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Physiological stressors : the effects of sleep disruption, energy restriction and prolonged exercise on thermoregulation and immune function

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PHYSIOLOGICAL STRESSORS: THE EFFECTS OF SLEEP DISRUPTION, ENERGY RESTRICTION AND PROLONGED EXERCISE ON THEMOREGULATION AND IMMUNE FUNCTION

by

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Summary

The purpose of this thesis was to examine; the effects of various stressors on human thermoregulation and immune function, a functional link between these two systems and the potential of novel measurement techniques.

A valid, un-obtrusive measure of skin temperature was desirable for a majority of studies in this thesis. Wireless iButtons® provided such a method. Both iButtons® and conventional thermistors displayed high validity correlation ($r > 0.999$) and mean bias (iButtons® = +0.121°C, thernistors = +0.045°C) with a reference thermometer.

A reliable measure of *in vivo* immunity in response to stressors, was also desirable. Contact sensitization with diphenylcyclopropenone proved a simple and robust method. Results demonstrate that exercise-induced-stress impairs both phases of *in vivo* T-cell-mediated immunity (oedema: induction -53%; elicitation -19%).

Two-nights total sleep deprivation, with or without energy-restriction did not impair thermoregulation during cold exposure, nor appear to increase the risk of hypothermia. Core temperature, skin temperature and time to reach 35.9°C core temperature were not significantly different between trials.

Three-nights sleep disruption (SDIS) did not affect core temperature at rest or during exercise-heat-stress. However, SDIS upper-body skin temperature was higher during exercise and forearm-sweating lower (-21%). Thus, alterations to heat-loss pathways were observed, but with no increase in thermal strain.

Concurrent examination of the effects of SDIS, demonstrated enhanced *in vivo* T-cell-mediated immunity (oedema +98%). *In vitro* markers were unaltered by SDIS, suggesting these markers may not reveal the true effect of stressors on the co-ordinated immune response.

Significant differences between sleep deprivation and control proximal and distal skin temperatures lends evidence to the functional, mechanistic coupling between circadian variations in skin temperature and skin immunity, as prolonged periods of increased skin blood flow

observed during sleep disruption provides enhanced maintenance of skin immunity and thus likely to account for the observed increase in T-cell-mediated immune responses.

The purpose of this thesis was to examine the effects of various stressors on thermoregulation and immune function, and to examine the functional link between these two systems, utilizing conventional, as well as examining the potential future usage of novel techniques and methods in humans.

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Publications

I was involved throughout every stage of all of the studies in this thesis, from idea, through, ethics proposals, implementation of methodology, logistics, phlebotomy, biochemistry, analyses and preparation of manuscripts for peer reviewed publication. Nevertheless, I also gratefully acknowledge input from the other named authors for their various contributions to each publication. The following is a list of publications arising from the material presented in this thesis.

Full Research Publications

Harper Smith, A.D., Crabtree, D.R., Bilzon, J.L.J. and Walsh, N.P. (2010). The validity of wireless iButtons® and thermistors for human skin temperature measurement. *Physiological Measurement*; **31**(1):95-114.

Costa RJS, Harper Smith AD, Oliver SJ, Walters R, Maassen N, Bilzon JLJ and Walsh NP (2010). The effects of two nights of sleep deprivation with or without energy restriction on immune indices at rest and in response to cold exposure. *European Journal of Applied Physiology*; **109**(3):417-28.

Harper Smith AD, Coakley S, Ward M, Macfarlane, A, Friedmann P, and Walsh NP (2011). Exercise-induced stress inhibits both the induction and expression phases of in vivo T-cell-mediated immune responses in humans. *Brain, Behavior and Immunity*; **25**(6):1136-1142.

Abstracts Published in Conference Proceedings

Harper Smith, A.D., Costa, R.J.S., Oliver, S.J., Bilzon, J.L.J. and Walsh, N.P. The effects of fifty-two hours of sleep deprivation with and without energy restriction on thermoregulation during cold stress. *Annual Conference of the British Association of Sport and Exercise Sciences*, Brunel University, London, U.K., May 2008.

Harper Smith, A.D., Crabtree, D.R., Bilzon, J.L.J. and Walsh, N.P. The validity of wireless iButtons® and thermistors for human skin temperature measurement, *Wales Institute of*

Sport Health and Exercise Science Annual Conference, Aberystwyth University, Aberystwyth, U.K., July 2009.

Harper Smith, A.D., Ward, M.D., Friedmann, P.S., Macfarlane, A.W. and Walsh, N.P. Prolonged moderate intensity exercise decreases the *in-vivo* immune response to Diphenylcyclopropenone, a novel experimental contact sensitizer. *International Society of Exercise and Immunology Symposium*. University of Tübingen, Tübingen, Germany, September 2009.

Table of Contents

Thesis Format

A literature review (**Chapter 2**) provides a brief background and proposes the broad aims of the research presented in the thesis. A general methods chapter follows, that outlines the common procedures and analyses performed in the subsequent experimental studies (**Chapter 3**). The thesis consists of four independent experimental studies. The first experimental study principally investigated the validity of wireless iButtons®, a novel alternative for the measurement of human skin temperature measurement (**Chapter 4**). The second experimental study investigated the effects of a systemic exercise-induced stress on both the induction and elicitation phases of the T-cell-mediated immune response to the novel antigen diphenylcyclopropenone (DPCP) by use of the experimental contact-hypersensitivity (CHS) method (**Chapter 5**). The third experimental study tested the hypotheses that two nights of total sleep deprivation impairs human thermoregulation during a cold-air test and that energy-restriction might have additive, detrimental effects, heightening the risk of hypothermia (**Chapter 6**). The fourth experimental study tested the hypotheses that three-nights of disrupted sleep impairs thermoregulation at rest as well as during subsequent exercise-heat-stress (**Chapter 7**). In the same experimental study, the effects of three nights of disrupted sleep on immune function at rest were examined in concert with the theory that there is a functional, mechanistic link between sleep, circadian skin temperature rhythms and skin immunity (**Chapter 8**). To conclude, a general discussion (**Chapter 9**) contains a summary of key findings and a concise critical analysis of the research programme, which highlights the conceived limitations and potential areas for future research. Throughout the thesis, abbreviations are defined at first use, or if in common scientific usage, are defined in the list of abbreviations preceding the experimental chapters. As such, for clarity, a list of tables, figures and abbreviations appears prior to **Chapter 1**. **Bold type** is used when referral to other sections elsewhere within this thesis is encouraged, for addition comprehension of the material referenced.

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List of Abbreviations

ANOVA	analysis of variance
ASTM	American Society for Testing and Material
AU	arbitrary units
BSI	British Standards Institution
CAT	cold-air test
$CD3^+$	cluster of differentiation 3
$CD4^+$	cluster of differentiation 4
$CD8^+$	cluster of differentiation 8
CI	confidence interval
cm	centimetre
CHS	contact hyper-sensitivity
CNS	central nervous system
CON	control (trial)
CV	co-efficient of variation
d	day
DEXA	dual energy x-ray absorptiometry
DPCP	2,3, di-phenyl cyclo-propenone / diphenlcyclopropenone
EDTA	ethylene di-amine tetra-acetic acid
EHI	exertional heat illness
EHSP	exercise heat-stress protocol
ELISA	enzyme-linked immunosorbent assay
ER	energy restriction (trial)
EX	exercise trial
g	gram
h	hour
HPA	hypothalamic-pituitary-adrenal
HR	heart rate
HR_{max}	maximal heart rate
ICC	intra-class correlation coefficient
IFN	interferon
ISO	International Organization for Standardization
Ig	immunoglobulin
IL	interleukin
kg	kilogram
kJ	kilojoule
km	kilometre
$km \cdot h^{-1}$	kilometers per hour
K_3EDTA	ethylene di-amine tetra-acetic acid
L	litre
LoA	limits of agreement
M	metabolic heat production
m	metre
mg	milligram
min	minute
$min \cdot night^{-1}$	minutes per night
MJ	mega joules
ml	millilitre
$ml \cdot kg^{-1} \cdot min^{-1}$	millilitres per kilogram per minute
$ml \cdot kg^{-1}NBM \cdot day^{-1}$	millilitres per kilogram of nude body mass per day

$ml \cdot min^{-1}$	millitres per minute
mm	millimetre
mmol	millimole
mOsmol	milliosmole
$m \cdot s^{-1}$	metres per second
m_{swf}	local forearm sweating rate
N	number of participants
NBM	nude body mass
NREM	non rapid eye movement (sleep)
PAR	primary allergic response
REM	rapid eye movement (sleep)
RER	respiratory exchange ratio
RH	relative humidity
RMR	resting metabolic rate
RPE	rating of perceived exertion
r	Pearson's moment-product correlation coefficient
s	second
S	heat balance (heat debt / heat storage)
SD	standard deviation
s_{diff}	standard deviation of the mean bias
SDEP	sleep deprivation (trial)
SDIS	sleep disruption (trial)
SEM	standard error of the mean
s-IgA	saliva immunoglobulin-A
t	time
T	temperature
T_{amb}	ambient temperature
T_b	(mean) body temperature
T_{db}	dry bulb temperature
TEE	typical error of the estimate
TEM	typical error of the measurement
TNF	tumour necrosis factor
T_{sk}	skin temperature
T_{re}	rectal temperature
TT	time trial
URTI	upper respiratory tract infection
v	velocity
$\dot{V}O_{2max}$	maximum oxygen uptake
$\dot{V}O_{2peak}$	peak oxygen uptake
W	watts
y	years
°C	degrees Celsius
µg	microgram
$\mu g \cdot cm^{-2}$	micrograms per square centimetre
µl	microlitre
µSv	microsievert
Σ	summed

CHAPTER ONE

General Introduction

Many occupational settings present physical, mental, emotional, nutritional and even traumatic stress. Stressors such as prolonged exercise, sleep loss and energy restriction are believed to negatively affect normal human thermoregulation and immune function in normo-thermic as well as extreme environments, for example, during military and athletic training (Castellani *et al.*, 2003; Gleeson, 2007; Shephard *et al.*, 1998). The findings from studies examining the effects of stressors such as sleep disruption can also be applied to populations such as shift workers and parents of neo-nates. It is also thought that methods currently used to assess certain aspects of thermoregulation and immune function can be improved upon (Albers *et al.*, 2005; van Marken Lichtenbelt *et al.*, 2006). Furthermore, it has been suggested that there is a functional, mechanistic role for the circadian modulation of thermoregulation, promoting, in particular, the maintenance of skin immune function (Van Someren, 2006).

It has been demonstrated that disrupted sleep was the second most common pre-disposing factor in both fatal and non-fatal casualties of exertional heat illness (EHI) in the Israeli Defence Forces (Rav-Acha *et al.*, 2004). In two separate investigations, one night of total sleep deprivation lowered resting core temperature (Sawka *et al.*, 1984) and reduced local sweat rate, sweating sensitivity (Sawka *et al.*, 1984) and cutaneous vasodilation (Kolka and Stephenson, 1988), affecting normal thermoregulation during exercise-heat stress. The effects of a longer period of sleep loss on thermoregulation in the heat, remains unknown. It has also been indicated that sleep deprivation, energy restriction and prolonged physical exertion can negatively impact thermoregulation during prolonged cold exposure, which may predispose individuals to hypothermia (Castellani *et al.*, 2003; Kolka and Stephenson, 1988; Young *et al.*, 1998).

However, due to the nature of the multi-stressor scenario involved in military operations and the studies that have examined them, the contribution of each stressor on disrupted thermoregulation is unclear. Thus, unfortunately, the results of prospective studies in the currently available literature do not clearly characterize whether, and how, each individual stressor might negatively affect thermoregulation. Furthermore, fundamental measurements in human thermal physiology, such as skin temperature (T_{sk}) (van Marken Lichtenbelt *et al.*, 2006) cannot easily be examined in free living, normal routine conditions with the currently favoured methodology, such as hard-wired thermistors or thermocouples. Thus, the utilization of a device capable of an inexpensive, autonomous, wireless, temperature monitoring and recording would considerably benefit human thermal physiology research (van Marken Lichtenbelt *et al.*, 2006).

Stress in a variety of forms is also widely acknowledged to modulate immune function (Dhabhar, 2002b) and susceptibility to infection (Cohen *et al.*, 1991). It has been suggested that the stress of sleep loss may evoke sub-optimal host defence which may increase the risk of illness, infection, decrease effective wound healing and increase the susceptibility to sepsis (Costa *et al.*, 2010; Shephard *et al.*, 1998). Although, it has been suggested that disturbed sleep may cause declines in natural and cellular immunity as well as alterations in the complex cytokine network (Irwin, 2002), unfortunately, the vast majority of research performed on sleep disturbances have only utilized *in vitro* methods of assessing immune function (Dinges *et al.*, 1994; Dinges *et al.*, 1995; Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). Measurements *In vitro*, Latin for "in glass", involve the analysis of immune markers isolated and removed from the living organism in its natural biological environment, such as cell counts in blood in a test tube, and as a consequence do not provide an accurate assessment of the systemic state of the immune system. In contrast, analyses *in vivo* (Latin for "in the living"), such as dermatological patch testing, take place on the whole, intact, living organism during normal

living situations, and as such, provide a better picture of the overall effects of the experimental conditions on the subject.

Prolonged exercise is also believed to adversely affect immune function (Gleeson, 2007). However, again, only a limited number of studies in the literature have utilized *in vivo* measures of immune function to assess the effects of prolonged exercise (Bruunsgaard *et al.*, 1997). This suggests that the results will not truly or accurately represent changes that naturally occur in the body post-exercise (Bruunsgard et al, 1997). As such, *in vivo* measures of immunity should bring about results that better represent an ongoing and coordinated, cell-mediated immune response (CMI), giving improved predictions of immune response (Gleeson, 2006). Without incorporating *in vivo* measures of immunity, it is difficult to determine the clinical significance of stress on immune function.

As suggested, the utilization of an *in vivo* measure of immune function, able to identify the effects of various stressors such as sleep deprivation and prolonged exercise, would therefore be a very useful addition to stress physiology research. And, as with currently available immune function measures, there is scope for improvement in the research methodology of thermoregulation, such as skin temperature measurement. Hopefully, through the application of novel measures of *in vivo* immune function and thermoregulation, the proposed functional, mechanistic link between the thermoregulatory and immune systems, whereby, the nocturnal period of increased skin blood flow, may support maintenance of the skin as a primary barrier in host defence (Krueger and Majde, 1990; Van Someren *et al.*, 2002), can now be explored more thoroughly.

CHAPTER TWO

Literature Review

2.1 What is stress?

Stress is a common component of modern life and is frequently discussed by the media and in everyday conversation. However, people's conception of what constitutes stress, varies widely; from the idea of 'good stress', which may be the excitement and positive pressure to perform a certain task or attain a certain goal, to the majority of people who consider stress, only to be 'bad stress'. This 'bad stress' tends to be associated with psychological negative conditions such as anxiety, fear, nervousness, irritability, dissatisfaction, depression and panic attacks. However, stress is not so easily characterized as 'good' or 'bad', and is not only a psychological condition. A clearer biological definition of stress is:

> "….a constellation of events, comprised of a stimulus (*stressor*), that precipitates a reaction in the brain (*stress perception*), which subsequently activates physiologic *fight/flight/fright* systems in the body (*stress response*)." (Dhabhar and McEwen, 1997)

The consequence of the body initiating a stress response, is the release of hormones and neurotransmitters that act as the brain's messengers to the body.

Another designation distinguishes the types of stress into seven different categories which represent the stimuli, each with their own examples of stressors (Haas and Levin, 2006):

1. PHYSICAL: intense exertion, manual labour, lack of sleep, travel

2. CHEMICAL: drugs, alcohol, caffeine, nicotine and environmental pollutants such as cleaning chemicals or pesticides
3. MENTAL: perfectionism, worry, anxiety, long work hours
4. EMOTIONAL: anger, guilt, loneliness, sadness, fear
5. NUTRITIONAL: food allergies; energy, vitamin and mineral deficiencies
6. TRAUMATIC: injuries or burns, surgery, illness, infections, extreme temperatures
7. PSYCHO-SPIRITUAL: troubled relationships, financial or career pressures, challenges with life goals, spiritual alignment and general state of happiness

Although these designations appear to comprise examples of stressors generally considered to be 'negative'; experienced during harmful, hazardous or unpleasant experiences, a physiological stress response is also triggered during pleasurable events such as sexual stimulation (Colborn *et al.*, 1991) and orgasm (Kruger *et al.*, 1998). Stress is also distinctly characterized by temporal designations. The duration of the experience will determine whether the stress is considered *acute stress* or *chronic stress*. Acute stress can last for intervals of seconds to hours, and chronic stress, is stress that persists on a daily basis for weeks, months or longer (Dhabhar, 2002a). Another critical distinction between the characteristics of acute and chronic stress is that the outcomes of the stress response in the short term can be beneficial and adaptive, whereas they are likely to be detrimental when the stress is ongoing and experienced long term (Dhabhar and McEwen, 1996; Dhabhar and McEwen, 1997). The dysregulation of the circadian rhythms of corticosterone in rodents (Dhabhar and McEwen, 1997) and cortisol in humans are key indicators of chronic stress (Sephton *et al.*, 2000).

2.2 New Terminology in Stress Research and Description of the Stress Response

The introduction of the concept of "stress" into general, as well as medical discourse is widely attributed to the Canadian–Hungarian medical scientist Hans Selye (1907–1983). The "fight or

flight" response embodies Selye's description of stress, which activates the emergency reaction of the adreno-cortical and sympathetic nervous systems (Selye, 1936). The general adaptation syndrome was proposed by Selye, which he postulated as being a three step physiological reaction to a given stressor. Phase one consists of the alarm reaction, in which the adrenal medulla releases adrenaline, and the adrenal cortex releases glucocorticoids, both of which aid in the restoration of homeostasis. Phase two represents the aftermath of the restoration of homeostasis, leading to resistance, in which defence and adaptation become optimally sustained. The final phase exists if the stressor(s) persist(s), leading to "exhaustion" and, as such, there is a termination of any adaptive response. In this construct, the outcome if situations lead to phase three of the adaptation syndrome, is infection and potentially death (Selye, 1936).

Selye's general adaption syndrome provides a good general framework for the stress response, but it should be noted that different stressors evoke different responses from the HPA axis and the adrenergic and nor-adrenergic nerves (Goldstein and Eisenhofer, 2000). Furthermore, the "fight or flight" stress response does not apply equally to both sexes, but rather applies more accurately to males of the species when under threat. Females of the animal species interpret stressors differently and have different behavioural and hormonal responses to "stress" than males, resulting in dissimilar physiological regulation of mediators of the stress response. Thus, although females do flee from extreme hazards, the difference in responses to non life-threatening danger has led to the female characterization of the stress response as "tend-and-befriend," rather than "fight or flight" (Taylor *et al.*, 2000). For example, the classical conditioning response to the stress of acute tail shock display is the opposite in male and female rodents, whereby males improve and females deteriorate in their conditioning performance (Wood and Shors, 1998). Furthermore, as stated previously, there is a temporal relationship between the body and its exposure to the mediators of the stress response, whereby, the time course of secretion and exposure will determine whether the effects will be protective or

damaging. In other words, the immediate effects of exposure to stress mediators is adaptive, whereas continual exposure to the same stressor eventually becomes mal-adaptive and detrimental. Current discoveries indicate that is not the 'exhaustion' of the protective mechanisms of the immune system, as proposed by Selye, *per se*, but rather that the mediators of the stress response ultimately themselves begin to assault the body of the host (Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1999; McEwen, 2005).

Recently, other authors have proposed more specific terminology to replace the terms "stress". Allostasis has been proposed as a term that attempts to clarify these ambiguities, and refers to the adaptive processes that maintain homeostasis through the production of such primary mediators as the hormones of the HPA axis, catecholamines, glucocorticoids and cytokines (McEwen, 2005). Homeostasis, constitutes the regulatory systems that are essential for life, whereas the distinction with allostasis is that the allostatic systems are those that support and allow these homeostatic, regulatory systems to maintain balance (McEwen, 2005). Allostatic load has been proposed as term to quantify the extent of stress experienced, with allostatic overload referring to 'chronic stress' and the associated symptomology (McEwen, 2005). However, these terms have not yet been adopted by the majority of the medical and scientific community or yet become common convention. As such, this thesis will continue to use the current conventional terminology in stress physiology, i.e., stress and stressors as defined above.

Publications in this literature review were located from extensive online searches using the scientific website databases of MEDLINE (www.pubmed.com) and ISI Web of Knowledge, which included examining citation reports for forward and back searches from the relevant literature identified. Only literature specifically relevant to the aims of the experimental chapters are included.

2.3 The Effects of the Stressors Sleep Disruption, Energy Restriction and Physical Exertion on Thermoregulation and a Potential Novel Measurement Method

2.3.1 A Novel Method for the Measurement of Skin Temperature

A mentioned in **Chapter 1**, human skin temperature (van Marken Lichtenbelt *et al.*, 2006) cannot easily be examined in free living, normal routine conditions, for example during exercise or when sleeping. This is because the currently used methods, such as thermistors and thermocouples are hard wired and thus restrict participants' movements, the wires can easily entangle in moving limbs and these wires are subject to breakage and detachment (Buono *et al.*, 2007; Van den Heuvel *et al.*, 1998). A device which shows particular promise for these purposes are iButtons®, which are capable of autonomous, wireless, temperature monitoring and recording (van Marken Lichtenbelt *et al.*, 2006). The iButton® devices were originally designed to monitor temperature-sensitive consignments during transport and have been utilized in relatively few scientific studies (Areas *et al.*, 2006; Davidson *et al.*, 2003; Fronczek *et al.*, 2006; Hubbart *et al.*, 2005; Johnson *et al.*, 2005; van Marken Lichtenbelt *et al.*, 2006), but are yet to be validated for human skin temperature measurement during exercise.

2.3.2 In the Cold

It has been indicated that sleep deprivation, energy restriction and physical exertion can negatively impact thermoregulation during prolonged cold exposure, which may predispose individuals to hypothermia (Castellani *et al.*, 2003; Kolka *et al.*, 1984; Young *et al.*, 1998). The few studies that have examined the effects of the combined stress of sleep deprivation, negative energy balance and exhaustive physical activity on thermoregulatory responses during cold exposure have shown impairment to core temperature responses and shivering thermogenesis that lead them to predict an increased risk of hypothermia in these conditions (Castellani *et al.*, 2001; Castellani *et al.*, 2003; Young *et al.*, 1998). Greater metabolic heat production was

required from shivering thermogenesis to maintain an equivalent body temperature in the cold, when participants were subjected to multiple stressors or inadequate recovery periods (Castellani *et al.*, 2001; Castellani *et al.*, 2003; Young *et al.*, 1998). Although it has been suggested that the combination of sustained sleep deprivation, energy restriction and fatiguing exercise affect normal thermoregulation during cold exposure (Castellani *et al.*, 2003; Young *et al.*, 1998), the relative influence of, and interaction between individual stressors is still unclear. The studies mentioned constituted a combination of the stressors sleep deprivation, energy restriction and prolonged physical exertion experienced at the same time, (Castellani *et al.*, 2003; Young *et al.*, 1998). The studies that have attempted to examine the independent effect of individual stressors on thermoregulation during cold exposure have presented equivocal conclusions as to whether an outcome of increased hypothermia risk would be evoked (Castellani *et al.*, 1999; Castellani *et al.*, 2001; Haman *et al.*, 2004; Landis *et al.*, 1998a; Savourey and Bittel, 1994). Negative energy balance during periods of cold exposure has itself been identified as a potential factor limiting shivering thermogenesis (Jacobs *et al.*, 1994; Wissler, 1985). Insufficient fuel supplies for heat production to offset the increases in heat loss associated with sleep deprivation are likely to amplify the susceptibility to hypothermia in a combined stressor state. Cold exposure and sleep deprivation are both stressors that can also affect thermoregulation via alterations of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Benzinger, 1969; Kioukia-Fougia *et al.*, 2002). As the Pre-Optic Anterior Hypothalamus is the central controller of thermoregulation, disruptions in the HPA axis can lead to modifications in neuro-endocrine responses that can then affect thermoregulation and even fuel utilization (Benzinger, 1969; Kioukia-Fougia *et al.*, 2002). Intriguingly, studies which have investigated the effects of only one night of total sleep deprivation on the risk of hypothermia revealed no differences in thermoregulatory variables in comparison to control conditions (Caine-Bish *et al.*, 2004; Landis *et al.*, 1998a; Savourey and Bittel, 1994; Young *et al.*, 1998). As one-night of total sleep deprivation did not appear to affect thermoregulation in previous studies (Caine-Bish *et al.*, 2004; Landis *et al.*, 1998a; Savourey

and Bittel, 1994; Young *et al.*, 1998), it is possible that a more prolonged period of sleep deprivation may be necessary to elicit significant and consistent findings, and fill this void in the literature.

Summary of the findings of relevant studies:

(Caine-Bish *et al.*, 2004)

- Participants: 12 men and women.
- Stressor(s): Sleep deprivation and cold exposure; the effects of 33 h of sleep deprivation on thermoregulation were determined during 180 min of cold exposure at 12°C.
- Results: There was no significant difference in rectal core temperature, mean skin temperature, metabolic heat production and tissue insulation between control and sleep deprivation trials

(Castellani *et al.*, 2001)

- Participants: 13 men (10 experimental and 3 control)
- Stressor(s): exercise and cold exposure (experimental group) - cold-wet walk up to 6 h in 5°C air three times; Controls - no exhaustive exercise was performed on any day.
- Results: mean skin temperature was higher in the experimental group, change in rectal temperature was greater, metabolic heat production similar between trials.

(Castellani *et al.*, 2003)

- Participants: 10 men
- Stressor(s): sleep deprivation, energy restriction and exhaustive physical exertion and cold exposure; participants' thermoregulatory responses were assessed during a cold air

test (CAT: 10°C for 180 min) after a control period and after 84 h of sustained military operations (SUSOPS).

- Results: CAT rectal temperature decreased to a greater extent and metabolic heat production was lower in SUSOPS compared to control. The mean body temperature-metabolic heat production relationship indicated that the threshold for shivering was lower after SUSOPS versus control. Mean weighted skin temperatures were lower during the initial 1.5 h of CAT in SUSOPS versus control. Heat debt was similar between trials.

(Kolka *et al.*, 1984)

- Participants: 6 men and 1 woman.
- Stressor(s): sleep deprivation, exercise and cold exposure; participants walked at low to moderate intensity on a treadmill (5.6 $km \cdot h^{-1}$) for 60 min in a cold environment (0°C) after normal sleep (control) and following a 50 h period of sleep deprivation.
- Results: resting rectal core temperature lower following the 50 h period of wakefulness versus control. Rectal temperature became not significantly different between trials after 15 min of exercise.

(Landis *et al.*, 1998b)

- Participants: 6 women.
- Stressor(s): Sleep deprivation, mild heat exposure; thermoregulatory responses were examined after one night of sleep deprivation and after one night of normal sleep during thermal challenges (32°C and 38°C).
- Results: Mean oesophageal and rectal temperatures were reduced due to one night of sleep deprivation. The mean threshold for sweating and skin blood flow were not altered by one night of sleep deprivation Peripheral vasodilation was attenuated when skin temperature was held at 38°C. Oesophageal and rectal temperatures decreased more

rapidly in sleep deprived participants skin temperature was cooled and held constant at 32°C.

(Savourey and Bittel, 1994)

- Participants: 12 men.
- Stressor(s): Sleep deprivation and cold exposure; thermoregulatory alterations were examined before and after 27 h sleep deprivation and during exposure to both 1 h temperate (25°C) and 2 h cold conditions (1°C).
- Results: Rectal temperature, mean skin temperature and metabolic heat production were not statistically different during exposure to temperate ambient temperatures. During cold exposure, rectal temperature did not change, but skin temperature and metabolic heat production were both higher after one night of sleep deprivation. The increase in metabolic heat production following sleep deprivation demonstrated a lower threshold and intensification of shivering. Higher subjective ratings of cold sensation were also observed following sleep deprivation.

(Young *et al.*, 1998)

- Participants: 8 men.
- Stressor(s): sleep deprivation, energy restriction and exhaustive physical exertion; 4 h cold air exposure (10°C) immediately after 61 d of strenuous military training (A), and again after short (48 h, SR) and long (109 d, LR) recovery.
- Results: Rectal temperature was lower before and during cold air exposure during A than SR and LR. Rectal temperature declined during cold exposure in A and SR but not LR. Mean weighted skin temperature during cold exposure was higher in A and SR than in LR. Metabolic rate increased during all cold exposures, but was lower during A and LR

than SR. Mean body temperature threshold for increased metabolism was lower during A than SR and LR.

2.3.3 In the Heat

It has been suggested that the stressors sleep disruption / deprivation and prolonged physical exertion can also impair thermoregulation and performance in adverse environmental conditions (Aakvaag *et al.*, 1978; Bugge *et al.*, 1979; Rognum *et al.*, 1986c). In a seminal study examining the incidence of exertional heat illness (EHI) through compilation of case studies over an entire decade in the Israeli Defence Forces (1992 to 2002), it was demonstrated that disrupted sleep was the most prevalent physiological pre-disposing factor, along with poor physical fitness, in fatal EHI cases (5 out of 6) and the second most common pre-disposing factor (29 of 72 cases) in non-fatal casualties (Rav-Acha *et al.*, 2004). However, due to the nature of the multi-stressor scenario involved in military operations and the studies that have examined them, it is unclear whether sleep disruption is simply a coincidental factor or a true pre-disposing factor.

In the few studies which have attempted to examine the effect of sleep disruption / deprivation on thermoregulation during exercise-heat stress, thermoregulation during exercise-heat stress did appear to be affected by total sleep deprivation. While resting core temperature was lowered (Sawka *et al.*, 1984) after 30 h of total sleep deprivation, local sweat rate and sweating sensitivity were decreased (Sawka *et al.*, 1984) and cutaneous vasodilation reduced (Kolka and Stephenson, 1988). Reduction in wet (evaporative) and dry (radiative and convective) heat loss due to complete sleep deprivation may therefore increase thermal strain during exercise-heat stress and increase the risk of EHI. These studies (Kolka and Stephenson, 1988; Sawka *et al.*, 1984) investigated the effect of one night of total sleep deprivation on thermoregulation during exercise-heat stress, and as such, the effects of a longer period of disrupted sleep (1 to 2 h sleep permitted per night) on these thermoregulatory responses remains unknown.

It has also been suggested (Bahr *et al.*, 1991; Opstad and Bahr, 1991), that the multi-stressor scenario of prior strenuous prolonged exercise, restricted sleep and energy intake deficiency may reduce set-point temperature, elevate resting metabolic rate and reduce mechanical efficiency during cycle-ergometry exercise. However, these two studies were not cross-over designed (Bahr *et al.*, 1991; Opstad and Bahr, 1991), with military personnel performing the laboratory-based cycle-ergometry exercise after sustained operations in the field and then again in a rested state two months later.

Summary of the findings of relevant studies:

(Kolka and Stephenson, 1988)

- Participants: Three men and three women.
- Stressor(s): sleep deprivation and heat exposure; following 33 h of wakefulness and one night of normal sleep (control), thermoregulatory measures were examined during cycle ergometry exercise (30 min at 60% $\dot{V}O_{2peak}$).
- Results: During steady-state exercise, oesophageal temperature was unchanged by sleep loss. The sensitivity of forearm skin blood flow to forearm skin blood flow was depressed an average of 30% after 33 h of wakefulness. Sleep loss did not alter the oesophageal temperature at which the onset of sweating occurred. Arm skin temperature was not different between control and sleep loss conditions.

(Opstad and Bahr, 1991)

- Participants: 15 men.
- Stressor(s): Sleep deprivation, energy restriction and exhaustive physical exertion; following 3-4 days of continuous military exercises (SUSOPS) and control conditions,

the thermoregulatory responses to cycle ergometry exercise for 30 min at 45% $\dot{V}O_{2max}$ were measured.

- Results: Resting rectal temperature was reduced following sustained military operations, but trunk and thigh temperatures were increased. Oxygen uptake during exercise increased 14-24% both before and during the exercise test in the stressed condition. During SUSOPS, skin temperatures were significantly increased.

(Sawka *et al.*, 1984)

- Participants: 5 men.
- Stressor(s): sleep deprivation, exercise, heat exposure; thermoregulatory measures were examined following 33 h of wakefulness (SDEP) and normal sleep (CON) when participants performed 40 min of cycle ergometer exercise (50% $\dot{V}O_{2peak}$).
- Results: Oesophageal temperature was increased during exercise during the sleep deprivation trial in comparison to the control trial. Total body sweat rate, calculated from Potter balance measurement was 27% less during SDEP than CON. Chest sweat rate and thermal conductance were both lower during the final 20 min of exercise in SDEP than CON. Final exercise chest sweat rate values were 19% lower in SDEP than CON. During SDEP, chest sweat rate sensitivity was 38% lower and chest thermal conductance 42% lower than CON.

(Symons *et al.*, 1988)

- Participants: 11 men.
- Stressor(s): sleep deprivation, exercise; Thermoregulatory responses were examined following both 60 h of wakefulness and 60 h of normal sleep, to a battery of physical tests including maximal isometric and isokinetic muscular strength and endurance of selected upper and lower body muscle groups, performance of the Wingate Anaerobic

Power Test, simple reaction time, the blood lactate response to cycle exercise at 70% $\dot{V}O_{2max}$ and the cardiovascular and respiratory responses to treadmill running at 70% and 80% $\dot{V}O_{2max}$.

- Results: Maximal isometric and isokinetic muscular strength and endurance of selected upper and lower body muscle groups, performance of the Wingate Anaerobic Power Test, simple reaction time, the blood lactate response to cycle exercise at 70% $\dot{V}O_{2max}$ and most of the cardiovascular and respiratory responses to treadmill running at 70% and 80% $\dot{V}O_{2max}$.

2.4 The Effects of the Stressors Sleep Loss and Prolonged Exercise on Immune Function and a Potential Novel Measurement Method

2.4.1 A Novel Method for the Measurement of in vivo Immunity

As mentioned in **Chapter 1**, *in vivo* measures of human immune function are of significant importance because they represent a multi-cellular response that is clinically relevant (Albers *et al.*, 2005; Walsh *et al.*, 2011b). A method that has shown potential in the *in vivo* measurement of skin immunity is contact hypersensitivity testing with the chemical hapten Diphenylcyclopropenone (DPCP) which has been used with success in various dermatological assessments (Kelly *et al.* 1998, Lesiak *et al.* 2007, Palmer and Friedman 2004, Sleijffers *et al.* 2001). These studies have been restricted primarily to the evaluation of the *in vivo* immune response at the skin to ultra-violet light exposure, but would appear potentially suited to the assessment of the immune response to other stressors such as sleep loss and exercise as well.

2.4.2 In Response to Sleep Loss

It has been suggested that the stress of total sleep deprivation or disruption of sleep may evoke sub-optimal host defence which may increase the risk of illness, infection, decrease effective wound healing and increase the susceptibility to sepsis (Costa *et al.*, 2010; Shephard *et al.*, 1998). The duration of sleep loss may be acute (for example, because of the anxiety associated with athletic competition, or the demands of extended military operations (Brenner *et al.*, 2000)), or chronic (due to pain, or the obstructed breathing associated with severe obesity or airway congestion due to respiratory infection) (Walsh *et al.*, 2011a). A recent study showed that 30 h of total sleep deprivation did not alter leukocyte trafficking, neutrophil degranulation or saliva S-IgA responses at rest (Costa *et al.*, 2009). Two to three nights of sleep deprivation has been shown to elicit a circulating leukocytosis, with decreased CD4+ T-cell subset counts (Dinges *et al.*, 1994) and a decrease in neutrophil and lymphocyte function (Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). T-lymphocyte subsets were also significantly lower in chronic insomniacs (Savard *et al.*, 2003), but the duration of sleep loss required to induce this reaction remains unclear. Although the clinical significance of a decrease in immune function with prolonged sleep deprivation in healthy individuals remains unclear, decreases in neutrophil function for *example* have been implicated in increased infection incidence in clinical populations (Ellis *et al.*, 1988; Smitherman and Peacock, 1995). In night shift workers, disturbed sleep was shown to increase the incidence of the common cold (Mohren *et al.*, 2002) and led to a greater risk of upper respiratory infections (Cohen *et al.*, 1997) and has also been documented by a higher occurrence of infection (cold symptoms) experienced by airline crew when working long haul flights on trans-meridian routes (Haugli *et al.*, 1994). However it is difficult to infer the clinical relevance of these studies as the conclusions were based solely on questionnaires (Cohen *et al.*, 1997; Haugli *et al.*, 1994; Mohren *et al.*, 2002).

Although it has been suggested that disturbed sleep may cause declines in natural and cellular immunity, as well as alterations in the complex cytokine network (Irwin, 2002), unfortunately, the vast majority of research performed on sleep disturbances have only utilized *in vitro* methods of assessing immune function (Dinges *et al.*, 1994; Dinges *et al.*, 1995; Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). Relatively few studies have examined the *in vivo* immune response to disrupted sleep. One study demonstrated that one night of sleep deprivation after vaccination with Hepatitis A significantly lowered antibody titre 28 days later (Lange *et al.*, 2003). Participants in this study who were allowed to sleep following vaccination displayed a distinct increase in the release of several immuno-stimulating hormones including growth hormone, prolactin, and dopamine, as well as lowered concentrations of thyrotropin, adrenaline and nor-adrenaline, but relatively little difference in white blood cell subset counts (Lange *et al.*, 2003). Another study demonstrated that sleep deprivation appears to cause up to a 50% decrease in antibody production following influenza virus vaccination (Spiegel *et al.*, 2002). One night of sleep deprivation also decreased skin barrier function recovery, assessed via tape stripping on the forearm, and increased plasma IL-1β, TNFα, and natural killer cell activity in women (Altemus *et al.*, 2001). Without incorporating *in vivo* measures such as these, as adjuncts *to in vitro* markers of immunity, it is difficult to determine the clinical significance of the stress of sleep loss on immune function.

Summary of the findings of relevant studies:

(Altemus *et al.*, 2001)

- Participants: 25 women.
- Stressor(s): Sleep deprivation, exercise and psychological stress; the effect of three different stressors, psychological interview stress (N = 25), one nights of sleep deprivation (N = 11), and a 3 d exercise protocol (N = 10), on several dermatologic

measures was examined: transepidermal water loss, recovery of skin barrier function after tape stripping, and stratum corneum water content (skin conductance). The effects of stress on plasma levels of several stress-response hormones and cytokines, natural killer cell activity, and absolute numbers of peripheral blood leukocyte were also measured.

- Results: Interview stress caused a delay in the recovery of skin barrier function, as well as increases in plasma cortisol, norepinephrine, IL-1β and IL-10, TNFα and an increase in circulating natural killer (NK) cell activity and NK cell numbers. Sleep deprivation decreased skin barrier function recovery and increased plasma IL-1β, TNFα and NK cell activity. The exercise stress did not affect skin barrier function recovery, but caused an increase in natural killer cell activity and circulating numbers of both cytolytic T lymphocytes and helper T cells. Cytokine responses to the interview stress were inversely correlated with changes in barrier function recovery. These results suggest that acute psychosocial and sleep deprivation stress disrupts skin barrier function homeostasis in women, and that this disruption may be related to stress-induced changes in cytokine secretion.

