germany) and adrenaline and noradrenaline from K_3EDTA plasma (Cat No. RE59242, IBL international, Hamburg, Germany). The intra-assay coefficient of variation was 1.9, 12.2, 16.2% for plasma cortisol, adrenaline and noradrenaline, respectively.

4.3.8. Statistical analysis

The required sample size for the main outcome measures (bacterially stimulated elastase release per neutrophil) was estimated to be between six and fourteen (G*Power, Version 3.1.2) using standard alpha (0.05) and beta (0.8) levels, and means and standard deviations from previous investigations in our laboratory that have examined the effect of cold exposure on immune function (Costa et al., 2011). All data analysis was completed using Prism 6 (GraphPad Software Inc, La Jolla, CA, USA). Data are reported as mean (SD). Paired t-tests were used to compare cold trial and control trial participant body mass, fat percentage, hydration status (urine specific gravity), rectal core temperature, energy expenditure and metabolic heat production. To determine the

effect of mild hypothermia on immune function each blood parameter obtained immediately after the lowest rectal core temperature was compared by paired t-test to the control trial sample collected at the nearest time of day (08:30 hours for bloods). To determine the effect of prolonged cold exposure and recovery from cold exposure, a fully-within groups factorial ANOVA was used to compare each immune parameter (two trials – Control and Cold x four to six measurements for time dependent on analysis of blood or saliva). Where data violated the assumption of normality it was log-transformed before completing the ANOVA. For post-hoc analysis of the fully-within groups factorial ANOVA we used Holm-Sidak-tests. Significance was accepted as $P < 0.05$. In addition to traditional hypothesis testing a standardised difference (Cohen's *d* effect size) was calculated for each outcome measure to compare cold and control immune parameters at individual time points. In these calculations the numerator was the mean difference between trials. The denominator was the control standard deviation. Effect sizes were interpreted as ≥0.2 small, ≥0.5 moderate and ≥0.8 large (Cohen, 1988).

4.4. Results

4.4.1. Pre trial body composition and hydration status

Before each experimental trial, body mass and hydration status, as assessed by urine specific gravity, were similar (Body mass, Cold 68.65 (6.43) kg, Control 68.73 (6.23) kg, P = 0.73; Body fat, Cold 10.9 (3.7) %, Control 11.4 (3.7) %, P = 0.10; Urine specific gravity, Cold 1.022 (0.005) g·mL^{-1}, Control 1.022 (0.004) g·mL^{-1}, P = 0.81).

4.4.2. Experimental trial thermoregulatory and plasma volume responses

Rectal core temperature and metabolic heat production were similar during the thermoneutral rest at the beginning of each trial (rectal core temperature, Cold 36.80 (0.29) °C, Control 36.78 (0.24) °C, P = 0.55; metabolic heat production, Cold 48 (11) W·m^2, Control 47 (12) W·m^2; P = 0.66). Immersing participants in cold water increased metabolic heat production (Cold 165 (58) W·m^2, Control 45 (13) W·m^2, P < 0.001) and reduced rectal core temperature to 36°C in 29 (14) minutes (**Figure 7.**).

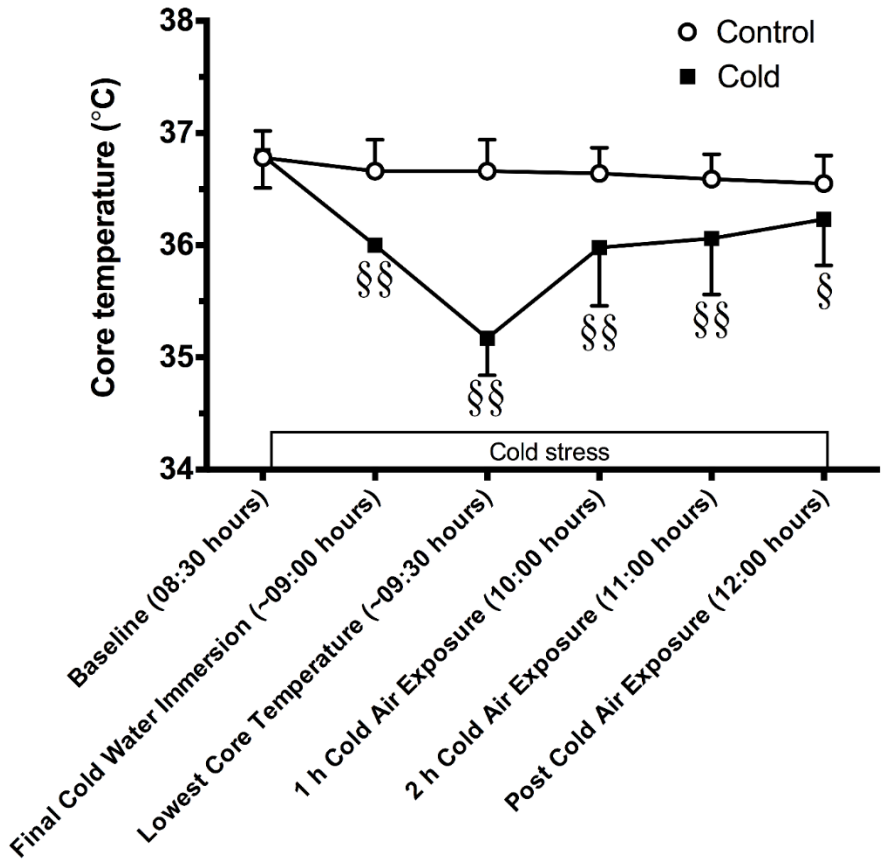

Fig. 7. Rectal core temperature during a cold exposure trial and thermoneutral control trial. Interaction post hoc statistics indicate a difference between trials at the time of day compared, § $P < 0.05$, §§ $P < 0.01$. Note the cold stress only occurred during the cold trial.

Rectal core temperature was lowest 21 (31) minutes into the cold air exposure (35.17 (0.33) °C, which was lower than control trial rectal core temperature at this time (36.66 (0.28) °C, $P < 0.001$). Rectal core temperature then rose slowly during the cold trial (cold air exposure 60 min 35.98 (0.52) °C, 120 min 36.06 (0.50) °C, 180 min 36.23 (0.41) °C). However, at 180 minutes it was still lower than in the control trial (36.55 (0.25) °C, $P = 0.01$). During the cold air exposure, metabolic heat production declined (60 min 120 (37) $W \cdot m^2$, 120 min 104 (36) $W \cdot m^2$, 180 min 100 (40) $W \cdot m^2$). However, at 180 minutes it was still greater than in the control trial (60 (23) $W \cdot m^2$, $P = 0.04$). Consequently the energy expenditure during the cold stress was 180% greater during

the cold trial than during the control trial (Cold 2692 (1111) kJ, Control 966 (442) kJ, $P < 0.001$).

Immediately after the lowest rectal core temperature, plasma volume was reduced by 12 (5) % ($P = 0.53$). Immediately after the cold exposure plasma volume continued to be lower in the cold trial relative to the control trial (Cold -10 (5) %, Control -2 (5) %: Interaction $F_{(3,39)} = 8.5$, $P < 0.001$). Plasma volume returned to within control values by 1 hour after cold exposure (Cold -4 (5) %, Control -1 (4) %).

4.4.3. Mild hypothermia, immune function and neurotransmitters

Table 1 shows that mild hypothermia caused immune modulations that included increases in circulating leukocytes (30%), decreases in antigen-stimulated IFN-γ production (44%), and no change in Neutrophil degranulation (bacterially stimulated elastase release per neutrophil), plasma elastase concentration and antigen-stimulated IL4. Additionally, Mild hypothermia increased plasma cortisol (39%), adrenaline (150%) and noradrenaline (357%).

	Number	Control	Mild hypothermia	Absolute difference (90% confidence intervals)	Effect size	*P* value
Leukocytes ($x10^9 \cdot L^{-1}$)	18	5.25 (0.93)	6.82 (1.81)	1.57 (0.90 to 2.23)	1.7 (large)	<0.001
Lymphocytes ($x10^9 \cdot L^{-1}$)	18	1.70 (0.42)	2.07 (0.60)	0.37 (0.16 to 0.59)	0.9 (large)	0.008
Monocytes ($x10^9 \cdot L^{-1}$)	18	0.48 (0.13)	0.64 (0.19)	0.17 (0.10 to 0.24)	1.2 (large)	<0.001
Neutrophils ($x10^9 \cdot L^{-1}$)	18	2.69 (0.74)	3.83 (1.43)	1.13 (0.59 to 1.67)	1.5 (large)	0.002
Bacterially stimulated elastase release per neutrophil ($fg \cdot cell^{-1}$)	17	591 (133)	566 (216)	-25 (-117 to 67)	-0.2 (small)	0.65
Antigen stimulated IFN-γ ($pg \cdot mL^{-1}$)	12	27 (30)	15 (14)	-12 (-22 to -3)	-0.4 (small)	0.04
Antigen stimulated IL-4 ($pg \cdot mL^{-1}$)	10	1.0 (1.0)	0.9 (0.7)	-0.1 (-0.4 to 0.2)	-0.1 (trivial)	0.46
Plasma elastase ($ng \cdot mL^{-1}$)	17	42 (14)	49 (24)	7 (-1 to 15)	0.5 (moderate)	0.14
Plasma cortisol ($nmol \cdot L^{-1}$)	18	358 (99)	498 (158)	140 (78 to 203)	1.4 (large)	0.001
Plasma adrenaline ($nmol \cdot L^{-1}$)	18	0.4 (0.3)	1.0 (0.5)	0.6 (0.4 to 0.7)	1.5 (large)	<0.001
Plasma noradrenaline ($nmol \cdot L^{-1}$)	18	2.3 (1.0)	10.5 (5.1)	8.2 (6.1 to 10.2)	3.6 (large)	<0.001

Table 1. Mild hypothermia effect on blood immune and hormone responses for each blood parameter mean and (SD) is presented for the control (08:30 hours) and mild hypothermia (T_{re} < 36 °C). Absolute differences are presented as the mean difference of mild hypothermia minus control with 90% confidence intervals in brackets. Effect sizes are standardised differences (Cohen's *d*) with a qualitative description in brackets. Effect sizes were interpreted as ≥0.2 (small), ≥0.5 (moderate) and ≥0.8 (large) (Cohen, 1988). Abbreviations: IFN-γ, interferon-gamma, IL-4, interleukin-4.

4.4.4. Three-hour cold air exposure effects on circulating immune responses

Relative to baseline, circulating leukocytes were increased by 50% immediately after cold exposure and throughout recovery were higher than during the control trial (Interaction $F_{(3,39)}$ = 11.8, P < 0.001: **Figure 8.A**). This was attributable to a 93% increase in neutrophils (Interaction $F_{(3,39)}$ = 14.7, P < 0.001: **Figure 8.B**), which contrasted a smaller 13% decrease in lymphocytes (Interaction $F_{(3,39)}$ = 3.9, P = 0.02: **Figure 8.C)**. This decline in lymphocytes was in contrast to the initial increase reported during mild hypothermia (**Table 1**). Lymphocytes returned to within control values 3 hours after cold air exposure. Monocytes were not altered from control values after the 3 hour cold exposure (Interaction $F_{(3,39)}$ = 1.2, P = 0.32), which differed from the initial increase observed with mild hypothermia (**Table 1**).

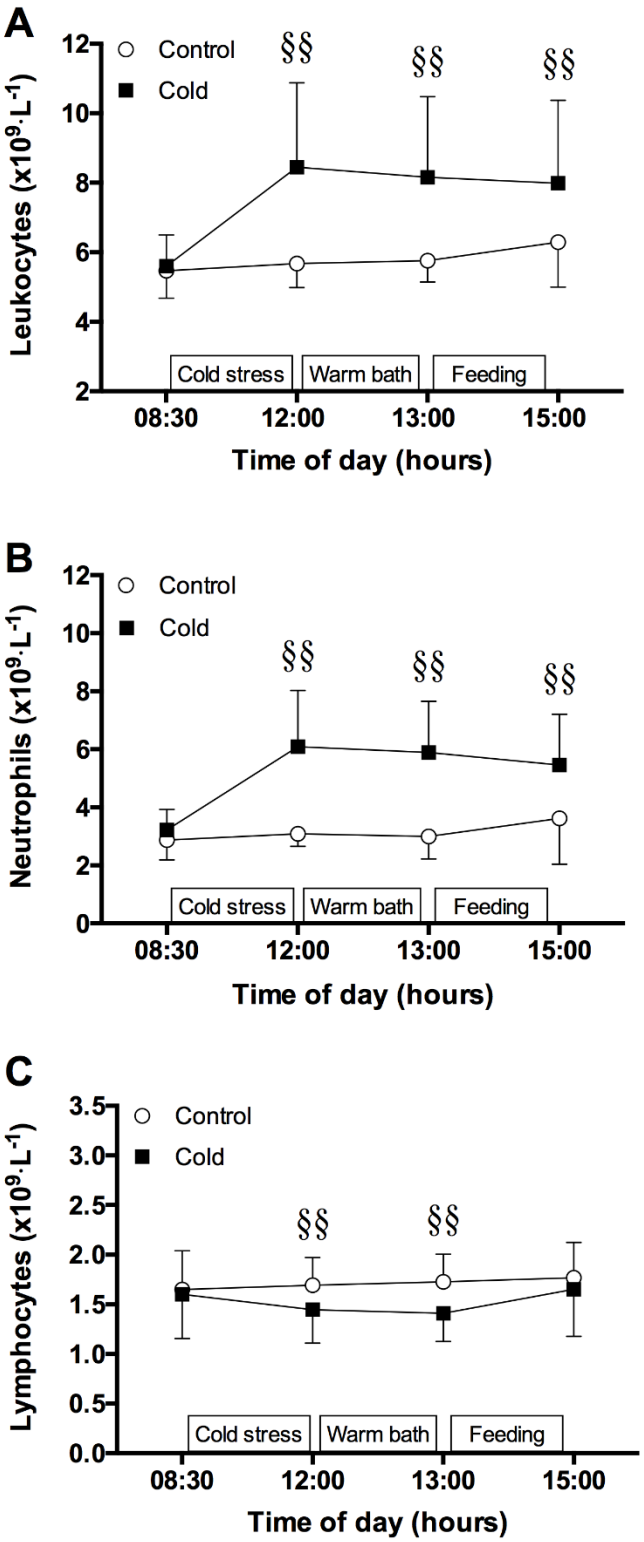

Fig. 8. Circulating leukocytes (A), neutrophils (B) and lymphocytes (C) during a cold exposure trial and thermoneutral control trial. Data are for the participants that completed the whole cold trial only (n = 14). Interaction post hoc statistics indicate a difference between trials at the time of day compared, §§ $P < 0.01$. Note the cold stress and warm bath only occurred on the cold trial.

Neutrophil function, as assessed by bacterially stimulated neutrophil degranulation, was increased at 13:00 from 08:30 hours (Main effect of time F = 9.4, $P < 0.001$). However, there was no difference between the trials (Interaction F = 1.8, $P = 0.33$; **Figure 9.**), which was consistent with observations during mild hypothermia (**Table 1**). Immediately after cold exposure, plasma elastase concentration was greater than in the control trial (Interaction F = 3.1, $P = 0.04$: **Table 2**).

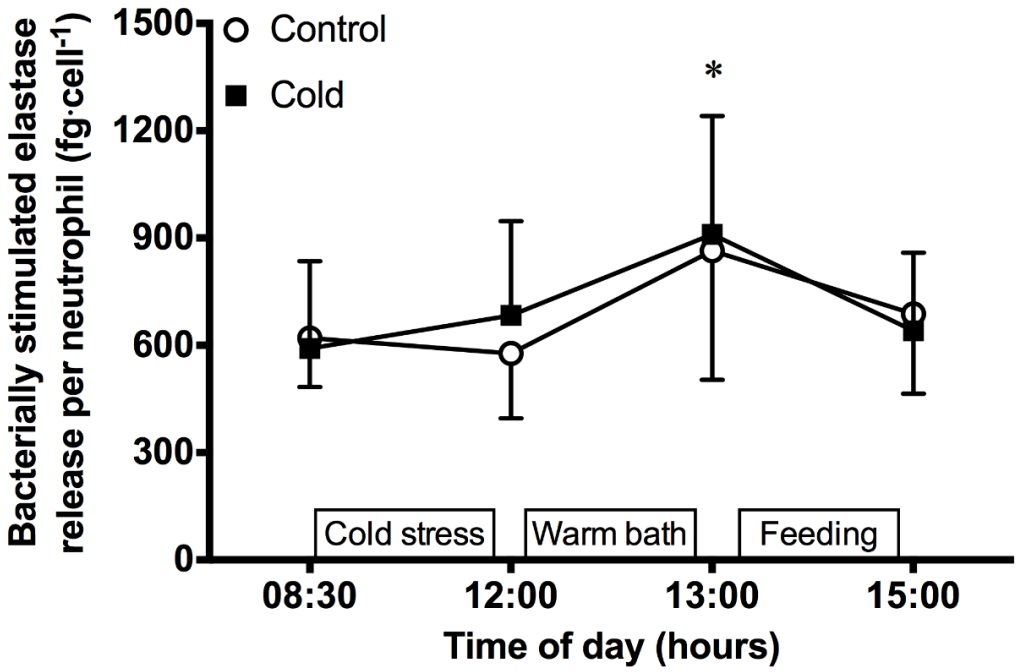

Fig. 9. Neutrophil degranulation during a cold exposure trial and a thermoneutral control trial. Data are for the participants that completed the whole cold trial only ($n = 13$). Main effect of time ($P < 0.01$): Post hoc statistics: * indicates that 13:00 hours was greater than all other times of day ($P < 0.01$). Note the cold stress and warm bath only occurred on the cold trial.

Antigen-stimulated IFN-γ production decreased immediately after cold exposure, being 69% lower than for the control trial throughout recovery (Interaction $F_{(3,21)} = 2.9$, $P = 0.06$: **Table 2**). In contrast, antigen-stimulated IL-4 production was not altered by cold exposure (Interaction $F_{(3,27)} = 1.7$, $P = 0.19$: **Table 2**), which was consistent during mild hypothermia (**Table 1**).

	08:30 hours Baseline	12:00 hours Post cold air	13:00 hours 1 h post cold air	15:00 hours 3 h post cold air
Antigen-stimulated IFN-γ ($pg \cdot mL^{-1}$)				
Cold	27 (23)	10 (9)§§	9 (10)§	11 (10)§§
Control	34 (34)	33 (37)	26 (32)	41 (45)
Effect size	-0.2 (small)	-0.6 (moderate)	-0.5 (moderate)	-0.7 (moderate)
Antigen-stimulated IL-4 ($pg \cdot mL^{-1}$)				
Cold	1.1 (0.9)	0.7 (0.7)	0.9 (0.7)	0.9 (0.8)
Control	1.0 (1.0)	0.9 (0.9)	1.1 (1.0)	1.0 (1.0)
Effect size	<0.1 (trivial)	0.2 (small)	0.2 (small)	-0.1 (trivial)
Plasma Elastase ($pg \cdot mL^{-1}$)				
Cold	582 (14)	759 (39)	798 (31)	688 (31)
Control	661 (24)	577 (39)	714 (30)	721 (68)

Table 2. Prolonged cold exposure effect on antigen-stimulated production interferon-gamma, plasma elastase and interleukin-four cytokines. Data are for the participants that completed the whole cold trial only (n = 8 for IFN-γ, plasma elastase and IL-4). Interaction post hoc statistics indicating a difference between trials at the time of day compared, § $P < 0.05$ and §§ $P < 0.01$. Effect sizes are standardised differences (Cohen's d) with a qualitative description in brackets. Effect sizes were interpreted as ≥0.2 (small), ≥0.5 (moderate) and ≥0.8 (large) (Cohen, 1988).

4.4.5. Three-hour cold air exposure effects on neurotransmitter

Plasma cortisol was greater during the cold trial than for the control trial (main effect of condition $F_{(1,13)}$ = 5.0, P = 0.05, interaction $F_{(3,39)}$ = 3.2, P = 0.03: **Table 3**). Differences were evident at baseline (**Table 3**: post hoc: P = 0.03, d = 0.8). However, differences between trials greatest at the lowest rectal core temperature (**Table 1**: P < 0.001, d = 1.4). Plasma cortisol returned to within control values during the cold trial after the cold exposure (**Table 3**). Plasma adrenaline and noradrenaline were greater in the cold than the control trial (adrenaline, main effect of condition $F_{(1,13)}$ = 5.0, P = 0.04, interaction $F_{(3,39)}$ = 3.5, P = 0.02; noradrenaline, main effect of condition $F_{(1,13)}$ = 63.5, P < 0.001, Interaction $F_{(3,39)}$ = 26.4, P < 0.001: **Table 3**). Differences were evident immediately after the cold exposure (**Table 3**: Post hoc: P < 0.01) and were similar to those at the lowest rectal core temperature (**Table 1**, d < 0.8, large effect size). Plasma adrenaline returned to within control values by 1 hour after the cold exposure (**Table 3**). There was, however a trend for noradrenaline to remain elevated during recovery 1 hour after the cold exposure (P = 0.09, d = 0.6), although this difference between trials was reduced after 3 hours recovery.

	08:30 hours Baseline	12:00 hours Post cold air	13:00 hours 1 h post cold air	15:00 hours 3 h post cold air
Plasma cortisol (nmol· L^{-1})†				
Cold	441 (122)§	348 (165)	265 (209)	308 (112)
Control	353 (105)	282 (174)	301 (189)	304 (102)
Effect size	0.8 (large)	0.4 (small)	-0.2 (small)	<0.1 (trivial)
Plasma adrenaline (nmol· L^{-1})†				
Cold	0.5 (0.4)	0.9 (0.7)§§	0.7 (0.4)	0.5 (0.3)
Control	0.5 (0.3)	0.4 (0.5)	0.5 (0.6)	0.6 (0.8)
Effect size	<0.1 (trivial)	1.0 (large)	0.3 (small)	-0.1 (trivial)
Plasma noradrenaline (nmol· L^{-1})†				
Cold	2.4 (0.8)	6.6 (2.1)§§	3.5 (0.7)	3.6 (1.2)
Control	2.2 (0.9)	2.5 (1.1)	2.7 (1.4)	3.2 (1.1)
Effect size	0.2 (small)	3.7 (large)	0.6 (moderate)	0.4 (small)

Table 3. Prolonged cold exposure effect on plasma adrenaline, noradrenaline and cortisol. Data are presented as mean (SD) and are for participants that completed the whole cold trial (n = 14). † Main effect of condition. Interaction post hoc statistics indicating a difference between trials at the time of day compared, § $P < 0.05$ and §§ $P < 0.01$. Effect sizes are standardised differences (Cohen's d). Effect sizes were interpreted as ≥0.2 (small), ≥0.5 (moderate) and ≥0.8 (large) (Cohen, 1988).

4.5. Discussion

The aim of the current study was to test the hypothesis that cold exposure, leading to mild hypothermia (rectal temperature ~35 °C) would impair immune function as assessed by neutrophil degranulation and antigen-stimulated cytokine production by whole blood culture (IFN-γ and IL-4 cytokine production) in healthy humans. Hypothermia is frequent in some occupational populations exposed to cold air and cold water during activities (e.g. military personnel, hikers and outdoor enthusiasts) (Beeley et al., 1993; Brannigan et al., 2009; Giesbrecht, 2000; Keatinge et al., 2001; Nuckton et al., 2000) and there is evidence of an increase of URTI incidence after cold exposure. In addition, trauma victims often suffer from hypothermia on arrival at hospital which is associated with a poorer prognosis and an increase in mortality rate (Lapostolle et al., 2012; Luna et al., 1987).

In the current study, cold-water immersion and cold-air exposure under controlled laboratory conditions were successful in inducing mild-hypothermia in healthy young males. In contrast to our hypothesis, immune indices such as neutrophil degranulation (bacterially stimulated elastase release per neutrophil), plasma elastase concentration and antigen stimulated IL-4 production were not compromised compared with a thermo-neutral control trial. However, in support of our hypothesis, whole-body cooling to mild hypothermia and cold exposure decreased antigen-stimulated IFN-ϒ production compared to a thermoneutral control trial. The decrease in antigen-stimulated IFN-ϒ production was evident from immediately after onset of mild hypothermia, and remained below thermoneutral control values even after rewarming restored normal body temperature and individuals had received approximately two-thirds of their daily energy requirements (Nieman, 2011). Moreover, an increase in circulating leukocytes, cortisol, adrenaline and noradrenaline was observed during mild hypothermia. These data suggest that mild-hypothermia might decrease immunity leading to an "open window" where immune function is suppressed. This window of opportunity may allow for an increase in susceptibility to URTI.

Compared with previous studies (Lackovic et al., 1998; Brenner et al., 1999; Wenisch et al., 1996; Costa et al., 2010; Beilin et al., 1998), a strength of the present research is that experimental procedures were carried out under controlled laboratory conditions and were specifically designed to enable us to be more specific regarding the influence of cold exposure and mild hypothermia on immune function. Indeed, the main limitation of the studies in the literature was the lack of a thermo-neutral control trial and therefore the effect of diurnal variation on outcomes could not be excluded. Moreover, other limitations such as multi-stressor experimental designs and the study of patient populations with existing comorbidities or undergoing surgery mean that prior to this study it was difficult to interpret the possible interaction between cold exposure and the immune system.

Additional strength to the present study design comes from protocol followed; removing participants from water immersion and cold-air exposure at pre-defined body temperature (water immersion T_{re} 36 °C, cold-air exposure T_{re} 35 °C), rather than a pre-defined time period, allowed for a more accurate assessment of the influence of cold exposure and mild hypothermia on immune indices. Furthermore, a cut off time of 3 hours of cold-air exposure was previously agreed to reflect the average time to rescue in practical context. Indeed, even if the time for rescues varies depending on weather conditions and location of the casualty, it has been reported that the time taken for rescues is likely to be at least 2.25 hours, and non-helicopter evacuations could be in the region of 3.5 hours (MacGregor, 1988; Grant et al., 2002). Lastly, even if participants did not reach a rectal core temperature of 35 °C in this time they were removed from the chamber.

A limitation of the present study is that mild hypothermia does not generally apply to the general population but affects those involved in outdoor activities during winter months (e.g. soldiers, climbers, athletes). In this respect, the data presented have to be seen as the first attempt to clarify if cold exposure and the subsequent mild-hypothermia is a potential factor in the increase of URTI reported during winter months.

During cold exposure, the body attempts to maintain a normal body temperature by increasing heat production and minimizing heat loss. This is accomplished through involuntary tonic muscular activity, rhythmic muscular activity (shivering), and peripheral vasoconstriction. Although primarily thermoregulatory, these mechanisms induce metabolic and hormonal changes such as SAM axis stimulation, which can subsequently affect immune function (Brenner et al., 1999; Castellani et al., 2002).

