DOCTOR OF PHILOSOPHY

The influence of sleep deprivation, cold exposure, exercise stress and nutritional intervention on selected immune responses

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Award date:
2009

Awarding institution:
Bangor University

THE INFLUENCE OF SLEEP DEPRIVATION, COLD EXPOSURE, EXERCISE STRESS AND NUTRITIONAL INTERVENTION ON SELECTED IMMUNE RESPONSES.

By

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A thesis submitted to

Bangor University

In fulfillment of the requirement of the degree of

Doctor of Philosophy

School of Sport, Health and Exercise Sciences

Bangor University

United Kingdom

31st December, 2009

Summary

The physiological stress induced by prolonged strenuous exercise, sleep-deprivation, exposure to environmental extremes, and nutrient deprivation has previously been reported to disturb host defences, with neuroendocrine responses being a key mechanism for this disturbance. Participating in activities that are associated with a combination of physiological stressors may amplify immune modulating neuroendocrine responses and induce greater disturbances to host defences.

With this in mind, the purpose of this thesis was to investigate the effects of: 1. one night of total sleep-deprivation on selected immune (circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva immunoglobulin A (IgA)) and stress hormone responses at rest and following prolonged strenuous exercise; 2. two nights of total sleep-deprivation with and without energy-restriction on selected immune and stress hormone responses at rest and after passive cold-exposure; 3. passive cold-exposure inducing modest reductions in whole-body core temperature on selected immune and stress hormone responses; and finally, 4. carbohydrate feeding with and without the addition of protein during recovery from prolonged strenuous exercise on selected immune, stress hormone and insulin responses.

One night of total sleep-deprivation did not alter circulating leukocyte trafficking and plasma cortisol response, or compromise bacterially-stimulated neutrophil degranulation and saliva secretory IgA (S-IgA) responses at either rest or following prolonged strenuous exercise.

Two nights of total sleep-deprivation with and without a 90% energy-restriction did not alter circulating leukocyte trafficking and plasma stress hormone responses, or compromise bacterially-stimulated neutrophil degranulation and saliva S-IgA responses at rest. Passive cold-exposure alone decreased circulating lymphocyte counts, bacterially-stimulated neutrophil degranulation and saliva S-IgA responses. The addition of two nights of total sleep-deprivation with or without a 90% energy-restriction prior to cold exposure did not further amplify these immune perturbations.

The ingestion of carbohydrate with and without the addition of protein immediately after prolonged strenuous exercise, in which only water was provided during exercise, prevented the decrease in bacterially-stimulated neutrophil degranulation during recovery. On the other hand, the provision of carbohydrate with and without protein immediately after prolonged strenuous exercise did not influence circulating leukocyte trafficking, stress hormone responses, or prevent the post-exercise decrease in saliva IgA concentration.

It is possible that insulin response, and unlikely that stress hormone (cortisol, catecholamines) responses, after carbohydrate ingestion with and without protein, may have a role in maintaining bacterially-stimulated neutrophil degranulation with immediate feeding after prolonged strenuous exercise, in which only water is provided during exercise.

I dedicate this thesis to my supportive, loving and beautiful wife Vera Camões-Costa, and to my Ph.D. research program supervisor and academic mentor Dr Neil Walsh.

Acknowledgements

First and foremost I gratefully acknowledge and thank my research supervisor Dr. Neil Walsh, for his academic mentorship and guidance throughout my time as a Ph.D. research student. His friendly nature, open mindedness and professional manner allowed for a pleasant and productive working environment. Additionally, his high academic standards enabled me to progress my research knowledge and skills, and for this I thank him. An enormous thank you and acknowledgment to Kevin Williams, for his technical support, true natured approach, and being a fundamental key in all the experiments within this thesis. A special thanks to all the undergraduate and postgraduate students who were involved in aspects of protocol design, data collection and data analysis. Their hard work and enthusiasm allowed for such demanding sleepless experimental protocols to be enjoyable, productive and professionally executed. A special thanks must also go to Dr. James Bilzon for his continued support and assistance with research design and planning; Robert Walters from Ysbyty Gwynedd for this hematological assistance; Dr. Hans-Peter Kubis as my thesis chair and for always providing me with a pleasant morning chat in the laboratory and during our evening runs; and Simon Worth for his assistance in the biochemistry laboratory. Thank you to all the research participants for their effort and enthusiasm with which they completed such demanding study protocols. Especially, Walter Staiano for his volunteering support and exuberant personality. Another special thank you to Tommy Du Preez for his charming nature as a study participant and for providing the coffee during the many "*all nighters*" and early morning starts. Finally, I would like to give my upmost thanks to my beautiful wife Vera for her unyielding endless support and love, which has been there for all the ups and downs of my Ph.D. research program.

The experimental trials within this thesis were funded by the Ministry of Defence's Headquarters Army Recruitment and Training Division, Upavon, UK.

Publications

The following list of publications arises from the material presented in this thesis. My contribution to these publications includes: involvement in protocol design, research team coordination, data collection, data analysis and preparation of manuscript for journal submission. I also gratefully acknowledge the input and involvement from other named authors for each publication.

Full papers

Costa, R.J.S., Harper-Smith, A.D., Oliver, S.J., Walters, R., Maassen, N., Bilzon, J.L.J., & Walsh, N.P. (2010). The effects of two nights of sleep deprivation with and without energy restriction on selected immune indices at rest and in response to cold exposure. *Eur.J.Appl.Physiol.*, 109, 417-428.

Oliver, S.J., Costa, R.J.S., Laing, S.J., Bilzon, J.L.J., & Walsh, N.P. (2009). One night of sleep deprivation decreases treadmill endurance performance. *Eur.J.Appl.Physiol.*, 107, 155-161.

Costa, R.J.S., Oliver, S.J., Laing, S.J., Walters, R., Williams, S., Bilzon, J.L.J., & Walsh, N.P. (2009). Influence of timing of post-exercise carbohydrate-protein ingestion on selected immune indices. *Int.J.Sport Nutr.Exerc.Metab.,* 19, 366-384.

Costa, R.J.S., Cartner, L., Oliver, S.J., Laing, S.J., Walters, R., Bilzon, J.L.J., & Walsh, N.P. (2008). No effect of a 30-h period of sleep deprivation on leukocyte trafficking, neutrophil degranulation and saliva IgA responses to exercise. *Eur.J.Appl.Physiol.,* 105, 499-504.

Abstracts

Costa, R.J.S., Walters, R., Bilzon, J.L.J., & Walsh, N.P. (2009). Neutrophil degranulation response to carbohydrate feeding with and without protein immediately after prolonged exercise. *ISEI Symposium,* pp. 101.

Harper-Smith, A.D., Costa, R.J.S., Oliver, S.J., Bilzon, J.L.J., & Walsh, N.P. (2008). The effects of fifty-three hours of sleep deprivation with and without energy restriction on thermoregulation during cold stress. *BASES Annual Conference.*

Costa, R.J.S., Cartner, L., Oliver, S.J., Laing, S.J., Bilzon, J.L.J., & Walsh, N.P. (2008). The effects of a 30 hour period of sleep deprivation on selected immune responses to exercise. *ECSS conference proceedings*, pp. 84.

Costa, R.J.S., Blanchfield, A., Oliver, S.J., Laing, S.J., Walters, R., Bilzon, J.L.J., & Walsh, N.P. (2008). Neutrophil degranulation response to carbohydrate and protein feeding after prolonged exercise. *ECSS conference proceedings*, pp. 26.

Costa, R.J.S., Blanchfield, A., Williams, E., Heaney, L., Bywater, L., Oliver, S.J., Laing, S.J., Walters, R., Bilzon, J.L.J., & Walsh, N.P. (2007). Circulating leukocyte and T-lymphocyte subset response to carbohydrate and protein feeding after prolonged exercise. *ISEI Symposium*, pp. 64.

Oliver, S.J., Cartner, L., Laing, S.J., Costa, R.J.S., Bilzon, J.L.J., & Walsh, N.P. (2007). The effect of a 30 hour period of sleep deprivation on pre-loaded treadmill time trial performance. *J.Sport Sci.,* 25, S10-11.

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Thesis Format

The general introduction (**Chapter 1**) and literature review (**Chapter 2**) provides background, rationale and justification for the aims of the research presented in this thesis. A description of the general methods follows (**Chapter 3**), which outlines the common procedures and analyses performed in the subsequent experimental studies. The thesis consists of four independent experimental studies. The first study investigated the effects of one night of total sleep-deprivation on selected immune (circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva IgA) and stress hormone responses at rest and in response to prolonged strenuous exercise (**Chapter 4**). The second study is a follow-on investigation, which studied the effects of two nights of total sleep-deprivation with and without energy-restriction on selected immune and stress hormone responses at rest and in response to cold-exposure (**Chapter 5**). The third study investigated the effects of the timing of post-exercise carbohydrate and protein ingestion on selected immune, stress hormone and insulin responses (**Chapter 6**). The final study is a follow-on investigation, which studied the effects of immediate post-exercise carbohydrate ingestion with and without the addition of protein on selected immune, stress hormone and insulin responses (**Chapter 7**). A general discussion (**Chapter 8**) contains a summary and critical analysis of the main findings of the research programme, highlighting limitations and potential areas for future research. Throughout the thesis, abbreviations are defined at first use, and for clarity a list of abbreviations, tables and figures appear prior to **Chapter 1**. Occasionally, overlap amongst chapters may occur due to existent links between chapters. Bold type is used when reference to sections elsewhere within this thesis is made.

List of Tables

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List of Abbreviations

ANOVA	Analysis of variance
BM	body mass
BL	Baseline
cAMP	cyclic adenosine monophosphate
CAT	cold air test
$CD3^+$	cluster of differentiation 3
$CD4^+$	cluster of differentiation 4
$CD8^+$	cluster of differentiation 8
$CD19^+$	cluster of differentiation 19
CHO	carbohydrates
CHO-PRO	carbohydrate and protein
CHO+PRO	carbohydrate with the addition of protein
CON	control
ConA	concanavalin A
CV	coefficient of variation
°C	degrees centigrade
dB	decibels
DEXA	dual-energy x-ray absorptiometry
DF	delayed feeding
DTHS	delayed-type hypersensitivity
EEG	electroencephalography
E.coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
EOG	electro-oculography
ER	energy-restriction
fg	femtogram
g	gram
g	g-force
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GH	growth hormone
h	hour

HAV	hepititus A virus
HPA	hypothalamic-pituitary adrenal axis
HR	heart rate
HR_{max}	maximal heart rate
IF	immediate feeding
IgA	immunoglobulin A
IFN-γ	interferon gamma
IL-1β	interleukin 1 beta
IL-1ra	interleukin 1 receptor antagonist
IL-4	interleukin 4
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-17	interleukin 17
IU	international units
Kcal	kilocalorie
K_3EDTA	potassium ethylenediaminetetraacetic acid
kg	kilogram
km	kilometre
LPS	lipopolysaccharide
L	litre
m	metre
MEOT	main effect of time
µg	microgram
µIU	micro-international units
µl	microlitre
µm	micrometre
µmol	micromole
µSv	microsievert
mg	milligram
min	minute
ml	millilitre
mmol	millimole
MOD	Ministry of Defense
NBM	nude body mass

NK	natural killer
NKCA	natural killer cytotoxic activity
ng	nanogram
nmol	nanomole
NREMS	non rapid eye movement sleep
PHA	phyotohemagglutinin
pIgR	polymeric immunoglobulin receptor
PRO	protein
PSG	polysomnography
PWM	pokeweed mitogen
REMS	rapid eye movement sleep
RH	relative humidity
RMR	resting metabolic rate
RPE	rating of perceived exhaustion
s	second
SAM	sympatheticoadrenal-medullary axis
SD	standard deviation
SDEP	sleep-deprivation
SEM	standard error of the mean
S-IgA	secretory immunoglobulin A
SS	steady state
TNFα	tumour necrosis factor alpha
T_{core}	core temperature
T_{re}	rectal temperature
tSC	transmembrane secretory component
TT	time trial
URI	upper respiratory tract illnesses
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_{2max}$	maximal oxygen uptake
$\dot{V}O_{2peak}$	peak oxygen uptake
VT	ventilatory threshold
W_{max}	maximal wattage output
w/v	water volume
y	year

CHAPTER ONE

General Introduction

The Ministry of Defence (MOD) employs over 207,000 military service personnel and 84,440 civilian staff. Recruitment is based on the requirement for operational, military and technical support required to "*deliver security and defence in a changing world*" (MOD, 2004). Defence services are provided by three main subgroups, comprising the Royal Navy, Armed Forces and the Royal Airforce. These subgroups, independently and synergistically, aim to monitor national and international affairs, develop security strategies, intervene where threat occurs, and in conjunction with other international organisations provide human resources for humanitarian aid and keeping peace. Similarly, the human body comprises its own MOD in the form of the immune system (*Immunitas*-freedom from). Comprised of a dynamic system of organs (e.g. bone marrow, thymus, lymphatic nodes and spleen), networks (e.g. circulatory and lymphatic systems), circulating cells (e.g. leukocytes) and molecules (e.g. cytokines, antibodies, complement, anti-microbial proteins); the immune system continuously monitors and defines self-components, in order to assess and reject what it considers to be foreign pathogens. Thus, maintaining a protective, supportive and peaceful relationship between the organism and its changing environment.

Regulation of the direction and degree of normal immune responses is predominantly dependent on pathogen presence, tissue damage, neuroendocrine control and/or circadian variations (Shephard *et al.*, 1998; Peake, 2002). Possibly, one of the key regulators of immune activity is neuroendocrine control. Stimulation of the Hypothalamic-Pituitary Adrenal (HPA) and Sympatheticoadrenal-Medullary (SAM) axes modulates immune

responses due to neurotransmitter, neuropeptide and neurohormonal receptor sites on lymphoid tissues and immune cells (Weigent & Blalock, 1987; McCarthy & Dale, 1988; Benschop *et al.*, 1996; Sternberg, 2006). Several reviews have previously discussed the influence of neuroendocrine stimulus induced by various physiological stressors including: prolonged strenuous exercise, sleep-deprivation, nutrient deprivation and exposure to environmental extremes, on host defences (Dinges *et al.*, 1995; Nieman, 1997; Shephard *et al.*, 1998; Walsh & Whitham, 2006; Gleeson, 2006). These reviews report either a depressed or enhanced immune status, which appears to be dependent on the severity and duration of the stress stimulus. It is however more frequently reported that these physiological stressors have the potential to induce suboptimal host defences driven by neuroendocrine mechanisms (Cupps & Fauci, 1982; Benschop *et al.*, 1996; Felten *et al.*, 1998; Madden, 2003).

Prolonged strenuous exercise, sleep-deprivation, exposure to environmental extremes and nutrient deprivation have all individually been reported to disturb immune homeostasis (Nieman, 1994; Dinges *et al.*, 1995; Shephard *et al.*, 1998; Walsh & Whitham, 2006). It is therefore plausible that participating in activities that are associated with a combination of stressors (e.g. sustained military training and routine operations, travelling across time zones for sports competitions, ultra-endurance events, adventure and exploration activities) may amplify immune modulating neuroendocrine responses and induce greater perturbations to host defences. Such outcomes may have clinical significance in increasing the risk of illness, infection, dampening effective wound healing and increasing susceptibility to sepsis following injury in the field; especially in unfamiliar foreign locations that may extensively be occupied by unfamiliar pathogens (Martinez-Lopez *et al.*, 1993; Shephard *et al.*, 1998).

Military-based field studies have reported depressed cellular and humoral mediated adaptive immunity, depressed oral-respiratory mucosal immunity, increases in pro-inflammatory cytokines and increases in infection rates leading to course failure whilst adhering to multi-stressor combat courses (Martinez-Lopez *et al.*, 1993; Boyum *et al.*, 1996; Gomez-Merino *et al.*, 2003; Gomez-Merino *et al.*, 2005; Tiollier *et al.*, 2005; Gundersen *et al.*, 2006). It is however difficult to determine which individual or combination of stressors is responsible for these immune perturbations, primarily due to limited research control. Additionally, carbohydrate intake during exercise has been reported to attenuate some of the immune perturbation associated with exercise stress (Nieman *et al.*, 1997; Gleeson *et al.*, 1998; Bishop *et al.*, 2002), whilst protein may also provide a favourable effect (Castell *et al.*, 1996; Bassit *et al.*, 2000; Bassit *et al.*, 2002).

Taking this into account, participating in activities associated with multiple physiological stressors may superimpose neuroendocrine responses inducing amplified or accumulative perturbations to host defences, possibly increasing the risk of unwanted health outcomes during this period of perturbed host defences (Martinez-Lopez *et al.*, 1993; Shephard *et al.*, 1998). The focus of this thesis was to assess selected immune responses (circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva IgA responses) to independent and combined physiological stress (prolonged strenuous exercise, total sleep-deprivation, energy-restriction, and cold-exposure), reported to be associated with perturbations in immune status, and identify whether nutritional strategies may prevent unwanted perturbation to host defences.

CHAPTER TWO

Literature Review

Sustained military training and routine operations, adventure and exploration activities, ultra-endurance competitions, and travelling across time zones for sports competitions are commonly associated with a variety of immune modulating physiological stress (e.g. prolonged strenuous exercise, sleep-deprivation, nutrient deprivation, exposure to environmental extremes). Accompanying perturbations to host defence may have significant clinical implications (Martinez-Lopez *et al.*, 1993; Shephard *et al.*, 1998), especially when activities are performed in unknown environments, and/or extensive contact with the surrounding population exists after stress stimuli, whereby an increased number of unrecognisable foreign opportunistic pathogens are present.

Substantial published evidence suggests that physiological stress may play a key role in causing impaired host defenses reported after adherence to the aforementioned activities. The following literature review will look at the current evidence behind the influence of individual and combined impact of physiological stress on immune responses, and the role which nutrition may play in modifying these immune responses. In **Section 2.1** the principal immune induces (circulating leukocyte trafficking, neutrophil degranulation and saliva IgA responses) measured within the experimental chapters will be reviewed. In addition, the role of stress hormones (catecholamines and cortisol) on immune modulation will also be reviewed. **Section 2.2** will follow and review the impact of multi-stressor field protocols on circulatory leukocyte trafficking, functional immune responses, oral-respiratory mucosal immunity and infection incidence. **Sections 2.3**, **2.4** and **2.5** will proceed and review the influence of prolonged strenuous exercise, sleep-deprivation and

cold-exposure, respectively, on circulatory leukocyte trafficking, functional immune responses and oral-respiratory mucosal immunity. Finally, **Section 2.6** will review the influence of energy-restriction on circulatory leukocyte trafficking, functional immune responses and oral-respiratory mucosal immunity, and additionally review the role of nutrients in attenuating stress induced immune perturbations.

2.1 Selected Immune Responses

2.1.1 Circulating Leukocyte Trafficking

Circulating leukocytes encompass granulocytes (50 - 80%), lymphocytes (25 - 50%), and monocytes (2 - 10%; Albers *et al.*, 2005). More than 90% of circulating granulocytes are phagocytic polymorphonuclear neutrophils (normal range 2 - 8 $x10^9 \cdot L^{-1}$). Circulating lymphocytes in lesser numbers (normal range 1 - 4 $x10^9 \cdot L^{-1}$) are characterised by their subsets including, thymus originated $CD3^+$ T-lymphocyte cells (comprised of $CD4^+$ T-lymphocyte helper cells and $CD8^+$ T-lymphocyte cytotoxic/suppressor cells) that focus on cellular mediated adaptive immunity, and bone marrow originated $CD19^+$ B-lymphocyte cells (comprising plasma and memory cells) that focus on humoral mediated adaptive immunity. Natural Killer (NK) cells are also classed as lymphocytes and make up 10-15% of circulating lymphocytes. Functioning as part of the innate immune system, NK cells direct their activities to cell disintegration or lysis of virus-infected cells (natural killer cytotoxic activity, NKCA).

Research into the effects of stress stimulus on the immune system is frequently conducted on leukocyte counts obtained from systemic circulation. Even though this parameter gives a good indication of general immune status (e.g. stress-induced perturbation to the circulatory leukocyte pool), it is only physiologically relevant by itself in extreme cases where clinically significant low levels are observed (e.g. disease-related malnutrition; Albers *et al.*, 2005). Minor alteration in circulating leukocytes should be carefully interpreted since it may only indicate leukocyte redistribution. For example, exercise and thermal stress have been reported to induce a circulating lymphopenia hypothesised as a redistribution of T-lymphocyte cells within lymphoid and non-lymphoid tissue, possibly to enhance immune surveillance and response, and not necessarily a negative immune

perturbation (Kruger & Mooren, 2007). In addition, circulating neutrophils and monocytes also enter into tissues when infection or tissue damage is present, further contributing to altered circulating leukocyte counts (Pyne, 1994; Albers *et al.*, 2005). Nevertheless, determining immune cell populations and trafficking is useful, since they provide essential background data for the interpretation of functional parameters. As an example, total plasma elastase concentration reflects the extent of neutrophil degranulation as a whole, which is dependent on circulating neutrophil counts. Whereas, elastase release per neutrophil gives a more specific indication of neutrophil degranulation status at a *per cell* basis (Robson *et al.*, 1999; Laing *et al.*, 2008a; Laing *et al.*, 2008b).

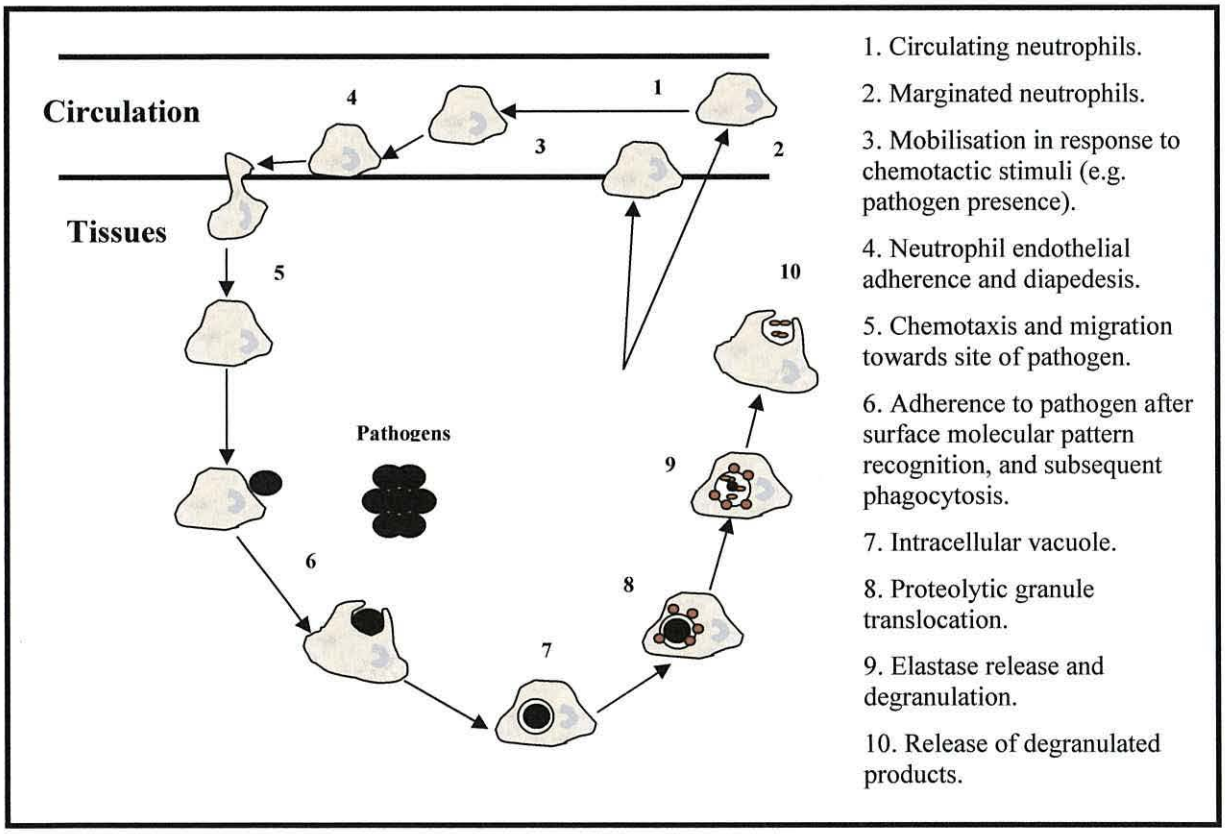
2.1.2 Neutrophil Function

Originating and maturing in the bone marrow, neutrophils are considered the predominant first line defensive phagocyte (Pyne, 1994). After maturation, they are released and remain in circulation for 4 to 10 h prior to marginating into tissues, where they reside with a half-life of 24 to 48 h (Smith, 1994). In circulation, neutrophils are attracted and migrate towards areas of infection and inflammation within tissues (chemotaxis), where they combine defence strategies with adjacent phagocytes in response to chemotactic signals (e.g. damage tissue fragments, molecular patterns on pathogen surfaces, cytokine responses), further amplifying innate immune responses (Smith, 1994; Smith & Pyne, 1997).

After chemotaxis into areas of pathogen presence, neutrophils induce destruction by multiple toxic agents (e.g. proteases, reactive oxygen species, and proteins that interfere with bacterial development; Borregaard & Cowland, 1997). One particular destructive mechanism, phagocytosis, allows neutrophils to engulf and ingest pathogens through

cytoplasmic extension (**Figure 2.1**). Ingested pathogens are held within intracellular vacuoles in the neutrophil cytoplasm. Intracellular granules fuse with the ingested vacuole and release degranulating elastase enzyme which proteolyses the ingested pathogen. At the same time an oxidative burst is initiated that generates highly reactive oxygen species, which aids in the killing and breakdown of ingested pathogens (Pyne, 1994; Peake, 2002).

Figure 2.1: Illustrative description of neutrophil phagocytosis and degranulation activity.



Created by thesis author, based on Pyne (1994), and Peake (2002).

The ability of neutrophils to engulf and digest foreign pathogens (bacterially-stimulated neutrophil degranulation) has been previously used to assess neutrophil function *in vitro* (Dufaux & Order, 1989; Gray & Haseman, 1994). Studies have reported increases in bacterially-stimulated plasma elastase concentration after stress stimulus (e.g. prolonged strenuous exercise; Robson *et al.*, 1999; Bishop *et al.*, 2003). These increases are

noticeably associated with the acute neutrophilia induced by the stress stimuli and not necessarily the increase in neutrophil cell function (Robson *et al.*, 1999; Laing *et al.*, 2008a). Previous stress research protocols have reported neutrophil function as bacterially-stimulated elastase release per neutrophil, which represents a more appropriate measure of assessing changes in neutrophil function, and has been shown to decrease after stress stimuli (Robson *et al.*, 1999; Bishop *et al.*, 2002; Li & Cheng, 2007; Laing *et al.*, 2008a). In regard to clinical relevance, depressed neutrophil phagocytic function, as measured *in vitro*, has been associated with increased risk of infection; albeit, this has been observed in clinical patients (Ellis *et al.*, 1988; Marhoffer *et al.*, 1992; Bessman & Sapico, 1992; Smitherman & Peacock, 1995; Alba-Loureiro *et al.*, 2007). It is therefore plausible that depressed neutrophil function may contribute to increased risk of illness and infection, and dampened effective tissue repair and wound healing amongst individuals that take part in stress inducing activities (Peake, 2002).

2.1.3 Oral-Respiratory Mucosal Immunity

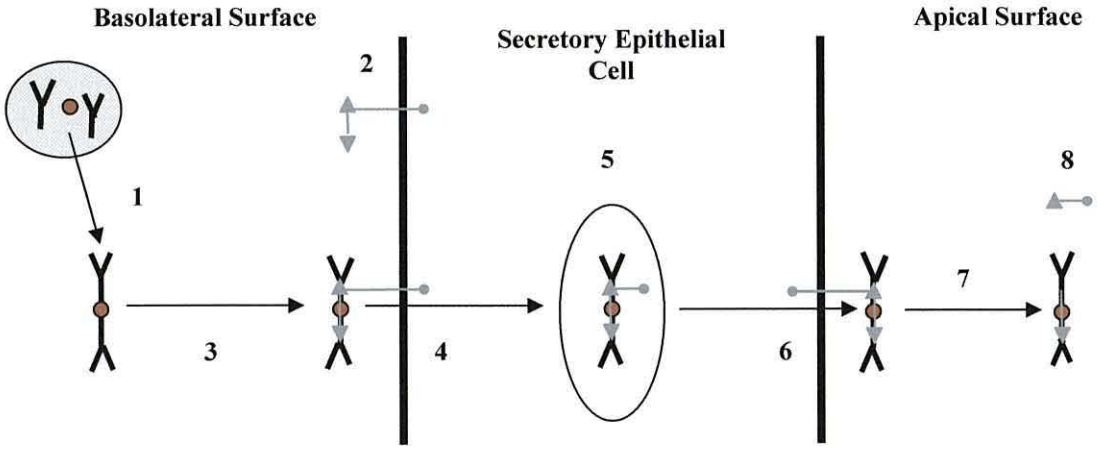
Saliva flow and mucosal secretions form a fundamental first line defence against invading foreign pathogens present in the oral-respiratory pathway. Taking into account that 95% of all oral-respiratory infections are initiated at the mucosal surface, the importance of mucosal immunity cannot be underestimated, especially during periods of induced stress, when oral-respiratory mucosal immunity may be altered (Gleeson & Pyne, 2000; Bosch *et al.*, 2003a; Bosch *et al.*, 2003b). Saliva flow provides a mechanical washing effect to protect the oral-respiratory mucosal surface, whilst mucosal secretions contain antibacterial and antiviral components (e.g. immunoglobulins, mucins, amylase, lactoferrin, lysozyme) that inhibit bacterial attachment to mucosal epithelium, neutralise viruses and prevent their replication (McNabb & Tomasi, 1981; Tenovuo, 1998; Dowd,

1999; West *et al.*, 2006). The importance of saliva flow is recognised in individuals suffering from xerostomia (a condition associated with low saliva flow), who present high incidence of oral and upper respiratory tract illnesses (URI; e.g. sore throat, oropharyngitis; Fox *et al.*, 1985; Fox, 2004).

IgA is the prevailing immunoglobulin in the oral-respiratory mucosal immune pathway. It is predominantly in the form of a polymeric S-IgA, comprising of an IgA dimer joined by a J chain, and acquires a polymeric Ig receptor (pIgR; more commonly known as transmembrane secretory component (tSC)) prior to translocation. The acquisition of the tSC occurs as the J-chain dimer passes the secretory epithelium (translocation) from the plasma cells residing in submucosa lymphoid and non-lymphoid tissues, to the oral-respiratory mucosal surface (Woof & Mestecky, 2005; **Figure 2.2**). S-IgA production by plasma cells requires activation of B-lymphocyte cells directly by antigens or indirectly by antigen presentation and/or cytokines (e.g. IFN-γ, TNF-α, IL-1β, IL-4; Glesson, 2000a; Kaetzel, 2005). The J-chain incorporated into S-IgA increases the efficacy of antigen-binding sites, making S-IgA suitable for agglutinating bacteria and viruses. It presents minimal complement-activating potential, allowing non-inflammatory protective activity, and most importantly, is involved in creating the binding site for tSC and promoting dimer IgA affinity with tSC, facilitating and optimising translocation (Johansen *et al.*, 2000). Moreover, tSC protects S-IgA from proteolysis after secretion in the oral-respiratory mucosal environment (Woof & Mestecky, 2005). Due to the processes involved in S-IgA synthesis and translocation, both the release of IgA from plasma cells and the availability of tSC for translocation are potential rate limiting factors in saliva secretion of S-IgA (Johansen & Brandzaeg, 2004). For example, substantially lower levels of mucosal S-IgA have been observed in mice with tSC deficiency (Shimada *et al.*, 1999).

S-IgA accounts for 60-80% of immunoglobulins in saliva and >90% of immunoglobulins in nasal-associated lymphoid tissues (Gleeson & Pyne, 2000). S-IgA exerts its protective action by excluding and clearing pathogens through immune complexes at the mucosal surface, and neutralisation of intra-epithelial viruses, preventing viral replication (Mazanec *et al.*, 1993; Brandtzaeg *et al.*, 1999; Norderhaug *et al.*, 1999). In regards to clinical significance, individuals with selective IgA deficiency have been reported to suffer high incidences of URI, whilst low levels of saliva IgA observed in healthy populations, and in athletic populations in response to strenuous exercise, are also related to increased prevalence of URI (Hanson *et al.*, 1983; Jemmott & McClelland, 1989; Gleeson & Pyne, 2000).

Figure 2.2: Illustrative description of saliva S-IgA synthesis and translocation.



1. Plasma cell synthesis of IgA dimer and addition of J-chain.
2. Basolateral epithelial surface synthesis of transmembrane secretory component- tSC (also known as polymeric immunoglobulin receptor- pIgR).
3. J-chain positive dimeric IgA binds to tSC.
4. Endocytosis of polymeric S-IgA into the secretory epithelial cell.
5. Transcytosis of polymeric S-IgA across the secretory epithelial cell *via* endocytic vacuole.
6. Transport of the polymeric S-IgA across the apical surface.
7. Cleavage of the tSC releases S-IgA onto the mucosal surface.
8. Free tSC and tSC bound to active S-IgA on the mucosal surface promotes host defenses.

Created by thesis author, based on Brandtzaeg *et al.* (1999), Norderhaug *et al.* (1999), and Johansen & Brandtzaeg (2004).

2.1.4 Stress Hormones and Immune Responses

Acute periods of physiological stress are associated with increases in plasma stress hormone release, namely cortisol and catecholamines (Opstad *et al.*, 1980; Opstad & Aakvaag, 1981; Ronsen *et al.*, 2001; Majde & Krueger, 2005; Walsh & Whitham, 2006). Both cortisol and catecholamines (e.g. adrenaline and noradrenaline) have the potential to directly and indirectly (through cytokine interaction) modulate circulatory leukocyte trafficking and functional immune responses (**Figure 2.3**; McCarthy & Dale, 1988; Hoffman-Goetz & Pedersen, 1994; Sternberg, 2006). This proposal is emphasized by the presence of glucocorticoid and adrenergic receptors on leukocytes and lymphoid tissues (Cupps & Fauci, 1982; Benschop *et al.*, 1996; Felten *et al.*, 1998; Madden, 2003). In addition, stress hormones have previously been labelled as immunosuppressive, affecting circulatory leukocyte maturation, neutrophil function and oral-respiratory mucosal immunity (Khansari *et al.*, 1990; Pederson *et al.*, 1997; Nieman, 1997; Gleeson & Pyne, 2000).

Glucocorticoid administration *in vivo* has been reported to suppress maturation, differentiation and proliferation of leukocytes, decrease neutrophil phagocytosis, inhibit the expression of cell-adhesion molecules involved in leukocyte trafficking (e.g. selectin), and inhibit the production and secretion of chemokines, for example IL-8, a predominant chemokine required for neutrophil recruitment and activation (Forslid & Hed, 1982; Cronstein *et al.*, 1992; Scheinman *et al.*, 1995; Williams *et al.*, 1999; Sacedon *et al.*, 1999; Porreca *et al.*, 1999; Sternberg, 2006).

Similarly, *in vivo* administration of adrenaline (5 $\mu l \cdot L^{-1}$ sodium chloride solution), but not noradrenaline (10 $\mu l \cdot L^{-1}$ sodium chloride solution), has been shown to decrease circulating

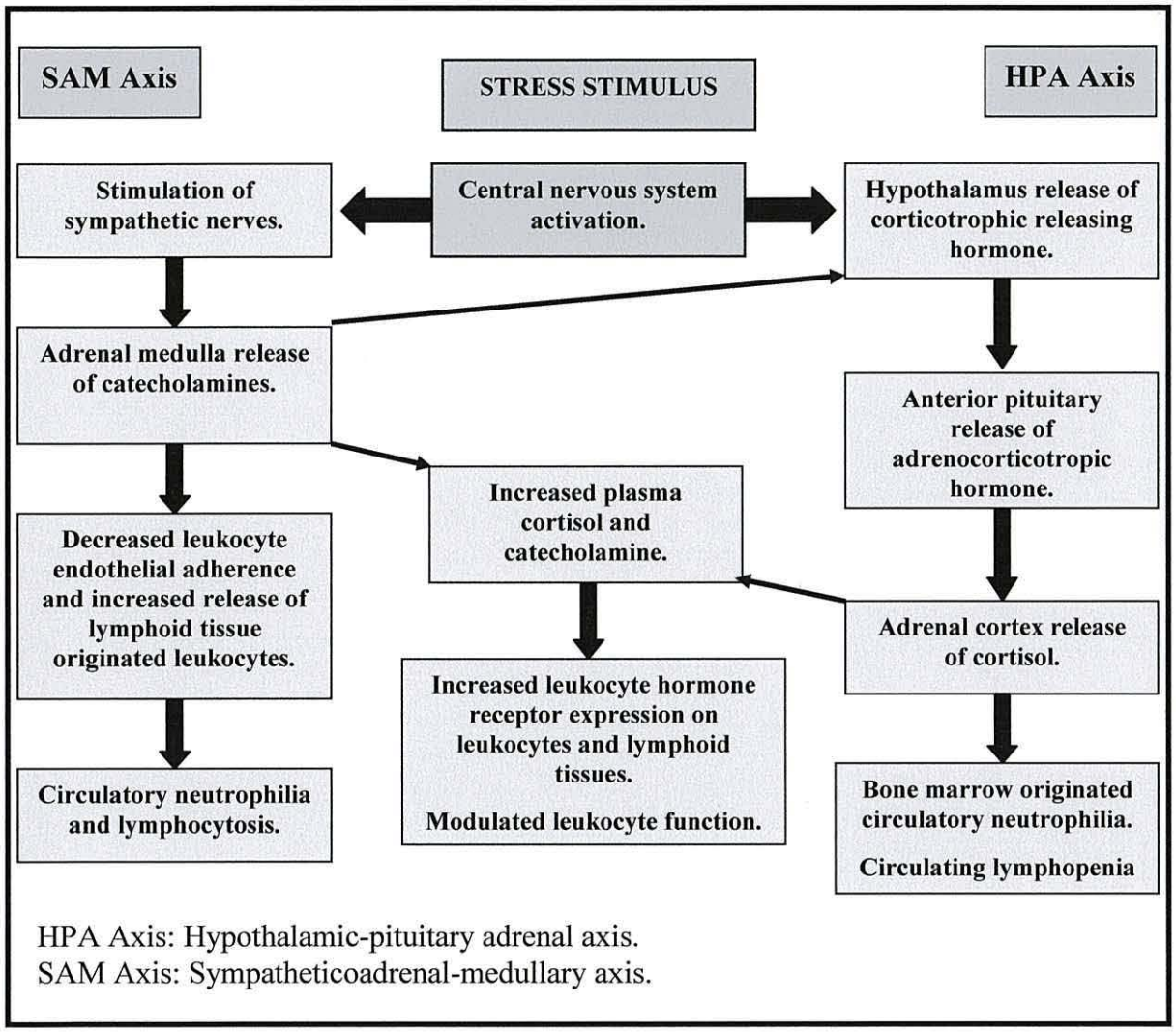
lymphocyte subsets through β_2 adrenergic receptor activation on lymphocytes (Schedlowski *et al.*, 1993), possibly inducing lymphocyte redistribution into lymphoid and non-lymphoid tissues (Kruger & Mooren, 2007). On the other hand, increases in noradrenaline observed without substantial increases in adrenaline or cortisol after cold exposure, were accompanied by reductions in lymphocyte proliferation response to phytohemagglutinin (PHA; Jurankova *et al.*, 1995). Moreover, administration of a 12 $\mu l \cdot L^{-1}$ adrenaline solution and a 120 $\mu l \cdot L^{-1}$ noradrenaline solution *in vivo* led to a 72% decrease in neutrophil phagocytosis (measured by neutrophil uptake of fluorescein isothiocyanate-labelled bacteria; Wenisch *et al.*, 1996b). The addition of 1 μmol of adrenaline to human neutrophils *in vitro* resulted in a reduced elastase release from N-formyl-methionyl-leucyl-phenylalanine activated neutrophils (Tintinger *et al.*, 2001). The addition of β adrenergic receptor antagonist propranolol prevented the decrease in elastase release. Investigators concluded that the depressive effects of adrenaline on activated neutrophil elastase release was due to down-regulation of neutrophils by increased levels of cAMP (Tintinger *et al.*, 2001). It therefore appears that stress hormones may play a role in modulating leukocyte trafficking and function.

In regards to oral-respiratory mucosal immunity, salivary glands are innervated by both parasympathetic and sympathetic nerve endings. Thus, alterations in catecholamine status have the potential to alter saliva flow and saliva protein secretions (Chicharro *et al.*, 1998; Bosch *et al.*, 2003; Teeuw *et al.*, 2004). For example, reductions in parasympathetic activation and subsequent increased sympathetic activation is reported to reduce saliva flow rate (Carpenter *et al.*, 1998; Tenovuo, 1998; Teeuw *et al.*, 2004). Additionally, glucocorticoid (e.g. dexamethasone) administration *in vivo* has been reported to suppress B-lymphocyte cells maturation, differentiation and proliferation, and inhibit the

transepithelial transport of IgA; subsequently reducing saliva IgA concentration in rodents (Wira *et al.*, 1990; Sabbadini & Berczi, 1995). Such findings may possibly account for the suppressed saliva IgA responses observed after stress stimuli (Saxon *et al.*, 1978; Forslid & Hed, 1982; Gleeson & Pyne, 2000).