(Lange *et al.*, 2003)

- Participants: 10 men and 9 women.
- Stressor(s): Sleep deprivation and vaccination; the effects of one night of sleep deprivation (SDEP) on the response to hepatitis A vaccination (HAV) were examined in comparison to responses in control (CON) participants allowed normal sleep (SDEP group, N = 9, 5 men and 4 women; CON group, N = 10, 5 men and 5 women). Participants had not previously been vaccinated with hepatitis A virus (HAV). HAV antibody titres were measured repeatedly until 28 d after vaccination. Plasma hormone

concentrations and white blood cell (WBC) Subset counts were determined on the night and day after vaccination.

- Results: CON participants displayed a nearly two-fold higher HAV antibody titre after 4 weeks than SDEP participants. Sleep after vaccination (CON) increased release of several immune-stimulating hormones including growth hormone, prolactin, and dopamine. Concentrations of thyrotropin, norepinephrine, and epinephrine were lowered by sleep, whereas sleep only marginally influenced WBC subset counts. Sleep compared with sleep deprivation on the night after vaccination improved the formation of antigen-specific immune defence as reflected by antibody production. Sleep presumably acts by inducing a hormonal environment in secondary lymphoid tissues, enhancing lymphocyte proliferation and differentiation as well as antibody synthesis.

(Rechtschaffen *et al.*, 2002)

- In this review, the results of a series of studies on total and selective sleep deprivation in murine models are integrated and discussed.
- Collective Results: Total sleep deprivation, paradoxical sleep deprivation, and disruption and/or deprivation of non-rapid eye movement (NREM) sleep induce a syndrome that included debilitated appearance, skin lesions, increased food intake, weight loss, increased energy expenditure, decreased body temperature during the late stages of deprivation, increased plasma norepinephrine, and decreased plasma thyroxine and death. These results lend evidence to the function of sleep in homeostatic maintenance of effective thermoregulation, which in turn may be required for the maintenance of skin immunity.

2.4.3 In Response to Prolonged Exercise

The importance of using *in vivo* measures to assess human immune function in stress research has recently been highlighted (Albers *et al.*, 2005) not least because the *in vivo* response to an antigenic challenge involves a multi-cellular response that is clinically relevant (Albers *et al.*, 2005; Walsh *et al.*, 2011b). The few studies that have examined the effects of different stressors (e.g. mental stress or physical exertion) on *in vivo* immune responses in humans have assessed the circulating antibody response to injection of either a novel antigen (e.g. keyhole limpet haemocyanin) (Smith *et al.*, 2004) or a previously encountered antigen (e.g. influenza vaccine) (Edwards *et al.*, 2006; Edwards *et al.*, 2010). Using keyhole limpet haemocyanin enables the investigator to identify the effects of stress on the unique cellular and co-stimulatory signals required for primary (induction) of the immune response that using previously encountered antigens does not (Walsh *et al.*, 2011b). T-cell-mediated memory responses (delayed type hypersensitivity) have also been assessed using the Mérieux CMI Multitest®: this test involves the intra-dermal injection of seven previously encountered antigens and measurement of the skin reaction 48 h later. One such study showed reduced skin reactions to the Mérieux CMI Multitest® in endurance athletes after prolonged exercise in comparison with rested controls (Bruunsgaard *et al.*, 1997). Research to date indicates that short-lasting mental stress or exercise (lasting minutes) can enhance cell-mediated immunity but that prolonged stress (e.g. physical exertion that lasts hours and activates the hypothalamic-pituitary-adrenal axis) decreases the response in humans (Bruunsgaard *et al.*, 1997; Dhabhar and Viswanathan, 2005; Edwards *et al.*, 2006; Walsh *et al.*, 2011a).

Summary of the findings of relevant studies:

(Bruunsgaard *et al.*, 1997)

- Participants: 44 men.

- Stressor(s): Exercise and vaccination; Vaccinations with tetanus and diphtheritis toroid and purified pneumococcal polysaccharide and a skin test with seven different antigens was applied on the forearm in 11 trained triathletes after a half Ironman triathlon (Group A), 11 trained triathletes that did not participate in the event (Group B) and 22 moderately trained, recreationally active men that also did not participate in the event (Group C). B and C were used as control groups Antibody titres were measured before and 2 weeks after the exercise. The skin test was read 48 h after the application.
- Results: Group A revealed a significantly lower skin test response to the tetanus antigen than both groups B and C. In group A, a smaller cumulative response (sum of the diameters of indurations and number of positive skin test spots) was found than in both groups B and C. No differences in antibody titres were found among the three groups. Thus, the *in vivo* cell-mediated immunity was impaired in the first days after prolonged, high intensity exercise, whereas there was no impairment of the *in vivo* antibody production measured 2 weeks after vaccination.

(Edwards *et al.*, 2006)

- Participants: 31 men and 29 women.
- Stressor(s): Exercise, mental stress and vaccination; the effects of acute exercise (a four step incremental cycle ergometer test, with each step lasting 4 min. The workloads performed at each step were 84, 133, 182, and 231 W for men and 70, 98, 126, and 154 W for women), mental stress and control conditions prior to influenza vaccination on the subsequent antibody response to each of the three viral strains of influenza were examined.
- Results: Women in both the exercise and mental stress conditions showed higher antibody titres at both 4 and 20 weeks than those in the control condition, while men

responded similarly in all conditions. IL-6 at +60 min recovery was found to be a significant predictor of subsequent A/Panama antibody response in women.

(Edwards *et al.*, 2010)

- Participants: 160 men and women.
- Stressor(s): Exercise and vaccination; participants were randomly assigned to a resting control group or one of three intervention groups, who exercised at an intensity of 60%, 85%, or 110% of their pre-determined concentric one repetition maxima. The exercise groups performed 50 repetitions of the eccentric portion of both bicep curl and lateral raise movements. All participants then immediately received a reduced dose (50% recommended dose) of influenza vaccine. Antibody titres to the three viral strains contained in the vaccine were measured at baseline and at 28 d post-vaccination.
- Results: Compared to the control group, exercise enhanced the antibody response to the least immunogenic of the three strains (B/Florida). The exercise groups showed an augmented response to the A/Uruguay strain compared to control; but, this effect was observed only in men. The intervention had no effect on the antibody responses to the most immunogenic strain, A/Brisbane. Finally, antibody responses were unrelated to the intensity of the exercise bout.

(Kohut *et al.*, 2001)

- Subjects: 32 male mice
- Stressor(s): Exercise and infection; 16 BALB/cJ mice ran on a treadmill (EX) at gradually increasing speeds until voluntary fatigue (~2.5 h), and 16 control mice were handled and remained next to the treadmill. Mice were infected with herpes simplex virus (HSV) 20 min after exercise. Mice were killed 2 or 7 d post-infection, and sera and spleens were taken for the determination of HSV-specific serum IgM, splenocyte

cytokine production during culture with HSV, and splenocyte natural killer cell cytotoxicity.

- Results: Type 1 T helper [interleukin (IL)-2, interferon-g, IL-12] and type 2 T helper (IL-10) cytokine production in spleen cell cultures, as well as natural killer cell cytotoxicity, decreased in EX on day 2 post-infection. On day 7 post-infection, there was no difference in HSV specific serum IgM or cytokine production by cells from control and EX mice, with the exception of decreased IL-12 in EX mice. These findings suggest that fatiguing exercise may alter the kinetics of antigen-specific cytokine production.

2.5 A functional link between nocturnal increase in skin blood flow and skin immune function?

Prior to sleep onset, the secretion of melatonin by the pineal gland in response to darkness, causes an intense increase in skin blood flow leading to an increase in heat loss (Van der Helm-Van Mil *et al.*, 2003). It is highly unlikely that this presents a fundamental thermoregulatory drive, as without behavioural measures such as warm bedding, there would be a high risk of hypothermia from this circadian phase of increased heat loss. So what other primary function might this represent? It has been proposed that this notable nocturnal increase in skin blood flow might rather be for the purpose of supporting the maintenance of the skin as a primary barrier in host defence against pathogens (Krueger and Majde, 1990; Van Someren *et al.*, 2002). The basis of this theory is outlined here.

The nocturnal increase in skin blood flow allows for increased numbers of the agents of the immune system to be transported to the skin. A similar response is observed during the vasodilation that occurs when the skin is irritated or injured (Chahl, 1988). A reduction in circulating numbers of monocytes, natural killer cells and lymphocytes in the blood that is

observed during sleep (Moldofsky *et al*., 1986; Born *et al*., 1997) has been suggested to represent up-regulated redistribution of lymphocytes into the interstitial compartment (Benca and Quintas, 1997; Dhabhar *et al.*, 1995; Dhabhar and McEwen, 1996; Ottaway and Husband, 1992). As efferent flow of fluid out of the blood and into the interstitial space is increased by greater blood flow, there is also an augmented availability of endogenous anti-microbial peptides, such as cat-helcidin and alpha and beta-defensins during this circadian phase of increased skin blood flow (Bensch *et al.*, 1995; Gallo *et al.*, 1994; Harder *et al.*, 1997; Harder *et al.*, 2001). However, migration of these immune agents from the extravascular tissues into the peripheral lymph is decreased during sleep (Engeset *et al.*, 1977). Taken together, these observations indicate an increased presence in the skin of mediators of the immune system during periods of nocturnal sleep.

Enhanced skin blood flow causes increased skin temperature, which, heightened by the action of behavioural thermoregulatory measures such as the use of insulative bedcoverings, promotes capillary permeability (Engeset *et al.*, 1977) and sweat rate; the latter of which is higher at night already. Again, increased sweating at night would not be beneficial in the absence of behavioural thermoregulatory measures, as it would lead to increased heat loss and risk of hypothermia. Furthermore, in the presence of these measures, evaporative heat loss through sweating becomes ineffective. Therefore, this phenomenon is also more likely for the promotion of skin immune function, as sweat contains the antibiotic peptide dermcidin (Schittek *et al.*, 2001), lending more evidence to the theory that there is a functional link between thermoregulation and immune function.

2.6 Thesis Aims

In light of the findings of the current body of research, the broad aims of the following experiments were: 1. To evaluate the methodology for human skin temperature measurement to determine if current methods could be improved upon, by examining the validity of wireless iButtons®, a novel alternative (**Chapter 4**). 2. To examine the effects of a systemic exercise-induced stress on both the induction and elicitation phases of the T-cell-mediated immune response to the novel antigen diphenylcyclopropenone (DPCP) by use of the experimental contact-hypersensitivity (CHS) method (**Chapter 5**). 3. To examine the effects of two nights of total sleep deprivation with and without energy restriction, on human thermoregulation at rest and during a cold-air test (**Chapter 6**). 4. To examine the effects of three nights of severely disrupted on thermoregulation at rest as well as during subsequent exercise-heat-stress (**Chapter 7**). 5. To examine the effects of three nights of disrupted sleep on immune function at rest and the potential functional, mechanistic link between sleep, circadian skin temperature rhythms and skin immunity (**Chapter 8**).

CHAPTER THREE

General Methods

3.1 Ethical approval

All studies received ethics approval from the Bangor University Ethics Committee (School of Sport, Health and Exercise Sciences, Bangor University, U.K.) and were conducted in accordance with the Declaration of Helsinki principles. All participants were fully verbally briefed on the nature and purpose of each study, were familiarized with all procedures involved and received a detailed written explanation and description of the study in which they participated (**Appendix A**). Each participant was made fully aware that they were free to withdraw from the study at any time and completed an informed consent form (**Appendix B**). Participants also completed a general medical health questionnaire (**Appendix C**). Participants were only eligible to participate in each study if they reported the absence of illness in the six weeks prior to commencing a study and were not taking any medication or dietary supplements. Participants also refrained from alcohol and caffeine for 72 h, and exercise for 24 h prior to preliminary testing and each experimental trial, during all studies. All participants were healthy, non-smoking, recreationally active males.

3.2 Anthropometry and body composition

Participants' height was recorded using a wall stadiometer (Bodycare Ltd, Warwickshire, UK) and nude body mass (NBM) by digital platform scales accurate to the nearest 50 g (Model 705, Seca, Hamburg, Germany). Body composition was assessed by dual energy x-ray absorptiometry (DEXA, Hologic QDR1500, software version 5.72, Bedford, USA). For this procedure,

participants removed all metallic objects and dressed in t-shirt and shorts only. Dual energy x-ray absorptiometry emits an effective dose of radiation equal to 3.6 μSv or approximately one fortieth of a chest x-ray (Njeh *et al.*, 1999).

3.3 Peak oxygen uptake ($\dot{V}O_{2peak}$)

In order to determine $\dot{V}O_{2peak}$ and subsequent trial exercise workloads, participants performed a continuous incremental exercise test to volitional exhaustion on either a motorized treadmill (**Chapters 5, 6, 7 and 8**, (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany), or a stationary cycle ergometer (**Chapter 4**, *Monark 814e, Varberg, Sweden*). Treadmill tests were based on a standard protocol (Bruce, 1977a), whereby, on completion of a 5 min warm-up period at 8 $km \cdot h^{-1}$, participants began running at 10 $km \cdot h^{-1}$ at a 1.0% gradient, with 2 $km \cdot h^{-1}$ incremental increases every 3 min, until 14 $km \cdot h^{-1}$ was reached, after which the gradient was increased by 2.5% every 3 min until fatigue. Cycle ergometer tests, were also based on a standard protocol (Winter *et al.*, 2006), whereby, on completion of a 5 min warm-up period at 70 W, participants began cycling at 175 W, with incremental increases of 35 W every 3 min until fatigue. During all $\dot{V}O_{2peak}$ tests, expired gas was analysed continuously using an on-line breath-by-breath system (Cortex Metalyser 3B, Biophysik, Leipzig, Germany) to determine the volumes of expired gas, oxygen and carbon dioxide. Criteria for attaining $\dot{V}O_{2peak}$ included the participant reaching volitional exhaustion, a HR within 10 $beats \cdot min^{-1}$ of age predicted HR_{max} and a respiratory exchange ratio greater than or equal to 1.15 (Bird and Davison, 1997). Descriptions of workloads during trials are given in each experimental chapter.

3.4 Sample collection and analysis

Resting blood samples (0 h) were obtained when participants were in a fasted state and after the participants had remained seated for 15 min. Urine samples were obtained after collection of blood samples.

3.4.1 Blood

Whole blood samples were collected into four separate vacutainer tubes (Becton Dickinson, Oxford, UK), two containing the anticoagulant ethylene di-amine tetra-acetic acid (K_3EDTA) and two containing the anticoagulant lithium heparin. Blood in one of the tubes containing K_3EDTA was stored at room temperature and analysed for haemoglobin concentration (**Chapters 6 and 8**), circulating total and differential leukocyte counts (**Chapter 8**), within 6 h of collection, using an automated haematology analyser (Gen S, Beckman Coulter, Fullerton, CA, USA). Haematocrit (heparinized blood) was determined in triplicate using the capillary method (**Chapter 6**), as previously described (Maughan *et al.*, 2001), and plasma volume changes were estimated (Dill and Costill, 1974). The remaining blood samples were centrifuged at 1500 *g* for 10 min at 4°C. Plasma and serum was then aspirated into Eppendorf tubes and stored at -80°C for further analysis.

Plasma concentration of glucose (heparinized plasma) was determined (**Chapter 6**) using a spectrophotometric kit (Randox, County Antrim, UK). An aliquot of heparinized plasma was also used for the determination (**Chapter 6**) of cortisol using an enzyme-linked immunosorbent assay (ELISA) kit (DRG Diagnostics, Marlburg, Germany). The intra-assay coefficient of variation was 3.4% for both plasma glucose and cortisol. Aliquots of K_3EDTA plasma were used for the determination of adrenaline and nor-adrenaline (**Chapter 6**) using high-pressure liquid

chromatography (Clinrep complete kit for catecholamines, Recipe Chemicals, Munich, Germany) as described previously (Chudalla *et al.*, 2006). The intra-assay coefficient of variation for plasma adrenaline and nor-adrenaline was 5.0 and 8.0%, respectively.

Standard flow-cytometric techniques (**Chapter 8**) were used to determine T-lymphocytes (CD3+), helper/inducer T-lymphocytes (CD4+) and cytotoxic/suppressor T-lymphocytes (CD8+) using the tetraCHROME murine monoclonal antibody reagent (Beckman Coulter, UK). A 100 µl volume of whole blood from one of the K_3EDTA tubes was added to a tube containing 10 µl reagent (FITC [CD45], PC5 [CD3], RD1 [CD4], and ECD [CD8]), and incubated in the dark at room temperature for 15 min and then lysed using a T-Q prep (Beckman Coulter, UK). The stained cell suspensions were enumerated on an Epics XL flow cytometer (Beckman Coulter, UK) with 10,000 events recorded. The lymphocyte population was determined by gating on CD45 versus side scatter. Counts for individual subsets (CD3+, CD4+, and CD8+) were obtained by multiplying the corresponding percentages of cells derived from the Epics XL flow cytometer (by quadrant analysis of dot plots) by the total lymphocyte counts on the Gen S Coulter counter. T-lymphocytes (CD3+), helper/inducer T-lymphocytes (CD4+) and cytotoxic/suppressor, as described previously (Laing *et al.*, 2008). All blood borne parameters were corrected for changes in plasma volume (Dill and Costill, 1974).

3.4.2 Urine

Participants' mid-flow urine samples were collected and provided in Universal tubes (**Chapters 6 and 8**). The specific gravity of the urine samples was determined by a handheld refractometer (Atago Uricon-Ne, NSG Precision Cells, New York, USA), was 1.012 $g{\cdot}L^{-1}$ (0.004) and urine colour was determined by a urine colour scale (Armstrong *et al.*, 1994). Urine specific gravity

and urine colour were used to confirm indicated euhydration based on published indices (Armstrong *et al.*, 1994).

3.5 Statistical analyses

The primary outcome measures of this thesis were the thermoregulatory (T_{re}, T_{sk}) and *in vivo* immune parameters (skin oedema and erythema). As these were novel measures and have chapters dedicated to them alone, the methodology is outlined in the relevant chapters themselves (skin temperature measurement utilizing wireless iButtons®, **Chapter 4** and *in vivo* skin immunity measurement via challenge with the antigen DPCP, **Chapter 5**), rather than here. Sample sizes were estimated using either data from previous studies (**Chapter 6**), or the typical error of the measure for the primary outcome variable(s) (**Chapter 4, 5, 7 and 8**). Further detail on sample size estimation is provided in the statistical section of each experimental chapter. Alpha and power levels were set at 0.05 and 0.8, respectively, both of which are standard estimates (Jones *et al.*, 2003).

In each experimental study, repeated measures analysis of variance (ANOVA) was the main statistical procedure used to identify main effects and interactions in the primary outcome measures. In cases where the assumptions of sphericity and normality were violated, appropriate adjustments to the degrees of freedom were made to the ANOVA using the Greenhouse-Geisser correction factor. Where significant main effects and interactions were found by ANOVA, Bonferroni corrected *t*-tests were used where appropriate, unless otherwise stated. Various other statistical techniques were also employed, which are detailed in the statistical analyses section of each experimental chapter. Unless otherwise stated, all data are presented as mean and SD. Statistical significance was accepted at $P < 0.05$. Analyses were performed on Statistical

Package for Social Sciences, (Version 14.2 , **Chapter 4, 5 and 6** and Version 15.0, **Chapter 7 and 8**; SPSS Inc., Chicago, IL, USA)

CHAPTER FOUR

The validity of wireless iButtons® and thermistors for human skin temperature measurement

4.1 Summary: Skin temperature is a fundamental variable in human thermo-physiology, and yet skin temperature measurement remains impractical in most free-living, exercise and clinical settings, using currently available hard-wired methods. *Purpose:* To compare wireless iButtons® and hard-wired thermistors for human skin temperature measurement. *Methods:* In the first of two investigations, iButtons® and thermistors monitored temperature in a controlled water bath (range: 10 to 40°C) and were referenced against a certified, mercury thermometer. In the second investigation, eight healthy males completed three randomized trials (Ambient temperature = 10, 20 and 30°C) while both devices recorded skin temperature at rest (in low and high wind velocities) and during cycle-ergometry exercise. *Results: Investigation 1.* Both devices displayed very high validity correlation with the reference thermometer (r > 0.999). Prior to correction, mean bias was +0.121°C for iButtons® and +0.045°C for thermistors. Upon calibration correction the mean bias for iButtons® and thermistors was not significantly different from zero bias. Interestingly, typical error of the estimate of iButtons® (0.043°C) was 1.5 times less than that of thermistors (0.062°C), demonstrating iButtons'® lower random error. *Investigation 2.* The offset between iButton® and thermistor readings was generally consistent across conditions, however, thermistor responses gave readings that were always closer to ambient temperature than iButtons®, suggesting potential thermistor drift towards environmental conditions. Mean temperature differences between iButtons® and thermistors during resting trials ranged from 0.261 to 1.356°C. Mean temperature differences between iButtons® and thermistors during exercise were 0.989°C (Ambient temperature=10°C), 0.415°C (Ambient temperature = 20°C) and 0.318°C (Ambient temperature = 30°C). Observed error estimates were within the acceptable limits for the skin temperature method comparison, with typical errors < 0.3°C, correlation coefficients > 0.9 and CV < 1% in all conditions. *Conclusion:* These findings indicate that wireless iButtons® provide a valid alternative for human skin temperature measurement during laboratory and field investigations particularly when skin temperature measurement using other currently available methods may prove problematic.

4.2 Introduction

Skin forms the barrier between the human body and the thermal environment, and as such skin temperature (T_{sk}) plays a fundamental role in thermoregulation (van Marken Lichtenbelt *et al.*, 2006). Hard-wired devices such as thermocouples and thermistors are often used to measure T_{sk} in both clinical and exercise physiology investigations (Fuller *et al.*, 1999; Imrie and Hall, 1990). However, wired skin temperature sensors can be awkward, since the recording equipment is substantial, the wires can easily entangle in moving limbs and these wires are subject to breakage and detachment (Buono *et al.*, 2007; Van den Heuvel *et al.*, 1998). In addition, the data loggers for wired skin temperature sensors are expensive (Davidson *et al.*, 2003), the equipment as a whole can hinder participants' movements and the sensors themselves, with the required wiring, can take considerable time to prepare and set up (Fuller *et al.*, 1999).

An inexpensive, autonomous, wireless, temperature monitoring and recording device would therefore considerably benefit human thermal physiology research, medical monitoring of various conditions such as cold and heat illness and injury, specialized surgical procedures and the assessment of certain challenging clinical populations. Such a device would enhance our ability to observe human thermal physiology during free-living, ambulatory and clinical settings (van Marken Lichtenbelt *et al.*, 2006). The device examined in the present study is the latest high-capacity model of wireless Thermochron iButton® data logger (DS1922L: **Figure 4.1**). The iButton® devices were originally designed to monitor temperature-sensitive consignments during transport and have been utilized in relatively few scientific studies (Areas *et al.*, 2006; Davidson *et al.*, 2003; Fronczek *et al.*, 2006; Hubbart *et al.*, 2005; Johnson *et al.*, 2005; van Marken Lichtenbelt *et al.*, 2006). The manufacturer's specifications for the DS1922L, state that this latest model will now function throughout the full human thermo-physiological range. The DS1922L provides digitized readouts, with a user-determined resolution, set at the time of

programming the devices, of either 0.5°C or 0.0625°C. Thermochron iButtons® are small (16.25 x 5.89 mm^2), inexpensive (iButton per unit price range = US$ 30 to 50), independent temperature sensors and data loggers enclosed in a watertight stainless steel package (Hubbart *et al.*, 2005). Currently, the only foreseeable disadvantage with the iButton® system is that temperature readings can only be viewed once the device has finished logging data. Thermochron iButtons® have many advantages over standard temperature sensor systems (Davidson *et al.*, 2003). Crucially, they are wireless, but unlike other wireless systems, iButtons® do not require an auxiliary, external, control-recording receiver that must remain within a certain radius of the transmitter. Therefore, in addition to the obvious logistical benefits, there are also no concerns about cross-talk interference, and thus multiple devices can be used in concert. This evidence indicates that iButtons® could be particularly valuable with regard to the continuous monitoring of T_{sk} during free-living and ambulatory conditions (Rutkove *et al.*, 2007), with relevance in many scientific fields, including environmental physiology, exercise physiology, sleep and circadian rhythm research (van Marken Lichtenbelt *et al.*, 2006). Furthermore, iButtons® may be viable for the T_{sk} monitoring of clinical populations, as certain patient groups do not comply well with the extensive wiring associated with skin thermocouples and thermistors, e.g. patients with neurodegenerative disorders, such as Alzheimer's dementia or Parkinson's disease as well as pediatric groups (van Marken Lichtenbelt *et al.*, 2006). The iButton® method may also prove valuable to the medical field for peri-operative temperature monitoring (Rajek *et al.*, 1999). The iButtons® are easy to sterilize and they are wireless, both attributes being advantageous for surgical applications, as they would not hinder the surgical procedure (Rajek *et al.*, 1999).

In the current literature, there has been only one previous study which attempted to provide a preliminary evaluation of an earlier iButton® model for scientific research on humans (van Marken Lichtenbelt *et al.*, 2006). This older model of iButton® did not function through the full

human thermo-physiological range however, and the evaluation (van Marken Lichtenbelt *et al.*, 2006) did not include a full battery of validity statistics, nor were comparative assessments during exercise included. Interestingly, iButtons® have not, thus far, been employed in exercise physiology research at all, and the most recent model, the DS1922L iButton®, has yet to be validated for these or any other purposes. Therefore, the initial aim of this study was to assess the validity of the DS1922L Thermochron iButton® for temperature measurement, by means of a water bath comparison with a certified, mercury, reference thermometer and subsequently, to compare these results with those of wired thermistors. Further, to assess the effect of correction of the iButton® and thermistor methods, through formulae derived from individual linear regression analyses, in meeting the international criteria (Altman and Bland, 1983; British Standards Institution, 1987) of the British Standards Institution (BSI) and the International Organization for Standardization (ISO). The second aim of the study was to assess the measurement agreement between iButtons® and thermistors during applications on human skin. This section of the study was designed to evaluate iButtons® during passive rest and exercise, through a range of environmental temperatures (10, 20 and 30°C) and conditions (low wind velocity, higher wind velocity). It was hypothesized that the latest model of iButton® and thermistors would both provide temperature measurement that met the relevant acceptable error limits for thermometry (ASTM, 2006; Kelechi *et al.*, 2006) of The American Society for Testing and Material (ASTM), when validity statistics were applied to the water bath and human skin application data.

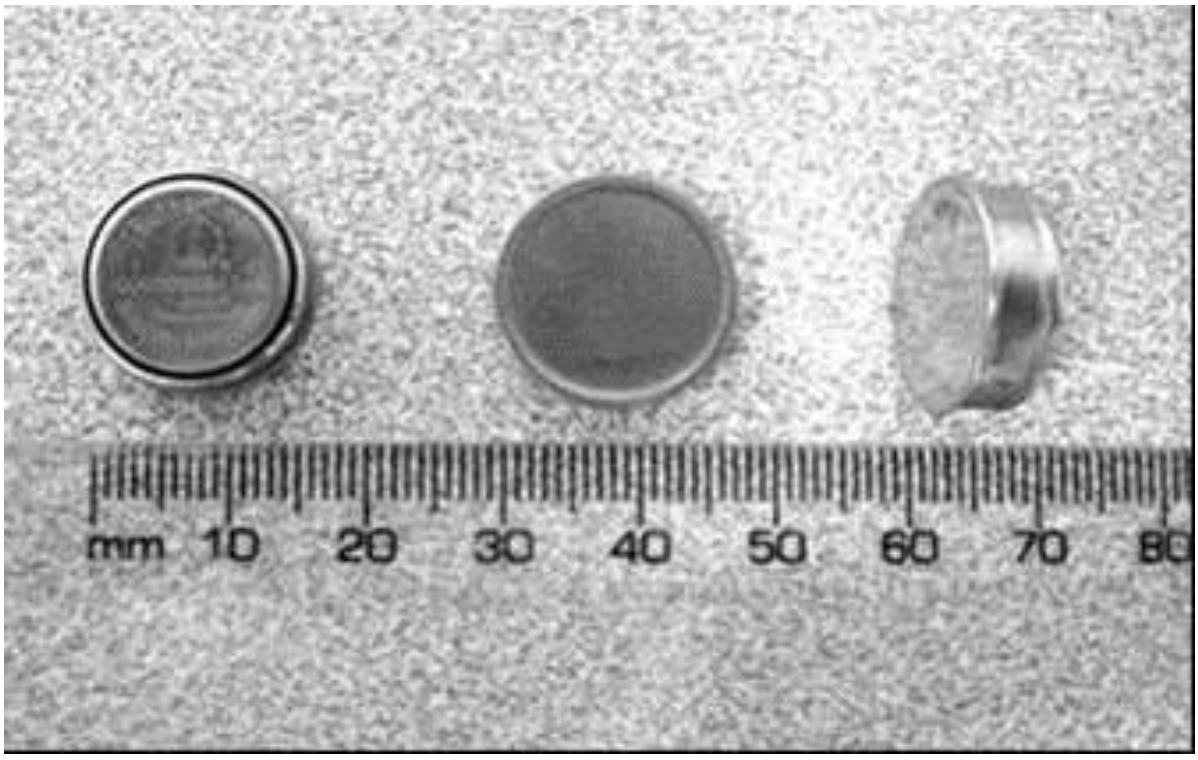

Figure 4.1 A photograph of iButtons®, showing scale. The sensor side is the side with the etched manufacturer's coding.

4.3 Methods

General Design

In order to accomplish the stated aims, the study was sectioned into two separate investigations. The first investigation examined the measurement agreement of the devices in a water bath with a mercury reference thermometer. From the results of the water bath investigation, individual *post hoc* corrections were applied to each of the 46 iButtons® and 28 thermistors, based on calibration equations from linear regression. The second investigation compared human T_{sk} measurements reported by the wireless iButtons® against those of the wired skin thermistors during passive exposures and cycle ergometry exercise during a range of environmental conditions.

4.3.1 Investigation 1: Water Bath Comparison

To examine the validity of the devices, the iButtons® (Maxim Integrated Products, Inc., Sunnyvale, CA, USA; resolution 0.0625°C) and thermistors (Grant EUS-U, Grant Instruments LTD, Cambridge, UK; resolution 0.01°C) were placed in a thermostatic circulator water bath (GD 120 R1, Grant Instruments, Cambridge, UK) for temperature measurement assessment and referenced against a multi-point calibrated, certified, mercury thermometer (BS593, Brannan Thermometers and Gauges, Cumbria, UK; temperature range: −0.5 to 40.5°C; division 0.1°C). The reference thermometer was certified by the United Kingdom Accreditation Service to have been calibrated at 10, 20, 30 and 40°C. Mercury-in-glass thermometers are the criterion reference for evaluations of practical temperature measurement devices.

The 46 iButtons® and 28 thermistors were placed into the water bath and assessed during 4 x 20 min stable, plateau temperatures (10, 20, 30 and 40°C). The temperature measurements of the

two practical methods were referenced against the partially immersed, calibrated, certified, reference thermometer. Temperature variation of the water bath was less than 0.025°C at all plateau measurement temperatures. The iButtons® were taped 1 cm apart (Hypafix, BSN Medical GmbH, Hamburg, Germany) to the inside of mesh net bags, which were suspended in the water bath. The tape was placed over the base of the iButton® which allowed the sensor side to be in full contact with the circulating water. The iButtons® were programmed prior to immersion via a USB computer interface (DS1402D-DR8 Blue Dot Receptor; DS9490R 1-Wire to USB Adapter, Maxim Integrated Products, Inc., Sunnyvale, CA, USA). At this time, the iButtons'® resolution was set at 0.0625°C and the iButton® real-time clock was also then synchronized with that of a laptop computer. The thermistor wires were also taped 1 cm apart to the vertical walls of the water bath, and hung in the water bath at an approximate 45° angle. In accordance with the European Cooperation for Accreditation of Laboratories (EAL-G31, 1997), it was also ensured that the iButtons® and thermistors remained thus separated and did not touch the walls of the water bath or one another. The thermistors were connected to a data logger (Squirrel 2020, Grant Instruments Ltd., Cambridge, UK) and the real-time clock was also synchronized with the laptop computer. The iButton® and thermistor data loggers were programmed to sample and log T_{sk} once every minute, a sample rate which would be relevant and practical in thermo-physiological investigations. Both devices were programmed to begin data collection prior to immersion and to stop logging data after removal from the water bath. Data was obtained from the iButtons® at the time they were programmed to stop logging when interfaced with a laptop computer via USB. At this time the data was also directly imported into a spreadsheet programme.

4.3.2 Investigation 2: Human Skin Application

Eight healthy, recreationally active males (means, (SD): age 27 years, (5); height 180 cm, (6); body mass 82 kg, (7); body fat 13%, (6); $\dot{V}O_{2peak}$ 51 $ml \cdot kg^{-1} \cdot min^{-1}$, (5)) volunteered to participate in the study. All participants received a briefing session that outlined the nature of the study, which fully informed them of all risks involved. Participants completed a medical questionnaire to ensure suitability to participate, and provided written informed consent.

Preliminary Measures. Each participant underwent anthropometric measurements, followed by a continuous incremental cycle ergometry test to determine $\dot{V}O_{2peak}$ based on a standard protocol (Winter *et al.*, 2006), detailed in **Chapter 3**. After a 20 min recovery period, participants cycled on the ergometer to allow verification of the workload, equivalent to 50% $\dot{V}O_{2peak}$, to be determined for the experimental trials.

Experimental Trials. The three ambient temperatures selected for the human experimental trials were chosen to represent typical cold (10°C), mild (20°C) and warm (30°C) environmental conditions. Participants completed all three experimental trials (10, 20 and 30°C) in an environmental chamber (Design Environment LTD, Gwent, UK), in a randomized order, 48 h apart. Participants were instructed to arrive at the laboratory in a euhydrated condition having consumed a light breakfast and 500 ml of water at least two hours prior. Participants refrained from exhaustive exercise, caffeine and alcohol in the previous 24 h. All three trials began at the same time of day, 1000 h.

Protocol. The three experimental trials on human skin (10, 20 and 30°C) were further divided into three 40 min segments under different ambulatory and wind velocity conditions. These segments consisted of (I) passive seated rest on an open-backed stool at low wind velocity (wind

v = 0.18 m·s^{-1}), (II) passive seated rest on an open-backed stool at a higher wind velocity (wind v = 2.30 m·s^{-1}) and (III) cycle ergometry exercise at 50% $\dot{V}O_{2peak}$ (wind v = 2.30m·s^{-1}). Relative humidity (RH) was set at 40% throughout. In order to produce the higher wind velocity during the passive higher wind and exercise conditions, a fan was set up 60 cm in front of each participant, whilst another was set up 60 cm behind each participant, each generating a wind velocity of 2.30 m·s^{-1}. A 30 min stabilization/normalization period at a dry bulb temperature (T_{db}) of 25°C separated each segment. During the 10 and 20°C trials, participants were warmed with blankets between segments to enable T_{sk} stabilization. Participants were clothed only in athletic shorts for the passive segments and then donned athletic shoes and socks for the exercise segment.

Preparation. Trials began with participants applying a rectal thermistor probe (YSI Series 400, Henley Medical Supplies Ltd, Herts., UK), in privacy, 10 cm beyond the external anal sphincter in order to monitor rectal core temperature (T_{re}) continuously during all conditions, purely as a safety precaution. Mean T_{sk} was measured by skin thermistors and iButtons®. The iButtons® were programmed before their application on human skin, as outlined by the manufacturer. The iButton® resolution was set at 0.0625°C and the iButton® real-time clock was synchronized with that of a laptop computer. The thermistors were connected to a data logger (Squirrel 2020, Grant Instruments LTD, Cambridge, UK) and the real-time clock was also synchronized with the laptop computer. The iButton® and thermistor data loggers were both programmed to sample and log T_{sk} once every minute. The participants received the same set of thermistors with the same accompanying data logger and site-specific iButton® set for each of the three trials.

The iButtons® and thermistors were affixed to the skin using the same strip of adhesive, water-resistant, medical-grade tape (Hypafix, BSN Medical GmbH, Hamburg, Germany), with a separation of approximately 1 cm. An additional strip of tape was placed directly below the

thermistors' sensor heads, attaching 5 cm of wiring from each thermistor to the skin at each site. This precaution was taken to try to minimize movement of the thermistors and ideally prevent detachment. In accordance with ISO-standard 9886 (ISO9886, 2004), mean T_{sk} was measured at 14 equally weighted body locations during the passive, seated exposure conditions, as represented by the following equation:

$$\textit{Mean } T_{sk} = (0.07 \cdot T_{forehead}) + (0.07 \cdot T_{right\ \text{scapula}}) + (0.07 \cdot T_{left\ upper\ chest}) + (0.07 \cdot T_{right\ arm\ in\ upper\ location}) + (0.07 \cdot T_{left\ arm\ in\ lower\ location}) + (0.07 \cdot T_{left\ hand}) + (0.07 \cdot T_{right\ anterior\ thigh}) + (0.07 \cdot T_{left\ calf}) + (0.07 \cdot T_{neck})\ (0.07 \cdot T_{left\ para\text{-}vertebral}) + (0.07 \cdot T_{left\ posterior\ thigh}) + (0.07 \cdot T_{right\ shin}) + (0.07 \cdot T_{right\ abdomen}) + (0.07 \cdot T_{right\ instep})$$

During the exercise condition, mean T_{sk} was measured at eight sites, in accordance with the formula and weighting of ISO-standard 9886 (ISO9886, 2004), as follows:

$$\textit{Mean } T_{sk} = (0.07 \cdot T_{forehead}) + (0.175 \cdot T_{right\ \text{scapula}}) + (0.175 \cdot T_{left\ \text{upper chest}}) + (0.07 \cdot T_{right\ arm\ in\ upper\ location}) + (0.07 \cdot T_{left\ arm\ in\ lower\ location}) + (0.05 \cdot T_{left\ hand}) + (0.19 \cdot T_{right\ anterior\ thigh}) + (0.2 \cdot T_{left\ calf})$$

The number of T_{sk} sites was reduced to eight during the exercise trial, as 14, hard-wired skin thermistors would not have been viable; this number of wires extending from the exercising limbs would have caused too great a hindrance to the participants.

Following the completion of each participants' visit, the iButtons® were interfaced with the laptop computer and programmed to discontinue data logging. The temperature data stored on the iButtons® was then exported directly into a spreadsheet programme for analysis. The temperature data stored on the thermistors' data logger was also exported into a spreadsheet programme for statistical analyses, after the data logger was programmed to stop logging.

Statistical Analyses

Definitions and usage of statistical terminology for method comparison studies were adopted from the published literature (Altman and Bland, 1983; Bland and Altman, 1986; Hopkins, 2000; Mantha *et al.*, 2000).

Linear Regression Correction. Independent linear regression analyses by the method of least squares were applied to determine the temperature measurement correlation between the reference thermometer and each individual iButton® and thermistor device, over the four water bath, plateau temperatures. The results of these analyses were then utilized to derive individual calibration equations and establish correction formulae for each device.

Mountain Plot. To represent the comparison of the agreement of the practical methods with the reference thermometer in the water bath investigation graphically, a mountain plot was created. A mountain plot is a complementary representation of the same data used to form Bland and Altman analyses (Krouwer and Monti, 1995). The mountain plot is particularly useful as it allows a visual comparison between multiple methods against the criterion method and provides information about systematic bias and the distribution of the differences between methods. Specifically, a mountain plot is an empirical, cumulative distribution that is folded on the fiftieth percentile (Krouwer and Monti, 1995). The tendency for the method to deviate significantly from the criterion method is represented by the width of the tails of that plot. When long tails are observed on a curve, this reflects large differences between the methods.