The relatively modest leucocytosis immediately after cold exposure is likely to be explained by demargination of neutrophils from the vascular endothelium. Although raised circulating noradrenaline after cold exposure might play a role in demargination of neutrophils from the vascular endothelium (Brenner et al., 1998), other mechanisms such as shear effect and adrenaline intervention results could be involved (Zhang et al., 2011; Bote, Giraldo and Peake, 2008, Laing et al., 2008, Ortega et al., 2005; Ramel and Peake, 2004; Ramel, 2003; Malpica et al., 2002). In this context, catecholamines are the most likely cause for the observed rise in circulating neutrophils because they are rapidly released in the blood, causing an immediate response in immune cells. On the contrary, the small increase in plasma cortisol (~12%) during cold exposure is unlikely to account for the changes observed in leukocyte trafficking due to the time required for cortisol itself to produce any immune responses (Goldstein-Golaire et al., 1970, Jefferies, 1991). This is supported by that a second delayed increase of blood leukocytes count due to cortisol-induced release of neutrophil from the bone marrow has not been observed a few hours after cold exposure (Gagnon et al., 2014). There is evidence that the second increase in leukocyte trafficking elicited by cortisol is dependent upon intensity and duration of the exercise (Fragala et al., 2011), and that only high-intensity exercise results in significant elevation of cortisol (200%) (Jacks et al., 2002), which is unlikely to be the case of present study. In addition, substantial biological effects of cortisol tend to be reported only when the plasma cortisol concentration exceeds the capacity of the corticosteroid-binding globulin (550 nmol l^{-1}) and free cortisol concentration increases (Costa et al., 2011).

Cold exposure and mild hypothermia did not compromise neutrophil function, as assessed by bacterially stimulated neutrophil degranulation. These results conflict with previous findings where modest whole body cooling (T_{re} 35.9 °C) was observed to decrease neutrophil degranulation (Costa et al., 2011). The decrease reported previously was explained by the demargination of more naïve intravascular neutrophils that responded less to the bacterial challenge. The discrepancy in the results might be explained by additional stressors in Costa et al.'s (2011) study before the cold exposure, which included two days of sleep and energy restriction with daily maximal exercise. The significant neutrophilia reported in our study is in line with evidence in the literature (Fedor and Fisher, 1959, Villalobos et al., 1955; Blair et al., 1969). An increase in circulating neutrophils was reported in septic patients who were cooled to 34 °C by Blair and colleagues (1969). To the contrary, in another study on animals, Biggar and colleagues (1983) found that hypothermia was associated with a marked reduction in the release of mature and immature neutrophils from the bone marrow into the circulation. Once again it is underlined that the mediators of the neutrophilia and decrease in neutrophil degranulation in response to stress (e.g. exercise) are not as clear as previously thought and require further research.

Lymphocytosis is observed during and after cold exposure, with the number of cells falling below pre-exercise levels during recovery. It has been previously reported that changes in T cell populations, and to a lesser extent B cell populations, are proportional to stress intensity and duration, although the effect of intensity is more marked (Gleeson et al., 2005). Research showed a 50% decrease in T cell population after a 2.5 h treadmill run at 75% VO_{2Max} compared with resting values (Gleeson et al., 2005). Due to the prolonged nature of that exercise bout present data can be compared. We acknowledge the limitation that we did not assessed maximal oxygen uptake before trials and therefore we are not able to determine the workload imposed by thermoregulatory responses. In addition, indirect assessment of the cost of thermogenic activity can be perform using the Metabolic Equivalent of Tasks (MET) providing us with

the information that the increased thermoregulatory demand during the three hours cold exposure can be equated with prolonged-moderate exercise (5.6 (2.0) MET) (Ainsworth et al., 2011).

Lymphocyte count acted in a similar fashion to that measured in response to prolonged exercise even if the percentage decrease observed during trials was smaller. In addition, T cell number returned to baseline levels after 3 h, similarly to the response to prolonged exercise (Robson et al., 1999). Raised circulating adrenaline might account for the lymphopenia during recovery after cold exposure possibly via alterations in adhesion molecules and surface receptors on lymphocytes (Costa et al., 2011; Kruger and Mooren, 2007). Adrenaline is thought to have a key role in governing lymphocyte trafficking during environmental stress (Costa et al., 2010). Moreover, adrenaline and noradrenaline indirectly mediate symphatetic influences on cardiac output, with the subsequent increase in shear stress associated with enhanced blood flow. T cells play a fundamental role in the orchestration and regulation of the cell-mediated immune response to pathogens (Shephard and Shek, 1999). One important consequence of a defect in T cell function is an increased incidence of viral infections (Fabbri et al., 2003).

Cold air exposure did not alter monocyte count after the initial increase with mild hypothermia. A plausible explanation of this increase could be that shivering and involuntary tonic contractions count as acute physical exercise which has been reported to result in a transient monocytosis and which most likely represents the shifting of monocytes from the marginated to the circualtion pool (Young et al., 2000). This could occur as a result of haemodynamic and/or cortisol or catecholamine-induced release from the vascular endothelium (Walsh et al., 2011). This explanation is supported by research which showed that administering the beta-blocker propranolol reduced exercise-induced monocytosis (Walsh et al., 2011). Moreover, reports showed that stressors can affect monocyte phenotype, cell surface protein, and cytokine expression. For example, in response to acute exercise, there is a preferred use of inflammatory monocytes (CD4+/CD16+) (Freidenreich and Volek, 2012). It is therefore conceivable

that these marginated cells have a more mature inflammatory function for entry into tissues and are knocked off the endothelium in response to exercise. Interestingly, the percentage of these CD14+/CD16+ cells is reduced during recovery, maybe indicating remarginalization or tissue recruitment. Acute exercise also reduces expression of TLRs 1,2 and 4 on inflammatory monocytes (Gleeson et al., 2011). However it remains to be elucidated the extent to which these changes reflect a true decrease versus monocyte population shift.

Some of the changes reported on innate immune markers are similar to the ones effected by exercise and this might partially be explained by the raised concentration of catecholamines observed in both scenarios. Indeed, under these conditions exercise could be seen as the thermogenic activity through which the body counteracts the cold environment (Walsh et al., 2006). Through involuntary tonic muscular activity and rhythmic muscular activity (shivering) up to 30% of aerobic capacity could be involved making reasonable expecting immune markers mimics the response to exercise (Young et al., 2000). It was previously been reported (Golden and Dudley, 1992) that shivering corresponded to 46% VO_{2Max} in 12 °C water immersion which is close to the water temperature used in this study (13 °C). In addition, metabolic rate during cold exposure was also found to be comparable to the ~40% VO_{2Max} observed results presented by Young et al. (2000) in participants who had a T_{re} of 35 °C and was.

A direct comparison between the rise in adrenaline and noradrenaline recorded in the present study and the raise induced by exercise can be performed using data provided by Galbo et al. (1975). The increase in catecholamines observed is similar to the one observed in response of a short bout of running (10 mins) at 75% VO_{2Max} intensity or repeated bouts of running performed at 75% VO_{2Max} intensity for prolonged periods of time. Therefore, this suggests that the changes observed on innate immunity might be partially explained by the thermoregulatory activity initiated by the body to counteract mild-hypothermia. In addition, it also helps to describe why neutrophil function was not affected by cold exposure even if neutrophilia has occurred. Furthermore,

catecholamines have a direct effect on cardiac output and the shear effect derived. This additionally contributes to justification of the observed changes in white blood cell count.

Antigen-stimulated IFN-ϒ production was lower compared with thermoneutral control trial values throughout cold exposure and did not recover to baseline levels after 3 hours despite participants having been fed and rewarmed. Similar results were found following a 2.5 h of cycling exercise at 65% VO_{2Max} (Lancaster et al., 2005). The release of cytokines such as interferon is mediated by Th1 cells which play an important role in defence against intracellular pathogens (e.g. viruses) (D'Elios et al., 2007) and they have been found to decrease in response to prolonged moderate-intensity exercise (Gleeson et al., 2005), this might explain the decreased production of IFN-ϒ. They are part of the acquired immunity which is designed to combat infections by preventing colonisation of pathogens and destroying invading micro-organisms (Gleeson et al., 2005). A disregulation of IFN-ϒ production has been reported in illness prone athletes compared with healthy counterparts (Clancy et al., 2006). This is the first study that reports that other stressors (e.g. cold) might affect interferon production. Present results suggest that the decreased capability of the adaptive immunity induced by cold exposure may increase the possibility of being affected by pathogens even during the recovery period. This is explained by the fact that the adaptive immunity response is initiated by the presentation of antigens to T helper lymphocytes within the peptide binding groove of major histocompatibility complex class II molecules on antigen presenting cells CD4+ T cells. This forms a key part of the cell-mediated immune response, since they orchestrate and direct the subsequent adaptive immune response to bacteria and viruses. A decreased production of IFN- ϒ might result in a suppressed response of the immune system to invading micro-organisms as they act with immunostimulatory and immunomodulatory effects or by directly inhibiting viral replication. This might, in part, explain of the increased number of URTI reported by athletes and outdoor enthusiasts during winter months.

IL-4 production was not altered by cold exposure. This finding is in line with evidence in the literature regarding IL-4 production in response to prolonged exercise at moderate intensity (Gleeson et al., 2005). The role played in a hypothermic scenario by this cytokine is not well established, but it is known to activate B cells and T cell proliferation and regulate adaptive immunity responses (Paul et al., 1997). It is usually coupled with another anti-inflammatory cytokine, IL-10. There is evidence that IL-10 production from microglia, decreases with mild-hypothermic state and that this decrease is proportional to the decrease in body core temperature (Matsui et al., 2004). This decrease is beneficial as excessively high levels of IL-10 are detrimental for the central nervous system.

In conclusion, cold exposure and mild-hypothermia did not alter neutrophil degranulation, or antigen-stimulated IL-4 production but did lower antigen-stimulated IFN-ɣ production. Despite rewarming and feeding, IFN-ɣ production did not return to control values within the 3-hour recovery period. These findings might therefore, at least in part, explain the increased susceptibility to upper respiratory tract infections reported after cold exposure.

CHAPTER FIVE

Muscle-damaging exercise increases heat strain during subsequent exercise heat stress

5.1. Abstract

It is still unclear if exercise-induced muscle damage (EIMD) increases heat strain during subsequent exercise heat stress, which in turn may increase the risk of exertional heat illness. We investigated heat strain during exercise heat stress 30 min after EIMD to coincide with the maximal increment in circulating pyrogens [e.g. interleukin (IL)-6] and 24 h after EIMD to correspond with the late muscle inflammatory response when a greater rate of metabolic energy expenditure ($\dot{M}$) and thus diminished economy might also increase heat strain.

Thirteen non–heat-acclimated males (mean(SD), age 20(2) years) completed exercise heat stress tests (running for 40 min at 65% VO_{2max} in 33 °C, 50% humidity) 30 min (HS1) and 24 h (HS2) after treatment, involving running for 60 min at 65% VO_{2max} on either -10% gradient (EIMD) or +1% gradient (CON) in a crossover design. Rectal (T_{re}) and skin (T_{sk}) temperature, local sweating rate, and $\dot{M}$ were measured throughout HS tests.

Compared with CON, EIMD elicited higher circulating IL-6 pre-HS1 ($P < 0.01$) and greater plasma creatine kinase and muscle soreness pre-HS2 ($P < 0.01$). The ΔT_{re} was greater after EIMD than CON during HS1 (0.35 °C, 95% confidence interval = 0.11 °C – 0.58 °C, $P < 0.01$) and HS2 (0.17 °C, 95% confidence interval = 0.07 °C –0.28 °C, $P < 0.01$). $\dot{M}$ was higher following EIMD throughout HS1 and HS2 ($P < 0.001$). Thermo-effector responses (T_{sk}, sweating rate) were not modified by EIMD. Thermal sensation and RPE were greater on EIMD after 25 min during HS1 ($P < 0.05$). The final T_{re} during HS1 correlated with the pre-HS1 circulating IL-6 concentration in EIMD trial ($r = 0.67$).
Heat strain was higher during endurance exercise in the heat conducted 30 min after and, to a lower degree, 24 h after muscle-damaging exercise. These data show that EIMD is a probable risk factor for exertional heat illness especially during exercise heat stress when behavioural thermoregulation clues, they might suggest to stop exercising, are neglected.

5.2. Introduction

Athletes and soldiers taking part in heavy training often have to perform repeated bouts of strenuous exercise on the same day, in hot environments, which may predispose them to EHI or the more severe form, and often incurable, EHS. Numerous conventional risk factors have been classified for EHI and EHS such as hot and humid environmental conditions, incongruous clothing, absence of heat acclimation, sleep disruption, high exercise intensity, obesity, poor physical fitness levels, and hidden medical conditions (Armstrong et al., 2007; Epstein et al., 1999; Rav-Acha et al., 2004). However, these conventional risk factors do not account for all EHI events, implying that other lesser known risk factors and pathways may play a role in many cases of EHI (Sawka et al., 2011). An additional pathway for the development of EHI has been suggested that includes a systemic inflammatory reaction (Bouchama, 1995; Lambert et al., 2001; Shephard and Shek, 1999), with an increment in pyrogenic cytokines [e.g., interleukin (IL)-1β, IL-6, and tumor necrosis factor-α], which may accelerate the progression of the person toward EHI and/or EHS (Walsh et al., 2011). In support of this assumption, the fever response to LPS injection was abolished in rodents pre-treated with anti-IL-6 antibodies (Cartmell et al., 2000; Rummel et al., 2006), and rodents injected with IL-1 receptor antagonist showed increased survival after experimental heatstroke (Chiu et al., 1996). Evidence also exists that exercise itself has a pyrogenic effect similar to that of fever. For example, post-exercise plasma taken from humans caused fever in rodents, opposite to pre-exercise plasma that did not cause the same effect (Pedersen et al., 2002). Furthermore, 6 days of integration with the cycloxygenase (COX) inhibitor rofecoxib attenuated the increase in body core temperature during prolonged exercise in humans, implying that a prostaglandin-mediated inflammatory process may also reinforce to increased heat strain during exercise (Bradford et al., 2007).

It still needs to be established whether exercise-induced muscle damage (EIMD) boosts heat strain during successive bouts of exercise heat stress, which consecutively may increase the risk of EHI.

Exercising muscle releases the pyrogenic cytokine IL-6 (Febbraio et al., 2002), and it has been showed that EIMD evokes a greater circulating IL-6 response than non–muscle-damaging concentric exercise (Braun and Dutto, 2003). Accordingly, it is possible that EIMD and correlated inflammation (increase in circulating pyrogenic cytokines) might increase heat strain at least in the short-term during consecutive exercise heat stress. The muscle soreness that commonly peaks 24–48 h after EIMD might also augment heat strain during walking and running by increasing the rate of metabolic energy expenditure (decreased economy) as a result of alterations in gait and motor unit recruitment because of muscle stiffness (Montain et al., 2000). To the best of our knowledge, only one published study has tried to elucidate these possibilities, althought if the experimental design of this investigation elicited only relatively modest heat strain (Montain et al., 2000). Core body temperature was 0.2–0.3 °C higher at 2 and 7 h but was not altered 26 h after lower body muscle-damaging exercise (eccentric component of leg press and leg curl) (Montain et al., 2000). The authors credit a large proportion of the EIMD-evoked increase in core body temperature during exercise heat stress to diminished economy but admitted that other elements such as the inflammatory response probably play a role. Regrettably, the muscle-damaging protocol did not evoke a significant increase in circulating inflammatory markers (e.g. the pyrogen IL-6) prior tothe exercise heat challenge 2 h after muscle injury and only a small increase 7 h after injury. Fundamentally, it is still unclear whether the acute circulating inflammatory response that follows EIMD exarcebates to greater heat strain during subsequent exercise heat stress. In addition, from a practical viewpoint, participants in the preceding study were heat acclimated (Montain et al., 2000), so the effect of EIMD on exercise heat strain in non–heat-acclimated individuals, in whom EHI is more common (Armstrong et al., 2007) remains unknown.

The aim of this study was to examine the effect of EIMD upon heat strain during successive exercise bouts in the heat. Therefore, we had non–heat-acclimated participants

perform muscle-damaging exercise on a downhill running model (10% gradient) and then assessed heat strain during two subsequent exercise heat stress running bouts. The first exercise heat stress bout was performed 30 min post-EIMD to coincide with the early inflammatory phase and expected increases in circulating pyrogens (e.g. IL-6). The second exercise heat stress bout was performed 24 h post-EIMD to coincide with the delayed muscle inflammatory response, i.e. when circulating creatine kinase (CK) values peak after downhill running (Clarkson and Hubal, 2002) and when lower economy might be expected to change heat strain. The EIMD responses to the two exercise heat stress bouts were compared with an energy-expenditure equivalent control, trial (treadmill running on a +1% gradient). We hypothesized that EIMD would increase core rectal temperature (T_{re}) during subsequent exercise heat stress compared with control and that the increase in T_{re} would be correlated with increases in circulating pyrogen 30 min post-EIMD.

5.3. Methods

5.3.1. *Participants*

Thirteen healthy, active males volunteered to participate in the study and provided fully informed written consent. The study received local ethical approval and was conducted within the standards required by the *Declaration of Helsinki*. Participant characteristics were as follows (mean (SD)): age, 20 (2) years; nude body mass (NBM), 70.5 (7.5) kg; height, 176 (5) cm; body mass index, 22.8 (2.0) $kg \cdot m^{-2}$; body surface area,1.86 (0.11) m^2; and maximal oxygen uptake (VO_{2max}), 60 (5) $mL \cdot kg^{-1} \cdot min^{-1}$. All participants were non–heat acclimated nor accustomed to downhill running or regular eccentric exercise.
Participants were non-smokers and free from any known immune, cardiovascular, or metabolic diseases and not taking any medication (e.g., anti-inflammatories). Participants were asked to refrain from exercise 72 h before and from alcohol and caffeine 24 h before all exercise bouts. All data were collected between February and December 2011.

5.3.2. *Study Design*

A counterbalanced experimental design in which the order of presentation of trials was randomized was used to test the hypothesis that previous muscle damage would increase rectal core temperature (T_{re}) during subsequent exercise heat stress tests.
To assess this, participants completed exercise heat stress tests, 30 min (HS1) and 24 h (HS2) after both muscle-damaging exercise (EIMD: running downhill on -10% gradient) and energy-expenditure equivalent exercise (CON: running at +1% gradient). The two trials were separated by 14 days, and an overview of the study design is depicted in **Figure 10**.

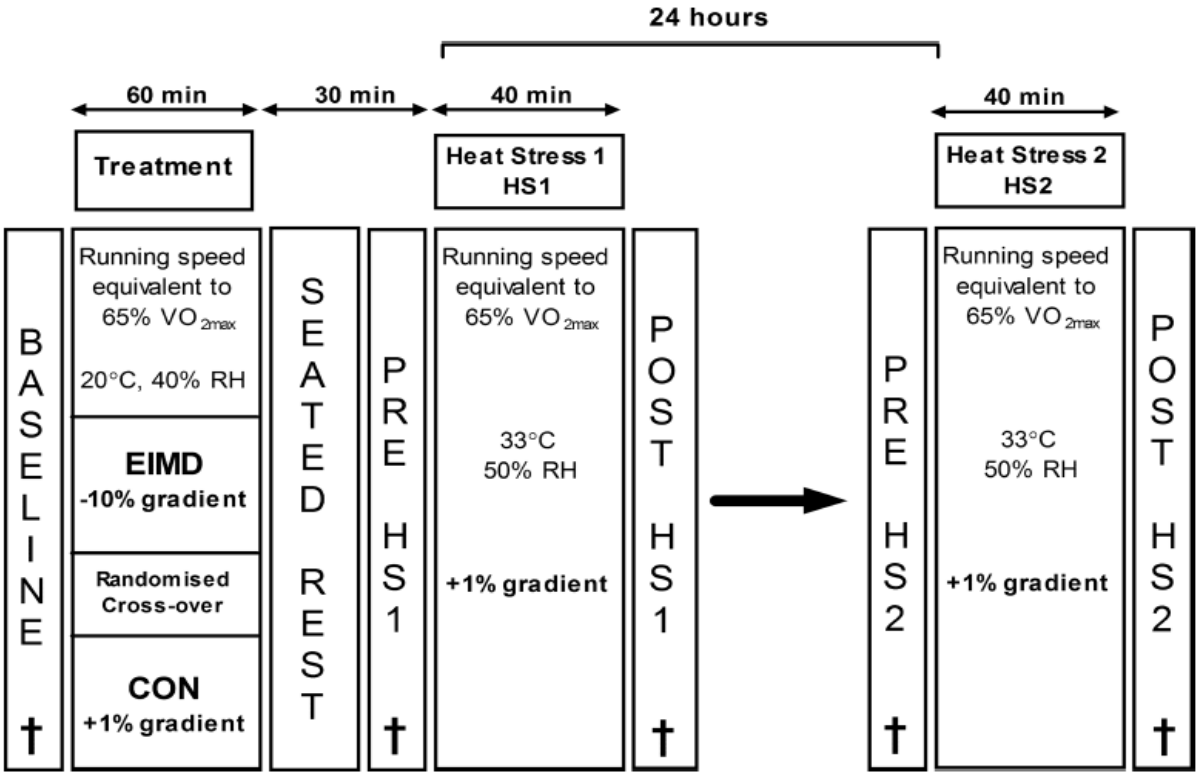

Fig. 10. Schematic of experimental procedures. EIMD, exercise-induced muscle damage; CON, energy-expenditure equivalent control exercise; RH, relative humidity.† Blood sampling time points.

5.3.3. *Fitness and familiarization*

Participant's VO_{2max} was determined by a continuous maximal incremental exercise test performed on a motorized treadmill (HP Cosmos Mercury 4.0, Nussdorf-Traunstein, Germany) to volitional exhaustion. Expired gas was analysed by an online breath by-breath system (Cortex Metalyser 3B; Biophysik, Leipzig, Germany). From this, the treadmill running speed which elicited 65% VO_{2max} running at +1% gradient was calculated by interpolation of the running speed–VO_2 relationship.

This running speed was subsequently verified and used as the running speed for the treatment on CON and for both heat stress tests in both trials. Participants were also asked to run downhill on a -10% gradient with expired gas analysed continuously, with the running speed adjusted accordingly until 65%VO_{2max} had been verified. This running speed was used for the treatment phase to elicit muscle damage on the EIMD trial.

Participants were also familiarized with all instrumentation and procedures used in the experimental trials. They were given diet diaries to record all food consumed during the 24 h before and during the first experimental trial and instructed to replicate this diet before and during the second trial.

5.3.4. *Experimental Trials*

On the day of the experimental trial, participants arrived at the laboratory [20 °C ambient temperature and 40% relative humidity (RH)] at 07:10 h fasted. Participants were provided fluids to consume (40 $mL \cdot kg^{-1}$ $NBM \cdot d^{-1}$) in the previous 24 h period to ensure they began exercise euhydrated, and this was verified by assessing urine specific gravity upon arrival (Atago Uricon-Ne refractometer; NSG Precision Cells, Farmingdale, NY). Participants were then weighed nude (NBM) on a digital platform scale accurate to the nearest 50 g (Model 705; Seca, Hamburg, Germany). On the basis of this body mass, participants were then provided with a small breakfast consisting of a cereal bar equivalent to 8.4 $kJ \cdot kg^{-1}$ NBM and water (5 $mL \cdot kg^{-1}$ NBM). At 08:15 h, baseline
NBM was again measured, and a rested blood sample was taken before the participant fitted a rectal thermistor probe and an HR monitor (Polar Electro, Kempele, Finland). The participant also rated their perceived muscle soreness at this point.

5.3.5. *Exercise Trial Treatment*

The exercise trial treatment (EIMD or CON) started at 08:30 h, with participants wearing standardized clothing, i.e. running shorts, socks, and shoes. Participants ran for 60 min at the predetermined speed that reflected 65% VO_{2max} on either +1% gradient (CON) or -10% gradient (EIMD). Rectal core temperature (T_{re}) and HR were measured every 10 min, and 60-s expired gas samples were collected by Douglas bag method at 20 and 40 min of exercise and analyzed for VO_2. Water (2 $mL \cdot kg^{-1}$ NBM) was provided every 15 min throughout treatment.

5.3.6. *Exercise Heat Stress 1*

Immediately after treatment, participants rested for 30 min during which skin thermistors were applied. A standardized amount of water (2.5 $mL \cdot kg^{-1}$ NBM) was also provided during this period. Pre heat stress 1 (pre-HS1) NBM was assessed, and a blood sample was collected just before the participant entered the environmental chamber (Delta Environmental Systems, Chester, UK), which was maintained at a dry bulb temperature of 33 °C, 50% RH, and 0.2 $m \cdot s^{-1}$ face-on wind velocity. Immediately upon entering the chamber, a ventilated capsule was attached to the forearm (for local sweating rate), and participants then began exercise heat stress (HS1) by running ,without fluids, on a motorized treadmill at +1% gradient for 40 min at the predetermined running speed that reflected 65% VO_{2max}. Throughout HS1, measurements taken were: local forearm sweating rate, HR, T_{re}, skin temperature (T_{sk}), RPE (Borg 6–20 scale (Borg, 1982)), thermal sensation (McGinnis 0–13 point (Hollies et *al*., 1977)), and 60-s expired gas samples by Douglas bag method VO_2. Immediately after HS1, participants were removed from the chamber, seated, and a blood sample was drawn (post-HS1). Post-HS1, NBM (after towel drying) was re-assessed. Fluids were provided to replace sweat losses during HS1, and participants were provided with a standardized meal and fluids to consume until they returned to the laboratory the following morning for the second heat stress test (HS2). Participants refrained from any exercise, caffeine or alcohol in the intervening period between the two HS bouts.

5.3.7. *Exercise Heat Stress 2*

HS2 was performed 24 h after the beginning of HS1. Participants reported to the laboratory and were provided with a standardized breakfast and fluids identical to the previous day. Participants rated their perceived muscle soreness just before HS2. For HS2, participants ran in the same environmental conditions and running speed as that during HS1. Before (pre-HS2) and after (post-HS2) exercise heat stress blood samples were drawn, and NBM was also assessed. All measures collected during HS2 were

identical to HS1. The participant left the laboratory and returned 14 days later to complete the remaining trial.

5.3.8. *Measurements and Instrumentation*

Body temperatures. T_{re} was measured using a flexible, sterile, disposable thermistor (Henleys Medical Supplies Ltd., Herts, UK) inserted 12 cm beyond the anal sphincter, with temperature recorded using a data logger (YSI model 4000A; YSI, Dayton, OH). Participants used the same thermistor in both trials. T_{sk} was measured at four sites on the left side of the body (on the chest at a point midway between the acromion process and the nipple, the anterior mid-bicep, the anterior mid-thigh, and the lateral calf) using insulated thermistors (Grant EUS-U, Cambridge, UK) fixed to the skin using surgical tape. Temperature data were registered using a portable data logger (Grant SQ2020, Cambridge, UK). Mean T_{sk} was calculated using a four-site–weighted equation (Ramanathan, 1964). Because of individual skin thermistors becoming detached during HS in three participants, T_{sk} mean data are presented for those with a complete data set (n = 10).

5.3.9. *Sweating responses*

Whole-body sweating rate was estimated from NBM losses during HS1 and HS2. Local forearm sweating rate was measured by dew point hygrometry.