The effects of physiological stress and subsequent increases in stress hormones have previously been linked with depressions in host defenses during the post-stress period (Pedersen *et al.*, 1997; Shephard *et al.*, 1998; Walsh & Whitham, 2006). For example, previous studies have reported that prolonged strenuous exercise (e.g. ≥1 h at ≥70% $\dot{V}O_{2max}$) induces alterations in leukocyte trafficking (lymphocytosis and neutrophilia immediately post-exercise, followed by a lymphopenia and bone marrow originated neutrophilia during recovery), decreases lymphocyte proliferation and phagocytic function, and decreases saliva IgA responses during the recovery period (Pederson *et al.*, 1997; Nieman, 1997; Robson *et al.*, 1999; Gleeson & Pyne, 2000). Researchers indicate that these immune perturbations are likely to be attributed to the increased stress hormone responses observed (**Figure 2.3**).

Figure 2.3: The influence of stress hormones cortisol and catecholamines (e.g. adrenaline and noradrenaline) on immune responses.



Created by thesis author, adapted from McCarthy & Dale (1988), Nieman (1994), and Pedersen *et al.* (1997).

2.2 Multi-Stressor Field Studies and Selected Immune Responses

Illness incidences are reportedly one of the primary causes of course failure amongst army ranger cadets during a sixty-two day military training course (Martinez-Lopez *et al.*, 1993). Taking this into account, field studies that have observed the effects of sustained military training courses, which included continuous loaded exercise, intermittent sleep-deprivation (1 to 3 h sleep·day^{-1}) and energy-restriction (>80% restriction of daily energy requirements), have reported immune disturbances featuring altered cellular and humoral mediated adaptive immunity, depressed oral-respiratory mucosal immunity and increases in infection rates (Boyum *et al.*, 1996; Gomez-Merino *et al.*, 2005; Gundersen *et al.*, 2006; Martinez-Lopez *et al.*, 1993; Tiollier *et al.*, 2005).

2.2.1 Multi-Stressor Field Studies, Circulating Leukocyte Trafficking and Functional Immune Responses

Five to seven days of continuous military combat course (with and without 3 weeks of physical fitness conditioning prior to the combat course), which included energy-restriction and intermittent sleep-deprivation was associated with a circulating leukocytosis, neutrophilia, and lymphopenia with accompanying reductions in T and B-lymphocyte counts (Boyum *et al.*, 1996; Gomez-Merino *et al.*, 2003; Gomez-Merino *et al.*, 2005; Gundersen *et al.*, 2006). In addition, after the combat course, an enhanced neutrophil chemotaxis, granulocyte-macrophage colony stimulating factor (GM-CSF; after 24 h), and lymphocyte blastogenesis response to lipopolysaccharide (LPS), but not to PHA and concanavalin-A (Con-A) was observed (Boyum *et al.*, 1996; Gundersen *et al.*, 2006). Even though the effects of the aforementioned stress protocols may appear to enhance phagocytic migration, the effects of multi-stressor protocols on specific phagocyte function (e.g. neutrophil degranulation) has not been determined.

Moreover, three weeks of physical fitness conditioning (repetitive bouts of exercise on consecutive days) prior to multi-stressor combat courses are reported not to alter circulating leukocyte trafficking or function (Gomez-Merino *et al.*, 2003; Gomez-Merino *et al.*, 2005; Tiollier *et al.*, 2005). However, chronic exercise training prior to a multi-stressor combat course may set the scene for amplified or accumulative immune perturbations when compared with initiating a multi-stressor combat course from rest. For example, even though immune perturbations were evident, Boyum *et al.* (2006) reported no increased incidence of infection during or up to five weeks following five to seven days multi-stressor military training course initiated from rest. Whereas, increased incidences of URI were reported during and after three weeks of physical fitness conditioning followed by five days multi-stressor combat course (Gomez-Merino *et al.*, 2005; Tiollier *et al.*, 2005), and a 35% reduced *in vitro* lymphocyte response to bacterial challenge has been reported after six weeks of basic cadet training (Lee *et al.*, 1992).

2.2.2 Multi-Stressor Field Studies and Saliva IgA Responses

Increased incidences of URI have been reported during sustained military operations in the Canadian Arctic winter (St Rose *et al.*, 1972; Sabiston & Livingstone, 1973). Previous studies have also observed decreases in saliva IgA concentration and increased incidences of URI after three weeks of physical fitness conditioning followed by five days multi-stressor combat course, which also included exposure to differing environmental conditions (4 to 25°C and 34 to 87% relative humidity (RH); Gomez-Merino *et al.*, 2003; Tiollier *et al.*, 2005). Furthermore, Tioller *et al.* (2005) reported a substantial increase in saliva cortisol after three weeks of physical fitness conditioning (16 $nmol \cdot L^{-1}$ pre *vs.* 31 $nmol \cdot L^{-1}$ post), which remained elevated for one week after cessation of five days multi-stressor combat course (23 $nmol \cdot L^{-1}$). Similarly, Gundersen *et al.* (2006) reported a substantial increase in

plasma cortisol on days two and four of a continuous multi-stressor ranger course. Interestingly, plasma cortisol levels were not significantly elevated on day seven, possibly due to substantial increases in plasma volumes observed, which were not corrected prior to analysis (Dill & Costill, 1974). Conversely, Gomez-Merino *et al.* (2003; 2005) reported increased plasma noradrenaline and decreased plasma cortisol following three weeks of physical fitness conditioning followed by five days multi-stressor combat course. The investigators attributed the depressed saliva S-IgA concentration to increased sympatheticoadrenergic activation, which possibly down-regulated S-IgA translocation (Woof & Mestecky, 2005).

The discrepancies in immune outcomes observed in multi-stressor field studies are possibly due to different protocols, populations (fitness status) and population numbers used. These factors have the potential to expose participants to differing degrees and durations of individual and combined physiological stress between and within studies. Such studies are unreliable in concluding the effects of individual and combined physiological stress on immune responses, mainly due to lack of adequate research control. A potential limitation within these studies, is the difficulty in determining which individual or combination of stressors are primarily responsible for the alterations in host defences and incidences of infection observed, whether an amplification or accumulative perturbing process is at play with combining stressors, and/or whether a particular physiological stressor counterbalances another to induce a neutral or immune enhancing outcome.

2.3 Prolonged Exercise and Selected Immune Response

The link between exercise and possible increased risk of infection was originally observed by Peters & Bateman (1983), who reported greater incidence of URI (33%; n= 141) two weeks after a 56 km running competition compared to live-in rest controls (15%; n= 124). These observations were supported by further studies that reported higher recalls of URI following endurance running events (Peters, 1990; Nieman *et at.*, 1990), whilst increased self-reported incidence of URI (n= 530) was found to correlate with exercise training load in runners (Heath *et al.*, 1991; Linde *et al.*, 1987). However, care is needed in interpreting these earlier subjective studies, since they are primarily based on participant recall or self-reported symptoms, rather than clinical verification (Pedersen & Nieman, 1988; Ekblom *et al.*, 2006).

From these observations, a "*J-shaped model*" for the relationship between infection (predominantly URTI) and exercise load was proposed by Nieman (1994), suggesting individuals who undertook large exercise volumes are at high risk of illness and infection (Nieman, 1998a). The J-shaped model is supported by substantial amount of evidence linking exercise-induced immune perturbations with possible predisposition to increased illness and infection susceptibility in active individuals (Nieman, 1994; Pederson & Bruunsgaard, 1995). Subsequently, the "*open-window*" hypothesis was proposed to explain this association (**Figure 2.4**). It suggests that viruses and bacteria may overcome host defences during the post-exercise recovery period, whereby exercise-induced neuroendocrine stimulated immune perturbations may weaken host defences (Pedersen *et al.*, 1994). Exercise-induced perturbation to circulating leukocyte trafficking, neutrophil degranulation and saliva IgA responses will be reviewed.

2.3.1 Prolonged Exercise and Circulating Leukocyte Trafficking

Prolonged strenuous exercise is associated with a biphasic circulating leukocytosis (**Figure 2.4**; McCarthy & Dale, 1988). The degree of post-exercise circulating leukocytosis is primarily dependant on both intensity and duration of the exercise load, whereby prolonged submaximal exercise has been shown to produce a larger circulating leukocytosis magnitude than short maximal intensity exercise (Robson *et al.*, 1999). The biphasic exercise-induced circulating leukocytosis is initiated by an acute phase lymphocytosis and neutrophilia predominantly due to cell demargination induced by the physiological properties of exercise stress. The lymphocytosis involves the demargination of all lymphocyte subsets, but predominantly T-lymphocyte cytotoxic/suppressor $CD8^+$, whilst the immediate neutrophilia is predominantly demargination of endothelium-adhered segmented mature neutrophils (McCarthy & Dale, 1988; Shek *et al.*, 1995; Peake, 2002).

The mechanisms responsible for the acute differential leukocyte demargination includes: exercise-induced increased cardiac output, amplified mechanical stress associated with enhanced blood flow redistribution on vascular walls, increased lymphatic flow, and increases in catecholamines (Foster *et al.*, 1986; Shephard, 2003). The release of marginated leukocytes from vascular endothelium appears to be correlated with heart rate (Foster *et al.*, 1986). In addition, haemodynamic and mechanical stress of increased blood flow through capillary structures of tissues containing marginated leukocytes (e.g. lungs, skeletal muscles) will also promote demargination of leukocytes (Foster *et al.*, 1986; Gannon *et al.*, 2001). Exercise-induced increases in lymphatic flow may also contribute to the acute phase exercise-induced leukocytosis through increasing lymphatic release of lymphocytes into circulation (McCarthy & Dale, 1988).

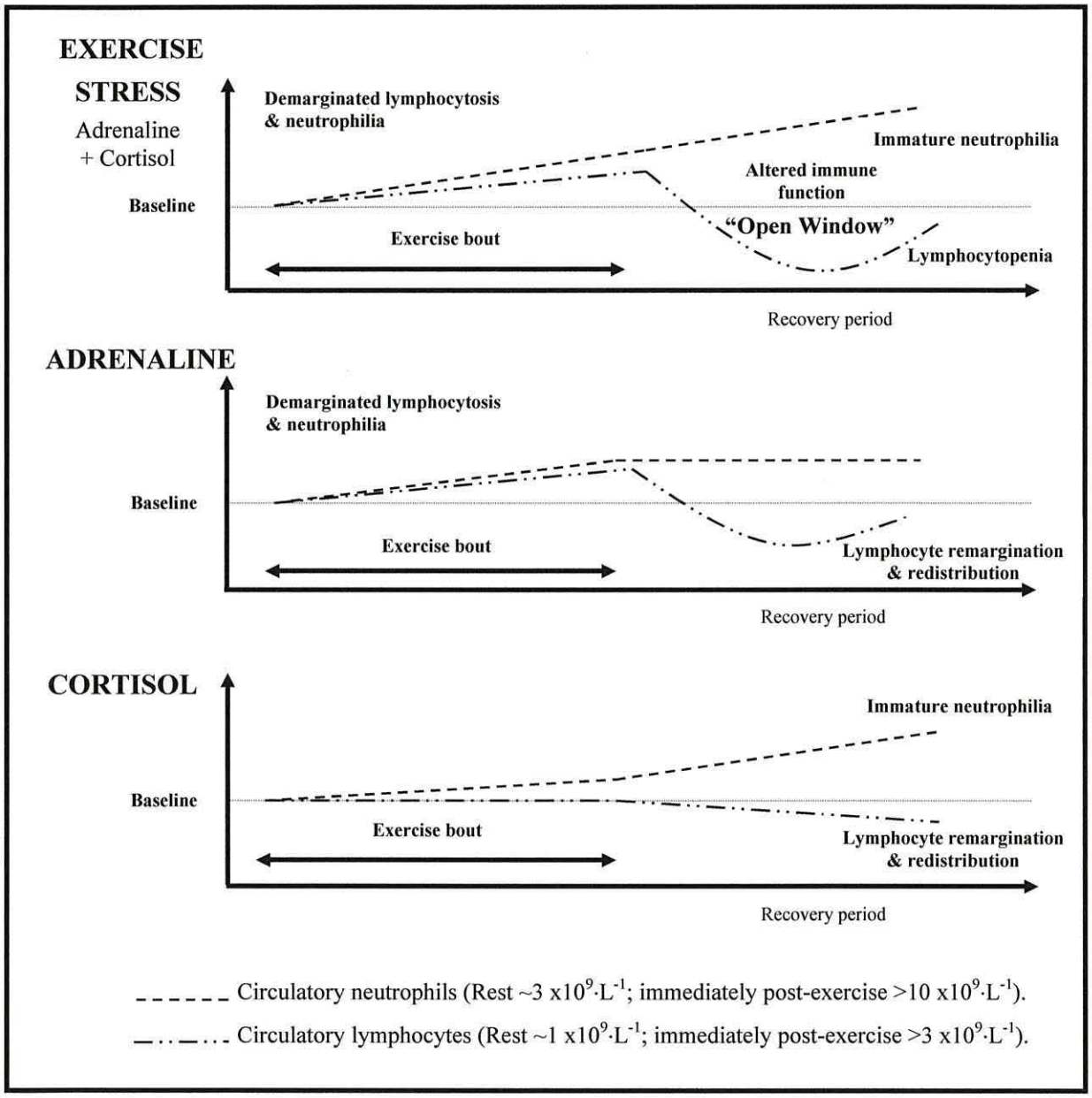
Increases in adrenaline associated with exercise stress also contributes to the post-exercise circulating leukocytosis (**Figure 2.4**; Field *et al.*, 1991; Tvede *et al.*, 1994). Adrenaline infusion has been shown to induce circulating leukocytosis and lymphocytosis to a similar degree as 1 h running exercise at 75% $\dot{V}O_{2max}$ (Tvede *et al.*, 1994; Iversen *et al.*, 1994; Shephard, 2003). Adrenaline promotes increased heart rate and skeletal muscle blood flow through β_1 and β_2 adrenergic receptor activation, respectively, thus enhancing leukocyte demargination (McCarthy & Dale, 1988). Additionally, adrenaline can further promote demargination by reducing adherence of leukocytes to vascular endothelium, by down-regulating adherence molecule expression on the surface of leukocytes and endothelial cells (Kurokawa *et al.*, 1995; Shephard, 2003). For example, adrenaline infusion and activation of β adrenergic receptors have been reported to reduce neutrophil endothelium adherence, mediated by increased levels of cAMP. The addition of β adrenergic receptor antagonist propranolol, blocks demargination by attenuating the activity of cAMP (Boxer *et al.*, 1980).

The delayed exercise-induced circulating leukocytosis, commonly seen during recovery from prolonged strenuous exercise, is predominantly due to cortisol-inducing neutrophil release from bone marrow, which is reported to last for up to 24 h after the cessation of prolonged strenuous exercise (**Figure 2.4**; Gabriel *et al.*, 1992; McCarthy *et al.*, 1992; Robson *et al.*, 1999). It appears that the duration of exercise, and subsequent degree of plasma cortisol increase, is an influential factor determining this response (Hansen *et al.*, 1991; McCarthy *et al.*, 1992; Robson *et al.*, 1999). Cortisol infusion produces a delayed neutrophilia (Fehr & Grossmann, 1979), associated with the circulatory release of immature band neutrophils (Toft *et al.*, 1994; Nakagawa *et al.*, 1998; Suzuki *et al.*, 1999), presenting lower functional capacity (degranulation and respiratory burst) to *in vitro*

bacterial stimulation, compared with fully matured neutrophils (Hetherington & Quie, 1985; Berkow & Dodson, 1986; Li & Gleeson, 2005b). Hetherington & Quie (1985) reported that infusion of adrenaline caused an increase in endothelium derived mature circulatory neutrophils, whereas hydrocortisone infusion stimulated the release of immature bone-marrow originated neutrophils. Peake (2002) therefore argued that exercise stress may potentially alter the proportion of functionally responsive neutrophils into circulation, possibly weakening host defences.

Simultaneously, cortisol also contributes to a post-exercise lymphopenia (**Figures 2.3** and **2.4**), which is commonly observed to start 30 to 60 min after cessation of prolonged strenuous exercise, and may last for up to 5 h post-exercise (Gabriel *et al.*, 1992). After prolonged strenuous exercise there is a remargination and redistribution of lymphocytes into lymphoid and non-lymphoid tissues, possibly due to the combination of post-exercise increases in cortisol, decreases in adrenaline, and increased trafficking into vascular compartments (McCarthy & Dale, 1988; Toft *et al.*, 1994; Kruger *et al.*, 2008). Glucocorticoid administration has been shown to induce combined circulatory neutrophilia and lymphopenia through inhibiting lymphocyte entry into circulation and promoting lymphocyte migration into tissues, both reaching maximum levels at 4 to 6 h after administration (Cupps & Fauci, 1982; Gabriel *et al.*, 1992; Calvano *et al.*, 1992). For example, 45 min treadmill running at 80% $\dot{V}O_{2max}$ increased circulating lymphocyte counts immediately post-exercise, subsequently 1 h into recovery, circulatory lymphocyte counts declined 45% below pre-exercise values and remained substantially lower for 3 h 30 min, predominantly due to declines in T-lymphocyte cytotoxic/suppressor $CD8^+$ (Nieman *et al.*, 1994).

Figure 2.4: Proposed neuroendocrine mechanisms to explain exercise-induced immune perturbations to circulating leukocyte trafficking.



Created by thesis author, adapted from McCathy & Dale (1988), Pedersen *et al.* (1997), Pedersen & Nieman (1998), and Peake (2002).

2.3.2 Prolonged Exercise and Neutrophil Function

Since exercise is commonly associated with tissue damage and possibly injury, the role of neutrophils in infection resistance and assistance in removing tissue debris during recovery is vital (Goodman *et al.*, 1997; Suzuki *et al.*, 1999; Butterfield *et al.*, 2006).

Neutrophils exert their main destructive activity within tissues, therefore their ability to migrate to surrounding tissues *via* endothelial adherence and chemotaxis is fundamental. Even though adrenaline induced reductions in neutrophil endothelium adherence have been reported (Boxer *et al.*, 1980), neutrophil adherence to the endothelium is not substantially suppressed by prolonged strenuous exercise, whilst chemotaxis may possibly be enhanced (Ortega *et al.*, 1993a; Ortega *et al.*, 1993b).

In regard to neutrophils' destructive capacity, plasma elastase concentration (a marker of neutrophil degranulation activity) has been shown to increase after prolonged strenuous exercise, and is positively correlated with the intensity and duration of exercise (Pyne, 1994; Blannin *et al.*, 1996; Peake, 2002). However, this correlation may primarily be attributed to the cortisol-induced delayed circulatory neutrophilia, and not necessarily the increase in functional response per neutrophil (Robson *et al.*, 1998; Peake, 2002). Taking into account cortisol-induced delayed circulatory neutrophilia, such immature neutrophils may present lower functional capacity compared with fully matured neutrophils (Pyne, 1994; Toft *et al.*, 1994; Nakagawa *et al.*, 1998). On the other hand, previous studies have reported no change in the neutrophil lobe index (proportion of segmented neutrophils) within circulation after prolonged strenuous exercise (Laing *et al.*, 2008a; Laing *et al.*, 2005a).

Studies observing the effect of prolonged strenuous exercise on bacterially-stimulated elastase release per neutrophil have actually reported no changes during and immediately post-exercise, but substantial decreases during the recovery period (Blannin *et al.*, 1996; Robson *et al.*, 1999; Walsh *et al.*, 2000; Li & Gleeson, 2004). For example, cycling exercise to exhaustion at 80% $\dot{V}O_{2max}$ (37 ± 19 min) and 55% $\dot{V}O_{2max}$ (164 ± 23 min) resulted in an increase in bacterially-stimulated plasma elastase concentration, but a

substantial decline in bacterially-stimulated elastase release per neutrophil, with 55% $\dot{V}O_{2max}$ exercise intensity producing the greatest reduction (Robson *et al.*, 1999). The observed reductions in bacterially-stimulated neutrophil degranulation are reported to remain depressed for 3 h post-exercise (Robson *et al.*, 1999). A more recent investigation conducted by Li & Cheng (2007) supports these findings. Additionally, the decrease in bacterially-stimulated elastase release per neutrophil observed during recovery from exercise may also be attributed to the desensitisation of neutrophils after exercise-induced activation. It has been reported that neutrophils may enter into a refractory period following activation, which recovers with time (Henson *et al.*, 1981; Prasad *et al.*, 1991; Pyne, 1994; Bishop *et al.*, 2003).

2.3.3 Prolonged Exercise and Saliva IgA Responses

The effects of exercise on saliva IgA responses was first reported in cross-country skiers, whereby lower saliva IgA concentrations were observed in elite athletes, with further reductions observed after competition (Tomasi *et al.*, 1982). The investigators speculated that an exercise-induced temporary antibody deficiency in the oral-respiratory surface may lead to susceptibility of viral and bacterial infection. More recent studies generally report that moderate exercise induces insignificant effects on saliva IgA responses (Gleeson, 2000b). Conversely, single and repeated bouts of prolonged strenuous exercise, especially in highly trained athletes, depresses saliva IgA responses immediately after exercise and during recovery (Mackinnon & Hooper, 1994; Nehlsen-Cannarella *et al.*, 2000; Gleeson, 2000b). There is however some discrepancy between studies. In short, decreased saliva IgA concentration has been reported after maximal and submaximal running exercise (McDowell *et al.*, 1992a; Mackinnon & Hooper, 1994), whereas increases in saliva IgA concentration have been seen in runners after marathon competition (Ljungberg *et al.*,

1997), and after two 90 min cycle ergometer exercise bouts at 60% $\dot{V}O_{2max}$ (Li & Gleeson, 2005a). In addition, no change in saliva IgA concentration was reported after 60 min of intermittent maximal cycle ergometer exercise (Walsh *et al.*, 1999), or after 2 h of submaximal cycle ergometer exercise at 60% $\dot{V}O_{2max}$ (Bishop *et al.*, 2000).

Discrepancies in results may be due to the methodology of saliva IgA measurements including: differing timing of samples (diurnal variation), saliva collection methods (stimulated *vs.* unstimulated), exercise protocols (mode, intensity, duration), storage of samples, differing fitness and psychological status of participants, and differing analytical methods used (Hucklebridge *et al.*, 1998; Gleeson, 2000b; Woof & Kerr, 2006). Consistency in methodology is likely to improve the reliability and validity of saliva IgA measurements. Furthermore, previous studies have failed to control the nutritional and hydration status of participant. Thus, the confounding effects of nutritional and hydration status have not been taken into consideration (Gleeson *et al.*, 2004). This is surprising, since athletes and military personnel may potentially present suboptimal nutrition and hydration status, especially during periods of increased workload (Barr & Costill, 1992; Edwards *et al.*, 1993; Thompson *et al.*, 1995; Trapper *et al.*, 1997; Carins & Booth, 2002).

Moreover, reporting saliva IgA as a concentration reflects the current protective status in the oral-respiratory pathway. However, one major source of variation in saliva IgA concentration is an alteration in saliva flow rate induced by stress stimulus. Saliva secretions are under neuroendocrine control (Teeuw *et al.*, 2004), so stress status has the potential to alter basal saliva flow rates, promoting either a concentrating or diluting effect (dependent on the neural tone) on saliva IgA concentrations (Ljungberg *et al.*, 1997; Valdimarsdottir & Stone, 1997; Chicharro *et al.*, 1998; Hugo *et al.*, 2008). Furthermore,

dehydration associated with physical stress (e.g. exercise) also has the potential to alter saliva secretions (Walsh *et al.*, 1999; Oliver *et al.*, 2008).

Expressing IgA as a secretion rate corrects for changes in saliva flow that may occur with stress stimulus, possibly reflecting a more appropriate method of reporting exercise-induced changes in IgA synthesis and translocation (Blannin *et al.*, 1998; Walsh *et al.*, 1999). For example, decreases in saliva IgA secretion rate have been observed after supra-maximal cycling exercise, kayak training, and triathlon competition (Mackinnon *et al.*, 1993; Mackinnon & Jenkins, 1993; Steerenberg *et al.*, 1997). Moreover, in a controlled laboratory setting where nutritional and hydration aspects were well controlled, a 34% decrease in saliva IgA secretion rate was observed after cycle ergometer exercise at 62% $\dot{V}O_{2max}$ (194 Watts) in thermoneutral (20°C, 76% RH) and hot (30°C, 60% RH) conditions (Laing *et al.*, 2005b). It has however been argued that both saliva flow rate and saliva IgA concentration are both influential factors in host defence, since they reflect the true nature of current oral-respiratory host defences. Reporting saliva flow rate, saliva IgA concentration and secretion rate is potentially the most rational way of reporting overall saliva IgA responses.

After prolonged strenuous exercise, local plasma cell S-IgA production in the submucosa may be suppressed through neuroendocrine mechanisms (Mackinnon & Hooper, 1994; Gleeson, 2000a). Glucocorticoid administration has been reported *in vivo* to suppress B-lymphocyte cell maturation, differentiation and proliferation, possibly accounting for the decrease in saliva IgA concentration and secretion rate observed following repetitive bouts of prolonged strenuous exercise (Saxon *et al.*, 1978; Forslid & Hed, 1982; Gleeson & Pyne, 2000). For example, the administration of the corticosteroid dexamethasone reduces

S-IgA concentration in rats (Wira *et al.*, 1990). Moreover, acute suppression in saliva S-IgA concentration and secretion rate observed after prolonged strenuous exercise may also be attributed to alteration in tSC production and/or S-IgA translocation, which are predominantly under neuroendocrine control, rather than B-lymphocyte cell synthesis, which requires many hours to days (Norderhaug *et al.*, 1999; Teeuw *et al.*, 2004; Woof & Mestecky, 2005).

In respect to clinical relevance, the relationship between URI with reduced levels of saliva immunoglobulins in active individuals was first reported by Levando *et al.* (1988) who observed suppressed saliva immunoglobulin levels with increased training load amongst the Russian Olympic team. URI are reported as the most common infection in elite athletes, with highest illness incidences occurring in endurance athletes who show low levels of saliva IgA (Nieman, 1997; Neville *et al.*, 2008). A meta-analysis on non-exercise related studies confirmed that low levels of saliva IgA are associated with increased incidence of illness (Jemmott & McClelland, 1989). Interestingly, a recent study indicated that symptoms of URI in athletes were not always associated with infectious aetiology. Less than half (n= 111) of athletes presenting upper respiratory symptoms had evidence of infection through pathogen presence (Cox *et al.*, 2010). The majority of athletes presenting upper respiratory symptoms responded to topical anti-inflammatory spray, indicating symptom origins of inflammatory nature and not pathogen aetiology (Cox *et al.*, 2010; Gleeson *et al.*, 2008). It has been reported that mechanical damage to the airway epithelium and subsequent inflammatory responses are likely to mimic episodes of URI (Bonsignore *et al.*, 2003; Gleeson *et al.*, 2008). This has been supported by Cox *et al.* (2008), who observed that only 57% of athletes reporting symptoms of URI were identified as having pathogen presence.

2.4 Sleep-Deprivation and Selected Immune Responses

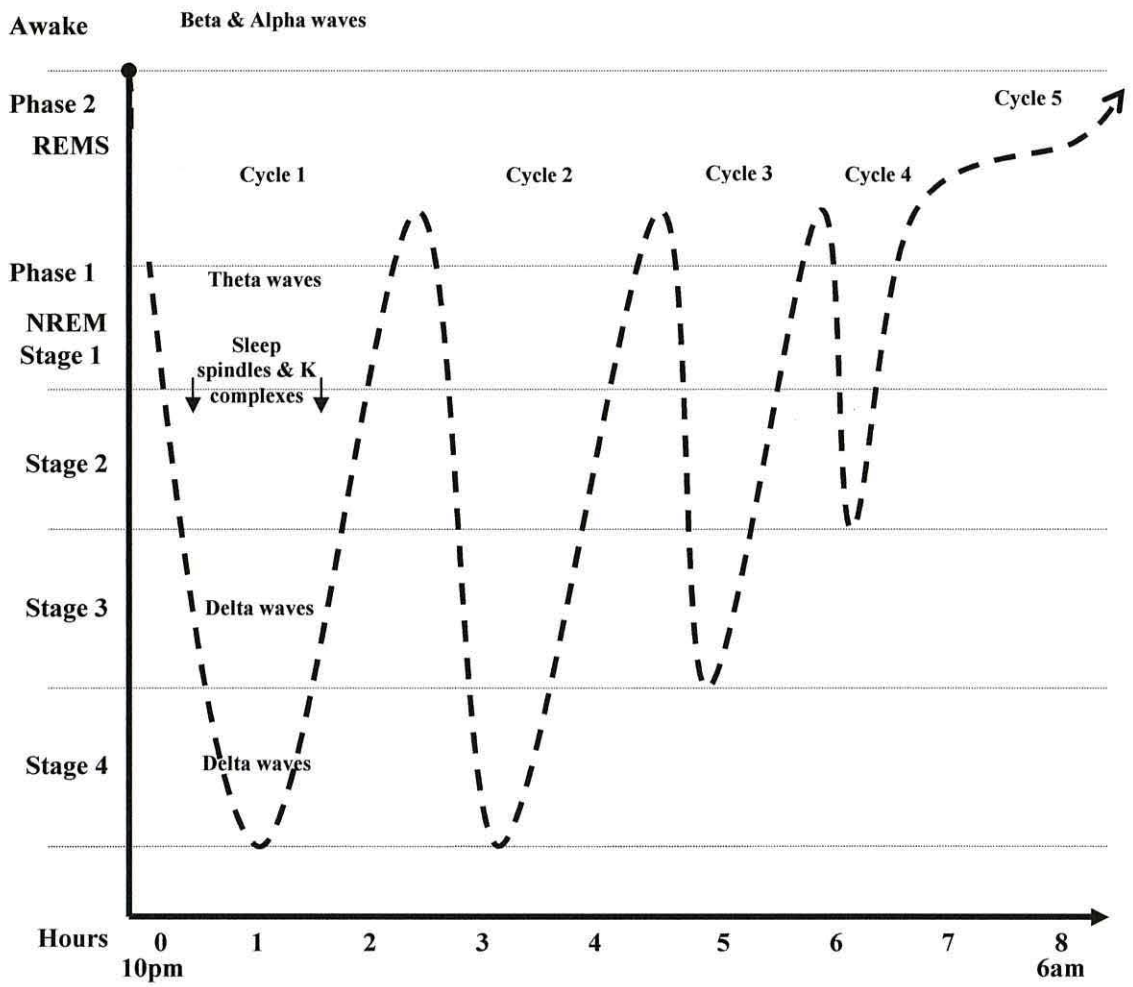
Sleep is considered a fundamental and enforced part of human biological rhythms. The exact functions of sleep are still unclear amongst chronobiology researchers. However, the increase in gross brain activity during sleep, as determined by electroencephalography, shows a highly active period, in which restoration to central nervous activity occurs (Horne, 1978; Andretic *et al.*, 2008). Reports in humans and animals suggest that disturbances to the sleep-wake cycle has drastic affects on health and cognitive function (Lieberman *et al.*, 2005). For example, in humans greater than eight days of total sleep-deprivation (complete absence of sleep) results in neurophysiological deficiencies, collapse and hospitalisation (Luby *et al.*, 1960; Kollar *et al.*, 1969). In rats, thirty-two days of total sleep-deprivation (platform over water method; Bergmann *et al.*, 1989) results in death by septicaemia and multi-organ failure (Everson, 2005; Zager *et al.*, 2007). Additionally, sleep appears to be essential in regulating, recovering and optimising host defences (Born *et al.*, 1997; Bryant *et al.*, 2004). Certainly, sleep loss has been proposed to impair host defences and predisposes the body to pathogen invasion (Krueger & Karnovsky, 1987; Krueger & Majde, 1990).

In humans, it has been reported that total sleep-deprivation for more than three nights may reach a state of clinical significance, whilst neurobehavioral deficits from total sleep-deprivation reach a significant symptomatic degree within two nights of total sleep-deprivation (Banks & Dinges, 2007). Previously, sleep research designs have used polysomnography (PSG) in determining sleep patterns (**Figure 2.5**). PSG includes the simultaneous application of electroencephalography (EEG, measuring brain wave activity), electromyography (EMG, measuring muscle contraction) and electro-oculography (EOG, measuring eye movements). However, due to the invasive nature of

this method, it created limitation of its applicability in laboratory and field based research designs (AASM, 1995; Sadeh *et al.*, 1995). Developments in technology (e.g. accelerometers) allowed for a more practical and convenient method of objectively assessing sleep patterns (Ancoli-Israel *et al.*, 2003). Accelerometers continuously record movements (due to the presence of minimal movements during sleep, wakefulness is operationally defined as >40 activity counts per epoch), indicating sleep-wake segments during the nocturnal period (Ancoli-Israel *et al.*, 2003). The validity and realiability of accelerometers has been assessed to be in line with PSG, and has been considered more reliable than sleep logs (e.g. Pittsburg sleep scale; Monk *et al.*, 1994), which rely on participant recall and are subjective in nature (Ancoli-Israel *et al.*, 2003). The fact that accelerometers can not define brain wave activity and specific stages of sleep is one limitation of this method compared with PSG.

Sleep consists of two main phases (**Figure 2.5**): 1. non rapid eye movement sleep (NREMS), which is more commonly known as slow wave or deep sleep, occurring at the onset of sleep; 2. rapid eye movement sleep (REMS), which is also known as paradoxical, occurring during the latter part of the sleep cycle. Evidence suggests that the absence of these sleep phases may impair both innate and adaptive immunity (Krueger & Karnovsky 1987; Krueger *et al.*, 2003).

Figure 2.5: Summary of the complexities of the sleep-cycle.



Rapid Eye Movement Sleep (REMS): Associated with heightened EEG, EOG, and diminished EMG activity, indicating muscle paralysis. This stage is associated with dreaming. REMS phase duration increase as the sleep cycle prolongs. Sleep cycle 5 is solely REMS until wakefulness.

Non Rapid Eye Movement Sleep (NREMS) Stage 1: Is characterised by increased theta waves and periodical increases in wave frequency (sleep spindles) and wave amplitude (K complexes). From wakefulness, these wave activities push sleep into stage 2 NREMS. Commonly known as light sleep, this stage only occurs at the onset of sleep (cycle 1) and lasts 5 to 10 min.

NREMS Stage 2: Associated with increased EEG and EMG activity. It is predominantly theta waves and is still considered light sleep. However, prepares the body for deep sleep stages 3 and 4.

NREMS Stage 3: Associated with continual increases in EEG and EMG activity. There is a progressive increase in delta wave activity, which is characterised by slow deep amplitude cerebral activity. This stage indicates the initial stages of deep sleep.

NREMS Stage 4: This stage is characterised by enhanced EEG and EMG activity. There is a predominance of delta waves activity (>50% cerebral activity). Thus, indicating an extensive period of deep sleep. The greatest duration of NREMS stage 4 occurs at the onset of sleep, and duration diminishes as the sleep cycle prolongs. Sleep cycles 3, 4, and 5 are relatively absent of NREMS stage 4.

EEG: Electroencephalogram, EMG: Electromyogram, EOG: Electro-oculogram.

Created by thesis author, adapted from Muller *et al.* (2006), and Susmakova & Krakovska (2008).

2.4.1 Sleep-Deprivation and Circulating Leukocyte Trafficking

An original study looking into the effects of sleep-deprivation on immune responses in humans reported that during and after a 77 h period of total sleep-deprivation, total and differential circulating leukocyte counts were not altered compared with resting values (Palmblad *et al.*, 1976). However, a later study conducted by Dinges *et al.* (1994) reported a significant increase in total and differential leukocyte counts, and a decrease in T-lymphocyte helper cells ($CD4^+$) after 64 h of total sleep-deprivation. The sleep deprivation protocol used by Palmblad *et al.* (1976) required participants to experience a simulated battlefield environment with quasi-continuous battle noise at 95 dB, with an additional requirement to actively fire an electronic rifle. It might be expected that the additional stress coupled with 77 h total sleep-deprivation would induce a greater leukocytosis compared with the inactive protocol of Dinges *et al.* (1994), but this was not the case.

More recently experimental studies have similarly reported discrepant findings. Compared with resting baseline values, both increases and no changes to total and differential circulating leukocyte counts have been reported after 24 h of total sleep-deprivation (Born *et al.*, 1997; Heiser *et al.*, 2000). Additionally, Born *et al.* (1997) reported an increase in circulating T-lymphocyte subsets after 24 h total sleep-deprivation, whereas suppressed T-lymphocyte subsets have been reported in individuals suffering from chronic insomnia (Savard *et al.*, 2003). So the effects of sleep-deprivation on circulating leukocyte trafficking is currently unclear. It is possible that differing protocols of sleep-deprivation, lack of research control on immune modulating cofactors (e.g. physical activity, nutritional intake, psychosocial factors), and analytical aspects may account for the discrepancies in research findings.

2.4.2 Sleep-Deprivation and Functional Immune Responses

Large discrepancies in research results have also been reported on the effects of sleep-deprivation on immune responses to *in vitro* bacterial challenge. After only 24 h into a 77 h period of total sleep-deprivation that included the addition of continuous simulated combat, granulocyte phagocytic function substantially declined, whilst PHA-induced lymphocyte blastogenesis declined after 48 h of total sleep-deprivation (Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). Both functional markers returned to baseline values after five days of recovery sleep. Similarly, Moldofsky *et al.* (1989a) reported a decrease in PHA-induced lymphocyte blastogenesis after 64 h period of total sleep-deprivation, however they also reported an increase in pokeweed mitogen (PWM)-induced lymphocyte blastogenesis. Interestingly, both functional measurements reduced below baseline after one night of recovery sleep. A follow-up study from the same research group reported a similar increase in PWM-induced lymphocyte blastogenesis, but no change in PHA-induced lymphocyte blastogenesis after a 40 h period of total sleep-deprivation (Moldofsky *et al.*, 1989b). A more recent study that followed up the previous work by Moldofsky *et al.* (1989a; 1989b) showed no changes in PHA, ConA and PWM-induced lymphocyte blastogenesis after 64 h total sleep-deprivation (Dinges *et al.*, 1994). Moreover, reduced NKCA has been reported after a 40 h and 64 h period of total sleep-deprivation (Moldofsky *et al.*, 1989a; Moldofsky *et al.*, 1989b), after partial sleep deprivation (Irwin *et al.*, 1994; Irwin *et al.*, 1996), and in individuals suffering from chronic insomnia (Irwin *et al.*, 2003).

The antibody response to vaccination has also previously been used as a measure of assessing adaptive immunity during sleep-deprivation protocols. Lange *et al.* (2003) measured inactivated hepatitis A virus (HAV) titres twenty-eight days after vaccination.

They observed lower inactivated HAV titres in subjects that were total sleep-deprived the night after vaccination, compared with subjects that had a regular sleep the night after vaccination. The investigators attributed these results to attenuated immune-stimulating growth hormone (GH) response and increased immunosuppressive catecholamines in total sleep deprived participants. Other research groups have found similar results, whereby total sleep-deprivation was found to affect the adaptive immune system's ability to respond appropriately to vaccination (Brown *et al.*, 1989; Spiegel *et al.*, 2002). Therefore, evidence suggests that sleep may be required to improve antigen-specific immune defences *via* increasing immune-stimulatory factors (e.g. GH and melatonin), which are reported to enhance lymphocyte proliferation, differentiation and antibody synthesis (Guerrero & Reiter, 2002; Lange *et al.*, 2003; Reiter, 2003).

2.4.3 Immune Modulating Sleep Regulators

Disturbances to sleep-wake cycles may pose a threat to human health through neurophysiological dysfunction (Luby *et al.*, 1960; Kollar *et al.*, 1969; Lieberman *et al.*, 2005; Banks & Dinges, 2007). The mechanisms by which disturbances to the sleep-wake cycle and neurophysiological dysfunction may affect immune status include: altered immune circadian rhythms, neuroendocrine responses, pro-somnogenic cytokine activity, and altered melatonin nocturnal responses, all of which are promoted by the elevated homeostatic drive to sleep (Dinges *et al.*, 1995).

The immune system is susceptible to modification by circadian variations. Disturbance to normal circadian immune activity through sleep-deprivation may subsequently alter immune function (Shephard & Shek, 1997). Additionally, disturbance to the sleep-wake cycle, and subsequent increased homeostatic drive to sleep, stimulates the SAM and HPA

axis to release immune-perturbating stress hormones (Pedersen *et al.*, 1997; Madden, 2003; Irwin *et al.*, 2003). Radomski *et al.* (1992) reported a significant stress hormone and altered circadian hormone response with two nights of total sleep-deprivation, which also included attenuated nocturnal increases in GH.

Pro-somnogenic cytokines (e.g. IL-1β, TNFα, IFNγ) have been implicated as sleep promoting factors (Benca & Quintas, 1997). The onset of NREM sleep in humans coincides with dramatic increases in IL-1β, TNFα and IFNγ levels (Moldofsky *et al.*, 1986; Krueger & Karnovsky, 1987). After sleep onset, reductions in these cytokines are reported (Dickstein & Moldofsky, 1999; Irwin, 2002). Therefore, it is not surprising that substantial chronic increases in pro-somnogenic cytokines (e.g. IL-1β, TNFα and IFNγ) have been observed after 24 h and 64 h of total sleep-deprivation in humans (Dinges *et al.*, 1994; Born *et al.*, 1997; Dimitrov *et al.*, 2004). Indeed, intravenous injection of IL-1β, TNFα and IFNγ have been reported to induce NREM sleep and diminished REM sleep in a variety of animal species (Shoham *et al.*, 1987; Krueger *et al.*, 2003; Opp & Toth, 2003). Conversely, individuals suffering from chronic insomnia are reported to present attenuated nocturnal increases in pro-somnogenic cytokines (Savard *et al.*, 2003). In addition, pro-somnogenic cytokine activity has also been implicated in driving acute immune responses during sleep-deprivation (Dinges *et al.*, 1995; Dickstein & Moldofsky, 1999; Majde & Krueger, 2005), promoting phagocytosis, T and B-lymphocyte cell proliferation, and inducing fever responses (Krueger *et al.*, 2003). It is plausible that these responses may be disturbed through pro-somnogenic cytokine dysfunction, with the prolonging of sleep-deprivation (Krueger *et al.*, 2003).