Fundamental Validity Statistics. Additional validity statistics were applied to the data. Absolute agreement between the methods for both investigations were assessed by determining the mean difference (bias) and 95% limits of agreement (95% LoA) as described by Bland and Altman

(Bland and Altman, 1986). Standard or typical error were also determined for both validity (typical error of the estimate, TEE) and reliability (typical error of the measurement, TEM), along with their associated coefficients of variation (CV) to assess absolute agreement (Atkinson and Nevill, 1998; Bland and Altman, 1986; Hopkins, 2000; Hopkins *et al.*, 2009), as appropriate. Summed statistics for the devices and 95% confidence intervals (95% CI) were determined for Investigation 1. In addition, relative agreement was assessed from Pearson's moment-product correlation coefficients (*r*) and type 2,1 intra-class correlation coefficients (ICC) based on published recommendations (Bland and Altman, 1995; Shrout and Fleiss, 1979).

The mean differences between the iButton®, thermistor and mercury thermometer methods during the water bath investigation were analysed using repeated-measures ANOVA. Statistically significant differences were followed up by *post hoc* two-tailed Dunnett multiple comparison tests. The Dunnett t-test treats one data set as a control (reference) and compares all other data sets against it, and thus was used for the water bath investigation. Here, the control data set was that of the calibrated, mercury thermometer. In Investigation 2, the difference between iButton® and skin thermistor measurements during each of the three human skin application trials were analysed using two-way (trial x time) repeated-measures ANOVA. Proportional error in the data were also examined to verify homoscedasticity, with data treatment and the statistical methods utilized, employed accordingly (Atkinson and Nevill, 1998). According to the BSI and ISO, a method is considered to show good repeatability if, (I) the agreement with the reference is not significantly different from zero and (II) 95% of the differences observed lie within 1.96 SD (the 95% LoA) of the mean difference (Altman and Bland, 1983; Bland and Altman, 1986; British Standards Institution, 1987; Mantha *et al.*, 2000). If a method meets these two criteria, it can be considered suitable for interchangeable usage with the reference. However, a more informed decision may be made from a composite assessment of a battery of validity statistics: very low bias, high correlation, low typical error, narrow 95%

LoA and a low CV demonstrates good agreement between methods. For the water bath assessment of Investigation 1, a systematic bias less than 0.2°C, typical error less than 0.1°C, 95% LoA less than 0.3°C, correlations greater than 0.9 and a CV less than 1% were considered acceptable and would therefore designate good validity of the method in that setting. These values are similar to other relevant studies that examined devices for measuring body temperature (Casa *et al.*, 2007; Gant *et al.*, 2006), meet the ASTM criteria (ASTM, 2006) for thermometry comparisons and take into account individual physiological variability inherent in skin temperature measurement, as well as the precision of the mercury thermometer criterion. In the absence of a true reference standard for human T_{sk} measurement in Investigation 2, acceptable error limits for the comparison between iButtons® and thermistors were also adopted *a priori* to meet ASTM criteria and were similar to other relevant studies (Kelechi *et al.*, 2006): typical error < 0.3°C, 95% LoA < 0.9°C. The validity cut-off values selected were suitable to reveal whether the differences between the methods would reach physiological significance.

The sample size for Investigation 2 (N = 8) was similar to, or larger than, other relevant studies (Buono *et al.*, 2007; Gant *et al.*, 2006; Hershler *et al.*, 1992; Matsukawa *et al.*, 2000; Van den Heuvel *et al.*, 2003; van Marken Lichtenbelt *et al.*, 2006). An estimation of the sample size for the human skin temperature investigation, with a crossover design, based on the precision of the mercury thermometer (0.1°C) and a typical error equal to the precision of the thermometer, indicated that a sample size of eight was likely to provide adequate statistical precision (Hopkins, 2000). Typical error of 0.1°C or less in the devices in Investigation 1, would confirm that a sample size of eight was sufficient for Investigation 2, based on published statistical calculations (Hopkins, 2000) and would enable the detection of differences of 0.1°C in skin temperature using this sample size in future studies.

4.4 Results

4.4.1 Investigation 1: Water Bath Comparison

4.4.1.1 Linear Regression Analyses

The linear regression analyses performed on the 46 iButtons® and the calibrated reference thermometer for temperature measurement showed very strong correlation, with all validity regression coefficients greater than 0.999. Individual thermistor linear regression analyses also showed high correlation (r > 0.999) with the reference mercury thermometer temperature measurements.

4.4.1.2 Mountain Plot

The mountain plot showed that the uncorrected iButtons® consistently reported higher temperatures compared to the reference thermometer (**Figure 4.2**). The uncorrected thermistors showed a wider distribution, with wider tails than did the uncorrected iButtons, suggestive of larger error in the thermistor method (**Figure 4.2**). Following the application of correction formulae, the median bias of both the iButtons® and thermistors became directly centred over the mean temperature difference of 0.00°C. The tails of the iButton® and thermistor plots also became contracted onto the zero bias line with correction and showed no perceptible deviation from the reference method. This indicated that the correction process greatly improved the agreement with the reference thermometer, such that there was no significant mean difference between the equivalent 28 corrected iButtons®, 28 corrected thermistors and the reference thermometer ($F_{2, 81}$ = 0.991; P = 0.376). Therefore, the corrected methods both demonstrated very close agreement with the criterion.

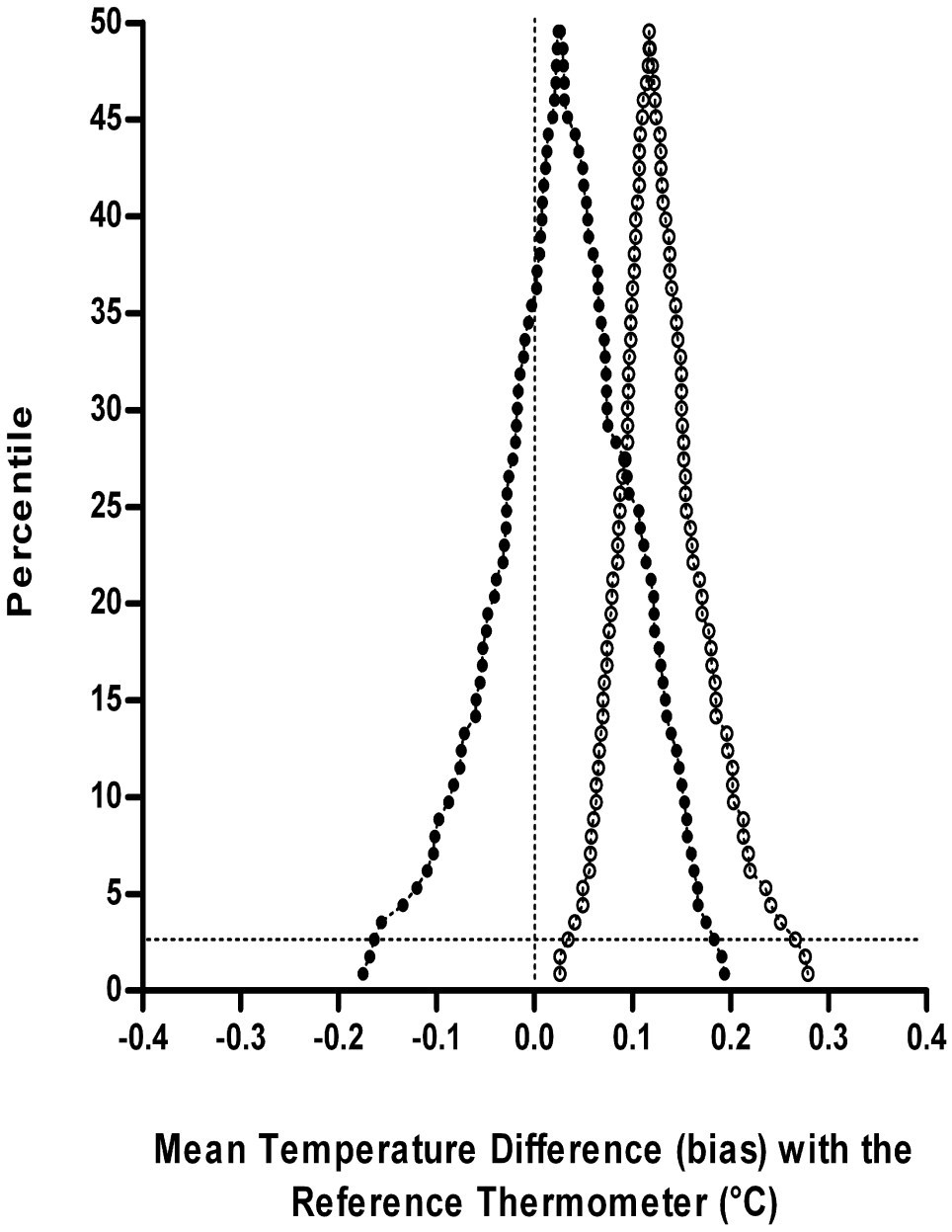

Figure 4.2 Mountain plot of two different devices for measuring temperature during a water bath comparison over a range of temperatures (10°C, 20°C, 30°C and 40°C): distribution of uncorrected iButton® (o) and uncorrected thermistor (●) readings compared with a certified mercury thermometer as the reference method. Vertical dashed lines represent zero bias with the criterion reference method. Horizontal dashed lines represent the 95% LoA.

4.4.1.3 Validity and Reliability

The iButtons® performed better than the manufacturer's specifications (accuracy = ±0.5°C) with a mean bias for all devices over all time points through the range of water bath temperatures of +0.121°C, but this bias was significantly greater ($P < 0.001$) than the reference bias of zero (**Table 4.1**). The thermistors also performed better than the manufacturer's specifications (accuracy = ±0.2°C) with a mean bias for all devices over all time points through the range of water bath temperatures of +0.045°C but this was also significantly greater ($P < 0.001$) than the reference bias of zero (**Table 4.1**). Following the application of individual correction formulae derived from linear regression analyses, the mean bias of the 46 corrected iButtons® (-3.61×10^{-5}°C; 95% CI, -1.06×10^{-4} to $+3.35 \times 10^{-5}$°C) was not significantly different ($P = 0.687$; 95% CI of the difference -2.06×10^{-4} to $+9.02 \times 10^{-5}$°C) from zero bias with the reference thermometer. After individual correction formulae were applied to the thermistor data, the mean bias was also not significantly different ($P = 0.992$; 95% CI of the difference -2.77×10^{-6} to $+3.37 \times 10^{-6}$°C) from zero bias, with a mean bias for the 28 corrected thermistors of $+3.37 \times 10^{-7}$°C (95% CI, -2.00×10^{-6} to $+2.68 \times 10^{-6}$°C). Thus, the correction procedure significantly improved iButton® and thermistor measurements, with a significantly smaller comparative mean bias after calibration ($P < 0.001$). With correction, the typical error of the iButtons® (1.70×10^{-4}°C) and the thermistors (4.47×10^{-6}°C) became negligible, as did the associated 95% LoA.

Device		*Mean Bias (°C)*	*s_{diff} (°C)*	*TEE (°C)*	*95% LoA (°C)*	*TEE CV (%)*	*TEM (°C)*	*TEM CV (%)*
	Acceptable Limits	**±0.20**	**0.15**	**0.10**	**0.30**	**1%**	**0.10**	**1%**
iButtons®	∑	+0.121***	0.061	0.043	0.119	0.169	0.031	0.186
	(95% CI)	(0.117 to 0.124)	(0.054 to 0.068)	(0.041 to 0.045)	(0.116 to 0.122)	(0.153 to 0.189)	(0.029 to 0.033)	(0.175 to 0.200)
Thermistors	∑	+0.045***	0.088	0.062	0.173	0.272	0.019	0.148
	(95% CI)	(0.040 to 0.050)	(0.086 to 0.091)	(0.060 to 0.065)	(0.168 to 0.178)	(0.245 to 0.305)	(0.017 to 0.020)	(0.139 to 0.158)

Table 4.1 Validity and reliability comparisons of mean iButton® and thermistor temperature recordings with a reference mercury thermometer during water bath measurements over a range of temperatures (10°C, 20°C, 30°C and 40°C). A tabular report of, validity statistics: mean bias, total error (standard deviation of the mean bias; s_{diff}), typical error of the estimate (TEE), Bland and Altman's 95% limits of agreement (95% LoA), TEE as a coefficient of variation (CV) and, reliability statistics: typical error of the measurement (TEM) and TEM as a CV. ∑, (95% CI) represents the mean data for the water bath temperature range with 95% confidence intervals. ***$P < 0.001$, mean bias significantly different from zero.

Even before correction, both devices demonstrated a mean bias in the water bath of less than 0.2°C, validity correlation coefficients greater than 0.999, validity and reliability typical errors less than 0.1°C with associated CV less than 1%. Overall, both methods met the *a priori* criteria for good absolute and relative validity and reliability in this setting, which improved even further with correction. However, the estimated error values for the thermistors were between 1.5 and 2 times greater than the observed error of the iButtons® prior to correction during all temperature conditions (**Table 4.1**). Data from Investigation 2 were corrected *post hoc* based on the calibration step performed in Investigation 1.

4.4.2 Investigation 2: Human Skin Application

The responses of the iButtons and the thermistors showed similar differences across all skin temperature sites, regardless of trial and condition, therefore only mean T_{sk} is reported. In the absence of a true reference method or 'gold standard' for human skin temperature measurement, absolute values are presented, with no directionality.

4.4.2.1 10°C Trial

The iButtons® consistently reported significantly higher mean T_{sk} measurements compared with the thermistors ($P < 0.05$) throughout the 40min passive rest, low wind condition (**Figure 4.3A**) and throughout the passive rest, higher wind condition. During exercise the mean T_{sk} reported by the iButtons® was not different ($P > 0.05$) from the mean T_{sk} reported by the thermistors from time point (t) = 0 min to t = 14 min and from t = 27 min to t = 40 min. Nevertheless, between t = 14 min and t = 27 min, the mean T_{sk} reported by the iButtons® was higher ($P > 0.05$) than the mean T_{sk} reported by the thermistors (**Figure 4.4A**).

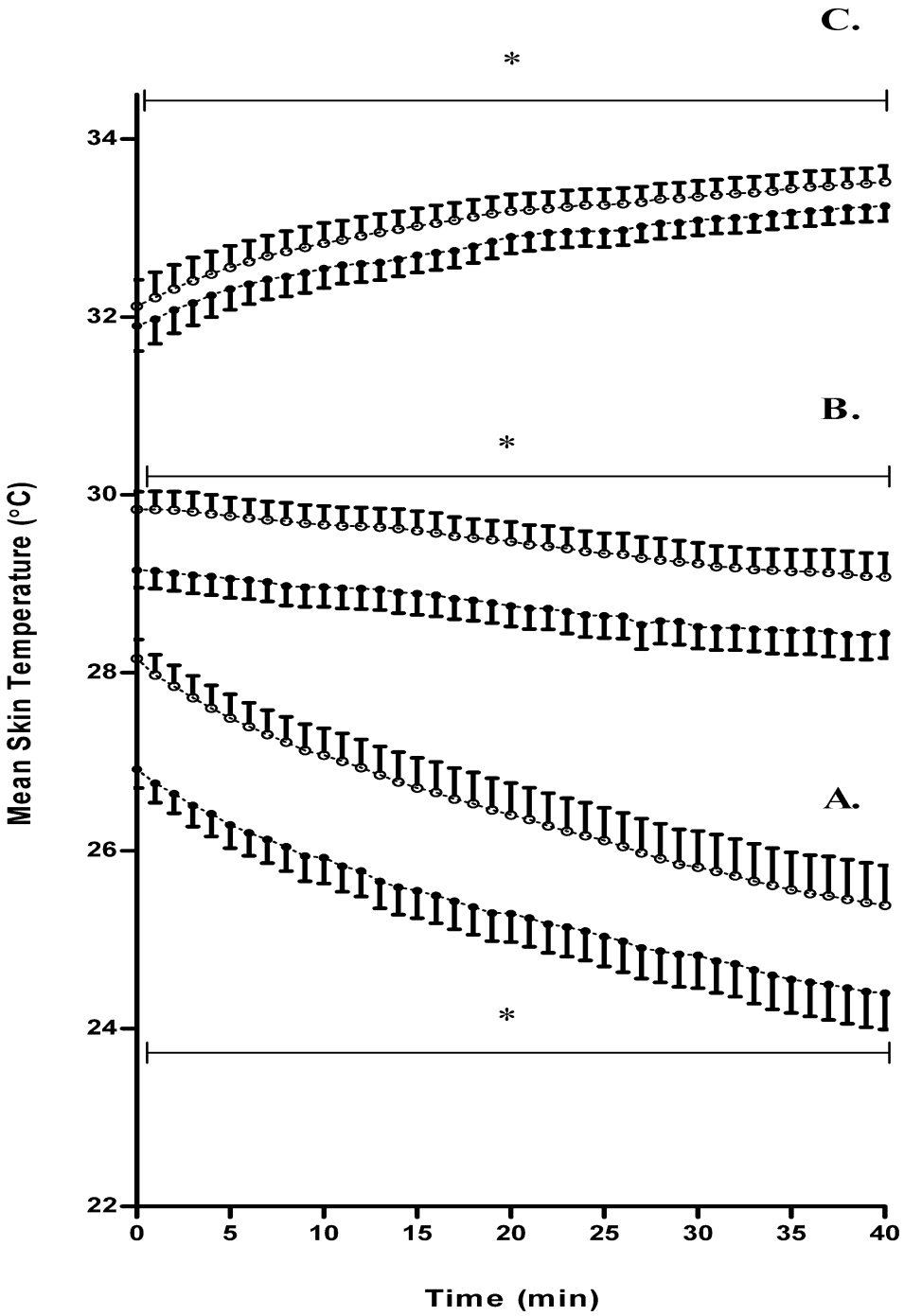

Figure 4.3 Comparison between mean T_{sk} measurements of iButtons® (o) and thermistors (●) during passive, low wind conditions at ambient temperature trials of (**A.**) 10°C, (**B.**) 20°C and (**C.**) 30°C. Values are means, (SD). *$P < 0.05$, significant differences between iButtons® and thermistors.

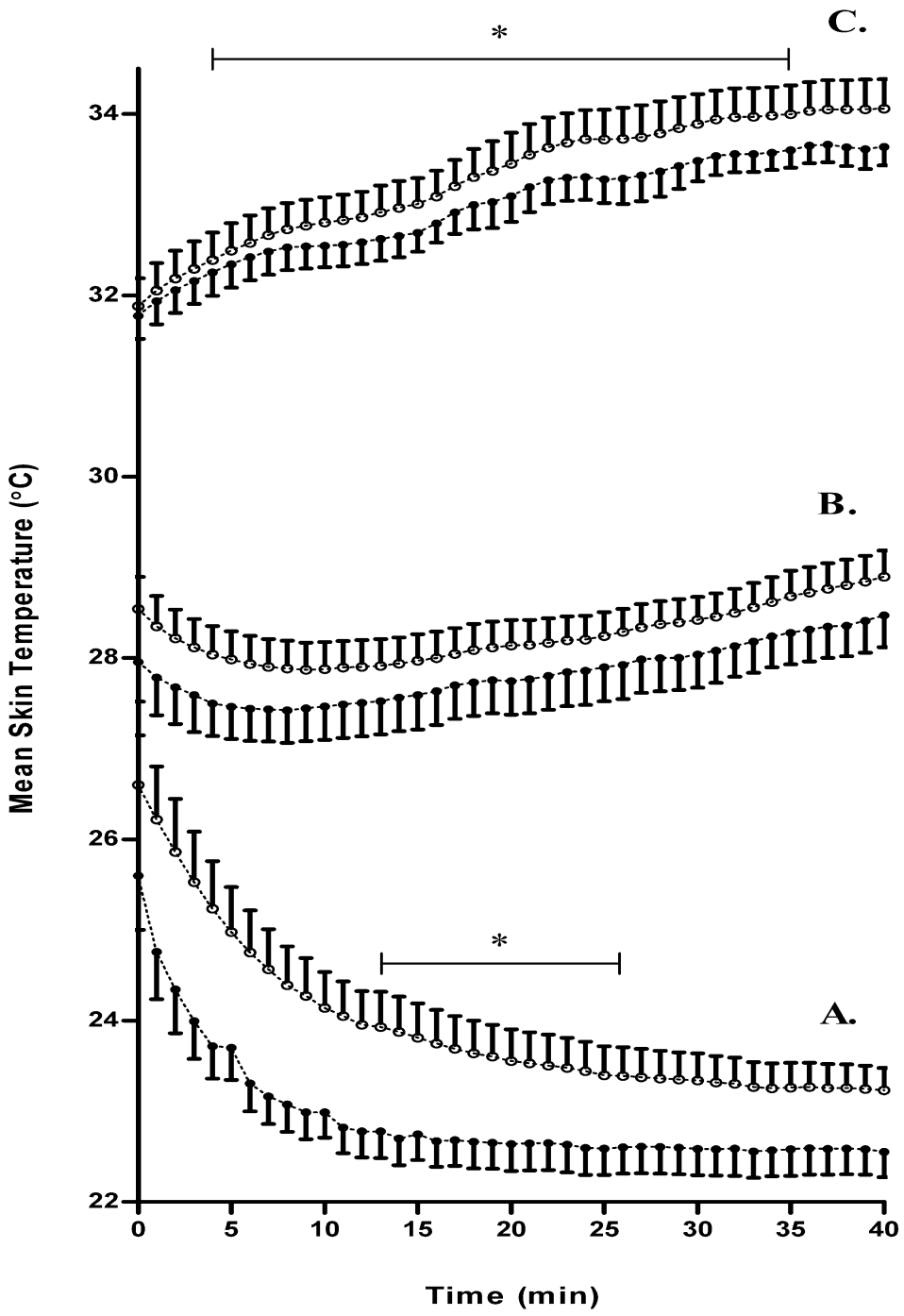

Figure 4.4 Comparison between mean T_{sk} measurements of iButtons® (o) and thermistors (●) during cycle ergometry exercise, high wind conditions at ambient temperature trials of (**A.**) 10°C, (**B.**) 20°C and (**C.**) 30°C. Values are means, (SD). *P < 0.05, significant differences between iButtons® and thermistors.

4.4.2.2 20°C Trial

Again, iButtons® consistently reported higher ($P < 0.05$) mean T_{sk} measurements compared with the thermistors throughout the 40min passive rest, low wind (**Figure 3B**) and throughout the passive rest, higher wind conditions. However, no significant differences ($P > 0.05$) were identified when the iButton® and thermistor mean T_{sk} recordings were compared over time during the exercise condition (**Figure 4.4B**).

4.4.2.3 30°C Trial

Once more, the iButtons® consistently reported higher ($P < 0.05$) mean T_{sk} measurements compared with the thermistors throughout the 40 min passive rest, low wind condition (**Figure 4.3C**) and throughout the passive rest, higher wind condition. During exercise, from t = 0 min to t = 5 min and from t = 35 min until t = 40 min, the mean T_{sk} reported by the iButtons® did not differ ($P > 0.05$) from the mean T_{sk} reported by the thermistors. Nevertheless, between t = 5 min and t = 35 min, the mean T_{sk} reported by the iButtons® was higher ($P < 0.05$) than the mean T_{sk} reported by the thermistors (**Figure 4.4C**).

There was a statistically significant mean difference between temperature measurement methods for all human skin application trials and conditions ($P < 0.05$), except during the 20°C exercise, high wind condition ($P = 0.057$). The iButtons® consistently reported higher temperature measurements than thermistors, with a mean difference over all the trial conditions of 0.690°C, (0.393). The mean temperature differences between the methods proved to be parallel and uniform across all the passive conditions with small deviations from uniformity during exercise in the cold and exercise in the heat (**Figure 4.3A&C**).

4.4.2.4 Validity and Reliability

The mean differences between temperature measurements varied substantially between trials and conditions (0.261 to 1.356°C). However, the correlation coefficients remained high (> 0.9) and the estimated typical error between the methods remained low (< 0.3°C) as did the 95% LoA (< 0.9°C) and CV (< 1%), which further suggested that the differences were uniform and parallel throughout each trial condition (**Tables 4.2, 4.3, 4.4**). Therefore, as the offset between the temperature measurements of the two devices was consistent and parallel across each individual trial condition, this can easily be accounted and adjusted for as necessary.

Trial and Condition		*Mean Difference (°C)*	*s_{diff} (°C)*	*R*	*ICC*	*Typical error (°C)*	*95% LoA (°C)*	*CV (%)*
	Acceptable Limits	**±0.50**	**0.45**	**> 0.9**	**> 0.9**	**0.30**	**0.90**	**1%**
Passive, low wind		1.107	0.083	0.999	0.999	0.027	0.075	0.108
(95% CI)		(1.081 to 1.183)	(0.066 to 0.114)	(0.998 to 1.000)	(0.999 to 1.000)	(0.022 to 0.035)		(0.089 to 0.139)
Passive. high wind		1.356	0.048	0.999	0.999	0.046	0.128	0.197
(95% CI)		(1.340 to 1.371)	(0.038 to 0.067)	(0.998 to 1.000)	(0.998 to 1.000)	(0.038 to 0.060)		(0.161 to 0.253)
Exercise, high wind		0.989	0.282	0.968	0.952	0.171	0.474	0.729
(95% CI)		(0.900 to 1.079)	(0.223 to 0.398)	(0.920 to 0.987)	(0.919 to 0.985)	(0.140 to 0.279)		(0.597 to 0.937)

Table 4.2 Comparisons between iButton® and thermistor methods for measurement of human skin temperature during 10˚C trials. Tabular report of mean difference, total error (standard deviation of the mean difference; s_{diff}), Pearson's product-moment correlation (*r*), Intra-class correlation coefficient (ICC), within-subject standard deviation (typical error), Bland and Altman's 95% limits of agreement (95% LoA) and typical error as a coefficient of variation (CV) for iButton® and thermistor comparisons during the 10°C trial. 95% CI are the 95% confidence intervals.

Trial and Condition		*Mean Difference (°C)*	*s_{diff} (°C)*	*r*	*ICC*	*Typical error (°C)*	*95% LoA (°C)*	*CV (%)*
	Acceptable Limits	**±0.50**	**0.45**	**> 0.9**	**> 0.9**	**0.30**	**0.90**	**1%**
Passive, low wind		0.697	0.022	0.997	0.994	0.019	0.053	0.065
(95% CI)		(0.690 to 0.704)	(0.01 to 0.031)	(0.992 to 0.999)	(0.991 to 0.997)	(0.015 to 0.024)		(0.053 to 0.084)
Passive, high wind		0.789	0.032	0.999	0.999	0.014	0.039	0.051
(95% CI)		(0.779 to 0.800)	(0.026 to 0.045)	(0.998 to 1.000)	(0.998 to 1.000)	(0.011 to 0.018)		(0.042 to 0.066)
Exercise, high wind		0.415	0.066	0.978	0.954	0.067	0.186	0.240
(95% CI)		(0.394 to 0.436)	(0.052 to 0.091)	(0.945 to 0.991)	(0.952 to 0.956)	(0.055 to 0.085)		(0.197 to 0.308)

Table 4.3 Comparisons between iButton® and thermistor methods for measurement of human skin temperature during 20˚C trials. Tabular report of mean difference (°C), total error (standard deviation of the mean difference; s_{diff}), Pearson's product-moment correlation (*r*), Intra-class correlation coefficient (ICC), within-subject standard deviation (typical error), Bland and Altman's 95% limits of agreement (95% LoA) and typical error as a coefficient of variation (CV) for iButton® and thermistor comparisons during the 20°C trial. 95% CI are the 95% confidence intervals.

Trial and Condition		*Mean Difference (°C)*	*s_{diff} (°C)*	*r*	*ICC*	*Typical error (°C)*	*95% LoA (°C)*	*CV (%)*
	Acceptable Limits	**±0.50**	**0.45**	**> 0.9**	**> 0.9**	**0.30**	**0.90**	**1%**
Passive, low wind		0.280	0.030	0.997	0.994	0.029	0.080	0.089
(95% CI)		(0.271 to 0.290)	(0.024 to 0.040)	(0.993 to 0.999)	(0.991 to 0.997)	(0.024 to 0.037)		(0.073 to 0.114)
Passive, high wind		0.261	0.020	0.997	0.994	0.019	0.053	0.058
(95% CI)		(0.255 to 0.268)	(0.016 to 0.028)	(0.992 to 0.999)	(0.991 to 0.997)	(0.015 to 0.024)		(0.048 to 0.075)
Exercise, high wind		0.318	0.108	0.999	0.998	0.029	0.080	0.089
(95% CI)		(0.284 to 0.352)	(0.087 to 0.147)	(0.997 to 0.999)	(0.997 to 0.999)	(0.024 to 0.037)		(0.073 to 0.115)

Table 4.4 Comparisons between iButton® and thermistor methods for measurement of human skin temperature during 30˚C trials. Tabular report of mean difference, total error (standard deviation of the mean difference; s_{diff}), Pearson's product-moment correlation (*r*), Intra-class correlation coefficient (ICC), within-subject standard deviation (typical error), Bland and Altman's 95% limits of agreement (95% LoA) and typical error as a coefficient of variation (CV) for iButton® and thermistor comparisons during the 30°C trial. 95% CI are the 95% confidence intervals.

The mean change in temperature was less than 0.02°C for both devices from a preliminary test-retest reliability assessment during the final 5min of trials in which thermal equilibrium was reached. Minute by minute changes in the mean temperature were not statistically different for either device for any of the trial conditions tested ($P > 0.05$). The reliability of both methods was extremely good for the 5 min periods of stable T_{sk} at the end of trials, as also indicated by correlation coefficients greater than 0.9, TEM (typical error) less than 0.1°C, 95% LoA less than 0.3°C and CV less than 1%.

4.5 Discussion

The findings from the comparison between iButtons® and thermistors supported the hypotheses that both methods would provide temperature measurements within acceptable error limits. However, the thermistors demonstrated higher random error than iButtons® in the water bath investigation. T_{sk} reported by iButtons® was consistently higher than thermistors during the investigation on human skin and these mean temperature differences were parallel and uniform in all of the passive conditions with only small deviations in the exercise setting. This suggested that the offset (i.e. mean difference) between the methods was consistent across individual trial conditions, which can therefore be corrected as necessary. In addition, wind velocity of the magnitude used in this study did not affect the differences in T_{sk} between the two methods. These findings indicate that wireless iButtons® provide a valid alternative for general temperature and T_{sk} measurement. The beneficial attributes of iButtons® therefore become particularly relevant during T_{sk} assessment and research during free-living and ambulatory conditions, in field studies, as well as in the monitoring of cold and heat illness and injury, where T_{sk} measurement using other currently available methods would be problematic. Investigators should still take into account though, the consistent T_{sk} difference between the iButtons® and thermistors when comparing studies using the two different methods. As previously stated, the only foreseeable disadvantage with the iButton® system is that temperature readings can only be viewed once the device has finished logging data. The iButton® logging periods can range from several seconds to several days, as determined by the user. So, if there is concern over waiting to obtain the data during longer logging periods, then the iButtons® can be removed and the data acquired. Then, the iButtons® can be re-programmed and re-applied for further measurement periods, all of which only takes a matter of minutes.

5.1 Investigation 1: Water Bath Comparison

Before correction, the iButtons® and thermistors both displayed a mean bias with the reference mercury thermometer that was significantly greater than zero. Thus, in their off-the-shelf state, neither method could be considered to have equivalent accuracy to the mercury thermometry method for temperature measurement. However, with the implementation of a calibration step, the iButton® and thermistor methods demonstrated accuracy that was equivalent to that of the reference thermometer. Although both methods demonstrated low typical errors, small 95% LoA and low CV, the validity typical error of the thermistor method was approximately 1.5 to 2 times greater than that of iButtons®. This suggested that there was larger random error in the thermistor measurement method. Both methods showed high validity correlation coefficients following linear regression calibration analyses ($r > 0.999$).

The findings from the water bath section of the study corroborate and expand upon previous studies which evaluated an older model of iButton® that was less suited for human T_{sk} measurement than the model used in the present study (Davidson *et al.*, 2003; Hubbart *et al.*, 2005; van Marken Lichtenbelt *et al.*, 2006). The present study and previous studies (Davidson *et al.*, 2003; Hubbart *et al.*, 2005; van Marken Lichtenbelt *et al.*, 2006) suggest that if thermal physiologists require temperature measurements which are in close agreement (≤ 0.125°C) with a calibrated reference thermometer, then corrections through formulae derived from individual linear regression, would be recommended for both methods. This individual correction can be considered as an 'in-house' calibration step. However, as one might imagine, the individual correction procedure is considerably time consuming; a highly specific water bath and calibrated reference thermometer are required, and these resources might not always be available or feasible. Consequently, if thermal investigators do not consider the demonstrated mean differences between the uncorrected iButton® or thermistor methods and the reference

thermometer large enough to be concerned about in the relevant setting and application (Tables 1-4), then correction may not be necessary.

4.5.2 Investigation 2: Human Skin Application

Despite calibrating iButtons® and thermistors in a water bath, iButtons® consistently reported higher mean T_{sk} than thermistors throughout the 40 min of all trials in 10-30˚C ambient conditions. This highlights a limitation of using a water bath to calibrate sensors for T_{sk} measurement, particularly when the heat transfer characteristics in water and air are so different. Clearly, the lack of a true reference method for T_{sk} remains a limitation in studies attempting to validate new devices for T_{sk} measurement. Encouraging though, is recent evidence indicating that infrared thermometry may hold promise as a reference method for T_{sk} measurement (Giansanti and Maccioni, 2007). The explanation for the observed differences between the calibrated iButtons® and calibrated thermistors could be attributed to one or more of the typical complications with contact thermometry (Giansanti and Maccioni, 2007). Of these complications, the most likely causes for the differences between the methods are that the iButton® and thermistor devices may differentially affect or be affected by: 1) the heat exchange between skin and the external environment, 2) the gas/vapour exchange with the environment and 3) the heat exchange between the skin and the sensor (Giansanti and Maccioni, 2007). Another possibility for the lower T_{sk} using calibrated thermistors compared to calibrated iButtons® is that the thermistors' smaller surface area and wired attachment increases the likelihood of the thermistors losing direct contact with the skin. Any loss of contact (e.g. small angular changes), might cause the thermistor measurements to tend towards ambient temperature and give false readings. Even when precautions are taken to prevent thermistor detachment, this can still occur, which is a major disadvantage of all hard-wired temperature measurement devices. It is also possible that the two devices respond to occlusion by tape differently. However, although occlusion by tape can alter the skin micro-environment and affect heat

transfer (Buono and Ulrich, 1998), the conditions were the same for both devices, so response differences due to taping are highly unlikely. The iButtons® and thermistors were both taped to the skin with the same section of tape and were within 1cm of one another at each site. Although there has been criticism of utilizing 'covered' temperature sensors (Buono and Ulrich, 1998) acrylic rings utilized in previous studies to keep the sensors 'uncovered' are no longer commercially available and in manufacturing one's own replica of these acrylic rings, there is always the risk of a loss of good thermal contact, which is less of a worry with proper taping. To clarify any existing uncertainties as to which measurement technique provides readings closer to a true value, a future study could compare iButtons® and thermistors with a device which might perform better during human T_{sk} applications, such as an infrared thermometer (Giansanti and Maccioni, 2007). A potential future study should also examine human T_{sk} in ambient conditions that are warmer than T_{sk} (e.g. 40˚C), to determine whether thermistors do indeed always tend or drift towards readings that are closer to ambient conditions, in comparison with iButtons®, as could be inferred from previous research (van Marken Lichtenbelt *et al.*, 2006). In the present study, the ambient conditions (10, 20 and 30˚C) were lower than T_{sk} readings and thermistors always gave lower T_{sk} readings than iButtons®. Future studies should also examine the reliability of iButtons® during repeated exposures to the same ambient conditions. It would also be of interest to determine how iButton® T_{sk} recordings fare with sensor usage and ageing (Hubbart *et al.*, 2005), and through this, establish how often re-correction procedures might need to be implemented.

In conclusion, these findings indicate that wireless iButtons® provide a valid alternative for T_{sk} as well as general temperature measurement during laboratory and field investigations particularly when T_{sk} measurement using other currently available methods may prove problematic.

CHAPTER FIVE

Exercise-induced stress inhibits both the induction and elicitation phases of *in vivo* T-cell-mediated immune responses in humans

5.1 Summary: Little is known about the influence of exercise on induction and elicitation phases of *in-vivo* immunity in humans. Experimental contact-hypersensitivity, a clinically relevant *in-vivo* measure of T cell-mediated immunity, was used to investigate the effects of exercise on induction and elicitation phases of immune responses to a novel antigen. The effects of 2 h moderate-intensity-exercise upon the induction (Study One) and elicitation of *in vivo* immune memory (Study Two) to diphenylcyclopropenone (DPCP) were examined. Study One: Matched, healthy males were randomly-assigned to exercise (N = 16) or control (N = 16) and received a primary DPCP exposure (sensitization), 20 min after either 2h running at 60% $\dot{V}O_{2peak}$ (EX) or 2h seated rest (CON). Four weeks later, participants received a low, dose-series DPCP challenge (elicitation) on their upper inner arm, which was read at 24 and 48 h as clinical score, oedema (skinfold thickness) and redness (erythema). Study Two: Pilot; Thirteen healthy males were sensitized to DPCP. Elicitation challenges were repeated every four weeks until responses reached a reproducible plateau. Then, N = 9 from the pilot study completed both EX and CON trials in a randomized order. Elicitation challenges were applied and evaluated as in Study One. Results demonstrate that exercise-induced stress significantly impairs both the induction (oedema -53% at 48h; $P < 0.001$) and elicitation (oedema -19% at 48h; $P < 0.05$) phases of the *in vivo* T-cell-mediated immune response. These findings demonstrate that prolonged moderate-intensity exercise impairs the induction and elicitation phases of *in-vivo* T-cell-mediated immunity. Moreover, the induction component of new immune responses appears more sensitive to systemic-stress-induced modulation than the elicitation component.

5.2 Introduction

Stress in a variety of forms, is a common component of everyday life and it is widely acknowledged that stress modulates immune function (Dhabhar, 2002b) and susceptibility to infection (Cohen *et al.*, 1991). In line with this, individuals undergoing heavy physical exertion exhibit decreases in circulating and mucosal immune function and increases in the number and severity of upper respiratory tract infection symptoms (Walsh *et al.*, 2011b). Much of the research into the immunological consequences of stress has relied on animal models, *in vitro* measures of immune function, or subjective self-reporting of infection (Dhabhar and McEwen, 1999; Walsh *et al.*, 2011b). Results from animal modelling are of questionable relevance and transferability to humans (Vukmanovic-Stejic *et al.*, 2006), *in vitro* measures of immune competence are of limited value in predicting clinically significant outcomes (particularly when sub-populations of immune cells are isolated from their normal environment), and data from self-reporting should be interpreted with caution. The importance of using *in vivo* measures to assess human immune function in stress research has recently been highlighted (Albers *et al.*, 2005) not least because the *in vivo* response to an antigenic challenge involves a multi-cellular response that is clinically relevant (Albers *et al.*, 2005; Walsh *et al.*, 2011b).