Anhydrous compressed nitrogen was passed through a 5 cm^2 capsule placed on the lower arm ventral surface (halfway between the antecubital fossa and carpus) and connected to a hygrometry system (DS2000; Alpha Moisture Systems, UK). Local forearm sweating rate was calculated using the difference in water content between effluent and influent air and the flow rate (1 $L \cdot min^{-1}$) and normalized for the skin surface area under the capsule (expressed in milligrams per square centimetre per minute). Sweating threshold and sensitivity were calculated by plotting individual relationships between local forearm sweating rate and T_{re} values. A simple linear regression equation for the initial 4 min of exercise was calculated, with the threshold T_{re} for active

thermoregulatory sweating defined as the T_{re} at which local forearm sweating rate = 0.06 mg·cm^{-2}·min^{-1}. Sweating sensitivity was calculated as the slope of the linear regression line for both (a) the exercise transient phase (when the rapid increase in sweating rate occurred during the initial 4 min of exercise) and (b) the plateau sweating phase (when sweating rate plateaued from 6 to 40 min of exercise).

5.3.10. Rate of metabolic energy expenditure ($\dot{M}$)

Oxygen consumption (VO_2) was calculated for 60 s expired air samples collected into a Douglas bag and analyzed for O2 and CO2 concentrations (Servomex, Crowborough, UK) and volume (Harvard Apparatus, Edenbridge, UK). $\dot{M}$ was calculated using VO_2 (L·min^{-1}) and RER in the following equation (Nishi, 1981): $\dot{M}$ (W) = VO_2 {21,166[0.23(RER) +0.77]} /60.

5.3.11. *Perceived leg muscle soreness*

Perceived leg muscle soreness was self-rated using two scales. First, participants rated leg muscle soreness using a 100-mm visual analog scale anchored on the left with the phrase ‘‘My leg muscles don’t feel sore at all’’ and anchored on the right with the phrase ‘‘My leg muscles feel so sore I don’t want to move them.’’ Participants gave their rating while performing a wall sit with their legs bent 90°. Participants also rated their leg muscle soreness using a seven-point validated Likert scale (Vickers, 2001) while walking up and down the laboratory.

5.3.12. *Blood collection, handling, and analysis*

Whole blood samples were collected, without venestasis, by venepuncture from an antecubital vein, and collected into one K_2EDTA and one lithium heparin vacutainer tube (BD, Oxford, UK).

Haematocrit (capillary method) using a micro-haematocrit reader (Hawksley & Sons Ltd., Lancing, UK) and haemoglobin concentration using a photometer (Hemocue, Sheffield, UK) were both analysed immediately on whole blood, in triplicate, with plasma volume change calculated (Dill and Costill, 1974). The remaining whole blood was spun in a

refrigerated centrifuged at 1500 *g* for 10 min, with the plasma aspirated, aliquoted into eppendorf tubes, and frozen at -80 °C for later analysis. CK activity pre-treatment and 24 h post-treatment was assessed as an indirect measure of muscle damage. Plasma CK activity was measured in duplicate, on lithium heparin-treated plasma using an enzyme reaction kit (EnzyChromi CK assay kit; BioAssay Systems, Hayward, CA). The intra-assay coefficient of variation for CK was 3.8%. Plasma IL-6 concentrations were determined at baseline and before and after HS1 and HS2 on EDTA-derived plasma in duplicate using a commercially available high sensitivity ELISA (Quantikine_ HS IL-6, HS600B; R&D Systems Europe, Abingdon, UK). Intra-assay coefficient of variation for plasma IL-6 concentration was 5.3%.

For all biochemical analyses, the participant's samples were always assayed on the same plate.

5.3.13. *Statistical Analysis*

A sample size calculation was performed (G*Power, 3.1.2) using mean and SD core temperature data from Montain et *al*. (Montain et *al.*, 2000). For a two-tailed test with alpha level set at 0.05, and power set at 0.8, recruiting 12 participants was deemed appropriate to detect a significant difference in core rectal temperature between the conditions during subsequent exercise in the heat. All data were checked for normality and sphericity and analysed using either paired t-tests or fully repeated-measures ANOVA with the Greenhouse–Geisser correction applied to the degrees of freedom if necessary. All F values reported are for the time – trial interaction unless otherwise stated. Tukey's HSD or Bonferroni-adjusted paired t-test post hoc procedures were used to determine within-subject differences where appropriate. To assess the contribution of acute circulating inflammation (IL-6) after muscle damage on altered heat strain during subsequent exercise heat stress, Pearson correlations were performed by correlating the pre-HS IL-6 concentration with the final T_{re} attained during HS. All data were analysed using SPSS version 14 software (IBM, NY). Statistical significance was accepted as $P < 0.05$.

5.4. Results

5.4.1. *Treatment Responses*

Both treatments [-10% (EIMD) and +1% gradient running (CON)] were conducted at the same exercise intensity with no between-trial differences in $\dot{M}$ (EIMD = 939 (111), CON = 941 (106) W, P = 0.90) or RER (EIMD = 0.92 (0.05), CON = 0.94 (0.05), P = 0.25). Despite being conducted at the same exercise intensity, ΔT_{re} was greater during 60 min -10% downhill running [EIMD = 1.88 °C (0.33) °C] than 60 min +1% gradient running [CON = 1.25 °C (0.27) °C; P < 0.001]. However, 30 min post-treatment and just before HS1, T_{re} was not different between trials (P = 0.52).

Sixty minutes of downhill running was successful in inflicting muscle damage because indirect markers of muscle damage, assessed by plasma CK and perceived muscle soreness (VAS and Likert scale), were all significantly greater on EIMD than CON 24 h post-treatment (P < 0.001, **Table 4**).

		Before	**24 h After**
Plasma CK activity ($U \cdot L^{-1}$)	EIMD	82 ± 82	253 ± 76[a]
	CON	83 ± 60	133 ± 70
Perceived muscle soreness (VAS)	EIMD	11 ± 10	52 ± 15[a]
	CON	6 ± 6	17 ± 9
Perceived muscle soreness (Likert scale)[b]	EIMD	1 ± 0.3	3.4 ± 1.4[a]
	CON	2 ± 0.4	7 ± 0.7

Table 4. Markers of muscle damage before and 24 h after either 60 min EIMD (-10% gradient running at 65% VO_{2max}) or 60 min of energy-expenditure equivalent control exercise (+1% gradient running at 65% VO_{2max}). Values are presented as mean ± SD; n = 13. [a]EIMD significantly higher than CON (P < 0.001); [b]Likert scale (0 – 6); VAS [visual analog scale (0 – 100 mm)].

Furthermore, all participants on EIMD had increases in plasma CK and perceived muscle soreness (VAS and Likert scale), and these responses were greater on EIMD compared with CON 24 h post-treatment for all participants. There were no baseline differences for any of these variables.

5.4.2. *Exercise Heat Stress Responses*

Rectal core and skin temperature.

Mean ΔT_{re}, T_{re}, and T_{sk} responses to HS1 and HS2 are presented in **Figure 11**.

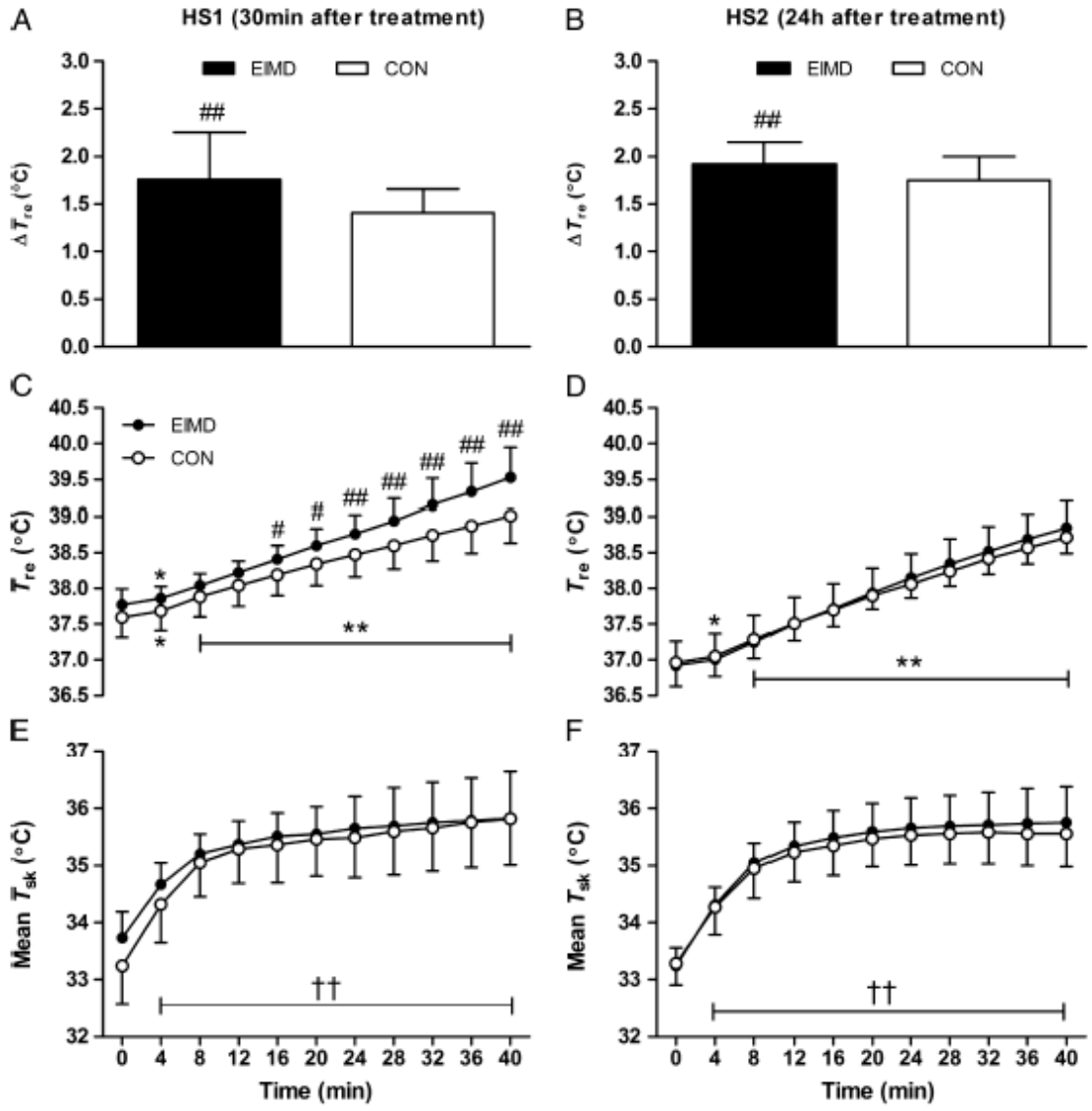

Fig. 11. Change in rectal temperature (ΔT_{re}, A and B), absolute temperature (T_{re}, C and D), and mean skin temperature (T_{sk}, E and F) responses during exercise heat stress tests, which involved running for 40 min at 65% VO_{2max} in 33 °C, 50 % RH conducted 30 min (HS1) and 24 h (HS2) after treatment, which involved either EIMD (-10% gradient running, EIMD, *filled bar and circles*) or energy equivalent controlexercise (+1% gradient running, CON *empty bar and circles*). EIMD was significantly greater than CON (# P < 0.05, ## P < 0.01). Significantly greater than time 0 on both trials (* P < 0.05, ** P < 0.01).†† Main effect of time, significantly greater than time 0 (P < 0.01). Data are presented as mean ± SD, n = 13 for T_{re} and n = 10 for T_{sk}.

T_{re} demonstrated an interaction during both HS1 (F = 8.8, P < 0.01) and HS2 (F = 6.3, P < 0.01). There was no significant difference in starting T_{re} between trials before HS1 or HS2. The ΔT_{re} during HS1 was greater after EIMD than CON (P < 0.01; **Fig. 11A**). During HS1, T_{re} was significantly higher after EIMD than CON from 16 min onward, resulting in a higher final T_{re} after 40 min of the same exercise heat stress (EIMD = 39.53 °C (0.42) °C, CON = 39.01 °C (0.38) °C, P < 0.01; **Fig. 11C**). Furthermore, five participants showed evidence of mild hyperthermia with a T_{re} 39.5 on EIMD compared with only one participant on CON. During HS2, ΔT_{re} was also significantly greater after EIMD than CON (P < 0.05; **Fig. 11B**). Although post hoc testing failed to show any significant differences between trials in actual T_{re} throughout HS2, there was a trend for a 0.13 °C higher final T_{re} on EIMD than CON (P = 0.08; **Fig. 11D**). Mean T_{sk} was not altered by previous muscle-damaging exercise during HS1 (F = 2.1, P = 0.17; **Fig. 11E**) or HS2 (F = 0.44, P = 0.56; **Fig. 11F**).

5.4.3. *Sweating rate and plasma volume change*

Whole body sweating rate was not different between trials during HS1 [EIMD = 23.1 (8.2), CON = 23.2 (5.0) $mL \cdot min^{-1}$, P = 0.98) or HS2 [EIMD = 21.4 (9.0), CON = 22.6 (5.0) $mL \cdot min^{-1}$, P = 0.62]. There was a small interaction for local forearm sweating rate during HS1 (F = 2.9, P < 0.05; **Fig. 12A**), with sweating rate only higher after 2 min of exercise on EIMD than CON (P < 0.05). Despite the threshold temperature for initiation of sweating visually appearing to occur at a higher T_{re} on EIMD than CON during HS1 (**Fig. 12C**), this was not significant (P = 0.36). There was no difference in sweating sensitivity between trials during the initial transient exercise phase (P = 0.50) nor later during exercise in HS1 (6–40min, P = 0.19). Throughout HS2, there was no effect of sweating threshold temperature (P = 0.95), sweating sensitivity during the initial transient phase of exercise (P = 0.69), or sweating sensitivity during the plateau sweating phase from 6 to 40 min of exercise (P = 0.17; **Fig. 12D**).

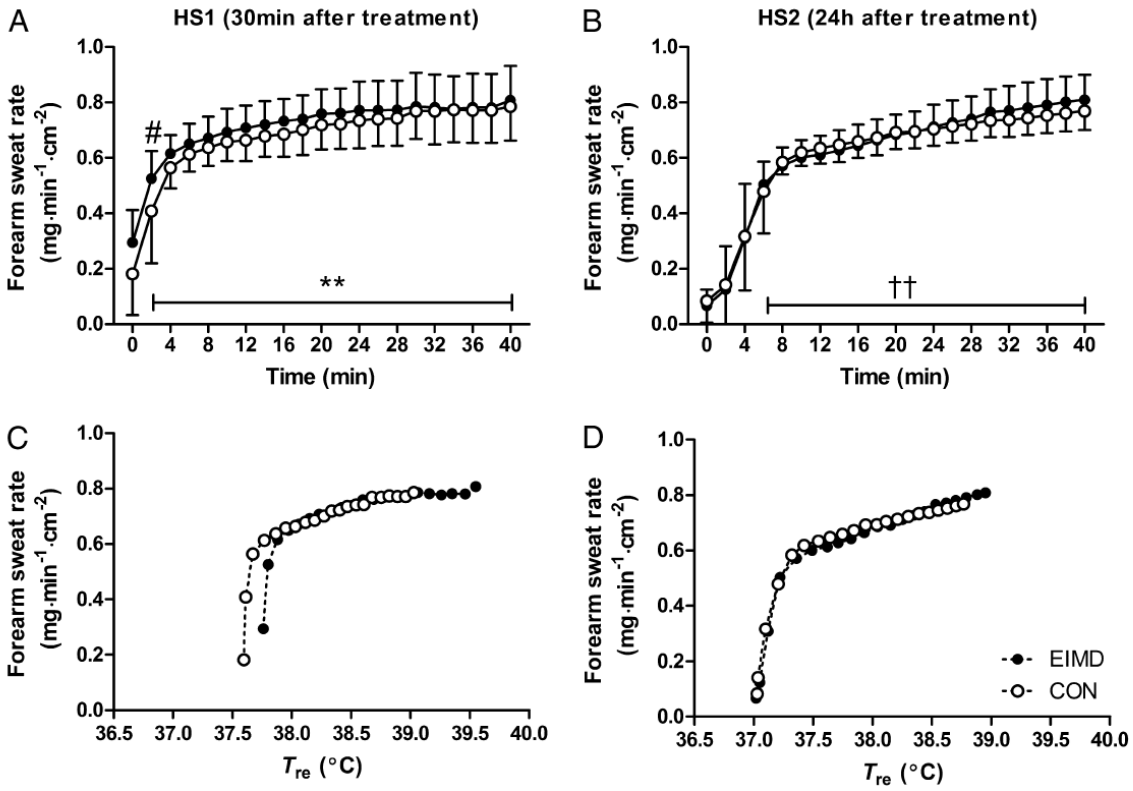

Fig. 12. Local forearm sweating rate reported as a function of time (A and B) and as a function of rectal temperature (T_{re}, C and D) during exercise heat stress tests, which involved running for 40 min at 65 % VO_{2max} in 33 °C, 50 % RH conducted 30 min (HS1) and 24 h (HS2) after treatment, which involved either EIMD (-10% gradient running, EIMD, *filled circle*) or energy equivalent control exercise (+1% gradient running, CON, *empty circle*). EIMD was significantly greater than CON (# $P < 0.05$). ** Significantly greater than time 0 on both trial ($P < 0.01$). †† Main effect of time, significantly greater than time 0 ($P < 0.01$). Data are presented as mean ± SD, n = 11.

There was no effect of muscle damage on plasma volume change during HS1 and HS2 (no interaction, $F = 1.7$, $P = 0.15$, or main effect of trial, $F = 0.1$, $P = 0.75$).

Rate of metabolic energy expenditure, RER, and HR.

Mean $\dot{M}$ was significantly higher after EIMD than CON throughout both HS1 and HS2 [HS1: EIMD = 1040 (115) W, CON = 970 (102) W, $P < 0.001$; HS2: EIMD = 986 (131) W, CON = 920 (113) W, $P < 0.001$]. These metabolic rates equated to 71 (6)% and 66 (6)% VO_{2max} for EIMD and CON, respectively, during HS1, and 67 (6)% and 62 (6)%

VO_{2max} for EIMD and CON, respectively, during HS2. There was no difference in mean RER between EIMD and CON throughout either HS1 [EIMD = 0.94 (0.05), CON = 0.92 (0.04), P = 0.35] or HS2 [EIMD = 0.94 (0.03), CON = 0.96 (0.07), P = 0.19]. Mean HR throughout both HS1 and HS2 was significantly greater on EIMD than CON [HS1: EIMD = 177 (12) beats·min^{-1}, CON = 168 (14) beats·min^{-1}, P < 0.01; HS2: EIMD = 164 (12) beats·min^{-1}, CON = 159 (11) beats·min^{-1}, P < 0.05].

5.4.4. *Thermal sensation and RPE*

Thermal sensation and RPE data are presented in **Figure 13**. Participants rated their thermal sensation significantly hotter on EIMD from 25 min onward than CON throughout HS1 ($F = 5.7$, $P < 0.01$; **Fig. 13A**). Throughout HS2, there was a trend for a higher thermal sensation during EIMD than CON (main effect of trial, $F = 4.4$, $P = 0.06$; **Fig. 13B**). Participant's RPE was higher from 25 min onward on EIMD during HS1 ($F = 4.1$, $P < 0.05$, main effect of trial $F = 7.4$, $P < 0.01$; **Fig. 13C**). Participants RPE was considerably higher on EIMD than CON 24 h post-treatment throughout HS2 (main effect of trial, $F = 11.4$, $P < 0.01$; **Fig. 13D**).

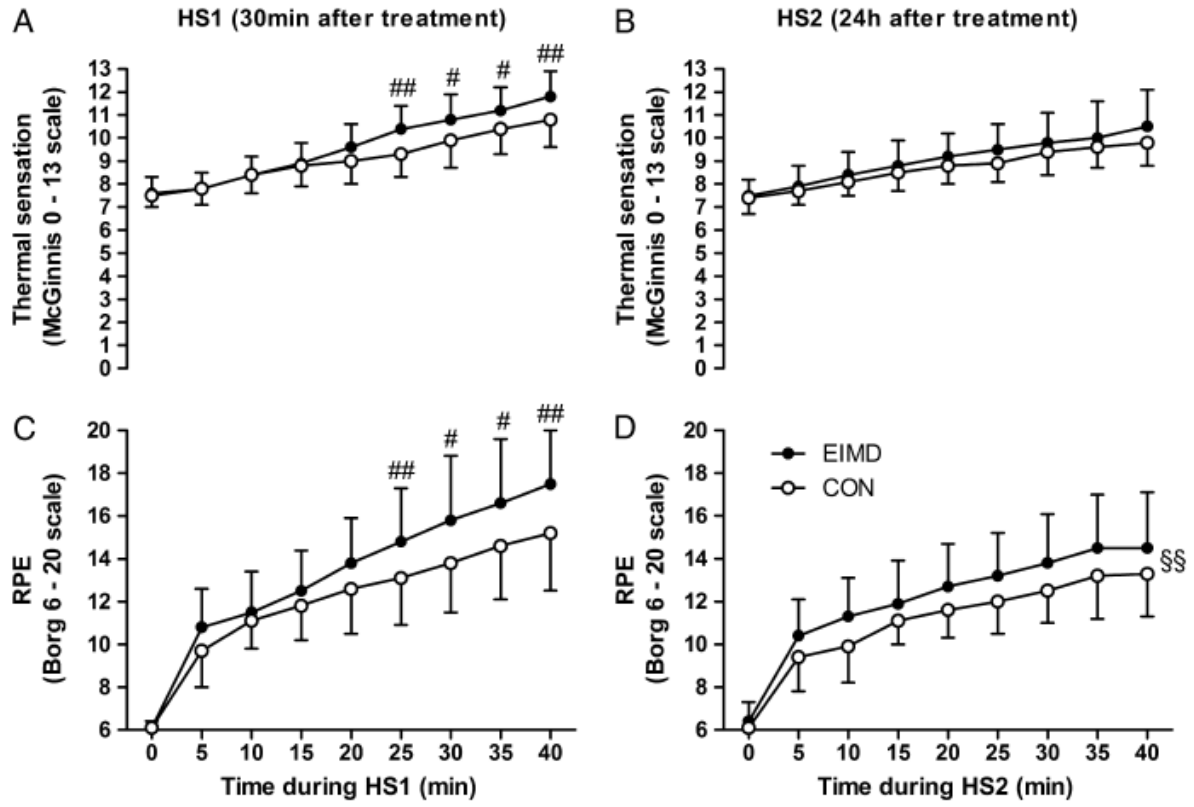

Fig. 13. Thermal sensation (A and B) and RPE (C and D) during exercise heat stress tests, which involved running for 40 min at 65% VO_{2max} in 33 °C, 50% RH conducted 30 min (HS1) and 24 h (HS2) after treatment, which involved either EIMD (-10% gradient running, EIMD, filled circle) or energy equivalent control exercise (+1% gradient running, CON, empty circle). EIMD was significantly greater than CON (# $P < 0.05$, ## $P < 0.01$). §§ Main effect of trial ($P < 0.01$). Data are presented as mean ± SD, n = 13.

5.4.5. *Plasma IL-6*

Plasma IL-6 concentration was measured as a circulating marker of inflammation. Plasma IL-6 concentration was not different between trials at baseline but responded differently to the treatment and heat stress tests ($F = 18.1$, $P < 0.001$; **Fig. 14**). Thirty minutes after treatment and immediately before HS1, plasma IL-6 concentration was significantly elevated above baseline on EIMD ($P < 0.001$) and CON ($P < 0.01$). Importantly, participants began HS1 on EIMD with a significantly higher plasma IL-6 concentration than CON ($P < 0.01$). In addition, the plasma IL-6 exercise response to HS1 was significantly greater on EIMD versus CON ($P < 0.01$). The acute circulating inflammatory response after treatment, measured as the pre-HS1 plasma IL-6 concentration, correlated with the final T_{re} attained after HS1 in EIMD trial ($r = 0.67$, $P < 0.01$). On day 2, immediately pre-HS2, plasma IL-6 concentrations on both trials had returned to baseline values with no difference between trials. As such, the preceding IL-6 response was not strongly associated with final T_{re} during HS2 ($r = -0.33$, $P = 0.10$). Plasma IL-6 concentration increased after HS2 on both EIMD and CON ($P < 0.01$) with no between-trial differences.

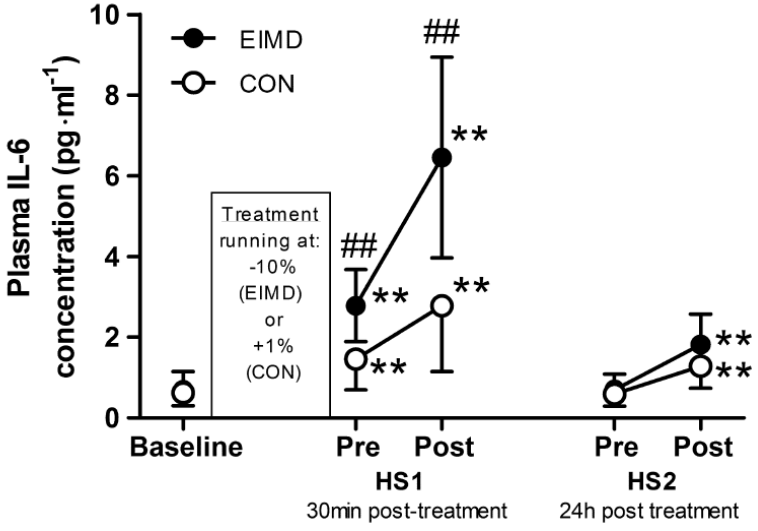

Fig. 14. Plasma IL-6 concentrations at baseline and before and after exercise heat stress tests, which involved running for 40 min at 65 % VO_{2max} in 33 °C, 50% RH conducted 30 min (HS1) and 24 h (HS2) after treatment, which involved either EIMD (-10% gradient running, EIMD, filled circle) or energy equivalent control exercise (+1% gradient running, CON, empty circle). ## EIMD significantly greater than CON ($P < 0.01$). ** Significantly greater than baseline ($P < 0.01$). Data are presented as mean ± SD, n = 13.

5.5. Discussion

In agreement with our hypothesis, in non–heat-acclimated males, ΔT_{re} is increased during exercise heat stress performed 30 min after moderate muscle-damaging exercise timed to coincide with the early inflammatory phase. This ended in a ~0.5 °C greater final T_{re} during HS1 in muscle damaged participants (final T_{re} ~39.5 °C) after just 40 min of moderate exercise heat stress, which under hot environmental conditions could perhaps expedite the progression toward EHI.

We have also shown that the preceding EIMD-evoked increase in plasma IL-6 concentration was associated (r = 0.67) with increased heat strain during HS1 conducted 30 min after exercise heat stress tests, (running for 40 min at 65% VO_{2max} in 33 °C, 50% RH) conducted 30 min (HS1) and 24 h (HS2) after EIMD (-10% gradient running) relative to energy equivalent control exercise (+1% gradient running).

When exercise heat stress was timed to coincide with the deferred muscle inflammatory phase 24 h after moderate muscle-damaging exercise, we also observed a meaningful, even if small, increase in ΔT_{re}. These results have realistic applicability, particularly for non–heat-acclimated athletes and soldiers who undertake repeated, and/or prolonged heavy training bouts, with an eccentric component, in hot environmental conditions. Thus actions that can decrease EIMD associated inflammation may mitigate the risk of EHI.