Sleep-deprivation may also alter immune responses through disturbances in the pineal gland's release of melatonin. Melatonin release is primarily driven by light-darkness ratio. As daylight fades the pineal gland increases its release of melatonin (Reiter, 2003). Prolonged sleep-deprivation (≥24 h) has the potential to disturb melatonin release, and subsequently alter its influence on immune activation (Reiter, 2003). Melatonin acts by directly regulating gene expression and production of pro-somnogenic cytokines, acts as a nocturnal free radical scavenger and broad-spectrum antioxidant, is a potent inhibitor of immune cell apoptosis, and is generally considered to be an immune-enhancer (Liu *et al.*, 2001; Guerrero & Reiter, 2002; Tan *et al.*, 2002). Melatonin is also presumed to stimulate T-lymphocyte helper ($CD4^+$) and monocyte cell activity, neutrophil degranulation and oxidative burst activity, NKCA, and acts as a protective mechanism against free radical damage to leukocytes (Tan *et al.*, 2002). Thus, disturbances to normal melatonin functioning, as seen during prolonged sleep-deprivation, may affect host defences (Guerrero & Reiter, 2002; Reiter, 2003).

The effects of sleep-deprivation on functional immune measures are currently limited and inconclusive. However, as previously discussed, studies have demonstrated declines in phagocytic and lymphocyte function after total and partial sleep deprivation. This suggests that sleep-deprivation may possibly play a role in impairing host defences. More specifically, the effects of sleep-deprivation on bacterially-stimulated neutrophil degranulation and saliva S-IgA responses have not been determined, and therefore warrants investigation.

2.5 Cold-Exposure and Selected Immune Responses

Exposure to a cold environment is relatively common amongst individuals undertaking alpine extreme sports, arctic and wilderness expeditions, ultra-endurance competitions, and in military personnel during sustained military training and operations, especially in polar regions and during wet winter months. There is a social belief that exposing the body to cold increases the risk of illness and infection, particularly URI. The current evidence for this relationship remains limited and inconclusive (Castellani *et al.*, 2002; Walsh & Whitham, 2006). Even so, high rates of URI have been reported during sustained military operations in the Canadian Arctic winter (St Rose *et al.*, 1972; Sabiston & Livingstone, 1973). However, it is unclear whether the cold-exposure *per se*, or other stress factors (e.g. nutrient restriction, prolonged exercise, sleep-deprivation or a combination) resulted in the increased appearance of URI. Moreover, cold-induced stimulation of the HPA and SAM axis, and subsequent increases in stress hormones, induced by initiation of shivering thermogenesis, increased sympathetic tone associated with peripheral vasoconstriction, and metabolically driven release of stress hormone for endogenous energy substrate release, may account for immune perturbations induced by cold-exposure (Walsh & Whitham, 2006).

2.5.1 Cold-Exposure and Circulating Leukocyte Trafficking

Increases in total and differential circulating leukocyte counts have been reported after 1 h mid-chest water immersion at 14°C (Jansky *et al.*, 1996), whilst similar increases in total circulating leukocyte, T-lymphocyte subsets, B-lymphocyte cell and NK cell counts were also observed after a 2 h cold air test (CAT) at 5°C, which was conducted after a 1 h mid-chest water immersion at 35°C (Brenner *et al.*, 1999). Interestingly, Brenner *et al.* (1999) only reported a 0.3°C drop in rectal temperature (T_{re}), which did not go below 36.0°C,

whilst no temperature measurements were made by Jansky *et al.* (1996). On the other hand, more forceful chilling protocols conducted on animals, reported decreases in T-lymphocyte subsets and lymphocyte responses to mitogens in rats after 3 min cold-water immersions in 4°C and 15°C repeated three times in a 24 h period (Shu *et al.*, 1993). In support of these findings, 5 min cold-water immersion at 1°C for eight consecutive days resulted in suppressed immune response to West Nile-25 encephalitic virus (70% mortality in cold exposure mice *vs.* control normothermic mice; Ben-Nathan *et al.*, 1996).

With regards to circulating neutrophil trafficking, increases in circulatory neutrophil adherence to vascular endothelium, and subsequent neutropenia, has been observed during surgery induced cold-exposure (Menasche *et al.*, 1995). A cold (26°C) cardiopulmonary bypass surgery room temperature resulted in increased neutrophil adhesion compared with a warm (32°C) surgery room temperature. However, the lack of core temperature (T_{core}) measurements in 20 case-matched patients is a limitation in this study design (Menasche *et al.*, 1995). Nevertheless, these results were supported by Nash *et al.* (2001), who observed *in vitro* increases in neutrophil adhesion to P-selectin at 0°C and 10°C compared with 37°C. Additionally, impaired neutrophil diapedesis (neutrophil flow through 8 μm pore) was also observed at 0°C and 10°C compared with 37°C (Nash *et al.*, 2001). The large discrepancies in temperatures within such experimental models must be interpreted with caution, since these temperatures are not realistic in practice and are a limitation when applying such findings to the practical setting.

2.5.2 Cold-Exposure and Functional Immune Responses

Perioperative hypothermia is common with anaesthesia and surgical procedures. Delayed recovery from surgery (e.g. impaired wound healing), with greater vulnerability and

frequency of infection, has been reported in hypothermic surgical patients (Birdi *et al.*, 1999). Substantially reduced PHA-induced lymphocyte proliferation 24 and 48 h post-surgery has been observed in a randomised abdominal surgery routine care group (≥1°C decrease in T_{core}), compared with an intraoperative forced-air warming group who maintained T_{core} (Beilin *et al.*, 1998). In addition, a 72% decreased *in vitro* phagocytosis (*vs.* preoperative) was reported in 6 hypothermic colorectal cancer surgery patients after mild intraoperative hypothermia (mean tympanic temperature 35°C; Wenisch *et al.*, 1996a). The, authors concluded that lowering T_{core} is associated with reductions in phagocytosis and oxidative burst activity in response to *Escherichia coli* (E.coli). A limitation to this study was the lack of a control group undergoing surgery in normothermic conditions.

Increases in plasma elastase concentration have been reported after cardiopulmonary bypass surgery at hypothermic (28°C) and mild hypothermic (32°C) surgical theatre ambient conditions, compared with normothermic (37°C) surgical theatre ambient conditions (Birdi *et al.*, 1999). However, elastase release per neutrophil was not reported, and therefore individual neutrophil functional responses with differing surgical theatre ambient temperature exposures cannot be substantiated. Furthermore, a previous study that reported an increase in plasma elastase concentration at 26°C cardiopulmonary bypass surgery theatre ambient temperature, compared with 32°C, attributed the outcomes to a cold-induced increase in adhesion triggered neutrophil degranulation response, and not necessarily a pathogen induced functional response (Menasche *et al.*, 1995). Additionally, it may be worth considering the amplified anxiety associated with hospital admissions and waiting for surgical procedures, and the influence of medications on immune responses. Appropriate control groups are essential for accurate interpretation in such research trials.

2.5.3 Cold-Exposure and Saliva IgA Responses

No investigation has observed the effects of rested cold-exposure on oral-respiratory mucosal immunity. Previous focus has been towards investigating the effects of exercising in cold environmental conditions on oral-respiratory mucosal immune responses. As previously mentioned, decreases in saliva IgA after a cross-country skiing competition have been observed (Tomasi *et al.*, 1982), whilst high rates of URI have also been reported during sustained military operations in the Canadian Arctic winter (St Rose *et al.*, 1972; Sabiston & Livingstone, 1973). It has been proposed that large inflows of cold air lower the temperature of mucous membranes, resulting in the attenuated oral-respiratory tract secretions, dysfunction of the mucosal epithelium and/or local plasma cells, which predisposes the oral-respiratory tract to pathogen invasion (Tomasi *et al.*, 1982; Giesbrecht, 1995; Teeuw *et al.*, 2004). Conversely, 2 h of cycle ergometer exercise at 70% $\dot{V}O_{2max}$ in -6.4°C resulted in no significant changes in saliva flow rate, saliva IgA concentration and secretion rate (Walsh *et al.*, 2002). Obviously, exercise stress induced by such protocols will attenuate the decreases in T_{core} and prevent the occurrence of hypothermia. Therefore, such research protocols can only assess the influence of cold air inflow and not the influence of reductions in T_{core} on oral-respiratory mucosal immunity.

Since saliva flow rates and saliva IgA translocation are under neural control (**Section 2.1.3** and **2.1.4**; Norderhaug *et al.*, 1999; Teeuw *et al.*, 2004; Woof & Mestecky, 2005), it is plausible that saliva IgA responses may be suppressed by cold-exposure through SAM and HPA axis stimulation (Castellani *et al.*, 2002; Walsh & Whitham, 2006). However, this may only be the case if substantial decreases in T_{core} (e.g. $\leq$36.0°C) are observed. Investigation into the effects of cold-exposure inducing reductions in T_{core} at rest on saliva IgA responses is warranted.

It appears that the effects of cold-exposure on selected immune responses are currently limited and inconclusive. The main reasons for this are possibly inadequate research control, the methodological aspects of research protocols, the lack of consistency in body temperature measurements, and failure to reach substantial reductions in whole-body temperature (e.g. $\leq$36.0°C T_{core}), which may possibly directly and indirectly influence immune responses (Shephard & Shek, 1998; Castellani *et al.*, 2002; Walsh & Whitham, 2006).

2.6 Influence of Nutrition on Selected Immune Responses

The role of nutrition in immune competence has been of interest to researchers in the last two decades. Various nutrients have been implicated in immune competence due to their essential role in immune cell metabolism and protein synthesis, cell and tissue integrity, directly or indirectly regulating lymphoid cell activation, and antioxidant defence systems. Thus, nutrients have been implicated as essential for immune cell proliferation and function (Bishop *et al.*, 1999; Calder, 2007). The nutrients known to play a role in immune competence include: carbohydrate (CHO; Neiman, 1998b; Braun & Von Duvillard, 2004); protein (PRO), especially amino acids glutamine, arginine and branch chained amino acids (Daly *et al.*, 1990; Chandra, 1992; Walsh *et al.*, 1998; Bassit *et al.*, 2000; Bassit *et al.*, 2002); fatty acids, particularly the omega 6 to omega 3 ratio (Venkatraman *et al.*, 2000; Venkatraman & Pendergast, 2002; Gleeson, 2006); micronutrients, which include antioxidants (e.g. retinol, ascorbic acid, tocopherol, β-carotene, selenium), water soluble vitamins (e.g. cobalamin, pyridoxine, folic acid), minerals (e.g. iron, zinc, copper, and manganese), and possibly nutriceutics (e.g. echinacea, probiotics, nucleotides, taurine, antioxidants; Bishop *et al.*, 1999b; Gleeson *et al.*, 2004; Calder, 2007).

Both macronutrient and micronutrient deficiencies induced by malnutrition, starvation, and/or illness, can impair immune function (Chandra & Kumari, 1994; Scrimshaw & SanGiovanni, 1997). Nutrient deficiencies have been directly associated with the suppression of various components of host defence including: plasma and secretory antibody production, T-lymphocyte cell subset distribution and maturation, proliferative responses to mitogens, bactericidal capacity of phagocytes, complement formation, pro and anti-inflammatory cytokine responses, and dysfunction of various components of the external innate immune systems (Scrimshaw & SanGiovanni, 1997; Calder & Jackson,

2000). Additionally, nutrient deficiencies have been indirectly associated with perturbations in host defences through activation of the SAM and HPA axis, and subsequent release of stress hormones (Pequignot *et al.*, 1980; Fichter *et al.*, 1986; Chandra, 1997; Braun & Von Duvillard, 2004; Gleeson, 2006).

Populations with high activity levels (e.g. athletes, military personnel, ultra-endurance and adventure/exploration groups) have greater difficulties in achieving nutritional requirements compared with inactive populations, due to greater food volumes required to achieve nutritional requirements, food/drink preparation time, scheduled exercise volumes, and exercise-induced changes in anabolic/catabolic metabolism and appetite regulation (Jequier & Tappy, 1999; de Graaf *et al.*, 2004; Park & Bloom, 2005). Marginal nutritional deficiencies have been identified in populations with high activity levels, predominantly in the form of macronutrient intake (especially, carbohydrate) not meeting exercise requirements (Burke *et al.*, 2004; Broad & Cox, 2008).

Indeed, carbohydrate is considered an important nutrient in promoting immune competence. As a key nutrient for leukocytes, carbohydrate is used directly as a glycolytic intermediate precursor in the nucleotide biosynthesis required for immune cell proliferation. It is also used as an energy source by lymphocytes, neutrophils and macrophages (Bishop *et al.*, 1999b). Additionally, carbohydrate is essential in fuelling phagocytosis (Newsholme & Newsholme, 1989; Blannin *et al.*, 1996; Bishop *et al.*, 1999b), and *in vitro* macrophage and lymphocyte proliferative responses to ConA have been reported to be dependent on blood glucose concentration (Hume & Weidemann, 1979). Furthermore, carbohydrate also indirectly influences host defence by attenuating SAM and HPA axis stimulation, and subsequent release of stress hormones (Hume &

Weidemann, 1979; Bishop *et al.*, 1999b; Braun & Von Duvillard, 2004; Gleeson, 2006). Experimental evidence suggests that the intake of adequate dietary carbohydrate during periods of heavy exercise loads (experimental range: 8 - 12 g CHO·kg·day^{-1}), and carbohydrate supplementation prior to and during prolonged strenuous exercise (experimental range: pre 17 - 75 g CHO; during 21 - 76 g CHO·h^{-1}) attenuates some of the exercise-induced transient immune perturbations associated with prolonged strenuous exercise (Gleeson *et al.*, 1998; Braun & Von Duvillard, 2004; Costa *et al.*, 2005; Gleeson, 2006).

Protein-energy malnutrition (PEM) induced by malnutrition, starvation, and/or illness also has the potential to impair immune function (Chandra, 1992; Chandra & Kumari, 1994; Scrimshaw & SanGiovanni, 1997; Calder & Jackson, 2000). Since protein is required for immune cell production and function, PEM has been shown to depress T-lymphocyte $CD4^+$ counts, phagocytic function, lymphocyte proliferative response to mitogens, cytokine production and complement formation (Daly *et al.*, 1990; Reynolds *et al.*, 1990; Gleeson *et al.*, 2004). However, chronic protein deficiencies are rare amongst healthy active individuals who are consuming sufficient energy to meet their activity demands (Rennie & Tipton, 2000; Tipton & Wolfe, 2004; Broad & Cox, 2008). Therefore, PEM is an unlikely contributor to reduced host defences observed in highly active individuals (Broad & Cox, 2008). Currently controversy exists around the role of protein in attenuating exercise-induced immune perturbations (Walsh *et al.*, 1998; Gleeson, 2006). Several authors have argued that supplementation with glutamine or branched chain amino acids may maintain host defence and aid immune recovery after strenuous exercise (Castell *et al.*, 1996; Bassit *et al.*, 2000; Bassit *et al.*, 2002). However, these conclusions require substantiation (Walsh *et al.*, 1998).

Micronutrient deficiencies are also reported to be rare amongst highly active healthy individuals consuming sufficient energy to meet their daily energy requirements (Clarkson, 1991; van der Beek, 1991; Clarkson & Haymes, 1994), so are also unlikely to contribute to reduced host defences. Meanwhile, the role of dietary fats in immune modulation and supporting host defences is less convincing. Evidence suggests that the involvement of dietary fat extends only in augmenting pro and anti-inflammatory immune responses, dependent on the type and quantity of essential fatty acids consumed (e.g. omega 3 to omega 6 ratio; Venkatraman *et al.*, 2000; Venkatraman & Pendergast, 2002; Gleeson, 2006).

2.6.1 Energy-Restriction and Selected Immune Responses

Acute periods of intensified training in military personnel and athletes contribute towards a negative energy status (Barr & Costill, 1992; Edwards *et al.*, 1993; Thompson *et al.*, 1995; Carins & Booth, 2002). Various studies involving energy restriction with and without the addition of other variables (e.g. prolonged strenuous exercise, fluid-restriction) have implicated energy deficits in immune dysfunctions. For example, 36 h to seven days of complete food abstinence has been reported to decrease circulating T-lymphocyte subset counts (Savendahl & Underwood, 1997; Walrand *et al.*, 1997). Similarly, soldiers' circulating T-lymphocyte subset and B-lymphocyte counts declined during twelve days of military training on dietary ration packs (1800 Kcal·day^{-1}; Booth *et al.*, 2003). Furthermore, a well controlled laboratory study involving 48 h of 90% energy restriction (290 Kcal·day^{-1} consumed), with and without a 75% fluid restriction (960 - 962 ml·day^{-1} consumed), resulted in decreased circulating leukocyte, T-lymphocyte $CD3^+$ and $CD4^+$ counts, which normalised after refeeding (Laing *et al.*, 2008b).

Additionally, lower circulating neutrophil counts has been reported after six weeks adhering to a very low energy diet (400 Kcal·day^{-1}; Field *et al.*, 1991). Similarly, lower circulating neutrophil counts have been reported in participants receiving a diet consisting only 75% of their daily energy requirements during seven consecutive days of 3 h·day^{-1} running exercise at 75% $\dot{V}O_{2max}$, when compared to participants who received 110% of their daily energy requirements (Galassetti *et al.*, 2006). Conversely, no change in circulating neutrophil counts was observed after seven days of complete food abstinence (Savendahl & Underwood, 1997), and after twenty days of 800-1500 Kcal·day^{-1} energy deficit in Judoists' attempting to reduce body weight to meeting competition weight criteria (Kowatari *et al.*, 2001). Similarly, no change in circulating neutrophil counts have been observed after 48 h of 90% energy restriction, with and without fluid restriction (Laing *et al.*, 2008b). Whereas, increased circulating neutrophil counts were observed after a 36 h period of complete food abstinence (Walrand *et al.*, 2001).

With regards to neutrophil function, reduced glucose availability and uptake by neutrophils' has been implicated in the decrease in neutrophil chemotaxis observed during three months of energy restriction in obese patients (McMurray *et al.*, 1990). More acutely, 36 h of complete food abstinence in healthy adults also resulted in a decreased neutrophil chemotaxis, but no significant change in stimulated neutrophil oxidative burst activity was observed (Walrand *et al.*, 2001). Similarly, no change in neutrophil oxidative burst was reported after seven consecutive days of prolonged strenuous exercise with a dietary intake equivalent to 75% of daily energy requirements (Galassetti *et al.*, 2006). Whilst, no change in bacterially-stimulated neutrophil degranulation was observed after 48 h of 90% energy restriction, with and without fluid restriction, and in response to subsequent 30 min treadmill time trial exercise bout (Laing *et al.*, 2008b). Conversely,

impaired phagocytic activity was reported in athletes restricting food and fluid intake to reduce weight to meet category criteria before competition (Kowatari *et al.*, 2001; Ohta *et al.*, 2002).

Chronic nutritional deprivation has also been implicated in affecting oral-respiratory mucosal immunity. It is plausible that energy deficits promote disturbance to saliva IgA responses by decreasing flow rate through SAM axis stimulation and decreased IgA synthesis and translocation through HPA axis stimulation (Saxon *et al.*, 1978; Wira *et al.*, 1990; Teeuw *et al.*, 2004). For example, increases in noradrenaline and cortisol have been reported following 15 h of complete food abstinence and during severe energy restriction in healthy adults (Pequignot *et al.*, 1980; Fichter *et al.*, 1986). Additionally, intestinal IgA concentration and tSC expression has been shown to be depress following severe energy restriction in mice (Ha & Woodward, 1998). Whilst, food and fluid abstinence for 24 h induced decreases in saliva flow rate and secretory proteins in adults (Ship & Fischer, 1997; Tenovuo, 1998), and reduced saliva IgA responses have been reported in malnourished children compared with well fed children (Watson *et al.*, 1985; Johansson *et al.*, 1994). In a well controlled laboratory study, 48 h of 90% energy restriction (289 $Kcal \cdot day^{-1}$ consumed) resulted in no changes to saliva flow rate, saliva IgA concentration and secretion rate (Oliver *et al.*, 2007). However, the addition of fluid restriction (962 $ml \cdot day^{-1}$ consumed), resulted in a significant decrease in saliva flow rate, and subsequently a reduction in saliva IgA secretion rate (Oliver *et al.*, 2007).

The discrepant findings in circulating leukocyte trafficking, neutrophil function and saliva IgA responses presented so far may be due to differing degrees and durations of energy restrictions, accompanying fluid deficits, the presence of confounding variables, or a

combination of these factors. Furthermore, differing population groups (fitness status), macronutrient distribution within restrictions, lack of research control for confounding variables, and timing of samples (circadian variations) may further account for immune discrepancies. Appropriate research design and control is required for accurate interpretation of the influence of nutritional restriction on circulating leukocyte trafficking, neutrophil function and saliva IgA responses.

2.6.2 Carbohydrate Intake Prior to and During Exercise and Selected Immune Responses

Over the past two decades, exercise immunology researchers have investigated the effects of carbohydrate intake prior to and during exercise on immune responses. An original study by Nieman *et al.* (1997), demonstrated that 750 ml of a carbohydrate solution (6% *w/v)* prior to and 250 ml of the same solution every 15 min during a 2 h 30 min running exercise at 75% $\dot{V}O_{2max}$ attenuated stress hormone release and promoted fewer perturbations in selected immune responses during the recovery period. Follow-up research into the effects of carbohydrate supplementation prior to and during exercise on selected immune responses reported: reduced post-exercise circulating leukocytosis, neutrophilia, monocytosis, lymphocytosis, increased NK cell counts, attenuated rise in the neutrophil to lymphocyte ratio, attenuated circulating lymphopenia during the recovery period, attenuated depression of the lymphocyte proliferative response to mitogen challenge, attenuated depression of total phagocytic activity and neutrophil degranulation response to bacterial challenge, enhanced oxidative burst activity, and attenuated depression of T-lymphocyte cellular immune responses (Nieman, 1998b; Braun & Von Duvillard, 2004; Gleeson, 2006). B-lymphocyte humoral immune responses appear not to be affected by carbohydrate intervention prior to or during exercise (Lancaster *et al.*,

2004). The role of carbohydrate supplementation prior to and during exercise on mucosal immunity remains less clear (Bishop *et al.*, 2000; Li & Gleeson, 2005a).

With regards to specific immune functional responses, ingesting 750 ml of a carbohydrate solution (6% *w/v*) prior to and 280 ml of the same solution every 15 min during 2½ h cycling at 85% ventilatory threshold (VT) attenuated post-exercise leukocytosis, neutrophilia and prevented the decrease in T-lymphocyte response to PHA (Green *et al.*, 2003). Also, ingesting 5 $ml \cdot kg^{-1}$BM of a carbohydrate solution (6.4% *w/v*) prior to and 2 $ml \cdot kg^{-1}$BM of the same solution every 15 min during high intensity intermittent exercise (Loughborough Intermittent Shuttle Test; Nicholas *et al.*, 2000) attenuated the decrease in bacterially-stimulated neutrophil degranulation observed on the placebo trial (Bishop *et al.*, 2002). The consumption of 50 $ml \cdot kg^{-1}$BM of a 6% (*w/v)* and 12% (*w/v)* carbohydrate solution throughout a 4 h cycling exercise bout at 70% $\dot{V}O_{2max}$ increased neutrophil oxidative burst activity compared with placebo (Scharhag *et al.*, 2002). On the other hand, studies have also reported no attenuation of exercise-induced immune depression on lymphocyte or neutrophil function with carbohydrate intervention (Nieman *et al.*, 1997; Henson *et al.*, 1998; Bishop *et al.*, 2003). The mode, duration and intensity of exercise protocols, method of presenting immune function (e.g. total phagocytic/ proliferative activity *vs.* per cell response), and differing bacterial stimulants (e.g. LPS, ConA, PHA, PWM, E.Coli, bacterial mixtures) may account for these discrepancies.

Carbohydrate supplementation during exercise appears to have minimal influence on saliva IgA responses compared with placebo (Bishop *et al.*, 2000; Li & Gleeson, 2005a). However, the frequent intake of carbohydrates during exercise in a fluid form appears to favour the maintenance of saliva flow rate compared with fluid restriction and fluid

volume equivalent of water (Bishop *et al.*, 1999a; Bishop *et al.*, 2000; Walsh *et al.*, 2004a; Walsh *et al.*, 2004b). Thus, even though saliva IgA concentration and secretion rate are not altered with carbohydrate feedings during exercise, the influence of carbohydrate intake during exercise on maintaining saliva flow rate may be of importance to oral-respiratory immune competence as described in **Section 2.1.3**.

Speculation into the beneficial effect of carbohydrate ingestion before and during prolonged exercise on immune responses is that it may primarily be due to the maintenance of plasma glucose levels, which potentially blunts stress hormone and cytokine responses (Gleeson, 2006). More recently, evidence has favoured a role for insulin in modulating immune function, due to insulin receptors being located on surface membranes of phagocytes and lymphocytes (Alba-Loureiro *et al.*, 2007). Hyperinsulinaemic-euglycaemic clamp studies have shown that raised plasma insulin, without rises in plasma glucose, increases neutrophil chemotaxis, phagocytosis and bactericidal capacity in healthy subjects (Walrand *et al.*, 2004; Walrand *et al.*, 2006; Stegenga *et al.*, 2008a). It is conceivable that marginal increases in plasma insulin concentration after carbohydrate ingestion during endurance or intermittent exercise may also be involved in attenuating exercise-induced immune perturbations, rather than simply attenuated stress hormone release as proposed by previous studies (Nieman *et al.*, 1997; Robson *et al.*, 1999; Bishop *et al.*, 2002).

From a practical viewpoint, the optimal amount of carbohydrate intake during exercise that attenuates immune perturbations (~60 g $CHO \cdot h^{-1}$; Gleeson, 2006) appears to be attainable in a laboratory setting, but rarely practised amongst individuals participating in endurance activity (e.g. distance runners, triathletes, ultra-endurance athletes, military

personnel). This is mainly attributed to exercise-induced gastrointestinal distress not allowing appropriate quantities of carbohydrate (in fluid or solid form) to be tolerated (Peters *et al.*, 1993), individual feeding tolerance and competition pacing strategies (Burke *et al.*, 2005; Kruseman *et al.*, 2005), and adherence to nutritional interventions, such as training fasted or with plain water, in the attempt to enhance endurance performance through selective gene expression and metabolic adaptations to promote enhanced fat oxidation (Civitarese *et al.*, 2005; Hawley *et al.*, 2006; De Bock *et al.*, 2008).

A recent study reported that 3 out of 18 highly trained runners instructed to consume ~1.0 g $CHO \cdot kg^{-1}BM$ with ~380 $ml \cdot h^{-1}$ of water during a half-marathon, complained of gastrointestinal discomfort and lost time at feeding stations resulting in a 105 s extension to their race time (Burke *et al.*, 2005). Whilst, less than half of the runners competing in a 44 km mountain marathon (18 out of 42 runners) consumed more than 30 g $CHO \cdot h^{-1}$ and no runner consumed the recommended 1.0 g $CHO \cdot kg^{-1}BM \cdot h^{-1}$ during the competition (Kruseman *et al.*, 2005). Furthermore, reports indicate that elite marathoners consume as little as ~200 $ml \cdot h^{-1}$ of fluids during competition (Noakes, 2003), whilst the British Ministry of Defence's nutritional recommendations indicates plain water as the sole fluid to be provided to military personnel during training, routine or special operations (**Section 2.2**; Casey & Messer, 2004). Consequently, if limited carbohydrate is available and consumed during immune-perturbating exercise, carbohydrate provisions with or without the addition of protein shortly after heavy prolonged exercise may be important for immune recovery. The role of carbohydrate, with and without the addition of protein, consumed after prolonged strenuous exercise has not been addressed and warrants investigation.

2.7 Thesis Aims

The presented literature suggests that exposure to a single physiological stressor has the potential to perturbate multiple host defenses including, circulating leukocyte trafficking, neutrophil function and saliva IgA responses. It is plausible that performing activities involving a combination of physiological stress has the potential to amplify or accumulate these immune perturbations.

With this information in mind, the broad aims of the experiments contained within this thesis were to investigate: 1. the effects of one night total sleep-deprivation on selected immune (circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva IgA responses) and stress hormone responses at rest and following prolonged strenuous exercise; 2. the effects of two nights total sleep-deprivation with and without energy-restriction on selected immune and stress hormone responses at rest and after passive cold exposure; 3. the effects of passive cold-exposure inducing modest whole-body cooling on selected immune and stress hormone responses; and finally, 4. the effects of carbohydrate feeding, with and without the addition of protein, during the recovery period after prolonged strenuous exercise on selected immune, stress hormone and insulin responses.

It was hypothesised that: 1. performing exercise after one night of total sleep-deprivation would evoke greater immune disturbances compared with performing exercise after one night of normal sleep; 2. two nights total sleep-deprivation would evoke greater immune disturbances compared with two nights of normal sleep, and that the addition of energy-restriction would amplify the immune disturbances; 3. passive cold-exposure inducing modest whole-body cooling would induce immune disturbances, and the addition of total-

sleep deprivation with and without energy-restriction would amplify the immune disturbances; and finally, 4. carbohydrate feeding immediately after prolonged strenuous exercise would attenuate exercise-induced immune disturbances during recovery, and the immune perturbations would recover even more rapidly with the addition of protein to the carbohydrate feeding.

CHAPTER THREE

General Methods

3.1 Ethical Approval

Approval was received from the Local Ethics Committee (*School of Sport, Health and Exercise Sciences, Bangor University*) prior to commencement of each experimental study. The nature and purpose of each study was fully explained to participants, both verbally and written *via* participant information sheets (**Appendix A**). Each participant was made fully aware that they were free to withdraw from any study at any time. Participants gave written informed consent and a health questionnaire was completed prior to the commencement of each experimental study (**Appendix B** and **C**). Participants were only eligible to initiate and complete the experimental procedure if they reported no symptoms of infection or illness, and no prescribed or non-prescribed medications or supplements were taken twelve weeks prior and during the study. Participants were asked to complete a health and training log during their time adhering to experimental procedures. Participants were also asked to refrain from alcohol and caffeine for 72 h, and exercise for 24 h prior to preliminary testing sessions and each experimental trial. All volunteers were non-smokers.

3.2 Anthropometry and Body Composition

Participants height was measured using a wall studio-meter (Bodycare Ltd, Warwickshire, UK), whilst nude body mass (NBM) was measure using digital platform scales to the nearest 50 g (Seca 705, Vogel & Halke, Hamburg, Germany for **Chapters 4** and **6**; STW-150KE, Hampel Electronics, Zhonghe, Taiwan for **Chapters 5** and **7**). Body composition was determined by whole body Dual-Energy X-ray Absorptiometry (DEXA; Hologic

QDR1500, software version 5.72, Bedford, USA). Participants were asked to remove all metal objects and dress in shorts and t-shirt only. DEXA procedure and subsequent radiation exposure, equal to 3.6 μSv, were explained to participants prior to scanning.

3.3 Maximal Oxygen Uptake

To determine maximal oxygen uptake ($\dot{V}O_{2max}$) and exercise workload for the experimental trials, participants performed a continuous incremental running exercise test to volitional exhaustion on a motorised treadmill (Woodway GmbH, D-79576, Weil am Rhein, Germany for **Chapters 4** and **6**; and h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany for **Chapters 5** and **7**). The running exercise $\dot{V}O_{2max}$ tests began with a treadmill speed of 8 $km \cdot h^{-1}$ and 1% inclination. Speed was increased by 2 $km \cdot h^{-1}$ every 3 min until reaching 16 $km \cdot h^{-1}$, upon which inclination was increased by 2.5% every 3 min until participants reached volitional exhaustion. Criteria for attaining $\dot{V}O_{2max}$ included participants reaching volitional exhaustion (rating of perceived exhaustion (RPE) at 20; Borg, 1982), a heart rate (HR) within 10 $beats \cdot min^{-1}$ of age predicted HR_{max} and a respiratory exchange ratio ≥1.15 (Bird & Davison, 1997). During the $\dot{V}O_{2max}$ tests, expired gas was continually analysed using an on-line breath-by-breath system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany). From the $\dot{V}O_2$-work rate relationship, the treadmill speed and gradient for the experimental trials exercise workload was extrapolated and verified. During the $\dot{V}O_{2max}$ tests, two fans were placed one metre from the treadmill at a speed of 2.3 $m \cdot s^{-1}$ (VelociCal, TSI, St Paul, USA).

3.4 Sample Collection and Analysis

For experimental consistency, throughout all experimental trials, venous blood samples were taken first, followed by unstimulated saliva samples, mid-flow urine samples, and

finally anthropometric measurements. Blood, saliva and urine samples were collected at least 15 min after fluid consumption. Additionally, baseline blood and saliva samples were collected after participants had remained seated for at least 10 min.

Blood collection and analysis. Whole blood samples were collected by venepuncture from an antecubital vein into two K_3EDTA vacutainer tubes (4 ml, 1.6 $mg \cdot ml^{-1}$ of ethylenediaminetetraacetic acid; Becton Dickinson, Oxford, UK) and two lithium heparin vacutainer tubes (4 ml, 1.5 $IU \cdot ml^{-1}$ of heparin; Becton Dickinson, Oxford, UK). For each sample taken, one K_3EDTA vacutainer tube was stored at room temperature prior to haematological analysis within 6 h of collection. Haematological analysis, which included haemoglobin concentration, total and differential circulating leukocyte counts were performed using an automated cell counter (Gen-S, Beckman Coulter, High Wycombe, UK) at the local hospital (Haematology Department, Ysbyty Gwynedd). Haematocrit was determined by capillary method (Maughan *et al.*, 2001) in triplicate using lithium heparin blood samples and a micro-haematocrit reader (Hawksley & Sons Limited, Lancing, UK). All blood borne immune and hormonal indices measured were corrected for changes in plasma volume (Dill & Costill, 1974). An additional 1 ml volume of lithium heparin blood was used to determine bacterially-stimulated neutrophil elastase release. The remaining lithium heparin and K_3EDTA samples were centrifuged (Rotina 35R, Hettich, Tuttlingen, Germany) at 1500 *g* for 10 min at 4°C within 15 min of sample collection. Plasma was aspirated into eppendorfs and stored at -80°C for further analysis.

As previously described (Robson *et al.*, 1999; Laing *et al.*, 2008), 1 ml volume of lithium heparin blood was added to 50 µl of 10 $mg \cdot ml^{-1}$ bacterial stimulant (nonviable bacterial extract; Sigma, Poole, UK) within 5 min of collection and gently vortex-mixed. Samples

were immediately placed in a water bath (Grant Instruments Ltd., Cambridge, UK), at 37°C for 60 min, and further mixed by gently inversion at 30 min. After 60 min, samples were centrifuged (Mikro 20, Hettich, Tuttlingen, Germany) at 5000 *g* for 2 min. The supernatant was then aspirated into eppendorfs and stored at -80°C for further analysis. Plasma elastase concentration and elastase release per neutrophil, which are markers of neutrophil degranulation activity (Robson *et al.*, 1999; Laing *et al.*, 2005), was determined by using Enzyme-Linked Immunosorbent Assay (ELISA; Biovendor Laboratory Medicine, Modrice, Czech Republic) in unstimulated and bacterially-stimulated lithium heparin plasma. All samples used to determine plasma elastase concentration were run on the same day, with standards and controls for each plate. For the experimental chapters within this thesis, the intra-assay Coefficient of Variation (CV) for plasma elastase concentration was 3.2% .

Plasma glucose was determined on lithium heparin plasma using a spectrophotometric kit (Randox, County Antrim, UK; **Chapters 5**, **6** and **7**). Aliquots of lithium heparin plasma were also used to determine plasma insulin (**Chapters 6** and **7**; DRG Diagnostics, Marburg, Germany) and cortisol concentrations using ELISA (DRG Diagnostics, Marburg, Germany; **Chapters 4**, **5**, **6** and **7**). All insulin and cortisol samples analysed were run on the same day, with standards and controls on each plate. Aliquots of K_3EDTA plasma were used for the determination of plasma adrenaline and noradrenaline concentrations using high-pressure liquid chromatography (Chapters **5** and **6**; Chromsystems, Munchen, Germany; analysis conducted by Hannover Medical School, Department of Sports & Exercise Physiology, Germany). For the experimental chapters within this thesis, the CV's for plasma glucose, insulin, cortisol, adrenaline and noradrenaline concentrations were 3.4%, 3.8%, 3.5%, 5.2% and 5.9%, respectively.

Saliva collection and analysis. Prior to saliva collection, participants were asked to thoroughly rinsing the mouth with sterile water and swallow in order to empty the mouth. Unstimulated whole saliva samples were collected by a dribbling method for 4 min into pre-weighed 30 ml universal tubes (HR 120-EC, A & D instruments, Tokyo, Japan). Participants were asked to lean forward and passively drool into the universal tube with minimal orofacial movements. All saliva samples were collected while participants remained quietly seated in the laboratory.

Saliva volume was measured by weighing the universal tube immediately after collection to the nearest milligram and saliva density was assumed to be 1 $g \cdot ml^{-1}$ (Cole & Eastoe, 1988). From this, saliva flow rate in $\mu l \cdot min^{-1}$ was determined by dividing the volume of saliva by the collection time. Aliquots of saliva were pipetted into eppendorfs and stored frozen at -80°C prior to analysis. After thawing, samples were centrifuged at 5000 *g* for 2 min, and subsequently used to determine saliva S-IgA concentration by polymeric-IgA-directed ELISA (**Chapters 4, 5** and **6**; Immundiagnostik, Bensheim, Germany), whilst saliva IgA concentration was determine by IgA antibody ELISA (**Chapter 7**; DiaMetra, Segrate, Italy). Saliva S-IgA and IgA secretion rates were calculated by multiplying the saliva flow rate by concentration. For the experimental chapters within this thesis, the CV's for saliva S-IgA and IgA concentrations were 2.9% and 4.2%, respectively.

Urine collection and analysis. All urine samples were collected mid flow into 30 ml universal tubes. Urine specific gravity (USG) determined by a handheld refractometer (Atago Uricon-Ne, NSG Precision Cells, New York, USA), urine osmolality determined by a handheld osmometer (Pocket Osmocheck, Vitech Scientific, West Sussex, UK), and urine colour were used to establish hydration status (Armstrong *et al.*, 1994).

3.5 Statistical Analysis

The primary variables of this thesis were circulating leukocyte trafficking (circulating total leukocyte, neutrophil and lymphocyte counts), neutrophil degranulation (unstimulated and bacterially-stimulated plasma elastase concentration, and elastase release per neutrophil), saliva IgA responses (saliva flow rate, saliva S-IgA/IgA concentration and secretion rate), plasma glucose and plasma hormone concentrations (insulin, cortisol, adrenaline and noradrenaline). The required sample size for variables measured was established to be between four and ten participants (www.dssresearch.com/toolkit/sscalc) using previous data examining the effects of physiological stress and carbohydrate feeding during exercise on selected immune responses (Bishop *et al.*, 2002; Oliver *et al.*, 2007; Laing *et al.*, 2008a; Laing *et al.*, 2008b). Alpha and beta levels were set at 0.05 and 0.8 respectively, both of which are standard estimates (Jones *et al.*, 2003). The acceptance level of significance was set at $P< 0.05$.

In each experimental study participants completed all trials. Subsequently, unless otherwise stated, repeated measures Analysis of Variance (ANOVA) was used to identify main effects and interactions in the dependent variables (SPSS version 16.0, Illinois, USA). Assumptions of homogeneity and sphericity were checked, and where appropriate adjustment to the degrees of freedom was made using the Greenhouse-Geisser correction method. Significant main effects were analysed using post hoc Tukey's HSD test, or Bonferroni corrected t-tests where appropriate. Data in text and tables are presented as mean value ± standard deviation (SD). For clarity, data in figures are presented as mean value ± standard error of the mean (SEM).

CHAPTER FOUR

The Effects of a 30 h Period of Total Sleep-Deprivation on Selected Immune Responses at Rest and in Response to Exercise

4.1 Summary: The aim was to determine the effect of one night without sleep on selected immune and plasma cortisol responses at rest and in response to subsequent prolonged strenuous exercise. Using a repeated measures cross-over design, on one occasion eleven male participants slept normally (CON) and on another occasion they were totally sleep deprived for 30 h (SDEP). After 30 h participants performed 30 min steady state (SS) treadmill exercise at 60% $\dot{V}O_{2max}$ followed by a 30 min treadmill time trial (TT). Circulating leukocyte counts, bacterially-stimulated neutrophil degranulation, saliva S-IgA and plasma cortisol responses were determined from blood and saliva samples collected at 08:00 h on day one (baseline (BL)), 30 h, post-SS, post-TT, 2 h post-TT and 18 h post-TT. No trial x time interactions were observed for selected immune and plasma cortisol responses. A circulating leukocytosis, neutrophilia, and lymphocytosis were observed post-TT compared with 30 h ($P< 0.01$). In addition, at post-TT compared with 30 h a decrease in bacterially-stimulated neutrophil degranulation (20%; $P< 0.05$) and an increase in saliva S-IgA concentration (83%) were observed ($P< 0.01$). Plasma cortisol concentration increased post-TT (62%) compared with post-SS ($P< 0.01$). In conclusion, a 30 h period of total sleep-deprivation does not alter circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation or saliva S-IgA responses either at rest or after submaximal and strenuous exercise.