The small number of studies that have examined the effects of different stressors (e.g. mental stress or physical exertion) on *in vivo* immune responses in humans have assessed the circulating antibody response to injection of either a novel antigen (e.g. keyhole limpet haemocyanin) (Smith *et al.*, 2004) or a previously encountered antigen (e.g. influenza vaccine) (Edwards *et al.*, 2006; Edwards *et al.*, 2010). Using keyhole limpet haemocyanin enables the investigator to identify the effects of stress on the unique cellular and co-stimulatory signals required for primary (induction) of the immune response that using previously encountered antigens does not (Walsh *et al.*, 2011b). T-cell-mediated memory responses (delayed type hypersensitivity) have

also been assessed using the Mérieux CMI Multitest®: this test involves the intra-dermal injection of seven previously encountered antigens and measurement of the skin reaction 48 h later. One such study showed reduced skin reactions to the Mérieux CMI Multitest® in endurance athletes after prolonged exercise in comparison with rested controls (Bruunsgaard *et al.*, 1997). Unfortunately, injecting recall antigens in this way permits only the assessment of pre-existing immunological memory and not the primary induction response. Research to date indicates that short-lasting mental stress or exercise (lasting minutes) can enhance cell-mediated immunity but that prolonged stress (e.g. physical exertion that lasts hours and activates the hypothalamic-pituitary-adrenal axis) decreases the response in humans (Bruunsgaard *et al.*, 1997; Dhabhar and Viswanathan, 2005; Edwards *et al.*, 2006; Walsh *et al.*, 2011a) .

Experimental contact sensitization with novel chemicals such as diphenylcyclopropenone (DPCP) may provide investigators with a simple, practical, sensitive and robust method for separate analysis of the effects of various systemic stressors on the induction and elicitation phases of the T-cell-mediated immune response (Albers *et al.*, 2005; Friedmann, 2007; Palmer and Friedmann, 2004). Contact sensitisation by small xenobiotic chemicals experienced via topical skin exposure normally induces a purely T cell-mediated immune response. In experimental contact sensitization, the immune response is initiated by application of a known sensitizing dose of the desired chemical, never previously encountered by the individual. Following a period, usually four weeks, to allow the establishment of immune memory, the vigour of antigen-specific *in vivo* immune reactivity is assessed by measuring the skin reaction (oedema and erythema) evoked by topical application of a concentration series of the contact sensitizer, e.g. DPCP. Deliberate contact sensitisation with specially synthesised chemicals such as DPCP has long been used for topical immunotherapy of conditions such as alopecia areata and warts (Buckley and Du Vivier, 2001). Experimental contact sensitization has also been used to assess the immuno-modulating effects of atopic eczema (Rees *et al.*, 1990), local ultraviolet

light exposure (Cooper *et al.*, 1992; Palmer and Friedmann, 2004; Sleijffers *et al.*, 2001), and to monitor immuno-competence in HIV-infected patients (Levis and Kaplan, 2005). However, experimental contact sensitization has not been used to evaluate the influence of systemic stress on both the induction and elicitation phases of *in vivo* cell-mediated immunity.

The objectives of the present study were to investigate the effects of a systemic stress (controlled physical exertion) on both the induction and elicitation phases of the T-cell-mediated immune response to a novel antigen by use of the experimental contact-hypersensitivity (CHS) method. This was achieved by performing two randomized, controlled studies. In a parallel-group, randomized, cross-sectional design (Study One), the effects of prolonged, moderate intensity exercise (a relevant stressor experienced by recreational runners and joggers) were investigated, immediately prior to the induction of the cell-mediated immune response to the contact sensitizing antigen DPCP. In a randomized, cross-over design (Study Two), the elicitation of the *in vivo* immune memory response to DPCP was investigated after the same exercise or after physical rest. It was hypothesised that exercise-induced stress would reduce both the induction (Study One) and elicitation (Study Two) of the *in vivo* cell-mediated immune response to DPCP.

5.3 Methods

In addition to the criteria stated in **Chapter 3**, participants were excluded if they had a history of atopy or any other immune-related or inflammatory dermatological conditions based on established criteria (Kelly *et al.*, 1998). Again, all participants were healthy, non-smoking, recreationally active males with no previous history of exposure to DPCP.

Summary of overall design

Study One investigated the effects of moderate exercise–induced stress on the induction of new T-cell-mediated immune responses. Participants were randomized to undertake 2 h of moderate exercise, running on a treadmill (EX) or to sit quietly for 2 h (CON) in the same laboratory, immediately after which they all received the same, single, sensitizing dose of DPCP. Four weeks later the strength of immune memory induction was quantified by measuring the immune responses (oedema and erythema) elicited by simultaneous topical challenge with a dose-series of DPCP. Study Two compared the effects of the same exercise-stress and the same control period, 2 h rest, on the elicitation phase of the cell-mediated immune response in pre-sensitized participants in a randomized cross-over design. Since this required each volunteer to receive the elicitation challenge on two separate occasions (after exercise and after rest), before commencing Study Two, a pilot study was performed to determine the number of repeated challenges necessary to achieve a reproducible plateau. In Study Two, participants from the pilot study randomly undertook either EX or CON immediately prior to the elicitation challenge with DPCP. Four weeks later, participants returned to the laboratory to complete the other arm of the trial (EX or CON) prior to an additional elicitation challenge with the dose-series of DPCP.

5.3.1 Study One: The effect of prior exercise on induction of antigen-specific immune memory

In a cross-sectional design, the male participants recruited for Study One were matched for age and aerobic fitness and then randomly assigned to either the EX (N = 16; means, (SD): age 26 years, (5); height 177 cm, (7); body mass 78 kg, (7); $\dot{V}O_{2peak}$ 59 ml·kg^{-1}·min^{-1}, (8)) or CON experimental group (N = 16; means, SD: age 27 years, (7); height 181 cm, (8); body mass 78 kg (8); $\dot{V}O_{2peak}$ 58 ml·kg^{-1}·min^{-1}, (8)). There were no significant differences between the groups for any of these physical and anthropometric characteristics.

Familiarization and Preliminary measures. Following written informed consent, all participants were familiarized with the laboratory equipment and procedures. Anthropometric measurements were then recorded (**Chapter 3**). In order to determine peak oxygen uptake ($\dot{V}O_{2peak}$), each participant performed a continuous incremental exercise test on a treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany), based on a standard protocol (Bruce, 1977b), as detailed in **Chapter 3**. A speed equivalent to 60%$\dot{V}O_{2peak}$ was then calculated and confirmed for each participant. All participants avoided alcohol, caffeine and strenuous exercise in the 24 h before, through the 48 h after all visits to the laboratory and completed a 4-day diet diary encompassing the period from 24 h before through the 48 h after initial exposure to DPCP. This 4 day diet was then replicated for the equivalent timing on all future visits. All participants arrived at the laboratory on every occasion at 1030 h having consumed a standardized light breakfast and 500 ml water at least 2 h in advance.

Experimental Procedures

Stress. Acute systemic exercise-stress involved 2h running on a treadmill in a laboratory at a speed equivalent to 60% $\dot{V}O_{2peak}$ in ambient conditions of 20°C, 40% RH, with a fan-generated

wind velocity of 2.3 $m \cdot s^{-1}$ beginning at 1100 h. Nude body mass (NBM) was recorded before and after exercise to determine water quota during exercise and fluid replacement following exercise. Participants were permitted 5 $ml \cdot kgNBM^{-1} \cdot h^{-1}$ water during exercise, with any additional exercise fluid loss replaced following exercise. Immediately after the trial, exercising participants showered and returned to the laboratory within 15 min of completion. The CON, non-stress condition consisted of 2 h seated, passive rest in the same laboratory, in the same ambient conditions at the same time of day, with a water intake of 2 $ml \cdot kgNBM^{-1} \cdot h^{-1}$.

Induction of Contact Sensitivity. The sensitizing exposure to the novel antigen DPCP, initiating the induction of antigen-specific immune memory, involved application of an occluded patch, constituting an 11 mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on Scanpor hypoallergenic tape containing a 10 mm filter paper disc. The patch was soaked in 22.8 μl of 0.125% DPCP in acetone (patch = 30 $\mu g \cdot cm^{-2}$ DPCP). After a 3 to 5 minute drying period, the patch was placed on the lower back and left in place for exactly 48h. Participants were sensitized to DPCP 20 minutes after completing either 2 h EX or 2 h CON conditions. All participants were sensitized at the same time of day (1300 h).

Primary Allergic Response (PAR). Sensitization to DPCP was confirmed 14 days after removal of the sensitizing patch by visual assessment of responses at that site; the mean redness was quantified with an erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark): triplicate measurements at the reaction site minus the mean of three triplicate background measurements at an adjacent site on the lower back. This time period was selected as previous research has suggested that the PAR will typically develop and be observable 7 to 14 days following sensitization (Friedmann, 1991).

Elicitation. The vigour of *in vivo* antigen-specific immune responsiveness was quantified by measuring the responses elicited by secondary exposure to the same antigen. Exactly four weeks after the initial sensitization to DPCP, all participants received a challenge with a dose-series of DPCP on individual patches, each comprising an 8mm aluminium Finn chamber on Scanpor hypoallergenic tape containing a 7 mm filter paper disc. Patches were applied to the volar aspect of the upper arm (10 μl of DPCP: 0.0048%, 1.239 $\mu g \cdot cm^{-2}$; 0.0076%, 1.982 $\mu g \cdot cm^{-2}$**;** 0.0122%, 3.172 $\mu g \cdot cm^{-2}$; 0.01953%, 5.075 $\mu g \cdot cm^{-2}$; 0.03125%, 8.120 $\mu g \cdot cm^{-2}$ and 10 μl 100% acetone control patch for background subtraction). This dilution series allowed for quantification of the linear portion of the sigmoidal dose-response curve (Friedmann *et al.*, 1983; Friedmann, 2006). Patches were applied in randomly allocated order at the local site in order to minimise any anatomical variability in responses. Elicitation patches were removed after exactly 6 h and the responses measured at 24 and 48 h.

Assessment of CHS responses. Mean skin-fold thickness was determined from triplicate measurements at each elicitation site using modified spring-loaded skin callipers (Harpenden Skin-fold Calliper, British Indicators, England). Skin-fold thickness was recorded to the nearest 0.1 mm by the same investigator by placing the jaws of the calliper at the outer diameter of the response site and measuring skin thickness only (no subcutaneous fat). As previously described (Cooper *et al.*, 1992; Friedmann *et al.*, 1983) this method provides an objective measure of skin oedema (inflammatory swelling). Mean skin erythema was determined from triplicate measurement at each elicitation site using an erythema meter. As previously described (Memon and Friedmann, 1996), this method provides an objective measure of skin redness. Mean background values were determined from triplicate measurements at the acetone patch site for both thickness and redness. In order to determine the increase in thickness and redness, the value from the acetone-only site was subtracted from each elicitation site value. The values for increase in skin-fold thickness and redness over all the doses were summed, which gave an

approximation of the area under the dose-response curve, representative of the overall reactivity of each participant to DPCP (Cooper *et al.*, 1992; Palmer and Friedmann, 2004).

5.3.2 Pilot Study: Investigation of change in responsiveness with repeated monthly administration of DPCP

Thirteen males (means, (SD): age 23 years, (2); height 181 cm, (4); body mass 81 kg, (12); $\dot{V}O_{2peak}$ 53 $ml \cdot kg^{-1} \cdot min^{-1}$, (7)) volunteered to participate in this study. One week after familiarizations were performed, all participants were sensitized with DPCP (as in Study One). The PAR was confirmed at fourteen days post-sensitization; after a further fourteen days, the first elicitation challenge was administered to participants (as in Study One). Elicitation challenges were repeated at intervals of four weeks, alternating arms on each occasion, until a plateau in response was observed. CHS responses were assessed 48 h after the elicitation challenge (as quantified in Study One).

5.3.2.1 Pilot Study Findings: Boosting of initial response through repeated monthly challenges with DPCP

Repeated monthly recall challenges with DPCP resulted in an initial significant amplification or 'boosting' of responses between Recall 1 and Recall 2, seen clinically at the higher doses as larger reactions which sometimes included vesicles or even bullae, (**Figure 5.1**: $P = 0.014$; 95% CI of the difference, -5.41 to -0.79). Responses to subsequent recall challenges did not show significant boosting (**Figure 5.1**). Following repeated recall challenges, erythema readings became less reliable for quantifying graded redness responses since stronger responses sometimes included vesicles and bullae that are in the yellow visual spectrum not the red. Therefore, erythema responses were not reported for the pilot study and not utilized in the

subsequent Study Two. It was established that a reproducible response plateau was attained by the third recall challenge with DPCP (**Figure 5.1**) but because of the possibility of vesicles or blisters at the top doses, the top two doses were omitted from Study Two and replaced by two lower concentrations at the bottom end of the dose response range.

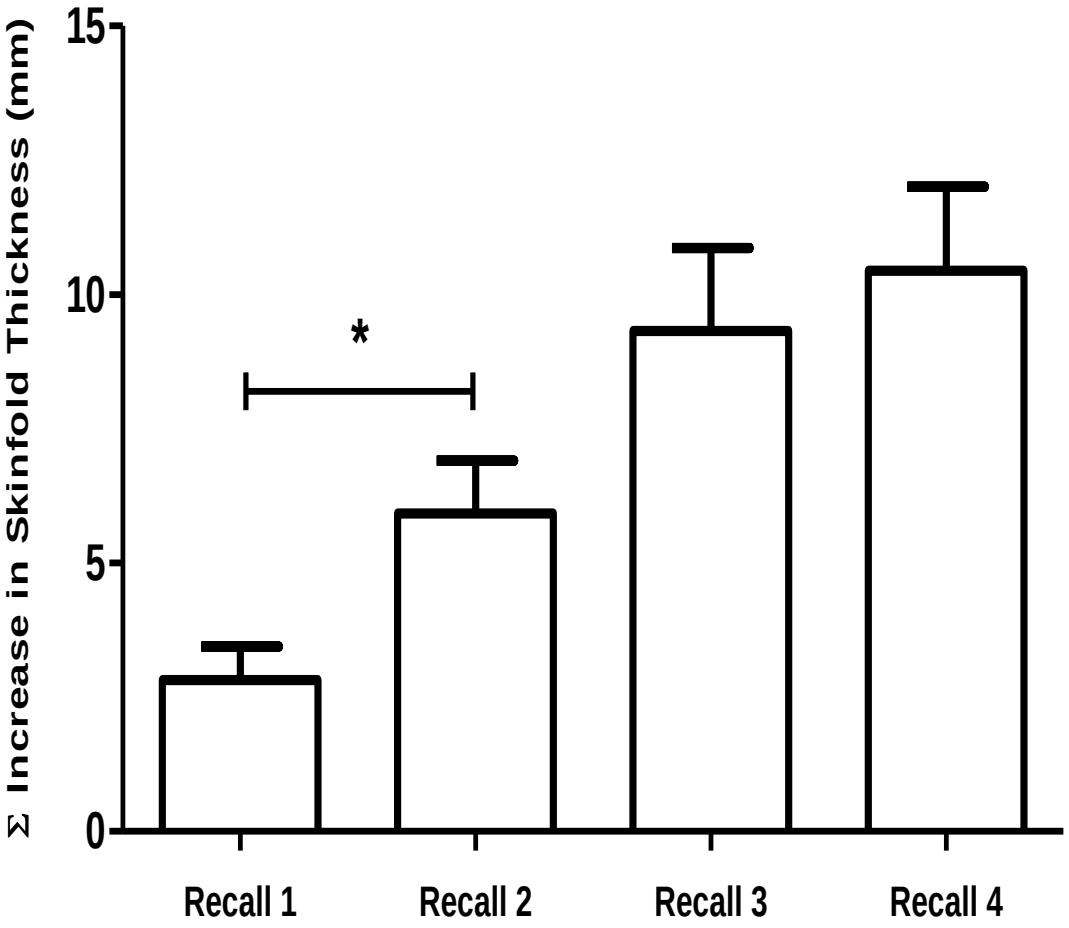

Figure 5.1 Augmentation of responses to DPCP by repeated monthly recall challenges. Pre-sensitized participants received repeated recall challenges with DPCP (5 doses) at four week intervals. Responses measured at 48 h as increase in skin-fold thickness relative to an acetone background control site, were summed (Σ) for the 5 challenge sites. Columns are means; Error bars are SD; * $P < 0.05$, significant difference between recall challenges.

5.3.3 Study Two: The effect of prior exercise on elicitation of antigen-specific immune memory

Nine of the participants from the pilot study, which measured the change of response with repeated challenges, volunteered to participate in Study Two. Four weeks after the final pilot-study challenge with DPCP (fourth elicitation challenge), in a repeated-measures cross-over design, participants completed the first (EX or CON) of two trials, and completed the other trial a further four weeks later, in a randomized order. Each trial was followed 20 minutes later by a fifth and sixth elicitation challenge respectively with DPCP. The elicitation patches were applied as in Study One, except that the top two DPCP doses were substituted by two lower dose patches (10 µl of DPCP: 0.00186%, 0.484 $\mu g \cdot cm^{-2}$; 0.00298%, 0.774 $\mu g \cdot cm^{-2}$). This modification was made to avoid excessively strong blistering reactions observed in a few high responders at the highest doses (due to the boosting of reactivity induced by the repeated challenges) in the pilot study. The alternating order of arms receiving the elicitation patches was continued from the pilot study. Assessments of CHS responses were taken at 24 and 48 h post-challenge (utilizing the same procedure as in Study One and the pilot study).

Sample Size. An estimation of sample size for Studies One and Two was calculated based on published formulae (Hopkins, 2000). Skin-fold thickness was the primary measurement variable, and thus the sample size calculation was obtained utilizing values for the graded division of the skin-fold callipers (0.20 mm) and the test-retest reliability typical error (0.18 mm) from a concurrent assessment of the reliability of this method. Typical error of 0.20 mm or less in the measurement meant that a sample size of two groups of sixteen in the cross-sectional designed Study One and a sample size of between eight and ten in the cross-over designed Study Two was sufficient for good statistical precision.

Statistical Analyses. The data from the pilot study were analysed using repeated-measures ANOVA. In Study One and Study Two, two-factor (trial x time) repeated measures ANOVA were used to analyse differences in the summed increase in responses to DPCP between EX and CON trials and two-factor (trial x dose) ANOVA were used to analyse the differences in dose responses to DPCP. The 95% confidence intervals (CI) of the differences were calculated where appropriate.

5.4 Results

5.4.1 Study One: The effect of prior exercise on induction of antigen-specific immune memory

5.4.1.1 Assessment of CHS responses

The experimental group (EX) sensitized with DPCP immediately after running on a treadmill for 2 h gave significantly reduced responses to challenge with a dose series of DPCP, four weeks post-sensitization compared with the group sensitized after a 2 h seated rest period (CON). The responses (skinfold thickness) summed from five challenge doses, revealed a significant trial x time interaction ($F(1,30) = 4.9$, $P < 0.05$). An overall main effect of trial ($F(1,30) = 13.0$, $P < 0.01$) on summed skinfold responses, demonstrated that CON responses were significantly greater than EX. Summed skinfold responses also increased significantly over time ($F(1,30) = 24.8$, $P < 0.0001$), with 48 h responses greater than the responses at 24 h. Bonferroni *post hoc* analyses revealed that the summed response to DPCP was significantly less (48%) in EX compared with CON at 24 h ($P < 0.05$; 95% CI of the difference, 0.05 to 2.96; **Figure 5.2A**) and a significant, 53% less than CON at 48 h ($P < 0.001$; 95% CI of the difference, 1.20 to 4.10; **Figure 5.2A**). Similarly, the summed erythema responses also revealed a significant trial x time interaction ($F(1,30) = 4.5$, $P < 0.05$). An overall main effect of trial ($F(1,30) = 14.4$, $P < 0.01$) on summed erythema responses also demonstrated that CON responses were significantly greater than EX. Summed erythema responses also increased over time ($F(1,30) = 55.4$, $P < 0.0001$), with 48 h responses greater than the responses at 24 h. Bonferroni *post hoc* analyses revealed that the summed response to DPCP was significantly reduced (60%) in EX compared with CON at 24 h ($P < 0.01$; 95% CI of the difference, 7.88 to 38.79; **Figure 5.2B**) and 46% reduced at 48 h ($P < 0.001$; 95% CI of the difference, 9.98 to 40.88; **Figure 5.2B**).

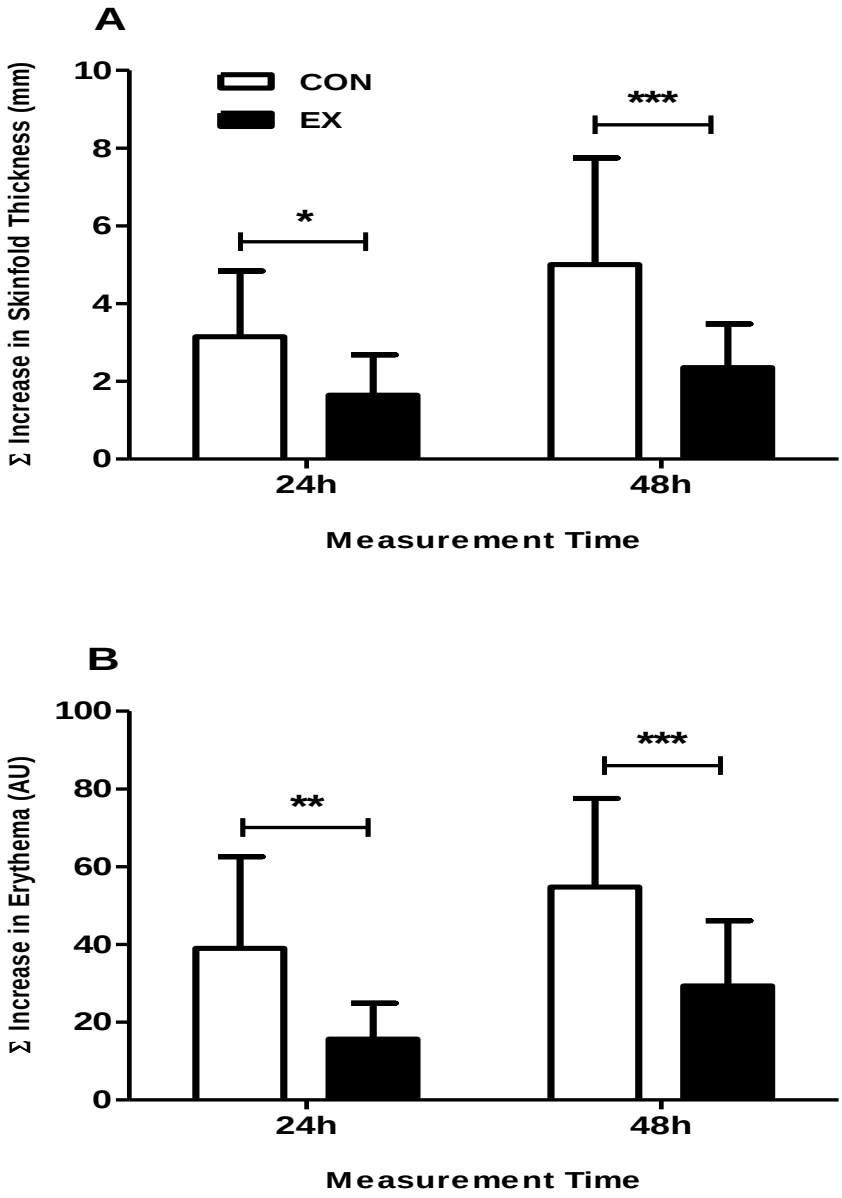

Figure 5.2 Exercise prior to the induction of contact sensitivity with DPCP results in reduced responses to elicitation challenge with the antigen DPCP. In participants sensitized with DPCP immediately after exercise-stress (EX), the inflammatory swelling (A) quantified as increase in skinfold thickness and the redness (B) quantified as increase in erythema, to five elicitation dose challenges with DPCP four weeks later, were significantly reduced, compared with the responses elicited from the control (CON) participants sensitized immediately after a period of seated rest. N = 16 per group; Columns are the means of summed responses (Σ) of the five doses of DPCP; Error bars are SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, significant difference between groups at that measurement time.

The reactivity to DPCP of each individual and each group was assessed over the full dose range of DPCP. This assessment was used to determine the dose response curve for each group at the 48 h peak response for increase in skinfold thickness (**Figure 5.3A**) and erythema (**Figure 5.3B**). Logarithmic transformation of the data allowed for the calculation of the x intercept when y = 0, utilizing linear regression on the linear portion of the dose response curve. By back transformation (anti-log), a threshold dose for a response to DPCP could be calculated for both CON and EX groups. The linear regression calculation indicated that the x-intercept when y = 0 (the threshold for a positive response), was 0.53 and 1.38 $\mu g \cdot cm^{-2}$ for the CON and EX groups respectively. This meant that for the EX group, a dose 2.58 times greater was necessary in order to elicit a positive response, in comparison to CON.

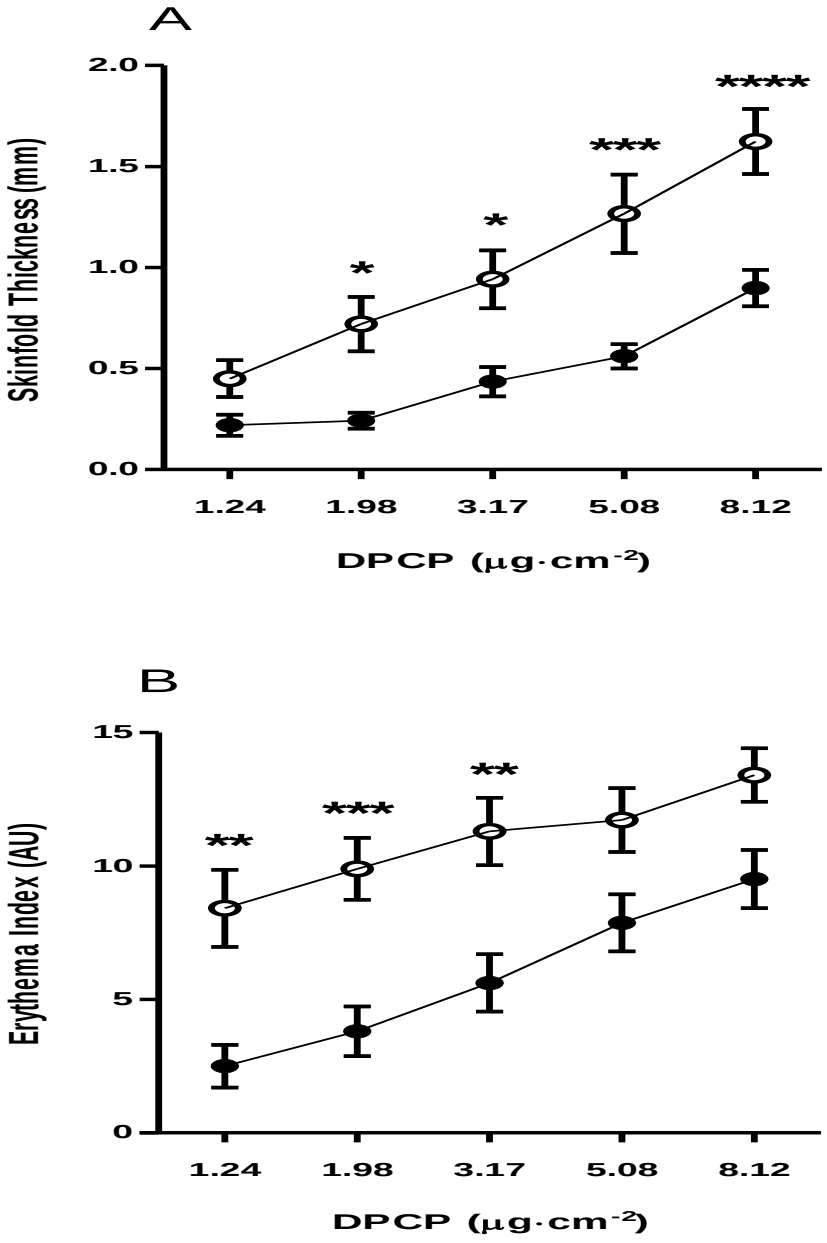

Figure 5.3 Exercise prior to the induction of contact sensitivity with DPCP results in reduced responses to elicitation challenge with the antigen DPCP. In participants sensitized with DPCP immediately after exercise-stress (●), the 48 h inflammatory swelling (A) (increase in skinfold thickness) and redness (B), (increase in erythema) to challenge with DPCP four weeks later were both significantly reduced compared with responses from the control participants (○). N = 16 per group; Points are means; Error bars are SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **** $P < 0.0001$, significant difference between groups at that dose per unit area.

5.4.2 Study 2: The effect of prior exercise on the elicitation of antigen-specific immune memory

5.4.2.1 Assessment of CHS elicitation responses

In participants with established immune reactivity to the antigen DPCP, the performance of moderate exercise-stress immediately prior to the administration of an elicitation challenge had a significant reductive effect on the recall response to DPCP. The inflammatory responses to challenge with five doses of DPCP were measured as increase in skinfold thickness and the readings for the five challenge sites were summed to approximate the area under the curve. The responses (skinfold thickness) summed from five challenge doses, revealed a significant trial x time interaction ($F(1,8) = 5.4$, $P < 0.05$). An overall main effect of trial ($F(1,8) = 17.5$, $P < 0.01$), demonstrated that CON responses were significantly greater than EX. Summed skinfold responses interestingly decreased over time ($F(1,8) = 15.55$, $P < 0.01$), with 24 h responses this time greater than the responses at 48 h. Bonferroni *post hoc* analyses revealed that the summed skinfold response to DPCP challenges performed immediately after exercise were reduced, 17%, at 24 h ($P < 0.05$; 95% CI of the difference, 1.16 to 4.98; **Figure 5.4**) and 19% at 48 h ($P < 0.05$; 95% CI of the difference, 0.99 to 3.32; **Figure 5.4**) in comparison to challenges in the same participants performed after the CON seated resting period.

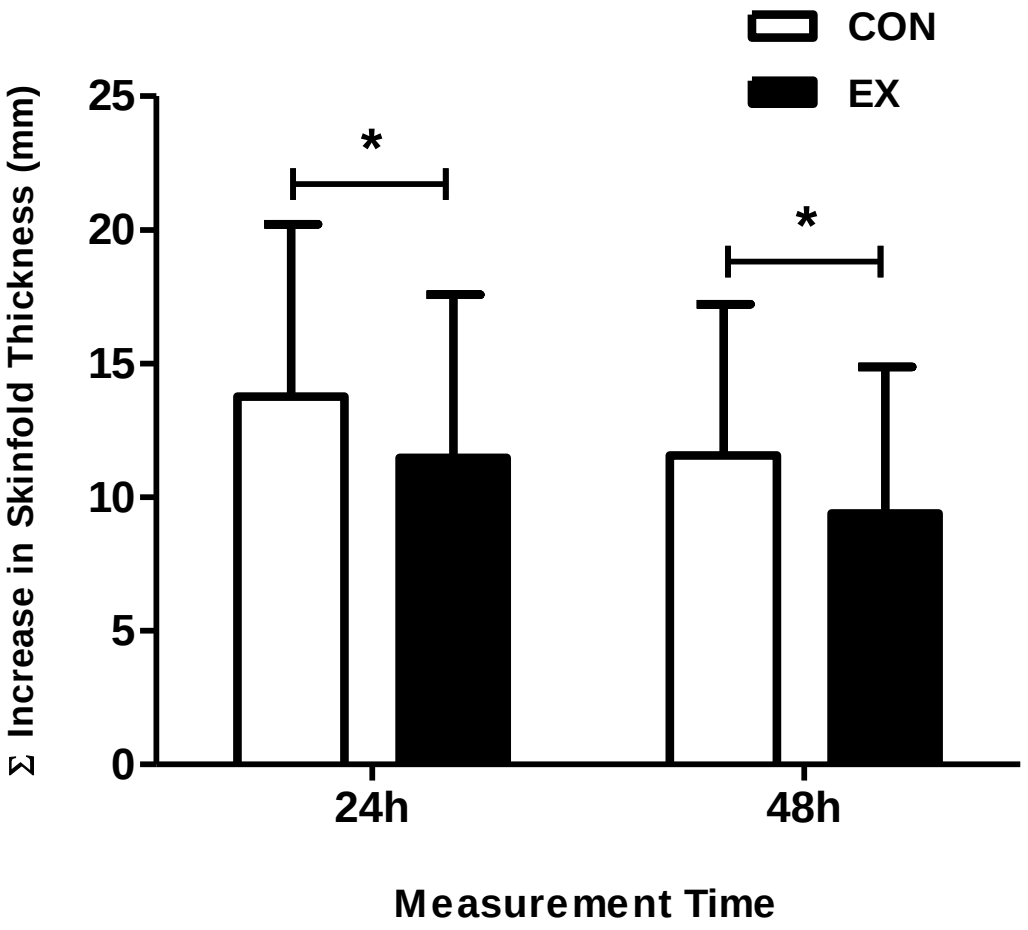

Figure 5.4 Exercise prior to elicitation challenge with DPCP results in reduced oedema response. In participants challenged with the DPCP immediately after the exercise-stress trial (EX), the inflammatory swelling (skinfold thickness) relative to a background control measure at both 24 h and 48 h was significantly reduced compared with the control trial (CON) challenged immediately after a period of seated rest. N = 9; Columns are the means of summed responses (Σ) at five doses of DPCP; Error bars are SD. * $P < 0.05$, significant difference between trials at that measurement time.

As in Study One, the reactivity to DPCP following each trial condition was assessed as increase in skinfold thickness over the full dose range of DPCP, at both the 24 h (**Figure 5.5A**) and 48 h response measurement times (**Figure 5.5B**).

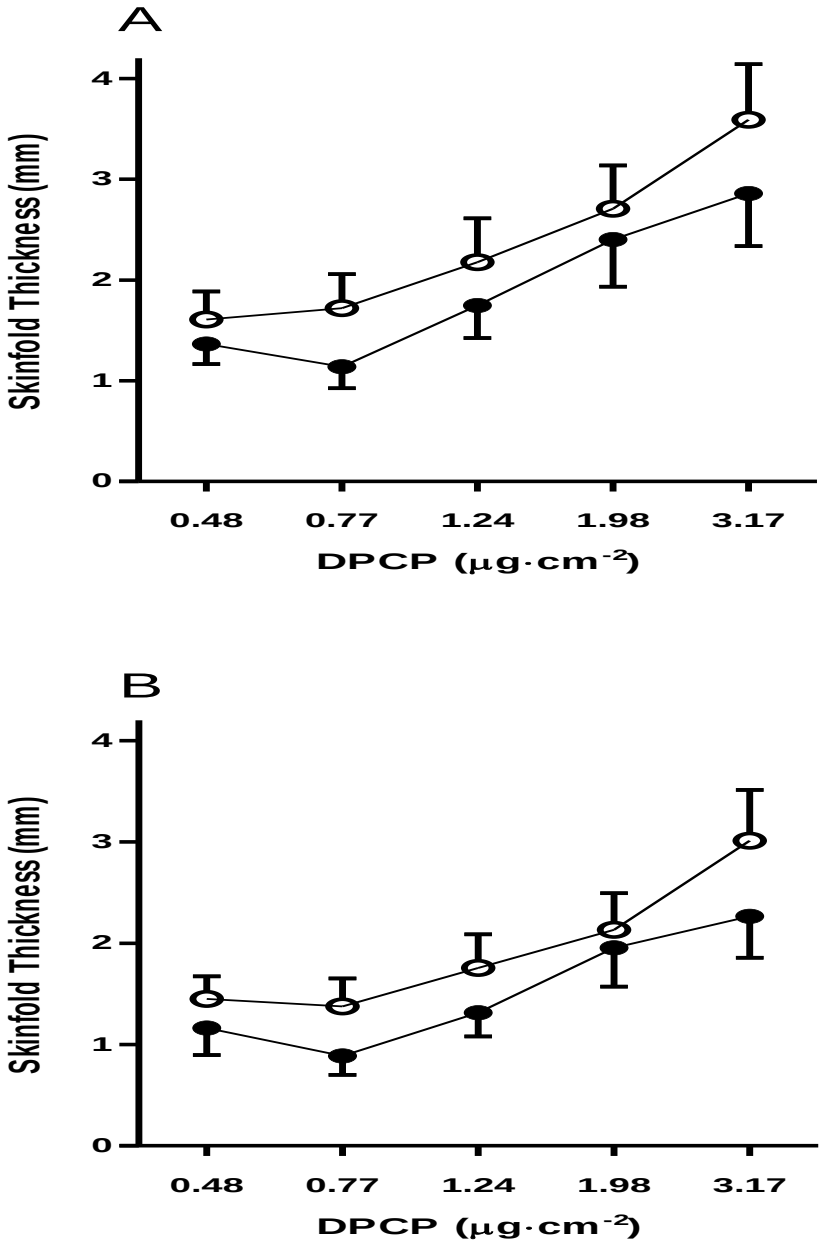

Figure 5.5 Exercise prior to elicitation challenge with DPCP results in reduced oedema over the entire dose range. In participants receiving a recall challenge with five doses of DPCP immediately after exercise-stress (●), the inflammatory swelling, quantified as increase in skinfold thickness, were significantly reduced compared with responses elicited during the control trial (○) at both **A.** 24 h (main effect of trial: $F(1,8) = 7.7$, $P = 0.024$) and **B.** 48 h (main effect of trial: $F(1,8) = 15.8$, $P = 0.004$) post-challenge. Participants had previously been sensitized to DPCP and received four monthly recall challenges with the antigen in a pilot study. N = 9; Points are means; Error bars are SEM.

5.5 Discussion

Using the method of experimental contact sensitization, this study is the first to show, in line with the hypotheses, that one single bout of prolonged moderate-intensity exercise impairs both the induction (-53% at 48 h: Study One) and elicitation (-19% at 48 h: Study Two) phases of the cell-mediated *in vivo* immune response in humans. These findings demonstrate that the induction component of new immune responses is more sensitive to exercise-stress-induced modulation than the elicitation component of T-cell-mediated memory responses.

The approach of using experimental contact sensitization gives the investigator a number of quantitative advantages: first, it allows precise control of the initial sensitizing exposure to an antigen never previously encountered by the host, so that experimental manipulations and procedures can be targeted at the induction phase of the response (Study One). Second, once clinical contact hypersensitivity is present, as reflected by the primary allergic response at the induction site, the strength of reactivity, or of sensitization, can be quantified relatively non-invasively by measurement of the inflammatory responses elicited by challenge with a dose series of the same antigen. Third, because of the relatively homogeneous responses of normal humans to these potent sensitizers, statistically robust data can be obtained with relatively small groups (Cohen *et al.*, 2001). The sensitizing stimulus, a concentration of 0.125% DPCP (30 $\mu g \cdot cm^{-2}$) was chosen as it was most likely to induce sensitization in all participants (Cooper *et al.*, 1992; Palmer and Friedmann, 2004; Sleijffers *et al.*, 2001) and was on the linear part of the sensitizing dose-response curve (Friedmann *et al.*, 1983). If an excessively powerful stimulus was used, that induced maximal sensitization and put responses on to the plateau of the dose-response range, subtle modulation of immune responses might not have been detected (Friedmann, 2006). The method has also allowed the inhibitory effects of prolonged moderate-intensity exercise-stress on the expression of pre-existing T-cell-mediated immune memory

responses (Study Two: **Figure 5.4**) to be demonstrated. A within-subject crossover design to obtain paired data comparing the expression of CHS after exercise-induced stress or rest in the same participants was chosen. Since this approach required consecutive re-exposures to DPCP, and it was known from previous work that the first re-exposure would boost reactivity at the second re-exposure, it was first necessary, in a pilot study, to perform serially repeated challenges with DPCP until responsiveness stabilized and became repeatable. This gave further data about the temporal relationships in human immune responses, due to repeated antigenic challenge, which had not previously been defined. In the pilot study, it was found that by the third challenge, given three months after the initial sensitizing exposure, reactivity had increased significantly and had reached a plateau (**Figure 5.1**). Hence, reproducible responses could be obtained at subsequent similar challenges indicating that the CHS method has utility in repeated-measures crossover designed studies.