Downhill running is often used in studies to evoke muscle damage (Braun and Dutto, 2003; Paulsen et *al.*, 2012; Peake et *al.*, 2005), and the aim of the model used here was to evoke moderate, realistic muscle damage that would not impair the participants ability to complete the subsequent exercise heat stress bouts. All participants were able to complete the HS bouts, and the degree of muscle damage was equivalent to experiencing ‘‘a light pain when walking’’ (Vickers, 2001). As anticipated, EIMD resulted in greater increases in plasma CK concentration than CON. Although we acknowledge that we did not assess muscle strength as an indicator of muscle damage and the

limitations of plasma CK and delayed onset of muscle soreness as markers of EIMD (Paulsen et *al.*, 2012), the mode of EIMD (downhill running) and severity of soreness perceived may mirror that encountered by soldiers and athletes performing unaccustomed exercise. Despite not being a central focus of the current study, an important finding in the context of EHI risk is the greater increase in T_{re} (+0.63 °C) during the EIMD treatment in thermo-neutral conditions. This took place even exercise intensity was carefully matched in CON and EIMD and is likely interpreted by altered muscle fibre recruitment and efficiency during eccentric exercise (Abbott and Wilkie, 1952). With our primary aim in mind, it is important that T_{re} was not significantly different between trials before commencement of HS1, 30 min later, or HS2, 24 h later.

To the best of our knowledge, there is only one published study that has examined heat strain during exercise heat stress after muscle injury, which showed a 0.2 °C – 0.3 °C greater core body temperature than control at 2 h and 7 h, but no effect 26 h after injury (Montain et *al.*, 2000). In line with our two phase approach, we noticed a 0.35 °C greater increment in T_{re} during exercise heat stress conducted 30 min post-EIMD timed to correspond with the early inflammatory phase, where participants exhibit evidence of mild hyperthermia (mean final T_{re}: EIMD = 39.5 °C, CON = 39.0 °C). The 0.17 °C greater increase in T_{re} during exercise heat stress conducted 24 h post-EIMD indicates a minor effect of the delayed muscle inflammatory phase on exercise heat strain. These considerations happened without any significant differences between trials in thermo-effector response measures (e.g., skin temperature, whole body and local sweating rate, and sweating sensitivity) or hydration (plasma volume change). We recognize the limitations with measuring local sweating rate at only one site, and using T_{re} to calculate sweating sensitivity as opposed to oesophageal temperature, which responds more quickly during exercise-induced hyperthermia (Gagnon et al., 2010). We felt that these were necessary compromises because of the practical constraints of the running mode of exercise and for participant comfort.

We also acknowledge that additional testing at later time-points post-exercise (48 h-120 h post) has not been performed. Indeed, many of the criterion measures may peak 48-

72 h post exercise. Therefore, assessment at these timepoints would have confirmed whether or not the criterion measures continued to rise and if muscle function was altered. Again we felt this was another necessary compromise to reflect real life situations where non–heat-acclimated athletes and soldiers undertake repeated, and/or prolonged heavy training bouts, in hot environmental conditions over short periods of time.

Eccentric exercise has been demonstrated to inhibit glycogen resynthesizes (Costill et al., 1990; O'Reilly et al., 1987), consequently one might hypothesize that modified substrate availability may have changed metabolism and contributed to increased heat strain, particularly during HS2. However, this is unlikely because we did not notice any differences between trials in RER during HS1 or HS2. On the basis of previous evidences (Montain et al., 2000), one might also assume a meaningful involvement of reduced economy in the increased heat strain after EIMD, possibly due to modified gait, greater motor unit recruitment, and/or muscle weakness due to the muscle-damaging protocol (Montain et al., 2000). In support of this assertion, $\dot{M}$, HR, and RPE were greater during HS1 and HS2 after EIMD. Muscle-damaging exercise in humans has been shown to reduce economy, with steady-state exercise VO_2 elevated 3% – 7% in the 3 d after downhill running (Braun and Dutto, 2003; Chen, Nosaka and Tu, 2007). Furthermore, rodent models have provided evidence that damaged muscle fibres also demonstrate diminished contraction economy (Warren et al., 1996).

We assessed circulating IL-6 concentration as a marker of acute inflammation and hypothesized that EIMD-evoked increases in circulating IL-6 would be associated with increased heat strain during consecutive exercise heat stress 30 min after muscle-damaging exercise. As expected, EIMD generated a significantly greater elevation in circulating IL-6 response than CON before HS1, although it is important that the circulating level of IL-6 after EIMD was relatively modest compared with the level usually observed after more prolonged, metabolically demanding endurance exercise (Nieman et al., 2001).

Nevertheless, it has been observed a moderate correlation between the previous circulating IL-6 concentration and the final T_{re} during HS1 in EIMD trial (r = 0.67). It is noteworthy to stress the sequential nature of this relationship, that is, it was the preceding, perhaps modest, early inflammatory response that was associated with subsequent increases in T_{re} during exercise heat stress. Additionally, these associations were not evident during HS2 when pre-HS2 IL-6 concentrations had returned to baseline values, with no difference between trials. Because IL-6 is a known pyrogen (Dinarello et al., 1991), it is plausible that acute increases in circulating IL-6 after muscle damage may act upon the cycloxygenase 2–mediated PGE2 pathway, ending-up in an increment in the thermoregulatory set point (Bradford et al., 2007; Rummel et al., 2006) and heat strain during exercise heat stress. However, we recognize the relatively moderate augmentation in the circulating IL-6 response after EIMD and that the concept of a thermoregulatory set point remains controversial (Romanovsky, 2007).

Future studies should examine the supposed contribution of pyrogenic pathways after muscle damage upon exercise heat strain. Because the exercise intensities on the two treatments were carefully matched, with no differences in RER, it is unlikely that modified metabolic demands or fuel utilization during treatment could account for the greater plasma IL-6 after EIMD. Whether the greater circulating IL-6 response after EIMD come from muscle cells, leukocytes, or other cells remains a topic of debate (Braun and Dutto, 2003; Montain et al., 2000; Paulsen et al., 2012).

Soldiers and athletes undergoing heavy training are generally expected to perform repeated bouts of arduous physical activity on the same day, often in hot environments that might predispose them to EHI. Irrespective of the underlying mechanisms, based on the evidences of the present study, it is expected that the non–heat-acclimated soldier or athlete may experience greater heat strain during a subsequent exercise heat stress bout performed shortly after, and to a much lesser extent, the following day after unaccustomed muscle-damaging exercise. It has been also noticed a greater increase in T_{re} during the -10% gradient downhill running treatment than the energy-expenditure matched +1% gradient running exercise. Taking this into account, individuals

undertaking a prolonged bout of exercise in the heat that includes an eccentric component, such as running or walking across mountainous terrain, might also experience greater heat strain. Given the increase in thermal sensation during HS1 and increase in RPE during HS1 and HS2 after EIMD, the effects of EIMD may be somewhat self-limiting; that is, we anticipate that in many cases, the exercising individual would lower their exercise intensity while exercising in the heat after EIMD. However, it is a common observation in military and athletic scenarios that very motivated and highly experienced individuals choose to ignore some of the normal behavioural thermoregulation cues (Sawka et al., 2011) so they would be at increased risk of EHI as a consequence of EIMD. Likewise, it might be predicted EIMD-associated increased heat strain during exercise in the heat when the exercise intensity is externally regulated, for example, during group exercises in military scenarios.

EIMD has not been proposed as a risk factor for EHI in recent reports (Armstrong et al., 2007; Casa et al., 2012), but our data show that EIMD should be given due consideration as a risk factor for EHI, specifically for exercise in hot environments. Because it has been shown an association, perhaps moderate, between previous acute circulating inflammation and subsequent exercise heat strain, a logical line of exploration is to identify strategies that can diminish inflammation, which in turn may mitigate the rise in T_{re} and diminish the risk of EHI during exercise heat stress. It remains to bedemonstrated, for example, whether the well known repeated bout effect for muscle-damaging exercise (Clarkson et al., 1992) or a period of heat acclimatization provides protection from the observed increase in heat strain during exercise heat stress after EIMD. Nonsteroidal anti-inflammatory drugs (e.g., Ibuprofen) are an unsuitable possibility because nonsteroidal anti-inflammatory drugs augment the plasma IL-6 response in ultramarathon runners (Nieman et al., 2006) and increase gastrointestinal permeability, which in turn might increase the risk of EHI through the immune sequelea that results from gastrointestinal endotoxin leakage (Lambert *et* al., 2001; Lim et al., 2006). Whether actions that dampen inflammation also curtail some of the important cell

signalling and associated physiological adaptations with training remains a debatable point.

It is quite credible that strategies to diminish inflammation may decrease the risk of EHI for athletes and soldiers performing in hot environments in the short term but paradoxically decrease some of the training adaptations in the long term (e.g., anti-inflammatory supplementation using antioxidants (Peternelj and Coombes, 2011; Ristow et al., 2009). Clearly, more evidence is required before we will know whether this is an ill-conceived concept.

In conclusion, these data show that a bout of EIMD, brought about by downhill running, increases heat strain during successive endurance exercise in the heat conducted 30 min after and, to a much lesser extent, 24 h after muscle damage. EIMD evoked increases in the circulating pyrogen IL-6 were associated with increased heat strain during exercise heat stress conducted 30 min after EIMD. These results have practical relevance, particularly for non–heat-acclimated athletes and soldiers undertaking either multiple, or prolonged bouts of heavy exercise with an eccentric component in the heat.

Consequently, it would be of interest to investigate, and adopt strategies that may reduce the risk of EHI following EIMD. Reducing the degree of muscle damage encountered might be a countermeasure to lessen the degree of heat strain encountered. One unexplored avenue of investigation is the well-known repeated bout effect of muscle-damaging exercise, where a single session of eccentric exercise induces adaptations resulting in less evidence of damage when the exercise bout is repeated.

CHAPTER SIX

The influence of the exercise-induced muscle damage repeated-bout-effect upon heat strain during subsequent exercise in the heat

6.1. Abstract

Exercise-induced muscle-damage (EIMD) has recently been shown to increase heat strain during exercise-heat-stress (HS), and thereby represents a risk factor for EHI. We hypothesised that a repeated-bout of EIMD blunts the increase in rectal temperature (T_{re}) during subsequent endurance exercise in the heat. Sixteen non-heat-acclimated males were randomly allocated to EIMD (n=9) or control (CON, n=7). EIMD performed a downhill running treatment at -10% gradient for 60 min at 65% $\dot{V}O_{2max}$ in 20°C, 40% RH. CON participants performed the same treatment but at +1% gradient. Following treatment, participants rested for 30 min, and then performed HS (+1% gradient running for 40 min at 65% $\dot{V}O_{2max}$ in 33°C, 50% RH) during which thermoregulatory measures were assessed. Both groups repeated the treatment and subsequent HS 14-days later. Isometric quadriceps strength was assessed at baseline, and 48-h post treatment. The decrease in leg strength 48-h post EIMD trial 1 (-7.5%), was absent 48-h post EIMD trial 2 (+2.9%) demonstrating a repeated-bout-effect. Final T_{re} during HS was lower following EIMD trial 2 (39.25 ± 0.47 ºC) compared with EIMD trial 1 (39.59 ± 0.49 ºC, P< 0.01), with CON showing no difference. Thermal sensation and the T_{re} threshold for sweating onset were also lower during HS on EIMD trial 2.

The repeated-bout-effect blunted the increase in heat strain during HS conducted after EIMD. Incorporating a muscle-damaging bout into training could be a strategy to reduce the risk of EHI and improve endurance performance in individuals undertaking heavy exercise with an eccentric component in the heat.

6.2. Introduction

Exercising in hot conditions poses one of the greatest challenges to homeostasis of the human body (Armstrong et al., 2007; Sawka et al., 2011). If heat production during exercise is not effectively dissipated, core body temperature can rise to dangerous levels, thereby placing the individual at risk of developing EHI (Armstrong et al., 2007) or the more serious and potentially fatal condition of EHS (Casa et al., 2012; Sawka et al., 2011). In addition to exercising in hot and humid environments, a number of risk factors for EHI/EHS have been identified, including: inappropriate clothing, inadequate heat-acclimation, high body mass index, poor physical fitness, dehydration, and underlying medical conditions (Armstrong et al., 2007; Epstein et al., 1999; Rav-Acha et al., 2004; Sawka et al., 2011). Other potential risk factors, which are less well supported include disruption of sleep, and the circulating inflammatory response (Bouchama, 1995; Lim and Mackinnon, 2006; Moore et al., 2013; Shephard and Shek, 1999). Highly motivated, well trained athletes, who may ignore normal thermoregulatory behavioural cues, and military personnel, in whom evaporative cooling is limited due to their occupational clothing, and where exercise intensity is often externally regulated, are two groups who may be at particular risk of EHI/EHS (DeMartini et al., 2014). A number of deaths in athletes and military personnel each year are attributed to EHS (Carter et al., 2005; Casa et al., 2012; Rav-Acha et al., 2004). These groups regularly perform multiple bouts of heavy exercise, often within a short time span and in hot environmental conditions, and often involving an eccentric component (e.g. walking downhill/jumping/lunging etc.).

As discussed previously (**Chapter 5**), it has been recently shown by Fortes et al. (2013), and others (Montain et al., 2000), that prior exercise-induced muscle-damage (EIMD) increases heat strain during exercise-heat-stress conducted shortly after muscle damage. For example, final rectal temperature (T_{re}) was 0.53 °C higher during 40 min of moderate exercise-heat-stress conducted 30 min after 1-hour of EIMD, compared to identical exercise-heat-stress conducted 30 min after 1-hour of non-muscle-damaging control exercise (Fortes et al., 2013). This increased heat strain suggests that

performing exercise with an eccentric component may represent an additional risk factor for EHI.

Clearly, it is of interest to investigate and adopt strategies that may reduce the risk of EHI. It is plausible that reducing the degree of muscle damage encountered will lessen the degree of heat strain encountered. One obvious, but unexplored avenue of investigation is the well-known repeated bout effect of muscle-damaging exercise, where a single session of eccentric exercise induces adaptations resulting in less evidence of damage when the exercise bout is repeated (Clarkson et al., 1992; Clarkson and Hubal, 2002; McHugh et al., 1999). The mechanism to explain this repeated bout effect is not fully understood, though likely involves neural, cellular and mechanical mechanisms (Clarkson et al., 1992; Clarkson and Hubal, 2002; McHugh et al., 1999). In our previous study (Fortes et al., 2013), increased circulating interleukin (IL)-6 concentration following EIMD was associated with increased heat strain during subsequent exercise heat stress, a pertinent finding due to a potential inflammatory role in the aetiology of EHI/EHS (Bouchama, 1995; Lim and Mackinnon, 2006; Shephard and Shek, 1999). A dampening of the inflammatory response is one proposed explanation for the repeated bout effect (Pizza et al., 1996), and given that there is some evidence that circulating IL-6 concentration is reduced following a second, repeated bout of EIMD compared with the first bout (Smith et al., 2007) this may provide a mechanism for a potential beneficial repeated bout effect in reducing heat strain. Also, circulating extracellular (e)Hsp 72 was measured as concentrations have been shown to increase following EIMD (Peake et al., 2005). Additionally, eHsp72 itself may have direct pyrogenic effects (Pastukhov et al., 2003), and it provides another measure of the inflammatory response due to its immune-stimulating capability (Asea et al., 2000; Whitham and Fortes, 2008).

With this in mind, the aim of this study was to investigate the influence of the EIMD repeated-bout-effect upon heat strain during subsequent exercise in the heat. To this end, we had non-heat-acclimated participants perform muscle-damaging exercise using

a downhill running model, and then assessed heat strain during an exercise-heat-stress test conducted 30 min post EIMD to coincide with the circulating inflammatory response. This procedure was then repeated 14 days later. We hypothesised that the second (repeated) bout of EIMD would blunt the increase in T_{re} during subsequent exercise-heat-stress compared with the first EIMD trial. In order to discount any potential temporary heat acclimation, or passage of time effects, a separate control group undertook all identical procedures, except that non-muscle-damaging exercise was performed instead of EIMD.

6.3. Methods

6.3.1. *Participants*

Sixteen healthy, physically active males volunteered to participate in the study, giving fully-informed consent in writing. The study received local ethical approval and was conducted according to the standards required by the *Declaration of Helsinki*. Characteristics of the participants were as follows: (Mean ± standard deviation): age, 21.9 ± 1.7 yr; nude body mass, 72.3 ± 7.5 kg; height, 176 ± 7 cm; body mass index, 23.1 ± 1.9 $kg \cdot m^{-2}$; body surface area, 1.88 ± 0.12 m^2, and maximal oxygen uptake (VO_{2max}) 59 ± 5 $mL \cdot kg^{-1} \cdot min^{-1}$. Participants were excluded if they were heat-acclimated, or regularly undertook downhill running or eccentric exercise as part of their normal training. Participants were non-smokers, and free from any known immune, cardiovascular or metabolic disease. To further confirm participants were free from upper-respiratory tract infections, on each of the 14 days preceding both main trials, participants completed an illness-specific questionnaire (WURSS-44) (Barrett et al., 2002). No participants were taking any medication (e.g. non-steroidal anti-inflammatory drugs) or generic ergogenic supplementation. Participants were asked to refrain from exercise 72 hours prior to, and from alcohol and caffeine 24 hours prior to all exercise bouts.

6.3.2. *Study design*

Participants were randomised to one of two protocols, EIMD or CON. Nine participants completed an exercise-heat-stress-test 30 min after a treatment of muscle-damaging exercise (EIMD: running downhill on -10% gradient) (trial 1). This protocol was repeated two weeks later (trial 2). A separate control group (CON, n = 7) performed energy expenditure equivalent non-muscle-damaging exercise treatment (CON: running at +1% gradient), followed 30 min later by the exercise-heat-stress test, with the protocol repeated 14 days later. Thermoregulatory measurements (T_{re}, skin temperature (T_{sk}), sweating rate), perceptual measures (thermal sensation, rating of perceived exertion), heart rate, and metabolic energy expenditure, were assessed throughout all heat stress tests. Blood samples were collected at pre-treatment, pre, post and 1-hour post HS on all trials and assessed for circulating inflammatory markers IL-6, IL-10, and heat shock protein (Hsp) 72 concentration. Circulating extracellular (e)Hsp72 was measured as concentrations have been shown to increase following EIMD (Peake et al., 2005). Additionally, eHsp72 itself may have direct pyrogenic effects (Pastukhov et al., 2003), and it provides an other measure of the inflammatory response due to its immune-stimulating capability (Asea et al., 2000; Whitham and Fortes, 2008). Isometric leg strength of the quadriceps, and perceived muscle soreness were determined at baseline and 48-hours post trials to assess the degree of muscle damage. There were no differences in anthropometric or fitness data between the groups. An overview of the study design is presented in **Figure. 15**.

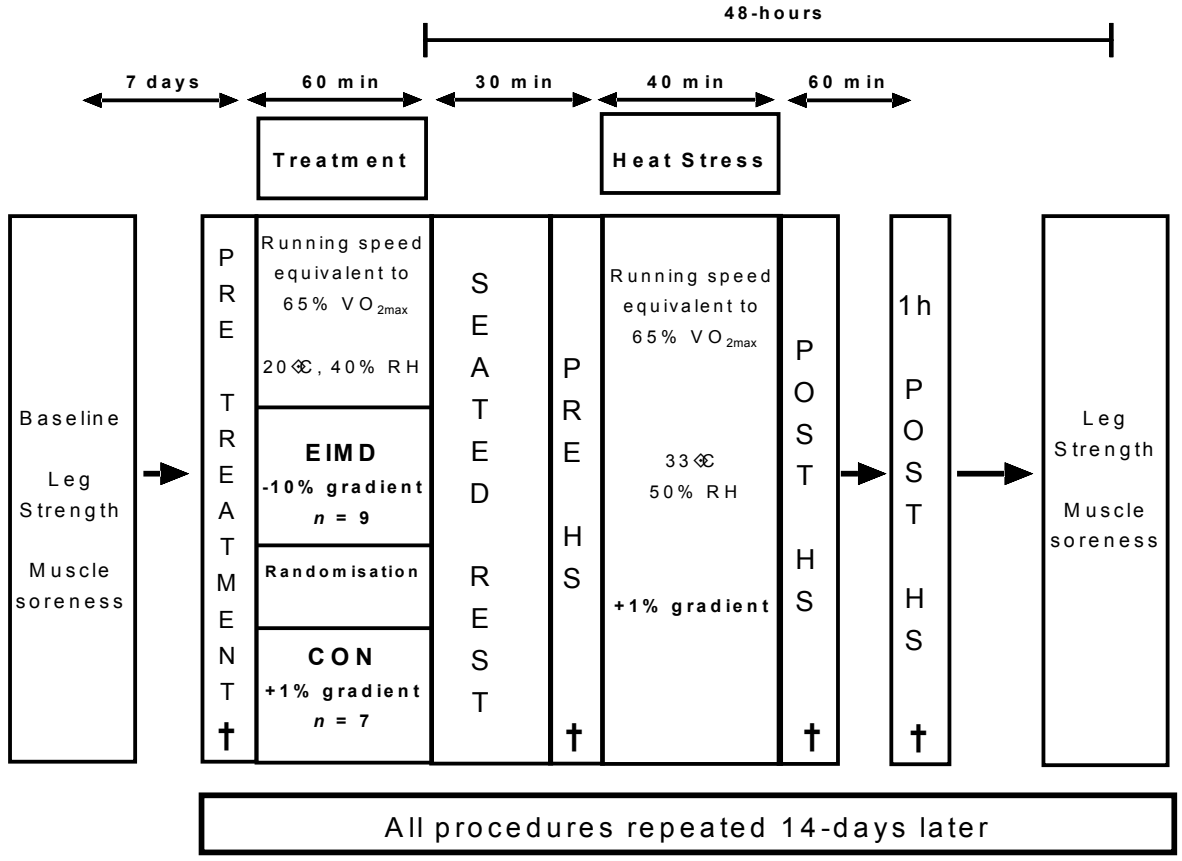

Fig. 15. Schematic of experimental procedures. EIMD, exercise-induced muscle damage; CON, energy expenditure equivalent control exercise; HS, exercise heat stress; RH, relative humidity. † denotes blood sampling time points. Isometric leg strength was assessed at baseline and 48-hours post HS.

6.3.3. *Preliminary testing and familiarisation*

The VO_{2max} for each participant was determined by a continuous maximal incremental exercise test to exhaustion performed on a motorised treadmill (HP Cosmos Mercury 4.0, Nussdorf-Traunstein, Germany) (Fortes et al., 2013). Expired gas was analysed by an on-line breath-by-breath system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany). After this, the treadmill running speed, which elicited 65%, VO_{2max} running at +1% gradient was obtained by interpolation of the running speed-VO_2 relationship. A running speed verification was then conducted and employed as the running speed for the treatment on the CON group, and for the heat stress test for both groups. A subsequent

verification was also performed on downhill running at -10% gradient with expired gas analysed on a continuous basis, and with the running speed adjusted accordingly until 65% VO_{2max} was attained. This downhill running verification took no more than 6 minutes, and the verified running speed was used during treatment on those assigned to muscle-damaging (EIMD) trials. Expired gas data were averaged every ten seconds over a period of two minutes during this process of verification. All instrumentation and procedures used in the experimental trials were also presented to participants at this time. Thirty minutes after this verification was completed, maximal isometric leg strength was measured to assess participants' baseline quadriceps leg-strength within 7 days of the first experimental trial. Diet diaries were provided to record all food consumed during the 24 hours prior to, and during the first experimental trial, and participants were instructed to replicate this before and during the second trial 14 days later.

6.3.4. *Experimental trials*

On the day of each experimental trial participants reported to the laboratory (ambient temperature 20 °C and relative humidity (RH) 40%) at 07:10 am having fasted overnight. Participants were provided fluids to consume (40 $mL \cdot kg^{-1} \cdot NBM \cdot d^{-1}$) in the prior 24-hour period to ensure they began exercise euhydrated. This was verified by checking that urine-specific gravity (Atago Uricon-Ne refractometer, Atago Co., Ltd. Tokyo, Japan.) upon arrival was less than 1.028 (Armstrong et al., 2010). Participants' fasted condition was verified by a finger-prick blood sample analysed for glucose and ensuring the reading was <6 $mg \cdot dl^{-1}$ (ACCU-CHECK Aviva, Roche, Basel, Switzerland). Participants' nude body mass (NBM) was then measured on a digital platform scale accurate to the nearest 50 g (model 705, Seca, Hamburg, Germany). A cereal bar equivalent to 8.4 $kJ \cdot kg^{-1}$ NBM and water (5 $ml \cdot kg^{-1}$ NBM) was subsequently provided to participants based on this body mass. At 08:15am, baseline NBM was measured, and a resting blood sample was taken before the participant fitted a rectal thermistor and heart rate monitor

(Polar Electro, Kempele, Finland). The participants also rated their perceived muscle soreness at this stage.

6.3.5. *Exercise trial treatment*

The exercise trial treatment (EIMD or CON) started at 08:30 am, with standardised clothes worn by participants (e.g. running shorts, socks and shoes). Participants ran for 60 min at the individualised predetermined speed that reflected 65% VO_{2max} on either +1 % gradient (CON) or -10% gradient (EIMD). Rectal core temperature and heart rate (HR) were assessed every 10 min, and 60 s expired gas samples were collected by Douglas bag method at 20 and 40 min of exercise, and analysed for VO_2. Water (2 $mL \cdot kg^{-1}$NBM) was provided throughout treatment every 15 min.

6.3.6. *Exercise heat stress*

After the treatment, participants sat for 30 min in a temperature-controlled environment (20 °C) during which skin thermistors were applied (Grant EUS-U, Cambridge, UK). Participants were provided with a standardised amount of fluids (2.5 $mL \cdot kg^{-1}$NBM) during this time. A blood sample was collected and pre-heat stress NBM was determined just before the participant entered an environmental chamber (Delta Environmental System, Chester, UK) which was kept at a dry bulb temperature of 33 °C, 50% RH, and 0.2 $m \cdot s^{-1}$ face-on wind velocity. Immediately upon entering the chamber, a ventilated capsule was fastened to the forearm (for local sweating rate). Participants then began exercise-heat-stress (HS) by running without fluids, on a motorised treadmill at +1% gradient for 40 min at the predetermined set running speed that reflected 65% VO_{2max}. Throughout HS, the following measurements were taken: local forearm sweating rate, HR, T_{re}, physiological strain index (Moran et al., 1998) and skin temperature (T_{sk}) continuously; rating of perceived exertion (RPE, Borg 6-20 scale (Borg, 1982)) and thermal sensation (McGinnis 0-13 points (Hollies and Goldman, 1977)) every 5 min; and 60 s expired gas samples by Douglas bag method (VO_2) every 10 min of exercise. Immediately after HS, participants were removed from the chamber, seated, and a blood sample was taken

after a standardised period of 3 min (post HS). Post HS NBM (following towelled drying) was also determined at this time. Water was provided to replace sweat losses encountered during HS. A further blood sample was taken 1-h post HS. Participants were then provided with a standardised meal (3290 kJ), and fluids to consume until they came back to the laboratory 48-h later for assessment of leg strength and perceived muscle soreness. Participants were required to refrain from any exercise, or ingesting caffeine or alcohol in this 48-h intervening period. The participant left the laboratory and returned 14 days later to repeat the trial (trial 2).