4.2 Introduction

In a similar manner to the acute immune response to strenuous exercise (Gleeson, 2007), two to three nights of total sleep-deprivation has been shown to elicit a circulating leukocytosis (Dinges *et al.*, 1994), and a decrease in neutrophil and lymphocyte function (Palmblad *et al.*, 1976; Palmblad *et al.*, 1979; Moldofsky *et al.*, 1989a; Moldofsky *et al.*, 1989b). Although the clinical significance of a decrease in immune function with prolonged sleep-deprivation remains unclear, decreases in neutrophil function for example have been implicated in increased infection incidence in clinical populations (Ellis *et al.*, 1988; Smitherman & Peacock, 1995). A stimulatory effect of sleep deprivation on stress hormones (Radomski *et al.*, 1992) may mediate the altered immune response, which in turn might increase susceptibility to infection (Dinges *et al.*, 1995; Irwin, 2002).

The effect of one night of total sleep-deprivation on immune function remains unclear due to limited and conflicting evidence, and a lack of experimental control in previous studies. For example, a one night period of total sleep-deprivation increased circulating leukocyte counts in one study (Born *et al.*, 1997), but had no effect in another (Heiser *et al.*, 2000). In addition, one study showed that only one night of a three night period of total sleep-deprivation was sufficient to decrease neutrophil phagocytosis (Palmblad *et al.*, 1976). However, the female participants were exposed to additional stressors that could account, at least in part, for the observed decrease in neutrophil function. It is plausible that exercise stress after one night of total sleep-deprivation may amplify stress hormone responses and associated exercise-induced immune perturbations (Gleeson, 2007). However, to date the influence of sleep-deprivation on immune indices in response to subsequent exercise has not been investigated.

Therefore, the aim of the present study was to determine the effects of one night of total sleep-deprivation on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva S-IgA and plasma cortisol responses at rest and following prolonged submaximal and strenuous exercise. It was hypothesised that performing exercise after total sleep-deprivation would evoke greater immune disturbances compared with performing exercise after one night of normal sleep.

4.3 Methods

Participants. Eleven healthy recreationally active males (age 20 ± 3 y; NBM 77.6 ± 7.8 kg; height 1.80 ± 0.05 m; body fat 13.2 ± 4.5 %; $\dot{V}O_{2max}$ 55.5 ± 5.6 $ml \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in the study. Ethical procedures were followed in accordance with **Section 3.1.**

Preliminary measurements. Prior to the first experimental trial, participants were asked to report to the laboratory where the preliminary measurements as described in **Section 3.2** and **3.3** were undertaken. The treadmill speed (9.6 ± 0.6 $km \cdot h^{-1}$) at 1% gradient that elicited 60% $\dot{V}O_{2max}$ for participants steady state (SS) exercise was extrapolated and verified. A time trial (TT) familiarisation was also performed. Blinded to the treadmill speed, participants controlled the speed of the motorised treadmill at 1% gradient, and were instructed to run as fast as possible for 30 min.

Experimental trials. Using a randomised cross-over design, participants completed two trials separated by seven days. On one occasion participants were allowed normal nocturnal sleep (CON: 496 ± 18 min) and on another occasion participants were totally sleep deprived for 30 h (SDEP). To control dietary and fluid intake prior to and during each experimental trial, participants were provided with food and fluid which catered for their estimated daily energy (3280 ± 209 $Kcal \cdot day^{-1}$; Cunningham, 1980) and fluid (35 $ml \cdot kg^{-1}BM \cdot day^{-1}$; Todorovic & Micklewright, 2004) requirements. Urine measurements as previously described in **Section 3.4**, indicated euhydration in all participants on arrival at the laboratory for each trial. For the experimental trials participants reported to the laboratory at 0600 h after a night of normal sleep (CON: 517 ± 19 min; SDEP: 513 ± 21

min) and resided in laboratory accommodation until 1800 h the following day. Nocturnal movements were monitored by an accelerometer (GT1M, ActiGraph LLC, Florida, USA).

At 1200 h on the second day of each trial participants began the exercise protocol. The exercise protocol was performed on a motorised treadmill and consisted of a 30 min SS run at 60% $\dot{V}O_{2max}$, followed by 15 min seated rest and then a 30 min TT (Oliver *et al.*, 2009). HR measured by short-range radiotelemetry monitor (Polar Electro, Kempele, Finland) was recorded every 5 min during SS and TT. During the SS and TT exercise bout participants were not permitted to drink fluids. Both exercise bouts were performed in ambient conditions of 20°C and 59% relative humidity with two fans placed one metre from the treadmill at a speed of 2.3 $m \cdot s^{-1}$. In addition, NBM measurements were taken at 0800 h on day one (baseline (BL)), 30 h, post-SS, post-TT, 2 h and 18 h post-TT.

Blood collection and analysis. Whole blood samples were collected as previously described in **Sections 3.4**, at BL, 30 h, post-SS, post-TT, 2 h and 18 h post-TT. Blood analysis was performed in accordance with **Section 3.4**. Bacterially-stimulated neutrophil degranulation was not measured at 18 h post TT (**Table 4.2**), since previous studies report bacterially-stimulated neutrophil degranulation returning to baseline values within 6 h after cessation of prolonged strenuous exercise (Robson *et al.*, 1999; Li & Cheng, 2007).

Saliva collection and analysis. Unstimulated whole saliva samples were collected as previously described in **Sections 3.4**, at BL, 30 h, post-SS, post-TT, 2 h and 18 h post-TT. Saliva analysis was performed in accordance with **Section 3.4**.

Statistical Analysis. Statistical analysis of data was performed in accordance with **section 3.5**. Due to participant specific incomplete sample set and data outliers for bacterially-stimulated neutrophil degranulation (**Table 4.2**) and saliva S-IgA responses (**Table 4.3**), the data set of three participants were removed prior to data analysis.

4.4 Results

There were no trial x time interactions, but there was a significant main effect of time (MEOT) for exercising HR (SS: $F(6,60)= 262.4$, $P< 0.01$; TT: $F(6,60)= 232.0$, $P< 0.01$), plasma volume change ($F(5,50)= 20.0$, $P< 0.01$), NBM ($F(5,50)= 30.3$, $P< 0.01$), and plasma cortisol concentration ($F(5,50)= 12.7$, $P< 0.01$). Mean exercising HR was similar during SS (CON: 143 ± 4 beats·min^{-1}; SDEP: 143± 5 beats·min^{-1}) and TT (CON: 183 ± 3 beats·min^{-1}; SDEP: 181 ± 3 beats·min^{-1}) between trials. Plasma volume change was -8.8 ± 1.8 % post-TT compared with BL. A -1.0 (± 0.2)% decrease in NBM was observed post-TT ($P< 0.01$ *vs.* 30 h). Plasma cortisol concentration decreased after 30 h (348 ± 39 nmol·L^{-1}) compared with BL (500 ± 47 nmol·L^{-1}: $P< 0.01$) and increased after the TT (440 ± 45 nmol·L^{-1}) compared with post-SS (271 ± 16 nmol·L^{-1}: $P< 0.01$).

Circulating leukocyte, neutrophil and lymphocyte counts. There were no trial x time interactions, but there was a significant MEOT for circulating leukocyte ($F(5,50)= 6.5$, $P< 0.01$), neutrophil ($F(5,50)= 35.4$, $P< 0.01$), and lymphocyte counts ($F(5,50)= 19.4$, $P< 0.01$) counts (**Table 4.1**). A leukocytosis, neutrophilia and lymphocytosis were observed after the TT ($P< 0.01$). However, lower circulating lymphocyte counts were observed after 30 h, post-SS and 2 h post-TT compared with BL ($P< 0.01$).

Bacterially-Stimulated Neutrophil degranulation. There were no trial x time interactions, but there was a significant MEOT for unstimulated plasma elastase concentration ($F(4,28)= 15.2$, $P< 0.01$), bacterially-stimulated elastase concentration ($F(4,28)= 61.4$, $P< 0.01$) and bacterially-stimulated elastase release per neutrophil ($F(4,28)= 6.3$, $P< 0.01$; **Table 4.2**). Unstimulated elastase concentration increased post-TT compared with BL ($P< 0.01$). Bacterially-stimulated elastase concentration was higher than BL from 30 h

onwards ($P< 0.05$), whilst bacterially-stimulated elastase release per neutrophil was lower post-TT (20%) and 2 h post-TT (17%) compared with 30 h, but was not significantly lower than BL during recovery.

Saliva S-IgA responses. There were no trial x time interactions or main effects for saliva flow rate or saliva S-IgA secretion rate (**Table 4.3**). However, a significant MEOT was observed for saliva S-IgA concentration ($F(5,35)= 4.1$, $P< 0.01$). A significant increase in saliva S-IgA concentration (83%) was observed post-TT compared with 30 h ($P< 0.05$; **Table 4.3**).

Table 4.1: Circulating leukocyte, neutrophil and lymphocyte count responses to 30 min steady state (SS) treadmill exercise at 60% $\dot{V}O_{2max}$ followed by a 30 min time trial (TT) after sleep (CON) and 30 h total sleep-deprivation (SDEP).

		BL	30 h (Pre-SS)	Post SS	Post TT	2 h post TT	18 h post TT
		(08:00 h day 1)	(12:00 h day 2)	(12:30 h day 2)	(13:15 h day 2)	(15:15 h day 2)	(07:15 h day 3)
					##**	##**	
Leukocyte	CON	6.5 ± 2.1	5.6 ± 2.0	6.9 ± 2.8	10.4 ± 4.4	9.8 ± 3.5	6.0 ± 1.9
($x10^9 \cdot L^{-1}$)	SDEP	5.9 ± 1.4	6.0 ± 1.6	7.1 ± 1.9	10.7 ± 2.9	10.1 ± 2.5	5.8 ± 1.3
					##*	##**	
Neutrophil	CON	3.6 ± 1.8	3.7 ± 1.9	5.1 ± 2.7	6.7 ± 3.9	8.1 ± 3.6	3.7 ± 1.6
($x10^9 \cdot L^{-1}$)	SDEP	3.0 ± 0.9	4.1 ± 1.3	5.1 ± 1.6	7.6 ± 2.7	8.3 ± 2.4	3.6 ± 1.1
			##	##	**	##	
Lymphocyte	CON	2.1 ± 0.7	1.3 ± 0.6	1.3 ± 0.5	2.4 ± 0.9	1.1 ± 0.5	1.5 ± 0.4
($x10^9 \cdot L^{-1}$)	SDEP	2.1 ± 0.5	1.4 ± 0.4	1.4 ± 0.5	2.4 ± 0.7	1.2 ± 0.4	1.5 ± 0.5

Mean ± SD (n= 11): MEOT ## P< 0.01 *vs.* BL; * P< 0.05 and ** P< 0.01 *vs.* 30 h.

Table 4.2: Unstimulated and bacterially-stimulated neutrophil degranulation responses to 30 min steady state (SS) treadmill exercise at 60% $\dot{V}O_{2max}$ followed by a 30 min time trial (TT) after sleep (CON) and 30 h total sleep-deprivation (SDEP).

		BL	30 h (Pre-SS)	Post SS	Post TT	2 h post TT
		(08:00 h day 1)	(12:00 h day 2)	(12:30 h day 2)	(13:15 h day 2)	(15:15 h day 2)
					##**	
Unstimulated plasma elastase ($ng \cdot ml^{-1}$)	CON	40 ± 14	43 ± 15	85 ± 40	272 ± 158	93 ± 27
	SDEP	36 ± 8	53 ± 34	74 ± 32	209 ± 118	70 ± 25
			#	##*	##**	##**
Stimulated plasma elastase ($ng \cdot ml^{-1}$)	CON	2529 ± 1073	3705 ± 1357	4704 ± 1628	5463 ± 1567	5759 ± 1047
	SDEP	2506 ± 938	2791 ± 679	3476 ± 1399	5027 ± 1405	5714 ± 878
					**	**
Stimulated elastase per cell ($fg \cdot cell^{-1}$)	CON	416 ± 93	496 ± 131	453 ± 124	358 ± 113	367 ± 125
	SDEP	428 ± 99	414 ± 102	399 ± 63	372 ± 111	389 ± 107

Mean ± SD (n= 8): MEOT # $P< 0.05$ and ## $P< 0.01$ *vs.* BL; MEOT * $P< 0.05$ and ** $P< 0.01$ *vs.* 30 h.

Table 4.3: Saliva flow rate, saliva S-IgA concentration and secretion rate responses to 30 min steady state (SS) treadmill exercise at 60% $\dot{V}O_{2max}$ followed by a 30 min time trial (TT) after sleep (CON) and 30 h total sleep-deprivation (SDEP).

		BL	30 h (Pre-SS)	Post SS	Post TT	2 h post TT	18 h post TT
		(08:00 h day 1)	(12:00 h day 2)	(12:30 h day 2)	(13:15 h day 2)	(15:15 h day 2)	(07:15 h day 3)
Saliva flow rate ($\mu l \cdot min^{-1}$)	CON	198 ± 135	236 ± 178	225 ± 135	179 ± 120	271 ± 175	172 ± 115
	SDEP	245 ± 185	253 ± 189	228 ± 163	224 ± 176	360 ± 170	277 ± 218
Saliva S-IgA concentration ($\mu g \cdot ml^{-1}$)	CON	355 ± 155	232 ± 77	406 ± 182	482 ± 289 *	231 ± 75	342 ± 237
	SDEP	328 ± 116	296 ± 61	371 ± 113	484 ± 310	226 ± 132	260 ± 86
Saliva S-IgA secretion rate ($\mu g \cdot min^{-1}$)	CON	67 ± 26	80 ± 49	104 ± 74	98 ± 78	78 ± 54	76 ± 47
	SDEP	88 ± 66	85 ± 64	86 ± 64	101 ± 86	80 ± 61	78 ± 68

Mean ± SD (n= 8): * P< 0.05 *vs.* 30 h.

4.5 Discussion

The effects of sleep deprivation on immune function in response to subsequent strenuous exercise has not previously been investigated. The current data does not support the hypothesis, as it shows that a 30 h period of total sleep-deprivation does not alter circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation or saliva S-IgA responses either at rest or after subsequent prolong strenuous exercise.

The observed alterations in circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva S-IgA in the present study most likely reflect circadian variations (Shephard & Shek, 1997), and the widely reported acute effects of strenuous exercise (Gleeson, 2007). The results agree with one study showing no change in circulating leukocyte counts after one night of total sleep-deprivation (Heiser *et al.*, 2000). Conversely, disagrees with other studies that showed an increase in circulating leukocyte counts (Born *et al.*, 1997), and a decrease in neutrophil phagocytosis after a night without sleep (Palmblad *et al.*, 1976). It is conceivable though that the decrease in neutrophil phagocytosis after one night of total sleep-deprivation shown previously (Palmblad *et al.*, 1976) was due to the additional stressors that participants were exposed to, these included battlefield noise and a rifle shooting task.

Typically, longer periods of total sleep-deprivation ranging from 40 to 77 h have been shown to elicit a circulating leukocytosis (Dinges *et al.*, 1994), and a decrease in lymphocyte and phagocyte function (Palmblad *et al.*, 1976; Palmblad *et al.*, 1979, Moldofsky *et al.*, 1989b). Therefore, it is possible that the relatively short period of total sleep-deprivation in the present study was not long enough to alter stress hormone and immune responses. Nevertheless, the results have practical relevance as they clearly

demonstrate that one night of total sleep-deprivation does not amplify stress hormone and associated immune responses to subsequent strenuous exercise. It remains to be shown if more prolonged periods of sleep-deprivation or more prolonged exercise following a similar period of sleep-deprivation to that used in the present study alters immune function or infection incidence. For example, it is very difficult to isolate an effect of sleep-deprivation, if any, on the decrease in immune function (e.g. decreased saliva S-IgA concentration) observed in military personnel during more prolonged five to seven days combat training exercises (Boyum *et al.*, 1996; Gomez-Merino *et al.*, 2003). In these studies, participants were exposed to a combination of stressors that could account for decreased immune function, including sleep-deprivation and prolonged physical exertion (Gleeson, 2007), but also including fluid and energy restriction (Oliver *et al.*, 2007; Laing *et al.*, 2008b), psychological stress (Keller *et al.*, 1981), and exposure to extreme environments (Walsh & Whitham, 2006).

In conclusion, these results show that a 30 h period of total sleep-deprivation does not alter circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva S-IgA responses or plasma cortisol concentration either at rest or following submaximal or strenuous exercise. A follow-up study should aim to identify the influence of more prolonged total sleep-deprivation with and without additional physiological stressors (e.g. energy-restriction, exposure to extreme environments) on selected immune responses.

CHAPTER FIVE

The Effects of Two Nights of Total Sleep-Deprivation With and Without Energy-Restriction on Selected Immune Responses at Rest and in Response to Cold-Exposure

5.1 Summary: The aims were to determine the effects of two nights of total sleep-deprivation with and without energy-restriction on selected immune and stress hormone responses at rest and in response to cold-exposure. Additionally, to determine the effects of passive cold-exposure on the same immune and stress hormone responses. On three randomised occasions ten males slept normally (436 ± 21 min·night^{-1}; CON), were totally sleep deprived (SDEP), or were totally sleep deprived and 90% energy restricted (SDEP+ER) for 53 h. After 53 h (1200 h) participants performed a seated cold air test (CAT) at 0.0°C until T_{re} decreased to 36.0°C. Circulating leukocyte counts, bacterially-stimulated neutrophil degranulation, saliva S-IgA and plasma stress hormone responses were determined from venous blood and unstimulated saliva samples collected at 08:00 h on day one (baseline (BL)), 24 h, 48 h, pre-CAT, post-CAT, 1 h and 2 h post-CAT. One night on SDEP increased bacterially-stimulated neutrophil degranulation (21%, $P< 0.05$), and two nights on SDEP and SDEP+ER increased S-IgA concentration (40% and 44%, respectively; $P< 0.01$). No other significant effects were observed for immunoendocrine measures prior to CAT. CAT duration was not different between trials (133 ± 53 min), and T_{re} decreased to 35.9°C. Modest whole-body cooling decreased circulating lymphocyte counts (25%; $P< 0.01$), S-IgA concentration (36%; $P< 0.01$), and secretion rate (24%; $P< 0.05$). A neutrophilia occurred post-CAT on CON and SDEP, and 2 h post-CAT on SDEP+ER ($P< 0.01$). Modest whole-body cooling also decreased bacterially-stimulated neutrophil degranulation on CON (22%), and SDEP (18%; $P< 0.05$). Plasma cortisol and noradrenaline increased post-CAT (31% and 346%, respectively; $P< 0.05$), but modest whole-body cooling did not alter plasma adrenaline. In conclusion, two nights of SDEP or SDEP+ER did not compromise selected immune responses at rest. However, modest whole-body cooling (T_{re} 35.9°C) decreased circulating lymphocytes, bacterially-stimulated neutrophil degranulation and S-IgA responses, but these responses were not amplified by prior SDEP or SDEP+ER.

5.2 Introduction

Prolonged strenuous exercise, energy-restriction, sleep-deprivation and exposure to environmental extremes are often encountered by those with extremely demanding occupations and pastimes. It is conceivable that impairment of immune responses may occur during activities involving a combination of physiological stressors. For example, military field studies incorporating five to seven days of heavy exertion, energy-restriction, sleep-deprivation and exposure to environmental extremes report decreased cellular, humoral and mucosal immunity, and increased infection rates leading to course failure (Martinez-Lopez *et al.*, 1993; Boyum *et al.*, 1996; Tiollier *et al.*, 2005). However, it is not possible to determine whether an individual stressor or a combination of stressors is responsible for the decreased immunity during these field studies, due to lack of adequate research control. It is likely that the interaction of these stressors on immune function is complex, further supporting the notion that investigations should identify both the independent and combined effects of stressors on immune function. Such an experimental approach is important because these stressors may be experienced alone or in combination in the field setting, and because the results from appropriately designed laboratory studies may provide important information about effective countermeasures.

Although heavy exertion and prolonged nutritional restriction are now widely recognised to decrease many aspects of immune function (Gleeson, 2007; Oliver *et al.*, 2007; Laing *et al.*, 2008b), the influence of sleep-deprivation and exposure to environmental extremes, particularly cold exposure, remain topics of debate (**Chapter 4**; Walsh & Whitham, 2006). **Chapter 4** demonstrated that a 30 h period of total sleep-deprivation did not alter circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation or saliva S-IgA responses either at rest or in response to prolonged strenuous exercise. However,

longer periods of total sleep deprivation (≥48 h) have been associated with a circulating leukocytosis (Dinges *et al.*, 1994) and decreases in neutrophil and lymphocyte function (Palmblad *et al.*, 1976; Palmblad *et al.*, 1979; Moldofsky *et al.*, 1989b). Surprisingly, there is also limited and somewhat conflicting information about the effects of cold-exposure on immune function (Walsh & Whitham, 2006). Evidence to date indicates that a very mild decrease in T_{core} (~0.5°C decrease) during short (30 min; Lackovic *et al.*, 1988) or prolonged (2 h; Brenner *et al.*, 1999) cold air exposure can actually have immune-stimulatory effects. Yet little information is available from tightly controlled laboratory studies about the effects of a decrease in T_{core} ≥1.0°C on immune function. It is conceivable that a continuum exists for the effects of core body cooling on immune function. Whereby, very mild decreases in T_{core} (e.g. ≤1.0°C decrease in T_{core}) have little or even stimulatory effects on immune function, while more severe decreases in T_{core} (≥1.0°C decrease in T_{core}) may have depressive effects on immune function. In line with this contention, one study showed that severe hypothermia in patients during surgery (~4.0°C decrease T_{re}) decreased aspects of neutrophil function, but the lack of experimental control presents limitations (Wenisch *et al.*, 1996a). It remains to be shown whether immune function is decreased by a more modest reduction in T_{core} (e.g. 1 to 2°C decrease in T_{re}), similar to that experienced during military, exploration, adventure and ultra-endurance activities, performed in cold and wet conditions.

With this information in mind, the purpose of the present study was to determine the effects of two nights of total sleep-deprivation with and without energy-restriction on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva S-IgA and stress hormone responses at rest and in response to cold-exposure. Additionally, to determine the effects of passive cold-exposure on the same selected immune responses. It

was hypothesised that total sleep-deprivation and energy-restriction would have additive detrimental effects on immune responses at rest. It was further hypothesised that a modest reduction in T_{core} ($\geq$1°C decrease in T_{re}) would depress immune responses, and that prior total sleep-deprivation and energy-restriction would amplify this response.

5.3 Methods

Participants. Ten healthy, recreationally active males (age 25.0 ± 6.3 y; height 178 ± 4 cm; NBM 79.4 ± 8.6 kg; body fat 17 ± 5 %; $\dot{V}O_{2max}$ 57.3 ± 8.4 $ml \cdot kg^{-1}BM \cdot min^{-1}$) volunteered to participate in the study. Ethical procedures were followed in accordance with **section 3.1.**

Preliminary measurements. Prior to the first experimental trial, participants were asked to report to the laboratory where preliminary measurements as described in **section 3.2** and **3.3** were undertaken. The treadmill walking speed (6.0 ± 0.5 $km \cdot h^{-1}$) and gradient (6.9 ± 2.0 %) that elicited 50% $\dot{V}O_{2max}$ for participants SS exercise was extrapolated and verified. A TT familiarisation was also performed. Blinded to the treadmill speed, participants controlled the speed of the motorised treadmill at 1% gradient, and were instructed to run 5 km as fast as possible. Additionally, resting metabolic rate (RMR) was determined by indirect calorimetry (Compher *et al.*, 2006), and adjusted by a general daily physical activity and diet induced thermogenesis factor coefficient of 1.2 (Todorovic & Micklewright, 2004). Estimated energy expenditure for SS exercise and the TT was predicted by indirect calorimetry (Weir, 1949), and was combined with the adjusted RMR data to predict total daily energy expenditure, and subsequent use in energy intake programming for the experimental trials. Finally, participants were also familiarised with a 20 min CAT, which was conducted in an environmental chamber (WIR Series, Design Environmental Ltd., Ebbw Vale, UK) set at 0.0°C and 40% RH.

Experimental trials. Using a randomly assigned repeated measures design, participants completed three experimental trials each separated by twelve days (**Figure 5.1**). The three experimental trials included; a control trial (CON), a total sleep-deprivation trial (SDEP),

and a total sleep-deprivation and energy-restriction trial (SDEP+ER). Participants were allowed normal nocturnal sleep on CON (436 ± 21 min·night^{-1}), but were total deprived of sleep on SDEP and SDEP+ER for 53 h. Dietary intake was provided to meet predicted total daily energy requirements (3316 ± 505 Kcal·day^{-1}, 54 ± 2 % carbohydrate, 31 ± 2 % fat, 14 ± 2 % protein) on CON and SDEP, but was 90% restricted on SDEP+ER for 53 h. Water was provided throughout each experimental trials at 2 h intervals (divided equally over 16 h; 0700 h to 2300 h), equivalent to 35 ml·kg^{-1}BM·day^{-1} (2785 ± 300 ml·day^{-1}; Todorovic & Micklewright, 2004), with estimated fluid losses during exercise added to this total. Daily physical activity was monitored by electronic pedometer (Digi-walker SW-200, Yamax, Tokyo, Japan), whilst participants sleep-wake cycles were monitored by an accelerometer (GT1M, ActiGraph LLC, Florida, USA; Ancoli-Israel *et al.*, 2003). Participants remained in the laboratory building living quarters for the entire experimental period during all trials, and were supervised by the research team at all times. In addition, NBM measurements were taken at 08:00 h on day one (baseline (BL)), 24 h, 48 h, pre-CAT, post-CAT, 1h and 2 h post-CAT.

Experimental procedures. The day prior to each experimental trial, to standardise nutrition, hydration and sleep-wake cycles, participants were provided with their predicted total daily energy and fluid requirements, and nocturnal sleep duration (444 ± 28 min·night^{-1}) was monitored as previously mentioned. After awakening at 0700 h, participants were transported to the laboratory for each experimental trial. Participants were requested to empty their bladder and bowels prior to BL measurements. Urine measurements as previously described in **Section 3.4,** indicated euhydration in all participants on arrival at the laboratory for each trial. The experimental trial began at 0800 h on day one. At 1200 h on days one and two, participants performed 90 min SS exercise,

followed by a 15 min seated rest period, then a 5 km TT. Both exercise bouts were performed in an air-conditioned laboratory (20 ± 1°C, 59 ± 7 % RH) with one fan placed one metre from the treadmill at a speed of 2.3 $m \cdot sec^{-1}$. Each TT was performed under standardised conditions in a quiet laboratory, with only information about distance completed provided to the participants. Water was consumed *ad libitum* during SS exercise in all trials, while no fluids were allowed during the TT.

At 1200 h on day 3, participants performed a CAT conducted in an environmental chamber. Prior to the CAT, participants were fitted with a HR monitor and a thermocouple was inserted 12 cm beyond the external anal sphincter (Grant REC soft insertion probe thermocouple; Grant 2020 Squirrel data logger, Shepreth, UK) to monitor T_{re}. Participants performed the CAT clothed in only athletic shorts, socks and trainers. Ambient conditions throughout the CAT were regulated at 0.0°C, 40% RH and 0.2 $m \cdot s^{-1}$ wind velocity. T_{re} was recorded prior to and every 5 min during the CAT. Whereas, HR, ratings of McGinnis 13-point thermal comfort (Hollies & Goldman, 1977) and 10-point pain sensation (Chen *et al.*, 1998) were recorded immediately prior to and every 10 min during the CAT.

Participants assumed a standardised seated position on a steal framed wooden laboratory chair and were instructed to minimise any movements, including behavioural thermoregulation (e.g. huddling, rubbing and/or fidgeting) during the CAT. Participants remained in the environmental chamber until their T_{re} reached 36.0°C or they achieved the cut-off criteria of 4 h exposure. As a health and safety precaution, recovery T_{re} was monitored every 5 min during the first hour after the CAT. Throughout this recovery period, participants remained seated and were wrapped in a blanket. One hour after the CAT participants were allowed to shower, re-clothe and consume a standard meal (1230

Kcal, 58% carbohydrate, 28% fat, 14% protein). After the experimental trial participants were transported to their homes.

Blood collection and analysis. Whole blood samples were collected as previously described in **Sections 3.4**, at BL, 24 h, 48 h, pre-CAT, post-CAT, 1h and 2 h post-CAT. Blood analysis was performed in accordance with **Section 3.4**. Plasma adrenaline and noradrenaline concentrations were not measured at 24 h (**Table 5.2**), since significant catecholamine responses have only been observed after two nights of total sleep-deprivation (Radomski *et al.*, 1992). Additionally, Plasma adrenaline and noradrenaline concentrations were not measured at 2 h post-CAT (**Table 5.2**), since previous studies report catecholamines returning to baseline values within 1 h of cessation from physiological stress stimuli (Laing *et al.*, 2008a).

Saliva collection and analysis. Unstimulated whole saliva samples were collected as previously described in **Sections 3.4**, at BL, 24 h, 48 h, pre-CAT, post-CAT, 1h and 2 h post-CAT. Saliva analysis was performed in accordance with **Section 3.4**.

Statistical Analysis. Statistical analysis of data was performed in accordance with **Section 3.5**. However, sleep quantity, TT performance and time to T_{re} 36.0°C during the CAT were examined using a one-way ANOVA.

Figure 5.1: The effects of two nights of total sleep-deprivation with and without energy-restriction on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva S-IgA responses at rest and in response to cold-exposure. Schematic of trial events.

PRE-TRIAL DAY	CONTROLLED PHYSICAL ACTIVITY, FOOD AND FLUID INTAKE	SLEEP

DAY 1 **0700 h awakening** **CON** **SDEP** **SDEP+ER**	**SAMPLES**	**BREAKFAST**	**90 min WALK @ 50% VO_{2max} + 5km TT**	**LUNCH**		**SNACK**		**DINNER**		**SNACK**		**SLEEP** **or** **SDEP**
DAY 2 **0700 h awakening on CON**	**SAMPLES**	**BREAKFAST**	**90 min WALK @ 50% VO_{2max} + 5km TT**	**LUNCH**		**SNACK**		**DINNER**		**SNACK**		**SLEEP** **or** **SDEP**
	0800h	0845h	12-1445h	1500h		1730h		2015h		2215h		2300-0700h

DAY 3 **0700 h awakening on CON**	**SAMPLES**	**BREAKFAST**	**SAMPLES**	**CAT (53h)** **T_{amb} 0.0°C + 40% RH**	**T_{re}: 36.0°C SAMPLES**	**RE-WARM**	**SAMPLES**	**RE-FEED**	**SAMPLES**	**LEAVE**
	0800h	0845h	1145h	12-1600h	13-1600h	13-1800h	14-1700h	14-1700h	15-1800h	15-1800h

Control (CON): nocturnal sleep (436 ± 21 min·night^{-1}); total sleep-deprivation (SDEP): 53 h total sleep-deprivation; total sleep-deprivation and energy-restriction (SDEP+ER): 53 h total sleep-deprivation and 90% energy-restriction. Samples: Nude body mass, antecubital venepuncture blood and unstimulated whole saliva. Time trial (TT). Cold air test (CAT).

5.4 Results

Nude body mass, daily physical activity and sleep quantity. A trial x time interaction was observed for NBM ($F(10,90)= 35.3$, $P< 0.01$) and daily physical activity ($F(6,54)= 7.3$, $P< 0.01$; **Table 5.1**). NBM gradually decreased throughout SDEP+ER ($P< 0.01$ *vs.* BL), whereas a significant decrease in NBM was only observed post-CAT for the CON and SDEP trials ($P< 0.01$ *vs.* pre-CAT). A significantly lower NBM was observed on SDEP+ER 24 h onwards compared with CON and SDEP ($P< 0.01$), whilst no significant difference in NBM was observed between CON and SDEP throughout the trials. Total pedometer counts were significantly higher on SDEP and SDEP+ER compared with CON ($P< 0.01$). No significant difference in sleep quantity was observed the night prior to each experimental trial and during CON (**Table 5.1**).

Cold air test measurements. There was no significant difference in pre-CAT T_{re} between trials (CON: 37.18 ± 0.19°C; SDEP: 37.12 ± 0.15°C; SDEP+ER: 37.03 ± 0.14°C). Nor was there a significant difference in time to T_{re} 36.0°C was observed between trials (CON: 116 ± 38 min; SDEP: 153 ± 65 min; SDEP+ER: 130 ± 51 min). Additionally, there were no trial x time interactions for HR, T_{re}, thermal comfort and pain sensation scales during the CAT, and T_{re} during the 1 h recovery after the CAT.

Plasma volume changes. A trial x time interaction was observed for plasma volume change ($F(4,36)= 2.6$, $P< 0.05$). Compared with BL, a significant increase in plasma volume was observed at 24 h on CON (4.0%; $P< 0.05$), and at 24 h (5.7%) and 48 h (9.3%) on SDEP ($P< 0.01$). Plasma volume change was significantly greater on SDEP at 48 h compared with CON and SDEP+ER ($P< 0.01$). The CAT also elicited a change in plasma volume (MEOT; $F(3,27)= 85.1$, $P< 0.01$), whereby decreases in plasma volume

were observed post-CAT (13.5%), 1 h (9.1%) and 2 h post-CAT (4.7%) compared with pre-CAT values ($P< 0.01$).

Table 5.1: Nude body mass changes, daily physical activity and sleep quantity during a 53 h period of normal sleep (CON), total sleep-deprivation (SDEP), and total sleep-deprivation with 90% energy-restriction (SDEP+ER) prior to a cold air test (CAT).

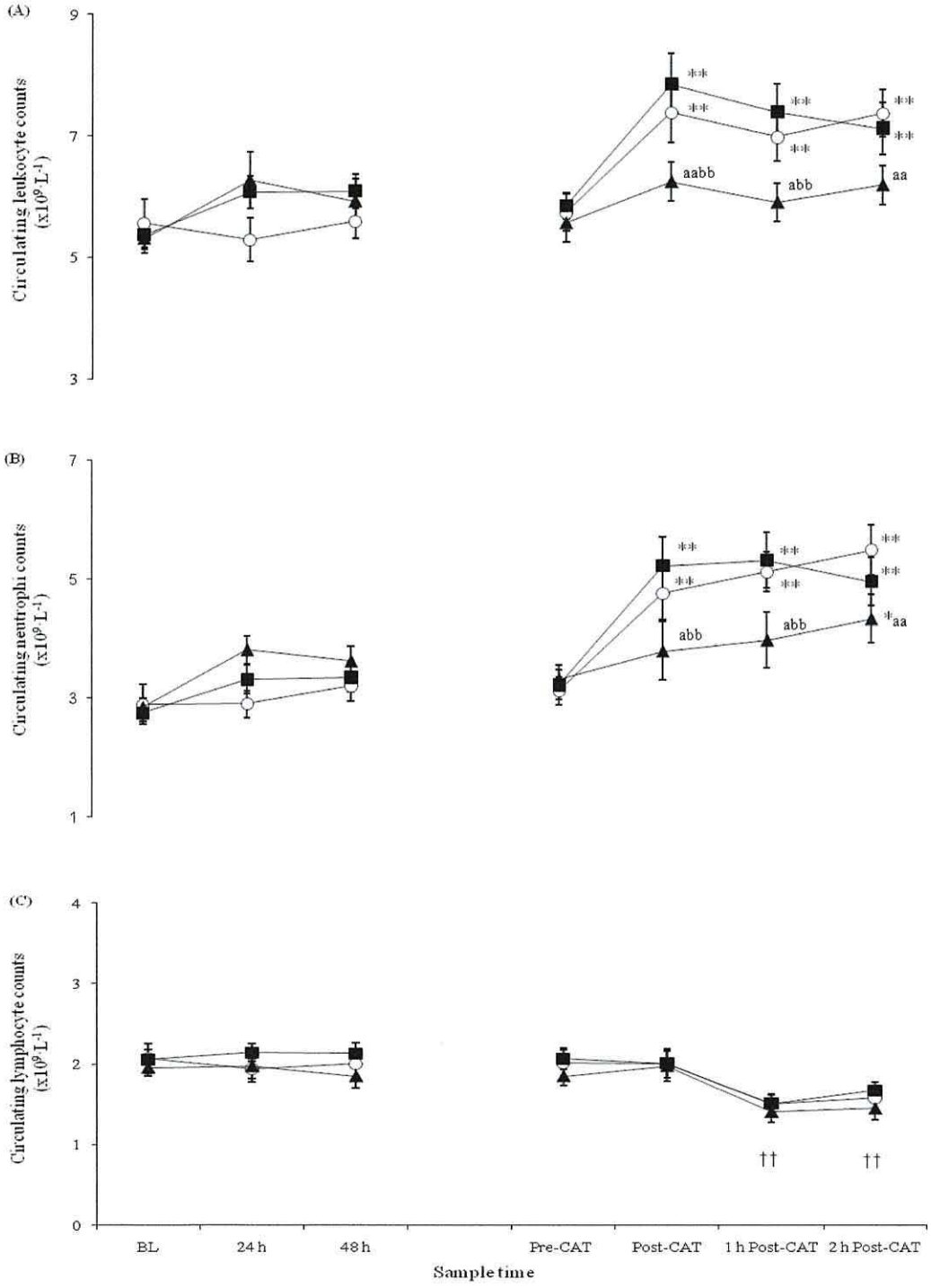
	CON	SDEP	SDEP+ER
Nude body mass (%)			
BL	78.8 ± 8.6 kg	78.7 ± 8.6 kg	79.0 ± 8.3 kg
24 h	-0.4 ± 0.4	-0.2 ± 0.6	-2.1 ± 0.7 ##aabb
48 h	-0.6 ± 0.5 #	-0.7 ± 0.6 #	-3.6 ± 0.8 ##aabb
Pre-CAT	0.0 ± 0.5	-0.4 ± 0.6	-3.8 ± 0.8 ##aabb
Post-CAT	-1.1 ± 0.7 ##**	-1.4 ± 0.7 ##**	-4.4 ± 0.9 ##**aabb
Physical activity (steps·day^{-1})			
Day one total	21384 ± 2236	23188 ± 4120	23351 ± 3049
Day two total	20425 ± 1946	24769 ± 3491 aa	25043 ± 4058 aa
Trial total (53 h)	43621 ± 4334	49798 ± 7571 aa	50184 ± 7230 aa
Sleep quantity (min·night^{-1})			
Pre-trial	437 ± 45	446 ± 21	449 ± 20
Night one	432 ± 25	0	0
Night two	440 ± 16	0	0

Mean ± SD (n= 10): # $P< 0.05$ and ## $P< 0.01$ *vs.* BL; ** $P< 0.01$ *vs.* pre-CAT; aa $P< 0.01$ *vs.* CON; bb $P< 0.01$ *vs.* SDEP.

Circulating leukocyte, neutrophil and lymphocyte counts. A trial x time interaction was observed for circulating leukocyte ($F(12,108)= 2.8$, $P< 0.01$; **Figure 5.2A**) and neutrophil counts ($F(12,108)= 3.0$, $P< 0.01$; **Figure 5.2B**), whereas a MEOT was observed for circulating lymphocyte counts ($F(6,54)= 19.4$, $P< 0.01$; **Figure 5.2C**). The CAT elicited a significant circulating leukocytosis and neutrophilia ($P< 0.01$) on CON and SDEP, which remained significantly elevated during recovery ($P< 0.01$). In contrast, a significant neutrophilia was only observed 2 h post-CAT on SDEP+ER ($P< 0.05$). Circulating leukocyte and neutrophil counts on SDEP+ER were significantly lower post-CAT and 1 h post-CAT compared with SDEP ($P< 0.01$), and post-CAT ($P< 0.05$), 1 h ($P< 0.05$) and 2 h post-CAT ($P< 0.01$) compared with CON. The CAT also induced a significant lymphopenia 1 h and 2 h post-CAT in all trials ($P< 0.01$ *vs.* pre-CAT).

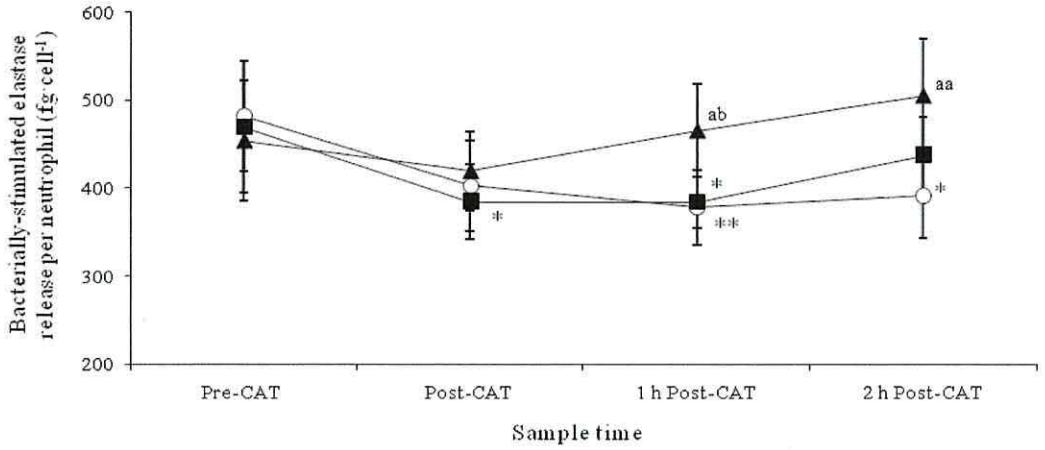
Bacterially-stimulated neutrophil degranulation. No main effects were observed for unstimulated plasma elastase concentration. A trial x time interaction was observed for bacterially-stimulated neutrophil degranulation (elastase release per neutrophil; $F(12,108)= 2.7$, $P< 0.01$; **Figure 5.3**), whereas a MEOT was observed for bacterially-stimulated plasma elastase concentration ($F(6,54)= 17.3$, $P< 0.01$). SDEP increased bacterially-stimulated neutrophil degranulation at 24 h only (21%; $P< 0.01$) compared with BL. Bacterially-stimulated plasma elastase concentration increased post-CAT (40%; $P< 0.01$ *vs.* pre-CAT) and remained significantly elevated during recovery ($P< 0.01$ *vs.* pre-CAT). The CAT also induced significant decreases in bacterially-stimulated neutrophil degranulation 1 h (22%; $P< 0.01$) and 2 h post-CAT (19%; $P< 0.05$) on CON, and post-CAT (18%) and 1 h post-CAT (18%) on SDEP ($P< 0.05$). Compared with SDEP+ER, bacterially-stimulated neutrophil degranulation was significantly lower 1 h ($P< 0.05$) and 2 h post-CAT ($P< 0.01$) on CON, and 1 h post-CAT on SDEP ($P< 0.05$).