Previous studies indicate that heavy exercise-stress is associated with a reduction in anti-microbial immune defences (Gleeson, 2007; Walsh *et al.*, 2011b) and an increase in self-reported number and severity of respiratory infections (Nieman, 1994; Peters and Bateman, 1983). A powerful CHS method was utilized to show that prolonged moderate-intensity exercise-stress has highly significant inhibitory effects on both the induction phase (-53%) and elicitation phase (-19%) of the cell-mediated *in vivo* immune response. Given the important role of T-cell-mediated immunity in anti-microbial protection, these results might explain, at least in part, the apparent increase in reported infections (particularly respiratory tract infections) in those undergoing heavy exercise stress. In Study One, where exercise took place immediately prior to the primary sensitization with DPCP it was demonstrated that in the EX group a threshold DPCP dose 2.58 times greater was necessary to evoke a similar elicitation response to that observed in the CON group. This indicates that the exercise-induced stress has attenuated the sensitisation by DPCP to the same extent as reducing the sensitising dose 4 to 6 fold

(Friedmann and Pickard, 2010). By contrast, exercise-induced stress immediately prior to elicitation of a pre-existing immune memory response had a much smaller inhibitory effect (Study Two). Thus, the challenge dose required to elicit a similar response to that in rested (CON) subjects was about 40% greater. The greater susceptibility of the induction phase of the cell-mediated immune response to suppression by prolonged moderate-intensity exercise-stress is in keeping with the greater suppression of induction seen with psychological stress (Smith *et al.*, 2004).

It is recognized that a limitation of the present study is that the mechanism(s) underlying the exercise-stress-induced reduction of *in vivo* T-cell-mediated immunity were not explored. It is speculated that there will be stress-related endocrine effects involving hormones such as gluco-corticoids (cortisol), catecholamines (e.g. adrenaline), pituitary/hypothalamus-derived neuropeptides (α-melanocyte stimulating hormone, prolactin, ACTH, or corticotrophin releasing hormone) (Dhabhar, 2002b; Dhabhar and McEwen, 1999), which could affect both phases of the immune response. The components of the immune system upon which the relevant mediators may act during the induction of immune memory might include dendritic cells and Langerhans cells in their role as processors and presenters of the immunogen, their interaction with T-cells in the regional lymph node or even their trafficking and migration to the lymph node. Stress-induced damage or disruption to the function of dendritic and Langerhans cells could cause T-cell clonal anergy and alterations in T_H2/T_H1 responses with a propensity for T_H2 expansion via negative signaling pathways, affecting the cutaneous immune response (Araneo *et al.*, 1989; Cooper *et al.*, 1992; Smith *et al.*, 2004). The greater reduction in the induction of immune memory (-53%) compared with the elicitation phase (-19%) is likely due to prolonged exercise-stress having a greater disruptive effect on antigen presentation and development of T-cell immune memory, but this remains unsubstantiated. The components of the immune system affected by prolonged exercise-stress during the elicitation phase might include antigen

presentation by Langerhans cells, antigen specific T-memory cell recognition, antigen specific T-cell clonal expansion (T-helper, T-cytotoxic and T-regulatory cells) and disruption of T-cell subset stimulation, action and secretion of cytokines (Edwards *et al.,* 2006). Further studies are required to unravel the mechanism(s) responsible for the observed reduction *in vivo* T-cell-mediated immunity with prolonged exercise stress.

In summary, this is the first demonstration, using the method of experimental contact sensitization, that a single bout of prolonged moderate intensity exercise-stress significantly reduces both the induction as well as the elicitation of *in vivo* cell-mediated immunity in humans. Clinical repercussions of the exercise-induced reduction in *in vivo* cell-mediated immunity include a potential increased risk of infection. These results also indicate that experimental contact sensitization with DPCP provides a simple, inexpensive, and robust method for research investigators to evaluate the effects of other forms of stress on both induction and elicitation of *in vivo* cell-mediated immunity. Furthermore, the data show that a plateau in CHS response can be attained with repeated serial administration of the antigen, allowing utilization of the CHS method in repeated-measures, cross-over designed research investigations.

CHAPTER SIX

Two nights of total sleep deprivation, with or without energy restriction, does not increase the risk of hypothermia during prolonged cold exposure in humans

6.1 Summary: The current study tested the hypotheses that 53 h sleep-deprivation (SDEP) impairs human thermoregulation during a cold-air test (CAT) and that energy-restriction (ER) has additive, detrimental effects, heightening hypothermia risk. In a randomized, repeated-measures design, ten healthy males (means, (SD): age 25 y, (6); body fat 17%, (5)) were monitored during CAT under three conditions, 12 d apart. Trials began at 0800 h with 53 h control (CON; sleep = 436min·night^{-1}), 53 h SDEP (sleep = 0 min·night^{-1}) or 53 h SDEP+ER (sleep = 0 min·night^{-1}; ER = 90%). Energy and fluid intake requirements were estimated during familiarizations. Semi-nude, participants began the CAT (0°C; 40%RH), 53 h into trials in a seated, resting position until rectal temperature (T_{re}) reached 35.9°C. Skin temperatures (T_{sk}) were recorded every 5 min. Expired gas was analysed every 10 min to determine metabolic heat production (M). Time to 35.9°C T_{re} tended (P = 0.06) to be longer on SDEP (153.3 min, (65.3)) than CON (116.4 min, (38.7)). T_{re} and mean weighted T_{sk} were not significantly different between trials (P > 0.05). The relationship between mean body temperature (T_b) and M indicated delayed shivering onset during SDEP (35.66°C, (0.64)) and SDEP+ER (35.82°C, (0.95)) versus CON (36.38°C, (0.65)) and higher M during SDEP and SDEP+ER required to maintain equivalent T_b during CAT. Plasma nor-adrenaline was higher (P = 0.03) during SDEP+ER than CON. In conclusion, 53 h total SDEP, with or without ER, did not significantly impair thermoregulation during cold exposure. Nevertheless, the relationship between M and T_b indicated potential underlying disturbances during prolonged whole body cooling. Two-nights SDEP with or without ER does not appear to increase the risk of hypothermia during prolonged cold exposure.

6.2 Introduction

Military personnel, ultra-endurance athletes, mountaineers and search and rescue teams often find themselves subject to a combination of environmental, physical and psychological stressors as part of their normal routine. It has been indicated that sleep deprivation, energy restriction and physical exertion can negatively impact thermoregulation during prolonged cold exposure, which may predispose individuals to hypothermia (Castellani *et al.*, 2003; Kolka *et al.*, 1984; Young *et al.*, 1998). Although it has been suggested that the combination of sustained sleep deprivation, energy restriction and fatiguing exercise affect normal thermoregulation during cold exposure (Castellani *et al.*, 2003; Young *et al.*, 1998), the relative influence of, and interaction between individual stressors is still unclear. It is possible that each of the individual stressors has a different degree of input, that the effects are synergistic, additive, or that some of the stressors have only a negligible effect on thermoregulation. The aforementioned multi-stressor studies (Castellani *et al.*, 2003; Young *et al.*, 1998) were performed on participants who had been in field training and operations, over a prolonged period of time and their thermoregulatory responses were then tested during laboratory based cold exposure. In the field, there could be little control over potential confounding variables such as hydration, nutritional status and relative exercise intensity. Trials were also always performed in the same order, a distinct design weakness (Castellani *et al.*, 2003; Young *et al.*, 1998). This makes it difficult to discern the individual effect and contribution of each of the joint stressors on thermoregulation in the cold. Furthermore, in certain of these studies (Young *et al.*, 1998) the 61 d U.S. Army Ranger training induced significant decreases in body mass (7.5 kg) and body fat (10%), which would have affected thermoregulatory responses to the cold, primarily by decreasing insulation and increasing conductive heat loss from the body core to the body surface shell (Young *et al.*, 1998). The few studies that have attempted to examine the independent effect of individual stressors on thermoregulation during cold exposure have presented equivocal conclusions as to whether an outcome of increased hypothermia risk would be evoked (Castellani *et al.*, 1999;

Castellani *et al.*, 2001; Haman *et al.*, 2004; Landis *et al.*, 1998a; Savourey and Bittel, 1994). It would therefore be of interest to ascertain the individual effect of sleep deprivation on thermoregulation during prolonged cold exposure, and whether energy restriction has synergistic, additive, detrimental effects that increase the risk of hypothermia, by utilizing a randomized, repeated measures design in a controlled laboratory setting.

Human core temperature displays a sinusoidal circadian rhythmicity, with peaks during the day and nadirs at night. The nocturnal thermoregulatory cascade (i.e. decrease in heat production and increase in heat loss which leads to a decrease in core temperature) commences in the early evening, prior to sleep onset (Van Someren, 2006). Interestingly, it has been suggested that even during periods of sleep deprivation, this rhythmic circadian modulation of skin blood flow and resting metabolic heat production is still maintained, with only limited blunting (Kräuchi, 2002; Kräuchi and Wirzjustice, 1994). Thus, as ambulatory sleep deprivation precludes the implementation of behavioural thermoregulatory measures (e.g. increased bedding for insulation and curling up), the normal nocturnal increase in skin temperature associated with the thermoregulatory cascade is likely to cause cumulative heat loss (Van Someren, 2006). This increased heat debt from prolonged sleep deprivation may cause a progressive lowering of core temperature, even in the presence of increased energy intake (Rechtschaffen *et al.*, 2002) and therefore increase the risk of hypothermia in cold conditions (Landis *et al.*, 1998a; Savourey and Bittel, 1994; Young *et al.*, 1998).

Negative energy balance during periods of cold exposure has itself been identified as a potential factor limiting shivering thermogenesis (Jacobs *et al.*, 1994; Wissler, 1985). Insufficient fuel supplies for heat production to offset the increases in heat loss associated with sleep deprivation are likely to amplify the susceptibility to hypothermia in a combined stressor state. Cold

exposure and sleep deprivation are both stressors that can also affect thermoregulation via alterations of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Benzinger, 1969; Kioukia-Fougia *et al.*, 2002). As the Pre-Optic Anterior Hypothalamus is the central controller of thermoregulation, disruptions in the HPA axis can lead to modifications in neuro-endocrine responses that can then affect thermoregulation and even fuel utilization (Benzinger, 1969; Kioukia-Fougia *et al.*, 2002). Intriguingly, studies which have investigated the effects of only one night of total sleep deprivation on the risk of hypothermia revealed no differences in thermoregulatory variables in comparison to control conditions (Caine-Bish *et al.*, 2004; Landis *et al.*, 1998a; Savourey and Bittel, 1994; Young *et al.*, 1998). The influence of two-nights of sleep deprivation on thermoregulation has great practical relevance to military personnel, ultra-endurance athletes, mountaineers and search and rescue teams as these individuals are often subjected to such durations of sleep loss. As one-night of total sleep deprivation did not appear to affect thermoregulation in previous studies (Caine-Bish *et al.*, 2004; Landis *et al.*, 1998a; Savourey and Bittel, 1994; Young *et al.*, 1998), it is possible that a more prolonged period of sleep deprivation may be necessary to elicit significant and consistent findings, and fill this void in the literature.

With this information in mind, the purpose of the present study was to examine the effects of 53 h total sleep deprivation and 53 h total sleep deprivation with energy restriction on thermoregulatory responses to subsequent prolonged cold exposure, in a controlled laboratory setting. In this setting, the effects of sleep deprivation could be isolated and with the addition of energy restriction in a separate trial, it could be determined whether the multi-stressor scenario of additional energy restriction would pose an amplified risk of hypothermia. It was hypothesised that two days (53 h) of total sleep deprivation would impair thermoregulatory responses to a cold-air test and that these impaired responses would be magnified with the addition of energy restriction, predictive of an increased risk of hypothermia.

6.3 Methods

Participants

Ten healthy, recreationally active males (means, (SD): age 25 years, (6); height 178 cm, (4); nude body mass (NBM) 79 kg, (9); body fat 17%, (5); $\dot{V}O_{2peak}$ 57 $ml \cdot kg^{-1} \cdot min^{-1}$, (8)) volunteered to participate in the study.

Preliminary Testing

Prior to the main experimental trials, a familiarization was performed. Participants were transported to the laboratory for the familiarization and arrived at 0800 h following an overnight fast. Subsequent to bowel and bladder voiding, participants' height, NBM (Seca 705, Hamburg, Germany) and body composition by dual-energy x-ray absorptiometry (QDR1500, Hologic QDR1500, Bedford, MA, USA) were obtained. 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 and Micklewright, 2004). Participants were then familiarized with a 20 min cold-air test (CAT), which was conducted in an environmental chamber (WIR Series, Design Environmental Ltd., Ebbw Vale, UK) set at 0.0°C and 40% relative humidity (RH).

After a 30 min rest period, peak oxygen uptake ($\dot{V}O_{2peak}$; Cortex Metalyser 3B, Biophysik, Leipzig, Germany) was estimated by means of a continuous incremental exercise test to volitional exhaustion on a motorized treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany), based on a standard protocol (Bruce, 1977a), as detailed in **Chapter 3**. The treadmill walking speed (6.0 $km \cdot h^{-1}$, (0.5)) and gradient (6.9%, (2.0)) that elicited 50% $\dot{V}O_{2max}$, for the participants' steady state exercise during trials, was then extrapolated and verified. In addition, each participant completed a time trial (TT) familiarization. Blinded to the treadmill speed,

participants controlled the speed of the motorized treadmill, set at a 1% gradient, and were instructed to run the 5 km as quickly as possible. Estimated energy expenditure for steady state exercise and the TT was predicted by indirect calorimetry (Weir, 1990), and was combined with the adjusted RMR data to predict total daily energy expenditure and subsequently used in energy intake prescription for the experimental trials.

Experimental trials

In a randomized, repeated-measures design, participants completed three experimental trials separated by twelve days (**Figure 6.1**). The three experimental trials involved 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 the CON trial (436 min·night^{-1}, (20)), but were totally deprived of sleep on SDEP and SDEP+ER trials for 53 h (0 min·night^{-1}). Dietary intake was provided to meet predicted total daily energy requirements (13.9 MJ, (0.7); carbohydrate 54%, (2), fat 32%, (1), protein 14% (1)) during CON and SDEP, with energy intake restricted by 90% (to mimic consignment of rations during prolonged survival during military operations or prolonged accidental environmental exposure) for 53 h during the SDEP+ER trial. Water was provided throughout each experimental trial at 2h intervals (divided equally over 16h; 0700 h to 2300 h), equivalent to 35 ml·kg^{-1}NBM·day^{-1} (2785 ml·day^{-1}, (300)); (Todorovic and Micklewright, 2004), with estimated fluid losses during exercise added to this total. Daily physical activity was monitored via electronic pedometers (Digi-walker SW-200, Yamax, Tokyo, Japan) and the participants' sleep-wake cycles were monitored by accelerometry (GT1M, ActiGraph LLC, Florida, USA); (Ancoli-Israel *et al.*, 2003). Participants remained in the laboratory building, complete with living quarters, for the entire duration of experimental trials and were supervised at all times. NBM measurements were taken at 0 h, 24 h, 48 h, pre-CAT, post-CAT, 1 h and 2 h post-CAT.

PRE-TRIAL DAY	CONTROLLED PHYSICAL ACTIVITY, DIETARY 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	**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	**SAMPLES**	**BREAKFAST**	**SAMPLES**	**CAT (53h)** **T_{amb} 0.0°C + 40% RH**	**T_{re}: 35.9°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

Figure 6.1 Schematic representation of timing schedule from the day prior to experimental trials, through day three of experimental trials (CON, SDEP and SDEP+ER).

Experimental procedures

The day prior to each experimental trial, participants were provided with rations equivalent to their predicted total daily energy and fluid intake requirements in order to standardize nutrition, hydration and sleep-wake cycles. The sleep duration on the night before trials was monitored by accelerometry (444 min·night^{-1}, (30)). After waking at 0700 h, participants were transported to the laboratory for each experimental trial. Upon arriving at the laboratory at 0800 h, following a 15 min seated rest period, a blood sample was collected, without venestasis, by venepuncture from an antecubital vein. Participants then voided their bladders into 24 h urine collection containers. Morning urine samples were used to confirm euhydration status based on published indices and procedures (Armstrong *et al.*, 1994). NBM was then recorded. Thereafter, blood and urine samples were collected at 24 h, 48 h, immediately pre-CAT, immediately post-CAT, 1 h post-CAT and 2 h post-CAT.

At 1200 h on days one (4 h) and two (28 h), participants performed a 90 min steady state walk at 50% $\dot{V}O_{2max}$, followed by a 15 min seated rest period and a 5 km TT (data not included in this manuscript). The exercise protocol was performed in an air-conditioned laboratory (20°C (1), 59% (7) RH) with a fan which generated a wind velocity of 2.3 m·s^{-1} placed one metre in front of the participant. Each TT was also performed under standardized conditions in a quiet laboratory, with only information about distance completed provided to the participants. Water was consumed *ad libitum* during the steady state walks in all trials. No fluids were allowed during the TT. Steady state exercise and time trial performance data has been published previously (Costa *et al.*, 2010).

Cold-Air Test

At 1100 h on day 3 (51 h) of all trials, participants were fitted with contact skin thermistors (Grant EUS-U thermistors; Grant 2020 Squirrel data logger, Shepreth, UK) affixed with tape to

the right side of the body at 4 sites (chest, thigh, biceps and calf). Participants inserted rectal thermistor probes, in privacy, 12 cm beyond the external anal sphincter (Grant REC soft insertion probe; Grant 2020 Squirrel data logger, Shepreth, UK) to monitor rectal temperature (T_{re}). Heart rate monitors (Polar Electro, Kempele, Finland) were also fitted at this time. After the pre-CAT blood draw, immediately prior to entering the environmental chamber, baseline skin temperatures and T_{re}, HR, as well as ratings of McGinnis 13 point (1 = so cold I am helpless to 13 = so hot I am sick and nauseated; 7 = comfortable) thermal comfort (Hollies and Goldman, 1977) and 10 point (1 = barely cool to 10 = unbearable pain) pain sensation to cold (Chen *et al.*, 1998) were recorded. These readings were taken after a 15 min seated rest period where participants were clothed only in athletic shorts, shoes and socks. A baseline 2 min resting expired gas sample was then collected into a 150 L Douglas bag and analysed.

In the same attire, at 1200 h (53 h), participants were assisted onto a standard wooden laboratory chair in the environmental chamber. Ambient conditions throughout the CAT were regulated at 0.0°C, 40% RH and 0.2 $m \cdot s^{-1}$ wind velocity. During the CAT, T_{re} was recorded at 5 min intervals. Skin temperatures, HR and Douglas bag expired gas samples were acquired at 10 min intervals, as were thermal comfort and pain sensation ratings. Participants were instructed to minimize any movement, including behavioural thermoregulation (i.e. huddling, fidgeting) during the CAT and assumed a standardized seated position, with hands on their knees. Participants remained in the environmental chamber until their T_{re} reached 35.9°C or they achieved the cut-off criteria of 4 h exposure.

As a health and safety precaution, recovery T_{re} was monitored and recorded every 5 min during the first hour after participants exited the environmental chamber. Throughout this 1 h recovery period, the participants were wrapped tightly in a blanket. Following the 1 h post-CAT recovery period, participants were allowed to shower, re-clothe and consume a standardized meal (5.15

MJ, 58% carbohydrate, 28% fat, 14% protein). After the 2 h post-CAT samples were taken, that trial was complete and participants were transported to their homes.

Calculations

Four skin surface temperatures were used to calculate mean skin temperature (mean T_{sk}) using an area-weighted 4-site equation, adjusted for regional proportions (Ramanathan, 1964):

$$Mean\ T_{sk} = (0.34 \cdot T_{chest}) + (0.33 \cdot T_{thigh}) + (0.18 \cdot T_{calf}) + (0.15 \cdot T_{\ biceps})$$

Mean body temperature (mean T_b) during the CAT was calculated based on the traditional two-compartment thermometry model (Burton, 1935), where x = 0.79 in a thermo-neutral environment, and x = 0.66 in the cold:

$$Mean\ T_b = x \cdot T_{re} + (1\text{-}x) \cdot T_{sk}$$

From the Douglas bag expired gas samples, values for respiratory exchange ratio (RER) and VO_2 were obtained and used along with body surface area (A_D) to estimate metabolic heat production (M) in $W \cdot m^{-2}$ during the CAT as follows (Gagge and Gonzalez, 1996):

$$M = (0.23[RER] + 0.77) \cdot (5.873[VO_2]) \cdot (60/A_D)$$

The balance between heat production and heat loss in $W \cdot m^{-2}$ was calculated by partitional calorimetry to determine heat debt (S) during cold exposure, using the following equation (Bittel *et al.*, 1989):

$$S = M – [W + L + E + K + (R + C)]$$

Here, W = work rate (0 as no exercise was performed). L = heat loss by respiration, convection and evaporation, equivalent to 0.08·M (Rennie *et al.*, 1962). E = evaporative heat loss [assumed to be constant at 4.1 $W \cdot m^{-2}$ in the cold, and limited to cutaneous perspiration (Boutelier *et al.*, 1982)]. K = heat loss by conduction (0 in this study). R + C = dry heat loss by radiation and convection, determined to be equivalent to $8.3 \cdot (T_{sk} - T_{amb})$, from previous calculations (Colin *et al.*, 1971).

Blood Analyses

Haematological parameters including haemoglobin, haematocrit, plasma volume change, glucose, cortisol, adrenaline and nor-adrenaline were measured as described in **Chapter 3**.

Statistical Analyses

Differences in pre-trial sleep duration, CAT exposure duration and relationships between thermoregulatory variables were analysed using repeated-measures analyses of variance (ANOVA). Repeated-measures two-factor (time × trial) ANOVA were used to examine differences in measurement variables during the duration of trials and during the CAT. Statistically significant differences were followed up by *post hoc* two-tailed Dunnett multiple comparison tests. The Dunnett t-test treats one data set as a control (reference) and compares all other data sets against it. Eta squared effect sizes (η^2) and 95% confidence intervals (95% CI) are presented for the main outcome variables of T_{re}, Mean T_{sk} and time to 35.9°C, where appropriate, with effect sizes interpreted as small (0.01), medium (0.09) or large (0.25) according to previous statistical prescriptions (Cohen, 1988). Additionally, Cohen's *d* effect size statistic (*d*) was calculated to examine the clinical significance of differences in exposure duration between trials. Cohen's *d* effect sizes can be interpreted as small (0.2), medium (0.5) and large (0.8) (Cohen, 1988), with a Cohen's *d* effect size statistic greater than 0.5 considered

to be clinically significant (Wolf, 1986). A sample size of ten participants was determined to have adequate statistical precision based on detectable core temperature differences in previous repeated measures design research examining the effects of sleep deprivation and energy restriction on thermoregulation in the cold (Castellani *et al.*, 2003; Young *et al.*, 1998). The method of least-squares linear regression was employed to determine relationships between thermoregulatory variables.

6.4 Results

6.4.1 Sleep Duration

The sleep duration on the night before trials, monitored by accelerometry, was not significantly different between trials ($P > 0.05$; pre-CON = 437 min·night^{-1}, (45), pre-SDEP = 446 min·night^{-1}, (21), pre-SDEP+ER = 449 min·night^{-1}, (20)). Sleep duration during CON trials averaged 432 min·night^{-1}, (25) on night one and 440 min·night^{-1}, (16) on night two. It was also confirmed from accelerometry data that during SDEP and SDEP+ER trials all participants endured 0 min·day^{-1} of sleep.

6.4.2 Body Mass

A trial x time interaction was observed for NBM ($P < 0.01$). NBM gradually decreased throughout SDEP+ER ($P < 0.01$ versus 0 h) whereas a decrease in NBM was only observed pre to post-CAT on CON and SDEP ($P < 0.01$ versus pre-CAT). NBM from 0800 h on day 1 (0 h) until immediately pre-CAT on day 3 decreased 0.04 kg, (0.38) on the CON trial, 0.34 kg, (0.50) on the SDEP trial and 2.84 kg, (0.52) on the SDEP+ER trial.

6.4.3 Thermoregulatory Responses

At 1200 h on day 3 (53 h), T_{re} immediately pre-CAT, was similar ($P > 0.05$; $\eta^2 = 0.17$) during all trials (CON: 37.18°C, (0.19); SDEP: 37.12°C, (0.15); SDEP+ER: 37.03°C, (0.14)). During the CAT, T_{re} was not significantly different ($P > 0.05$; $\eta^2 = 0.01$) between trials (**Figure 6.2A**). Pre-CAT mean T_{sk} were also similar between trials ($P > 0.05$; $\eta^2 = 0.13$), even though CON mean T_{sk} was 1.04 to 1.10°C higher than SDEP and SDEP+ER mean T_{sk} respectively, immediately pre-CAT. Mean T_{sk} was not significantly different ($P > 0.05$; $\eta^2 < 0.01$) between trials during the CAT (**Figure 6.2B**). There was also no significant difference in individual sites' T_{sk} ($P > 0.05$) between trials during the CAT. The thermal gradient (T_{re} - mean T_{sk}) during the CAT was not

significantly different between trials ($P > 0.05$). Heart rates showed no significant differences between trials during the CAT ($P > 0.05$). Thirteen-point thermal comfort ratings also showed no significant differences between trials during the CAT ($P > 0.05$), but subjective ratings of comfort decreased to the same extent with exposure duration on all trials from comfortable to severe cold discomfort (subjective rating = 7, (1) immediately pre-CAT to 2, (1) at CAT completion). Similarly, there were no differences in 10-point pain sensation ratings between trials ($P > 0.05$), although there was a tendency for the ratings to be the lowest in the CON trial ($P = 0.105$). Again, subjective ratings of pain sensation to cold did increase with cold exposure duration, from barely cool on all trials (subjective rating = 1, (0) immediately pre-CAT) to nearly unbearable pain (subjective rating = CON: 8, (2); SDEP: 7, (2); SDEP+ER: 8, (1) at CAT completion).

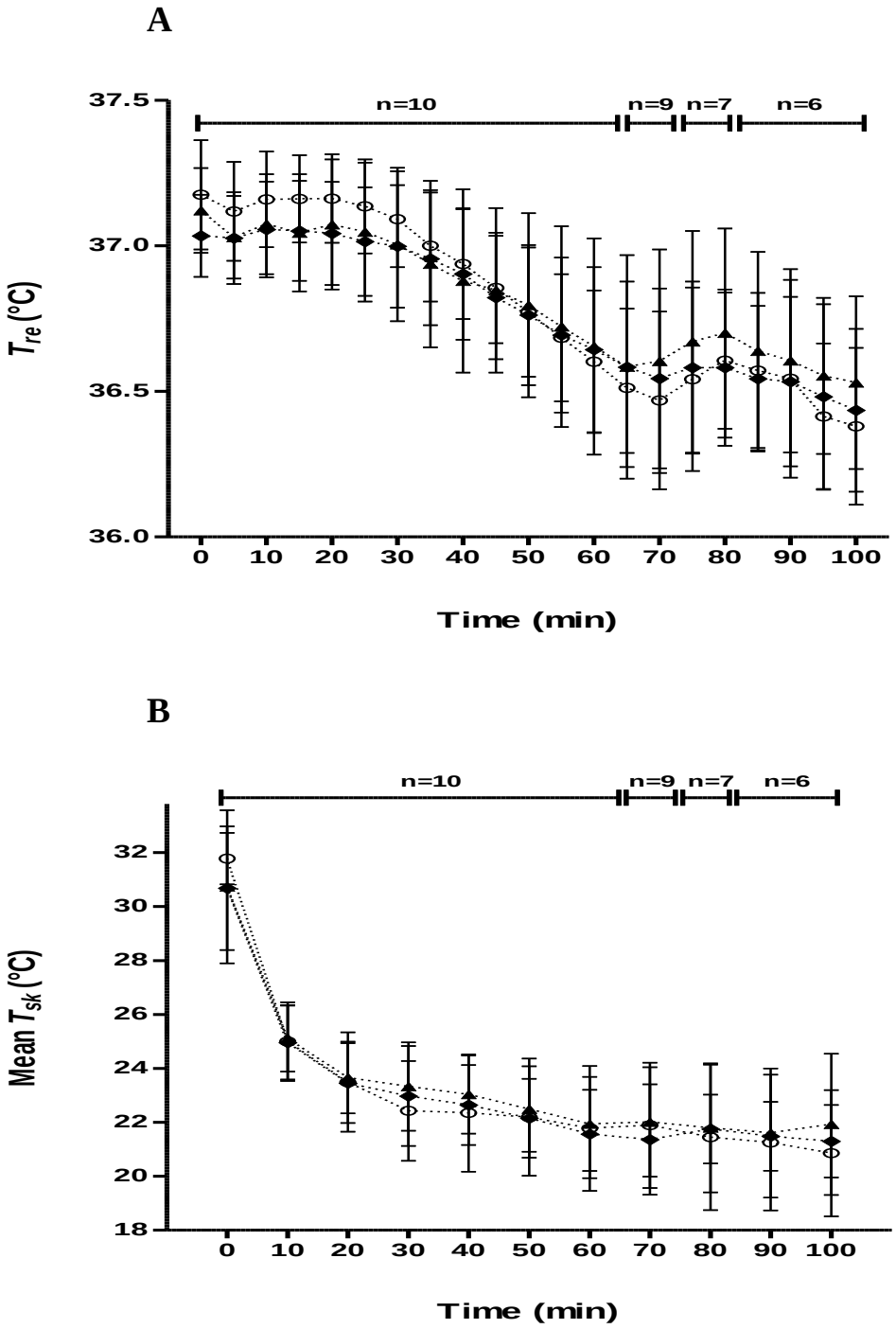

Figure 6.2 **A.** T_{re} against time for the first 100 min of the CAT. **B.** Mean T_{sk} against time for the first 100 min of the CAT, in an environmental chamber at 0°C and 40% RH following either 53 h of CON (○), SDEP (▲) or SDEP+ER (♦). Values are means, SD.

Time to reach 35.9°C T_{re} was not significantly different between trials (P = 0.063; η^2 = 0.27). However, it tended to take longer for participants' T_{re} to reach 35.9°C in both the SDEP trial (95% CI of the difference +1.7 to +71.9 min; d = 0.72) and the SDEP+ER trial (95% CI of the difference -21.9 to +48.4 min; d = 0.31) compared with the CON trial (**Figure 6.3**). Time to reach 35.9°C T_{re} was greater for nine out of the ten participants during the SDEP trial (153.3 min, (65.3); 95% CI +106.5 to +200.0 min) and eight of the ten participants during the SDEP+ER trial (129.7 min, (51.2); 95% CI +93.1 to +166.3 min) in comparison with the CON trial (116.4 min, (38.7); 95% CI +88.7 to +144.1 min).

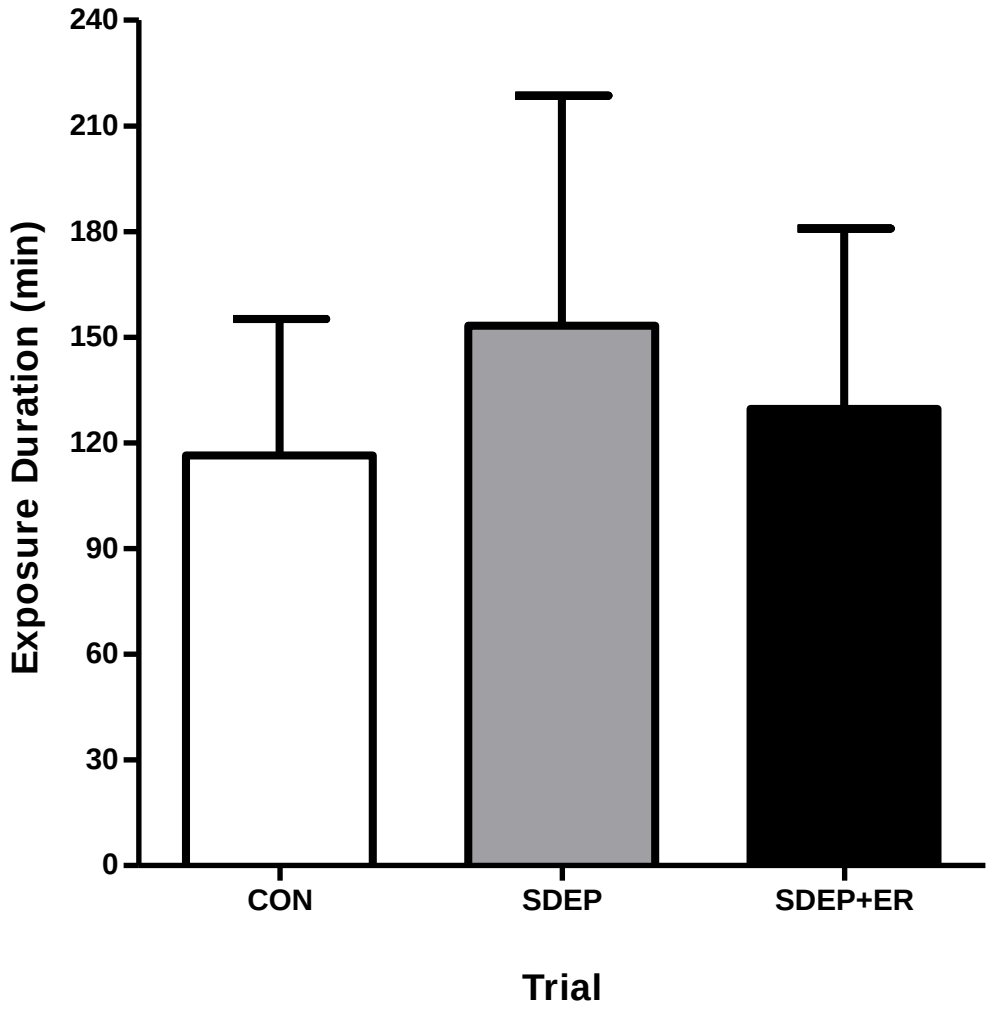

Figure 6.3 Time in minutes for participants to reach 35.9°C T_{re} during cold exposure in an environmental chamber at 0°C and 40% RH following a 53 h period of either CON, SDEP or SDEP+ER. Values are means, SD.

Metabolic heat production (**Figure 6.4A**), an indicator of shivering thermogenesis, was not significantly different between trials ($P > 0.05$). Individual expired gas responses, used in calculations of M, were not significantly different during the CAT, with comparable mean RER (CON: 0.89, (0.09); SDEP: 0.87, (0.07); SDEP+ER: 0.83, (0.13)) and mean VO_2 (CON: 648 $ml \cdot min^{-1}$, (253); SDEP: 669 $ml \cdot min^{-1}$, (272); SDEP+ER: 682 $ml \cdot min^{-1}$, (264)) between trials ($P > 0.05$).

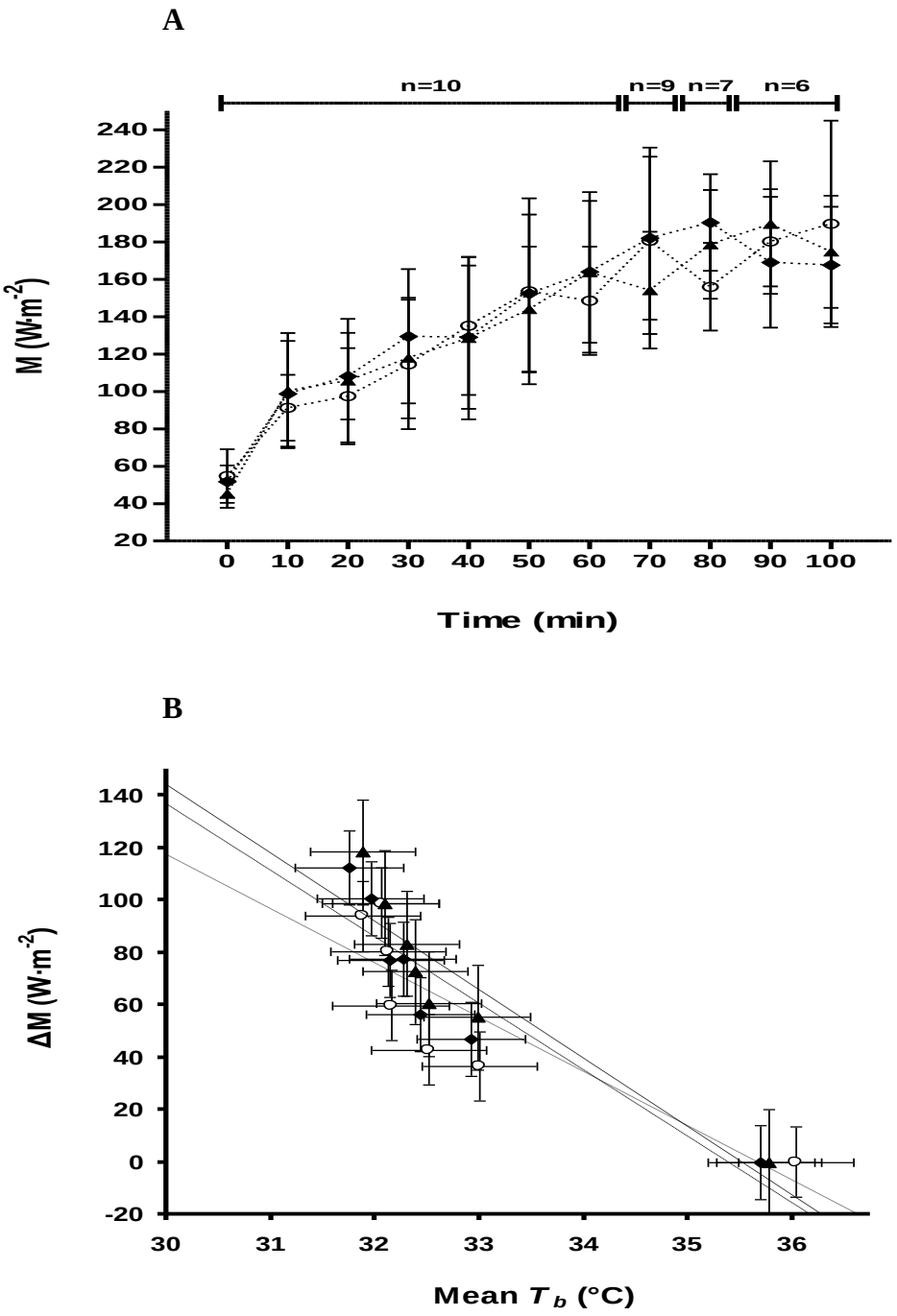

Figure 6.4 A. Metabolic heat production (M) against time for the first 100 min of the CAT; **B.** Change in Metabolic heat production (ΔM) against T_b, in an environmental chamber at 0°C and 40% RH following either 53 h of CON (○), SDEP (▲) or SDEP+ER (♦). R values for CON, SDEP and SDEP+ER linear regression were 0.86, 0.93 and 0.92 respectively. Values are means, SD.