6.3.7. *Assessment of muscle damage*

Isometric right-leg quadriceps strength was assessed on a dynamometer (HUMAC norm, CSMi, Stoughton, MA, USA). Participants were sat upright and strapped in on their upper extremities to limit excess motion. The chair was adjusted so that the pivot was located on the lateral epicondyle of their right leg. A lever was applied to the right leg and adjusted so that the pad was located on the inferior part of the tibialis anterior. This lever was locked in at an angle of 70 ° leg extension. A warm-up protocol consisted of six sub-maximal repetitions (two repetitions at 25%, 50% and 75% of perceived effort) with a 30 s recovery between each repetition. Following a 2 min rest period, participants were required to exert maximal isometric force against the lever pad. Verbal encouragement was given throughout each repetition. Isometric leg strength of the quadriceps was determined by the peak torque elicited from four maximal 5s contractions (with a 90 s recovery between each). A separate pilot study conducted within our laboratory showed that there was no learning effect for this test, and that peak torque determined from this test has a day-to-day co-efficient variation of 5.5%. Perceived leg muscle soreness was self-rated by participants on a seven-point validated Likert scale (Vickers, 2001), whilst walking up and down the laboratory. The participant was blinded to the scores they had previously reported.

6.3.8. *Body temperatures*

Rectal core temperature was assessed using a standard flexible, sterile, disposable thermistor (Henleys Medical Supplies Ltd, Herts, UK) inserted 12 cm beyond the anal sphincter, with temperature recorded using a data logger (YSI model 4000A, YSI, Dayton, USA). In both repeated trials participants used the same thermistor. Insulated thermistors (Grant EUS-U, Cambridge, UK) were attached to the skin via surgical tape which conducted heat and allowed evaporation (Hypafix, BSN medical GmbH, Hamburg, Germany), and were used to measure T_{sk} at four sites on the left side of the body (on the chest at a point equi-distant to the acromion process and the nipple, the anterior mid-bicep, the anterior mid-thigh, and the lateral calf). The amount of tape used was standardised both between and within participants. Temperature data were registered using a portable data logger (Grant SQ2020, Cambridge, UK). Mean T_{sk} was calculated using a four-site weighted equation (Ramanathan, 1964).

6.3.9. *Sweating responses*

Whole-body sweating rate was estimated from nude body mass losses during HS. Local forearm sweating rate was measured by dew-point hygrometry. Anhydrous compressed nitrogen was passed through a 5 cm^2 capsule placed on the lower arm ventral surface (half-way between the antecubital fossa and carpus), and connected to a hygrometry system (DS2000, Alpha Moisture System, UK). Local forearm sweating rate was calculated using the difference in water content between effluent and influent air and the flow rate (1 $L \cdot min^{-1}$), and normalised for the skin surface area under the capsule (expressed in $mg \cdot cm^{-2} \cdot min^{-1}$). Sweating threshold and sensitivity were calculated by plotting the individual relationship between local forearm sweating rate and T_{re} values. A simple linear regression equation for the first 6 min of exercise was calculated, with the threshold T_{re} for active thermoregulatory sweating defined as the T_{re} at which local forearm sweating rate = 0.06 $mg \cdot cm^{-2} \cdot min^{-1}$ (Buettner, 1959). Sweating sensitivity during the exercise transient phase was calculated as the slope of this linear regression line.

6.3.10. *Rate of metabolic energy expenditure ($\dot{M}$)*

Oxygen consumption (VO_2) was calculated from 60 s expired air samples collected into a Douglas bag and analysed for O_2 and CO_2 concentrations by a paramagnetic and infrared gas analyser (Servomex 5200 Multipurpose, Crowborough, UK), and volume (Harvard Apparatus, Edenbridge, UK). Respiratory exchange ratio (RER) was calculated, and $\dot{M}$ was determined using VO_2 ($L \cdot min^{-1}$) and RER in the following equation (Nishi, 1981):

$\dot{M}$ (W) = VO_2 (21166 [0.23 (RER) + 0.77])/60.

6.3.11. *Blood collection, handling and analysis*

Whole blood samples were collected by venepuncture from an antecubital fossa vein and gathered into two K_2EDTA and one lithium-heparin vacutainer tubes (BD, Oxford, UK). Haematocrit (capillary method in triplicate) using a micro-haematocrit reader (Hawksley & Sons Ltd., Lancing, UK) and haemoglobin concentration using a photometer (Haemocue, Sheffield, UK) were both analysed immediately on heparinised whole blood in triplicate with plasma volume change calculated (Dill and Costill, 1974). The remaining whole blood was spun in a refrigerated centrifuge at 1500 *g* for 10 min, with the plasma aspirated, aliquoted into reaction tubes, and frozen at -80 °C for subsequent analysis. Circulating IL-6 and IL-10 concentrations were assessed on EDTA derived plasma using commercially available high-sensitivity ELISAs (Quantikine® HS IL-6, HS600B; Quantikine® HS IL-10, HS100C, R&D Systems Europe, Abingdon, UK). Circulating eHsp72 concentration was also assessed by commercially available ELISA (Hsp72 EIA kit, ADI-EKS-700B, Enzo Life Sciences LTD, Farmingdale, USA). Intra-assay co-efficient of variation for circulating IL-6, IL-10 and eHsp72 concentration was 3.2 %, 5 %, and 3 % respectively. For all biochemical analyses, all participants' samples were always assayed on the same plate.

6.3.12. *Statistical analysis*

A sample size calculation was performed (G*Power, 3.1.5) using mean and standard deviation T_{re} data from our previous study (Fortes et al., 2013). For a two-tailed test with alpha level set at 0.05, and power set at 0.8, it was deemed appropriate to recruit a minimum of 7 participants in each group to detect a meaningful difference in final T_{re} during subsequent exercise in the heat. Due to recruitment randomisation, 9 participants were recruited into the EIMD group, and 7 into CON. All data were checked for normality and sphericity. Data were analysed within condition (i.e. EIMD trial 1 *vs* trial 2), using two way repeated measures ANOVA or paired t-tests as appropriate. Bonferroni-adjusted paired t-test post-hoc procedures were used to determine within-subject differences. Pearson product moment correlation was also performed to assess the relationship between change in leg strength 48h after EIMD with change in T_{re} during HS. All data were analysed using SPSS version 20 software (IBM, NY, USA). Data are presented as means ± SD. Statistical significance was accepted as $P < 0.05$.

6.4. Results

6.4.1. Treatment responses

The experimental model was successful in demonstrating a repeated bout effect of muscle damage, since the decrease in isometric leg strength 48h following EIMD trial 1 (-7.5 % *vs* baseline), was absent 48 h post EIMD 2 (+2.9 % *vs* baseline) ($P < 0.01$ versus trial 1, **Fig. 16A**), and the increase in perceived muscle soreness 48-h post EIMD in trial 1, was blunted in trial 2 ($P < 0.05$, **Fig**. **16C**). The control group did not demonstrate any differences in leg strength ($P = 0.37$, **Fig. 16B**) or muscle soreness ($P = 0.60$, **Fig. 16D**) between trial 1 and trial 2. There were no differences between trials 1 and 2 in either EIMD or CON for ΔT_{re} during treatment (EIMD trial 1, 1.84 ± 0.35, trial 2 = 1.77 ± 0.43 °C, $P = 0.53$; CON trial 1 = 1.36 ± 0.49, trial 2 = 1.48 ± 0.43 °C, $P = 0.42$).

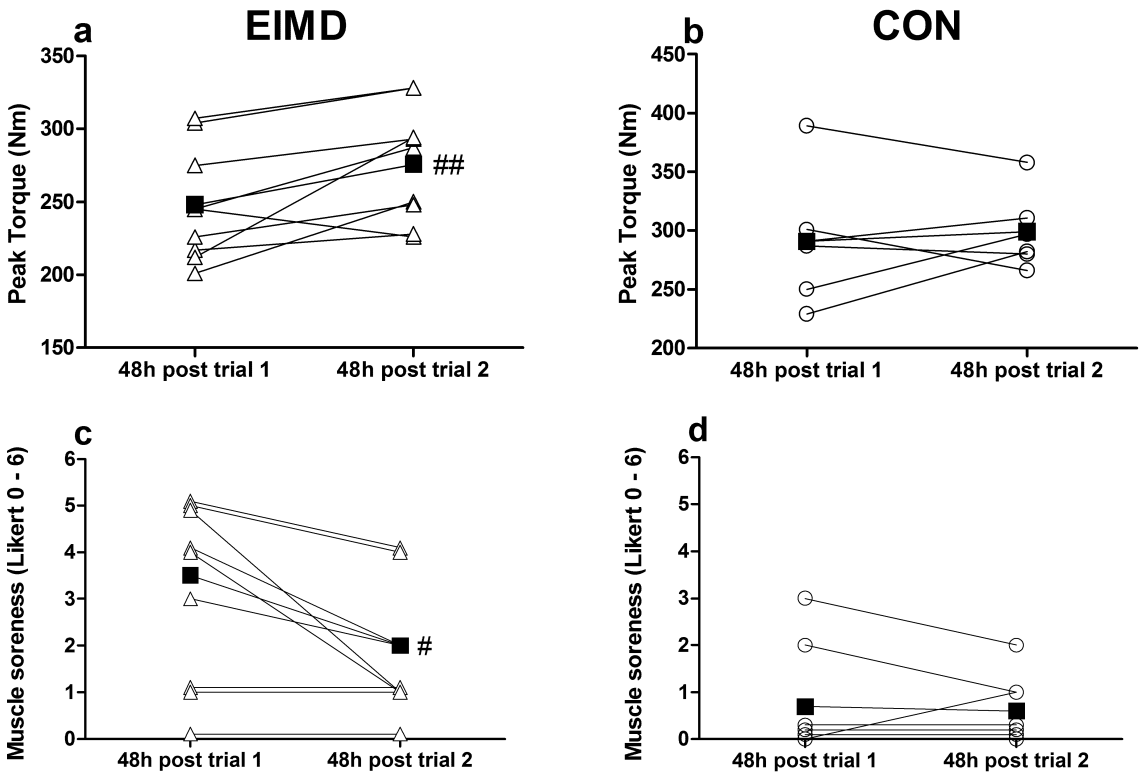

Fig. 16. Markers of muscle damage (leg strength peak torque (a and b) and muscle soreness (c and d)) assessed 48-h after either 60 min exercise-induced muscle damage (-10% gradient running at 65% VO_{2max}, EIMD, triangles, *n* = 9, a and c) or 60 min of control exercise (+1% gradient running at 65% VO_{2max}, CON, circles, *n* = 7, b and d). Trial 1 represents the first bout, trial 2 represents the repeated bout performed 14 days later. Individual responses are shown, with mean data shown as solid black squares. # $P < 0.05$, ## $P < 0.01$ *vs* trial 1.

6.4.2. Exercise heat stress responses

Rectal temperature, skin temperature and physiological strain index

Rectal core and T_{sk} responses to HS are presented in **Fig. 17**. There were no significant differences in T_{re} and T_{sk} responses to HS between CON trial 1 and CON trial 2 (**Figs. 17B, 17D, 17F**), confirming that there was no temporary heat acclimation effect of the first HS test on the second HS test or time effect on heat strain responses to HS. All participants on EIMD had a lower final T_{re} during HS on EIMD trial 2 (39.25 ± 0.47 °C)

compared with EIMD trial 1 (39.59 ± 0.49 °C, P < 0.01, **Fig. 17A**), with only two participants showing evidence of mild hyperthermia during HS on EIMD trial 2 (final T_{re} > 39.5 °C) compared with seven on EIMD trial 1. Physiological strain index was lower during HS on EIMD trial 2 (6.8 ± 2.8) compared with EIMD trial 1 (7.3 ± 3.1, P < 0.01). There was no significant difference in starting T_{re} between EIMD trials 1 and 2 before HS, but T_{re} was significantly lower on EIMD trial 2 compared with EIMD trial 1 throughout HS (main effect of trial, F = 22.2, P < 0.01), with a trend for an interaction (F = 2.9, P = 0.09, **Fig. 17C**). The ΔT_{re} during HS was also lower after EIMD trial 2 (1.61±0.33 °C) than trial 1 (1.81±0.40 °C, P<0.05) and the ΔT_{re} during HS correlated moderately with leg strength loss 48-h post EIMD (r = 0.46, P = 0.05). There was no repeated bout effect on mean T_{sk} during HS following EIMD (P = 0.32), **Fig. 17E**).

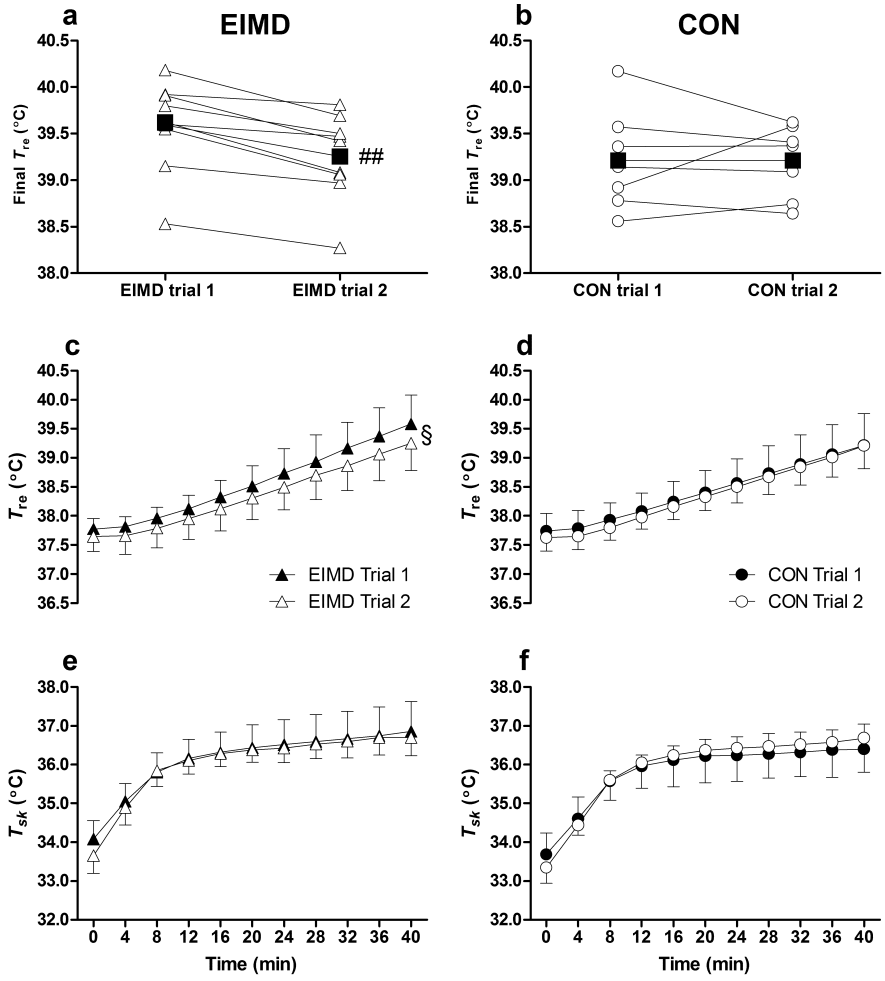

Fig. 17. Final rectal temperature (T_{re}, a and b), absolute rectal temperature (T_{re}, c and d) and mean skin temperature (T_{sk}, e and f) responses during exercise-heat-stress tests which involved running for 40 min at 65% VO_{2max} in 33°C, 50% RH conducted 30 min after treatment which involved either exercise-induced muscle damage (-10% gradient running, EIMD, triangles, n = 9) or control exercise (+1% gradient running, CON, circles, n = 7). Note: due to individual skin thermistors becoming detached during HS, T_{sk} data (figs. E and F) are presented in those with a full data set (n = 7 EIMD, n = 6 CON). Individual responses are shown for final T_{re} (a and b), with mean data shown as solid black squares (## P < 0.01 versus trial 1). For panels c-f, mean ± SD data are shown, with trial 1 representing the first bout (filled symbols), and trial 2 representing the repeated bout performed 14d later (open symbols). § Trial 2 significantly lower than trial 1 (main effect of trial, P < 0.05).

6.4.3. *Sweating rate and plasma volume change*

Whole body sweating rate was not different between the EIMD trials during HS (EIMD trial 1 = 23.3 ± 5.0, EIMD trial 2 = 23.0 ± 7.6 $mL \cdot min^{-1}$, $P = 0.89$), but there was a tendency for a higher forearm local sweating rate on EIMD trial 2 ($F = 2.6$, $P = 0.08$) during HS on EIMD trials (**Fig. 18A**). Although there was no difference in sweating sensitivity between EIMD trials during the initial transient exercise phase ($P = 0.17$), the threshold temperature for sweating onset was significantly lower on EIMD trial 2 than EIMD trial 1 (EIMD trial 1 = 37.74 ± 0.16 °C, EIMD trial 2 = 37.58 ± 0.24 °C, $P < 0.05$; **Fig. 18B**). No differences were observed during HS for sweating rate, sweating onset, or sweating sensitivity between CON trial 1 and 2. There was no effect on plasma volume change following HS on EIMD or CON trials.

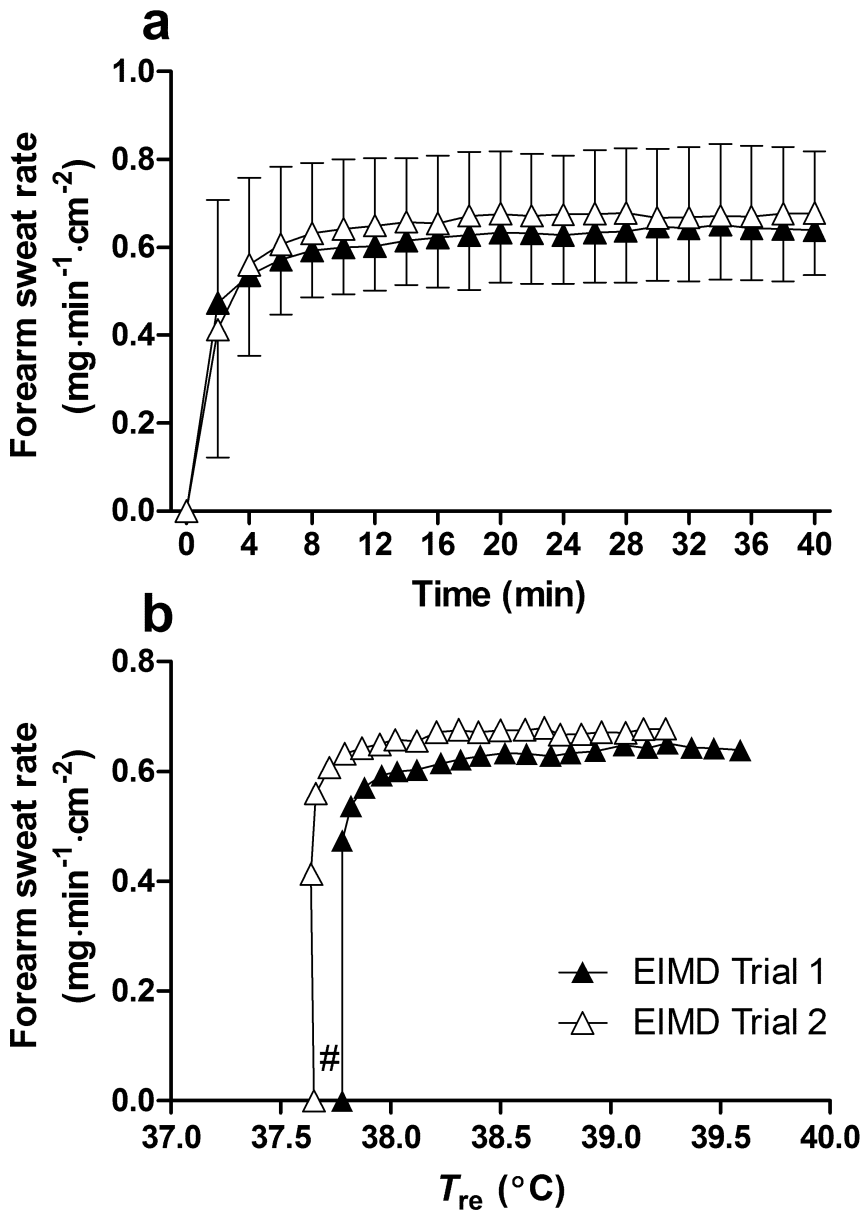

Fig. 18. Local forearm sweating rate reported as a function of time (a), and as a function of rectal temperature (T_{re}, b) during exercise-heat-stress tests which involved running for 40 min at 65% VO_{2max} in 33 °C, 50% RH conducted 30 min after exercise-induced muscle damage (-10% gradient running, n = 9). Trial 1 represents the first bout (filled triangles), trial 2 represents the repeated bout performed 14d later (open triangles). Values are means (±SD in fig A). # Significantly different T_{re} for sweating onset (P < 0.05).

6.4.4. *Rate of metabolic energy expenditure, RER and HR*

There were no between-trial differences on EIMD for mean $\dot{M}$ (trial 1 =1045 ± 115, trial 2 =1030 ± 128 W, P=0.21), indicating no improvement in running economy between trials. These metabolic rates equated to 75% ± 5% and 74% ± 6% VO_{2max} for EIMD trial 1 and trial 2 respectively, during HS. No difference was observed in mean RER or HR between EIMD trial 1 and EIMD trial 2 during HS (RER: EIMD trial 1 = 0.88 ± 0.03, EIMD trial 2 = 0.88 ± 0.04, P = 0.80, HR EIMD trial 1 = 176 ± 14 beats·min^{-1}, EIMD trial 2 = 175 ± 12 beats·min^{-1}, P = 0.54). No differences were observed during treatment for mean $\dot{M}$ between CON trial 1 and 2.

6.4.5. *RPE and thermal sensation*

Thermal sensation and RPE data for EIMD are presented in **Fig. 19**. Participants rated their thermal sensation lower throughout HS following EIMD trial 2 compared with trial 1 (main effect of trial, F = 6.5, P < 0.05, **Fig. 19A**). There was a trend (main effect of trial, F = 3.7, P = 0.09) for participants to rate their perceived exertion lower during HS following EIMD trial 2 compared with EIMD 1 (**Fig. 19B**). No differences were observed during HS for RPE or thermal sensation between CON trial 1 and 2.

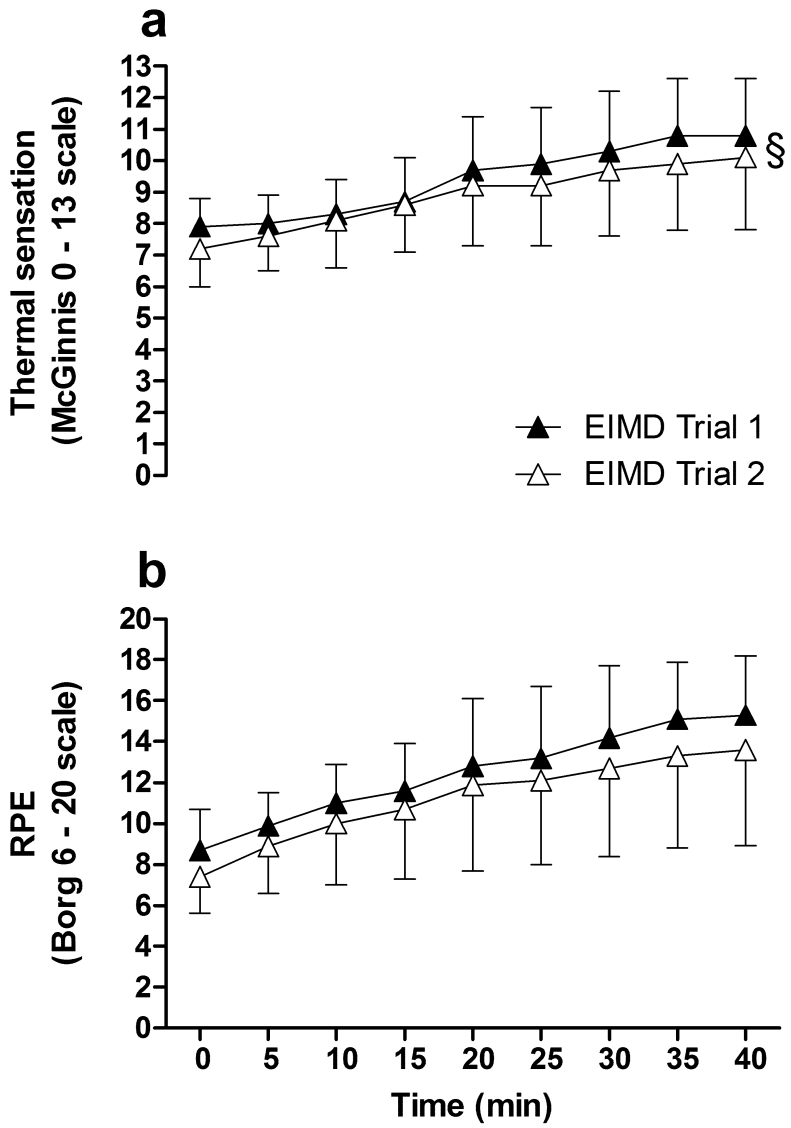

Fig. 19. Thermal sensation (a), and rating of perceived exertion (RPE, b) during exercise-heat-stress tests which involved running for 40 min at 65% VO_{2max} in 33 °C, 50% RH conducted 30 min after exercise-induced muscle damage (-10% gradient running, n = 9). Trial 1 represents the first bout (filled triangles), trial 2 represents the repeated bout performed 14d later (open triangles). § Trial 2 significantly lower than trial 1 (main effect of trial, $P < 0.05$). Data are mean ± SD.

6.4.6. *Plasma IL-6 and IL-10, and eHsp72*

Circulating concentrations of IL-6, IL-10 and eHsp72 responses to EIMD are depicted in **Fig. 20**. There was no EIMD repeated bout effect on the inflammatory response between trial 1 and 2 for IL-6 (F = 2.3, P = 0.11), IL-10 (F = 0.7, P = 0.45), or eHsp72 concentrations (F = 0.8, P = 0.45). Similarly, there were no between trial effects on CON for IL-6 (F = 0.9, P = 0.44), IL-10 (F = 0.2, P = 0.73), or eHsp72 concentration (F = 2.2, P = 0.15).