Figure 5.2: Circulating leukocyte (A), neutrophil (B), and lymphocyte count (C) responses to a cold air test (CAT) after a 53 h period of normal sleep (CON o), total sleep-deprivation (SDEP ■), and total sleep-deprivation with 90% energy-restriction (SDEP+ER ▲).



Mean ± SEM (n= 10): MEOT †† P< 0.01 *vs.* pre-CAT; ** P< 0.01 *vs.* pre-CAT; a P< 0.05 and aa P< 0.01 *vs.* CON; bb P< 0.01 *vs.* SDEP.

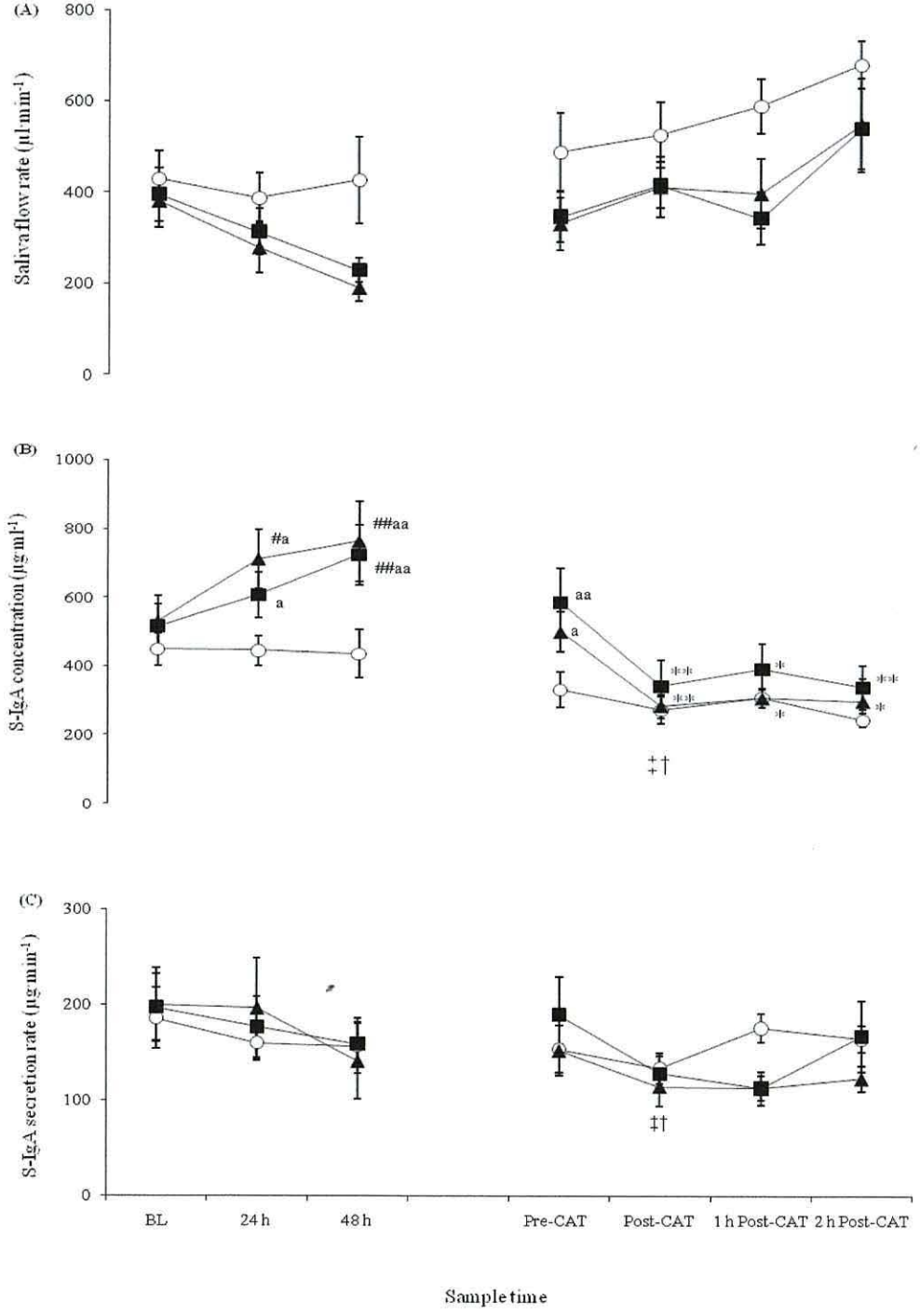
Figure 5.3: Bacterially-stimulated elastase release per neutrophil response to a cold air test (CAT) after a 53 h period of normal sleep (CON O), total sleep-deprivation (SDEP ■), and total sleep-deprivation with 90% energy-restriction (SDEP+ER ▲).



Mean ± SEM (n= 10): * P< 0.05 and ** P< 0.01 *vs.* pre-CAT; a P< 0.05 and aa P< 0.01 *vs.* CON; b P< 0.05 *vs.* SDEP.

Saliva S-IgA responses. A trial x time interaction was observed for saliva S-IgA concentration (F(12,108)= 2.0, P< 0.05; **Figure 5.4B**), whereas a MEOT was observed for saliva S-IgA secretion rate (F(6,54)= 2.5, P< 0.05; **Figure 5.4C**). Saliva S-IgA concentration was significantly higher at 48 h on SDEP (40%; P< 0.01), and at 24 h (34%; P< 0.05) and 48 h (44%; P< 0.01) on SDEP+ER compared with BL. Saliva S-IgA concentration was significantly higher on SDEP and SDEP+ER at 24 h (P< 0.05), 48 h (P< 0.01) and pre-CAT (P< 0.05) compared with CON. Furthermore, a trend (P= 0.07) was apparent for saliva flow rate, whereby a progressive decrease in saliva flow rate was observed by 48 h on SDEP (38%) and SDEP+ER (50%; **Figure 5.4A**). The CAT induced significant decreases in saliva S-IgA concentration on SDEP and SDEP+ER post-CAT (P< 0.01), 1 h and 2 h post-CAT (P< 0.05) compared with pre-CAT values. Additionally, saliva S-IgA secretion rate significantly decreased post-CAT compared with pre-CAT (24%; P< 0.05). Both saliva S-IgA concentration (40%) and secretion rate (35%) was significantly lower post-CAT compared with BL values (MEOT; P< 0.05).

Figure 5.4: Saliva flow rate (A), saliva S-IgA concentration (B), and saliva S-IgA secretion rate (C) responses to a cold air test (CAT) after a 53 h period of normal sleep (CON O), total sleep-deprivation (SDEP ■), and total sleep-deprivation with 90% energy-restriction (SDEP+ER ▲).



Mean ± SEM (n= 10): MEOT ‡ $P< 0.05$ *vs.* BL; MEOT † $P< 0.05$ *vs.* pre-CAT; # $P< 0.05$ and ## $P< 0.01$ *vs.* BL; * $P< 0.05$ and ** $P< 0.01$ *vs.* pre-CAT; a $P< 0.05$ and aa $P< 0.01$ *vs.* CON.

Plasma glucose, cortisol, adrenaline and noradrenaline. There were no trial x time interactions, however a MEOT was observed for plasma glucose ($F(2,27)= 58.2$, $P< 0.01$), cortisol ($F(2,27)= 8.9$, $P< 0.01$), and noradrenaline concentration ($F(4,36)= 65.7$, $P< 0.01$; **Table 5.2**). No main effects were observed for plasma adrenaline concentration. Plasma glucose concentration significantly increased 2 h post-CAT ($P< 0.01$ *vs.* pre-CAT), while plasma cortisol (31%) and noradrenaline (346%) concentrations significantly increased post-CAT ($P< 0.05$ and $P< 0.01$, respectively *vs.* pre-CAT). Plasma cortisol concentration returned to pre-CAT levels 1 h into recovery, and plasma noradrenaline concentration remained significantly above pre-CAT levels 1 h into recovery ($P< 0.05$). Furthermore, a trend ($P= 0.09$) was apparent for plasma noradrenaline concentration, whereby SDEP+ER induced a higher noradrenaline response post-CAT and during recovery compared with SDEP and CON.

Table 5.2: Plasma glucose, cortisol, adrenaline and noradrenaline responses to a cold air test (CAT) after a 53 h period of normal sleep (CON), total sleep-deprivation (SDEP), and total sleep-deprivation with 90% energy-restriction (SDEP+ER).

	BL (08:00 h day 1)	24 h (08:00 h day 2)	48 h (08:00 h day 3)	Pre-CAT (12:00 h day 3)	Post-CAT (13-16:00 h day 3)	1 h Post-CAT (14-17:00 h day 3)	2 h Post-CAT (15-18:00 h day 3)
Glucose (mmol·L^{-1})							††
CON	4.7 ± 1.0	4.9 ± 0.8	4.9 ± 0.8	4.9 ± 1.1	5.1 ± 1.1	5.0 ± 1.6	7.5 ± 2.4
SDEP	4.6 ± 1.1	4.7 ± 1.0	5.0 ± 0.8	4.6 ± 0.9	4.8 ± 1.3	5.1 ± 1.0	7.7 ± 1.5
SDEP+ER	4.6 ± 1.3	4.2 ± 1.3	4.6 ± 0.8	4.3 ± 1.2	4.7 ± 0.9	4.5 ± 1.0	7.0 ± 1.7
Cortisol (nmol·L^{-1})				‡	†	‡‡	‡‡
CON	468 ± 181	507 ± 171	550 ± 160	351 ± 96	450 ± 175	256 ± 101	286 ± 105
SDEP	491 ± 168	497 ± 184	535 ± 210	320 ± 90	392 ± 181	261 ± 104	258 ± 85
SDEP+ER	487 ± 169	584 ± 215	552 ± 191	291 ± 79	421 ± 186	305 ± 121	355 ± 117
Adrenaline (nmol·L^{-1})							
CON	0.4 ± 0.2	--------	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	--------
SDEP	0.3 ± 0.2	--------	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.2	--------
SDEP+ER	0.4 ± 0.2	--------	0.3 ± 0.2	0.4 ± 0.3	0.5 ± 0.3	0.3 ± 0.2	--------
Noradrenaline (nmol·L^{-1})					††	†	
CON	1.2 ± 0.5	--------	1.5 ± 0.4	1.7 ± 0.7	7.4 ± 2.6	3.1 ± 0.7	--------
SDEP	1.2 ± 0.3	--------	1.6 ± 0.5	1.7 ± 0.7	8.2 ± 3.5	3.5 ± 1.1	--------
SDEP+ER	1.1 ± 0.3	--------	1.9 ± 0.7	2.3 ± 0.8	9.8 ± 3.3	4.6 ± 1.4	--------

Mean ± SD (n= 10): MEOT ‡ P< 0.05 and ‡‡ P< 0.01 *vs.* BL; MEOT † P< 0.05 and †† P< 0.01 *vs.* pre-CAT.

5.5 Discussion

The aims of the current study were, firstly to determine the effects of two nights of total sleep-deprivation with and without energy-restriction on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva S-IgA and stress hormone responses at rest and in response to passive cold-exposure. Secondly, to determine the effects of passive cold-exposure on selected immune and stress hormones responses. Compared with multi-stressor field studies, a particular strength of the present study is that the experimental procedures carried out under controlled laboratory conditions enabled better determination of the influence of sleep-deprivation alone or sleep-deprivation with energy-restriction on selected immune responses. In addition, by removing participants from the CAT at a pre-defined core body temperature (T_{re} ≤36.0°C), rather than after a pre-defined time period, allowed for a more accurate assessment of the influence of modest whole-body cooling on selected immune responses. In contrast to two of the hypotheses, two nights of total sleep-deprivation with and without 90% energy-restriction did not compromise selected immune responses at rest, or amplify responses to subsequent modest whole-body cooling. In support of one of the hypotheses, modest whole-body cooling (T_{re} 35.9°C) decreased circulating lymphocytes, bacterially-stimulated neutrophil degranulation and saliva S-IgA responses. These data suggest that modest whole-body cooling poses a greater threat to immune status than two nights of total sleep-deprivation alone or two nights of total sleep-deprivation in combination with 90% energy-restriction.

In the current study, two nights of total sleep-deprivation alone or with 90% energy-restriction failed to induce significant alterations in resting circulating leukocytes. These findings extend to those reported in **Chapter 4**, which showed that one night of total sleep-deprivation did not alter resting circulating total or differential leukocyte counts. On

the contrary, a recent study reported that 48 h of 90% energy-restriction alone decreased circulating leukocyte counts mainly due to a decrease in circulating lymphocyte counts (Laing *et al.*, 2008b). This discrepancy highlights that an individual stressor known to decrease aspects of immune function (e.g. energy-restriction) does not necessarily have the same effect when combined with another stressor (e.g. sleep-deprivation). It remains unclear why the addition of sleep-deprivation to energy restriction prevented the decrease in circulating lymphocytes shown previously (Laing *et al.*, 2008b).

The relatively modest leukocytosis immediately post-CAT on SDEP and CON is likely explained by demargination of neutrophils from the vascular endothelium (McCarthy & Dale, 1988). Raised circulating noradrenaline observed post-CAT (**Table 5.2**) may have played a role in neutrophil demargination from vascular endothelium. However, other mechanisms must also be at work since similar increases in circulating noradrenaline were observed on all trials post-CAT; although a 2 h delay in neutrophilia was observed when energy-restriction was super-imposed onto sleep-deprivation (SDEP+ER). This once again highlights the complex interaction of various stressors on immune indices. Cold-exposure is widely acknowledged to increase plasma noradrenaline due to SAM axis stimulation, which in-turn promotes vasoconstriction, shivering thermogenesis and endogenous energy substrate breakdown (Castellani *et al.*, 2002). Raised circulating noradrenaline might account for the lymphopenia during recovery after the CAT, possibly *via* alterations in adhesion molecules and surface receptors on lymphocytes thought to have a key role in governing lymphocyte trafficking during environmental stress (Kruger & Mooren, 2007). The small but significant increase in plasma cortisol from pre-CAT (12 noon) to post-CAT is unlikely to account for the observed changes in circulating leukocyte trafficking, because post-CAT plasma cortisol was similar to 0 h (0800 h). 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 $nmol \cdot L^{-1}$) and free cortisol concentration increases (McCarthy & Dale, 1988).

Two nights of total sleep-deprivation alone or with 90% energy restriction did not compromise bacterially-stimulated neutrophil degranulation at rest. This finding extends to those reported in **Chapter 4** and a previous study, whereby one night of total sleep-deprivation alone, and two days of 90% energy-restriction alone did not alter resting bacterially-stimulated neutrophil degranulation (Laing *et al.*, 2008b). It was also shown that under controlled laboratory conditions, inducing modest whole-body cooling decreased bacterially-stimulated neutrophil degranulation. It is possible that the decrease in bacterially-stimulated neutrophil degranulation after the CAT on SDEP and CON can be explained by the demargination of intravascular neutrophils of different maturity status to those already in circulation (Hetherington & Quie, 1985; Berkow & Dodson, 1986). Additionally, the significant neutrophilia on SDEP and CON immediately after the CAT, but not on SDEP+ER where there was no decrease in bacterially-stimulated neutrophil degranulation after the CAT, lends support to this possibility. However, the widely accepted mediators of neutrophil response to acute stress (**Section 2.1.4**) do not provide adequate explanations for the current findings, since CAT-evoked stress hormone responses were similar between trials (Toft *et al.*, 1994; Nakagawa *et al.*, 1998).

Moreover, two nights of total sleep-deprivation alone or with 90% energy-restriction did not compromise oral-respiratory mucosal immunity at rest in the present study. These findings extend those reported in **Chapter 4** and a previous study, whereby one night of total sleep-deprivation alone and two days of 90% energy-restriction alone (Oliver *et al.*,

2007) did not lower resting saliva IgA concentration or secretion rate. However, the current study shows that two nights of total sleep-deprivation alone or with 90% energy-restriction tended to decrease saliva flow rate (P= 0.07) with concomitant increases in saliva S-IgA concentration, possibly attributed to an alterations in parasympathetic tone associated with the stress of total sleep-deprivation (Gleeson, 2000; Bosch *et al.*, 2003a).

Little is currently known about the effects of cold exposure that evokes a decrease in T_{core} on saliva IgA responses (Walsh & Whitham, 2006). A novel finding in the current study is that modest whole-body cooling was associated with a decrease in saliva S-IgA responses. The time course of the response indicates that modest whole-body cooling decreased S-IgA translocation, as synthesis of S-IgA may take many hours to days (Hucklebridge *et al.*, 1998). Plausible explanations for the decrease in saliva S-IgA observed with modest whole-body cooling includes, the effects of cold air decreasing the temperature of the mucosal membranes, a possible drying effect of cold air, and/or whole-body cooling evoked neuroendocrine regulation of transepithelial S-IgA translocation (Giesbrecht, 1995; Nieman, 1997), which is supported by the noradrenaline responses post-CAT observed in the current study.

In conclusion, two nights of total sleep-deprivation with and without 90% energy-restriction did not alter circulating leukocyte trafficking or compromise bacterially-stimulated neutrophil degranulation and saliva S-IgA responses at rest. However, modest whole-body cooling alone (T_{re} 35.9°C) decreased circulating lymphocytes, bacterially-stimulated neutrophil degranulation and saliva S-IgA responses. These responses were not amplified by prior total sleep-deprivation alone or total sleep-deprivation with 90% energy-restriction.

CHAPTER SIX

Influence of Timing of Post-Exercise Carbohydrate and Protein Ingestion on Selected Immune Responses

6.1 Summary: The aim was to determine the influence of immediate and 1 h delayed carbohydrate and protein (CHO-PRO) feeding following prolonged exercise on selected immune, plasma stress hormone and insulin responses. Nine male runners completed three feeding interventions in randomised order after 2 h running at 75% $\dot{V}O_{2max}$. During control (CON) participants received water (12 ml·kg^{-1}BM) immediately and 1 h post-exercise. During immediate feeding (IF) participants received a CHO-PRO solution equal to 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM immediately and water 1 h post-exercise. During delayed feeding (DF) participants received water immediately and CHO-PRO solution 1 h post-exercise. Circulating leukocyte counts, bacterially-stimulated neutrophil degranulation, saliva S-IgA, plasma stress hormone and insulin responses were determine from venous blood and unstimulated saliva and samples collected pre-exercise, immediately post-exercise and every 20 min until 140 min post-exercise. No significant interactions were observed for circulating leukocytes counts, saliva S-IgA secretion rate, plasma cortisol, adrenaline and noradrenaline concentration. Bacterially-stimulated neutrophil degranulation decreased during recovery on CON and DF (24% and 31% at 140 min, respectively; $P< 0.01$), but not on IF. Compared with CON, bacterially-stimulated neutrophil degranulation was higher on IF at 100 min post-exercise and higher on IF than DF at 80 min ($P< 0.05$) and 100 min onwards post-exercise ($P< 0.01$). Ingestion of a CHO-PRO solution immediately after, but not 1 h after, prolonged strenuous exercise prevented the decrease in bacterially-stimulated neutrophil degranulation, but did not alter circulating leukocyte trafficking, or saliva S-IgA and stress hormone responses.

6.2 Introduction

It has been reported that consuming carbohydrates prior to and during prolonged exercise maintains plasma glucose concentration, and attenuates stress hormone responses and associated immune perturbations in overnight fasted individuals (Nieman, 1998b; Bishop *et al.*, 2002). Ingesting 750 ml of a carbohydrate solution (6% *w/v*) prior to and 280 ml every 15 min during 2.5 h cycling at 85% ventilatory threshold attenuated the post exercise leukocytosis, neutrophilia and prevented the decrease in T-lymphocyte response to PHA (Green *et al.*, 2003). Also, ingesting 5 $ml \cdot kg^{-1}$BM of a carbohydrate solution (6.4% *w/v*) prior to and 2 $ml \cdot kg^{-1}$BM of the same solution every 15 min during high intensity intermittent exercise attenuated the decrease in bacterially-stimulated neutrophil degranulation observed on the placebo trial (Bishop *et al.*, 2002). The investigators speculated that the beneficial effect of carbohydrate ingestion before and during prolonged exercise on immune responses was due to blunted stress hormone and cytokine responses. More recent evidence supports a primary role for insulin modulating neutrophil function (Walrand *et al.*, 2006; Alba-Loureiro *et al.*, 2007). However, a role for insulin in modulating neutrophil responses after prolonged strenuous exercise with carbohydrate feeding has not been determined. Evidence is less convincing that the beneficial effect of carbohydrate ingestion before and during prolonged exercise extends to oral-respiratory mucosal immune responses (Bishop *et al.*, 2000).

Current recommendations for carbohydrate intake during exercise for energy provision and attenuated immune perturbations (~60 g $CHO \cdot h^{-1}$; Jeukendrup & Jentjens, 2000; Gleeson, 2006) whilst attainable in a laboratory setting, are rarely practiced by high level endurance athletes during training or competition (Noakes, 2003). For example, it has been reported that elite marathoners average only ~200 $ml \cdot h^{-1}$ fluid intake (Noakes,

2003). Besides the gastro-intestinal discomfort of consuming food and fluid at high exercise intensities; dietary and pacing strategies during competition/training have also been identified as an issue (Burke *et al.*, 2005; Kruseman *et al.*, 2005). Furthermore, many endurance athletes train without consuming carbohydrate during exercise, particularly in the off-season, in an attempt to increase metabolic adaptation to endurance exercise (Civitarese *et al.*, 2005; Hawley *et al.*, 2006; De Bock *et al.*, 2008). Consequently, if limited carbohydrate is consumed during competition and training in high level endurance athletes, carbohydrate provision shortly after heavy exercise may be important for immune recovery.

Moreover, some studies have shown that post-exercise provision of protein supplements may aid immune recovery and decrease the risk of URI after heavy exercise (Castell *et al.*, 1996; Bassit *et al.*, 2002). Although these studies provide support for protein supplementation after heavy exercise, this remains controversial because others show no beneficial effect of protein supplementation on post-exercise recovery of immune function (Walsh *et al.*, 2000). Nevertheless, endurance athletes are recommended to consume carbohydrate (1.2 $g \cdot kg^{-1}BM$) and protein (0.4 $g \cdot kg^{-1}BM$) immediately after heavy exercise to promote glycogen resynthesis (Ivy *et al.*, 2002) and tissue repair (Tipton & Wolfe, 2004). The effects of this recommended feeding regimen on immune recovery after endurance exercise remains unknown.

Therefore, the aim of the current study was to determine the influence of immediate and 1 h delayed carbohydrate and protein feeding following prolonged strenuous exercise on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva S-IgA responses, plasma insulin and stress hormone responses in trained runners. It was

hypothesised that carbohydrate and protein feeding would attenuate exercise-induced immune perturbations during recovery from prolonged strenuous exercise, and that immune perturbations would recover more rapidly when carbohydrate and protein is consumed immediately rather than 1 h after exercise.

6.3 Methods

Participants. Nine healthy competitive endurance runners (age 33 ± 12 y; NBM 71.7 ± 5.9 kg; height 1.77 ± 0.07 m; body fat 11.2 ± 3.4%; $\dot{V}O_{2max}$ 61 ± 5 ml·kg^{-1}·min^{-1}) volunteered to participate in the study. All participants were club-level road and fell runners with an average of 15 ± 7 y racing experience. Ethical procedures were followed in accordance with **section 3.1.**

Preliminary measurements. Prior to the first experimental trial, participants were asked to report to the laboratory where preliminary measurements as described in **section 3.2** and **3.3** were undertaken. From the $\dot{V}O_2$-work rate relationship the treadmill speed at 75% $\dot{V}O_{2max}$ was extrapolated and verified (11.3 ± 1.0 km·h^{-1} and 1% gradient).

Experimental trials. To control dietary intake, prior to each experimental trial a 24 h diet was provided which catered for the participants estimated energy requirements (2986 ± 144 Kcal; Harris & Benedict, 1919; WHO, 1985), and provided 69% carbohydrate, 16% protein, 15% fat, and 35 ml·kg^{-1}BM·day^{-1} of water (2511 ± 208 ml·day^{-1}; Todorovic & Micklewright, 2004). On three occasions separated by one week, participants reported to the laboratory at 0800 h following an overnight fast. Urine measurements as previously described in **Section 3.4,** indicated euhydration in all participants on arrival at the laboratory for each trial. After pre-exercise measurements and sample collection, participants ran on a motorised treadmill for 2 h at the previously determined treadmill speed that elicited 75% $\dot{V}O_{2max}$. The 2 h exercise bout was performed in ambient conditions of 20°C and 59% RH with two fans placed one metre from the treadmill at a speed of 2.3 m·s^{-1}. HR and RPE were collected every 10 min during exercise. Participants were given water at a rate of 3 ml·kg^{-1}BM·h^{-1} (215 ± 18 ml·h^{-1}) during exercise.

After the immediate post-exercise blood and saliva sample collections, NBM was recorded, and in randomised order participants performed one of three recovery drink interventions, where they ingested the same fluid volume (12 ml·kg^{-1}BM) at equivalent times (immediately and 1 h after exercise) during recovery. The recovery drink consisted of either water or a CHO-PRO drink (Science in Sport, Blackburn, UK) made to a 10% carbohydrate solution, which provided 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM. During one trial participants received water immediately after and 1 h after exercise (CON). During another trial participants received the CHO-PRO drink immediately after exercise and water 1 h after exercise (IF- immediate feeding). During another trial participants received water immediately after and the CHO-PRO drink 1 h after exercise (DF- delayed feeding). Participants remained seated for 140 min during the recovery period in which samples were collected.

Blood collection and analysis. Whole blood samples were collected as previously described in **Sections 3.4**, pre-exercise, immediately post-exercise, and at 20 min intervals post-exercise for 140 min. Blood analysis was performed in accordance with **Section 3.4**. Plasma adrenaline and noradrenaline concentrations were not measured from 100 min post-exercise onwards (**Table 6.4**), since previous studies report catecholamines returning to baseline values within 1 h of exercise cessation (Laing *et al.*, 2008a).

Saliva collection and analysis. Unstimulated whole saliva samples were collected as previously described in **Sections 3.4**, pre-exercise, immediately post-exercise, and at 20 min intervals post-exercise for 140 min. Saliva analysis was performed in accordance with **Section 3.4**.

Statistical Analysis. Statistical analysis of data was performed in accordance with **Section 3.5**. However, body mass loss was assessed using a one-way ANOVA.

6.4 Results

There was no significant interaction for HR (exercise mean 154 ± 5 bpm) and RPE (exercise mean 13 ± 1), and no difference in body mass loss between trials (mean of trials: 1.9 ± 0.3 kg). There was a significant trial x time interaction for plasma volume change ($P< 0.01$). Plasma volume decreased post-exercise on IF (-3.8 ± 6.3 %) and DF (-3.2 ± 3.3 %), but the decrease on CON (-2.6 ± 3.3 %) did not reach significance. Significant increases in plasma volume were observed from 60 min onwards on IF, 100 min onwards on DF, and at 40, 60 and 100 min onwards on CON ($P< 0.01$).

Circulating leukocyte, neutrophil, lymphocyte counts and neutrophil:lymphocyte ratio. There were no trial x time interactions for circulating leukocyte, neutrophil or lymphocyte counts. However, there was a significant MEOT for circulating leukocyte ($F(2.2,17.6)= 39.1$, $P< 0.01$), neutrophil ($F(2.0,16.3)= 55.3$, $P< 0.01$), and lymphocyte counts ($F(1.2,11.0)= 11.5$, $P< 0.01$; **Table 6.1**), and the neutrophil:lymphocyte ratio ($F(1.8,14.7)= 51.3$, $P< 0.01$). A significant circulating leukocytosis was observed following exercise and remained significantly elevated throughout recovery. The increase in circulating leukocyte counts during the recovery period was mainly attributed to the significant sustained neutrophilia ($P< 0.01$). Circulating lymphocyte counts increased immediately post-exercise, but this failed to reach significance. However, a significant lymphopenia ($P< 0.01$) was observed from 60 min post-exercise onwards. Compared with pre-exercise values (CON: 1.4 ± 0.4; IF: 1.6 ± 0.6; DF: 1.6 ± 0.6), the neutrophil:lymphocyte ratio significantly increased by 20 min post-exercise ($P< 0.01$) and continued to progressively increase throughout recovery (140 min post-exercise; CON: 8.1 ± 3.2; IF: 9.4 ± 3.6; DF: 7.0 ± 2.3).

Table 6.1: Circulating leukocyte, neutrophil and lymphocyte count responses to immediate carbohydrate and protein feeding (IF), 1 h delayed carbohydrate and protein feeding (DF), and water (CON) during recovery from 2 h running at 75% $\dot{V}O_{2max}$.

Counts ($x10^9 \cdot L^{-1}$)	Pre	Post	Recovery Period (min) 20	40	60	80	100	120	140
Leukocyte		††	††	††	††	††	††	††	††
CON	5.4 ± 1.3	9.4 ± 3.3	8.7 ± 2.8	9.8 ± 3.2	10.1 ± 3.0	10.9 ± 2.8	11.6 ± 3.1	12.0 ± 2.9	11.8 ± 3.2
IF	5.1 ± 1.1	9.8 ± 3.6	9.1 ± 3.6	10.1 ± 3.8	10.8 ± 3.8	11.4 ± 3.6	12.2 ± 3.3	12.3 ± 3.0	12.3 ± 3.0
DF	5.0 ± 1.0	9.1 ± 2.8	9.0 ± 2.7	10.0 ± 2.9	10.3 ± 3.0	10.8 ± 2.6	11.3 ± 2.6	12.0 ± 2.3	11.8 ± 2.4
Neutrophil		††	††	††	††	††	††	††	††
CON	2.6 ± 0.7	6.2 ± 2.2	6.3 ± 2.3	7.5 ± 2.8	8.0 ± 2.7	8.8 ± 2.6	9.5 ± 2.9	9.9 ± 2.7	9.5 ± 3.0
IF	2.6 ± 0.6	6.8 ± 3.0	6.8 ± 3.0	8.2 ± 3.5	9.0 ± 3.6	9.4 ± 3.4	10.1 ± 3.2	10.2 ± 3.0	10.2 ± 2.9
DF	2.6 ± 0.6	6.4 ± 2.3	6.6 ± 2.5	7.9 ± 2.7	8.3 ± 2.6	8.8 ± 2.4	9.4 ± 2.2	9.9 ± 2.0	9.6 ± 2.3
Lymphocyte					†	†	†	†	†
CON	2.0 ± 0.7	2.3 ± 1.0	1.7 ± 0.5	1.5 ± 0.4	1.4 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.3
IF	1.8 ± 0.6	2.2 ± 0.9	1.6 ± 0.5	1.3 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.1 ± 0.2
DF	1.8 ± 0.5	2.0 ± 0.6	1.7 ± 0.4	1.5 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.2 ± 0.2	1.3 ± 0.2	1.4 ± 0.2

Mean ± SD (n= 9): MEOT † $P< 0.05$ and †† $P< 0.01$ *vs.* pre.

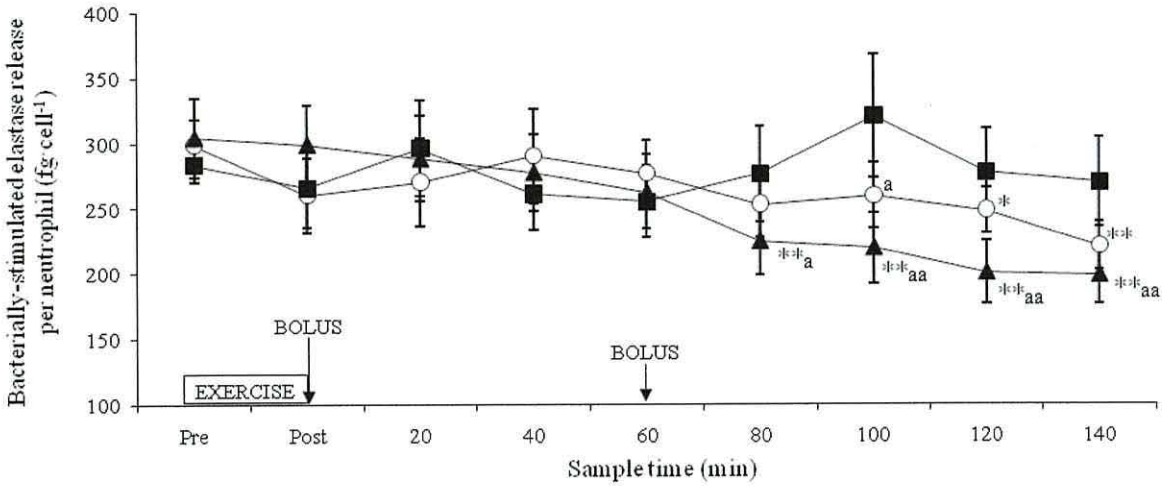
Bacterially-stimulated neutrophil degranulation. A significant MEOT was observed for unstimulated plasma elastase concentration ($F(1.2,10.0)= 16.9$, $P< 0.01$; **Table 6.2**). Unstimulated plasma elastase concentration increased immediately post-exercise ($P< 0.01$) and remained significantly elevated 40 min post-exercise ($P< 0.05$). A significant trial x time interaction was observed for bacterially-stimulated plasma elastase concentration ($F(16,128)= 3.5$, $P< 0.01$; **Table 6.2**), and bacterially-stimulated neutrophil degranulation (elastase release per neutrophil; ($F(16,128)= 3.0$, $P< 0.01$; **Figure 6.1**). In all trials bacterially-stimulated plasma elastase concentration significantly increased post-exercise ($P< 0.01$), and remained elevated throughout recovery. Compared with CON, bacterially-stimulated plasma elastase concentration was significantly higher at 100 min post-exercise on IF ($P< 0.05$). Bacterially-stimulated plasma elastase concentration was also significantly higher on IF than DF at 80 min post-exercise ($P< 0.05$) and 100 min onwards post-exercise ($P< 0.01$). IF prevented the decrease in bacterially-stimulated neutrophil degranulation, which was observed during recovery on CON (24% decrease at 140 min post-exercise; $P< 0.01$ *vs.* pre-exercise), and DF (31% decrease at 140 min post-exercise; $P<0.01$ *vs.* pre-exercise). Compared with CON, bacterially-stimulated neutrophil degranulation was significantly higher on IF at 100 min post-exercise ($P< 0.05$), and higher on IF than DF at 80 min ($P< 0.05$) and 100 min onwards post-exercise ($P< 0.01$).

Table 6.2: Unstimulated and bacterially-stimulated plasma elastase concentration responses to immediate carbohydrate and protein feeding (IF), 1 h delayed carbohydrate and protein feeding (DF), and water (CON) during recovery from 2 h running at 75% $\dot{V}O_{2max}$.

	Recovery Period (min)								
	Pre	Post	20	40	60	80	100	120	140
Unstimulated plasma elastase ($ng \cdot ml^{-1}$)		††	††	†					
CON	33 ± 9	118 ± 60	75 ± 37	67 ± 28	64 ± 20	62 ± 23	59 ± 19	60 ± 18	58 ± 15
IF	39 ± 20	143 ± 88	77 ± 34	74 ± 35	67 ± 23	67 ± 31	62 ± 25	61 ± 13	63 ± 15
DF	28 ± 7	111 ± 64	68 ± 35	61 ± 29	63 ± 22	57 ± 24	61 ± 22	65 ± 28	61 ± 30
Stimulated plasma elastase ($\mu g \cdot ml^{-1}$)									
CON	1.4 ± 0.4	2.9 ± 1.2**	2.9 ± 1.1**	3.6 ± 1.2**	3.6 ± 0.9**	3.8 ± 1.0**	4.1 ± 1.1**a	4.0 ± 0.6**	3.6 ± 0.7**
IF	1.4 ± 0.4	3.2 ± 1.0**	3.5 ± 1.2**	3.4 ± 1.0**	3.6 ± 0.8**	4.0 ± 0.6**	4.9 ± 1.1**	4.4 ± 1.0**	4.2 ± 1.2**
DF	1.4 ± 0.5	3.3 ± 1.2**	3.0 ± 0.9**	3.5 ± 0.9**	3.6 ± 1.1**	3.3 ± 1.1**a	3.3 ± 1.1**aa	3.2 ± 0.9**aa	3.1 ± 0.6**aa

Mean ± SD (n= 9): MEOT † P< 0.05 and †† P< 0.01 *vs.* pre; ** P< 0.01 *vs.* pre; a P< 0.05 and aa P< 0.01 *vs.* IF.

Figure 6.1: Bacterially-stimulated elastase release per neutrophil response to immediate carbohydrate and protein feeding (IF ■), 1 h delayed carbohydrate and protein feeding (DF ▲), and water alone (CON o) during recovery from 2 h running at 75% $\dot{V}O_{2max}$.



Mean ± SEM (n= 9): * P< 0.05 and ** P< 0.01 *vs.* pre; a P< 0.05 and aa P< 0.01 *vs.* IF.

Saliva S-IgA responses. No main effects were observed for saliva flow rate and saliva S-IgA secretion rate (**Table 6.3**). A significant trial x time interaction was observed for saliva S-IgA concentration (F(16,128)= 1.9, P< 0.05; **Table 6.3**). Compared with pre-exercise values, a significant decrease in saliva S-IgA concentration was observed 20 min and 80 min onwards post-exercise for CON and IF (P< 0.05), and 80 min onwards for DF (P< 0.01). Furthermore, significant acute decreases in saliva S-IgA concentration occurred in all trials after bolus ingestion at 20 min post-exercise (P< 0.01), and on CON (P< 0.05) and DF (P< 0.01) at 80 min post-exercise. Interestingly, saliva S-IgA concentration at 20 min post-exercise was significantly lower in IF with CHO-PRO bolus ingestion compared with water bolus ingestion on DF (P< 0.05). Likewise, at 80 min post-exercise saliva S-IgA concentration was significantly lower in DF with CHO-PRO bolus ingestion compared with water bolus ingestion on IF (P< 0.05). However, compared with CON, no

significant difference in saliva S-IgA concentration was observed at 20 min or 80 min post-exercise on IF and DF.

Plasma glucose and insulin concentration. A significant trial x time interaction was observed for plasma glucose ($F(16,128)= 14.7$, $P< 0.01$), and insulin concentration ($F(16,128)= 26.8$, $P< 0.01$; **Figure 6.2**). Plasma glucose and insulin concentration was unaffected by 2 h running at 75% $\dot{V}O_{2max}$ in all trials. Plasma glucose and insulin concentration significantly increased after the CHO-PRO bolus ingestion on IF and DF ($P< 0.01$), but did not change after the water bolus on CON and DF. The increase in plasma glucose concentration at 40 min post-exercise on IF remained significantly elevated 120 min post-exercise ($P< 0.01$ *vs.* pre). On DF the increase in plasma glucose concentration at 100 min post-exercise remained significantly elevated until the end of the recovery period measured ($P< 0.01$ *vs.* pre). The increase in plasma insulin concentration at 40 min post-exercise on IF and 80 min post-exercise on DF remained significantly elevated until the end of the recovery period measured ($P< 0.01$ *vs.* pre).