The relationship between mean T_b and M (**Figure 6.4B**) indicated a delayed shivering onset (T_b when $\Delta M = 0$) during SDEP (35.66°C, (0.64)) and SDEP+ER (35.82°C, (0.95)) versus CON (36.38°C, (0.65)), where shivering tended to be initiated at a higher mean T_b, though these differences did not reach statistical significance ($P > 0.05$). The gradient (slope) of the T_b to M relationship (**Figure 6.4B**) suggested that higher M during SDEP and SDEP+ER was required to maintain equivalent T_b at all temperatures during the CAT in comparison to the CON trial. The SDEP slope of the primary shivering response was 17% greater than CON and the SDEP+ER slope of the primary shivering response was 12% greater than CON. However, these differences did not reach statistical significance ($P > 0.05$).

Heat debt (negative heat balance), determined by partitional calorimetry, was not significantly different between trials ($P > 0.05$). On examining the T_{re} to S relationship, there were no significant differences in the gradients in the range of T_{re} assessed ($P > 0.05$). Nevertheless, the tendency was again similar to the T_b to M relationship, where there was better conservation of heat and therefore less cumulative heat debt at any given T_{re} during the CAT in the CON trial. Insulation and conductance were also not significantly different between trials ($P > 0.05$).

6.4.4 Blood Analyses

There were no trial x time interactions, but a main effect of time was observed for plasma glucose ($P < 0.01$), cortisol ($P < 0.01$), and nor-adrenaline concentration ($P < 0.01$; **Table 6.1**). No main effects were observed for plasma adrenaline concentration. Plasma glucose concentration significantly increased 2 h post-CAT after the standardised meal ($P < 0.01$ versus pre-CAT; **Table 6.1**). While, plasma cortisol (31%) and nor-adrenaline (346%) concentrations significantly increased post-CAT ($P < 0.05$ and $P < 0.01$, respectively versus pre-CAT; **Table 6.1**). Plasma cortisol concentration returned to pre-CAT levels 1 h into recovery, while plasma nor-adrenaline concentration remained significantly above pre-CAT levels 1 h into recovery (*P*

< 0.05; **Table 6.1**). Furthermore, a trend (P = 0.09) was apparent for plasma nor-adrenaline concentration, whereby, SDEP+ER induced a higher nor-adrenaline response post-CAT and during recovery compared with SDEP and CON.

	0 h	24 h	48 h	Pre-CAT	Post-CAT	1 h Post-CAT	2 h Post-CAT
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)	
Nor-Adrenaline (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)	

Table 6.1 Plasma glucose, cortisol, adrenaline and nor-adrenaline responses (N = 10) during a 53 h period of normal sleep (CON), total sleep deprivation (SDEP), and total sleep deprivation and 90% energy-restriction (SDEP+ER), followed by a cold-air test (CAT). Tabular report of mean, (SD). Main effect of time, ‡ $P < 0.05$ and ‡‡ $P < 0.01$, significant decrease from 0 h; Main effect of time, † $P < 0.05$ and †† $P < 0.01$, significantly greater that pre-CAT.

6.5 Discussion

The current study examined the effects of the isolated stress of two nights of SDEP (53 h) and with the addition of energy restriction on thermoregulation during a subsequent cold-air test in a controlled laboratory setting. It was determined that 53 h SDEP, with or without ER, did not significantly impair core temperature, skin temperature, metabolic heat production responses or insulation, conductance and heat debt during cold exposure. It was also determined that 53h SDEP, with or without ER, did not significantly affect the time to reach T_{re} 35.9°C. Interestingly however, SDEP had a large positive (η^2) and clinically significant (Cohen's *d*) effect on time to reach T_{re} 35.9°C, with participants' mean exposure duration 37 minutes longer during the SDEP trial in comparison to the CON trial. The relationship between metabolic heat production and mean body temperature, as well as between heat debt and core temperature indicated that there may be underlying thermoregulatory alterations due to SDEP, with and without ER, during prolonged whole body cooling (T_{re} < 35.9°C), but these differences were not statistically significant. The current findings do not support the hypotheses that two days (53 h) of complete sleep deprivation would impair thermoregulatory responses to a cold-air test and that these impaired responses would be magnified with the addition of energy restriction. These data indicate that that military personnel, ultra-endurance athletes and search and rescue teams are at no greater risk of hypothermia during cold exposure of less than 4h when sleep deprived, even with the addition of energy restriction. However, the time to reach T_{re} 35.9°C and the relationship between thermoregulatory variables results provide a paradox, whereby it could be suggested that the acute stress of two nights of sleep deprivation enhances thermoregulatory responses during short duration cold exposure (< 4h), whereas these responses may be impaired during longer duration exposure to the cold.

The few studies that have examined the effects of the combined stress of sleep deprivation, negative energy balance and exhaustive physical activity on thermoregulatory responses during

cold exposure have shown impairment to core temperature responses and shivering thermogenesis and have predicted an increased risk of hypothermia in these conditions (Castellani *et al.*, 2001; Castellani *et al.*, 2003; Young *et al.*, 1998). Greater metabolic heat production was required from shivering thermogenesis to maintain an equivalent body temperature in the cold, when participants were subjected to multiple stressors or inadequate recovery periods (Castellani *et al.*, 2001; Castellani *et al.*, 2003; Young *et al.*, 1998). Again, it is very difficult to differentiate and isolate out the effects of the individual stressors as the period of stress prior to cold exposure was not performed under controlled laboratory conditions in these three studies (Castellani *et al.*, 2001; Castellani *et al.*, 2003; Young *et al.*, 1998). Strengths of the current study are that the isolated effects of SDEP alone and with the addition of energy restriction on thermoregulation in the cold in a controlled laboratory setting were examined and that the study was performed with a repeated-measures cross-over design which the previous three studies (Castellani *et al.*, 2001; Castellani *et al.*, 2003; Young *et al.*, 1998) were not. The current study lends some evidence to the hypothesis that there could be underlying thermoregulatory disturbances due to SDEP and SDEP+ER during long duration cold exposure (> 4h, T_{re} < 35.9°C). The relationship between mean T_b and metabolic heat production (**Figure 6.4B**) indicated a modest delayed shivering onset (T_b when $\Delta M = 0$) during SDEP and SDEP+ER versus CON, where shivering was likely to be initiated at a higher mean T_b. The gradient (slope) of the T_b to M relationship (**Figure 6.4B**) suggested, as before, that higher M during SDEP and SDEP+ER was required to maintain equivalent T_b at all temperatures during the CAT in comparison to the CON trial as well. On examining the T_{re} to heat debt relationship, the tendency here was again similar to the T_b to M relationship, where there was better conservation of heat and therefore less cumulative heat debt at any given T_{re} during the CAT in the CON trial. Although these observed differences under the current conditions did not reach statistical significance, taken together and with predictive extrapolation, it would appear that normal thermoregulation in the cold may be affected by SDEP and SDEP+ER during this

primary phase of the shivering response (Xu *et al.*, 2005). Jointly, the findings from the relationships between thermoregulation variables in the current study suggest that disruptions to thermoregulation due to SDEP and ER stress may exist during more prolonged cold exposure. However, whether these stressors pose an increased risk of hypothermia during prolonged cold exposure remains a matter of debate.

The findings of the current study are in line with earlier studies which investigated the effects of a single night of sleep deprivation on the risk of hypothermia and revealed no differences in thermoregulatory variables in comparison to control conditions (Caine-Bish *et al.*, 2004; Landis *et al.*, 1998a; Savourey and Bittel, 1994; Young *et al.*, 1998). The current study attempted to clarify whether a longer duration of sleep deprivation would adversely affect cold thermoregulation, but it was determined that the longer period of two nights of SDEP still did not significantly impair the core temperature, skin temperature and shivering responses during one to four hours of cold exposure. In the present study, there were no observable additive, detrimental effects on thermoregulation from the addition of ER as a stressor, even though negative energy balance had been identified as a potential factor limiting shivering thermogenesis (Jacobs *et al.*, 1994; Wissler, 1985). It had been suggested that insufficient fuel supplies for heat production, to offset the increases in heat loss associated with sleep deprivation, are likely to amplify the susceptibility to hypothermia in a combined stressor state. However, studies which have examined the effects of 'glycogen-depletion' on heat production present another interesting paradox (Haman *et al.*, 2005): although glycogen becomes the major fuel source at higher shivering intensities, the body's ability to respond to greater heat production demands does not appear to be compromised by decreases in carbohydrate availability (Haman *et al.*, 2004; Young *et al.*, 1989). It is possible that the effects of limited substrate availability on human thermoregulation and shivering fatigue may only be seen during the combined associated extremes of cold exposure duration (> 5h), very high shivering intensities (> 75% shivering

peak) and low ambient (< 10°C) temperatures (Haman *et al.*, 2005; Haman, 2006). In fact, the effects of limited substrate availability on thermoregulation in the cold are never, on ethical grounds, likely to be observed in a laboratory setting and could only realistically occur during accidental exposure in the natural environment.

Thermoregulatory models for the prediction of long term cold exposure suggest that the primary shivering response is a linear response and shivering intensity increases as T_{re} and T_{sk} decrease (Tikuisis and Giesbrecht, 1999), until a maximal shivering metabolism is attained (Xu *et al.*, 2005). This maximum shivering metabolism plateaus at T_{sk} between 17 and 20°C and at T_{re} as low as 32°C (Xu *et al.*, 2005). Therefore, all laboratory studies examining thermoregulatory and shivering responses to cold air exposure in humans are examining only basal metabolism and the primary shivering response, due to ethical constraints. Therefore, although the current study only produced a modest decrease in core temperature (T_{re} 35.9°C), albeit with exposure durations at 0.0°C of up to four hours, it remains unnecessary to subject participants to whole body cooling which might reach severe hypothermic levels, as the response is linear and can be predicted and extrapolated down to much lower core temperatures. It would also appear that although there may be differences in the T_{re} to M relationship with SDEP and SDEP+ER, this may simply mean that maximum shivering metabolism might be reached at lower T_{re} than under CON conditions and may not affect thermoregulatory responses during the secondary shivering response. In fact, participants' mean time to reach T_{re} 35.9°C was on average 37 minutes longer during SDEP and 14 minutes longer on SDEP+ER than under CON conditions, likely due to the increased primary shivering response during these trials. By examining the effect size of these differences in exposure duration during each trial, SDEP had a large positive (η^2) and clinically significant (Cohen's *d*) effect on time to reach T_{re} 35.9°C. It is therefore likely that the study was underpowered due to a small sample size, as these effect size results suggest that with a larger sample size, it is probable that the difference in exposure duration between the SDEP and CON

trial would become significant. Whether differences in sub-maximal shivering metabolism might affect the risk of hypothermia will require further modelling from the data of studies such as this one and future studies, and until such time, it remains unknown whether differences in the primary shivering response will affect maximum shivering metabolism, the secondary shivering response, the termination of shivering which normally occurs at a T_{re} ~30°C (Xu *et al.*, 2005) and ultimately the risk of hypothermia. In light of this evidence, future studies examining disturbances to cold thermoregulation due to potential stressors, may wish to focus on the potential mechanisms for any thermoregulatory disruption associated with stressors such as sleep deprivation. Future studies might also like to examine the termination phase of shivering by utilizing drugs such as Meperidine which pharmacologically inhibit shivering (Logan *et al.*, 2011), in order to determine whether SDEP and SDEP with ER increases the risk of fatal hypothermia.

In summary, two nights without sleep, with or without 90% restriction in energy intake, did not significantly impair core temperature, skin temperature and heat balance responses during cold exposure up to four hours. Time to reach a rectal core temperature of 35.9°C during cold exposure was also not statistically different between trials, but two nights of sleep deprivation did have a large positive and clinically significant effect on exposure duration, which would likely become statistically significant with a larger sample size. The relationship between metabolic heat production and mean body temperature, as well as between heat debt and core temperature potentially indicate underlying alterations to normal thermoregulation during more prolonged whole body cooling (> 4h, T_{re} < 35.9°C) in the primary shivering response phase. It would therefore appear that military personnel, ultra-endurance athletes and search and rescue teams are under no greater risk of hypothermia from cold exposure of up to four hours in duration when sleep deprived for two nights, even with the addition of 90% energy restriction, than when they are well rested and well fed.

CHAPTER SEVEN

Influences of three nights of sleep disruption on thermoregulation during exercise in the heat

7.1 Summary: Sleep disruption (SDIS) has been implicated as a stressor that impairs thermoregulation and increases the risk of heat illness. The current study tested the hypotheses that three-nights SDIS impairs thermoregulation at rest and during subsequent exercise-heat-stress. Eleven male endurance athletes, un-acclimated to the heat, were monitored during two randomized trials, 14 d apart. Trials consisted of three-nights control (CON; sleep = 480 min·night^{-1}) and three-nights SDIS (sleep = 2 x 60 min·night^{-1}), followed by an exercise-heat-stress protocol (EHSP), in an environmental chamber (33°C; 40%RH): 45 min seated rest, followed by 45 min treadmill running at 60%$\dot{V}O_{2peak}$. Rectal temperature (T_{re}), 8-site skin temperature (T_{sk}) and forearm sweating were continuously measured, and expired gas analysed during EHSP. T_{re} was not significantly different ($P > 0.05$) between trials, Pre-EHSP (CON: 37.13°C, (0.21); SDIS: 37.17°C, (0.25)), during exercise, nor at the end of exercise (CON: 39.12°C, (0.44); SDIS: 39.09°C, (0.46)). SDIS T_{sk} was significantly elevated ($P < 0.05$) at several sites during seated rest (chest, hand, thigh) and 6-site weighted upper-body mean T_{sk} was significantly higher during SDIS exercise ($P < 0.001$). From 5 min into exercise onwards, forearm sweating was significantly lower in the SDIS compared to CON trial ($P < 0.05$), with an overall 21% reduction throughout exercise. Heat storage, metabolic rate, heart rate, RPE and thermal sensation were not significantly different between trials ($P > 0.05$). These findings show no effect of three-nights of SDIS on core temperature at rest or in response to exercise-heat-stress. However, SDIS does appear to alter heat loss pathways, with blunted forearm sweating (evaporative) and increases in upper-body skin temperature (radiative), with no increase in thermal strain.

7.2 Introduction

Military personnel, ultra-endurance athletes, search and rescue teams as well as shift workers, are often subjected to a multitude of stressors as part of their normal routine. These stressors can include prolonged physical exertion and periods of total deprivation or disruption of sleep, and it has been suggested that these stressors can impair thermoregulation and performance in adverse environmental conditions (Aakvaag *et al.*, 1978; Bugge *et al.*, 1979; Rognum *et al.*, 1986b). In a seminal study examining the incidence of exertional heat illness (EHI) through compilation of fatal and non-fatal case studies over an entire decade in the Israeli Defence Forces, it was demonstrated that disrupted sleep was the second most common pre-disposing factor in both fatal and non-fatal casualties (Rav-Acha *et al.*, 2004). However, due to the nature of the multi-stressor scenario involved in military operations and the studies that have examined them, it is unclear whether sleep disruption is simply a coincidental factor or a true pre-disposing factor. Unfortunately, the results of prospective studies in the currently available literature do not clearly characterize whether, and how, sleep disruption might negatively affect thermoregulation during exercise-heat stress.

The effect of sleep disruption on thermoregulation during exercise-heat stress is not well documented, with only two studies in the literature attempting to characterize the effect of the stressor on the fundamental thermoregulatory variables (Kolka and Stephenson, 1988; Sawka *et al.*, 1984). In these investigations, thermoregulation during exercise-heat stress did appear to be affected by total sleep deprivation. While resting core temperature was lowered (Sawka *et al.*, 1984) after 30 h of total sleep deprivation, local sweat rate and sweating sensitivity were decreased (Sawka *et al.*, 1984) and cutaneous vasodilation reduced (Kolka and Stephenson, 1988). Reduction in wet (evaporative) and dry (radiative and convective) heat loss due to complete sleep deprivation may therefore increase thermal strain during exercise-heat stress and

increase the risk of EHI. These studies (Kolka and Stephenson, 1988; Sawka *et al.*, 1984) investigated the effect of one night of total sleep deprivation on thermoregulation during exercise-heat stress, and as such, the effects of a longer period of disrupted sleep (1 to 2 h sleep permitted per night) on these thermoregulatory responses remains unknown.

It has also been suggested (Bahr *et al.*, 1991; Opstad and Bahr, 1991), that the multi-stressor scenario of prior strenuous prolonged exercise, restricted sleep and energy intake deficiency may reduce set-point temperature, elevate resting metabolic rate and reduce mechanical efficiency during cycle-ergometry exercise. However, these two studies were not cross-over designed (Bahr *et al.*, 1991; Opstad and Bahr, 1991), with military personnel performing the laboratory-based cycle-ergometry exercise after sustained operations in the field and then again in a rested state two months later. Apart from this design weakness, in the field, there could be little control over potential confounding variables such as hydration, nutritional status and relative exercise intensity. This makes it difficult to discern the individual effect and contribution of each of the joint stressors on thermoregulation.

In light of the scarcity of knowledge regarding the effects of sleep disruption on thermoregulation during exercise-heat stress and the applied relevance of obtaining this knowledge, the present study aimed to examine the influence of disrupted sleep, on thermoregulation at rest and during subsequent exercise-heat stress, in a controlled laboratory environment. The proposed model for the sleep disruption trial was 3 nights of severely disrupted sleep, whereby two one-hour blocks of sleep would be permitted each night, presenting a more ecologically valid model to a wider proportion of the population than a total sleep deprivation model. It was hypothesized that sleep disruption would alter resting core temperature and skin temperature, impair thermoregulatory responses to exercise-heat stress and increase thermal strain during exercise in the heat.

7.3 Methods

Participants

Eleven healthy, collegiate male endurance athletes (means, (SD): age 21 years, (1); height 179 cm, (9); nude body mass (NBM) 72 kg, (9); body fat 14%, (3); $\dot{V}0_{2peak}$ 63 $ml \cdot kg^{-1} \cdot min^{-1}$, (4)) volunteered to participate in the study. Participants did not undergo any formal heat acclimation programme prior to participation in the study. As collegiate endurance athletes, participants had been physically active as part of their normal weekly routine in the lead-in period to study participation. The study took place between December 2009 and March 2010, where maximum ambient temperatures ranged from 7.6 to 12.2°C.

Preliminary testing

Prior to the main experimental trials, participants performed a two-phase familiarization. Following bowel and bladder voiding, participants' height, NBM (Seca 705, Hamburg, Germany) and body composition by dual-x-ray absorptiometry (DXA; Hologic QDR1500, Bedford MA, USA) were obtained. Participants' fat free mass determined by DXA scanning was used to estimate resting metabolic rate (RMR) (Compher *et al.*, 2006). The RMR for each participant was multiplied by a coefficient of 1.7, which adjusts the individual RMR to account for general daily physical activity and diet induced thermogenesis, in order to more accurately determine individualized daily energy intake requirements (Todorovic and Micklewright, 2004). Each participant's $\dot{V}0_{2peak}$ was then determined by means of a continuous incremental exercise test on a motorized treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany) based on a standard protocol (Bruce, 1977b), as detailed **Chapter 3**. The treadmill speed that elicited 50 and 60% $\dot{V}0_{2peak}$ at a 1% gradient was then extrapolated and verified. Seven days after the first phase of familiarization, participants then undertook the exercise-heat stress protocol (EHSP) that would be used during the experimental trials and were familiarized with all the

procedures that would be involved during the main experimental trials, as subsequently described.

Experimental design

In a randomized, repeated-measures, crossover design, participants completed two experimental trials (**Figure 7.1**). Fourteen days separated the start of each trial. The two experimental trials involved a control trial (CON) and a sleep disruption trial (SDIS). Participants were allowed normal nocturnal sleep on the CON trial (480 $min \cdot night^{-1}$), but during the SDIS trial, participants were restricted to two blocks of one hour sleep per night for three consecutive nights. Allotted sleep during the SDIS trial took place between 2345 h and 0045 h and again between 0445 h and 0545 h. Participants remained in the laboratory building, complete with living quarters, for the entire duration of each experimental trial and were supervised at all times. At 1100 h on day four of both trials, all participants underwent an EHSP, as subsequently described.

Figure 7.1 Schematic representation of timing schedule from the two days prior to experimental trials, through day four of experimental trials (CON and SDIS).

Experimental procedures

The day prior to each experimental trial, participants were provided with food and water equivalent to their predicted total daily energy and fluid intake requirements in order to standardize nutrition, hydration and sleep-wake cycles. Sleep duration on the night before trials was monitored by accelerometry, as previously described (Ancoli-Israel *et al.*, 2003). Participants were transported to the laboratory and arrived euhydrated at 0800 h after an overnight fast. Participants were requested to empty their bladder and bowels prior to 0 h measurements. The participants' urine specific gravity, determined by a handheld refractometer (Atago Uricon-Ne, NSG Precision Cells, New York, USA), was 1.012 $g \cdot L^{-1}$ (0.004) on arrival at the laboratory, which indicated euhydration based on published indices (Armstrong *et al.*, 1994). Urine samples were also collected from participants at 24 h, 48 h, 72 h, and pre-EHSP in order to confirm maintenance of euhydration status, based on published indices and procedures (Armstrong *et al.*, 1994). Participants' NBM was recorded at 0 h, 24 h, 48 h, 72 h, pre-EHSP and post-EHSP.

Dietary intake was provided to meet predicted individual daily energy requirements (11.8 MJ, (0.81); Carbohydrate, 57%, (3); Fat, 25%, (3); Protein, 19%, (4)) during both CON and SDIS. Water was provided throughout each experimental trial at 3 h intervals, equivalent to 40 $ml \cdot kg^{-1} NBM \cdot day^{-1}$, with estimated fluid losses during exercise added to this total. Daily physical activity was monitored via electronic pedometers (Digi-walker SW-200, Yamax, Tokyo, Japan) and the participants' sleep-wake cycles were monitored by accelerometry (GTIM, ActiGraph LLC, Florida, USA) (Ancoli-Israel *et al.*, 2003). Participants were allowed to perform normal light daily activities such as playing board games, playing video games, watching television, and performed an hour of low intensity exercise each day. Exercise took place at 1445 h on day one, two and three of both trials, with participants performing a 60 min steady state walk at 50% $\dot{V}0_{2peak}$. The exercise protocol was performed in a temperature-controlled laboratory (20^{o}C, (1);

50% RH, (7)) with a fan generated wind velocity of 2.3 $m \cdot s^{-1}$ placed one metre in front of participants. Water was consumed *ad libitum* during exercise, for all participants in both trials.

Exercise Heat Stress Protocol (EHSP)

On both trials, prior to commencing the EHSP, participants were fitted with heart rate monitors (Polar Electro, Kempele, Finland) and wireless iButtons® to measure skin temperature (T_{sk}). Eight iButtons® were affixed to the skin using porous medical-grade tape (Hypafix, BSN Medical GmbH, Hamburg, Germany) at the following sites: forehead, right scapula, left upper chest, right upper arm, left lower arm, left hand, right anterior thigh and left calf. The iButtons® were programmed to log data in 0.0625ºC increments before application, as described in **Chapter 4**. Participants then applied rectal thermistor probes, in privacy, 12 cm beyond the external anal sphincter (Grant REC soft insertion probe thermocouple; Grant 2020 Squirrel data logger, Shepreth, UK) to monitor rectal temperature (T_{re}). Throughout the EHSP, participants wore standardized clothing, consisting of athletic shorts, shoes and socks. The EHSP began at 1100 h on day four of each trial with participants entering an environmental chamber, which was heated to 33°C and 40% RH with 0.2 $m \cdot s^{-1}$ wind velocity. The EHSP consisted of two phases, as follows:

Stabilization

The EHSP began with a 45 min stabilization phase. This passive pre-heating stage consisted of participants sitting upright on a plastic laboratory chair, while T_{sk} and T_{re} were continuously monitored. Participants were instructed to minimize any movement during stabilization and assumed a standardized seated position, with hands on their knees. Heart rate, McGinnis 13-point thermal comfort ratings (1 = so cold I am helpless to 13 = so hot I am sick and nauseated, 7 = comfortable; (Hollies and Goldman, 1977) were recorded every five minutes. Two-minute expired gas samples were collected in 150 L Douglas bags between 20 to 22 and 40 to 42

minutes into each trial and immediately analysed. Water intake during stabilization was equivalent to 3 $ml \cdot kg^{-1}$ NBM during both trials.

Exercise

The second phase of EHSP began immediately after the 45 min stabilization period, with participants mounting a motorized treadmill and commencing a 45 min run at 60% $\dot{V}O_{2peak}$. T_{sk}, T_{re} and heart rate were continuously monitored, with McGinnis 13-point thermal comfort ratings and ratings of perceived exertion (RPE) (Borg, 1982) recorded every five minutes. Local forearm sweating rate (m_{swf}) was monitored continuously with a dry-nitrogen ventilated sweat-cell capsule connected to a dew point hygrometry system (DS2000, Alpha Moisture Systems, Bradford, U.K.; (Graichen *et al.*, 1982)) and the responses were evaluated by comparing the average sweating rate over 5 min intervals throughout exercise. Due to manufacturing constraints, N = 6 of N = 11 participants' m_{swf} was monitored with the dew point hygrometry equipment. Two-minute expired gas samples were collected in 150 L Douglas bags at the mid-point of exercise and immediately analysed. Water intake during exercise was equivalent to 5$ml \cdot kg^{-1}$ NBM during both trials. Participants were continuously monitored during the EHSP and were removed from the environmental chamber if their T_{re} reached 39.9°C.

Immediately after completing the EHSP, participants were monitored during seated rest for an additional two hours in the laboratory (20°C, (1); 50% RH, (7)). T_{re} was monitored continuously and recorded every 5 min during the two hours after participants exited the environmental chamber. At 1h post-EHSP, participants were allowed to shower, re-clothe and consume a standardized meal (5.7 MJ, (0.5): Carbohydrate, 61%, (2); Fat, 20%, (4); Protein, 20%, (2)). Estimated fluid losses during the EHSP were determined, and replacement fluid was divided into two and provided in equal proportions in the two-hour post-EHSP period. After the 2 h post-EHSP samples were obtained, the trials were complete.

Calculations

Mean T_{sk} was measured at eight sites, in accordance with the formula and weighting of ISO-standard 9886 (ISO9886 2004), as follows:

Mean $T_{sk} = (0.07.T_{forehead}) + (0.175.T_{right\ scapula}) + (0.175.T_{left\ upper\ chest}) + (0.07.T_{right\ arm\ in\ upper\ location}) + (0.07.T_{left\ arm\ in\ lower\ location}) + (0.05.\ T_{left\ hand}) + (0.19.T_{right\ anterior\ thigh}) + (0.2.T_{left\ calf})$

Mean body temperature (T_b) was calculated from T_{re} and Mean T_{sk} data, utilizing a respective ratio of 9:1. Rate of body heat storage (S) was calculated from the change in T_b every 5 minutes of exercise using the following equation:

$$S = (0.965 \cdot m/A_D) \cdot dT/dt\ [W \cdot m^{-2}]$$

In the equation for determining S, m is body mass, AD is body surface area, dT is the change in mean body temperature, dt is time in hours, and 0.965 is the specific heat of the body in $W \cdot h \cdot °C^{-1} \cdot kg^{-1}$(Gagge and Gonzalez, 1996). Douglas bag expired gas samples were analysed to determine respiratory exchange ratio (RER) and VO_2 and thereby calculate the energy cost of locomotion using the following equation:

$$M = (0.23 \cdot RER + 0.77) \cdot 5.873 \cdot VO_2 \cdot 60\ [W]$$ (Gagge and Gonzalez, 1996)

Mean whole body sweat rate was calculated from the change in pre and post-EHSP NBM measurements on a standardized scale (Seca 705, Hamburg, Germany), subtracting respiratory water lost (0.029 g/kJ) and body mass loss due to the exchange of oxygen and carbon dioxide (0.026 ml/kJ).

The m_{swf} to T_{re} relationship in the first five minutes of exercise was used to calculate sweating thresholds for each trial. Regression equations for the m_{sw} to T_{re} relationship for each participant were determined, and the T_{re} for the threshold initiation of sweating was calculated where m_{swf} = 0.06 $mg \cdot cm^{-2} \cdot min^{-1}$ (Buettner, 1959).

Statistical Analyses

The method of least-squares linear regression was employed to determine relationships between thermoregulatory variables and rate of increase in local forearm sweating. Differences in pre-trial ActiGraph data and relationships between thermoregulatory variables were analysed using paired-samples t-tests. Repeated-measures two factor (time x trial) ANOVA were used to examine differences in measurement variables during the EHSP of trials.

7.4 Results

7.4.1 Sleep Duration, NBM & Physical Activity

Sleep duration on the night before trials, monitored by accelerometry, was not significantly different between trials ($P > 0.05$; pre-CON: 476 min·night^{-1}, (4); pre-SDIS: 474 min·night^{-1}, (4). Sleep duration during CON trials averaged 478 min·night^{-1}, (3). It was also determined from accelerometry data that during the SDIS trial, participants endured a mean sleep duration of 60 min, (1), during the first bout of sleep (2345 h to 0045 h) and 56 min, (6), during the second bout of sleep (0445 h to 0545 h). There were no significant differences between trials for NBM or daily physical activity ($P > 0.05$).

7.4.2 EHSP Thermoregulatory Responses

Following a 75 h period of CON and SDIS, resting T_{re} immediately prior to entering the environmental chamber for the EHSP was not significantly different between trials ($P > 0.05$; CON: 37.13°C, (0.21); SDIS: 37.17°C, (0.25)). During the EHSP, Stabilization T_{re} was not significantly different between trials or over time ($P > 0.05$). Exercise T_{re} was also not significantly different ($P > 0.05$) between trials (**Figure 7.2**), but did increase significantly over time, during both trials (main effect of time: $F(1, 45) = 439.0$; $P < 0.0001$). Immediately post-EHSP, T_{re} was not significantly different ($P > 0.05$) between the CON (39.12°C, (0.44)) and SDIS (39.09°C, (0.46)) trials.

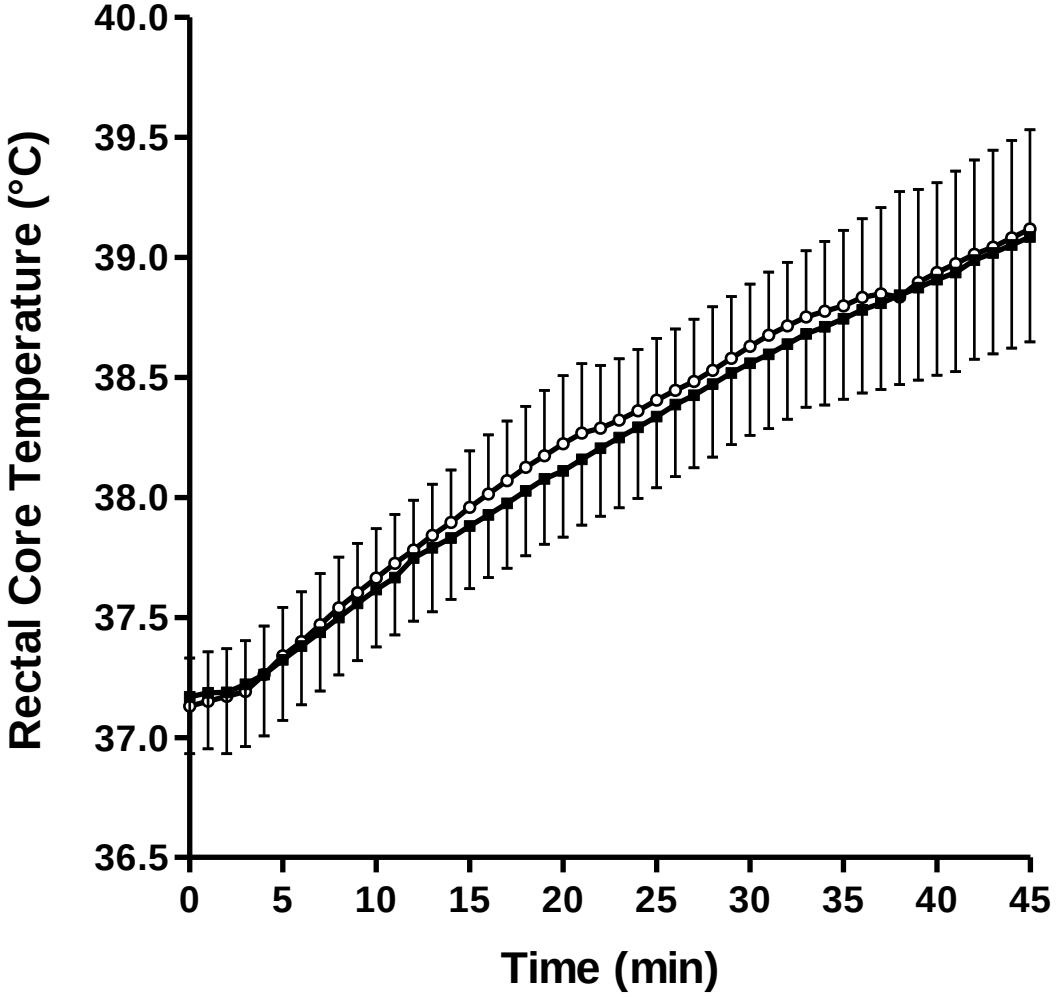

Figure 7.2 Rectal core temperatures during 45 min exercise-heat stress (33°C and 40%RH) following three-nights of normal sleep (○) and three-nights of sleep disruption (■). N = 11. Values are means, SD.

Following a 75 h period of CON and SDIS, resting 8-site mean T_{sk} immediately prior to entering the environmental chamber, was similar between trials (CON: 31.68°C, (0.69); SDIS: 32.02°C, (0.52)), with a tendency for SDIS to be higher than CON (P = 0.19). The 8-site mean T_{sk} was similar between trials during the 45 min EHSP stabilization period, with SDIS again tending to be higher than CON (P = 0.15). The 8-site mean T_{sk} was not significantly different between trials during exercise (P > 0.05) (**Figure 7.3A**), but a significant increase in 8-site mean T_{sk} did occur during both trials over time in both the stabilization (main effect of time: $F(1,45) = 623.2$; $P < 0.001$) and exercise phases (main effect of time: $F(1,45) = 29.3$; $P < 0.001$). Nevertheless, upon closer examination, individual T_{sk} sites did display significant differences between trials during both phases of the EHSP. Upper chest, hand and thigh T_{sk} were all significantly higher during the SDIS trial stabilization period (P < 0.05), with a trend for forearm T_{sk} to also be higher during SDIS stabilization (P = 0.090). Forehead, hand, thigh and calf T_{sk} were all significantly higher during the SDIS exercise phase (P < 0.05) than during CON (**Table 7.1**). Furthermore, a weighted mean by regional proportions of the six upper body T_{sk} sites, evaluated at 5 min intervals, also revealed that the entire region displayed a significantly higher temperature from the tenth minute of exercise in the heat onwards during the SDIS trial in comparison to the CON trial (trial x time interaction: $F(1,45) = 2.8$; $P < 0.001$; **Figure 7.3B**). With the weighting by body surface area for these sites, this meant that 42 to 49% of the regional surfaces of the skin presented with higher skin temperatures during the SDIS stabilization phase and 61% during the exercise phase in comparison with CON.

	Exercise time		
	0 min T_{sk} **(°C)**	**45 min** T_{sk} **(°C)**	***P* value**
Skin site			
Forehead			
CON	36.0, (0.3)	34.2, (2.5)	< 0.001[a] ***
SDIS	36.1, (0.2)	35.0, (1.9)	
Hand			
CON	34.2, (0.6)	35.3, (1.2)	0.006[a] **
SDIS	34.6, (0.7)	35.8, (0.7)	0.029[b] *
Thigh			
CON	34.9, (0.7)	36.2, (0.4)	0.019[a] *
SDIS	35.3, (0.7)	36.2, (0.8)	
Calf			
CON	34.3, (0.7)	36.4, (0.7)	0.002[a] **
SDIS	34.2, (0.4)	36.5, (0.6)	
Upper body			
CON	35.5, (0.3)	35.4, (1.1)	< 0.001[a] ***
SDIS	35.5, (0.4)	35.8, (0.9)	

Table 7.1 Regional skin temperatures during 45 min exercise-heat stress at 60% $\dot{V}O_{2peak}$ (33°C and 40% RH) following three-nights of normal sleep (CON) and three-nights of sleep disruption (SDIS). Mean skin temperature values (T_{sk}) are means, SD at the start and finish of exercise for CON and SDIS trials. Upper body is the weighted mean of the six upper body skin sites. *P* values are [a]trial x time interaction, [b]main effect of trial. All sites displayed a main effect of time $P < 0.001$, with T_{sk} increasing significantly over time, except the forehead site that showed a significant decrease in T_{sk} over time. Significant difference at: * $P < 0.05$ level, ** $P < 0.01$ level and *** $P < 0.001$ level. N = 11 participants.

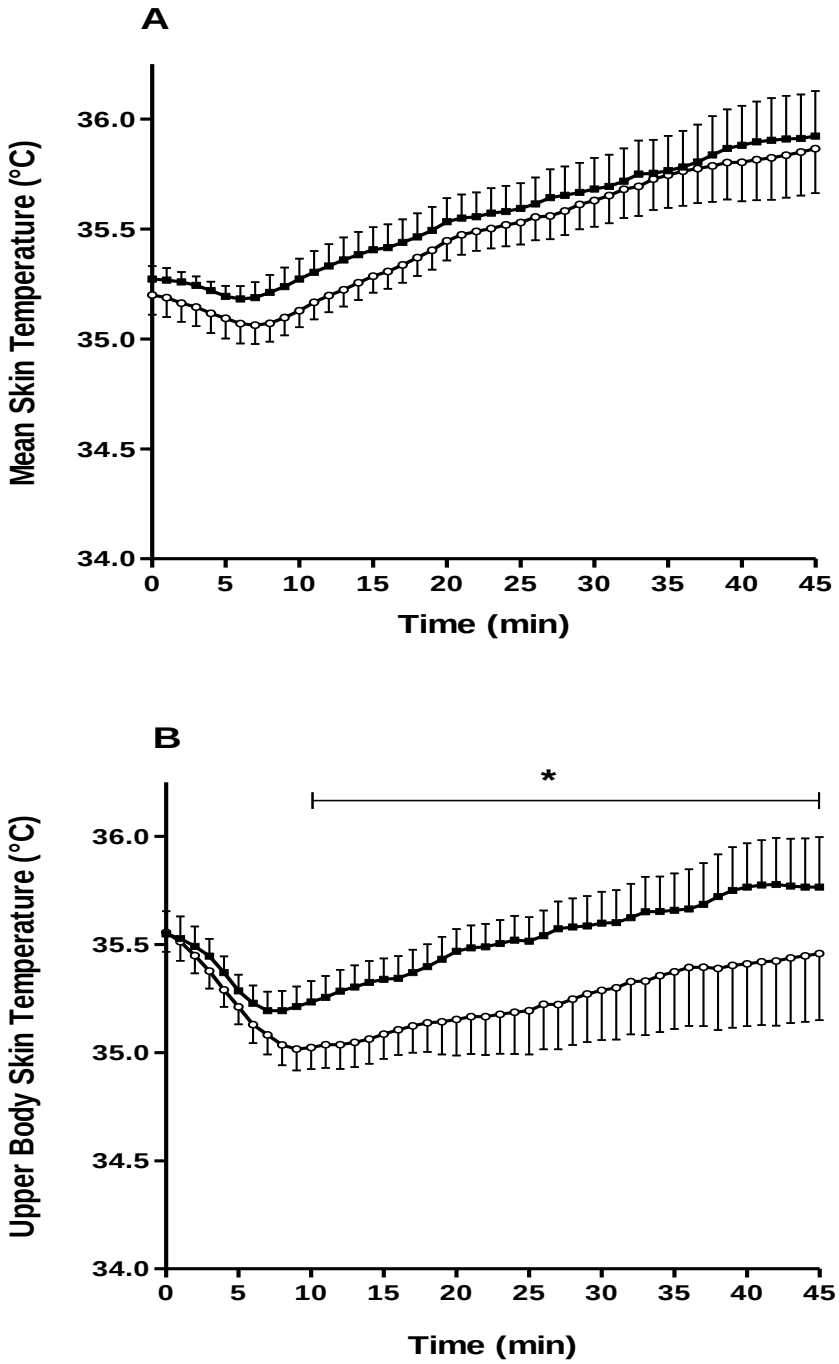

Figure 7.3 Whole body 8-site mean skin temperatures (**A**) and upper body 6-site mean skin temperatures (**B**) during 45 min exercise-heat stress (33°C and 40%RH) following three-nights of normal sleep (○) and three-nights of sleep disruption (■). N = 11. Values are means, SEM. * $P < 0.05$, Significant difference in skin temperatures between trials from ten minutes into exercise until the end of exercise.