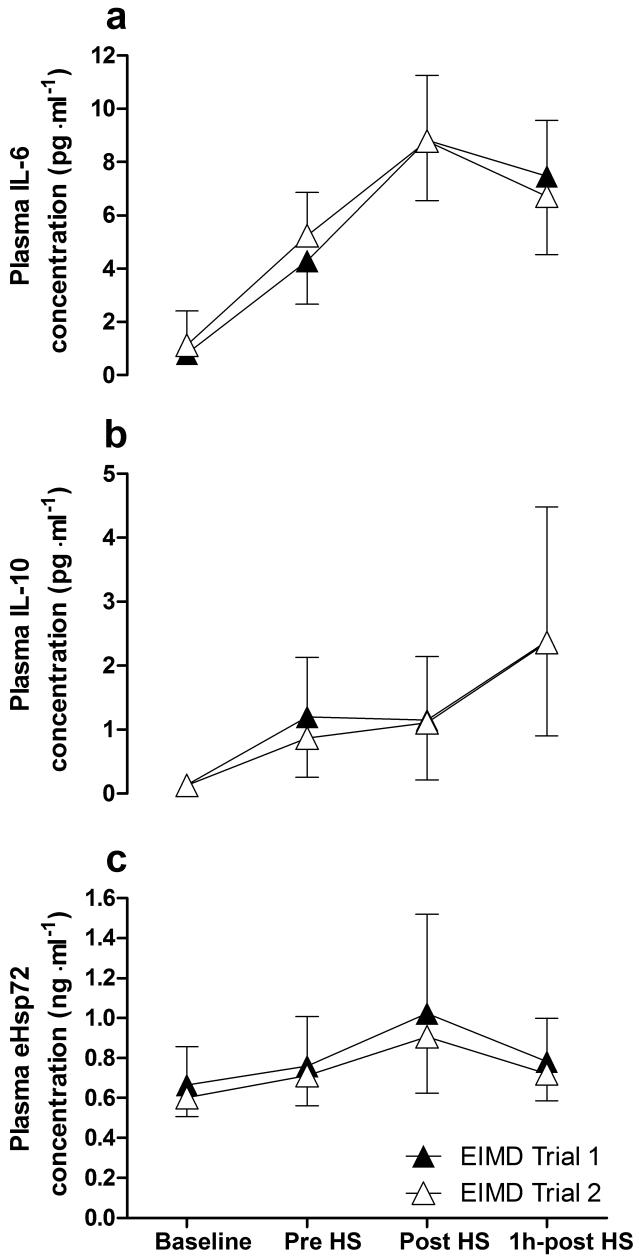

Fig. 20. Plasma interleukin (IL)-6 (a), plasma IL-10 (b), and extracellular heat shock protein (Hsp) 72 (c) concentrations at baseline, pre, post and 1h-post exercise-heat stress (HS), which involved running for 40 min at 65% VO_{2max} in 33°C, 50% RH, conducted 30 min after exercise-induced muscle damage (-10% gradient running, *n* = 9). Trial 1 represents the first bout (filled triangles), trial 2 represents the repeated bout performed 14d later (open triangles). No significant effects. Data are mean ± SD.

6.5. Discussion

We have recently shown that an acute bout of EIMD increases heat strain during subsequent exercise heat stress (HS), and thus, EIMD likely poses a risk factor for EHI (Fortes et al., 2013). The aim of the present study was to investigate if the well-known repeated bout effect for EIMD (Clarkson et al., 1992; McHugh et al., 1999) would blunt the increase in heat strain during subsequent exercise-heat-stress. In line with our hypothesis, heat strain was reduced (0.34 °C lower final T_{re}) during HS following the second EIMD bout, compared with the first bout. Furthermore, 7 of 9 participants showed evidence of mild hyperthermia (final T_{re} > 39.5 °C) during HS on EIMD trial 1 compared with only 2 of 9 participants during HS following EIMD trial 2. In addition, we observed a lower temperature threshold for sweating onset, a lower thermal sensation and a lower physiological strain index during HS after the second EIMD bout. This lessened heat strain during HS on the repeated EIMD trial was not due to temporary heat acclimation as a consequence of the first HS bout, nor due to passage of time effects, since no difference in heat strain was observed in the control group. By blunting the increase in thermal strain during exercise-heat-stress, incorporating a muscle-damaging bout into training could be used as a practical strategy to reduce the risk of EHI, and improve exercise performance, in individuals undertaking subsequent heavy exercise bouts with an eccentric component in the heat.

Unaccustomed and/or heavy eccentric exercise is known to result in EIMD, and is characterised by decreased muscle strength, and increased muscle soreness in the days following EIMD (Clarkson et al., 1992; Clarkson and Hubal, 2002). We utilised a downhill running model to evoke moderate, realistic muscle damage, evidenced by significant decreases in leg strength (-7.5%), and increases in muscle soreness 48-h post EIMD trial 1. This degree of muscle damage is equivalent to experiencing 'a light pain when walking' (Vickers, 2001) and therefore likely reflects the degree of muscle damage that may be encountered by soldiers and athletes undergoing unaccustomed exercise with an eccentric component. Research has consistently shown, that a prior

muscle-damaging bout produces a protective adaptation that results in lesser symptoms of EIMD when the muscle-damaging bout is repeated (e.g. blunted losses of leg strength, and reductions in muscle soreness) (Byrnes et al., 1985; Clarkson et al., 1992; McHugh et al., 1999). The success of the repeated bout effect was demonstrated herein, since leg strength was restored to baseline levels, and muscle soreness was significantly reduced 48-h following the repeated bout of EIMD.

Heat strain during HS was reduced following the second, repeated bout of EIMD compared with the first, with the ΔT_{re}, and final T_{re} reduced 0.2 °C and 0.34 °C respectively during HS on EIMD trial 2 compared with trial 1. The final T_{re} on EIMD trial 2 was similar to the final T_{re} observed in the control group during HS on both CON trials (**Fig. 17 c** and **d**), although we acknowledge that these groups were composed of different individuals, so are not directly comparable. In our previous study (Fortes et al., 2013), final T_{re} was 0.53 °C higher during HS following EIMD compared to control non-muscle-damaging exercise in a repeated measures design, whilst Montain et al. (2000), also demonstrated ~0.3 °C greater body temperature during HS following muscle-damaging exercise compared to control exercise. It therefore appears that a prior bout of EIMD provides a significant degree of protection against the increased heat strain that EIMD elicits during subsequent exercise-heat-stress. It is acknowledged the limitation that it has not been established a performance benefit due to the reduced heat strain but anticipate a delay in reaching a critical core temperature (purported to be ~40 °C (Gonzalez-Alonso et al., 1999)) after repeated EIMD. Further research should investigate whether the observed reduction in thermal strain with repeated EIMD translates into a meaningful performance improvement. In this regard, its noteworthy that a similar reduction in final exercising T_{re} (-0.3 °C) during exercise-heat-stress after 5 d heat acclimation brought about a 14% improvement in endurance performance during exercise in the heat (Garrett et al., 2009). Additional confidence in our findings could be added, since a particular strength of the current study is that a comparable control group was used in order to discount confounding heat acclimation or passage of

time effects. Previous research has also shown no effect of a single prior heat stress bout on subsequent thermoregulatory responses during exercise heat stress conducted 14 days later (Barnett and Maughan, 1993).

The circulating IL-6 response to muscle-damaging exercise has been shown to be higher compared with non-muscle-damaging exercise (Bruunsgaard et al., 1997), a finding supported by our previous study (Fortes et al., 2013) where the elevated circulating IL-6 concentration following EIMD correlated with the final T_{re} attained during subsequent HS. An elevated circulating IL-6 prior to heat stress is significant, given potential involvement of the acute inflammatory response in the aetiology of EHI (Bouchama, 1995; Lim and Mackinnon, 2006; Shephard and Shek, 1999; Walsh et al., 2011). A "dampening" of the inflammatory response is one proposed explanation for the repeated bout effect (Pizza et al., 1996). Indeed, a previous study has shown reduced pyrogenic cytokines (IL-6), and increased anti-inflammatory cytokines (IL-10) in the 12 hours following a repeated bout of muscle damage evoked by downhill running (Smith et al., 2007), although this finding is not supported by studies that have used different models of muscle damage (e.g. isokinetic knee extensor exercise (Croisier et al., 1999; Willoughby et al., 2003)). Therefore, it was of intereste in whether the hypothesised reduction in heat strain could be accounted for by an altered circulating inflammatory response following EIMD. However, it was not observed any difference in pyrogenic IL-6 or anti-inflammatory IL-10 response either prior to, or after heat stress between the two EIMD bouts. It should be noted that in the study by Smith et al. (2007), cytokine concentrations were averaged over the 12 hour period following EIMD, thereby not providing useful information on the kinetics of the early inflammatory response immediately following EIMD. eHsp72 concentration was also assesed, which has been shown to be elevated following downhill running (Peake et al., 2005), and is known to act as a "danger signal" activating the immune system (Horowitz and Robinson, 2007; Whitham and Fortes, 2008) including IL-6 (Asea et al., 2000). Furthermore, eHsp72 itself may have direct pyrogenic effects (Pastukhov et al., 2003). Nevertheless, we did

not observe a difference in circulating eHsp72 concentration between EIMD trials. It is noteworthy, however, that the circulating IL-6 and eHsp72 responses prior to HS during EIMD trial 1 were modest when compared with more metabolically challenging activities such as marathon running (Fehrenbach et al., 2005; Nieman et al., 2001), and therefore provided little margin for dampened responses.

Muscle-damaging exercise increases steady state exercising VO_2 following downhill running (Braun and Dutto, 2003), and rodent models have shown decreased contraction economy in damaged muscle fibres (Warren, III et al., 1996). One might therefore anticipate improved running economy during HS on the second EIMD trial. However, this was not the case, since $\dot{M}$ during HS was similar on both EIMD trials. EIMD has also been shown to inhibit glycogen resynthesis (O'Reilly et al., 1987), yet altered metabolism is also an unlikely mechanism since RER was not different between EIMD trials. Whilst there was no effect on T_{sk}, we did observe a lower temperature threshold for sweating onset, and a lower thermal sensation during HS on the second EIMD bout. The mechanism(s) for how a muscle-damaging bout of exercise 14 d earlier decreased the threshold T_{re} for sweating onset during HS following a repeated-bout of EIMD remains unclear. There may be shared pathways with the concept of cross-tolerance, where adaptations to one stressor confer protection via molecular, cellular and neural interactions to other forms of stressor (Horowitz, 2014; Horowitz and Robinson, 2007). This process is thought to be mediated, in part, by intra-cellular Hsp's, and it has been suggested that acquired thermotolerance (the accumulation of protective Hsp's within cells following heat exposure), and systemic adaptations to heat acclimation share a common pathway (Kuennen et al., 2011). This may be pertinent since EIMD has been shown to increase Hsp72 and Hsp27 expression within muscle cells following EIMD (Paulsen et al., 2009; Thompson et al., 2001), and that the changes we observed during HS on EIMD trial 2, reflect similar adaptations to those observed during heat acclimation (reduced exercising T_{re}, earlier sweating onset, reduced thermal sensation). Furthermore, a smaller intracellular heat shock response is shown in heat-intolerant

military personnel (Moran et al., 2006). It is acknowledged a limitation of the current study is that it has not been assessed intra-cellular Hsp's. It is possible that increases in Hsp's within muscle cells following the first EIMD bout confer some degree of heat acclimation evident 14 d later but future work is required to investigate this. It remains to be shown whether muscle-damaging exercise provides cross-tolerance against other novel stressors.

Non-heat-acclimated individuals undertaking heavy training bouts with an eccentric component in the heat are likely at increased risk of developing EHI (Fortes et al., 2013; Montain et al., 2000) e.g. athletes performing unaccustomed exercise, or soldiers running or walking across mountainous terrain. Considered alongside our present findings this work might help to explain why EHS in athletes, soldiers and labourers typically occurs in the first four days of unaccustomed heavy exertion in the heat (Armstrong et al., 2007; Cluver, 1932). The present findings indicate that this risk can be mitigated by incorporating a bout of muscle-damaging exercise into the training regimen, prior to travel/deployment to hot environments, but this requires further investigation. Further research should also investigate the duration of the demonstrated protective effect and confirm whether other models of eliciting EIMD, e.g. drop-jumps, have a comparable protective effect to that shown here with downhill treadmill running, with the added practical benefit of allowing a large number of individuals to be treated at once.

In conclusion, the repeated-bout-effect blunted the increase in heat strain during exercise-heat-stress conducted after EIMD, as shown by reduced exercising T_{re}, reduced thermal sensation, and earlier sweating onset. Incorporating a muscle-damaging bout into training could be a strategy to reduce the risk of EHI and improve endurance performance in individuals undertaking heavy exercise with an eccentric component in the heat.

CHAPTER SEVEN

General Discussion

7.1. Background

Extreme temperature exposure or single-day, multiple strenuous exercise bouts (singly or in any combination) are common in active populations (e.g. military personnel, athletes, outdoor enthusiasts). These individuals can resultantly have an increased susceptibility to URTI or be predisposed to EHI and EHS.

Anecdotal evidence points towards cold exposure increasing one's susceptibility to infections. Evidence to support such a link between cold exposure and an increased infection incidence is notably lacking in available human studies (Castellani and Rhind, 2002). The few human studies to investigate the effect of cold exposure on immune function report conflicting information that is dependent on the degree of hypothermia, the health of the individual, and the immune parameters studied (Lackovic et al., 1988; Brenner et al., 1999; Wenisch et al., 1996; Costa et al., 2010). Collectively, these studies suggest that lowering core temperature (core body temperature decrease > 1 °C) can disrupt circulating immune function, which may partly explain the increased URTI reported after cold exposure. Limitations of previous research are that they included unhealthy populations (e.g. patients undergoing surgery) and they did not perform a thermoneutral control trial (Wenisch et al., 1996; Beilin et al., 1998). Therefore it is unclear whether surgery, anaesthesia, existing comorbidity and diurnal variation have independent effects for the altered immune function reported. It therefore remained to be shown in healthy humans whether immune function is impaired when core temperature is reduced (e.g. core temperature decrease of 1–2 °C).

Oppositely, active populations such as athletes and soldiers taking part in heavy training are also expected to perform multiple bouts of strenuous exercise in hot environments, which may predispose them to EHI or the more severe form, and EHS. Conventionally classified risk factors for EHI and EHS include hot and humid environmental conditions, unsuitable clothing, sleep disruption, absence of heat acclimation, high exercise intensity, poor physical fitness levels (or obesity) and undiagnosed medical conditions (Armstrong et al., 2007; Epstein et al., 1999; Rav-Acha et al., 2004). These conventional risk factors

do not however explain all EHI events, this begs the question: "Which lesser known risk factors or pathways might play important roles in many cases of EHI?" (Sawka et al., 2011)

One further pathway was suggested for EHI progression; this relates to a systemic inflammatory reaction with an incremental increase in pyrogenic cytokines [e.g., interleukin (IL)-1β, IL-6, and TNF -α] (Bouchama, 1995; Lambert et al., 2001; Shephard and Shek, 1999). This may result in quicker progression toward EHI and/or EHS (Walsh et al., 2011). If firm evidence of a pyrogenic cytokines role in the development of EHI is uncovered, it would be of great interest to investigate, and develop strategies of avoiding or managing EHI.

Reducing the amount of muscle damage and inflammation would plausibly decrease heat strain. Indeed, evidence showed that over 80% of all EHI casualties reported during Marine recruits' training occurred in the first 3 days of unaccustomed exercise in the heat (Kark et al., 1996). This is also supported by data collected during Operation TELIC in the summer of 2003 where a heat illness rate of 50/1000 was recorded during the first 10-14 days of deployment among troops that were performing arduous physical activity prior the commencement of this doctorate (Bolton et al., 2006). One obvious, but as yet un-investigated avenue is the well-known repeated bout effect in muscle-damaging exercise, where a single session of eccentric exercise causes adaptations which in-turn result in decreased damage from subsequent training (Clarkson et al. 1992; Clarkson and Hubal 2002; McHugh et al. 1999).

This thesis, therefore aimed to investigate:

1. The effect of mild hypothermia and prolonged cold exposure on healthy humans using measures of immune function.
2. Whether exercise-induced muscle damage (EIMD) exacerbates heat strain during subsequent exercise-heat-stress, which may in due course increase the subject's risk of exertional heat illness.

3. Whether a repeated-bout of EIMD lower the increase in rectal temperature (T_{re}) experienced during subsequent endurance exercise under determined heat conditions.

7.2. Summary of main findings

Firstly, during mild hypothermia we observed a decreased antigen-stimulated whole-blood culture IFN-ϒ production and an increase in circulating leukocytes, and plasma cortisol, adrenaline and noradrenaline levels in healthy humans. Conversely, neutrophil degranulation (bacterially stimulated elastase release per neutrophil), plasma elastase concentration and production of antigen stimulated IL-4 were unchanged. Despite rewarming and feeding the adaptive immunity was suppresed for at leat 3-hours (**Chapter 4**). To the best of our knowledge, for the first time it has been showed that cold exposure and mild-hypothermia do not alter neutrophil degranulation, or antigen-stimulated IL-4 production but did lower antigen-stimulated IFN-ϒ production. In addition, despite rewarming and feeding, IFN-γ production did not return to control values within the 3-hour recovery period. Collectively, these findings might therefore, at least in part, explain the increased susceptibility to upper respiratory tract infections reported after cold exposure. Future research should confirm present findings and should investigate strategies to blunt the immune suppression following cold exposure and mild hypothermia in order to decrease the risk of infections for those involved in activities in cold environments.

Secondly, we found evidence that, in non–heat-acclimated males, the ΔT_{re} increased during exercise heat stress when performed 30 min after moderate muscle-damaging exercise (timed to match the early inflammatory phase). This resulted in a ~0.5 °C rise in final T_{re} during 40 min of moderate exercise heat stress (HS1) for the muscle damaged participants (final T_{re} ~39.5 °C).

It was also found that the preceding EIMD evoked an increase in plasma IL-6 concentration which was associated (r = 0.67) with increased heat strain during HS1.

It was also observed a meaningful, albeit small, increase in ΔT_{re} when exercise heat

stress was timed to coincide with deferred muscle inflammatory phase (24 h after moderate muscle-damaging exercise (**Chapter 5**). Data presented here, therefore show that a bout of EIMD, brought about by downhill running, increases heat strain during successive endurance exercise in the heat conducted 30 min after and, to a much lesser extent, 24 h after muscle damage. In line with previous literature, EIMD evoked increases in the circulating pyrogen IL-6 were associated with increased heat strain during exercise heat stress conducted 30 min after EIMD. These results have practical relevance, particularly for non–heat-acclimated athletes and soldiers undertaking either multiple, or prolonged bouts of heavy exercise with an eccentric component in the heat. Consequently, it has been of interest to investigate, and provide strategies that could reduce the risk of EHI following EIMD. Quite naturally, it has been thought that reducing the degree of muscle damage encountered could be a countermeasure to lessen the degree of heat strain encountered. One unexplored avenue of investigation was the well-known repeated bout effect of muscle-damaging exercise, where a single session of eccentric exercise induces adaptations resulting in less evidence of damage when the exercise bout is repeated.

We recorded reduced heat strain (0.34 °C lower final T_{re}) during HS post a second EIMD bout, compared to after the initial bout. Other observations include a lower temperature threshold for sweating onset, decreased thermal sensation and a decreased physiological strain index during HS after the second EIMD bout. No difference in pro-inflammatory IL-6 or anti-inflammatory IL-10 response was observed either prior to or after heat stress between EIMD bouts. In addition, an assessment of eHsp72 concentration showed it to be elevated following downhill running (Peake et al., 2005). Nevertheless, no difference was observed between EIMD trials in the concentration of circulating eHsp72 (**Chapter 6**). As showed by this data the repeated-bout-effect blunted the increase in heat strain during exercise-heat-stress conducted after EIMD, as shown by reduced exercising T_{re}, reduced thermal sensation, and earlier sweating onset. Therefore, incorporating a muscle-damaging bout into training could be a strategy to reduce the

risk of EHI and improve endurance performance in individuals undertaking heavy exercise with an eccentric component in the heat.

Future research should investigate where improved acclimation protocols or cross-acclimation adaptations would further improve safety for those at risk of EHI.

7.3. Effect of cold exposure and mild hypothermia on circulating leukocyte counts and their function

Circulating leukocytes were increased by 50% immediately following cold exposure and were higher than in the thermo-neutral control group throughout the recovery phase. This rather modest leucocytosis could possibly be explained by neutrophil demargination from the vascular endothelium, a consequence of shearing and increased concentrations of neuro-hormones (Brenner et al., 1998; Malpica et al., 2002; Ramel, 2003; Ramel and Peake, 2004; Ortega et al., 2005; Bote et al., 2008; Laing et al., 2008; Zhang et al., 2011).

The circulating leukocytes increase was primarily due to a 93% neutrophil increase. This significant neutrophilia has been reported previously (Fedor and Fisher, 1959; Villalobos et al., 1955; Blair et al., 1969). Indeed, Blair and colleagues (1969) reported a circulating neutrophil increase in septic patients when they were cooled to 34°C. On the contrary, in an animal study Biggar et al. (1983) found hypothermia to be associated with a notable reduction in mature and immature neutrophil release into circulation from the bone marrow.

We measured neutrophil function by bacterially stimulating neutrophil degranulation. This increased after cold exposure, however no differences were observed between trials. This observation is inconsistent with previous findings, where these had shown that a modest cooling of the whole body (T_{re} 35.9 °C) has lead to decreased neutrophil degranulation (Costa et al., 2011). This discrepancy underlines how unclear the neutrophilia mediators and cold responsive decrease in neutrophil degranulation are:

they are simply not as defined as had previously been thought and this requires further research.

A lymphocyte decline (-13%) was observed both during and after exposure to cold air with a return to control values after 3 hours. Previous studies reported that T cell population changes are proportional to stress duration and intensity, and that this is to a lesser extent true of changes in B cell populations (Gleeson et al., 2005). T cells are fundamental to the orchestration and regulation of cell-mediated immune responses to pathogens (Shephard and Shek, 1999). One important consequence of deficient T cell function is increased viral infections incidence (Fabbri et al., 2003). The lymphocyte decrease might contribute to the increased URTI susceptibility widely reported during winter. It is yet to be seen whether this decline is merely due to cold exposure or if it stems from shivering and involuntary tonic contractions, which count as acute physical exercise (Rhind et al., 2001).

The monocyte count showed a short initial increase as mild hypothermia set in, but beyond this it was unaltered by cold air exposure. One explanation of this rise could be the thermogenic activity, which is regarded to be exercise-stress. Indeed, transient monocytosis has been reported during physical exercise. The most likely cause is the shifting of monocytes into the circualtion pool (Rhind et al., 2001).

7.4. Effect of cold exposure and mild hypothermia on circulating citokines

Antigen-stimulated whole-blood culture IFN-γ production decreased immediately after cold exposure and remained 69% lower than the thermo-neutral control trial throughout the 3-hours recovery. Antigen-stimulated IL-4 production was unaltered by cold exposure. Th1 cells mediated the release of cytokines (such as interferons) and they are important to defence against intracellular pathogens (D'Elios et al., 2007). Prolonged moderate-intensity exercise has been found to depress Th1 cell production (Gleeson et al., 2005). This was the first study to report that other stress factors (e.g. cold) might adversely affect interferon production. Present results might indicate that a cold

exposure-induced decrease in adaptive immunity capability might increase the likelihood pathogen susceptibility, even during recovery. This is due to the fact that the adaptive immunity response follows an initiation process. The antigen is presented to T helper lymphocytes at the peptide binding groove on major histocompatibility complex class II molecules on antigen-presenting CD4+ T cells. This is key to the cell-mediated immune response, since they direct and orchestrate the adaptive immune response to viruses and bacteria. Decreased IFN- ϒ production might result in suppressed cellular immune system response to invading micro-organisms, since they act with immunostimulatory and immunomodulatory effects and directly inhibit viral replication (Gleeson et al., 2011).

As mentioned above, IL-4 productions remained unaltered by cold exposure and this is in line with literature evidence that IL-4 can be produced in response to prolonged, moderate intensity exercise (Gleeson et al., 2005). The steady concentration of this cytokine could be indicating that leukocyte function was unaffected during cold exposure. It is known that monocytes, lymphocytes and B cells have roles in IL-4 production, and that a combination of this and IL-10 can interrupt Type-1 T-cell cytokine production (Steensberg, 2003). On study showed that strenuous exercise decreases circulating type-1 T-cells, whereas the percentage of Type-2 T-cells was unaffected. Cortisol and adrenaline both decrease Type-1 T-cell cytokine production, whereas IL-6 stimulates Type-2 T cell cytokine production (**Chapter 4**).

The role played by IL-4 and IL-6 in a hypothermic scenario is as yet not well established. What is known is that it activates B cells and T cell proliferation and regulates adaptive immunity responses (Paul et al., 1997). This is usually coupled with IL-10, another anti-inflammatory cytokine. There is evidence that IL-10 production from microglia, on the contrary, decreases proportionally to the decrease in body core temperature (Matsui et al., 2004). This decrease is beneficial, since excessive levels of IL-10 are detrimental to the central nervous system.

7.5. Effect of cold exposure and mild hypothermia on neurotransmitters

Innate immunity is affected by adrenaline, noradrenaline and cortisol as outlined above. Plasma noradrenaline increase upon exposure to cold due to SAM axis stimulation. This promotes shivering thermogenesis, vasoconstriction and endogenous substrate breakdown (Castellani et al., 2002).

Neurotransmitters are also known to regulate both innate immune cell function and leucocytosis by bone marrow release and demargination (Ortega, 2003). In this context, the main effectors of the quick rise in circulating neutrophils seem to be catecholamines since they are rapidly released into the blood, and stimulate an immediate immune cells response (Brenner et al., 1998). The modest increase observed in plasma cortisol (~12 %) from cold exposure is not thought likely to account for the observed changes in leukocyte trafficking considering the time required for cortisol to affect immune responses (Goldstein-Golaire et al., 1970; Jefferies, 1991). Supporting this is the fact that a few hours after cold exposure there was no delayed blood leukocytes increase due to cortisol-induced release of neutrophils from the bone marrow (Gagnon et al., 2014). That the second increase in leukocyte trafficking elicited by cortisol is dependent upon exercise intensity and duration has been shown (Fragala et al., 2011) and high-intensity exercise results in significant cortisol elevation (200% from Jacks et al., 2002), although this is unlikely to be the case in the present study. Additionally, any significant cortisol effects tend to be reported once the plasma cortisol concentration exceeds the capacity of the corticosteroid-binding globulin (550 nmol l^{-1}) and the free cortisol concentration increases (Costa et al., 2011). This is not the case in the present study where the highest concentration of plasma cortisol recorded was 498 ± 158 nmol l^{-1} during mild-hypothermia. This additionally supports the idea that cortisol did not play a main role in the observed changes in leukocyte trafficking.

The present study directly compared the rise in adrenaline and noradrenaline. The increase in neurotransmitters, induced by exercise can be replicated using data provided by Galbo in 1975 (Galbo et al., 1975). The observed catecholamines rise is similar to

that observed in response to a short bout of running (10 mins) at 75 % VO_{2Max} intensity or to repeated bouts of running at 75 % VO_{2Max} intensity for prolonged time periods. This suggests that the innate immunity changes observed might be, in part, explained by the body initiating thermoregulatory activity which counteracts mild-hypothermia. That would help to explain why neutrophil function was unaffected by cold exposure even if neutrophilia had occurred. Notably, catecholamines have a direct effect on cardiac output and the shear effect derived, and this might explains the observed changes in white blood cell numbers.