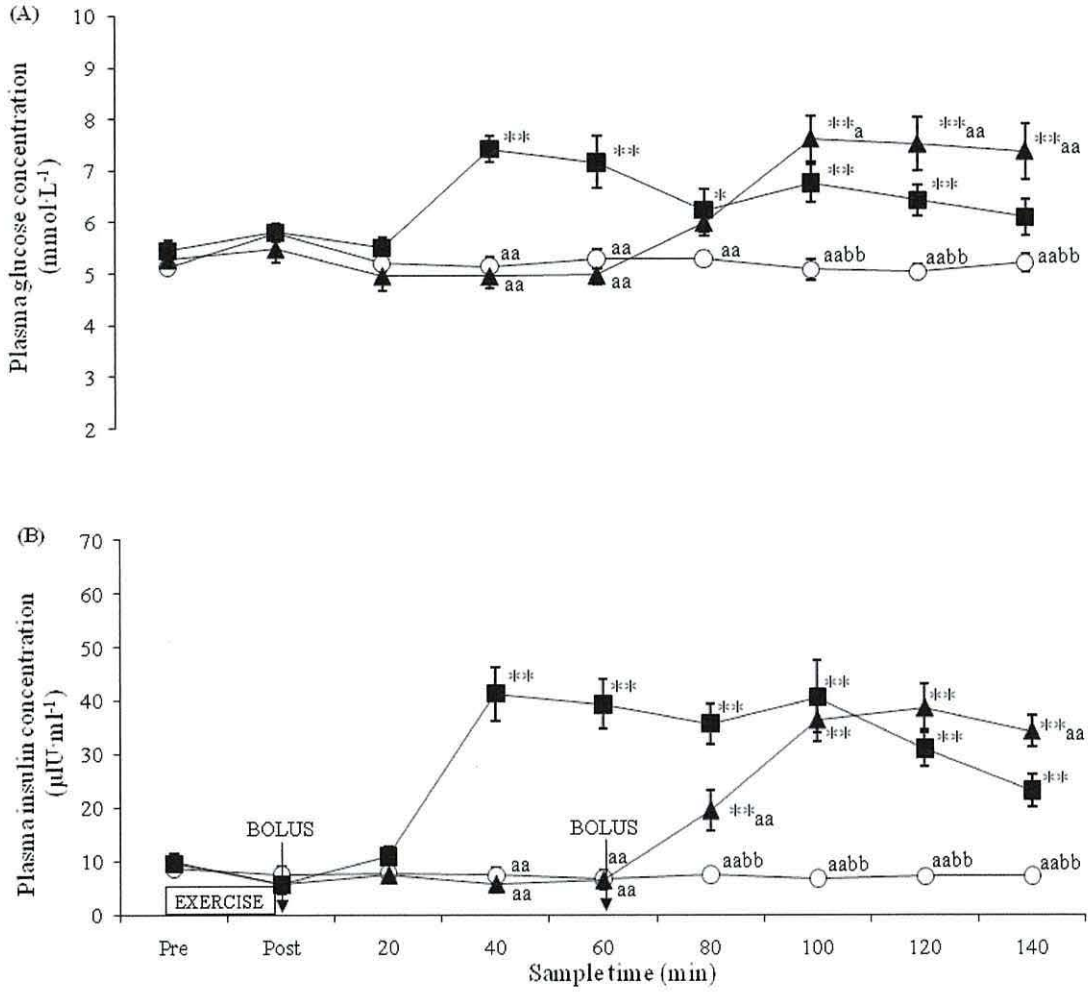
Plasma cortisol, adrenaline and noradrenaline concentration. No trial x time interaction was observed for plasma cortisol, adrenaline or noradrenaline concentrations (**Table 6.4**). However, a significant MEOT was observed for plasma cortisol ($F(8,56)= 9.1$, $P< 0.01$), adrenaline ($F(1.5,10.8)= 20.3$, $P< 0.01$), and noradrenaline concentrations ($F(1.6,11.1)= 44.3$, $P< 0.01$). Plasma cortisol concentration increased immediately post-exercise peaking at 20 min post-exercise ($P< 0.05$ *vs.* pre) and returned to pre-exercise levels by 100 min post-exercise. Plasma adrenaline and noradrenaline concentration significantly increased immediately post-exercise ($P< 0.01$ *vs.* pre) and were not significantly different from pre-exercise thereafter.

Table 6.3: Saliva flow rate, saliva S-IgA concentration and saliva S-IgA secretion rate responses to immediate carbohydrate and protein feeding (IF), 1 h delayed carbohydrate and protein feeding (DF), and water (CON) during recovery from 2 h running at 75% $\dot{V}O_{2max}$.

				Recovery Period (min)						
		Pre	Post	20	40	60	80	100	120	140
Saliva	CON	535 ± 402	408 ± 294	469 ± 462	577 ± 346	535 ± 415	599 ± 496	594 ± 551	658 ± 561	686 ± 571
flow rate	IF	522 ± 360	364 ± 310	453 ± 401	413 ± 382	414 ± 337	448 ± 345	525 ± 460	531 ± 377	541 ± 366
($\mu l \cdot min^{-1}$)	DF	545 ± 426	503 ± 413	574 ± 494	540 ± 434	620 ± 447	689 ± 499	682 ± 440	605 ± 495	711 ± 448
Saliva S-IgA	CON	462 ± 303	598 ± 295*	336 ± 140*§§	370 ± 179	390 ± 126	263 ± 124$^{**¥}$	246 ± 101**	242 ± 96**	269 ± 112**
concentration	IF	480 ± 211	596 ± 320	247 ± 80**§§	396 ± 120	424 ± 111	332 ± 178**	295 ± 101**	324 ± 125**	340 ± 110*
($\mu g \cdot ml^{-1}$)	DF	496 ± 316	527 ± 368	381 ± 250$^{\S\S a}$	392 ± 268	409 ± 228	199 ± 69$^{**¥¥a}$	250 ± 112**	287 ± 107**	284 ± 137**
Saliva S-IgA	CON	202 ± 163	226 ± 180	154 ± 159	198 ± 117	198 ± 140	131 ± 96	118 ± 75	151 ± 115	153 ± 106
secretion rate	IF	232 ± 166	213 ± 218	102 ± 78	177 ± 184	179 ± 156	138 ± 113	142 ± 113	164 ± 109	192 ± 151
($\mu g \cdot min^{-1}$)	DF	216 ± 164	217 ± 167	184 ± 168	168 ± 139	223 ± 178	137 ± 125	151 ± 102	155 ± 112	188 ± 118

Mean ± SD (n= 9). * P< 0.05 and ** P< 0.01 *vs.* pre; §§ P< 0.01 *vs.* immediately post-exercise; ¥ P< 0.05 and ¥¥ P< 0.01 *vs.* 60 min post-exercise; a P< 0.05 *vs.* IF.

Figure 6.2: Plasma glucose (A) and insulin (B) responses to immediate carbohydrate and protein feeding (IF ■), 1 h delayed carbohydrate and protein feeding (DF ▲), and water alone (CON o) during recovery from 2 h running at 75% $\dot{V}O_{2max}$.



Mean ± SEM (n= 9): * P< 0.05 and ** P< 0.01 *vs.* pre; a P< 0.05 and aa P< 0.01 *vs.* IF; bb P< 0.01 *vs.* DF.

Table 6.4: Plasma cortisol, adrenaline and noradrenaline responses to immediate carbohydrate and protein feeding (IF), 1 h delayed carbohydrate and protein feeding (DF), and water (CON) during recovery from 2 h running at 75% $\dot{V}O_{2max}$.

			Recovery Period (min)						
	Pre	Post	20	40	60	80	100	120	140
Cortisol ($nmol \cdot l^{-1}$)			†						
CON	401 ± 84	497 ± 190	556 ± 225	522 ± 212	451 ± 154	429 ± 164	416 ± 173	354 ± 116	310 ± 90
IF	378 ± 124	475 ± 226	529 ± 228	464 ± 195	454 ± 148	427 ± 107	386 ± 52	336 ± 31	296 ± 34
DF	356 ± 64	482 ± 116	500 ± 80	446 ± 73	401 ± 75	346 ± 63	328 ± 57	325 ± 93	314 ± 94
Adrenaline ($nmol \cdot l^{-1}$)		††							
CON	0.6 ± 0.1	2.0 ± 1.2	1.1 ± 0.4	0.9 ± 0.4	0.4 ± 0.2	0.4 ± 0.2	--------	--------	--------
IF	0.6 ± 0.2	2.1 ± 1.2	0.9 ± 0.5	0.9 ± 0.5	0.6 ± 0.3	0.4 ± 0.2	--------	--------	--------
DF	0.5 ± 0.2	1.8 ± 0.6	0.8 ± 0.4	0.7 ± 0.3	0.4 ± 0.1	0.5 ± 0.3	--------	--------	--------
Noradrenaline ($nmol \cdot l^{-1}$)		††							
CON	3.6 ± 0.9	12.2 ± 5.1	4.5 ± 1.9	4.3 ± 1.7	4.2 ± 1.5	5.3 ± 3.0	--------	--------	--------
IF	3.1 ± 1.3	11.5 ± 4.0	5.1 ± 2.6	4.5 ± 1.3	5.9 ± 2.9	5.5 ± 2.3	--------	--------	--------
DF	4.3 ± 2.3	10.8 ± 6.3	4.9 ± 2.6	4.8 ± 2.8	4.3 ± 2.5	5.2 ± 1.8	--------	--------	--------

Mean ± SD (n= 8): MEOT † $P< 0.05$ and †† $P< 0.01$ *vs.* pre.

6.5 Discussion

In support of the hypothesis, these results show that the ingestion of a CHO-PRO bolus providing 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM immediately after, but not 1 h after, prolonged strenuous exercise prevents the decrease in bacterially-stimulated neutrophil degranulation. In contrast to the hypothesis, CHO-PRO feeding immediately or 1 h after exercise did not alter circulating leukocyte trafficking and saliva S-IgA responses.

The effects of post-exercise feeding regimens (Ivy *et al.*, 2002; Tipton & Wolfe, 2004) on immune recovery after prolonged exercise have not previously been investigated. The findings of the present study may be important for endurance athletes who consume less than the recommended carbohydrate intake during exercise (Noakes, 2003; Kruseman *et al.*, 2005; Hawley *et al.*, 2006) required to off-set immune perturbations induced by exercise (Gleeson, 2006). Nevertheless, it is recognised that a limitation of the present study is that the current nutritional intervention does not allow the independent effect of carbohydrate and protein on immune parameters measured during recovery to be identified.

Nevertheless, these findings show that CHO-PRO ingestion early during recovery can prevent the decrease in bacterially-stimulated neutrophil degranulation often reported after prolonged strenuous exercise (Robson *et al.*, 1999; Bishop *et al.*, 2002; Laing *et al.*, 2008a). The mechanism(s) responsible for the decrease in bacterially-stimulated neutrophil degranulation after prolonged strenuous exercise on CON and DF in the present study remains unclear. However, a novel aspect in the present study is that even if a possible involvement for blood-borne factors in the decrease in bacterially-stimulated

neutrophil degranulation after heavy exercise is accepted, these results show that nutrient provision early during recovery can counter this effect.

An involvement of stress hormones is unlikely, as CHO-PRO feeding did not influence stress hormone responses during recovery (**Table 6.4**). Given the heavy reliance of neutrophils on glucose for fuel (Furukawa *et al.*, 2000), a direct effect of decreased glucose availability on neutrophil degranulation is also unlikely, because decreases in blood glucose concentration after exercise were not observed (pre: 5.3 ± 0.5 *vs.* post: 5.7 ± 0.6 $mmol \cdot l^{-1}$; **Figure 6.2**). Whereas, increased availability of glucose after CHO-PRO feeding (e.g. 7.4 ± 0.8 $mmol \cdot l^{-1}$ 40 min post-exercise on IF; **Figure 6.2**) might logically be implicated in preventing the decrease in bacterially-stimulated neutrophil degranulation observed on CON and DF. However, such a stimulatory effect of increased glucose availability on neutrophil degranulation seems unlikely because hyperglycaemia has been shown to decrease neutrophil degranulation, this however was shown in diabetes mellitus patients with poor glycaemic control (Alba-Loureiro *et al.*, 2007; Stegenga *et al.*, 2008a).

Recently there has been considerable interest in a potential role for insulin modulating neutrophil function (Walrand *et al.*, 2006; Stegenga *et al.*, 2008b). Hyperinsulinaemic-euglycaemic clamp have shown that raised circulating insulin increased neutrophil chemotaxis, phagocytosis and bactericidal capacity in healthy subjects (Walrand *et al.*, 2004; Walrand *et al.*, 2006). It is conceivable that the sustained increase in plasma insulin during recovery on IF (**Figure 6.2**) may be involved in preventing the decrease in bacterially-stimulated neutrophil degranulation observed on CON and DF. Additionally, because of the blood sample time points, a delayed insulin-induced recovery of

bacterially-stimulated neutrophil degranulation after the last blood sample on DF (140 min) may have been missed.

Additionally, the present study shows that nutrient availability during the short-term after prolonged strenuous exercise does not alter circulating leukocyte trafficking or saliva S-IgA responses during recovery. This is not surprising as neuroendocrine regulation may account at least partly for circulating leukocyte trafficking responses (McCarthy & Dale, 1988; Laing *et al.*, 2008a), and largely for saliva S-IgA responses (Bishop & Gleeson, 2009) to prolonged exercise, and an influence of refeeding on stress hormone responses was not observed. Although a fluid restriction trial was not included, these results indicate that fluid bolus ingestion decreases saliva S-IgA concentration during recovery. Moreover, there was a tendency for CHO-PRO to elicit a more pronounced decrease in saliva S-IgA concentration after fluid bolus ingestion compared with water.

In conclusion, these results show that the ingestion of a CHO-PRO solution equal to 1.2 g $CHO \cdot kg^{-1}BM$ and 0.4 g $PRO \cdot kg^{-1}BM$ immediately after, but not 1 h after, prolonged strenuous exercise prevents the decrease in bacterially-stimulated neutrophil degranulation, but does not alter circulating leukocyte trafficking, saliva S-IgA or stress hormone responses. A follow-up study should aim to identify the independent effects of carbohydrate and protein feeding during recovery from prolonged strenuous exercise on selected immune responses.

CHAPTER SEVEN

Effects of Immediate Post-Exercise Carbohydrate Ingestion With and Without the Addition of Protein on Selected Immune Responses

7.1 Summary: The purpose of this study was to determine the influence of carbohydrate intake immediately after prolonged exercise, with and without protein, on selected immune, plasma cortisol and insulin responses. Twelve male runners completed three feeding interventions in randomised order after 2 h running at 75% $\dot{V}O_{2max}$. On one occasion participants received a placebo solution (CON), on another occasion received a carbohydrate solution equal to 1.2 g CHO·kg^{-1}BM (CHO), and on another occasion received carbohydrate and protein solution equivalent to 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM (CHO+PRO) immediately post-exercise. All solutions were flavour and water volume equivalent (12 ml·kg^{-1}BM). Circulating leukocyte counts, bacterially-stimulated neutrophil degranulation, saliva IgA responses, plasma cortisol and insulin responses were determined from venous blood and unstimulated saliva samples collected pre-exercise, immediately post-exercise and every 30 min until 180 min post-exercise. Post-exercise circulating leukocytosis, neutrophilia and lymphopenia were similar throughout the recovery period on all trials (MEOT, $P< 0.01$). Bacterially-stimulated neutrophil degranulation significantly decreased during recovery on CON (23% at 180 min; $P< 0.01$ *vs.* pre-exercise), but remained above pre-exercise levels on CHO and CHO+PRO. An increase in saliva IgA concentration was observed immediately post-exercise, followed by a decreased during recovery (45% at 90 min; MEOT, $P< 0.01$). No changes in saliva IgA secretion rate were observed. In conclusion, carbohydrate ingestion with and without protein immediately after prolonged strenuous exercise prevented the decrease in bacterially-stimulated neutrophil degranulation during recovery, but did not alter leukocyte trafficking or saliva IgA responses. It is possible that insulin, and unlikely that cortisol, may have a role in maintaining bacterially-stimulated neutrophil degranulation with immediate carbohydrate feeding, with and without protein, after prolonged strenuous exercise.

7.2 Introduction

Prolonged strenuous exercise is associated with depletion of muscle glycogen, tissue damage and reductions in host defence (Nieman, 1997; Jentjens & Jeukendrup, 2003; Tipton & Wolfe, 2004). The consumption of carbohydrate and protein immediate after prolonged strenuous exercise is recommended and commonly practiced amongst endurance athletes in the attempt to aid glycogen and tissue protein resynthesis (Ivy *et al.*, 2002; Burke *et al.*, 2004; Tipton, 2008). This dietary strategy may also play a role in the maintenance and/or recovery of immune function after prolonged strenuous exercise (Gleeson, 2006).

Chapter 6 demonstrated that carbohydrate and protein intake (1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM) immediately and 1 h after 2 h of running exercise at 70% $\dot{V}O_{2max}$ in a fasted state did not influence exercise-induced perturbations to circulating leukocytes or saliva S-IgA responses during recovery. However, consuming carbohydrate and protein immediately after exercise prevented the decrease in bacterially-stimulated neutrophil degranulation observed on control (-24%) and with 1 h delayed carbohydrate and protein feeding (-31%; **Chapter 6**).

The mechanism(s) responsible for the maintenance of bacterially-stimulated neutrophil degranulation with immediate post-exercise carbohydrate and protein feeding remains unclear. However, there is recent considerable interest in the potential role that insulin may have in modulating neutrophil function, since hyperinsulinaemic-euglycaemic clamp has shown to increase neutrophil chemotaxis, phagocytosis and bactericidal capacity in healthy subjects (Walrand *et al.*, 2004; Walrand *et al.*, 2006). Therefore, it is conceivable that increases in plasma insulin during recovery from exercise may be involved in

preventing the decrease in neutrophil function, rather than attenuated stress hormone release as proposed by previous studies (Robson *et al.*, 1999; Bishop *et al.*, 2002).

Whilst carbohydrate and protein intake immediately after prolonged strenuous exercise may prevent the decrease in bacterially-stimulated neutrophil degranulation during recovery (**Chapter 6**); it still has not been determined whether it is the carbohydrate *per se*, or the combination of carbohydrate with protein, which prevent the decrease in bacterially-stimulated neutrophil degranulation. To date, no study has investigated the effects of carbohydrate ingested with and without protein after prolonged strenuous exercise on immune responses. If insulin does play a role in the maintenance of immune function during recovery from prolonged strenuous exercise, the addition of protein to a carbohydrate beverage may be more beneficial than carbohydrate alone. Studies have reported greater insulin responses with carbohydrate and protein ingestion, compared with carbohydrate alone, after glycogen depleting exercise protocols (van Loon *et al.*, 2000a; van Loon *et al.*, 2000b).

Therefore, the aim of the current study was to determine the influence of carbohydrate feeding, immediately following prolonged strenuous exercise, either with or without the addition of protein, on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva IgA responses in trained endurance runners; and in addition its influence on changes to plasma cortisol and insulin concentration during recovery from prolonged strenuous exercise. It was hypothesised that carbohydrate feeding with and without the addition of protein would attenuate exercise-induced immune perturbations after exercise. Additionally, fewer exercise-induced immune perturbations would be seen during the recovery period, when carbohydrate with the addition of protein was consumed, rather than carbohydrate alone.

7.3 Methods

Participants. 12 healthy competitive endurance runners (age 29 ± 7 y; NBM 77 ± 8 kg; height 1.78 ± 0.05 m; body fat 15 ± 4 %; $\dot{V}O_{2max}$ 63 ± 5 $ml \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in the study. All participants were club-level road and fell runners with an average of 10 ± 7 y competition experience. Ethical procedures were followed in accordance with **Section 3.1.**

Preliminary measurements. Prior to the first experimental trial, participants were asked to report to the laboratory where preliminary measurements as described in **Section 3.2** and **3.3** were undertaken. From the $\dot{V}O_2$-work rate relationship, the treadmill speed at 75% $\dot{V}O_{2max}$ was extrapolated and verified (11.5 ± 1.2 $km \cdot h^{-1}$ and 1% gradient).

Experimental trials. To control dietary intake, prior to each experimental trial a 24 h diet was provided which catered for the participants estimated energy requirements (2963 ± 193 Kcal; Harris & Benedict, 1919; WHO, 1985), and provided 60% carbohydrate, 23% fat, 17% protein, and 35 $ml \cdot kg^{-1}$BM of water (2698 ± 285 ml; Todorovic & Micklewright, 2004). On 3 occasions separated by one week, participants reported to the laboratory at 0700 h following an overnight fast, where a standard breakfast (526 Kcal; 118 g carbohydrate, 9 g protein, 2 g fat) was provided 2 h prior to the onset of exercise. Urine measurements as previously described in **Section 3.4,** indicated euhydration in all participants on arrival at the laboratory for each trial. After pre-exercise measurements and sample collection, participants ran on a motorised treadmill for 2 h at the previously determined treadmill speed that elicited 75% $\dot{V}O_{2max}$. The 2 h exercise bout was performed in ambient conditions of 20°C and 59% RH with two fans placed one metre from the treadmill at a speed of 2.3 $m \cdot s^{-1}$. HR and RPE were collected every 10 min during

exercise. Participants were given water at a rate of 3 ml·kg^{-1}BM·h^{-1} (231 ± 24 ml·h^{-1}) during exercise.

After the post-exercise blood and saliva sample collections, NBM was recorded, and in randomised order participants consumed a recovery solution involving the same fluid volume (12 ml·kg^{-1}BM), flavour (strawberry) and timing (immediately after exercise). On one occasion participants received a placebo (CON; 0 g of carbohydrate and protein), on another occasion received a carbohydrate solution (CHO; 10% carbohydrate solution providing 1.2 g CHO·kg^{-1}BM; Science in Sport, Blackburn, UK), and on another occasion received a carbohydrate and protein solution (CHO+PRO; 10% carbohydrate solution providing 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM; Science in Sport, Blackburn, UK). Participants received a further placebo bolus 1 h post-exercise in all trials. Participants remained seated for 180 min during the recovery period in which samples were collected.

Blood collection and analysis. Whole blood samples were collected as previously described in **Sections 3.4**, pre-exercise, immediately post-exercise, and at 30 min intervals post-exercise for 180 min. Blood analysis was performed in accordance with **Section 3.4**.

Saliva collection and analysis. Unstimulated whole saliva samples were collected as previously described in **Sections 3.4**, pre-exercise, immediately post-exercise, and at 30 min intervals post-exercise for 180 min. Saliva analysis was performed in accordance with **Section 3.4**.

Statistical Analysis. Statistical analysis of data was performed in accordance with **Section 3.5**. However, body mass loss was assessed using a one-way ANOVA.

7.4 Results

There were no significant main effects for HR (exercise mean: 156 ± 14 bpm) and RPE (exercise mean: 12 ± 1), and no significant difference in body mass loss was observed between trials (mean of trials: 2.4 ± 0.4 %). There was a significant MEOT for plasma volume change ($F(7,77)= 17.7$, $P< 0.01$). A significant decrease (-5.3% ± 5.2) in plasma volume was observed post-exercise compared with pre-exercise ($P< 0.01$).

Circulating leukocyte, neutrophil, lymphocyte counts and neutrophil:lymphocyte ratio

There were no trial x time interactions for circulating leukocyte, neutrophil, lymphocyte counts or the neutrophil:lymphocyte ratio. However, a significant MEOT was observed for circulating leukocyte ($F(2.1,22.9)= 51.4$, $P< 0.01$), neutrophil ($F(1.9,20.9)= 64.6$, $P< 0.01$), and lymphocyte counts ($F(1.3,14.7)= 36.0$, $P< 0.01$), and the neutrophil:lymphocyte ratio ($F(1.5,16.5)= 44.2$, $P< 0.01$). A significant circulating leukocytosis ($P< 0.01$; **Table 7.1**) and neutrophilia ($P< 0.01$; **Figure 7.1**) were observed following exercise and remained significantly elevated throughout the recovery period measured. Circulating lymphocyte counts significantly increased immediately post-exercise ($P< 0.01$), whilst a significant lymphopenia was observed at 90 min and 120 min post-exercise ($P< 0.05$; **Table 7.1**). Compared with pre-exercise values (CON: 1.9 ± 0.8; CHO: 1.5 ± 0.4; CHO+PRO: 1.5 ± 0.3), the neutrophil:lymphocyte ratio significantly increased by 30 min post-exercise ($P< 0.01$) and continued to progressively increase throughout recovery, peaking at 120 min post-exercise (CON: 7.4 ± 2.6; CHO: 6.1 ± 2.0; CHO+PRO: 7.4 ± 3.6).

Table 7.1: Circulating leukocyte and lymphocyte count responses to placebo (CON), carbohydrate alone (CHO), and carbohydrate with protein (CHO+PRO) feeding immediately after 2 h running at 75% $\dot{V}O_{2max}$.

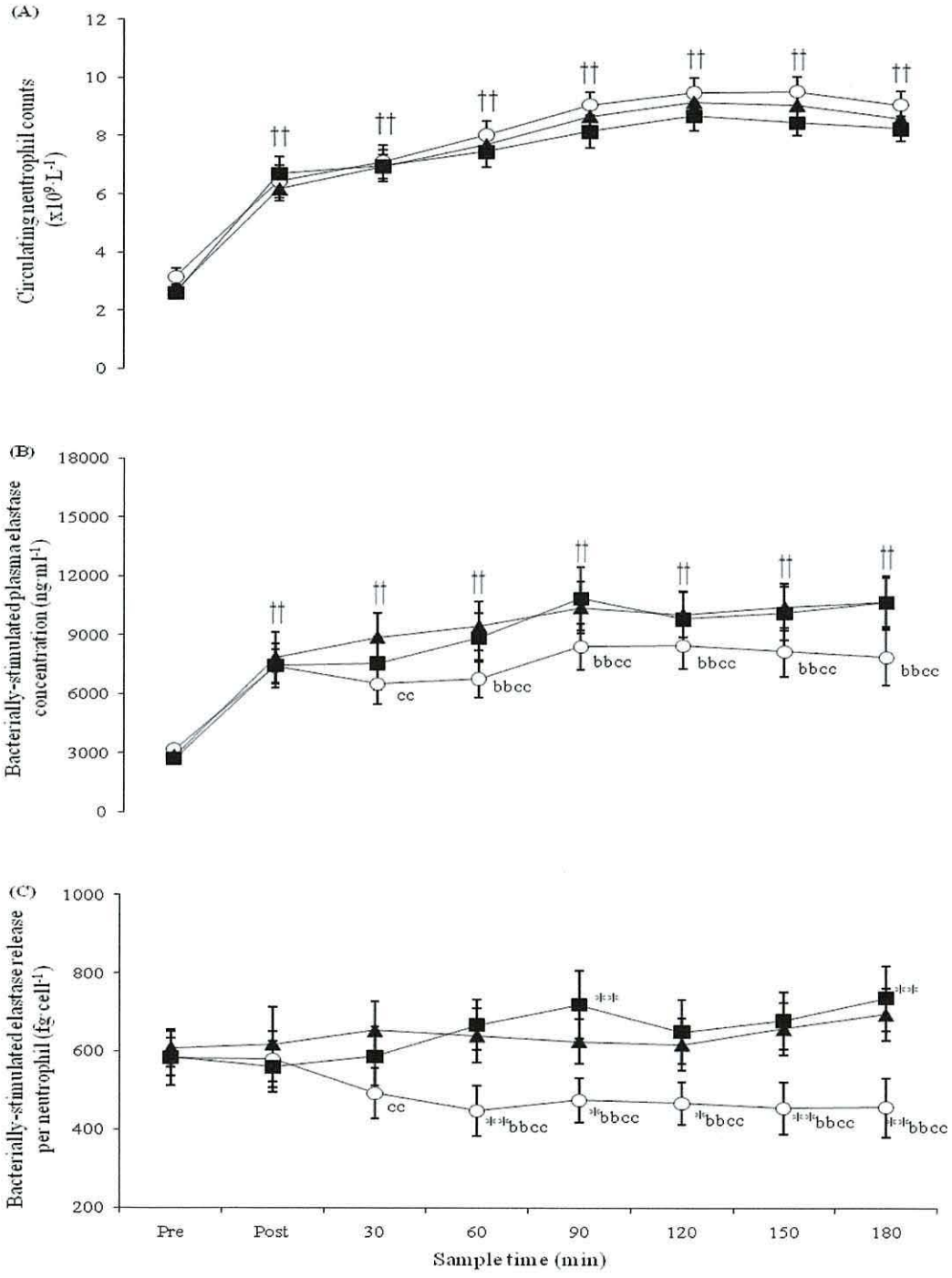
Counts ($x10^9 \cdot L^{-1}$)	Pre	Post	Recovery Period (min) 30	60	90	120	150	180
Leukocyte		††	††	††	††	††	††	††
CON	5.4 ± 0.8	9.8 ± 2.2	9.3 ± 2.2	10.1 ± 1.7	11.0 ± 1.4	11.3 ± 1.8	11.5 ± 1.6	11.2 ± 1.6
CHO	5.1 ± 0.6	10.2 ± 2.0	9.2 ± 2.0	9.5 ± 1.8	10.4 ± 1.9	10.9 ± 1.9	10.8 ± 1.7	10.7 ± 1.7
CHO+PRO	5.0 ± 0.9	9.7 ± 1.6	9.2 ± 1.6	9.8 ± 1.3	10.7 ± 1.7	11.3 ± 1.6	11.3 ± 1.7	11.2 ± 1.6
Lymphocyte		††			†	†		
CON	1.8 ± 0.4	2.8 ± 0.8	1.7 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	1.5 ± 0.3	1.6 ± 0.3
CHO	1.8 ± 0.3	2.8 ± 0.6	1.6 ± 0.3	1.5 ± 0.3	1.4 ± 0.4	1.5 ± 0.3	1.6 ± 0.3	1.7 ± 0.3
CHO+PRO	1.8 ± 0.3	2.8 ± 0.7	1.7 ± 0.4	1.4 ± 0.4	1.4 ± 0.3	1.4 ± 0.3	1.5 ± 0.4	1.8 ± 0.5

Mean ± SD (n= 12): MEOT † $P< 0.05$ and †† $P< 0.01$ *vs.* pre.

Bacterially-stimulated neutrophil degranulation. A significant MEOT was observed for unstimulated plasma elastase concentration ($F(3.2,35.4)= 30.2$, $P< 0.01$). Compared with pre-exercise values (CON: 54 ± 33 $ng \cdot ml^{-1}$; CHO: 46 ± 9 $ng \cdot ml^{-1}$; CHO+PRO: 49 ± 31 $ng \cdot ml^{-1}$; $P< 0.01$), unstimulated plasma elastase concentration significantly increased immediately post-exercise (CON: 219 ± 88 $ng \cdot ml^{-1}$; CHO: 178 ± 51 $ng \cdot ml^{-1}$; CHO+PRO: 229 ± 104 $ng \cdot ml^{-1}$). Significant trial x time interaction was observed for bacterially-stimulated plasma elastase concentration ($F(14,140)= 2.4$, $P< 0.01$; **Figure 7.1**). In all trials, bacterially-stimulated plasma elastase concentration significantly increased post-exercise ($P< 0.01$) and remained elevated throughout the recovery period measured. Compared with CON, bacterially-stimulated plasma elastase concentration was significantly higher 60 min post-exercise onwards on CHO ($P< 0.01$), and 30 min post-exercise onwards on CHO+PRO ($P< 0.01$).

Significant trial x time interaction was also observed for bacterially-stimulated neutrophil degranulation (elastase release per neutrophil; $F(14,140)= 4.0$, $P< 0.01$; **Figure 7.1**). Bacterially-stimulated neutrophil degranulation significantly decreased at 60 min post-exercise on CON (-23%) and remained significantly lower throughout the recovery period measured (-23% at 180 min post-exercise; $P< 0.01$ *vs.* pre-exercise). CHO and CHO+PRO prevented the decrease in bacterially-stimulated neutrophil degranulation observed on CON. Interestingly, a significant increase in bacterially-stimulated neutrophil degranulation was observed on CHO at 90 min (22%) and 180 min (28%) post-exercise ($P< 0.01$ *vs.* pre-exercise). Compared with CON, bacterially-stimulated neutrophil degranulation was significantly higher 60 min post-exercise onwards on CHO ($P< 0.01$), and at 30 min post-exercise onwards on CHO+PRO ($P< 0.01$).

Figure 7.1: Circulating neutrophil count (A), bacterially-stimulated plasma elastase concentration (B), and bacterially-stimulated elastase release per neutrophil responses to placebo (CON o), carbohydrate alone (CHO ■), and carbohydrate with protein (CHO+PRO ▲) feeding immediately after 2 h running at 75% $\dot{V}O_{2max}$.



Mean ± SEM (n= 12). MEOT †† P< 0.01 *vs.* pre; * P< 0.05 and ** P< 0.01 *vs.* pre; bb P< 0.01 *vs.* CHO; cc P< 0.01 *vs.* CHO+PRO.

Saliva IgA responses. A significant trial x time interaction was observed for saliva flow rate ($F(14,154)= 1.9$, $P< 0.05$), and a MEOT was observed for saliva IgA concentration ($F(2.0,22.4)= 18.4$, $P< 0.01$), whereas no main effect was observed for saliva IgA secretion rate (**Table 7.2**). Saliva flow rate significantly decreased immediately post-exercise on CON (-27%) and CHO+PRO (-26%) only ($P< 0.01$ *vs.* pre-exercise). However, during recovery saliva flow rate significantly increased 90 min onwards on CON ($P< 0.01$ *vs.* pre-exercise) and CHO+PRO ($P< 0.05$ *vs.* pre-exercise), and 90 min until 150 min on CHO ($P< 0.05$ *vs.* pre-exercise). Furthermore, saliva flow rate was significantly higher on CON at 30 min and 60 min post-exercise compared with CHO+PRO ($P< 0.01$), and at 30 min post-exercise compared with CHO ($P< 0.01$). Compared with pre-exercise values, a significant increase in saliva IgA concentration was observed immediately post-exercise in all trials (32%; $P< 0.05$). Conversely, saliva IgA concentration significantly decreased at 90 min (-45%) and 120 min (-38%) post-exercise in all trials ($P< 0.01$ *vs.* pre-exercise).

Table 7.2: Saliva flow rate, saliva IgA concentration and saliva IgA secretion rate responses to placebo (CON), carbohydrate alone (CHO), and carbohydrate with protein (CHO+PRO) feeding immediately after 2 h running at 75% $\dot{V}O_{2max}$.

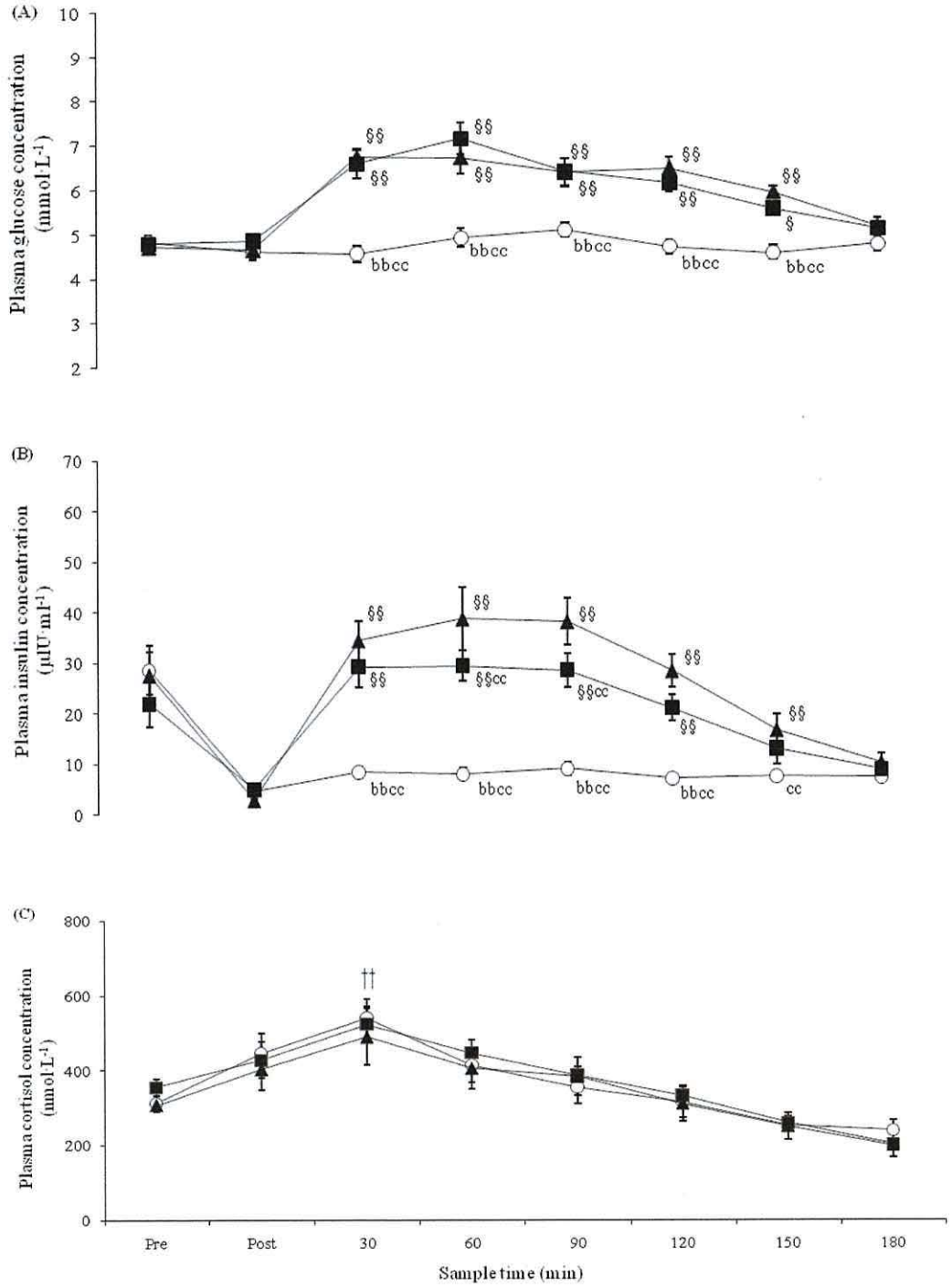
		Recovery Period (min)							
		Pre	Post	30	60	90	120	150	180
Saliva flow rate ($\mu l \cdot min^{-1}$)	CON	539 ± 286	391 ± 258 **	625 ± 338	644 ± 368	754 ± 416 **	729 ± 400 **	679 ± 365 **	715 ± 334 **
	CHO	493 ± 304	415 ± 258	456 ± 364 aa	562 ± 396	637 ± 406 **	636 ± 348 **	625 ± 329 *	599 ± 264
	CHO+PRO	516 ± 344	382 ± 246 **	458 ± 299 aa	490 ± 332 aa	647 ± 407 *	709 ± 475 **	719 ± 434 **	696 ± 372 **
			†			††	††		
Saliva IgA concentration ($\mu g \cdot ml^{-1}$)	CON	64 ± 21	94 ± 41	50 ± 27	60 ± 34	36 ± 20	40 ± 23	46 ± 23	50 ± 18
	CHO	68 ± 23	89 ± 45	75 ± 26	67 ± 27	36 ± 21	45 ± 22	49 ± 24	47 ± 21
	CHO+PRO	74 ± 40	91 ± 49	66 ± 19	81 ± 31	43 ± 19	43 ± 22	53 ± 18	54 ± 24
Saliva IgA secretion rate ($\mu g \cdot min^{-1}$)	CON	33 ± 18	34 ± 26	27 ± 13	35 ± 20	23 ± 11	26 ± 17	28 ± 13	33 ± 14
	CHO	31 ± 17	33 ± 26	28 ± 16	31 ± 20	21 ± 17	29 ± 22	32 ± 26	27 ± 14
	CHO+PRO	33 ± 24	31 ± 24	31 ± 22	35 ± 22	25 ± 15	26 ± 15	33 ± 15	34 ± 15

Mean ± SD (n= 12): MEOT † $P< 0.05$ and †† $P< 0.01$ *vs.* pre; * $P< 0.05$ and ** $P< 0.01$ *vs.* pre; aa $P< 0.01$ *vs.* CON.

Plasma glucose, insulin and cortisol concentration. A significant trial x time interaction was observed for plasma glucose ($F(14,154)= 7.7$, $P< 0.01$), and insulin concentration ($F(14,154)= 10.0$, $P< 0.01$). Whereas a significant MEOT was observed for plasma cortisol concentration ($F(1.6,17.9)= 19.0$, $P< 0.01$; **Figure 7.2**). Plasma glucose concentration was unaffected by 2 h running at 75% $\dot{V}O_{2max}$ in all trials. Significant increases in plasma glucose concentration were observed after CHO and CHO+PRO bolus ingestion only ($P< 0.01$ *vs.* post-exercise), which remained elevated until 150 min post-exercise (**Figure 7.2**). Plasma glucose concentration was significantly higher on CHO and CHO+PRO at 30 min until 150 min post-exercise compared with CON ($P< 0.01$).

Similarly, significant increases in plasma insulin concentration were observed after CHO and CHO+PRO bolus ingestion only ($P< 0.01$ *vs.* post-exercise; **Figure 7.2**), which remained elevated until 120 min post-exercise on CHO ($P< 0.01$), and 150 min post-exercise on CHO+PRO ($P< 0.01$). Plasma insulin concentration was significantly higher than CON at 30 min until 120 min post-exercise on CHO ($P< 0.01$) and 30 min until 150 min post-exercise on CHO+PRO ($P< 0.01$). Plasma cortisol concentration increased immediately post-exercise in all trials, peaking at 30 min post-exercise (59%; $P< 0.01$ *vs.* pre-exercise) and returned to pre-exercise levels by 120 min post-exercise.

Figure 7.2: Plasma glucose (A), insulin (B) and cortisol (C) responses to placebo (CON o), carbohydrate alone (CHO ■), and carbohydrate with protein (CHO+PRO ▲) feeding immediately after 2 h running at 75% $\dot{V}O_{2max}$.



Mean ± SEM (n= 12). MEOT †† P< 0.01 *vs.* pre; § P< 0.05 and §§ P< 0.01 *vs.* post; bb P< 0.01 *vs.* CHO; cc P< 0.01 *vs.* CHO+PRO.