Local forearm sweat rate (m_{swf}) in the SDIS trial was significantly lower than the CON trial from five minutes into exercise onwards (trial x time interaction: $F(1,90) = 1.4$; $P = 0.013$; **Figure 7.4**), notably from 25 min into exercise onwards. Overall, forearm sweating was suppressed by 21% throughout exercise during SDIS in comparison to CON. The slope of the rate of increase in sweating, from linear regression, in the first five minutes was significantly greater during the CON trial ($P = 0.014$), with a 36% steeper slope than during the SDIS trial (**Figure 7.4**). The relationship between mean T_{re} and m_{swf} indicated no statistical difference ($P > 0.05$) in the threshold T_{re} for initiation of sweating between the CON (37.14°C, (1.14)) and SDIS (37.08°C, (0.80)) trials, however, the mean gradient of the slope was 24% reduced during SDIS. Mean whole body sweat rates were not statistically different ($P > 0.05$) between the CON (22.56 $ml \cdot min^{-1}$, (7.01)) and SDIS (21.06 $ml \cdot min^{-1}$, (6.62)) trials during the exercise phase.

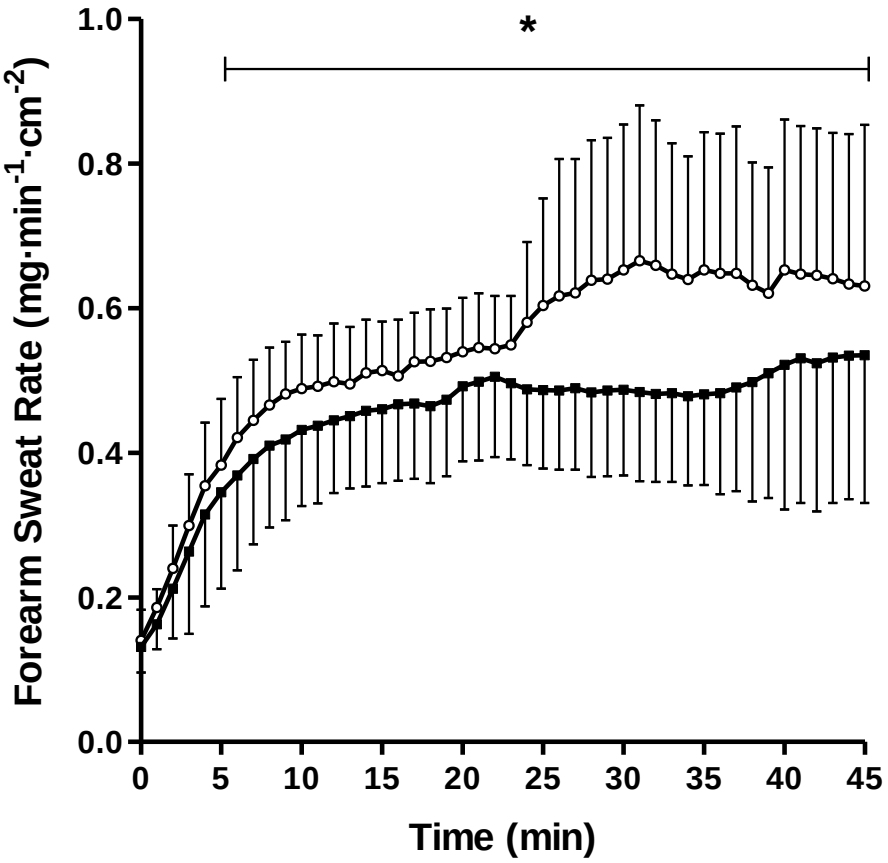

Figure 7.4 Forearm sweat rates during 45 min exercise-heat stress (33°C and 40%RH) following three-nights of normal sleep (○) and three-nights of sleep disruption (■). N = 6. Values are means, SD. * $P < 0.05$, Significant difference in sweating rates between trials from five minutes into exercise until the end of exercise.

Mean body temperature and thermal gradient (T_{re} - mean T_{sk}) during the EHSP were not significantly different between trials ($P > 0.05$). Heart rates showed no significant differences between trials during the EHSP ($P > 0.05$). McGinnis 13-point thermal comfort ratings and RPE also showed no significant differences between trials during the EHSP ($P > 0.05$). Subjective ratings of heat discomfort (EHSP beginning to end range: subjective rating 7 to 13) and RPE (EHSP beginning to end range: subjective rating 6 to 17) did increase with exercise duration on both trials ($P < 0.05$). It was determined that exercising metabolic rate was not significantly different ($P > 0.05$) between CON (1066.0 W, (144.0); 15.1 $W \cdot kg^{-1}$, (2.0)) and SDIS (1027.0 W, (151.0); 14.6 $W \cdot kg^{-1}$, (2.1)) trials in this study. Individual expired gas responses, used in calculations of M, were not significantly different during the EHSP, with comparable RER ($P > 0.05$) and $\dot{V}O_2$ ($P > 0.05$) between trials. Heat storage, S, was not significantly different between trials during the exercise bout ($P > 0.05$), but a significant increase in S occurred during both trials over the course of the exercise phase (main effect of time: $F(1,8) = 12.1$; $P < 0.0001$; **Figure 7.5**).

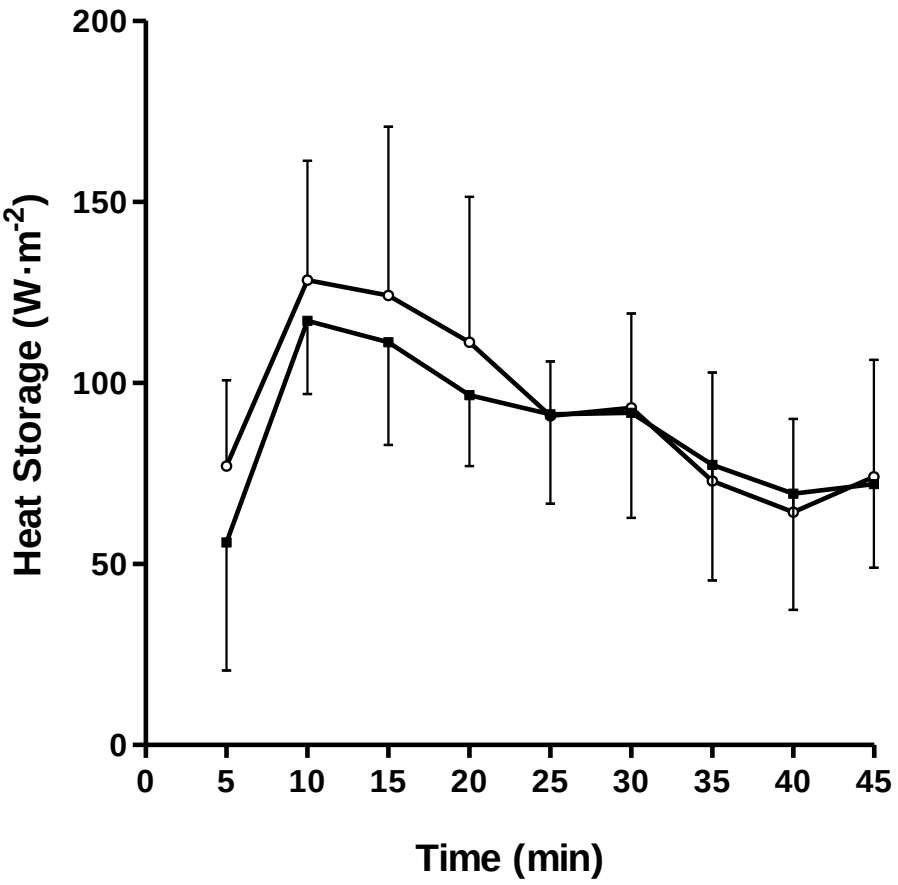

Figure 7.5 Rate of body heat storage during 45 min exercise-heat stress (33°C and 40%RH) following three-nights of normal sleep (○) and three-nights of sleep disruption (■). N = 11. Values are means, SD.

7.5 Discussion

Disrupted sleep has been implicated as a stressor that impairs thermoregulation and increases the risk of exertional heat illness (Kolka and Stephenson, 1988; Rav-Acha *et al.*, 2004; Sawka *et al.*, 1984). The aim of the current study was to determine whether three nights of severely disrupted sleep would impair resting thermoregulation and thermoregulatory responses to exercise-heat stress. In order to achieve this objective, participants performed two EHSP that included a run on a treadmill in an environmental chamber heated to 33°C and 40%RH; once after 75 h control conditions (CON) and once where sleep was severely disrupted for 75 h, with only two blocks of one hour sleep being permitted between 2345 and 0045 h and again between 0445 and 0545 h (SDIS). SDIS did not significantly affect core temperature, 8-site mean skin temperature or metabolic rate at rest or during exercise and did not increase thermal strain. However, SDIS did result in significant increases in discrete skin temperatures at various local sites during rest and during exercise, with SDIS 6-site upper body mean skin temperature significantly higher than CON from five minutes into exercise until the end of exercise in the heat. Furthermore, the local forearm sweating response to exercise in the heat was also significantly blunted by SDIS. Of note, is that these significantly lowered sweating responses due to SDIS during exercise in the heat do not appear to affect thermal strain. These disturbances to sweating responses by SDIS seem to be managed, or at least offset, by compensatory increases in dry heat loss through significant increases in regional upper body skin temperature, thus maintaining very similar core temperature and heat balance as CON conditions and preventing SDIS from increasing thermal strain. These results did not support the hypothesis that SDIS would affect thermoregulation during exercise-heat stress, but do expose underlying alterations in heat loss pathways due to SDIS.

In the current study, it was have shown that three nights of severely disrupted sleep did not affect resting core temperature. This is in contrast to previous studies which reported lowered resting core temperatures after one and two nights of total sleep deprivation (Kolka and Stephenson, 1988; Young *et al.*, 1998), although another study did not find any differences between control and sleep deprivation conditions following one night of total sleep deprivation (Sawka *et al.*, 1984). No difference was found in core or body temperatures during exercise-heat stress in the current study, and the rate of heat storage was also similar between trials, presenting findings similar to studies which have examined total sleep deprivation (Kolka and Stephenson, 1988; Sawka *et al.*, 1984).

Previous research suggests that a multi-stressor scenario of prior strenuous prolonged exercise, restricted sleep and energy intake deficiency may reduce set-point temperature (Opstad and Bahr, 1991). A reduced temperature set-point would result in decreased core temperatures at rest and during exercise as well as a lower core temperature at the onset of sweating. The findings showed none of these to be in effect during the independent stress of three nights of severely disrupted sleep. In the previous study, even though initial core temperature was seemingly lowered by 0.6°C due to sustained military operations, during exercise, the difference between trials became minimal, whereby there was only a 0.1 to 0.2°C difference in rectal temperatures after 30 min of exercise. Again, it is difficult to isolate the effects of individual stressors from the results of multi-stressor studies. Thus, the alteration in resting core temperature could be due to another stressor, or the effect of the combination of stressors. When this initial resting core temperature discrepancy between the earlier study and the current study is taken into account, the rectal core temperature responses to exercise become very similar between studies.

Sweat rate on the other hand, was significantly lower during the SDIS exercise-heat stress, decreasing the capacity for evaporative, wet heat loss pathways. These findings, using the model

of severely disrupted sleep are similar to previous reports on the effects of total sleep deprivation, that indicated lowered sweating sensitivity and absolute sweat rate (Kolka and Stephenson, 1988; Sawka *et al.*, 1984) during exercise in 35°C and 28°C conditions, respectively. In the current study, SDIS forearm sweating responses suggest that the rate of increase in sweating in response to the stimulus of exercise in the heat was suppressed from the first five minutes of exercise onwards and minute-by-minute sweating rates were lowered by 21% in comparison to CON. The difference in forearm sweat rate was particularly marked from approximately 25 minutes into exercise onwards. Although, it is unclear why this difference in sweat rates increased at this point into exercise. However, why these alterations in sweating responses do not appear to negatively affect thermoregulation during conditions of severe sleep loss, and do not appear to increase heat storage, thermal strain and core temperature does not seem to have been previously explored.

During exercise-heat stress under SDIS conditions, forehead, hand, thigh and calf T_{sk} were all significantly higher than CON. Upon closer examination, the 6-site mean weighted skin temperature of the entire upper body was significantly greater during SDIS from ten minutes into exercise until the end of exercise at forty-five minutes. These regional increases in T_{sk} during SDIS allow for an increased capacity for radiative, dry heat loss pathways during exercise in the heat. It is likely then, that this increase in radiative heat loss at greater than 60% of the body surfaces during exercise in the heat, at least in part, compensates for the reduction in evaporative heat loss elsewhere. It appears from the evidence of the 8-site whole body and 6-site upper body mean T_{sk} data that thermoregulatory alterations due to SDIS are more pronounced in the upper body during running exercise. This upper body versus lower body paradox may carry over to sweating responses and account for the lack of statistical difference in whole body sweating between trials, which requires further examination.

It was determined that exercising metabolic rate was not significantly different between CON and SDIS trials in this study. This finding is in contradiction to a previous study that demonstrated that a three to four day period of sleep disruption, prolonged work, and severe energy restriction resulted in an 18% higher metabolic cost of exercise (Bahr *et al.*, 1991; Opstad and Bahr, 1991), increasing the thermal strain of work in hot environments. However, these results are in agreement with the outcomes of studies examining the independent effects of sleep deprivation (Kolka and Stephenson, 1988; Martin and Gaddis, 1981; Sawka *et al.*, 1984; Symons *et al.*, 1988) on the metabolic responses to exercise. One explanation for the different findings between the current study and the earlier multi-stressor military operations study is that metabolic rate could have been affected by the combination of stressors and not simply due to sleep loss alone. In the previous articles, derived from the same study, (Bahr *et al.*, 1991; Opstad and Bahr, 1991) there could be little control over various factors which could confound the results, including nutrition and hydration status. Also, the participants had been performing continuous work immediately prior to the exercise protocol on a cycle ergometer which could generate metabolic drift (Epstein *et al.*, 1988; Hamilton *et al.*, 1991; Michael *et al.*, 1961). Thus, the decreased mechanical efficiency following three to four days of continuous simulated combat exercises with no organized sleep and virtually no food intake, reported by Opstad and colleagues (Bahr *et al.*, 1991; Opstad and Bahr, 1991) was likely due to the nutrition, hydration and exercise history during the hours preceding measurement rather than the effect of sleep disruption alone.

Storage of body heat was not significantly different between trials, in spite of the blunted sweating during SDIS. The increased skin temperatures might suggest that there are compensatory increases in dry heat loss through increases in skin blood flow responses to exercise during SDIS, which would explain the lack of difference in heat storage. An increase in skin blood flow responses would appear to be in contrast to the findings of a previous study

which showed a reduction in the sensitivity of forearm blood flow to oesophageal temperature in response to exercise in the heat following one night without sleep (Kolka and Stephenson, 1988). Although, it is unclear from the results of this study whether the response of the individual skin blood flow variable itself was significantly different between trials, or simply the relationship to oesophageal temperature (Kolka and Stephenson, 1988). Furthermore, a different model of three nights of disrupted sleep rather than total sleep deprivation was used, which could account for any differences between the two studies. In addition, from past experience and the experience of others (Karlsson *et al.*, 2002; Oberg, 1999; Rajan *et al.*, 2009), the results of any study which has utilized the laser Doppler skin blood flow method during applications which might incur any movement must be viewed with caution, as the method is extremely sensitive to any movement, with slight vibrations anywhere nearby creating movement artifacts. This makes the measurement of skin blood flow almost impossible during any mode of exercise and thus the surrogate skin temperature measurement is one of the best alternatives for discerning potential alterations in skin blood flow and vasodilation during ambulatory conditions. The core to skin temperature gradient largely determines heat transfer from the core to the skin as well as skin blood flow responses. Heat storage was similar during SDIS and CON, even after normalization for the thermal gradient between the core and the skin. As previously discussed, this similarity in heat storage between trials is also likely because forearm sweating rate was lower and regional skin temperatures higher during SDIS. Thus, more heat was transferred away from the body by radiative pathways by means of skin blood flow during SDIS compared to the CON trial, offsetting any increase in core temperature or thermal strain, allowing heat balance between trials to remain similar. Thus, it appears that SDIS does not affect core temperature or thermal strain, which were both similar between trials, but rather affects the heat transfer pathways, with greater reliance on dry heat loss during SDIS.

In summary, the current study determined that three nights of severely disrupted sleep did not affect resting core temperature; nor, during exercise-heat stress, were core temperature, 8-site whole body mean skin temperature, metabolic rate, heat storage and thermal strain affected. However, three nights of severely disrupted sleep did result in significant increases in skin temperature at several local sites, at rest and during exercise-heat stress, as well as a significant increase in 6-site upper body mean skin temperature, blunting of sweating responses and likely increases in skin blood flow during exercise-heat stress. From the above observations, it appears that in order to maintain similar core temperature, heat balance and thermal strain to those experienced during conditions of normal sleep, upper body radiative, dry heat loss is increased to compensate and offset the decrease in regional evaporative, wet heat loss experienced during the conditions of sleep disruption experienced in this study. Whether these heat loss pathways are regulated at the local or central level, remains to be examined. These results did not support the hypothesis that three nights of severely disrupted sleep would affect thermoregulation during exercise-heat stress, but do expose underlying alterations in heat loss pathways due to sleep disruption.

CHAPTER EIGHT

Influences of three nights of sleep disruption on: human *in vivo* and *in vitro* immune indices at rest, and the functional coupling between circadian skin temperature rhythms and immune function

8.1 Summary: Sleep disruption (SDIS) has been implicated as a stressor that impairs immune function. The current study tested the hypotheses that; three-nights SDIS impairs immune function at rest and that there is a functional, mechanistic link between sleep, circadian skin temperature rhythms and skin immunity. Eleven healthy human males (Mean, (SD): age 21 y, (2); nude body mass 72 kg, (9); body fat 15%, (2); $\dot{V}O_{2peak}$ 61 ml·kg^{-1}·min^{-1}, (3)) were monitored during two randomized trials, 14 d apart. Trials consisted of three-nights control (CON; sleep = 480 min·night^{-1}) and three-nights SDIS (sleep = 2 x 60min·night^{-1}). Eight weeks prior to trials, participants were sensitized to diphenylcyclopropenone (DPCP). Four weeks later, participants received the first of four weekly DPCP elicitation challenges, to achieve a reproducible *in vivo* baseline plateau response. At 48 h into all trials, participants received a low, dose-series DPCP elicitation challenge on their upper inner arm, which was read 24 h later, 72 h into each trial, as clinical score, oedema (skinfold thickness) and redness (erythema). Circulating lymphocyte, leukocyte, neutrophil, monocyte counts, T-lymphocyte sub-sets and CD4:CD8 ratios were determined at 0 h, 24 h, 48 h and 72 h. Participants were fitted with wireless iButtons® to measure skin temperature (T_{sk}) at proximal and distal locations, which logged data for 96 h at 10 min intervals, beginning at 0800 h on day one. Results demonstrate that three-nights of sleep deprivation stress significantly enhances the elicitation phase of the *in vivo* T-cell-mediated immune response (oedema +98% at 24 h post challenge; $P < 0.05$). Circulating lymphocyte, leukocyte, neutrophil, monocyte counts, T-lymphocyte sub-sets and CD4:CD8 ratios were unaltered by SDIS. Daytime and night-time proximal and distal T_{sk} were significantly different between trials ($P < 0.05$). In conclusion, three-nights disrupted sleep significantly enhances *in vivo* T-cell-mediated immunity, but does not affect individual *in vitro* immune indices, and suggests that *in vitro* immune indices may not reveal the true effect of stressors on the co-ordinated immune response. Furthermore, the alterations in T_{sk} due to sleep disruption lends evidence to the functional, mechanistic coupling between circadian variations in T_{sk} and skin immune function, whereby prolonged periods of increased skin blood flow provides for the maintenance and enhancement of skin immune function.

8.2 Introduction

Military personnel, ultra-endurance athletes, search and rescue teams, shift workers and parents of neo-nates, as well as other members of the normal population are often subjected to varying durations of sleep loss. It has been suggested that the stress of total sleep deprivation or disruption of sleep may evoke sub-optimal host defence which may increase the risk of illness, infection, decrease effective wound healing and increase the susceptibility to sepsis (Costa *et al.*, 2010; Shephard *et al.*, 1998).

The duration of sleep loss may be acute (for example, because of the anxiety associated with athletic competition, or the demands of extended military operations (Brenner *et al.*, 2000)), or chronic (due to pain, or the obstructed breathing associated with severe obesity or airway congestion due to respiratory infection) (Walsh *et al.*, 2011a). A recent study showed that 30 h of total sleep deprivation did not alter leukocyte trafficking, neutrophil degranulation or saliva S-IgA responses at rest (Costa *et al.*, 2009). Two to three nights of sleep deprivation has been shown to elicit a circulating leukocytosis, with decreased CD4+ T-cell subset counts (Dinges *et al.*, 1994) and a decrease in neutrophil and lymphocyte function (Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). T-lymphocyte subsets were also significantly lower in chronic insomniacs (Savard *et al.*, 2003), but the duration of sleep loss required to induce this reaction remains unclear. Although the clinical significance of a decrease in immune function with prolonged sleep deprivation in healthy individuals remains unclear, decreases in neutrophil function for example have been implicated in increased infection incidence in clinical populations (Ellis *et al.*, 1988; Smitherman and Peacock, 1995). A stimulatory effect of sleep deprivation on sleep-regulating cytokines (e.g. IL-1β, TNFα and IFNγ) and stress hormones (Dickstein and Moldofsky, 1999; Hu *et al.*, 2003; Radomski *et al.*, 1992) may mediate the

altered immune response which in turn might increase susceptibility to infection (Dinges *et al.*, 1995; Irwin, 2002).

Although it has been suggested that disturbed sleep may cause declines in natural and cellular immunity, as well as alterations in the complex cytokine network (Irwin, 2002), unfortunately, the vast majority of research performed on sleep disturbances have only utilized *in vitro* methods of assessing immune function (Dinges *et al.*, 1994; Dinges *et al.*, 1995; Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). Relatively few studies have examined the *in vivo* immune response to disrupted sleep. One study demonstrated that one night of sleep deprivation after vaccination with Hepatitis A significantly lowered antibody titre 28 days later (Lange *et al.*, 2003). Participants in this study who were allowed to sleep following vaccination displayed a distinct increase in the release of several immuno-stimulating hormones including growth hormone, prolactin, and dopamine, as well as lowered concentrations of thyrotropin, adrenaline and nor-adrenaline, but relatively little difference in white blood cell subset counts (Lange *et al.*, 2003). Another study demonstrated that sleep deprivation appears to cause up to a 50% decrease in antibody production following influenza virus vaccination (Spiegel *et al.*, 2002). One night of sleep deprivation also decreased skin barrier function recovery, assessed via tape stripping on the forearm, and increased plasma IL-1β, TNFα, and natural killer cell activity in women (Altemus *et al.*, 2001). Without incorporating *in vivo* measures such as these, as adjuncts *to in vitro* markers of immunity, it is difficult to determine the clinical significance of the stress of sleep loss on immune function.

Prior to sleep onset, a decrease in heat production and an increase in heat loss commences a nocturnal thermoregulatory cascade, due to an intense increase in skin blood flow. This increase in skin blood flow causes notable skin warming and crucially affects sleep propensity (Van Someren, 2006). However, it is unlikely that this circadian phase of decreased heat production

and increased heat loss presents a fundamental thermoregulatory drive, as without behavioural measures such as warm bedding; there would be a high risk of hypothermia. Rather, it is likely that there is a there is functional, mechanistic link between the thermoregulatory and immune systems, whereby, the period of increased skin blood flow, may support maintenance of the skin as a primary barrier in host defence (Krueger and Majde, 1990; Van Someren *et al.*, 2002). This interesting link between the two systems has not yet been explored, but presents an intriguing avenue for current and future research.

In light of the scarcity of knowledge regarding the effects of sleep disruption on immune function at rest and the applied relevance of obtaining this knowledge, the present study aimed to examine the influence of severely disrupted sleep, on *in vivo* and *in vitro* immune indices at rest in a controlled laboratory environment. The proposed model for the sleep disruption trial was 3-nights of severely disrupted sleep, whereby two one-hour blocks of sleep would be permitted each night, presenting a more ecologically valid model to a wider proportion of the population than a total sleep deprivation model. By measuring proximal and distal skin temperature continuously during this period, the mechanistic link between circadian variation in skin temperature and skin immunity could be explored. It was hypothesized that sleep disruption would impair resting indices of *in vivo* and *in vitro* immune function and that differences in skin temperature during sleep disruption would lend evidence to the theory linking thermoregulation and immune function.

8.3 Methods

Participants

The study was run concurrently with the study described in **Chapter 7**, and as such, the same eleven healthy, non-smoking, recreationally active human males participated (means, (SD): age 21 years, (1); height 179 cm, (9); nude body mass (NBM) 72 kg, (9); body fat 14%, (3); $\dot{V}0_{2peak}$ 63 $ml \cdot kg^{-1} \cdot min^{-1}$, (4)). Participants had no previous history of exposure to DPCP. Participants were excluded based on the criteria stated in **Chapter 3**, and also if they had a history of atopy or any other immune-related or inflammatory dermatological conditions based on established criteria (Kelly *et al.*, 1998).

Preliminary testing

Prior to the main experimental trials, participants performed a familiarization as described in **Chapter 7.**

Experimental design

In a randomized, repeated-measures, crossover design, participants completed two experimental trials. Fourteen days separated the start of each trial. The two experimental trials involved a control trial (CON) and a sleep disruption trial (SDIS). Participants were allowed normal nocturnal sleep on the CON trial (480 $min \cdot night^{-1}$), but during the SDIS trial, participants were restricted to two blocks of one hour sleep per night for three consecutive nights. Allotted sleep during the SDIS trial took place between 2345 h and 0045 h and again between 0445 h and 0545 h. Participants remained in the laboratory building, complete with living quarters, for the entire duration of each experimental trial and were supervised at all times. For a schematic representation of the timing schedule of experimental trials (CON and SDIS), see **Figure 7.1**.

Experimental procedures

The day prior to each experimental trial, participants were provided with food and water equivalent to their predicted total daily energy and fluid intake requirements in order to standardize nutrition, hydration and sleep-wake cycles. Sleep duration on the night before trials was monitored by accelerometry, as previously described (Ancoli-Israel *et al.*, 2003). Participants were transported to the laboratory and arrived euhydrated at 0745 h after an overnight fast. Participants were requested to empty their bladder and bowels prior to 0h measurements. The participants' urine specific gravity, determined by a handheld refractometer (Atago Uricon-Ne, NSG Precision Cells, New York, USA), was 1.012 $g \cdot L^{-1}$ (0.004) on arrival at the laboratory, which indicated euhydration based on published indices (Armstrong *et al.*, 1994). Urine samples were also collected from participants at 24 h, 48 h and 72 h in order to confirm the maintenance of euhydration status, based on published indices and procedures (Armstrong *et al.*, 1994). Participants' NBM was recorded at 0 h, 24 h, 48 h and 72 h. On both trials, at 0750 h, participants were fitted with wireless iButtons® to measure skin temperature (T_{sk}) at proximal (umbilicus) and distal (right wrist) locations, in accordance with previous research (van Marken Lichtenbelt *et al.*, 2006). The iButtons® were affixed to the skin using porous medical-grade tape (Hypafix, BSN Medical GmbH, Hamburg, Germany) and programmed before application to begin logging data at 0800 h on day one at 10 min intervals for 96 h, in 0.0625ºC increments, as described in **Chapter 5**.

Dietary intake was provided to meet predicted individual daily energy requirements (11.8 MJ, (0.81); Carbohydrate, 57%, (3); Fat, 25%, (3); Protein, 19%, (4)) during both CON and SDIS. Water was provided throughout each experimental trial at 3h intervals, equivalent to 40 $ml \cdot kg^{-1} NBM \cdot day^{-1}$, with estimated fluid losses during exercise added to this total. Daily physical activity was monitored via electronic pedometers (Digi-walker SW-200, Yamax, Tokyo, Japan)

and the participants' sleep-wake cycles were monitored by accelerometry (GTIM, ActiGraph LLC, Florida, USA) (Ancoli-Israel *et al.*, 2003). Participants were allowed to perform normal light daily activities such as playing board games, playing video games, watching television, and performed an hour of low intensity exercise each day. Exercise took place at 1445 h on day one, two and three of both trials, with participants performing a 60 min steady state walk at 50% $\dot{V}0_{2peak}$. The exercise protocol was performed in a temperature-controlled laboratory (20ºC, (1); 50% RH, (7)) with a fan generated wind velocity of 2.3 $m{\cdot}s^{-1}$ placed one metre in front of participants. Water was consumed *ad libitum* during exercise, for all participants in both trials.

In vivo DPCP methodology and analysis

Induction of Contact Sensitivity/Sensitization. As described in **Chapter 5**, a sensitizing exposure to the novel antigen DPCP, initiating the induction of antigen-specific immune memory, involved the application of an occluded patch, constituting an 11 mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on Scanpor hypoallergenic tape containing a 10 mm filter paper disc. The patch was soaked in 22.8 µl of 0.125% DPCP in acetone (patch = 30 $\mu g{\cdot}cm^{-2}$ DPCP). After a 3 to 5 minute drying period, the patch was placed on the lower back and left in place for exactly 48 h. All participants were sensitized at the same time of day (1300 h). Sensitization occurred eight weeks prior to the commencement of trials.

Primary Allergic Response (PAR). Sensitization to DPCP was confirmed 14 days after removal of the sensitizing patch by visual assessment of responses at that site. This time period was selected as previous research has suggested that the PAR will typically develop and be observable 7 to 14 days following sensitization (Friedmann, 1991).

Elicitation.

Four weeks after sensitization, the first of four weekly pre-trial elicitation challenges were administered to participants. Pre-trial elicitation consisted of a single occluded patch, constituting a 19mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on Scanpor hypoallergenic tape containing an 18 mm filter paper disc. The patch was soaked in 60.29 µl of 0.0125% DPCP in acetone (patch = 3.412 $\mu g \cdot cm^{-2}$ DPCP). After a 3 to 5 minute drying period, the patch was placed on the lower back and left in place for exactly 6 h. The location of patches alternated sides each week and patches were not placed over the same region of skin as previous challenges. Participants' elicitation challenges took place at the same time of day each week. These pre-trial elicitation challenges were carried out in order to achieve a baseline response plateau based on previous findings, described in **Chapter 5**.

Elicitation during trials. Trials commenced one week after the final pre-trial elicitation challenge. During both trials, the vigour of *in vivo* antigen-specific immune responsiveness was quantified by measuring the responses elicited by another elicitation challenge to the same antigen. At 48 h into both trials, all participants received an elicitation challenge with a dose-series of DPCP on individual patches, each comprising an 8 mm aluminium Finn chamber on Scanpor hypoallergenic tape containing a 7 mm filter paper disc. Patches were applied to the volar aspect of the upper arm (10 µl of DPCP: 0.0048%, 1.239 $\mu g \cdot cm^{-2}$; 0.0076%, 1.982 $\mu g \cdot cm^{-2}$**;** 0.0122%, 3.172 $\mu g \cdot cm^{-2}$; 0.01953%, 5.075 $\mu g \cdot cm^{-2}$; 0.03125%, 8.120 $\mu g \cdot cm^{-2}$ and 10 µl 100% acetone control patch for background subtraction). This dilution series allowed for quantification of the linear portion of the sigmoidal dose-response curve (Friedmann *et al.*, 1983; Friedmann, 2006). Patches were applied in randomly allocated order at the local site in order to minimize any anatomical variability in responses. Elicitation patches were removed after exactly 6 h and the responses measured at 24 h post-challenge (72 h into each trial). Responses were measured at 24 h post-challenge, as it was previously determined that this was the time of peak response, when participants had been administered multiple challenges (**Chapter 5**).

Assessment of CHS responses. Mean skin-fold thickness was determined from triplicate measurements at each elicitation site using modified spring-loaded skin callipers (Harpenden Skin-fold Calliper, British Indicators, England). Skin-fold thickness was recorded to the nearest 0.1 mm by the same investigator by placing the jaws of the calliper at the outer diameter of the response site and measuring skin thickness only (no subcutaneous fat). As previously described (Cooper *et al.*, 1992; Friedmann *et al.*, 1983) this method provides an objective measure of skin oedema (inflammatory swelling). Mean skin erythema was determined from triplicate measurement at each elicitation site using an erythema meter. As previously described (Memon and Friedmann, 1996), this method provides an objective measure of skin redness. Mean background values were determined from triplicate measurements at the acetone patch site for both thickness and redness. In order to determine the increase in thickness and redness, the value from the acetone-only site was subtracted from each elicitation site value. The values for increase in skin-fold thickness and redness over all the doses were summed, which gave an approximation of the area under the dose-response curve, representative of the overall reactivity of each participant to DPCP (Cooper *et al.*, 1992; Palmer and Friedmann, 2004).

Blood collection and analysis

Haematological parameters including haemoglobin, haematocrit, plasma volume change, circulating total and differential leukocyte counts as well as T-lymphocytes ($CD3^+$), helper/inducer T-lymphocytes ($CD4^+$) and cytotoxic/suppressor T-lymphocytes ($CD8^+$) were measured as described in **Chapter 3**.

Sample Size. An estimation of sample size was calculated based on published formulae (Hopkins, 2000). Skin-fold thickness was the primary *in vivo* measurement variable, and thus the sample size calculation was obtained utilizing values for the graded division of the skin-fold

callipers (0.20 mm) and the test-retest reliability typical error (0.18 mm) from a previous assessment of the reliability of this method, detailed in **Chapter 5**. Typical error of 0.20 mm or less in the measurement meant that a sample size of between eight and ten in this cross-over designed study was sufficient for good statistical precision.

Statistical Analyses. Two-way paired sample *t*-tests were used to analyse significant differences in summed skin-fold and erythema responses to DPCP between SDIS and CON trials, as well as pre-trial sleep quantity. Repeated-measures two factor ANOVA were used to analyse the differences in dose responses to DPCP (dose x trial) and circulating lymphocyte counts, circulating leukocyte counts, T-lymphocyte subsets ($CD3^+$,$CD4^+$,$CD8^+$) and CD4:CD8 ratio as well as proximal and distal skin temperatures (time x trial). The 95% confidence intervals (CI) of the differences were calculated where appropriate.

8.4 Results

8.4.1 Sleep Duration, NBM and Physical Activity

Sleep duration on the night before trials, monitored by accelerometry, was not significantly different between trials ($P > 0.05$), as detailed in **Chapter 7**. There were also no significant differences in NBM or daily physical activity between trials ($P > 0.05$).

8.4.2 Plasma Volume Changes

There were no significant differences in plasma volume changes between trials ($P > 0.05$).

8.4.3 Circulating Leukocytes, Neutrophils, Monocytes, Lymphocyte and T-lymphocyte Subsets

There were no significant differences in circulating lymphocyte counts, circulating leukocyte counts, circulating monocyte counts, circulating neutrophil counts, T-lymphocyte sub-sets and CD4:CD8 ratio between trials ($P > 0.05$). There was a main effect of time for mean CD4+ counts, such that at 24h, 48h and 72h CD4+ counts were lower when compared to 0 h, baseline measurements (**Table 8.1**).

	0 h	24h	48h	72h
Leukocytes (x 10^9 L^{-1})				
CON	6.4 (2)	6.9 (3)	6.7 (2)	5.9 (1)
SDIS	5.9 (1)	5.8 (1)	6.2 (1)	6.4 (1)
Neutrophils (x 10^9 L^{-1})				
CON	3.7 (2)	4.3 (3)	4.3 (2)	3.4 (1)
SDIS	3.2 (1)	3.4 (1)	4.1 (1)	4.0(1)
Monocytes (x 10^9 L^{-1})				
CON	0.5 (0.3)	0.6 (0.3)	0.6 (0.4)	0.5 (0.3)
SDIS	0.5 (0.2)	0.5 (0.1)	0.5 (0.1)	0.5 (0.2)
Lymphocytes (x 10^9 L^{-1})				
CON	1.8 (0.6)	1.7 (0.5)	1.6 (0.4)	1.7 (0.4)
SDIS	1.9 (0.6)	1.8 (0.5)	1.7 (0.5)	1.7 (0.5)
CD3 + (x 10^9 L^{-1})				
CON	1.4 (0.4)	1.2 (0.4)	1.1 (0.3)	1.2 (0.3)
SDIS	1.4 (0.5)	1.3 (0.4)	1.3 (0.4)	1.2 (0.4)
CD4 + (x 10^9 L^{-1})		*	*	*
CON	0.8 (0.3)	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)
SDIS	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.5 (0.2)
CD8 + (x 10^9 L^{-1})				
CON	0.4 (0.2)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)
SDIS	0.5 (0.3)	0.3 (0.1)	0.4 (0.2)	0.4 (0.2)
CD4:CD8 (x 10^9 L^{-1})				
CON	1.95 (0.5)	1.64 (0.5)	1.99 (0.5)	1.73 (0.6)
SDIS	1.67 (0.6)	1.85 (0.4)	1.76 (0.6)	1.70 (0.7)

Table 8.1 Circulating leukocyte, neutrophil, monocytes, lymphocyte counts, T-lymphocytes (CD3+), helper/inducer T-lymphocytes (CD4+) and cytotoxic/suppressor T-lymphocytes (CD8+) determined each morning after sleep (CON) and post SDIS (SDIS). Means (SD). *$P < 0.05$ versus 0 h.

8.4.4 In vivo Skin Immunity Assessed by Erythema and Oedema Responses to DPCP

In participants with established immune reactivity to the antigen DPCP, the stress of two nights of sleep deprivation immediately prior to the administration of an elicitation, challenge had a significant enhancing effect on the recall response to DPCP. The inflammatory responses to challenge with five doses of DPCP were measured as increase in skinfold thickness and the readings for the five challenge sites were summed to approximate the area under the curve. The two 4h post-challenge responses (skinfold thickness), summed from five challenge doses, demonstrated that SDIS responses were significantly greater than CON ($t = 2.44$, 7 d.f., $P = 0.04$; 95% CI of the difference, -5.64 to -0.088). This revealed that the summed skinfold response to DPCP challenges performed after two nights of sleep disruption were enhanced 98% at 24 h in comparison to challenges in the same participants performed during the CON trial (**Figure 8.1A**). The reactivity to DPCP following each trial condition was assessed as increase in skinfold thickness over the full dose range of DPCP, at the 24 h measurement times (**Figure 8.2**). There were no significant differences ($P > 0.05$) in erythema responses between trials (**Figure 8.1B**).