In addition, increased circulating adrenaline might account for the lymphopenia during recovery from cold exposure which is possibly via alterations in the adhesion molecules and surface receptors on the lymphocytes (Kruger and Mooren, 2007; Costa et al., 2011). Adrenaline is now thought to play a key role in regulating lymphocyte trafficking during environmental stress (Costa et al., 2010). Furthermore, adrenaline and noradrenaline sympathetically mediate influences on cardiac output indirectly, which this alters the subsequent increase in enhanced blood flow associated shear stress.

7.6. Similarities between immune response following cold exposure and after physical exercise

An indirect method was used to calculate the energy cost of thermoregulatory responses during cold exposure as suggested by the Compendium of Physical Activities (Ainsworth et al., 2011). Indeed, using Metabolic Equivalent of Task (MET) (MET = 3.5 mL oxygen • kg^{-1} body weight • min^{-1}), described as the ratio of the metabolic rate for an activity divided by a standardized expression of the rest metabolic rate, showed that thermogenic activity during cold exposure can be equated with prolonged bouts of moderate exercise (5.6 (2.0) MET) (**Chapter 4**).

This explains why some innate immune marker changes are similar to those from exercise. Under these conditions exercise can be looked upon as the thermogenic activity

the body counteracts a cold environment with (Walsh et al., 2006). It is reasonable to expect immune markers to mimic an exercise response (Young et al., 2000).

Lymphocytosis was observed during and after cold exposure. The number of cells during recovery fell below pre-exercise levels (**Chapter 4**). It has previously been reported that T cell population changes are proportional to stress intensity and duration, although the intensity of effect is more marked B cell populations (Gleeson et al., 2005). Research showed the T cell population decreased by 50 % after a 2.5 h of treadmill running at 75 % VO_{2Max} (control value: resting values. Gleeson et al., 2005). The lymphocyte count acted in a similar fashion to the response to prolonged exercise, although the decrease observed was smaller during our trials. The T cell number regained baseline levels after 3 h, which mirrors lymphocytic responses to prolonged exercise (Robson et al., 1999). Cold air exposure failed to alter monocyte count above a small initial increase associated with mild hypothermia. One plausible explanation for this rise could be shivering and involuntary tonic contractions. Young et al. (2001), looked on this as acute physical exercise, and others have reported it as resulting in transient monocytosis. That would most likely represent the migration of monocytes from the marginated to the circulation pool. This could be the result of, singly or in combination, a haemodynamic and/or cortisol or catecholamine-induced release at the vascular endothelium (Walsh et al., 2011). This is supported by a research study which asserted that administering the beta-blocker propranolol resulted in lessened exercise-induced monocytosis (Walsh et al., 2011). Beyond this, reports show that stressors can affect monocyte phenotype, cytokine expression and cell surface proteins (Freidenreich and Volek, 2012). For example, there is a preferrance for the use of inflammatory monocytes following accute exercise (CD4+/CD16+) (Freidenreich and Volek, 2012). It is conceivable, therefore, that marginated cells exhibit a more mature inflammatory function for entry into tissues and are seperated from the endothelium by exercise. It is interesting to note that the percentage of these CD14+/CD16+ cells reduces during recovery which may indicate

remarginalization or tissue recruitment. Acute exercise also reduces the inflammatory monocyte's expression of TLRs 1,2 and 4 (Gleeson et al., 2011).

Antigen-stimulated whole-blood culture IFN-ϒ production was lower throughout cold exposure and did not recover to baseline levels after 3 hours despite participants having been fed and rewarmed (see **Chapter 4**). This mirrored the rise seen after 2.5 h of cycling at 65 % VO_{2Max} (Lancaster et al., 2005). The release of IFN-ϒ (as with other cytokines) is mediated by Th1 cells. They are important in intracellular pathogen defence (e.g. against viruses. see: D'Elios et al., 2007). Prolonged moderate-intensity exercise has been found to decrease Th1 cells (Gleeson et al., 2005), which might explain a decreased IFN-ϒ production.

7.7. Study limitations and future directions (**Chapter 4**)

While we have provided some novel and thought-provoking preliminary findings on immune responses to mild-hypothermic state and cold exposure (**Chapter 4**), further research is still needed to clarify the nature and extent of the immune mechanisms involved. It is specifically suggested that an extended number of adaptive immunity markers would be measured. This would provide more compelling evidence on how, and for how long, adaptive immunity is suppresed post induced hypothermia. Further to this, the role of thermo-genic activity on innate immune markers during cold exposure needs clarification. Indeed, most of the measured markers responded similarly to moderate intensity, prolonged exercise. Some evidence suggests that increased metabolic response to cold exposure is equal to prolonged exercise performance. An *in vitro* investigation, or another study design that could measure or exclude the effects of thermogenic-activity on innate immune markers, should be conducted to clarify the effect of cold on human immune markers.

7.8. The effect of muscle-damaging exercise on heat strain during subsequent exercise heat stress (**Chapter 5**)

In agreement with our hypothesis, we showed that in non–heat-acclimated males ΔT_{re} is increased during exercise heat stress performed 30 min after moderate muscle-damaging exercise timed to coincide with the early inflammatory phase. This resulted in a ~0.5 °C greater final T_{re} during HS1 in muscle damaged participants (final T_{re} ~39.5 °C) after just 40 min of moderate exercise heat stress.

These results might have real-world applicability, particularly for non–heat-acclimated athletes and soldiers who undertake repeated and/or prolonged heavy training bouts with an eccentric component in hot environmental conditions. Actions that can decrease EIMD associated inflammation may mitigate the risk of EHI.

Downhill running is often used in studies to evoke muscle damage (Braun and Dutto, 2003; Paulsen et al., 2012; Peake et al., 2005), and the aim of the model used here was to evoke moderate, realistic muscle damage that would not impair the participants ability to complete subsequent exercise heat stress bouts.

7.9. Effect of muscle-damaging exercise on cytokines plasmatic concentrations and their subsequent effect on heat strain during exercise heat stress

We identified circulating IL-6 concentration to be a marker for acute inflammation, and assessed this for changes. We hypothesized that EIMD-evoked increases in circulating IL-6 and that this would be associated with increased heat strain during consecutive exercise heat stress (30 min after muscle-damaging exercise).

As expected, EIMD significantly increased circulating IL-6 relative to CON prior to the first exercise-heat-stress bout. Importantly the circulating level of IL-6 after EIMD was relatively modest compared to the level usually observed following prolonged, metabolically demanding endurance exercise (Nieman et al., 2001). As such, the post EIMD IL-6 increase showed wide variability (**Chapter 5**). Nonetheless, we noted a moderate correlation between the circulating concentration of IL-6 after treatment and the subsequent change in T_{re} during HS1 in EIMD trial (r = 0.67). The sequential nature

of this relationship is noteworthy; an early, modest inflammatory response was associated with subsequent increases in T_{re} during exercise heat stress. Furthermore, these associations did not follow the second exercise-heat-stress bout, when pre-HS2 IL-6 concentrations had returned to baseline values, with no difference between trials. Since IL-6 is known to be a pyrogen (Dinarello et al., 1991), acute increases in circulating IL-6 after muscle damage might plausibly act on the cycloxygenase 2–mediated PGE2 pathway, resulting in in an incremental change to the thermoregulatory set point (Rummel et al., 2006; Bradford et al., 2007) and heat strain during exercise heat stress. All things considered, we recognize the augmentation in the circulating IL-6 response after EIMD to be relatively moderate and understand that the mooted concept of a thermoregulatory set point is controversial (Romanovsky, 2007).

We showed an association, albeit moderate, between prior acute circulating inflammation and a subsequent exercise heat strain. Accordingly, a logical line of exploration would be to identify strategies that could diminish inflammation which may, in turn, mitigate rising T_{re} figures and lower EHI risk during exercise heat stress (**Chapter 6**).

Muscle-damaging exercise has previously been shown to cause a higher circulating IL-6 response, compared to non-muscle-damaging exercise (Bruunsgaard et al., 1997), and that finding was supported by our previous study (Fortes et al., 2013 – **Chapter 5**).

Therefore the elevated circulating IL-6 concentration following EIM correlated to the final T_{re} attained during a subsequent HS. Elevated circulating IL-6 before heat stress is significant as it is potentially involved in an acute inflammatory response in the aetiology of EHI (Bouchama, 1995; Shephard and Shek, 1999; Lim and Mackinnon, 2006; Walsh et al., 2011). A "dampening" of the inflammation response has been proposed as one explanation for the repeated bout effect (Pizza et al., 1996). Although our study did not observe any differences to the pro-inflammatory IL-6 response between the two EIMD bouts either before or after heat stress. Notably, Smith and colleagues reported that cytokine concentrations were averaged over the 12 hour period following EIMD. Therefore this figure could not be expected to garner closely time-referenced information on the kinetics of early inflammatory response following EIMD (Smith et al., 2007).

The concentration of circulating IL-10 exhibited no EIMD repeated bout effect. The chief role of IL-10 is to oppose inflammatory responses in order to down-regulate adaptive immune effector responses and to minimise tissue damage under microbial challenges. Additionally, IL-10 inhibits several pro-inflammatory cytokines and other soluble mediators. A previous study conducted during marathon competition in 1997 found that the dramatic increase in the inflammation responsive cytokine IL-6 was balanced by the release of the anti-inflammatory cytokine IL-10 (Ostrowski et al., 1999). Indeed, IL-6 appears to be responsible for subsequent rises in the circulating levels of anti-inflammatory cytokines (IL-10 and IL-1ra) and that it also stimulates the cortisol release from the adrenal glands (Steensberg, 2003). In this context, regulatory T cells, Th2 cells, monocytes, CD8+ T cells, macrophages and Th1 cells are the principle parties in IL-10 production. In our study, we observed a modest rise in IL-6 which might explain the lack of changes of IL-10 concentration between trials (**Chapters 5** and **6**). In effect, a substantial increase in IL-6 plasmatic concentration might be needed to trigger the subsequent rise of anti-inflammatory cytokines but further research is needed to clarify this process.

7.10. Heat Shock Protein 72 (Hsp 72)

We assessed eHsp72 concentration, which was shown by Peake et al. (2005) to be elevated following downhill running. It is also known to act as a "danger signal", activating the immune system and IL-6 (Horowitz and Robinson, 2007; Whitham and Fortes, 2008; Asea et al., 2000). Furthermore, eHsp72 has been suggested to have direct pyrogenic effects (Pastukhov et al., 2003). Regardless, our study did not observe a difference in circulating eHsp72 concentration between EIMD trials. Yet it should be kept in mind that during EIMD trial 1 the circulating IL-6 and eHsp72 responses prior to HS were modest when compared with marathon running or other more metabolically challenging activities (Nieman et al., 2001; Fehrenbach et al., 2005). The slight margin for dampened responses is clear when viewed in this light.

7.11. Exercise-induced muscle damage and the repeated bout effect (**Chapter 6**)

Our downhill running model evoked moderate, realistic muscle damage. Indeed, significant decreases in leg strength (-7.5 %), and increases in muscle soreness 48-h post EIMD trial 1 were seen. This muscle damage is equivalent to 'a light pain when walking' (Vickers, 2001) and therefore reflects that reportedly encountered by soldiers and athletes subjected to unaccustomed exercise with an eccentric component. Research has consistently shown that a prior muscle-damaging bout produces a protective adaptation, and results in lesser symptoms of EIMD when the muscle-damaging bout is repeated (e.g. diminished leg strength losses and reductions in muscle tenderness) (Byrnes et al., 1985; Clarkson et al., 1992; McHugh et al., 1999). The repeated bout effect was successfully demonstrated here, as leg strength regained baseline levels and muscle soreness was significantly reduced 48-h after the repeated bout of EIMD. The decrease in leg strength observed seems to support the theory which states that the repeated bout effect is due to fragile muscle fibres loss during the first bout of exercise and their subsequent replacement with less damage-susceptible fibres during recovery. Indeed, when the muscle-damaging exercise was repeated during EIMD trial 2 no further leg-strength decrease was observed. Conversely, if this fibre loss continued during EIMD trial 2 a significant decline in muscle strength would have occurred. Therefore, a plausible explanation is that damaged fragile fibres were repaired over time and that these repaired fibres are enhanced and less susceptible to damage. In conclusion, these findings do not fully explain the process behind the repeated bout effect but provided evidence that the first bout of damage produced an adaptation that lasted for at least 14 days, time that has occurred between trials during our study.

7.12. Studies limitations (Chapters 5 and 6)

Future studies need to examine the contribution of pyrogenic pathways after muscle damage upon exercise heat strain in more depth. Whether or not the greater proportion of circulating IL-6 response after EIMD (**Chapters 5** and **6**) come from leukocytes, muscle cells, or other cells remains to be shown (Montain et al., 2000; Braun and Dutto,

2003; Paulsen et al., 2012). However, our study did not observe any difference in the responses of either pyrogenic IL-6 or anti-inflammatory IL-10 prior to, or following heat stress between the two EIMD bouts (**Chapter 6**).

A previous study has shown reduced pyrogenic cytokines (IL-6), and increased anti-inflammatory cytokines (IL-10) in the 12 h following a repeated bout of muscle damage evoked by downhill running (Smith et al., 2007), although this finding is not supported by studies that have used different models of muscle damage (e.g. isokinetic knee extensor exercise (Croisier et al., 1999; Willoughby et al., 2003)). This hypothesised reduction in heat strain could be accounted for by an altered circulating inflammatory response following EIMD, as a "dampening" of the inflammatory response is one proposed explanation for the repeated bout effect (Pizza et al., 1996). Our findings do not, support this explanation for the repeated bout effect but we acknowledge that further research with an extended post-exercise time-frame (48 h-120 h post) is required (**Chapter 5**). Indeed, many of the criterion measures may peak 48-72 h post exercise. Data of this breadth would confirm whether or not the criterion measures rose, peaked or fell, and whether muscle function was altered.

We did not assess intra-cellular HSPs (**Chapter 6**). It is possible that increased muscle cell HSPs following the first EIMD bout would confer some degree of heat acclimation and this would be evident up to 14 d later. Indeed, EIMD has been previously showed to increase HSP72 and HSP27 expression within muscle cells (Paulsen et al., 2009; Thompson et al., 2001), and a smaller intracellular heat shock response is shown in heat-intolerant military personnel (Moran et al., 2006). During our study (**Chapter 6**), we did observe a lower thermal sensation during HS on the second EIMD bout and a lower temperature threshold for sweating onset which might open-up to the idea that muscle-damaging exercise provided cross-tolerance against other novel stressors (Horowitz, 2007). Another good question is whether, and if so how, the mechanism(s) for a muscle-damaging bout of exercise 14 d earlier would decrease the threshold T_{re} for sweating onset during HS following a repeated-bout of EIMD. Some pathways may

share responsibility with the cross-tolerance, i.e. adaptations to one stressor might confer protection to other forms of stressor via molecular, cellular and neural interactions (Horowitz, 2014; Horowitz and Robinson 2007). In this context, previous evidence suggested that acquired thermo-tolerance (post heat exposure cellular HSP accumulation) and systemic heat acclimation adaptations share a common pathway (Kuennen et al., 2011) and this process is expected to be mediated (at least in part) by intra-cellular Hsp. The changes we observed during HS on EIMD trial 2, reflect similar adaptations to those observed in heat acclimation (reduced exercising T_{re}, earlier sweating onset, reduced thermal sensation). Therefore, we strongly recommend that studies will be performed to identify the changes induced by muscle-damaging exercise on HSPs concentration at intracellular level and the subsequent effect on thermoregulation. If novel research on cross-tolerance will be inspired by the suggestions here presented new strategies to protect from EHI and EHS those involved in physical activity in hot environments may be developed. Also, heat-acclimation is currently known to require repeated exposures to heat over a considerable duration (Armstrong et al., 1991; Pandolf et al., 1998). Our findings suggest that one bout of EIMD could be a strategy to induce cross-tolerance and by reducing the amount of time and training required for acclimatization. This might be greatly beneficial to athletes and soldiers taking part in activities in hot environments.

7.13. Conclusions

The major conclusions drawn from this thesis are:

1. Cold exposure and mild-hypothermia did not alter neutrophil degranulation bacterially stimulated elastase release per neutrophil, or antigen-stimulated production of IL-4.
2. A leucocytosis, mainly caused by a neutrophil increases, has been observed during and following cold exposure, which was probably in response to neurotransmitters increase.
3. The increased release of noradrenaline and adrenaline might respond to increased metabolic needs.
4. However, lowered IFN-ϒ production was noted in the hours following the cold exposure presenting "open window" in the immune systems ability to respond to bacterial challenges. The prolonged period of adaptive immunity suppresion continued despite rewarming and feeding. This might partially explain the increased upper respiratory tract infection susceptibility aneqdotely reported by outdoor enthusiast during cold weather.
5. A bout of EIMD increases heat strain during successive endurance exercise in the heat conducted 30 min after muscle damage was confirmed by the data. This is also true, although to a much lesser extent, 24 h after the EIMD.
6. EIMD also evoked increases in circulating pyrogenic cytokine IL-6. This was associated with an increased severity of heat strain during exercise heat stress trials conducted 30 min after the EIMD.
7. Repeated-bout-effect lessened the onset of heat strain during exercise-heat-stress conducted after EIMD. This was shown by a reduced exercising T_{re}, reduced thermal perception, and an earlier onset of sweating during HS 2. Training programs that incorporate a muscle-damaging bout could be an effective strategy

in reducing EHI risk. This could also improve endurance performance in hot conditions when individuals undertake heavy exercise with an eccentric component.

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APPENDIX A:

Bangor University

School of Sport, Health and Exercise Sciences

Subject Information Sheet

Project Title: An Experimental Study of Practical Field Methods for Cold Casualty Protection and Treatment during Prolonged Cold Exposure

Research Co-ordinators: Dr Sam Oliver and Dr Neil Walsh

Tel: 01248 383965 **Email:**s.j.oliver@bangor.ac.uk

Additional Investigators: Dr Matt Fortes, Dr Gavin Lawrence**,** Jenny Brierley, Alberto Dolci, Phil Heritage, Aaron Burdett, Robin Wilson, Beth Palmer and James Firman

Invitation to take part

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being conducted and what will be required of you should you agree to be involved. Please take time to read the following information carefully and discuss it with the investigators. Ask us if there is anything that is not clear or if you would like more information.

Do I have to take part?

This is entirely your decision. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. **If you decide to take part you are still free to withdraw at any time without giving a reason.** If you decide not to participate or withdraw during the study, your decision will not affect your relationship with the School of Sport, Health and Exercise Sciences, or any of the investigators

involved in the study. All information collected during the study will be treated confidentially and if you choose to withdraw then your data will be deleted from our records.

Background

Prolonged cold exposure and becoming mildly cold is associated with impaired health and performance. Accidents in cold and wet environments can lead to hypothermia. This is especially the case when casualties remain stationary or are suffering from trauma with blood loss (for example, mountaineering or car accident). In the UK average mountain rescue times are typically at least 2 hours. It is therefore important that easily administrable and transportable cold protection methods are developed which can be used whilst casualties wait for emergency services rescue. Ultimately, mortality rates will be decreased if cold protection methods can be developed and shown to reduce or maintain a casualty's core temperature. Current field cold protection methods have been shown to be relatively ineffective (e.g. polythene survival bag), or their effectiveness is presently unknown (e.g. hot drinks and Blizzard Survival bags). Therefore the present investigation aims to determine the effectiveness of polythene survival bags with hot drinks and Blizzard survival bags with and without heat pads.

Objective

To assess which field method offers optimal protection for casualties in a cold environment.

What will be expected of you?

If you decide to take part in this study there will be a number of constraints placed upon you. During the day prior to completing a trial you will be expected:

- To avoid drinking alcohol or caffeinated drinks (i.e. coffee, tea, coke and diet coke).

- To avoid participating in moderate to high intensity exercise.

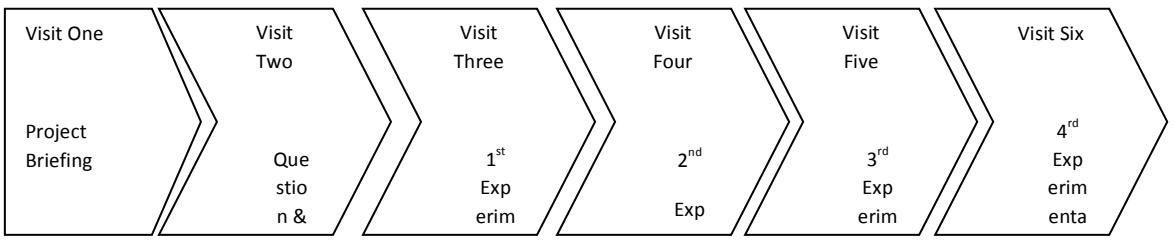
Whilst completing the experimental trials in the laboratory you will be expected:

- To spend one whole day in the laboratory on four separate occasions.
- To eat and drink only what is prescribed to you.
- Arrive to the laboratory after an overnight fast (10-12 hours)
- To follow the study's timetable.
- To be weighed nude (behind screens to maintain privacy).
- To have four blood samples taken during each one day trial (~65 ml) totaling 16 blood samples during the 4 week study period (~260 ml).
- To provide saliva and urine samples.
- To perform four cold water immersions (~13°C for a maximum of 60 minutes).
- To perform four 3 hour cold air tests in 0°C.
- To complete a health log 2 weeks prior, during and 2 weeks following the last trial.

You will be excluded if you are a smoker, asthmatic, diabetic, have a pacemaker or any heart condition or you are currently taking specific types of medication or supplements.

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP 34 HOURS OF YOUR TIME.

Summary of visits



Visit One: Project briefing and familiarisation (1 hour)

At this meeting you will be fully briefed about the requirements of the project. You will be talked through the subject information sheet by an investigator and given the opportunity to ask any questions. You will then leave with the information sheet so as to allow you time to discuss your possible involvement in the study with significant others. This will also allow you additional time to think of questions.

You will be asked to refrain from exercise and alcohol and caffeine consumption for 24 hours prior to each visit. At this visit you will be familiarised with the procedures for collection of saliva and venous blood. All blood samples will be collected by a qualified member of staff from a forearm vein using a small needle (~16 ml of blood at each collection). A saliva sample will be obtained by asking you to dribble into a plastic container for 5 minutes. You will also be familiarised with the cognitive function tests and also the health log which you will be asked to complete at the end of each day.

Visit Two: Question and answer session (optional)

Visit two is provided as an additional opportunity for you to ask questions. Once you are fully satisfied with the information and on agreement to take part in the study you will be asked to complete an informed consent form, a medical questionnaire and make arrangements for the following visits.

Experimental trials:

You will be required to complete four experimental trials in a random order each separated by 7 days. Each experimental trial will consist of an 8 hour period where you will be in the laboratory under treatment conditions (Visit three, four, five and six). **Including the briefing meeting, familiarisation visit and all four experimental trials you will be required to visit the laboratory on seven occasions for a total of ~34 hours.**

Visit Three, Four, Five and Six: (8 hours)

Prior to arriving at the laboratory

You will be instructed to consume your normal daily food intake and keep a food diary of what you ate the day before the trial. We will also provide you with your daily water requirements (approximately 2.5 L for a 70 kg person), which will help to ensure that you are hydrated prior to each trial. We ask that you arrive at the physiology laboratory with the appropriate clothing (i.e. swim shorts, a second pair of dry shorts and a towel) following an overnight fast at 07:00 hours.

Experimental procedures

On arrival to the laboratory and after completely emptying your bladder and bowels, your body composition will be estimated by a non-invasive bio-electrical impedance analysis; which requires you to stand on a set of weighing scales. On arrival at the laboratory you will be given a rectal probe to fit, and a universal container with which to collect a urine sample. The rectal probe will be used to monitor rectal core temperature. You will also be asked to wear a chest strap so that we can monitor your heart rate. You will then sit quietly for 30 minutes in the laboratory (ambient temperature ~20°C) after which blood and saliva samples will be obtained and you will complete the cognitive function tests. You will then enter the cold water bath (~13°C) and be immersed up to your shoulders until your core temperature has decreased to 36°C (~40 minutes). At this point you will be withdrawn from the water. After drying and putting on your dry shorts you will enter the environmental chamber (0°C) with one of the four survival bags. You will then be asked to sit on a chair whilst inside the survival bag. You will be randomly assigned at each trial to one of the four practical cold protection methods: 1. Polythene survival bag; 2. Polythene survival bag with hot drink; 3. Blizzard Survival bag and 4. Blizzard Survival Heat bag. After getting into the survival bag and at 1 and 2 hours you will be provided with a flavoured drink. The quantity of this drink will depend

on your body weight but will be similar to a large mug of tea. On one trial (trial 2) this drink will be hot, i.e. similar to that temperature of regular tea or coffee. You will be asked to consume these drinks within 15 min. Regular measurements of thermoregulation will be made and core temperature will be continually monitored. Cognitive function tests and saliva samples will be obtained at one hour intervals. The experimental trials will end and you will be removed from the chamber at 3 hours or if core temperature decreases to 35°C. Following removal from the environmental chamber (0°C), a blood and saliva sample will be obtained and you will be wrapped in blankets. You may be immersed in warm water to aid rewarming. One and two hours following the cold exposure we will obtain further blood and saliva samples during which time you will sit in a comfortable ambient temperature ~20°C with blankets or dressed in your normal clothes. During this time, your core temperature will be monitored and you will be supervised by experimenters. In the second hour of this recovery you will be provided with a large meal and warm drink. Following the final blood and saliva sample you will be allowed to eat and drink freely and you will be permitted to leave when your core temperature returns to with 0.5°C of your initial core temperature. Transport home will be provided if required.

Advantages of taking part

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, of your body composition (e.g. body fat %) and blood measures (e.g. immune function). This feedback should help you with planning and monitoring your athletic training program. The feedback you will receive regarding your body composition is similar to that which many testing facilities provide as a fee-paying service. Advantages for Undergraduates from within SSHES are that you will gain a valuable insight into the procedures and work involved in a 3rd year project. Additionally participation in this project can be used for skills units of the Undergraduate portfolio.

Disadvantages of taking part

The disadvantages of taking part in this study, which you will probably be most concerned about, are: 1. cold exposure test 2. blood samples; and 3. time commitment.

1. **Cold exposure**: Cold tests lasting two to three hours have been safely used to stress the body's ability to maintain core body temperature by our research group and many others. During these cold air tests you will likely experience some peripheral numbness and mild discomfort in your hands and feet. To minimise this discomfort, we will provide you with thermal gloves and socks.

A number of military studies have exposed individuals to freezing and/or wet conditions following periods of prolonged physical exercise, inadequate nutrition and sleep loss, and no medical complications were reported after rewarming in individuals whose core temperature decreased to 35°C). In these investigations no voluntary 'drop out' or medical withdrawal of subjects was reported. Additionally, in contrast to these previous investigations in which subjects sat uncovered, the provision of survival bags in the proposed study will provide insulation.

In the current investigation you will be removed from the climate chamber if your core temperature decreases to 35°C. It is important to note that 35°C is considered the upper limit of mild hypothermia (35-32°C), and following the removal of cold stress your thermoregulatory mechanisms are likely to steadily recover normal core temperature (~37°C). For example, in a study using cold water immersion to reduce core temperature to 33°C participants left to shiver post immersion were able to recover core temperature to 36°C within 1 hour.