7.5 Discussion

The aim of the current study was to determine the influence of carbohydrate feeding, immediate following prolonged strenuous exercise, either with or without the addition of protein, on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, and saliva IgA responses. In support of the hypothesis, these results show that the ingestion of a carbohydrate solution providing 1.2 g CHO·kg^{-1}BM or a carbohydrate and protein solution providing 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM immediately after prolonged strenuous exercise prevents the decrease in bacterially-stimulated neutrophil degranulation. In contrast to the hypothesis, carbohydrate feeding with and without protein immediately after prolonged strenuous exercise did not alter circulating leukocyte trafficking and saliva IgA responses. These results are in accordance with those described in **Chapter 6**, and may be important for individuals partaking in endurance activities who are unable to consume sufficient carbohydrate during exercise (Noakes, 2003; Kruseman *et al.*, 2005; Gleeson, 2006), or athletes who train whilst fasted and without carbohydrate intake during exercise in an attempt to improve training adaptations (Civitarese *et al.*, 2005; Hawley *et al.*, 2006; De Bock *et al.*, 2008).

Decreases in neutrophil function have been frequently reported after the cessation of prolonged exercise (Robson *et al.*, 1999; Peake, 2002; Laing *et al.*, 2008a). The current findings indicate that the consumption of carbohydrate *per se* early into recovery may prevent the exercise-induced decrease in bacterially-stimulated neutrophil degranulation from occurring, irrespective of exercise being initiated after a substantial carbohydrate meal (**Chapter 7**) or in a fasted state (**Chapter 6**). Previous studies have speculated various blood-borne mechanisms (e.g. decreased plasma glucose concentration, increases in plasma cortisol) may be responsible for the decrease in bacterially-stimulated neutrophil

degranulation, as observed on CON (Nieman *et al.*, 1997; Johnson *et al.*, 1998; Robson *et al.*, 1999; Bishop *et al.*, 2002). However, supportive evidence for these mechanisms is lacking, since continued declines in bacterially-stimulated neutrophil degranulation have been observed despite unsubstantial changes in proposed mechanisms (Li & Gleeson, 2005b; Laing *et al.*, 2008a; **Chapter 6**).

In support of this, in the current study, exercise stress did not evoke significant decreases in plasma glucose concentration in all trials (mean of trials: pre 4.8 ± 0.5 *vs.* post 4.7 ± 0.6 $mmol \cdot L^{-1}$), and plasma glucose concentration remained within normal range during the recovery period on CON (4.8 ± 0.6 $mmol \cdot L^{-1}$). Moreover, plasma cortisol concentration peaked at 30 min post-exercise and returned to pre-exercise values by 60 min post-exercise in all trials. A novel aspect of the current study is that the key nutrient, carbohydrate, provided early during recovery may counteract the possible involvement of blood-borne factors in the decrease in neutrophil degranulation seen after heavy exercise. Recently, the potential role for insulin modulating neutrophil function has been proposed (Walrand *et al.*, 2004; Walrand *et al.*, 2006; Stegenga *et al.*, 2008b). It is thus conceivable that the sustained increase in plasma insulin during recovery on CHO and CHO+PRO may be involved in preventing the decrease in bacterially-stimulated neutrophil degranulation observed on CON.

In accordance with findings described in **Chapter 6,** the present study demonstrated that carbohydrate with and without protein shortly after prolonged strenuous exercise does not alter circulating leukocyte trafficking or saliva IgA responses during recovery. Nutritional intervention in the current study had no effect on plasma stress hormone levels. These findings provide additional supports that carbohydrate with and without protein is unlikely

to influence circulating leukocyte trafficking or saliva IgA responses, since neuroendocrine regulation contributes to both circulating leukocyte trafficking (McCarthy & Dale, 1988; Laing *et al.*, 2008a), and saliva IgA responses (Bishop & Gleeson, 2009). Interestingly, placebo ingestion on CON promoted a significantly greater saliva flow rate at 30 and 60 min compared with CHO and CHO+PRO bolus ingestion. This indicates that nutrient availability within fluids may suppress saliva secretions *per se* in the short term. However, the decrease in saliva IgA concentration observed in all trials during the end stages of the recovery period measured, may be due to the dilution effect of substantial increases in saliva flow rate also observed in all trials. This may possibly be due to the 12 $ml \cdot kg^{-1}$BM placebo bolus which was taken at 60 min. Similar patterns in saliva IgA profile was observed in **Chapter 6**. Although a fluid abstinence trial was not included, our results indicate that fluid bolus ingestion decreases saliva IgA concentration during recovery, which may have clinical significance.

In conclusion, these results show that the ingestion of a carbohydrate bolus providing 1.2 g $CHO \cdot kg^{-1}$BM or a carbohydrate and protein bolus providing 1.2 g $CHO \cdot kg^{-1}$BM and 0.4 g $PRO \cdot kg^{-1}$BM immediately after prolonged strenuous exercise prevents the decrease in bacterially-stimulated neutrophil degranulation, but does not alter circulating leukocyte trafficking or saliva IgA responses. It is possible that insulin, and unlikely that cortisol, may have a role in maintaining bacterially-stimulated neutrophil degranulation with carbohydrate feeding, either with or without the addition of protein, immediately after prolonged strenuous exercise.

CHAPTER EIGHT

General Discussion

8.1 Background

Prolonged strenuous exercise, sleep-deprivation, exposure to environmental extremes and nutrient deprivation have all individually been reported to disturb immune responses, through neuroendocrine mechanisms. It is plausible that participating in activities that are associated with a combination of these physiological stressors (e.g. sustained military training and routine operations, travelling across time zones for sports competitions, ultra-endurance events, adventure and exploration activities), may amplify immune modulating neuroendocrine responses and induce greater perturbations to host defences. This outcome may have clinical significance in increasing the risk of illness, infection, dampen effective wound healing and increasing susceptibility to sepsis following injury in the field; especially in unfamiliar foreign locations, which may be occupied by unfamiliar pathogens (Martinez-Lopez *et al.*, 1993; Shephard *et al.*, 1998).

The broad aims of this thesis were therefore to investigate: 1. the effects of one night total sleep-deprivation on selected immune (circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation, saliva IgA) and stress hormone responses at rest and following prolonged strenuous exercise; 2. the effects of two nights total sleep-deprivation with and without energy-restriction on selected immune and stress hormone responses at rest and after passive cold-exposure; 3. the effects of passive cold-exposure inducing modest whole-body cooling on selected immune and stress hormone responses; and finally, 4. the effects of carbohydrate feeding, with and without the addition of protein, during the recovery period from prolonged strenuous exercise on selected immune, stress hormone and insulin responses.

8.2 The influence of total sleep-deprivation with and without energy-restriction on selected immune responses

The experimental trials in **Chapters 4** and **5** presented a novel aspect, as they were the first to determine the effects of total sleep-deprivation with and without energy-restriction on bacterially-stimulated neutrophil degranulation and saliva IgA responses at rest (**Chapter 5**) and in response to exercise (**Chapter 4**). Previous studies have indicated that a 24 h period of total sleep-deprivation is sufficient to induce a circulating leukocytosis and reduce phagocytic function (Palmblad *et al.*, 1976; Born *et al.*, 1997), whilst a 48 h period of total sleep-deprivation was needed to reduce lymphocyte function (Palmblad *et al.*, 1979). Additionally, disturbed sleep-wake cycles may have contributed to the depressed saliva IgA responses and increased incidence of URI reported during and after military combat training courses (Gomez-Merino *et al.*, 2003; Tiollier *et al.*, 2005; Gomez-Merino *et al.*, 2005). Conversely, results from the controlled laboratory experimental trials conducted in **Chapters 4** and **5** demonstrate that one and two nights of total sleep-deprivation does not alter circulating leukocyte trafficking (**Table 4.1**; **Figure 5.2**) and plasma stress hormone responses (**Table 5.2**), or compromise bacterially-stimulated neutrophil degranulation (**Table 4.2**; **Figure 5.3**) and saliva S-IgA responses (**Table 4.3**; **Figure 5.4**) at rest (**Chapter 5**), and in response to exercise (after one night of total sleep-deprivation; **Chapter 4**). Even though previous studies have found that energy-restriction has implications in reducing host defences (Walrand *et al.*, 2001), the addition of a 90% energy restriction during total sleep-deprivation in **Chapter 5** did not further perturbate or compromise immune responses or exacerbate stress hormone responses.

Taking into account that circulating leukocyte trafficking and neutrophil function is under neuroendocrine control (McCarthy & Dale, 1988; Hoffman-Goetz & Pedersen, 1994;

Sternberg, 2006). It would appear that disturbance to sleep-wake cycles and increased homeostatic drive to sleep, which stimulates SAM and HPA axis release of stress hormones, may subsequently alter circulating leukocyte trafficking and function (Uthgenannt *et al.*, 1995; Pedersen *et al.*, 1997; Madden, 2003; Irwin *et al.*, 2003). For example, Radomski *et al.* (1992) reported increases in stress hormones and altered circadian hormone responses after two nights of total sleep-deprivation. However, experimental **Chapters 4** and **5** demonstrate that one and two nights of total sleep-deprivation failed to induce substantial change in plasma stress hormone levels (**Section 4.4**; **Table 5.2**). Therefore, it is not surprising that no alterations in circulating leukocyte trafficking or neutrophil function were observed. Results from the current thesis are in agreement with one study showing no change in circulating leukocyte counts after one night of sleep deprivation (Heiser *et al.*, 2000). Whereas, they are in contrast with studies reporting a circulating leukocytosis and depressed neutrophil phagocytosis after only 24 h of total sleep-deprivation (Palmblad *et al.*, 1976; Born *et al.*, 1997). However, plasma levels of stress hormones were not measured in these studies. It is conceivable that the decrease in neutrophil phagocytosis was possibly due to the additional stress of continuous rifle shooting task and battlefield noise exposure in one of these studies (Palmblad *et al.*, 1976).

Discrepant findings in immune measures between sleep-deprivation studies most likely reflects differences in duration of sleep-deprivation and circadian variations (Shephard & Shek, 1997). Typically, longer periods of sleep deprivation ($\geq$ two nights) have been shown to elicit more pronounced immune perturbations (Dinges *et al.*, 1995). Therefore, it is conceivable the time period of sleep-deprivation used in the studies presented in **Chapters 4** and **5** were not long enough to substantially alter stress hormone and

circulating immune responses. However, from a practical viewpoint, total sleep-deprivation for more than two nights is not commonly practiced amongst humans. Individuals involved with activities which may include sleep disturbances (military, ultra-endurance, adventure, expedition and shift work activities) are more likely to adhere to intermittent sleep patterns or partial sleep-deprivation for more the two nights. The effects of this type of sleep pattern on immune function has not been determined and warrants further investigation. Furthermore, pre-trial sleep duration in **Chapters 4** and **5** (mean of trials; **Chapter 4**: 515 ± 20 min (bedtime 21:30 to 06:30); **Chapter 5**: 444 ± 28 min (bedtime 22:30 to 07:30), which did not significantly differ between trails in each experimental chapter, were measured to verify sleep quantity prior to the experimental trials. This research control measure was not used in previous studies. It is possible that the pre-trial sleep quantity may have been an influential factor in results obtained in these previous studies.

Previous studies looking into the effects of energy-restriction on circulating leukocyte trafficking and function (McMurray *et al.*, 1990; Field *et al.*, 1991; Walrand *et al.*, 2001; Booth *et al.*, 2003; Galassetti *et al.*, 2006) have presented conflicting findings, possibly due to the level and duration of energy-restriction and variations in research control. A well conducted laboratory study looking into the effects of a 48 h period of 90% energy-restriction reported a decrease in circulating leukocyte count mainly attributed to a mild reduction in circulating lymphocyte counts (Laing *et al.*, 2008b). However, in the same study, energy-restriction had no effect on circulating neutrophil counts or bacterially-stimulated neutrophil degranulation. The addition of two nights of total sleep-deprivation to a 90% energy-restriction, as described in **Chapter 5**, did not alter circulating leukocyte trafficking (**Figure 5.2**) and stress hormones responses (**Table 5.2**), or compromise

bacterially-stimulated neutrophil degranulation (**Figure 5.3**). The discrepancies found in circulating lymphocyte counts may highlight that an individual stressor previously reported to perturbate immune responses (e.g. energy-restriction alone) does not necessarily have the same effect when combined with another stressor (e.g. sleep-deprivation). It remains unclear why the addition of sleep-deprivation may have prevented the decrease in circulating lymphocyte counts observed previously (Laing *et al.*, 2008b). Possibly, the influence of sleep-deprivation on circadian modulation of peripheral blood flow (Van Someren, 2006), or sleep-deprivation induced increases in melatonin levels may have modulated peripheral blood flow (Salin-Pascual *et al.*, 1988; Guerrero & Reiter, 2002) and induce alteration in lymphocyte redistribution (Kruger & Mooren, 2007). However, this remains to be substantiated.

The studies presented in **Chapters 4** and **5** were also novel in respect to determining the effects of one and two nights of total sleep-deprivation with and without energy-restriction on saliva IgA responses at rest (**Chapter 5**), and in response to exercise (after one night of total sleep-deprivation; **Chapter 4**). Previous studies observing the effects of sleep-deprivation on saliva IgA responses were military based field studies, involving intermittent sleep protocols (1-3 h sleep·day^{-1} for 5-7 days). Decreases in saliva IgA concentration and increased incidence of URI were observed in military personnel after five days combat training course that was preceded by three weeks of physical fitness conditioning (Boyum *et al.*, 1996; Gomez-Merino *et al.*, 2003). In these studies, participants were exposed to additional stressors that could also account for decreased saliva IgA responses; including: exercise (Gleeson, 2007), fluid restriction (Oliver *et al.*, 2007), anxiety (Keller *et al.*, 1981), and exposure to extreme environments (Walsh & Whitham, 2006).

Up to two nights of total sleep-deprivation with and without 90% energy restriction appears to not compromise saliva IgA responses (**Table 4.3**; **Figure 5.4**). These findings extend to those recently showing that two days of 90% energy-restriction alone did not lower resting saliva IgA concentration or secretion rate (Oliver *et al.*, 2007). However, results from **Chapter 5** indicate that two nights of sleep-deprivation (with and without energy-restriction) tended to suppress saliva flow rate; which consequently, promoted substantial increases in saliva S-IgA concentration due to a concentrating effect (**Figure 5.4**). This is further supported by no observed changes in saliva S-IgA secretion rate, possibly indicating a minimal influence of total sleep-deprivation on S-IgA synthesis and translocation.

Moreover, since saliva secretions are under tight neural control (Teeuw *et al.*, 2004), the trend for decreases in saliva flow rate during sleep-deprivation is possibly due to reductions in parasympathetic tone induced by the anxiety associated with sleep-deprivation and the increased drive to sleep (Shephard & Shek, 1997). Even though saliva IgA responses were not compromised, the trend towards reduced saliva flow rate may have clinical implications, since saliva flow *per se* forms a fundamental first line defence (mechanical washing of the oral-respiratory mucosal surface and fluid base for anti-viral/bacterial mucosal secretion distribution) against foreign airborne pathogens (McNabb & Tomasi, 1981; Tenovuo, 1998; Dowd, 1999; West *et al.*, 2006). The importance of saliva flow is noticed in individuals suffering from xerostomia, who have a high incidence rate of oral and URI (Fox *et al.*, 1985; Fox *et al.*, 2004).

From a practical viewpoint, disturbed sleep patterns, combined with various degrees of energy-restriction, for more than 48 h is a commonly practiced in military, ultra-

endurance, adventure and expedition activities. The effect of considerable energy-restriction (>90%) in conjunction with disturbed sleep pattern (intermittent sleep or partial sleep-deprivation) for a more prolonged time period (e.g. ≥ one week) on circulating leukocyte trafficking, neutrophil function, saliva IgA responses and other immune indices has not been determined and warrants further investigation. Future studies should include, for example, assessment of *in vivo* responses (e.g. DTH, vaccine challenge), and assess the clinical significance of any reductions of immune function on infection incidence and tissue healing (Albers *et al.*, 2005).

8.3 The influence of cold-exposure on selected immune responses

Due to experimental protocol designs (e.g. temperature used, degree of T_{core} reduction, wet or dry conditions, type of cold exposure: water bath *vs.* CAT), and lack of research control (e.g. not monitoring T_{core}, differences in monitoring body temperature, no control group), previous studies investigating the effects of cold exposure on immune responses have failed to clearly determine cold-induced immune outcomes (Menasche *et al.*, 1995; Jansky *et al.*, 1996; Wenisch *et al.*, 1996a; Brenner *et al.*, 1999; Birdi *et al.*, 1999). The experimental trials in **Chapters 5** presented a novel aspect, as they were the first to determine the effects of passive cold exposure, resulting in modest whole-body cooling (35.9°C T_{re}), with and without prior physiological stress, on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva S-IgA responses. The results suggest that cold air exposure (0°C) resulting in modest whole-body cooling promotes a circulating leukocytosis, neutrophilia and lymphopenia (**Figure 5.2**), and decreases bacterially-stimulated neutrophil degranulation (**Figure 5.3**) and saliva S-IgA responses (**Figure 5.4**). The addition of a 53 h period of total sleep-deprivation without 90% energy-restriction prior to cold-exposure did not further amplify or compromise immune responses. However, the combination of a 53 h period of total sleep-deprivation with 90% energy-restriction prior to cold-exposure delayed circulating neutrophilia (**Figure 5.2**), and prevented the reduction in bacterially-stimulated neutrophil degranulation (**Figure 5.3**) during recovery from cold-exposure.

Cold exposure is known to stimulate the HPA and SAM axis to initiate shivering thermogenesis, increase sympathetic tone for peripheral vasoconstriction, and increase the release of endogenous energy substrate, with the aim of conserving and producing body heat (Shephard & Shek, 1998; Castellani *et al.*, 2002; Walsh & Whitham, 2006).

Therefore, the circulating leukocytosis and lymphopenia observed in **Section 5.4** (**Figure 5.2**) is likely to be attributed to HPA and SAM axis stimulated release of stress hormones (**Table 5.2**), as described in **Section 2.1.4**. In this instance, noradrenaline appears to be the main endocrine mechanism, since the biological effects of cortisol tend to be reported only when plasma cortisol concentration exceeds the capacity of corticosteroid-binding globulin (>550 $nmol \cdot L^{-1}$), and the CAT fail to induce cortisol change to a substantial degree in all trials (CAT induced cortisol peak; CON: 450 $nmol \cdot L^{-1}$; SDEP: 392 $nmol \cdot L^{-1}$; SDEP+ER: 421 $nmol \cdot L^{-1}$).

Due to milder perturbation to circulating leukocyte trafficking observed after cold-exposure compared with exercise stress, it is likely that stress hormone induced increases in endothelial demargination of neutrophils and redistribution of lymphocytes into lymphoid and non-lymphoid tissues would account for the changes observed (Castellani *et al.*, 2002; Kruger & Mooren, 2007). Additionally, alterations in adhesion molecules and surface receptors on lymphocytes, thought to have a key role in governing lymphocyte trafficking during environmental stress, may have also accounted for the post-CAT circulating lymphopenia (Kruger & Mooren, 2007). Moreover, a significantly delayed neutrophilia was observed post-CAT on SDEP+ER. Possibly, minor fluctuations in localised peripheral temperatures induced by the addition of 90% energy-restriction to a 53 h period of total sleep-deprivation prior to cold-exposure may have attenuated neutrophil demargination *via* change in neutrophil adhesion properties (Menasche *et al.*, 1995; Wenisch *et al.*, 1996a; Birdi *et al.*, 1999). However, this has not been substantiated.

Modest whole-body cooling with and without a 53 h period of total sleep-deprivation decreased bacterially-stimulated neutrophil degranulation (**Figure 5.3**). It is plausible that

this decrease can be explained by cold stress induced demargination of intravascular endothelial neutrophils with less ability to respond to bacterial challenge (McCarthy & Dale, 1988; Shephard, 2003; Foster *et al.*, 1986). Moreover, the maintained bacterially-stimulated neutrophil degranulation observed post-CAT after a 53 h period of total sleep-deprivation with 90% energy-restriction may reflect the reduced demargination of intravascular endothelial neutrophils (observed delayed circulating neutrophilia; **Figure 5.3)**. However, the mechanism(s) responsible for the neutrophilia and decreased degranulation is not clear and requires further clarification. For example, the widely accepted stress hormone mediators for neutrophilia and decreased neutrophil function to acute stress do not provide adequate explanation for these observations, since no differences in CAT-induced plasma cortisol and catecholamines were observed between trials (**Table 5.2**).

No previous studies have investigated the effects of passive cold exposure on saliva IgA responses. Studies that have observed the effects of cold environmental stress on saliva IgA responses have done so during exercise protocols that did not evoke a reduction in T_{core} (Tomasi *et al.*, 1982; Walsh *et al.*, 2002). Therefore, a novel finding in **Chapter 5** was that modest whole-body cooling was associated with a decrease in saliva S-IgA responses (**Figure 5.4**). The addition of total sleep-deprivation with or without 90% energy restriction did not further comprised saliva S-IgA responses (**Figure 5.4**). The time course of these responses indicated that modest whole-body cooling decreases S-IgA translocation since synthesis of S-IgA takes many hours to days (Saxon *et al.*, 1978; Hucklebridge *et al.*, 1998). Plausible explanations for the decrease of saliva S-IgA responses include the effects of cold air breathing in decreasing the temperature of mucosal membranes, a possible drying effect of cold air, and/or modest whole-body

cooling evoked neuroendocrine regulation of transepithelial S-IgA translocation (Giesbrecht, 1995; Shephard *et al.*, 1998).

From a practical viewpoint, military personnel and individuals partaking in ultra-endurance, adventure and expedition activities in cold ambient environments experience cold exposure to a level that induces considerable reductions in T_{core}. These individuals are further susceptible to cold induced injuries (e.g. frostbite, non-freezing cold injury and upper respiratory tract lesions), which may have clinical significance if immunocompromised (Shephard *et al.*, 1998). The effects of cold exposure evoking a substantial reduction in T_{core} ($\leq$35.0°C) on circulating leukocyte trafficking, neutrophil function, saliva IgA responses and other immune indices has not been investigated and warrants further investigation. Future studies should assess the effects of varying cold exposure techniques (e.g. water bath, cold air test, moist skin surface) on immune responses, include *in-vivo* techniques, in clinically hypothermic individuals (T_{core} $\leq$35.0°C), and assess the clinical significance of any reductions in immune function on infection incidence and tissue healing from specific cold-induced damage (Albers *et al.*, 2005). Additionally, further studies should investigate the separate effects of cold air breathing and cold exposure inducing decreases in T_{core}, on saliva IgA responses.

8.4 The influence of carbohydrate provisions, with and without protein, after prolonged strenuous exercise on selected immune responses

The experimental trials in **Chapters 6** and **7** presented a novel aspect, as they were the first to determine the effects of post-exercise consumption of carbohydrate with and without the addition of protein on circulating leukocyte trafficking, bacterially-stimulated neutrophil degranulation and saliva IgA responses. Previous studies have indicated that carbohydrate consumption prior to and during exercise maintains plasma glucose during exercise, attenuates stress hormone responses, and subsequently attenuates post-exercise circulating leukocytosis, neutrophilia and lymphopenia, prevents the decrease of neutrophil and lymphocyte function, and attenuates pro-inflammatory cytokine responses (Nehlsen-Cannarella *et al.*, 1997; Nieman *et al.*, 1997; Bishop *et al.*, 2002; Green *et al.*, 2003). Albeit, the role of carbohydrate intake prior to and during exercise on maintaining saliva IgA responses during recovery is less convincing (Bishop *et al.*, 2000). Additionally, protein supplementation post-exercise has also been reported to aid immune recovery (Castell *et al.*, 1996; Bassit *et al.*, 2000; Bassit *et al.*, 2002); however this remains inconclusive (Walsh *et al.*, 2000).

The amount of carbohydrate endurance athletes are advised to consume during exercise to attenuate exercise-induced immune perturbations (~60 g CHO·kgbw·h^{-1}; Gleeson *et al.*, 2006; Jeukendrup, 2008) is not consistently practiced by endurance runners, ultra-marathon runners, adventure athletes, triathletes and military personnel (Peters *et al.*, 1993; Noakes, 2003; Kruseman *et al.*, 2005; Casey & Messer, 2004; Burke *et al.*, 2005; Baar & McGee, 2008). Therefore, the provision of post-exercise nutritional recommendations (Tipton & Wolfe, 2004; Burke *et al.*, 2004; ACSM, 2009) may be of importance in immune recovery during the post-exercise period. The well controlled

laboratory experimental trials conducted in **Chapters 6** and **7** demonstrate that carbohydrate consumption with and without the addition of protein immediate after prolonged strenuous exercise prevented the decrease in bacterially-stimulated neutrophil degranulation normally observed during the recovery period (**Figure 6.1**; **Figure 7.1**). Conversely, this feeding strategy did not influence circulating leukocyte trafficking (**Table 6.1** and **7.1**) or prevent the decrease in saliva IgA concentration during recovery (**Table 6.3** and **7.2**).

The exercise stress of 2 h running at 75% $\dot{V}O_{2max}$ evoked a significant increase in circulating cortisol (**Table 6.4**; **Figure 7.2**), adrenaline and noradrenaline concentration (**Table 6.4**) in all trials. Subsequently, increases in circulating neutrophil and lymphocyte counts were observed post-exercise, as described in **Section 2.3.1**. Interestingly, carbohydrate ingestion with and without the addition of protein immediately after exercise did not assist the restoration of circulating leukocytes to resting counts, and throughout the recovery period measured leukocytes counts remained significantly elevated (140 min **Chapter 6**; 180 min **Chapter 7**). This is not surprising given that no differences in stress hormone responses were observed between trials in both **Chapters 6** and **7**. This is contrary to other studies that observed attenuated stress hormone responses and lower circulating leukocyte perturbation with carbohydrate ingestion prior to and during similar exercise intensity protocols (Nieman *et al.*, 1998; Starkie *et al.*, 2000). It is possible that carbohydrate intake prior to and during exercise limits the degree the stress hormone responses and subsequent leukocyte mobilisation. Whereas, if ingestion is delayed until the post-exercise period the full stress hormone and leukocyte mobilisation responses to the exercise stress has already been initiated. Recovery of such immune perturbations is possibly independent of the stress hormone concentration, and likely to depend on

leukocyte (predominantly neutrophil) remargination (McCarthy & Dale, 1988; Peake, 2002).

Depressed *in vitro* neutrophil phagocytic function has been associated with increased infection incidence in the clinical setting (Ellis *et al.*, 1988; Bessman & Sapico, 1992; Marhoffer *et al.*, 1992; Smitherman & Peacock, 1995; Alba-Loureiro *et al.*, 2007). The experimental trials in **Chapters 6** and **7** demonstrated that carbohydrate ingestion with and without the addition of protein immediately after 2 h running at 75% $\dot{V}O_{2max}$ prevented the decrease in bacterially-stimulated neutrophil degranulation, which was observed when water/placebo was consumed immediately after exercise (**Figure 6.1** and **7.1**), or a carbohydrate and protein solution was consumed 1 h after exercise (**Figure 6.1**). Although the clinical significance of this maintenance amongst healthy active individuals is still unclear, neutrophil degranulation plays a fundamental first-line defence against invading foreign pathogens. Therefore, suboptimal neutrophil function may contribute to illness and infection risk during recovery from exercise stress (Peake, 2002). Especially if athletes come into contact with foreign environments, and subsequently foreign pathogens (e.g. close contact with other individuals or animals, populated areas, travel into unfamiliar areas, sharing drinks and food, open tissue injury) during this period.

Previous studies have speculated that various blood-borne mechanisms (e.g. decreased plasma glucose concentration, increases in plasma cortisol, catecholamines and IL-6) maybe responsible for the decrease in neutrophil function (Nieman *et al.*, 1997; Robson *et al.*, 1999; Bishop *et al.*, 2002). Whilst neutrophils rely heavily on glucose for energy supply (Furukawa *et al.*, 2000), a direct effect of decreased glucose availability on reduced bacterially-stimulated neutrophil degranulation is unlikely; because decreases in plasma

glucose concentration after exercise were not observed, with (**Chapter 7**) and without (**Chapter 6**) the provision of a carbohydrate meal 2 h prior to exercise. Additionally, plasma glucose concentration remained within normal range during the recovery period on CON in both **Chapters 6** and **7** **(Figure 6.2** and **7.2)**. A stimulatory effect of increased glucose availability on neutrophil degranulation, with carbohydrate ingestion with and without protein, is also unlikely, because hyperglycaemia has been shown to decrease neutrophil degranulation in Diabetes Mellitus patients with poor glycaemic control (Alba-Loureiro *et al.*, 2007; Stegenga *et al.*, 2008a).

Furthermore, it is unlikely that attenuated stress hormones played a role in the maintenance of neutrophil function in **Chapters 6** and **7**. Plasma cortisol (**Chapters 6** and **7**) and catecholamine (**Chapter 6**) concentrations peaked within 30 min post-exercise and returned to similar pre-exercise value by 60 min post-exercise in all trials **(Table 6.4; Figure 7.2)**. Whilst decreases in bacterially-stimulated neutrophil degranulation were still observed at the end of the recovery period measured in **Chapters 6** and **7** (**Chapter 6**: CON, DF (**Figure 6.1**); **Chapter 7**: CON (**Figure 7.1**)). These findings are in accordance with previous studies that also reported declines in bacterially-stimulated neutrophil degranulation despite unsubstantial changes in proposed mechanisms (Li & Gleeson, 2005b; Laing *et al.*, 2008a).

It is plausible that increased insulin concentration may contribute to the maintained bacterially-stimulated neutrophil degranulation with immediate post-exercise consumption of carbohydrate, with and without protein. Studies using a hyperinsulinaemic-euglycaemic clamp have shown that raised plasma insulin concentration increases neutrophil chemotaxis, phagocytosis and bactericidal capacity in healthy subjects (Walrand *et al.*,

2004; Walrand *et al.*, 2006). Additionally, clinical studies have reported that intravenous invoked hyperinsulinaemia, or insulin therapy in Type 1 Diabetes Mellitus patients, is associated with elevations in neutrophil elastase release and improved neutrophil phagocytic function (Collier *et al.*, 1990; Rassias *et al.*, 2002; Walrand *et al.*, 2005; Alba-Loureiro *et al.*, 2007). A recent study reported that untreated Type I Diabetes Mellitus patients consistently present lower neutrophil phagocytic activity compared with insulin treated patients (Bilgic *et al.*, 2008). This response can be attributed to insulin receptor sites on polymorphonuclear neutrophil cell membranes that when stimulated have a priming effect on neutrophil function (Walrand *et al.*, 2005; Walrand *et al.*, 2006).

No previous studies have investigated the effects of post-exercise ingestion of carbohydrate with and without protein on saliva IgA responses. A novel finding in **Chapters 6** and **7** was that carbohydrate ingestion, with and without the addition of protein, did not prevent the decrease in saliva IgA concentration in response to prolonged strenuous exercise (**Table 6.3** and **7.2**). This is in accordance with studies that have also reported no influence of carbohydrate ingestion prior to and during exercise on saliva IgA responses (Bishop *et al.*, 2000; Li & Gleeson, 2005a). However, the presence of macronutrients (carbohydrate, protein) within a fluid solution appears to acutely influence the degree of saliva flow rate (**Table 7.2**) and the degree of saliva S-IgA concentration decrease (**Table 6.3**), which may have clinical significance (**Section 2.1.3**). In addition, the observed similarities in saliva IgA responses between trials in **Chapters 6** and **7** are not surprising, as neuroendocrine regulation may account largely for saliva IgA responses to prolonged exercise (Bishop & Gleeson, 2009), as described in **Sections 2.1.4** and **2.3.3.** An influence of post-exercise nutrient intake on stress hormone responses was not observed in both experimental chapters (**Table 6.4**; **Figure 7.2**).

Nevertheless, these results further indicate that fluid bolus ingestion decreases saliva IgA concentration during recovery (**Chapters 6** and **7**), with a tendency for the nutrient dense boluses to produce a more pronounced decrease in saliva S-IgA concentration (**Table 6.3**). This may be indicative of a dilution effect, since no significant changes in saliva IgA secretion rate were observed in **Chapters 6** and **7**. Clearly, the saliva IgA responses observed in these experimental chapters indicate that saliva IgA responses to exercise are influenced by fluid intake and drink composition (Bishop *et al.*, 2000; Oliver *et al.*, 2007). These results also support the argument that saliva flow rate, saliva IgA concentration and IgA secretion rate responded uniquely to different refeeding regimens. Therefore, justifies the presentation of saliva IgA responses both as concentration and secretion rate (Walsh *et al.*, 1999; Oliver *et al.*, 2007; Bishop & Gleeson, 2009).

From a practical viewpoint, many endurance athletes do not consume sufficient carbohydrates during exercise to avoid immune perturbations, and this may potentially have a clinical (e.g. illness and infection) and performance (e.g. unsustainable training loads, decrements in performance) significance. There is scope in the literature for future research to investigate the effects of different timings and concentrations of carbohydrate ingestion, with and without the addition of protein, on *in vitro* (e.g. bacterial challenge) and *in vivo* (e.g. DTH, vaccine challenge) immune function, and assess the clinical significance of any reductions of immune function on infection incidence (Albers *et al.*, 2005). Additionally, the effects that different timings and concentrations of post-exercise carbohydrate provisions and fluid volumes have on other aspects of oral-respiratory mucosal immune responses (e.g. salivary lactoferrin, amylase and lysozyme), reported to be modified by exercise, and may respond differently to nutrient availability in the oral

cavity (Ljungberg *et al.*, 1997; Chicharro *et al.*, 1998; Ward *et al.*, 2002; Li & Gleeson, 2005a; Allgrove *et al.*, 2008), warrants further investigation.

Conclusions

The major conclusions from this thesis are:

1. A 30 h period of total sleep-deprivation does not alter circulating leukocyte trafficking and plasma cortisol response, or compromise bacterially-stimulated neutrophil degranulation and saliva S-IgA responses at either rest or following submaximal or strenuous exercise.

2. Two nights of total sleep-deprivation with and without a 90% energy-restriction does not alter circulating leukocyte counts and plasma stress hormone responses, or compromise bacterially-stimulated neutrophil degranulation and saliva S-IgA responses at rest.

3. Modest whole-body cooling (T_{re} 35.9°C) decreases circulating lymphocyte counts, bacterially-stimulated neutrophil degranulation, and saliva S-IgA responses, possibly induced by noradrenaline response.

4. A 53 h period of total sleep-deprivation, with and without a 90% energy-restriction, prior to modest whole-body cooling (T_{re} 35.9°C), does not further perturbate circulating leukocyte trafficking and plasma stress hormone responses, or further compromise bacterially-stimulated neutrophil degranulation and saliva S-IgA responses to modest whole-body cooling.

5. The ingestion of a carbohydrate and protein solution equal to 1.2 g CHO·kg^{-1}BM and 0.4 g PRO·kg^{-1}BM immediately after, but not 1 h after, prolonged strenuous exercise prevents the usual decrease in bacterially-stimulated neutrophil degranulation. However, does not alter circulating leukocyte trafficking, saliva S-IgA and stress hormone responses.

6. The ingestion of a carbohydrate bolus alone providing 1.2 g CHO·kg^{-1}BM immediately after prolonged strenuous exercise prevents the decrease in bacterially-stimulated neutrophil degranulation, but does not alter circulating leukocyte trafficking, saliva IgA and plasma cortisol responses.

7. Increases in plasma insulin, rather than attenuated stress hormone responses, is a likely mechanism by which solutions ingested immediately after prolonged strenuous exercise that contain carbohydrate alone, or with the addition of protein, may maintain bacterially-stimulated neutrophil degranulation during the recovery period.

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Appendix A

Bangor University
School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title: The effects of one night without sleep on thermoregulation, hydration, immune function and exercise performance.

Research Supervisor: Dr. Neil Walsh **Tel :** 01248 383480
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Research Investigators: Ricardo Costa, Dr. Samuel Oliver, Dr. Stewart Laing, Louise Cartner. Joanna Carter, David Evans, Edward McDermott, Alex McGregor, Owen Mugridge, Jonathan Senior, and Polly Veazey-French.

Technical Assistance: Kevin Williams.

Invitation to take part
You have being invited to take part in a research investigation. Before you do so, it is important for you to understand why the research is being conducted and what will be required to do once you agree to be involved. Please read the following information carefully. You should 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 an 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 (SSHES), or any of the investigators involved in the study.

All information collected during the study will be coded and treated confidentially.

Background
It is thought that an individual's health and physical performance may be negatively affected by sleep deprivation. Therefore, it is important to be able to identify the level of disruption to health and performance that sleep deprivation may have. The consequences of sleep deprivation regularly affect both military personnel, who may have to stay alert and functional during long periods of sleep deprivation, and athletes travelling across time zones to compete in international events. It remains unclear whether one night without sleep influences body temperature regulation and hydration status whilst at rest and during

exercise. In addition, we do not know if one night without sleep affects exercise performance.

This study will also examine if one night without sleep decreases the ability of your immune system to fend off invaders (e.g. viruses) which may in turn raise the likelihood of you suffering opportunistic infections such as the common cold. Scientists know little about the effects of restricted sleep on immune function. In summary, the aims of this study are to examine the effects of one night without sleep on temperature regulation, hydration status, immune function and exercise performance.

What will be expected of you?

If you decide to take part in this study there will be a number of constraints placed upon your normal everyday life and activities.

The day prior to completing a trial you will be expected to:

- Avoid drinking alcohol, caffeinated drinks (i.e. coffee, tea, coke and diet coke) and avoid participating in any exercise for 24 h before you arrive at the laboratory.
- Wear a movement measurement device (Actigraph) in an arm band on your non-dominant arm. This will help us monitor your sleep patterns during the night before you arrive at the laboratory.

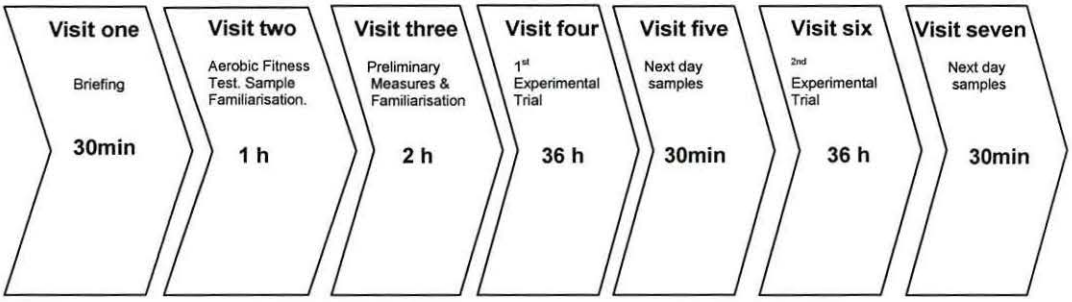
Whilst completing the experimental trials you will be expected to:

- Reside in the laboratory, under supervision, from 6am until 6pm the following day (36 h) on two separate occasions.
- Spend approximately 76 h in total in the laboratory in SSHES.
- Stay awake all night in the laboratory on one of these occasions (sleep deprivation trial) and sleep in a bed in the laboratory on another occasion (control trial).
- Eat and drink only what is given to you the day before, during and immediately after the trials.
- Follow the study's daily timetable, including going to bed and waking from sleep when asked.
- Perform one maximal aerobic test (10-15 min), two "light intensity" 30 min treadmill tests (one for each trial) and two self-paced 30 min time trials (one for each trial).
- Wear a rectal probe for the measurement of body temperature during exercise;
- Weigh yourself nude (behind screens to maintain privacy).
- Have 3 needle pricks for the collection of blood samples during each trial (98 ml blood collected during each trial) totalling 6 needle pricks during both sleep deprivation and control trials.

You will be excluded if you are a smoker or if you are on specific types of medication or supplements (i.e. Creatine). Asthmatics and Diabetes patients are also prohibited.

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~76 H OF YOUR TIME.

Summary of visits



Visit one: Project briefing (~30 min)
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.

Visit two: Fitness tests & sampling familiarisation. (~1 h)
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.

Fitness test: A 10-15 min treadmill test will be used to measure your aerobic fitness (maximal oxygen uptake). Briefly, the treadmill speed and gradient will be increased every 3 min until you reach volitional exhaustion. This test will require you to run at your maximal aerobic capacity for approximately one minute. We will require you to wear a face mask during this fitness test so that we can make measurements on your expired air: the face mask is not uncomfortable and will not impede your breathing.

Sample familiarisation: Following the fitness test a venous blood sample, saliva sample and urine sample will be collected to ensure you are fully familiar with these collection methods. All blood samples will be collected by a qualified member of staff from a forearm vein (14 ml of blood at each collection). A saliva sample will by collected by dribbling into a collection pot. Urine will be collected in privacy by passing urine into a plastic container. In order to complete the investigation these samples are a necessity.

Visit three: Preliminary measures and time trial familiarisation. (~ 2 h)
On arrival at the laboratory, measures of height and weight will be obtained; these will be followed by **a whole body scan using a DEXA** machine to determine your body composition. The DEXA is painless and involves you lying down on a flat bed for 20 min. The DEXA does emit a very small dose of ionising radiation. The dose of radiation you will receive is the same as an additional day of natural background radiation. This is substantially less than the radiation associated with a normal X-ray (approximately one twentieth of a chest x-ray).

Time trial familiarisation: Following this you will be required to exercise at a low intensity for 30 min on a treadmill whilst wearing a face mask. After a recovery period, the test will be followed by a familiarisation of the 30 min treadmill time trial that will be

used in the main experimental trials. The time trial will require you to run as far as possible in 30 min on a treadmill. You will control the speed of the treadmill throughout the time trial. Completing a practice time trial will help you to pace yourself during the time trial in the main experimental trials.

Visit four: Experimental trial one (~36 h)
You will be required to spend approximately 36 h residing in SSHES for each of the two experimental trials. The experimental trials will be performed in a random order and involve you being kept awake all night on one trial (sleep deprivation) and sleeping normally (control) on the other trial: the order of the trials may be the other way around for you. On the day prior to testing you will not be allowed to exercise and we will provide you with the food and drink to consume for the entire day.

Day 1: You will be woken at around 6am at your home and driven to SSHES by a member of the team. Upon arrival you will record your nude body mass (behind a screen for privacy). We will then collect baseline samples of blood, urine and saliva. We will provide you with all your food and drink whilst you are residing in the laboratory. You are free on this day to attend lectures and we only ask that you be available for nude body mass measurements approximately every 4 h. Two short walks will also be performed during the day on the control trial.

Overnight: During the night on the sleep deprivation trial we will keep you entertained with board games, TV, DVD's and a games console. On the control trial you will sleep in comfortable beds in a quiet room in SSHES. You will be under close supervision at all times.

Day 2: You will be required to provide samples of urine and saliva at 6 am after spending 24 h in SSHES. A standard breakfast will be provided. You will then rest quietly in SSHES during the morning before commencing the exercise testing.

Exercise testing: This will begin at 12 noon. We will require you to fit a rectal probe before the exercise testing (behind screens to maintain privacy) so that we can monitor body temperature during exercise. The rectal probe is a thin flexible cable that you insert approximately 10 cm past your anal sphincter. It is important that we closely monitor your body temperature during exercise to ensure you do not get too hot: we will withdraw you from exercise if this happens. Nude body mass, blood, urine and saliva samples will be collected before exercise. The blood samples before and in the hours after exercise will be collected using a cannula. The cannula will be inserted, by an experienced phlebotomist, into a forearm vein and a tap connected so that we can take a blood sample before, immediately after, 2 and 4 h after exercise without having to use separate needle pricks. The first exercise bout requires you to exercise on a treadmill for 30 minutes at a moderate level of exertion (60% of your aerobic capacity). We will require you to wear a face mask during this 30 min run so that we can make measurements on your expired air. Measures of perceived exertion (how hard you find the exercise) and thermal sensation (how hot your body feels) will be taken from you at 5 min intervals.

Immediately after the exercise, further blood, nude body mass, urine and saliva samples will be collected.

You will then be asked to step back onto the treadmill and perform a 30 min, self paced time trial as practised during the familiarisation. The aim of the time trial is to run as far as you can in 30 min. Measures of perceived exertion and thermal sensation will be taken at 5 min intervals.

Immediately after the time trial, further blood, nude body mass, urine and saliva samples will be collected.

Recovery: After you have had a shower we will provide you with sufficient sports drink to fully rehydrate you in the first 2 h of recovery. Further samples (blood, urine and saliva) will be collected 2 h and 4 h after exercise. The cannula will be removed from the forearm vein immediately after the 4 h blood collection. We will then provide you with a meal to consume and afterwards one of the team will drive you home.

Overnight recovery: It is very important that you stay at home and rest during the overnight recovery after both the sleep deprivation and control trial. Sleep deprivation may affect your concentration and coordination so you should remain rested during the overnight recovery and not leave your house. To ensure you remain rested overnight after both trials we will record your activities by having you wear the Actigraph movement measurement device in an arm band.

Visit five (~30 min)
After a sound nights sleep you will be required to visit the laboratory to provide further post exercise samples (blood, urine and saliva) the following morning at 9am. You must avoid eating breakfast as we will provide you with breakfast after the samples are collected.

Visit six: Experimental trial two (~36 h)
We will collect you from your home at approximately 6am and bring you to SSHES to complete the remaining experimental trial. The procedures outlined in visit four will be repeated.

Visit seven (~30 min)
We will ask that you visit SSHES for further samples at 9am the morning after visit six. The procedures are outlined in Visit five.

Advantages of taking part
A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, for your fitness level, body composition (e.g. body fat %) and blood measures (immune function). This feedback should help you with planning and monitoring your athletic training programme. The feedback you will receive regarding your fitness level is similar to that which many testing facilities provide as a fee-paying service. Advantages for those subjects who are Undergraduates from the SSHES department are that they gain 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.

Disadvantages of taking part

The disadvantages of taking part in this study, which you will probably be most concerned about, are: 1. sleep deprivation; 2. blood samples; 3. physical rigours and 4. time commitment.

1. Sleep deprivation: Many scientific investigations, and our pilot work, show that 1 night of sleep deprivation has very modest effects on physical and psychological function. Indeed, military personnel regularly endure 4-5 day operations without sleep and with the additional stress of limited food, fluid and constant activity. Field studies do not report medical complications even in military personnel on these severe operations. In addition, at all times you will be closely supervised by an experimenter trained in First-Aid.

2. Blood samples: Only qualified Phlebotomists with experience at performing this procedure will collect blood samples. To ensure you are completely happy with giving a blood sample we will familiarise you with the blood sample collection procedure on your second visit to the laboratory. The blood samples will be taken using a very small needle without the pressure of having a tourniquet around your upper arm. Although the first blood sample (6am at the start of an experimental trial) and the sample the morning after each trial (visit five and seven) will be collected using separate needle pricks, we will insert a small cannula with a tap into a forearm vein to collect five samples before and after exercise to avoid the inconvenience of many needless needle pricks. This means that for each trial you will only have three needle pricks rather than seven. We will collect 98 ml of blood during each experimental trial giving a total blood volume of 196 ml. This small blood loss will not impact upon your normal physical functioning.

3. Physical rigours: The physical components of this study include the aerobic fitness test, the two 30 min treadmill tests and the two treadmill time trials. Firstly, the fitness test will only require you to run at your maximal capacity for approximately one minute. You most probably experience the same physical sensations of fatigue regularly during your exercise training. Secondly, the 30 min treadmill tests will be performed at a moderate level of exertion (equivalent to a jog pace). Although the two treadmill time trials require you to run as far as you can in 30 min, you will control the speed of the treadmill and pace yourself how you see fit during the sleep deprivation and control trials. Finally, you will be fully familiarised with both running on the treadmill (e.g. controlling the speed and the emergency stop procedure) and the two 30 min treadmill tests before embarking upon the experimental trials. You will be closely supervised during the exercise trials and by monitoring your body temperature we will stop the trial should your body temperature rise too quickly.

4. Time commitment: To complete all aspects of the study we will require you to visit the laboratory on 7 occasions for a total of approximately 76 h. We understand that this study will ask a lot of you as a participant and so we are offering an incentive. As a participant you will be eligible for a £25 reward on completion of the first experimental trial and a further £25 reward on completion of the study. This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime.

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

Bangor University
School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title: Immune and thermoregulation responses to cold exposure after two nights of sleep-deprivation with or without energy-restriction in active males.

Research Supervisor: Dr. Neil Walsh **Tel :** 01248 383480
Email: n.walsh@bangor.ac.uk

Research Co-ordinators: Ricardo Costa and Dr. Samuel Oliver.

Additional Investigators: Edward Cooper-McDermott, Rachel Elsley, Adam Harper-Smith, Charlie Parker, Suzan Parry, Rob Samuel, Tom Williams, Iona Jones and Gavin Price.

Technical Assistance: Kevin Williams.

Invitation to take part
You are being invited as an athletic male to take part in a research investigation. 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 (SSHES), or any of the investigators involved in the study.

All information collected during the study will be coded and treated confidentially. The information will be kept in a locked filing cabinet and on the Research Co-ordinator's computer for 5 years before being confidentially destroyed.

Background
Athletes, emergency and armed forces personnel frequently experience sleep loss and energy restriction (e.g. military recruits on survival training) whilst still being expected to perform in extreme environments (e.g. freezing conditions). Previous research collected from military settings suggests that sleep deprivation and food restriction may compromise your body's ability to control temperature (thermoregulation) and protect against the invasion of foreign bodies like bacteria and viruses. Consequently, sleep deprivation and food restriction may compromise an individual's health by increasing their susceptibility to cold injury and opportunistic infections (e.g. common cold).

Nevertheless, as these investigations involved a combination of many stressors (e.g. fluid and food deficits, sleep loss, prolonged exercise, psychological) it is difficult to attribute altered thermoregulation and immune function to any one particular stressor. The main aim of this study is therefore to identify the effect of sleep deprivation and food restriction in a controlled laboratory setting on thermoregulatory control to a cold air exposure challenge. This study will also examine if two day period of sleep deprivation with or without food restriction and subsequent cold exposure decreases immune function which in turn may increase susceptibility to infections (e.g. colds).

What will be expected of you?
If you decide to take part in this study there will be a number of constraints placed upon your normal everyday life and activities.

The day prior to commencing a trial you will be expected to:

- Avoid drinking alcohol, caffeinated drinks (i.e. coffee, tea, coke and diet coke) and avoid participating in any exercise for 24 hours before you arrive at the laboratory;
- Wear a movement measurement device in an arm band on your non-dominant arm. This will help us monitor your sleep patterns during the night before you arrive at the laboratory.

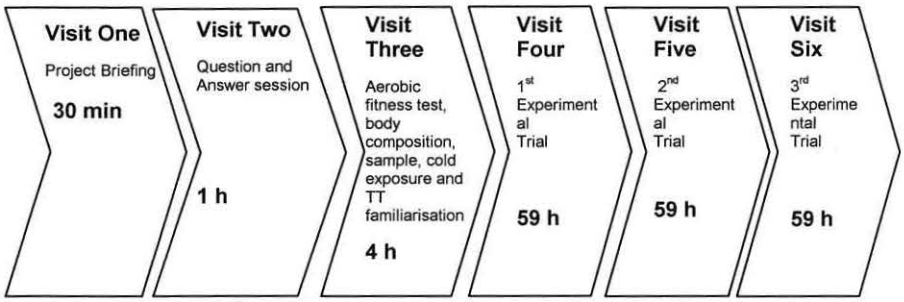
Whilst completing the experimental trials you will be expected to:

- Spend three consecutive days in the laboratory on three separate occasions.
- Live and sleep in the laboratory during each three day trial (soft furnishings, bedding, computers, TV, video, DVD will be provided and living temperature will be between 17-22°C).
- Stay awake for two nights in the laboratory on two of these occasions (sleep deprivation trial and a sleep + food restriction trial) and sleep in a bed in the laboratory on another occasion (control trial).
- Eat and drink only what is given to you the day before and during the trials.
- Follow the study's daily timetable, including going to bed and waking from sleep when asked.
- Perform prescribed physical activity (two 90 min brisk walks and two self paced 5 km time trials on each trial).
- Wear skin thermisters and a rectal probe for the measurement of body temperature (i.e. skin and core) during exercise and the cold air tests.
- Weigh yourself nude (behind screens to maintain privacy);
- Have seven blood samples taken during each trial (~140 ml during each trial) totalling 18 blood samples during study (total blood ~420 ml)
- Have twelve saliva samples collected during each trial totalling 36 samples.
- Perform a maximal aerobic test.

You will be excluded if you are a smoker or if you are on specific types of medication or supplements (i.e. Creatine). Asthmatics and Diabetes patients are also prohibited.

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~182.5 HOURS OF YOUR TIME.

Summary of visits



Visit one: Project briefing (~30 minutes)
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 involvement in the study with significant others. This will also allow you additional time to think of questions.

Visit two: Question and answer session (~1 hour)
Visit two is provided as an additional opportunity for you to ask questions. Once you are fully satisfied with the information and on verbal agreement to take part in the study you will be asked to complete an informed consent form, medical questionnaire and make arrangements for further visits.

Visit three: Familiarisation, body composition and aerobic fitness tests (~4 hours)
Body composition: On arrival at the laboratory, measures of height and weight will be obtained; these will be followed by a whole body scan using an x-ray machine to determine your body composition. The x-ray is painless and involves you lying down on a flat bed for approximately 20 min. The x-ray does emit a very small dose of ionising radiation. The dose of radiation you will receive is the same as an additional day of natural background radiation. This is substantially less than the radiation associated with a normal x-ray (~ 1/20 of a chest x-ray).

Fitness test: An incremental treadmill test will be used to measure your aerobic fitness (maximal oxygen uptake). This test will require you to run at your maximal aerobic capacity for approximately one minute. We will require you to wear a face mask during this fitness test so that we can make measurements on your expired air: the face mask is not uncomfortable and will not impede your breathing. After a brief rest you will be asked to remount the treadmill to enable us to calculate your walking speed for the 90 min experimental trial walks.

Time trial familiarisation: Following the incremental test and after a short recovery period you will be asked to complete 5 km on a treadmill as fast as you possible can (i.e. a 5 km time trial). You will control the speed of the treadmill throughout this time trial. Completing a practice time trial will help you to pace yourself during the time trial in the main experimental trials.

Sample familiarisation: Following the fitness test a venous blood sample, saliva sample and urine sample will be collected to ensure you are fully familiar with these collection methods. All blood samples will be collected by a qualified member of staff from a forearm vein (20 ml of blood at each collection). A saliva sample will by collected by dribbling into a collection pot. Urine will be collected in privacy by passing urine into a plastic container. In order to complete the investigation these samples are a necessity.

Chamber familiarisation: You will be seated in a climate chamber for approximately 20 minutes at 0°C in shorts, socks and trainers (no T-shirt). During this time you be required to practice breathing through a mouth piece and be fitted with rectal and skin thermisters.

Visit four, five and six: Experimental trials

You will be required to complete three experimental trials in a random order each separated by four days. Each experimental trial will consist of a 59 hour period where you will reside in the laboratory under one of treatment conditions prior to completing a cold air test. The trials are as follows: control trial, sleep deprivation trial and a sleep deprivation + energy restriction trial. The control trial will involve you consuming a range of foods and fluids that meet your energy, nutrient and fluid requirements (~3000 kcal and ~3 litres per day) whilst sleeping for approximately eight hours per night. The sleep deprivation trial will involve you consuming the same food and fluids as the control trial; however, you will be deprived of sleep for two nights. The sleep deprivation + energy restriction trial will involve you being deprived of sleep for two nights whilst you consume a range of foods providing 10% of your daily estimated energy requirements (~300 kcal per day). During this trial you will be provided with your daily water requirements (~3 litres per day).

On the day prior to each experimental trial you will be asked to refrain from exercise and only consume the food and drink we provide you. You will also be asked to wear an activity monitor overnight to enable us to record your sleep quality and quantity prior to the trials. On day 1 of each trial you will be collected at home around 7 am and driven to SSHES by one of the investigators. On each trial on both day 1 and day 2 you will be asked to perform a 90 min brisk uphill walk (4 mph/6.4 kph) and a 5 km time trial. For the remainder of each day you will be free to complete normal light habitual activities (e.g. office works, watching TV). We will provide you with all your fluid and foods in accordance with the appropriate trial (breakfast ~9 am, lunch ~2 pm and dinner ~7 pm). On the control trial you will sleep in comfortable beds in a quiet room in SSHES for approximately eight hours each night (11 pm to 7 am). On trials with sleep deprivation we will keep you entertained throughout the night period (11 pm to 7 am) with board games, TV, DVD's and a games console. You will be under close supervision at all times.

On day 3 at 12 noon, after 53 hours of an experimental trial, you will be required to perform a cold air test. During this test you will be seated wearing shorts, socks and trainers at 0°C until your core temperature declines to 36°C. Although dependent on the individual our pilot investigations have shown it will take approximately 90 min for you to reach this core temperature. Upon reaching a core temperature of 36°C you will be immediately removed from the chamber and begin the recovery period. For your safety throughout the cold air test and recovery period your core temperature will be continuously monitored. During the first hour after the cold air test, you will be provided with blankets and slowly rewarmed. After 1 hour you will be provided with a large

recovery (~1200 kcal). After fours hours of recovery (~6 pm) you will provide a blood, urine and saliva sample and then be transported by an investigators back to your home.

It is very important that you stay at home and rest during the overnight recovery after each of the trials. **Sleep deprivation may affect your concentration and coordination so you should remain rested during the overnight recovery and not leave your house.** To ensure you remain rested overnight after each of the trials we will record your activities by having you wear the activity monitor measurement. You will be given and asked to complete a simple health and sickness log for the duration of the study where for each day you are asked to record the occurrence of any health problems (e.g. cold symptoms). We will ask that you bring the health and sickness log to the laboratory the on each of your visits. On day 4, after a night of normal sleep a saliva sample will be collected by an investigator at your home.

During each trial you will have blood, urine an saliva samples collected on day 1 at 8 am, day 2 at 8 am, day 3 at 8 am, 12 noon, immediately post cold air test, 1 hour post cold air test and following refeeding (**7 blood samples in each trial**). In addition saliva samples will be obtained before and after the exercise on day 1 and day 2 of each experimental trial and at 8 am on day 4.

Four days after visit four you will be required to return to complete one of the two remaining experimental trials. You will be required to repeat this cycle until you have completed all three experimental trials.

Advantages of taking part

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, for your fitness level, 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 programme. The feedback you will receive regarding your fitness level 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 cover both the open and personal performance 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. sleep deprivation and energy restriction; 2. cold air test 3. blood samples; and 4. time commitment. At all times you will be closely supervised by an experimenter trained in First-Aid.

1. Sleep deprivation and energy restriction: Many scientific investigations, including those completed in our laboratory, show that 1 to 2 days of sleep deprivation or energy restriction have very modest effects on physical and psychological function. Indeed, military personnel regularly endure 4 to 5 day operations which involve constant activity, psychological stress, sleep loss and insufficient fluid and food. Field studies do not report medical complications even in military personnel on these severe operations.

2. Cold air test: Numerous scientific experiments have exposed individuals to freezing and/or wet conditions and reported no medical complications whilst decreasing individuals' core temperature to 35°C. It should be noted that even at 35°C which is considered the upper limit of mild hypothermia (35-32°C) very few medical complications are noted in healthy fit individuals. In this experiment your core temperature will be decreased to 36°C; which is within standard SSHES ethical guidelines. Following removal from the chamber you will be re-warmed with blankets, food and warm liquids. For your safety throughout the cold exposure and in recovery your core temperature will be monitored continuously.

3. Blood samples: Only qualified phlebotomists with experience at performing this procedure will collect blood samples. To ensure you are completely happy with giving a blood sample we will familiarise you with the blood sample collection procedure on your first visit to the laboratory. The blood samples will be taken using smaller needle than is typically used by doctors in your local surgery or hospital.

4. 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 182.5 hours. We understand that this study will ask a lot of you as a participant and so we are offering an incentive. As a participant you will be eligible for a £25 reward for each of the first two trials and on the completion of the third experimental trial a further £100 reward (total £150). This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime.

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

Bangor University
School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title: Does the timing of post-exercise carbohydrate and protein feeding alter immune responses during recovery from prolonged exercise?

Research Supervisor: Dr. Neil Walsh **Tel :** 01248 383480
Email: n.walsh@bangor.ac.uk

Research Co-ordinators: Ricardo Costa.

Additional Investigators: Lucy Bywater, Eifion Williams, Jennifer Heaney, Anthony Blanchfield, Dr. Stewart Laing and Dr. Samuel Oliver.

Technical Assistance: Kevin Williams.

Invitation to take part
You have been invited to take part in a research investigation. 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 read the following information carefully. You should 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 an information sheet to keep and be asked to sign a subject 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 (SSHES), or any of the researchers involved with the study.

All information collected during the study will be coded and treated confidentially.

Background
The immune system is responsible for defending our body against invading invaders. When our immune system is running low, we are more prone to being infected by foreign invaders; such as, bacteria and viruses (e.g. common cold). Past research has demonstrated that prolonged high intensity exercise can depress immune function, leaving the body open to illness and infection. Further studies have tried to determine which of the body's mechanisms are responsible for suppressing the immune system, and there has been a strong link between nutritional intake and how the immune system works. Some of the most impressive results have been found on carbohydrate content in normal dietary habits and carbohydrate feeding during exercise. These results indicate that a diet consistently high in carbohydrates and carbohydrate feedings during exercise can blunt the fall in immune function after prolonged high intensity exercise. However, little is known about

the timing of feeding on immune responses after exercise. Therefore, the aim of this study is to examine if the timing of post-exercise carbohydrate and protein feeding alters immune response during recovery from prolonged exercise?

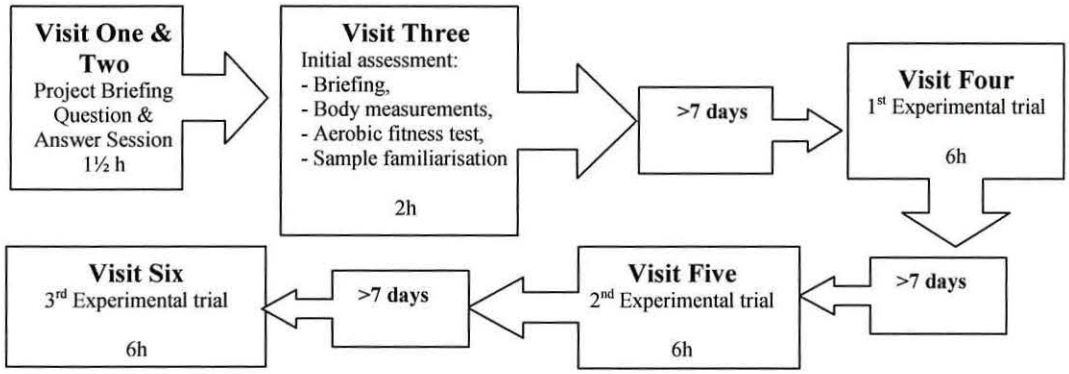
What will be expected of you?

If you decide to take part in this study there will be a number of constraints placed upon your normal everyday life and activities; which include:

- Complete a training log during the experimental exercise trials.
- The day prior to the initial assessment and each exercise trial, you will be expected;
  - to avoid caffeinated beverages (e.g. coffee, tea, colas).
  - to avoid taking part in any form of exercise.
- Avoid alcoholic beverages 2 days prior to the initial assessment and exercise trials.
- Consume a balanced diet that provides sufficient energy to meet your requirements. We will provide this food and drink the day prior to each experimental trial.
- You should also avoid taking any nutritional supplements one week prior to the exercise trials.
- On the initial assessment day, you will be required to be present at the laboratory at the SSHES for 2 h.
- On the 3 experimental trial days, you will be required to be present at the laboratory of the SSHES for 08:00 h, fasted. Your activities in the laboratory will finish at approximately 14:00 h.
- Whilst completing the experimental trials you will be expected:
  - To stay in the laboratory of the SSHES for the duration of the initial assessment and 3 experimental trials.
  - On one occasion perform a ~15 min maximal exercise test on an electric treadmill. On 3 separate occasions perform a 2 h run at 75% of your maximal exercise capacity on an electric treadmill.
  - Consume the fluids and foods provided during and after the exercise trial.
  - Weigh yourself nude (behind screen to maintain privacy). Have your height taken, and skin fold measurements performed.
  - To have blood samples taken from the antecubital vein by a trained researcher. These will occur prior to the exercise trial, immediately after the exercise trial and every 20 min after exercise for 2 h 20 min. This totals 27 blood samples for the 3 main experimental exercise trials. Thus, provide 128 ml blood at each of the 3 experimental trials (a total of 384 ml of blood taken during the whole study).
  - To provide a saliva sample before and after exercise, and at 20 min intervals after exercise via a dribble method that will be explained to you. This will total 9 saliva samples for each experimental exercise trial (27 saliva samples for the whole study).
  - To provide a urine sample prior to and after each experimental exercise trial. This will total 2 urine samples for each experimental exercise trial (6 urine samples for the whole study).

If you have or recently had (within three months) an infection, illness, and/or, injury you will be excluded from taking part in this study.

Summary of Visits.



Visit 1 (~30 min)
Project briefing
At this meeting you will be fully briefed about the requirements of the research project. You will be talked through the subject information sheet by a researcher and given opportunity to ask any questions. You will then leave with the information sheet to allow you time to discuss your possible involvement in the study with others. This will also allow you additional time to think of any further questions.

Visit 2 (~1 h)
Project briefing
Visit two is provided as an additional opportunity for you to ask questions. Once you are fully satisfied with the protocol and information about the research study, and agree to take part in the study, you will be asked to complete an informed consent form, a medical and health questionnaire, and arrange for the following visits.

Visit three (~2 h):
Initial assessment
On an agreed day you will be required to attend the laboratory in the SSHES for initial assessments. The procedures will be explained to you before any measurements are taken. The assessments are as follows:

Body measurements
Height will be measured. Nude body weight measurements will be taken: this will be done behind a screen for privacy. Skin fold measurements will be taken from nine body sites (chest, midaxillary, abdominal, iliac crest, suprailiac, subscapular, biceps, triceps, midthigh) by a trained researcher. These measurements will only create a small squeezing sensation at each skin fold site.

Aerobic fitness test
A 10-15 min incremental exercise test performed on an electric treadmill will be performed to measure your aerobic fitness (estimated maximal oxygen uptake). The treadmill speed and/or gradient will increase every 3 min until you reach voluntary exhaustion. This test will require you to run at your maximal aerobic capacity for approximately 1 min. You will be required to wear a face mask during the test so we can measure gases in your expired air. The face mask is not uncomfortable and will not impede your breathing or exercise performance.

Sample familiarisation
Following the fitness test a venous blood sample, saliva sample, and urine sample will be collected to ensure you are fully familiar with these collection methods. All blood samples will be collected by a phlebotomy trained researcher from the forearm vein (14 ml of blood). A saliva sample will be collected by dribbling method into a collection pot. Urine will be collected in privacy by passing urine into a plastic container. In order to complete the investigation these samples are a necessity. You will also be given a training log to complete during the entire duration of the study.

Experimental trials
To investigate the effects of the timing of feeding after exercise on immune status, you will be required to complete three experimental trials in a random order, each separated by seven days. Each experimental trial will consist of a period of ~6 h were you will reside in the laboratory of the SSHES and complete a 2 h exercise bout at 75% of your maximal exercising effort. On each experimental trial you will receive a different recovery drink (~800 ml) 15 min after and 75 min after exercise. On one occasion you will receive water alone 15min after and 75 min after exercise. On another occasion you will receive a carbohydrate/protein drink at both time points, and on another occasion you will receive water 15 min after exercise and a carbohydrate/protein drink 75 min after exercise. **Including the briefing visits, initial assessment and three experimental exercise trials, you will be required to visit the laboratory on six occasions for a total of ~21½ h.**

Visit four, five, and six (~8 hrs):
On a day planned, after your initial assessment, a researcher will deliver to you, your total food and fluid to be consumed during the day prior to the experimental exercise trial.
You will be required to arrive at the laboratory for 08.00 h. At 08:30 h we will collect base line nude body weight, blood, saliva, and urine samples. You will then start exercising at 9:00 h at 75% of your maximal exercise effort on a treadmill, and this will last for 2 h. You will be provided with ~350 ml of water each hour. Your heat rate and RPE (rating of perceived exertion) will be monitored every 10min.

Immediately after exercise, nude body weight, blood, saliva, and urine samples will be collected. Blood and saliva samples will then be collected every 20 min for 2 h 20 min. You will receive ~800 ml of fluid to drink at 15 min and 75 min after the cessation of exercise. In accordance with the experimental protocol this will either be water or a carbohydrate and protein drink. We will provide you with food and fluid after the last blood and saliva sample is taken.

You will be required to repeat the experimental trial on two further occasions, with the time period of one week between trials. Similar to the first experimental exercise trial, a researcher will deliver to you, your total food and fluid to be consumed during the day prior to the each experimental exercise trial.

Advantages of taking part
The results from this study will provide practical information about the timing of feeding after exercise and the speed of recovery of the immune system. Indeed, immune function appears to be lowered after heavy exercise, possibly accounting for the increased infection incidence at this time; as such, any information about how to improve immune recovery after exercise would be most welcome. This information will be particularly useful to

sports scientists and those involved in organising the training and dietary schedules of athletes and military personnel.

The benefits of taking part in this study are that you will receive comprehensive feedback, with full explanations; for your; fitness level, body composition, and immune system status. This information will help you with planning and monitoring your training programme. The feedback you will receive regarding your fitness level is similar to that which many testing facilities provide as a fee-paying service.

Disadvantages of taking part

The disadvantages of taking part in this study, which you will probably be most concerned about are: blood sampling, physical exertion, and time commitment.

1. Blood sampling

Only qualified phlebotomy trained researchers with experience at performing this procedure will collect blood samples. To ensure you are completely comfortable with giving blood samples, we will familiarise you with the blood sample collection procedure during the initial assessment. We will insert a small syringe into the forearm vein on several occasions for the multiple blood collections before and during the recovery period. The amount of blood collected during each experimental exercise trial (126 ml) will not impact upon your normal physical functioning.

2. Physical exertion

The physical components of this study include; the incremental aerobic fitness test, and the three 2 h exercise bouts at 75% of maximal effort. The incremental aerobic fitness test will only require you to run at your maximal capacity for approximately one minute. You most probably feel that same type of exhaustion and fatigue for longer periods during your normal training and competition habits. The three 2 h exercise bouts at 75% of maximal effort may promote post-exercise fatigue. However, they are of a similar volume and intensity of effort to that experienced during your training and competitions. As such, the 2 h runs can also be seen as useful intensive and prolonged training sessions.

3. Time commitment

To complete all aspects of the study we will require you to visit the laboratory on 6 occasions for a total of approximately 21½ h. We understand that is study will ask of you to break from you normal lifestyle routines; therefore, we are offering an incentive. As a participant you will be eligible for a £7.50 reward on the completion of the first experimental trial, a further £7.50 reward on the completion of the second experimental trial; and finally, a further £15 reward on the completion of the third experimental trial. This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime.

In addition, in case of emergencies there will be a researcher qualified in First Aid present through-out each aspect of the study.

If you have any further question or queries, please contact Dr Neil Walsh or any of the study researchers.

Bangor University
School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title: Does the addition of protein to a carbohydrate beverage ingested after prolonged exercise enhance recovery of selected immune indices?

Research Supervisor: Dr. Neil Walsh **Tel :** 01248 383480
Email: n.walsh@bangor.ac.uk

Research Co-ordinators: Ricardo Costa.

Additional Investigators: Francis Adams, Tom Williams and Katherine Richardson.

Technical Assistance: Kevin Williams.

Invitation to take part
You have been invited to take part in a research investigation. 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 read the following information carefully. You should 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 an information sheet to keep and be asked to sign a subject 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 (SSHES), or any of the researchers involved with the study.

All information collected during the study will be coded and treated confidentially.

Background
The immune system is responsible for defending our body against invading invaders. When our immune system is running low, we are more prone to being infected by foreign invaders; such as, bacteria and viruses (e.g. common cold). Past research has demonstrated that prolonged high intensity exercise can depress immune function, leaving the body open to illness and infection. Further studies have tried to determine which of the body's mechanisms are responsible for suppressing the immune system, and there has been a strong link between nutritional intake and how the immune system works. Some of the most impressive results have been found on carbohydrate content in normal dietary habits and carbohydrate feeding during exercise. These results indicate that a diet consistently high in carbohydrates and carbohydrate feedings during exercise can blunt the fall in immune function after prolonged high intensity exercise. Recently, our research team has also found that the intake of carbohydrate and protein immediately after prolonged

intensive exercise can also prevent certain aspects of the immune system from lowering during the recovery period. However, it is not known whether this prevention is due to the carbohydrates, protein, or the combination of both nutrients. Therefore, the aim of this study is to examine the effects of the addition of protein to a carbohydrate beverage ingested after prolonged exercise on selected immune parameters.

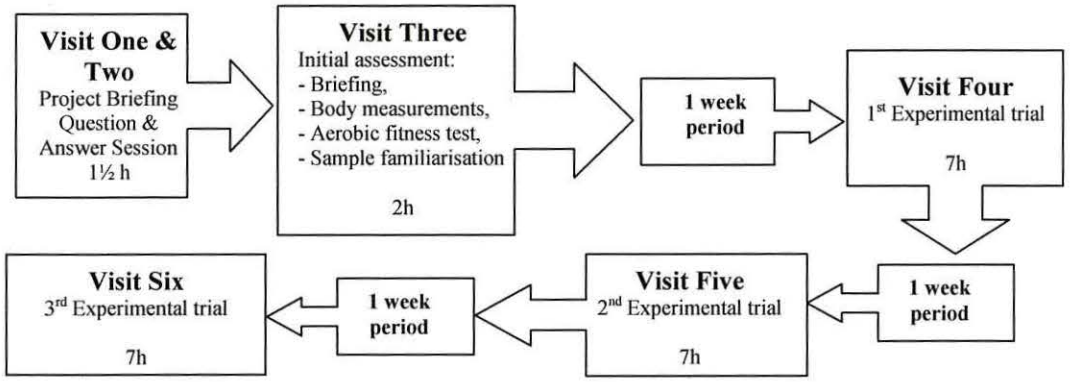
What will be expected of you?

If you decide to take part in this study there will be a number of constraints placed upon your normal everyday life and activities; which include:

- Complete a training log during the experimental exercise trials.
- Prior to the initial assessment and each exercise trial, you will be expected;
  - To avoid caffeinated and alcoholic beverages (e.g. coffee, tea, colas) for 3 days prior.
  - To avoid taking part in any form of exercise the day prior.
- Consume a balanced diet that provides sufficient energy to meet your requirements. We will be provided with this food and drink the day prior to each experimental trial.
- You should also avoid taking any nutritional supplements one month prior to the exercise trials.
- On the initial assessment day, you will be required to be present at the School of Sport, Health & Exercise Science (SSHES) laboratory for 2 h on the time arranged.
- On the 3 experimental trial days, you will be provided with a standard breakfast to be consumed at 0700 h, and you will be required to be present at the laboratory of the SSHES for 0800 h. Your activities in the laboratory will finish at approximately 1500 h.
- Whilst completing the experimental trials you will be expected:
  - To stay in the laboratory of the SSHES for the duration of the initial assessment and 3 experimental trials.
  - On one occasion perform a ~15 min maximal exercise test on an electric treadmill. On 3 separate occasions perform a 2 h run at 75% of your maximal exercise capacity on an electric treadmill.
  - Consume the fluids and foods provided during and after the exercise trial.
  - Weigh yourself nude (behind screen to maintain privacy). Have your height taken, and a DEXA scan performed by a trained researcher.
  - To have blood samples taken from the antecubital vein by a trained researcher. These will occur prior to the exercise trial, immediately after the exercise trial and every 30 min after exercise for 3 h. This totals 24 blood samples for the 3 main experimental exercise trials. To provide 128 ml blood at each of the 3 experimental trials (a total of 384 ml of blood taken during the whole study).
  - To provide a saliva sample before and after exercise, and at 30 min intervals after exercise via a dribble method that will be explained to you. This will total 8 saliva samples for each experimental exercise trial (24 saliva samples for the whole study).
  - To provide a urine sample prior to and after each experimental exercise trial. This will total 2 urine samples for each experimental exercise trial (6 urine samples for the whole study).

If you have or recently had (within one month) an infection, illness, injury, and/or taking medication you will be excluded from taking part in this study.

Summary of Visits.



Visit 1 (~30 min)
Project briefing
At this meeting you will be fully briefed about the requirements of the research project. You will be talked through the subject information sheet by a researcher and given opportunity to ask any questions. You will then leave with the information sheet to allow you time to discuss your possible involvement in the study with others.

Visit 2 (~1 h)
Project briefing
Visit two is provided as an additional opportunity for you to ask questions. Once you are fully satisfied with the protocol and information about the research study, and agree to take part in the study, you will be asked to complete an informed consent form, a medical and health questionnaire, and arrange for the following visits.

Visit three (~2 h):
Initial assessment
On an agreed day you will be required to attend the laboratory in the SSHES for initial assessments. The procedures will be explained to you before any measurements are taken. The assessments are as follows:

Body measurements
Height and nude body weight will be measured. This will be done behind a screen for privacy. DEXA body scan will be performed by a trained researcher. The DEXA body scan produced a radiation exposure equal to a normal day's background radiation.

Aerobic fitness test
A 10-15 min incremental exercise test performed on an electric treadmill will be performed to measure your aerobic fitness (estimated maximal oxygen uptake). The treadmill speed and/or gradient will increase every 3 min until you reach voluntary exhaustion. This test will require you to run at your maximal aerobic capacity for approximately 1 min. You will be required to wear a face mask during the test so we can measure gases in your expired air. The face mask is not uncomfortable and will not impede your breathing or exercise performance.

Sample familiarisation
Following the fitness test a venous blood sample, saliva sample, and urine sample will be collected to ensure you are fully familiar with these collection methods. All blood samples

will be collected by a phlebotomy trained researcher from the forearm vein (16 ml of blood). A saliva sample will be collected by dribbling method into a collection pot. Urine will be collected in privacy by passing urine into a plastic container. In order to complete the investigation these samples are a necessity. You will also be given a training log to complete during the entire duration of the study.

Experimental trials
To investigate the effects of ingesting carbohydrate with and without protein after exercise on immune status, you will be required to complete three experimental trials in a random order, separated seven days. Each experimental trial will consist of a period of ~7 h where you will reside in the laboratory of the SSHES and complete a 2 h exercise bout at 75% of your maximal exercising effort. On each experimental trial you will receive a different recovery drink (~900 ml) immediately after and 1 hour after exercise. The recovery drink will be either a placebo, a carbohydrate or a carbohydrate and protein drink.

Including the briefing visits, initial assessment and three experimental exercise trials, you will be required to visit the laboratory on six occasions for a total of ~24½ h.

Visit four, five, and six (~7 hrs):
One week after your initial assessment, a researcher will deliver to you, your total food and fluid to be consumed during the day prior to the experimental trials. After breakfast the following morning (this will be your last meal until after the exercise trial), you will be required to arrive at the laboratory for 0800 h. At 0915 h we will start collect base line nude body weight, blood, saliva, and urine samples. You will then start exercising at 0930 h at 75% of your maximal exercise effort on a treadmill, and this will last for 2 h. You will be provided with ~350 ml of water each hour. Your heart rate and RPE (rating of perceived exertion) will be monitored every 10 min.

Immediately after exercise, nude body weight, blood, saliva, and urine samples will be collected. Blood and saliva samples will then be collected every 30 min for 3h. You will receive ~900 ml of fluid to drink immediately after and 1 h after the cessation of exercise. In accordance with the experimental protocol this will either a placebo, a carbohydrate, or a carbohydrate and protein drink. We will provide you with food and fluid after the last blood and saliva sample is taken.

You will be required to repeat the experimental trial on two further occasions, with the time period of one week between trials. Similar to the first experimental exercise trial, a researcher will deliver to you, your total food and fluid to be consumed during the day prior to the each experimental exercise trial.

Advantages of taking part
By taking part in this study you will contribute to our knowledge about the effects of nutrition on recovery of the immune system after exercise. Your participation will help us determine whether carbohydrate alone or carbohydrate with additional protein can help recover the immune system after exercise. Indeed, immune function appears to be lowered after heavy exercise, possibly accounting for the increased infection incidence at this time; as such, any information about how to improve immune recovery after exercise would be most welcome. This information will be useful to sports scientists and those involved in organising the training and dietary schedules of athletes and military personnel.

The benefits of taking part in this study are that you will receive comprehensive feedback, with full explanations; for your; fitness level, body composition, and immune system status. This information will help you with planning and monitoring your training programme. The feedback you will receive regarding your fitness level is similar to that which many testing facilities provide as a fee-paying service.

Undergraduates in SSHES will gain valuable insight in to the procedures and work involved in a 3rd year project. In addition, participants in this project can cover both the open and personal performance 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: blood sampling, physical exertion, and time commitment.

1. Blood sampling
Only qualified phlebotomy trained researchers with experience at performing this procedure will collect blood samples. To ensure you are completely comfortable with giving blood samples, we will familiarise you with the blood sample collection procedure during the initial assessment. We will insert a small syringe into the forearm vein on several occasions for the multiple blood collections before and during the recovery period. The amount of blood collected during each experimental exercise trial (126 ml) will not impact upon your normal physical functioning.

2. Physical exertion
The physical components of this study include; the incremental aerobic fitness test, and the three 2 h exercise bouts at 75% of maximal effort. The incremental aerobic fitness test will only require you to run at your maximal capacity for approximately one minute. You most probably feel that same type of exhaustion and fatigue for longer periods during your normal training and competition habits. The three 2 h exercise bouts at 75% of maximal effort may promote post-exercise fatigue. However, they are of a similar volume and intensity of effort to that experienced during your training and competitions. As such, the 2 h runs can also be seen as useful intensive and prolonged training sessions.

3. Time commitment
To complete all aspects of the study we will require you to visit the laboratory on 6 occasions for a total of approximately 24½ h. We understand that is study will ask of you to break from you normal lifestyle routines. Therefore, we are offering an incentive. As a participant you will be eligible for a £15 reward on the completion of the first experimental trial, a further £15 reward on the completion of the second experimental trial; and finally, a further £30 reward on the completion of the third experimental trial. This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime.

In addition, in case of emergencies there will be a researcher qualified in First Aid present through-out each aspect of the study.

If you have any further question or queries, please contact Dr Neil Walsh or any of the study researchers.

Appendix B

Bangor University
SCHOOL OF SPORT, HEALTH AND EXERCISE SCIENCES

1	Title of project	
2	Name and e-mail address(es) of all researcher(s)	

Please tick boxes

1	I confirm that I have read and understand the Information Sheet dated ..... for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights being affected.	
3	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason. If I do decide to withdraw I understand that it will have no influence on the marks I receive, the outcome of my period of study, or my standing with my supervisor, other staff members of with the School.	
4	I understand that I may register any complaint I might have about this experiment with the 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 the standard report forms.	
5	I agree to take part in the above study.	

Name of Participant .....

Signature ..... Date .....

Name of Person taking consent.....

Signature ..... Date .....

WHEN COMPLETED – ONE COPY TO PARTICIPANT, ONE COPY TO RESEARCHER FILE

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 ..... Date .....
Participant

Signature ..... Date .....
Experimenter