Following removal from the chamber you will be wrapped in blankets. In addition, if your core temperature reduces below 35°C you will be immersed up to the axilla in a warm water bath (38-42°C). This technique has previously been used in a number of studies to rewarm participants in experiments where core temperature is decreased to between 33-

34°C. For your safety your core temperature will be monitored by a rectal probe every minute throughout the cold exposure protocol and during the first hour of recovery period or until your core temperature returns to within 0.5°C of their starting core temperature.

The results of this study will ensure people can be better informed as to the most effective method of cold protection in extreme environmental conditions.

2. **Blood samples**: The blood samples will be taken using a smaller needle than is typically used by doctors in your local surgery or hospital. Therefore you will likely experience only very mild discomfort i.e. like a scratch. The quantity of blood we are taking in each visit is small (i.e. approximately one tenth of that which would be obtained when you give blood). Additionally, qualified phlebotomists experienced at performing this procedure will collect blood samples. To ensure you are completely happy with giving blood samples, we will familiarise you with the blood sampling procedure on your first visit to the laboratory.

3. **Time commitment**: To complete all aspects of the study we will require you to visit the laboratory on six occasions for a total of approximately 33 hours. Thus, participation in this project satisfies the requirement in hours for the Undergraduate portfolio skills unit.

At all times you will be closely supervised by an experimenter trained in First-Aid.

Any further questions will be happily answered by Dr Sam Oliver or any of the additional investigators.

School of Sport, Health and Exercise Sciences,

Bangor University,

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The influence of exercise induced muscle damage upon subsequent thermoregulation during exercise in the heat.

Participant Information sheet

Project Investigators

Dr Matthew Fortes	01248 383687	m.fortes@bangor.ac.uk
Dr Neil Walsh	01248 383480	n.walsh@bangor.ac.uk
Umberto Di Felice	01248 388288	pexa04@bangor.ac.uk

Michael Crockford Alberto Dolci

Lindsey Jankowski Megan Butterworth

Ben Terzza Ryan Hillier-Smith

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Invitation to take part

You are being invited to take part in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, family and your GP if you wish. If there is anything that is not clear or if you would like more information, please do not hesitate to ask us. Take time to decide whether or not you wish to take part.

What is the purpose of this study?

Military personnel are often expected to perform repeated bouts of arduous physical activity on the same day, often in hot environments which may increase their risk of exertional heat illness (EHI) or the more serious and potentially fatal condition termed exertional heat stroke (EHS). Whilst a number of traditional risk factors have been identified that increase the risk of EHI and EHS in military personnel (for example, hot and humid environmental conditions, inappropriate clothing, inadequate acclimatisation, sleep disruption, high exercise intensity, high % body fat, and low physical fitness), it has also been suggested that muscle damaging exercise similar to that encountered during unaccustomed exercises such as downhill walking and running may adversely affect an individual's normal body temperature response to exercise in the heat. Additionally, alterations in immune function as a result of muscle damage may also pay a role in EHI and EHS. It is the purpose of this study to investigate the influence of muscle damage evoked by downhill running on the ability of an individual to thermoregulate, and on their immune system during subsequent exercise bouts conducted in a hot but safe environment.

Why have I been chosen?

You have been chosen because you are a recreationally active male, aged between 18 and 40 years of age, free from infection and, and not currently taking medication.

Do I have to take part?

This is entirely your decision. If you decide to take part you will be asked to sign a consent form. You will be given a copy of this form to keep for your information. You will be free to withdraw at any time without giving a reason. If you decide not to participate, it will not affect your relationship with the School of Sport, Health and Exercise Sciences or any of the researchers. All the information collected will be treated confidentially.

What will happen to me if I take part?

The study will involve you making eight visits to the School of Sport, Health and Exercise Sciences. The purpose and time commitment of each visit are outlined below:

Visit 1) You will be talked through the protocol by an investigator and given the opportunity to ask any questions. If you decide to take part, you will be asked to sign a consent form (visit length ~30 mins).

Visit 2) During this visit you will be asked to perform an incremental exercise test on a treadmill, whilst wearing a mask, which will last approximately 20 minutes. This test starts off easy (fast walking pace) and gets progressively harder until you cannot continue. By the end, you will be running very quickly. By measuring your expired air, we will be able to calculate your maximal oxygen uptake (a good indicator of your overall fitness) and to calculate the intensity at which you will exercise during the experimental trials. Following this test, you will have a short recovery period (approx 10 mins), and then you will be asked to run at a moderate speed on the treadmill for approximately 20 minutes in order for us to verify the correct exercise intensity for the subsequent experimental trials. We will ask you to run on a flat gradient, and then on a downhill gradient. Since you may not be used to running downhill, we will give you ample opportunity to practice, by starting off slowly and gradually increasing the running speed (visit length ~ 1 hour).

Visits 3- 8). There are 3 experimental trials, each one requiring 2 visits on consecutive days. The 3 experimental trials consist of a muscle damage trial (MD), control trial (CON) and a carbohydrate trial (CHO);

MD trial

You will be asked to report to the laboratory at 07:15h having not eaten that morning. We will provide you with a snack and a set amount of fluids. At 08.00h you will be asked to provide a urine sample, and to weigh yourself nude behind screens (for your privacy) for a body mass measurement. You will then be prepared for the exercise bout i.e. you will be asked to fit a rectal thermistor so that we can monitor your core temperature, and we will fit 4 skin thermistors that measure your skin temperature, and a heart rate monitor. You will then provide a blood sample which will be taken by venepuncture of a forearm vein by a qualified phlebotomist. You will be provided with a small amount of water before performing the first exercise bout. This exercise bout will be run on a motorized treadmill at a moderate exercise intensity (65% maximal oxygen uptake) with the gradient set at -10% downhill in order to induce muscle damage. You will be provided with small amounts of fluid during this exercise bout which will last for 1-hour. Additionally, you will be asked to breathe into a bag at 2 time points during this bout so that we can determine your exercise intensity. Upon completion, you will be asked to sit quietly in the laboratory for 30 mins during which time you will weigh yourself nude and another blood and urine sample will be collected. After this 30 min period, you will enter an environmental chamber set to hot conditions (33ºC and 50% relative humidity). You will run on the treadmill at a moderate intensity (65% maximal oxygen uptake) at a level gradient for 40 minutes, during which time you will not be allowed to consume fluids. During this exercise bout, you will be asked to breathe into a bag every 10 minutes, and to rate your perceived exertion and how hot you feel every 5 minutes. Once you have completed this second exercise bout, further nude body mass measures will be made, and a blood and urine sample will be collected. You will then sit quietly for 1-hour in the laboratory during which time your recovery core and skin temperatures will

be assessed continuously. You will be provided with recovery fluids during this time. A further blood and urine sample will be collected after this 1-hour recovery period. You will then be allowed to shower, and we will then provide you with a small lunch. A final body mass, blood and urine sample will be collected 2 hours after you finished exercising. You will then be free to leave the department, but will be asked to return at 08:30h the following day to complete one further exercise bout in the environmental chamber at the same intensity and duration (40 mins) and under the same environmental conditions (33ºC and 50% relative humidity),with nude body mass measured, and blood and urine samples collected, pre, immediately post and 1 and 2-hours after the exercise bout. You will also be asked to rate your muscle soreness. In the 21-hour period between the two exercise heat bouts, we will ask you to record what you eat, and to replicate this on the other two experimental trials. In total, this experimental trial will involve 2 visits – the first one ~5.5 hours, and the second one ~4 hours (~9.5 hours total).

Control trial

This experimental trial will follow exactly the same procedures and timings as the MD trial, except that the first exercise bout will be conducted on a level treadmill gradient (not downhill). (~9.5 hours total).

Carbohydrate trial

This experimental trial will follow the same procedures and timings of the MD trial, except that instead of consuming water prior and during the first exercise bout, you will consume a carbohydrate containing drink. Additionally, the first exercise bout will be conducted on a level treadmill gradient (not downhill). (~9.5 hours total).

These trials will be performed in a randomized order, and 14 days apart to allow you to recover. In total the study will require you to give 27 blood samples, 27 nude body mass measurements, 27 urine samples, and will take up 30 hours of your time.

What do I have to do before each visit?

We ask that you try to consume plenty of fluids in the 24 hours prior to visit 2, and we will provide you with a set amount of fluid to drink in the 24 hours prior to visits 3 -8. We also ask that in the 24 hours prior to visits 3-8 you do not perform any exercise, drink alcohol or consume any form of diuretics or tobacco. We will also ask you to record what you eat on the day before visits 3 and 4, and to replicate these meals on the night before visit 5 - 8. You may also wish to bring a towel and change of clothes for each visit.

What are the possible disadvantages and risks of taking part?

The disadvantages of taking part in this study are the discomfort associated with the use of temperature thermisters, blood sampling, muscle damage and exercising in a hot environment. For your safety your core temperature will be monitored by a rectal temperature probe inserted 10 cm beyond the anal sphincter. These probes are commonly used in thermoregulation research and most people do not notice them once they have started exercising. Blood sampling will be performed by qualified phlebotomists using a disposable small needle. We will collect 14ml of blood four times on each of the 6 exercise trial days. Removing this amount of blood will not affect your performance or health in any way. However, you may experience bruising of the area around the venepuncture site. Any discomfort you experience will be short lived. The muscle damaging protocol (downhill running) is routinely used in exercise physiology research and you will experience muscle soreness in the 1-3 days following this exercise bout. This soreness will be comparable to the sort of soreness you might experience after performing a new type of exercise for the first time. However, this soreness will pass in 3 – 5 days and will not leave any lasting damage. There are standard risks associated with performing intense exercise in hot conditions. Accordingly, all experimenters are trained in recognizing and treating signs and symptoms of exertional heat illness. Additionally, persons trained in CPR and AED will be readily available or present at all exercise trials. However, it should be noted that measures are in place to

minimize the risk of exertional heat illness, and accordingly the risk of this occurring is very small.

What are the possible benefits of taking part?

Taking part in this study will enable you to receive comprehensive feedback, with full explanations of your fitness level. The feedback you will receive regarding your fitness level is similar to that which many fitness testing centres provide as a fee-paying service. Advantages for undergraduates from within SSHES are that you will gain a valuable insight into the procedures and work involved in a 3rd year project. Additionally participation in this project can cover both the open and personal performance skills units of the undergraduate portfolio. Finally, you will be paid £50 cash upon completion of all experimental procedures.

What if something goes wrong?

If you are harmed whilst taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study you may contact the Head of the School of Sport, Health and Exercise Sciences, Professor Michael Khan.

Will my taking part in this study be kept confidential?

All information collected about you during the course of the study will be kept strictly confidential. It will be anonymised so that only the following people will have access to your records:

Dr Neil Walsh (University lecturer), Dr Matthew Fortes (Postdoctoral researcher), Umberto Di Felice (PhD student) and Alberto Dolci (PhD student).

Your data will be stored on a password protected computer and in locked filing cabinets. This data will be destroyed 10 years after the conclusion of the study.

What will happen to the results of the research study?

The results from this study may be published in a leading international physiology journal. Your blood samples will be stored for a maximum period of six years and may be used in future investigations in this area of research. Again, you will not be able to be identified from this. A written report will be given to any participants who are interested in the results of the study.

Who is organising and funding the research?

This research is organised by the named investigators above, and funded by Bangor University.

Who has reviewed the study?

The study has been approved by the School of Sport, Health and Exercise Sciences ethics committee of Bangor University.

Contact for further information

If you require any further information about the study please contact any of the project investigators whose contact details are displayed below:

Dr Matthew Fortes	01248 383687	m.fortes@bangor.ac.uk
Dr Neil Walsh	01248 383480	n.walsh@bangor.ac.uk

Thank you very much for taking the time to read this information sheet.

If you choose to participate in this study you will be given a copy of the information sheet and a signed consent form to keep

School of Sport, Health and Exercise Sciences,

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The influence of repeated bouts of muscle damaging exercise upon thermoregulation during exercise in the heat.

Participant Information sheet

Project Investigators

Alberto Dolci	01248 388288	pepc2e@bangor.ac.uk
Dr Matthew Fortes	01248 383687	m.fortes@bangor.ac.uk
Prof. Neil Walsh	01248 383480	n.walsh@bangor.ac.uk

Adnan Haq

Faye Walker

Invitation to take part

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What is the purpose of this study?

Athletes and military personnel are often expected to perform repeated bouts of arduous physical activity on the same day, often in hot environments which may increase their risk of exertional heat illness (EHI). A number of traditional risk factors have been identified that increase the risk of heat illness in athletes and military personnel (for example, hot and humid environmental conditions, wearing lots of clothing, lack of acclimatisation to hot conditions, poor sleep, high exercise intensity, high amount of body fat, and poor fitness). We have recently shown that downhill running, which can damage the leg muscles resulting in soreness, makes people get hotter when they perform an exercise run in the heat 30 mins later. This increase in body temperature may be due to alterations in the immune system, and could result in an increased risk of heat illness. It is also known that performing one bout of this type of muscle damaging exercise can lessen the negative effects, and reduce soreness after further muscle damaging type exercise bouts. It is the purpose of this current study to investigate if repeated muscle damage exercise (downhill running) results in people getting "less hot" whilst exercising in the heat. To assess this, you will be expected to perform either 1) muscle damaging exercise (60 min running downhill), followed by a 40 min exercise run in the heat. You will then repeat this 2 weeks later, and we will compare your body temperature responses between these two trials, or 2) non-muscle damaging exercise (60 min running on the flat), followed by a 40 min exercise run in the heat. You will

then repeat this 2 weeks later, and we will compare your body temperature responses between these two trials

Why have I been chosen?

You have been chosen because you are a recreationally active male, aged between 18 and 40 years of age, free from infection and, and not currently taking medication.

Do I have to take part?

This is entirely your decision. If you decide to take part you will be asked to sign a consent form. You will be given a copy of this form to keep for your information. You will be free to withdraw at any time without giving a reason. If you decide not to participate, it will not affect your relationship with the School of Sport, Health and Exercise Sciences or any of the researchers. All the information collected will be treated confidentially.

What will happen to me if I take part?

The study will involve you making ten visits to the School of Sport, Health and Exercise Sciences. The purpose and time commitment of each visit are outlined below:

Visit 1) You will be talked through the protocol by an investigator and given the opportunity to ask any questions. If you decide to take part, you will be asked to sign a consent form. Then you will be randomly assigned to the control trial (CON) or to exercise-induced muscle damage trial (EIMD) (visit length ~30 mins).

Visit 2) During this visit you will be asked to perform an incremental exercise test on a treadmill, whilst wearing a mask, which will last approximately 20 minutes. This test starts off easy (fast walking pace) and gets progressively harder until you cannot continue. By the end, you will be running very quickly. By measuring your expired air, we will be able to calculate your maximal oxygen uptake (a good indicator of your overall fitness) and to calculate the intensity at which you will exercise during the experimental trials. Following this test, you will have a short recovery period (approx 10 mins), and then you will be asked to run at a moderate speed on the treadmill for

approximately 20 minutes in order for us to verify the correct exercise intensity for the subsequent experimental trials. We will ask you to run on a flat gradient, and then depending on which group you have been assigned, on a downhill gradient. Since you may not be used to running downhill, we will give you ample opportunity to practice, by starting off slowly and gradually increasing the running speed (visit length ~ 1½ hours).

Visit 3) Using fitness machine similar to that found in a gym, we will assess your leg strength using 4 four maximal contractions after a warm-up consisting of six isometric contractions at 25%, 50% and 75% of intensity (visit length ~ 30 min).

Visit 4) The day after visit 3, you will be asked to report to the laboratory at 07:15h having not eaten that morning. We will provide you with a snack and a set amount of fluids. At 08.00h you will be asked to provide a urine sample, and to weigh yourself nude behind screens (for your privacy) for a body mass measurement. You will then be prepared for the exercise bout i.e. you will be asked to fit a rectal thermistor so that we can monitor your core temperature, and we will fit 4 skin thermistors that measure your skin temperature, and a heart rate monitor. You will then provide a blood sample which will be taken by venepuncture of a forearm vein by a qualified phlebotomist. You will be provided with a small amount of water before performing the first exercise bout. This exercise bout will be run on a motorized treadmill at a moderate exercise intensity (65% maximal oxygen uptake) with the gradient set at -10% downhill in order to induce muscle damage if you are on the EIMD trial or with the gradient set at +1% if you are on the CON trial. You will be provided with small amounts of fluid during this exercise bout which will last for 1-hour. Additionally, you will be asked to breathe into a bag at 2 time points during this bout so that we can determine your exercise intensity. Upon completion, you will be asked to sit quietly in the laboratory for 30 mins during which time you will weigh yourself nude and another blood sample will be collected. After this 30 min period, you will enter an environmental chamber set to hot conditions (33ºC and 50% relative humidity). You will run on the treadmill at a moderate intensity (65% maximal oxygen uptake) at a level gradient for 40 minutes, during which time you will

not be allowed to consume fluids. During this exercise bout, you will be asked to breathe into a bag every 10 minutes, and to rate your perceived exertion and how hot you feel every 5 minutes. Once you have completed this second exercise bout, a further nude body mass measure will be made, and a blood sample will be collected. You will then sit quietly for 1-hour in the laboratory during which time your recovery core and skin temperatures will be assessed continuously. You will be provided with recovery fluids during this time. A further blood sample will be collected after this 1-hour recovery period. You will then be allowed to shower, and we will then provide you with a small lunch, after which you will be free to leave the department. (visit length ~4.5 hours total).

Visit 5 and 6) These will be exactly the same as visit 3 (to assess your leg strength), and will be performed 24 hours, and 48 hours after visit 4 respectively. (visit length ~ 30 min each).

Visit 7, 8, 9, and 10) These visits will consist of exactly the same procedures and timings as per visits 3-6, but will be performed 2 weeks after the start of visit 3. (visit lengths ~ 30 min, 4.5 hours, 30 min and 30min each respectively).

In total the study will require you to give 8 blood samples, 8 nude body mass measurements, 2 urine samples, and will take up 14 hours of your time.

What do I have to do before each visit?

We ask that you try to consume plenty of fluids in the 24 hours prior to all visits, and we will provide you with a set amount of fluid to drink in the 24 hours prior to visits 4 and 8. We also ask that in the 24 hours prior to visits 4 and 8 you do not perform any exercise, drink alcohol or consume any form of diuretics or tobacco. We will also ask you to record what you eat on the day before visits 4, and to replicate this meal on the night before visit 8. You may also wish to bring a towel and change of clothes for each visit.

What are the possible disadvantages and risks of taking part?

The disadvantages of taking part in this study are the discomfort associated with the use of temperature thermistors, blood sampling, muscle damage and exercising in a hot environment. For your safety your core temperature will be monitored by a rectal temperature probe inserted 12 cm beyond the anal sphincter. These probes are commonly used in thermoregulation research and most people do not notice them once they have started exercising. Blood sampling will be performed by qualified phlebotomists using a disposable small needle. We will collect 12ml of blood three times on each of the 2 exercise trial days (visits 4 and 8). Removing this amount of blood will not affect your performance or health in any way. However, you may experience bruising of the area around the venepuncture site. Any discomfort you experience will be short lived. The muscle damaging protocol (downhill running) is routinely used in exercise physiology research and you will experience muscle soreness in the 1-3 days following this exercise bout. This soreness will be comparable to the sort of soreness you might experience after performing a new type of exercise for the first time. However, this soreness will pass in 3 – 5 days and will not leave any lasting damage. There are standard risks associated with performing intense exercise in hot conditions. Accordingly, all experimenters are trained in recognizing and treating signs and symptoms of exertional heat illness. Additionally, persons trained in CPR and AED will be readily available or present at all exercise trials. However, it should be noted that measures are in place to minimize the risk of exertional heat illness, and accordingly the risk of this occurring is very small.

What are the possible benefits of taking part?

Taking part in this study will enable you to receive comprehensive feedback, with full explanations of your fitness level. The feedback you will receive regarding your fitness level is similar to that which many fitness testing centers provide as a fee-paying service. Advantages for undergraduates from within SSHES are that you will gain a valuable insight into the procedures and work involved in a 3rd year project. Additionally

participation in this project can cover both the open and personal performance skills units of the undergraduate portfolio.

What if something goes wrong?

If you are harmed whilst taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study you may contact the Head of the School of Sport, Health and Exercise Sciences, Dr Tim Woodman.

Will my taking part in this study be kept confidential?

All information collected about you during the course of the study will be kept strictly confidential. It will be anonymised so that only the following people will have access to your records:

Prof. Neil Walsh (University lecturer), Dr Matthew Fortes (Postdoctoral researcher), and Alberto Dolci (PhD student).

Your data will be stored on a password protected computer and in locked filing cabinets. This data will be destroyed 10 years after the conclusion of the study.

What will happen to the results of the research study?

The results from this study may be published in a leading international physiology journal. Your blood samples will be stored for a maximum period of six years and may be used in future investigations in this area of research. Again, you will not be able to be identified from this. A written report will be given to any participants who are interested in the results of the study.

Who is organising and funding the research?

This research is organised by the named investigators above, and funded by Bangor University.

Who has reviewed the study?

The study has been approved by the School of Sport, Health and Exercise Sciences ethics committee of Bangor University.

Contact for further information

If you require any further information about the study please contact any of the project investigators whose contact details are displayed below:

Alberto Dolci	01248 388288	pepc2e@bangor.ac.uk
Dr Matthew Fortes	01248 383687	m.fortes@bangor.ac.uk
Prof. Neil Walsh	01248 383480	n.walsh@bangor.ac.uk

APPENDIX B:

INFORMED CONSENT TO PARTICIPATE

IN A RESEARCH PROJECT OR EXPERIMENT

Title of Research Project:

The influence of repeated bouts of muscle damaging exercise upon thermoregulation during exercise in the heat.

The researcher conducting this project subscribes to the ethics conduct of research and to the protection at all times of the interests, comfort, and safety of participants. This form and the information sheet have been given to you for your own protection and full understanding of the procedures**.** Your signature on this form will signify that you have received information which describes the procedures, possible risks, and benefits of this research project, that you have received an adequate opportunity to consider the information, and that you voluntarily agree to participate in the project.

Having been asked by Alberto Dolci of the School of Sport, Health and Exercise Sciences at Bangor University to participate in a research project experiment, I have received information regarding the procedures of the experiment.

I understand the procedures to be used in this experiment and any possible personal risks to me in taking part.

I understand that I may withdraw my participation in this experiment at any time.

I also understand that I may register any complaint I might have about this experiment to Dr Tim Woodman, Head of the School of Sport Health and Exercise Sciences, and that

I will be offered the opportunity of providing feedback on the experiment using standard report forms.

I may obtain copies of the results of this study, upon its completion, by contacting:

AlbertoDolci pepc2e@bangor.ac.uk

I confirm that I have been given adequate opportunity to ask any questions and that these have been answered to my satisfaction. ☐

I have been informed that the research material will be held confidential by the researcher. ☐

☐

I agree to participate in the study

NAME (please type or print legibly):

_____

ADDRESS:

(Optional)_____

PARTICIPANT'S SIGNATURE: _____ **DATE**:

_____

RESEARCHER'S SIGNATURE: _____ **DATE**:

_____

Two sheets should be completed - one for the participant and one for the researcher

APPENDIX C:

Bangor University

SCHOOL OF SPORT, HEALTH AND EXERCISE SCIENCES

Name of Participant ……..……..

Age ……

Are you in good health? ☐ YES ☐ NO

If no, please explain

How would you describe your present level of activity?

Tick intensity level and indicate approximate duration.

Vigorous		Moderate		Low intensity	

Duration (minutes)…….

How often?

< once per month		4-5 times per week	
once per month		> 5 times per week	
2-3 times per week			

Have you suffered from a serious illness or accident? ☐ YES ☐ NO

If yes, please give particulars:

Do you suffer, or have you ever suffered from:

	YES	NO		YES	NO
Asthma			Epilepsy		
Diabetes			High blood pressure		
Bronchitis					

Are you currently taking medication? ☐ YES ☐ NO

If yes, please give particulars:

Are you currently attending your GP for any condition or have you consulted your doctor in the last three months? ☐ YES ☐ NO

If yes, please give particulars:

Have you, or are you presently taking part in any other laboratory experiment?

☐ YES ☐ NO

PLEASE READ THE FOLLOWING CAREFULLY

Persons will be considered unfit to do the experimental exercise task if they:

- have a fever, cough or cold, or suffer from fainting spells or dizziness;
- have suspended training due to a joint or muscle injury;
- have a known history of medical disorders, i.e. high blood pressure, heart or lung disease;
- have had hyper/hypothermia, heat exhaustion, or any other heat or cold disorder;
- have anaphylactic shock symptoms to needles, probes or other medical-type equipment;
- have chronic or acute symptoms of gastrointestinal bacterial infections (e.g. Dysentery, Salmonella);
- have a history of infectious diseases (e.g. HIV, Hepatitis B); and if appropriate to the study design, have a known history of rectal bleeding, anal fissures, haemorrhoids, or any other condition of the rectum.

DECLARATION

I agree that I have none of the above conditions and I hereby volunteer to be a participant in experiments/investigations during the period of ……20……

My replies to the above questions are correct to the best of my belief and I understand that they will be treated with the strictest confidence. The experimenter has explained to my satisfaction the purpose of the experiment and possible risks involved.

I understand that I may withdraw from the experiment at any time and that I am under no obligation to give reasons for withdrawal or to attend again for experimentation.

Furthermore, if I am a student, I am aware that taking part or not taking part in this experiment, will neither be detrimental to, or further, my position as a student.

I undertake to obey the laboratory/study regulations and the instructions of the experimenter regarding safety, subject only to my right to withdraw declared above.

Signature (participant) ……..……. Date ……..

Print name ……..

Signature (experimenter) …….……. Date ……..

Print name …….

APPENDIX D:

The reproducibility of the Isometric right-leg extension strength test

Purpose. To assess the reproducibility of the Isometric right-leg extension strength test

Methods. Nine males (mean ± SD: age, 22 ± 2 years; height, 175 ± 8 cm; BM, 72 ± 7.2 kg; VO_{2max} 59.4 ± 5.1 mL· kg^{-1}·min^{-1}) performed three Isometric right-leg extension strength test spaced over a three days period. Within-subject variation was minimised by testing at a similar time of day. In addition, on the day prior to each test, participants refrained from exercise and consumed a similar diet and water equal to 40 mL·kg^{-1} of BM. All Isometric right-leg extension strength test were performed as previously described in **Chapter 6**. Typical error of the measurement, expressed as an absolute value (standard error of the measurement) and CV, were obtained as described elsewhere (Hopkins, 2000).

Results. Participants completed 317 ± 57.3, 329.3 ± 92.5 and 312.3 ± 66.9 Nm on trials 1, 2 and 3 respectively. The standard error of the measurement between trials 1-2 and 2-3 were 12.3 and -8.0 Nm. A CV of 5.8% was noted between trials, highlighting the importance of allowing participants a single familiarisation session. In addition, no order or learning effect was identified as ANOVA revealed no significant differences between trials 1, 2 and 3 ($P > 0.05$).

Conclusion. The CV of 5.8% is much smaller than the CV reported by results included in **Chapter 6**.