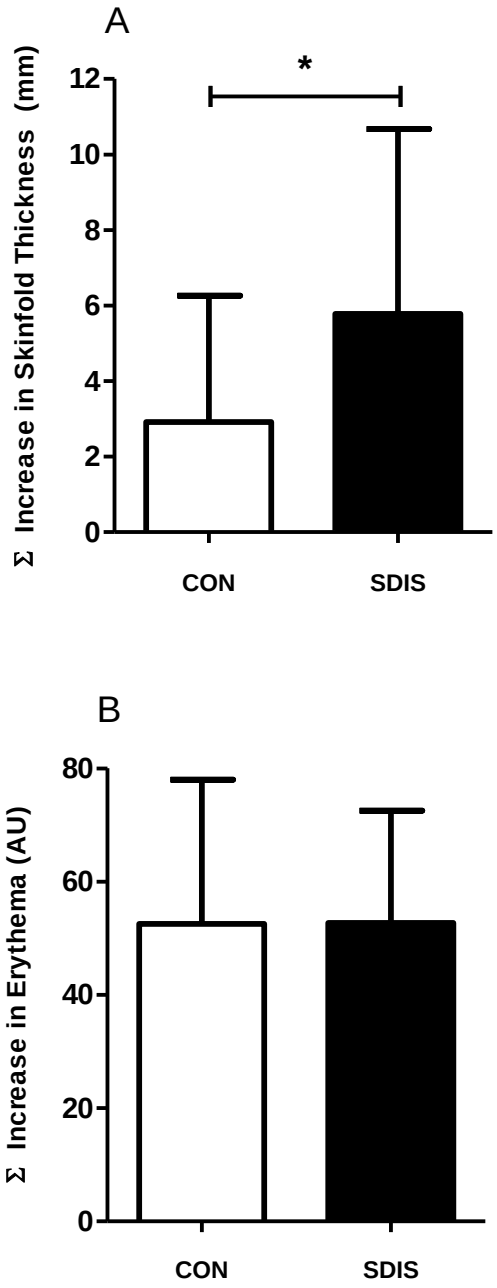

Figure 8.1 Two nights of disrupted sleep prior to elicitation challenge with DPCP results in enhanced oedema response (**A**), but not erythema response (**B**). In participants challenged with DPCP after two nights of disrupted sleep (SDIS), the inflammatory swelling (skinfold thickness) at 24 h post challenge was significantly enhanced compared with the control trial (CON), challenged after two nights of normal sleep, but redness, quantified as erythema was not significantly different between trials. Columns are the means of summed responses (Σ) at five doses of DPCP minus the acetone background control measure; Error bars are SD. * $P < 0.05$, significant difference between trials.

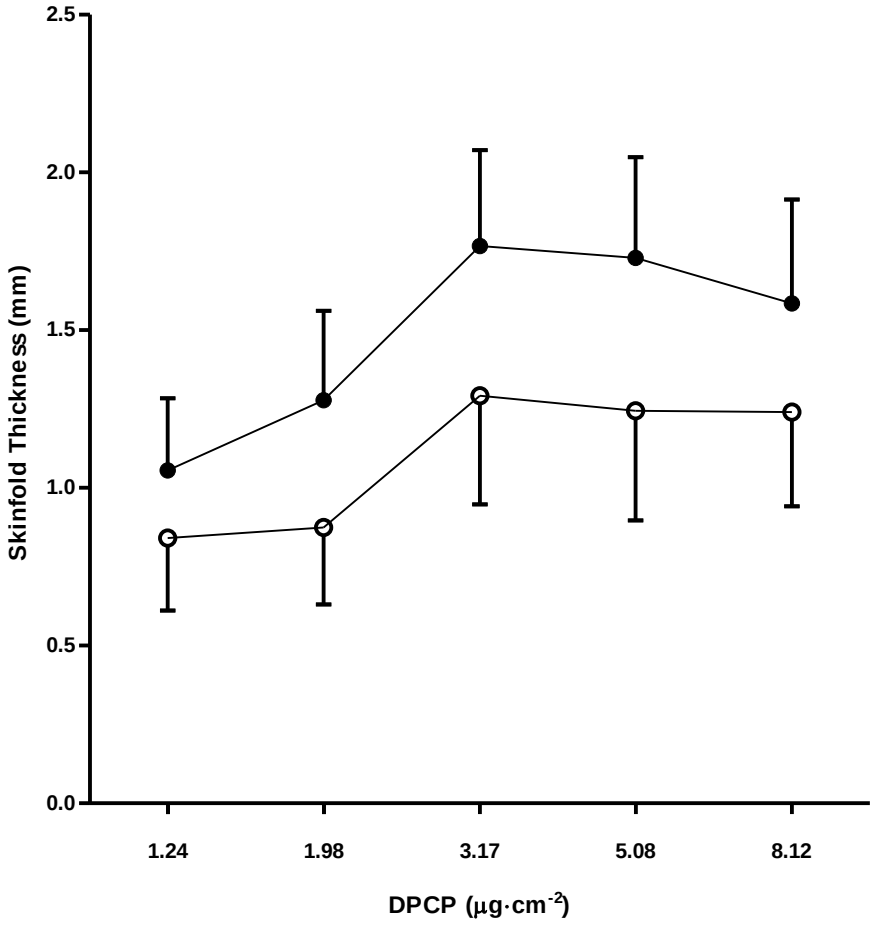

Figure 8.2 Two nights of disrupted sleep prior to elicitation challenge with DPCP results in enhanced oedema response over the entire dose range. In participants receiving a recall challenge with five doses of DPCP after two nights of disrupted sleep (●), the inflammatory swelling, quantified as increase in skinfold thickness, were significantly enhanced compared with responses elicited during the control trial (○) at 24 h post challenge. Participants had previously been sensitized to DPCP and received four weekly recall challenges with the antigen prior to the start of the study. Points are means; Error bars are SEM.

8.4.5 Proximal and Distal Skin Temperature

Daytime and night-time proximal and distal T_{sk} were significantly different between CON and SDIS trials ($P < 0.05$). Proximal T_{sk} was higher from the night of day one onwards during the CON trial in comparison to the SDIS trial, except during the permitted one hour of exercise each day, and during the two x one-hour bouts of sleep permitted during the SDIS trial (**Figure 8.3**). Distal T_{sk} was higher during the three nights of the CON trial in comparison to the SDIS trial, except during the during the two x one hour bouts of sleep permitted during the SDIS trial, where distal T_{sk} was very similar between trials (**Figure 8.4**). During the day, however, distal T_{sk} was higher during the daytime periods of the SDIS trial in comparison to the CON trial, except during, and immediately following, the permitted one hour of exercise during both trials, where distal T_{sk} was very similar (**Figure 8.3**).

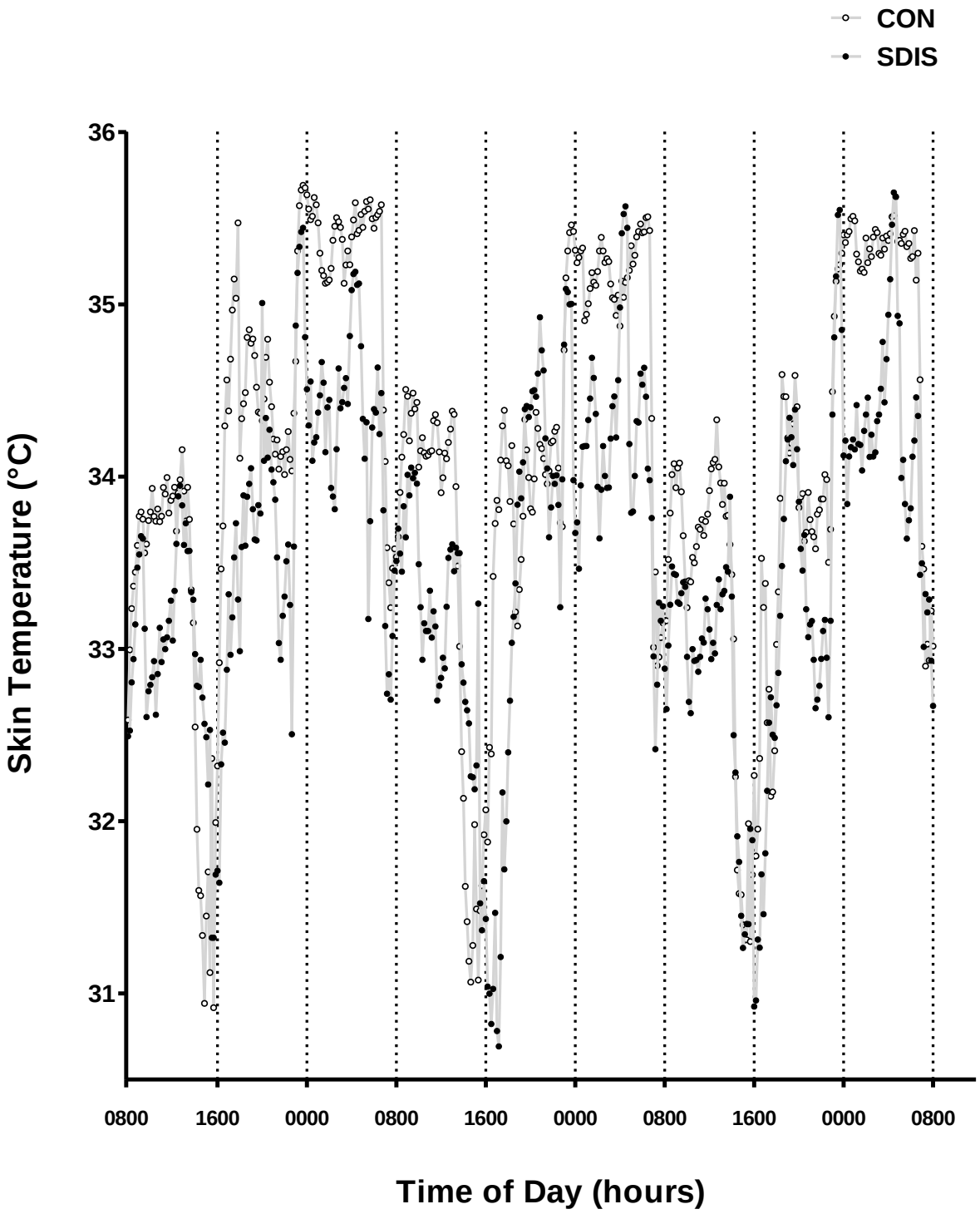

Figure 8.3 Proximal skin temperature at the umbilicus during three-nights of normal sleep (○) and three-nights of sleep disruption (●) during two separate trials (CON and SDIS) in the same participants. Dotted lines break each day into 8 h segments. N = 11. Values are means. Due to the complexity of the graphical representation, significant differences are discussed in the results text.

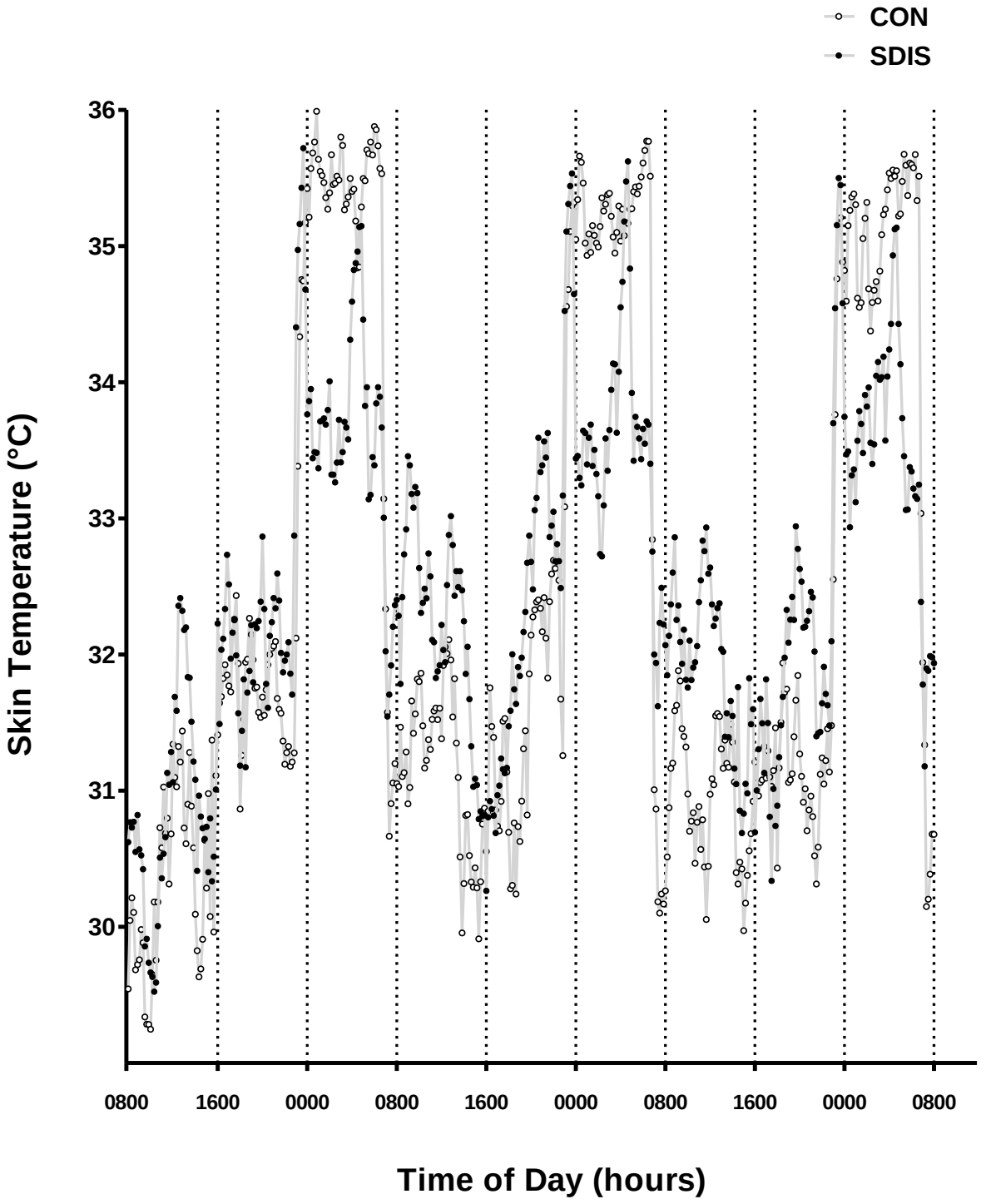

Figure 8.4 Distal skin temperature at the wrist during three-nights of normal sleep (○) and three-nights of sleep disruption (●) during two separate trials (CON and SDIS) in the same participants. Dotted lines break each day into 8 h segments. N = 11. Values are means. Due to the complexity of the graphical representation, significant differences are discussed in the results text.

8.5 Discussion

The aims of the current study were two-fold. The first aim was to determine the effects of three nights of sleep disruption on selected *in vitro* and *in vivo* immune indices at rest. The second aim was to determine whether there is a potential mechanistic link between circadian variation in skin temperature and skin immunity. Compared with multi-stressor studies in the field, a particular strength of the present study is that the experimental procedures were carried out under controlled laboratory conditions, enabling better determination of the influence of sleep disruption, in isolation, on immune indices. Another major strength of the current study was the utilization of an *in vivo* measure of immune function, able to provide a better insight into the clinical relevance of the effects of the stressor than the *in vitro* markers of immune function, utilized almost exclusively in the vast majority of stress physiology research. In contrast to the first hypothesis, three nights of disrupted sleep did not compromise *in vitro* immune indices at rest, but more pertinently, enhanced *in vivo* immune responses to the antigen DPCP. This could suggest that *in vitro* immune indices may not reveal the true effect of stressors on the co-ordinated immune response. In support of the second hypotheses, the alterations in proximal and in particular distal skin temperature, due to acute sleep disruption, lends strong evidence to the functional, mechanistic coupling between circadian variations in skin temperature and skin immune function, whereby prolonged periods of increased skin blood flow provides for the maintenance and enhancement of skin immune function.

In the current study, three nights of sleep disruption did not induce significant alterations in resting circulating leukocytes. These findings extend those of recent studies showing that one and even two nights of sleep deprivation did not alter resting circulating leukocyte or differential leukocyte counts (Costa *et al.*, 2009; Costa *et al.*, 2010). It is unclear why the three nights of sleep disruption did not affect circulating lymphocyte counts, and function, shown previously (Dinges *et al.*, 1994; Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979).

Possibilities include the influence of sleep disruption on circadian modulation of regional blood flow (Van Someren, 2006) and the likely knock-on effects on lymphocyte redistribution (Kruger and Mooren, 2007). Increases in circulating melatonin during periods of sleep loss (Salin-Pascual *et al.*, 1988) might modulate regional blood flow and therefore account for an alteration in lymphocyte redistribution (Guerrero and Reiter, 2002), but this remains unsubstantiated.

Circulating neutrophil, leukocyte and monocyte counts at rest also to remained unchanged in response to three nights of sleep disruption in the current study. The finding of the studies mentioned above also presented alternate findings regarding neutrophil function, which was decreased, and circulating lymphocyte counts, which were also shown to be reduced, following sleep deprivation (Moldofsky *et al.*, 1989; Palmblad *et al.*, 1976; Palmblad *et al.*, 1979). In previous studies in the laboratory, however, it was have observed that one and two nights of sleep deprivation did not compromise bacterially-stimulated neutrophil degranulation at rest, nor significantly affect the stress hormones cortisol, adrenaline and nor-adrenaline (Costa *et al.*, 2009; Costa *et al.*, 2010). The lack of alteration of the stress hormone response could partially account for the unaltered *in vitro* markers of immune function, whereby three nights of sleep disruption may not provide a great enough stress to affect the gross neuro-endocrine pathways. In another study, circulating leukocyte counts were also unchanged after one night of sleep deprivation (Heiser *et al.*, 2000), similar to these findings. Contrary to this though, a marked leukocytosis was demonstrated after 64 h (Dinges *et al.*, 1994) and 24 h of sleep deprivation (Born *et al.*, 1997), but with higher numbers of monocytes, natural killer cells, and counts of all lymphocyte subsets during sleep deprivation in the latter study (Born *et al.*, 1997). T-lymphocyte subsets and the CD4:CD8 ratio were similarly not statistically different between trials in the current study, yet previous studies have exposed a reduction in T-lymphocyte counts after one and two nights of sleep deprivation (Born *et al.*, 1997; Dinges *et al.*, 1994).

These conflicting 'postulations' highlight the importance of *in vivo* measures of immune reactivity, which are much more likely to shed light on the clinical significance and co-ordinated immune response to any given stressor than the 'pick it out of a hat' approach taken with *in vitro* 'markers'. These ambiguities also re-inforce the previous statement that *in vitro* immune markers may not reflect *in vivo* discoveries (Vedhara *et al.*, 1999). Similarly, stimulated *ex vivo* measures may not necessarily mirror the complexity of an *in vivo* immune response either (Gleeson, 2007). In the current study, it was demonstrated that three nights of severely disrupted sleep, surprisingly, enhanced the *in vivo* oedema response to the novel antigen DPCP, even though there were no changes in *in vitro* indices of immunity. It has been previously proposed that acute exposure to stressors evokes an immuno-enhancing response, whereas chronic exposure to stressors causes an immuno-suppressive response (Dhabhar *et al.*, 2000; Dhabhar, 2002b; Dhabhar and McEwen, 1996; Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1999). Thus, although it was hypothesised that three nights of severely disrupted sleep would evoke a stress response similar to that experienced during chronic exposure to stress, it appears that this duration of sleep loss is actually immuno-stimulating and evokes a response observed in acute exposure to stressors. It remains to be determined what the threshold number of days without sleep is in order to induce an immuno-suppressive response. Alternatively, put another way, when acute stress becomes chronic stress.

In experimental animals, it has been suggested that sleep deprivation reduces the antibody response to viruses, and after approximately 20 consecutive days without sleep, results in lethal bloodstream infection and mortality (Brown and Dougherty, 1956; Everson, 1993; Rechtschaffen *et al.*, 2002; Toth *et al.*, 1993). One of the first signs of immuno-deficiency in murine models is the appearance of ulcerative and hyper-keratotic lesions of the skin, principally at the extremities (Everson, 1995; Rechtschaffen *et al.*, 2002; Rechtschaffen and Bergmann, 1995), making the *in vivo* CHS challenge with DPCP used in the current study to test skin immunity, of particular relevance and utility. In murine models, along with lesions of the skin,

total sleep deprivation, and disruption and/or deprivation of non-rapid eye movement (NREM), induces a progressive syndrome with symptoms that included debilitated appearance, increased food intake, weight loss, increased energy expenditure, decreased body temperature; and during the late stages of deprivation, increased plasma nor-adrenaline, and decreased plasma thyroxine, and eventual death (Rechtschaffen *et al.*, 2002). These studies provide a good model for the time course of the pathology associated with prolonged sleep loss, but must be considered with caution, as it is known that rats and mice have subtle differences to humans in their skin immunity (Bos *et al.*, 1990). What this time period of pathology is in humans, again, still remains unclear.

In recognition of the limitation of previous research (**Chapter 5**), where the mechanism(s) underlying stress-induced immuno-modulation of *in vivo* T-cell-mediated immunity were not able to be explored, a battery of tests of *in vitro* immune indices were also included. However, intriguingly, even though it was discovered stress-induced immuno-modulation of *in vivo* T-cell-mediated immunity in this study, no significant differences between trials in any of the *in vitro* immune indices was discovered. It was speculated that there would be stress-related endocrine effects involving hormones such as gluco-corticoids (cortisol), catecholamines (e.g. adrenaline), pituitary / hypothalamus-derived neuropeptides (α-melanocyte stimulating hormone, prolactin, ACTH, or corticotrophin releasing hormone) (Dhabhar, 2002b; Dhabhar and McEwen, 1999), which could affect the *in vivo* T-cell-mediated immune response. Which, while this may be true, it brings into question cause and effect. Why are these immune disturbances occurring and how? It had been proposed that a possible mechanism for an enhanced cell-mediated immune response might be due to elevated circulating leukocyte counts (Dhabhar, 2002b), but this seems unlikely in light of current evidence. Again, what would cause this leukocytosis? As dendritic cells, Langerhans calls and keratinocytes are the primarily mediators involved in antigen presentation, initiating the cell-mediated immune response at the skin (Schroeder *et al.*, 2006; Sullivan *et al.*,

1985; Tamaki *et al.*, 1981), it is likely that alterations to their presence, numbers and function are mechanistically linked to any immuno-modulation at the skin. For example, skin diseases such as atopic dermatitis are associated with abnormal epidermal Langerhans cells and Langerhans cell-like dendritic cells (Reich *et al.*, 2001). Lymphocytes on the other hand have a secondary role in skin immunity (Schroeder *et al.*, 2006). Again, as previously stated, the components of the immune system upon which the relevant mediators may act during the induction of immune memory are likely to include dendritic cells and Langerhans cells in their role as processors and presenters of the immunogen, their interaction with T-cells in the regional lymph node or even their trafficking and migration to the lymph node. Stress-induced modification of the function of dendritic and Langerhans cells could cause alterations in T_H2/T_H1 responses, affecting the cutaneous immune response (Araneo *et al.*, 1989; Cooper *et al.*, 1992; Smith *et al.*, 2004). The stress of sleep loss might in some way affect one or more of the following: the function of Langerhans cells in antigen presentation, antigen specific T-memory cell recognition, antigen specific T-cell clonal expansion (T-helper, T-cytotoxic and T-regulatory cells) and disruption of T-cell subset stimulation, action and secretion of cytokines (Edwards *et al.*, 2006). Further studies are still required to unravel the mechanism(s) responsible for the observed enhancement of *in vivo* T-cell-mediated immunity with this duration of disrupted sleep.

Although an increase in the oedema responses to an in vivo immune challenges with DPCP following three nights of severely disrupted sleep was demonstrated, erythema (redness) responses were unaffected by the same stress. However, both strong and weak reactions to CHS testing can confound the results of erythema testing. With weak reactions, there is little distinction between measurements, and with strong reactors, bullae can develop, which completely changes the colour of the skin at the reaction site from a red scale to a yellow, which is detected as a lower reading by the erythema meter, even though the reaction is likely to be

larger (Friedmann, 2007). These factors could account for the lack of statistical difference in erythema responses discovered in the current study.

These results are in contradiction to a few previous studies which examined the effects of sleep loss on *in vivo* measures of skin immunity (Altemus *et al.*, 2001; Lange *et al.*, 2003; Mostaghimi *et al.*, 2005; Spiegel *et al.*, 2002). However in one of the studies (Altemus *et al.*, 2001), the participants were women, yet they did not control for menstrual cycle phase, despite the fact that the phasic hormonal variations / fluctuations are known to have distinct effects on the immune system (Haus and Smolensky, 1999), potentially confounding the results and conclusions of that study. In the studies that examined the effects of sleep loss on antibody production following vaccination (Lange *et al.*, 2003; Spiegel *et al.*, 2002), the disparate findings could be attributed to the differences in B and T cell biology, and development of B and T cell immunity. In another of the studies, which showed that deprivation of rapid eye movement (REM) sleep in rats after five days (Mostaghimi *et al.*, 2005), the incongruous findings could be attributed to the fact that disruption / deprivation of NREM sleep, as opposed to REM sleep, is known to have a much greater effect on immune responses (Rechtschaffen *et al.*, 2002). In addition, the present study did not specifically interfere with REM sleep. Furthermore, the immune response to wound healing differs from the T-cell mediated immune response, for instance, T-lymphocytes are involved at a much later stage in the wound healing process (Schaffer and Barbul, 1998).

It has been proposed that there is a functional, mechanistic link between the thermoregulatory and immune systems, whereby, the nocturnal circadian phase of increased skin blood flow, may support maintenance of the skin as a primary barrier, and the first line of defence, against environmental, pathogenic microbes (Krueger and Majde, 1990; Van Someren *et al.*, 2002). Paradoxically, the results of the present study lend evidence to support this theory. Unexpectedly, the acute stress of three nights of severely disrupted sleep enhanced *in vivo* T-cell

mediated skin immunity on the upper arm and this can be attributed to the greater number of hours where distal skin temperature was higher in the SDIS trial in comparison to the CON trial. Except during exercise, distal skin temperature at the wrist was up to 2.5°C higher from 0745 h until 2345 h during the SDIS trial and proximal skin temperature at the umbilicus was up to 1.5°C higher. Distal skin temperature at the wrist and proximal temperature at the umbilicus was only higher in the CON trial for the 6 hours when SDIS participants were prohibited from sleeping where the differences were reversed. These extended periods of time when distal skin temperature was higher during SDIS, due to increased distal skin blood flow, may allow for greater redistribution of systemic dendritic cells to the periphery where they are more likely to become involved in the *in vivo* immune response to the antigenic challenge with DPCP. Similarly, other agents of the immune system, such a leukocytes, will also be preferentially transported via the bloodstream during periods of increased blood flow, and in so doing, are given optimum access to the areas of immune challenge where they are required in the immune response to exogenous antigens (Van Someren, 2006). Furthermore, this increase in skin blood flow will provide supplemental nourishment and maintenance to the cells of the immune system, resident in these areas of primary host defence, optimizing their function. The progressive sleep deprivation / sleep disruption syndrome in the murine model indicates that, at a certain arbitrary stage, there is a decrease in body temperature and eventual decrease in peripheral skin temperature / skin blood flow, likely due to consistent heat loss and the inability after time to maintain homeostasis of normal circadian skin temperature rhythms (Rechtschaffen *et al.*, 2002). Although acute sleep deprivation, such as the three nights of severe sleep disruption in the current study displayed heightened daytime distal skin temperatures, eventually, after days of prolonged sleep loss, it is highly probable that a threshold is reached whereby these responses are severely dampened, become chronic, and there is an ultimate reduction in skin blood flow to the extremities. At this stage, skin immunity would become compromised. As seen in the present study, both proximal and distal night-time skin temperatures reach levels that are rarely, if ever

attained, during wakefulness, not even during periods of exercise. This evidence suggests that enhanced skin immunity due to long durations of increased distal skin temperature / distal skin blood flow augments the *in vivo* immune response to a model of pathogenic challenge from the acute stress of three nights of sleep disruption in the current study. As stated previously, the body's response to acute stress in often to enhance immune function, which was observed here, and it would appear that this is through the central control of skin temperature and skin blood flow. Therefore, although there is a heightening of the immune response during this duration of sleep disruption, this is only an acute response to short-term stress. This unlikely to be the case during chronic sleep loss, where there will be a loss of central drive whereby increased skin temperature and skin blood flow can no longer be maintained, and skin immunity deteriorates, as seen in chronically sleep deprived murine models (Brown and Dougherty, 1956; Everson, 1993; Rechtschaffen *et al.,* 2002; Toth *et al.,* 1993). This would therefore still suggest that a function of sleep, when skin temperature and skin blood flow are higher is to maintain and enhance skin immune function. Thus, circadian variation in skin blood flow, represented by significant increases in distal skin temperature during night-time sleep, represents an immunological rather than thermoregulatory drive.

In summary, three nights of severely disrupted sleep significantly enhanced *in vivo* T-cell-mediated immunity, but did not affect individual *in vitro* immune indices, and suggests that *in vitro* immune indices may not reveal the true effect of stressors on the co-ordinated immune response. Furthermore, the alterations in skin temperature due to sleep disruption lend evidence to the functional, mechanistic coupling between circadian variations in skin temperature and skin immune function, whereby prolonged periods of increased skin blood flow provides for the maintenance and enhancement of skin immunity.

CHAPTER NINE

General Discussion

9.1 Background

Military personnel, ultra-endurance athletes, search and rescue teams as well as shift workers and many other professions are often exposed to various stressors as part of their normal routine. It has been indicated that sleep loss, energy restriction and physical exertion can negatively impact thermoregulation, as well as immune function, in both temperate and adverse environmental conditions (Aakvaag *et al.*, 1978; Bugge *et al.*, 1979; Castellani *et al.*, 2003; Gleeson, 2007; Kolka and Stephenson, 1988; Rognum *et al.*, 1986a; Shephard *et al.*, 1998; Young *et al.*, 1998). It has been proposed that sleep loss may pre-dispose individuals to be more susceptible to EHI (Rav-Acha *et al.*, 2004) as well as increase the risk of hypothermia when combined with the stressors of energy restriction and prolonged physical exertion (Castellani *et al.*, 2003; Kolka *et al.*, 1984; Young *et al.*, 1998). Although many of these investigations have examined the effects of sleep loss, energy restriction and prolonged exercise on thermoregulation in the cold, the independent and combined effects of sleep loss and energy restriction on thermoregulation in the cold remains unclear. It has also been suggested that the stress of sleep loss may evoke sub-optimal host defence which may in turn increase the risk of illness, infection, decrease effective wound healing and increase the susceptibility to sepsis (Costa *et al.*, 2010; Shephard *et al.*, 1998). It is also thought that methods currently used to assess certain aspects of thermoregulation and immune function can be improved upon (Albers *et al.*, 2005; Lichtenbelt *et al.*, 2006). There is a distinct paucity of research that has utilized *in vivo* measures of immune function to assess the effects of stressors, preventing clear conclusions to be drawn regarding the clinical significance of stress on immune function. The utilization of an *in vivo* measure of immune function, able to identify the effects of various stressors would

therefore be a very useful addition to stress physiology research. The current methodology used to measure skin temperature puts great constraints on when this variable can be assessed. Therefore, an inexpensive, autonomous, wireless, temperature monitoring and recording device would considerably benefit human thermal physiology research, particularly during free living conditions (van Marken Lichtenbelt *et al.*, 2006). In addition, the effects of periods of sleep loss longer than one night in humans, on thermoregulation and immune function, are either unclear or unknown. Furthermore, it has been suggested that there is a functional, mechanistic role for the circadian modulation of thermoregulation, promoting, in particular, the maintenance of skin immune function (Van Someren, 2006). The circadian phase of decreased heat production and increased heat loss is unlikely to present a fundamental thermoregulatory drive, as without behavioural measures such as warm bedding, there would be a high risk of hypothermia. The broad aims of the thesis were therefore to investigate: 1. The validity of iButtons®, a novel, wireless method for the measurement of human skin temperature. 2. The effects of exercise on the induction and elicitation phases of the immune responses to a novel antigen, utilizing experimental contact-hypersensitivity, a clinically relevant *in-vivo* measure of T cell-mediated immunity. 3. The independent and combined effects of sleep deprivation lasting 53h on thermoregulation at rest and during prolonged cold exposure. 4. The effects of three nights of sleep disruption on thermoregulation and immune function at rest, and on thermoregulation during subsequent exercise-heat-stress. 5. The potential functional, mechanistic link between circadian variation in skin temperature and skin immune function.

9.2 Summary of Main Experimental Findings

First, although the thermoregulatory and immune systems are often considered in isolation, there is naturally a fundamental link between all the bodily systems. In this thesis, the first evidence is presented to show that there is a functional, mechanistic link between circadian variations in skin temperature / skin blood flow and skin immune function, testing previous hypotheses (Van Someren, 2006). Further, evidence is also given to the theory that the effects of various stressors display a temporal relationship, whereby acute stress may be adaptive and long term exposure may be mal-adaptive and harmful (Dhabhar, 2002a; Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1999). The time course determining whether the stress response displays acute or chronic characteristics also appears to vary between stressors. For example, only two hours of moderate exercise-induced stress reduces *in vivo* indices of skin immunity, whereas three nights of severely disrupted sleep increases the immune response. This can partially be explained by the fact that different stressors evoke different responses from the HPA axis and the adrenergic and nor-adrenergic nerves (Goldstein and Eisenhofer, 2000)

Novel methodology for the measurement of skin temperature demonstrated the validity and reliability of wireless iButtons® during laboratory and potential field investigations, with particular utility when skin temperature measurement, in many circumstances, using other currently available methods would prove problematic (**Chapter 4**).

This is the first demonstration, using the method of experimental contact sensitization, that a single bout of prolonged moderate intensity exercise-stress significantly reduces both the induction as well as the elicitation of *in vivo* cell-mediated immunity in humans. Clinical repercussions of the exercise-induced reduction in *in vivo* cell-mediated immunity include a potential increased risk of infection. These results also indicate that experimental contact sensitization with DPCP provides a simple, inexpensive, and robust method for research

investigators to evaluate the effects of other forms of stress on both induction and elicitation of *in vivo* cell-mediated immunity. Furthermore, the data showed that a plateau in CHS response can be attained with repeated serial administration of the antigen, allowing utilization of the CHS method in repeated-measures, cross-over designed research investigations (**Chapter 5**).

Two nights without sleep, with or without 90% restriction in energy intake, did not significantly impair core temperature, skin temperature and heat balance responses during cold exposure up to four hours. Time to reach a rectal core temperature of 35.9°C during cold exposure was also not statistically different between trials, but two nights of sleep deprivation did have a large positive and clinically significant effect on exposure duration, which would likely become statistically significant with a larger sample size. The relationship between metabolic heat production and mean body temperature, as well as between heat debt and core temperature potentially indicate underlying alterations to normal thermoregulation during more prolonged whole body cooling (> 4h, T_{re} < 35.9°C) in the primary shivering response phase. It would therefore appear that military personnel, ultra-endurance athletes and search and rescue teams are under no greater risk of hypothermia from cold exposure of up to four hours in duration when sleep deprived for two nights, even with the addition of 90% energy restriction, than when they are well rested and well fed (**Chapter 6**).

Three nights of severely disrupted sleep did not affect resting core temperature; nor, during exercise-heat stress, were core temperature, 8-site whole body mean skin temperature, metabolic rate, heat storage and thermal strain affected. However, three nights of severely disrupted sleep did result in significant increases in skin temperature at several local sites, at rest and during exercise-heat stress, as well as a significant increase in 6-site upper body mean skin temperature, blunting of sweating responses and likely increases in skin blood flow during exercise-heat stress. From these observations, it appears that in order to maintain similar core temperature,

heat balance and thermal strain to those experienced during conditions of normal sleep, upper body radiative, dry heat loss is increased to compensate and offset the decrease in regional evaporative, wet heat loss experienced during the conditions of sleep disruption experienced. Whether these heat loss pathways are regulated at the local or central level, remains to be examined. These results did not support the hypothesis that three nights of severely disrupted sleep would affect thermoregulation during exercise-heat stress, but do expose underlying alterations in heat loss pathways due to sleep disruption (**Chapter 7**).

Three nights of disrupted sleep, interestingly, significantly enhanced *in vivo* T-cell-mediated immunity, but did not affect individual *in vitro* immune indices, suggesting that *in vitro* immune indices may not reveal the true effect of stressors on the co-ordinated immune response. Furthermore, the alterations in skin temperature due to sleep disruption lend evidence to the functional, mechanistic coupling between circadian variations in skin temperature and skin immune function, whereby prolonged periods of increased skin blood flow provides for the maintenance and enhancement of skin immunity (**Chapter 8**).

9.3 Limitations and Direction for Future Research

9.3.1 Novel Measures of Thermoregulation and Immune Function

9.3.1.1 Human Skin Temperature Measurement

Future research will hopefully implement iButtons® for the measurement of human skin measurement. In individuals who excessively sweat during exercise, there was sometimes difficulty in keeping the iButtons® in place, an issue also experienced with thermistors. Thus, it would be favourable if future studies might be able to discover a reliable method of retaining the iButtons® in position in areas where there is an excessive amount of sweat in exercising conditions, to prevent any criticism of confounding factors. It would also be favourable for complete clarity, if examinations, utilizing this method, could be performed at environmental extremes beyond the boundaries of those listed in the current study (**Chapter 4**).

9.3.1.2 Human in vivo Measurement of Immune Function

Ideally, future research will be able to identify the relevant mechanism(s) behind modulation of the *in vivo* immune response, which was not able to be determined in this study. Utilizing current *in vitro* measures of immunity, an attempt was made to discover the mechanistic explanation for alterations in immune function, but this was unsuccessful. It is hoped that future studies will be able to examine the causal mechanisms via skin punch biopsies, sentinel node biopsies, extraction and examination of bullae fluid, molecular targeting and/or antigen-specific tracer technology, or some combination of all of these methodologies.

9.3.2 The Effects of Certain Stressors on Thermoregulation and Immune Function

9.3.2.1 Thermoregulation in the Cold

The main limitation of the study was the cut-off core temperature. Hopefully, with ethical consent, future studies should utilize a similar model to attempt to examine any potential effects of sleep deprivation on thermoregulation in the cold when participants become clinically hypothermic (T_{re} < 35.0°C). In light of this evidence, future studies examining disturbances to cold thermoregulation due to potential stressors, may wish to focus on the potential mechanisms for any thermoregulatory disruption associated with stressors such as sleep deprivation. Future studies might also like to examine the termination phase of shivering by utilizing drugs such as Meperidine which pharmacologically inhibit shivering (Logan *et al.*, 2011), in order to determine whether SDEP and SDEP with ER increases the risk of fatal hypothermia.

9.3.2.2 Thermoregulation in the Heat

Unfortunately, due to manufacturing constraints, only local sweat rate responses in six participants were able to be assessed. Even though significant differences between trials in the local forearm sweat rate during exercise in the heat due to sleep disruption were discovered, future studies may wish to examine this response with a larger sample size. With ethical consent, future studies may also wish to examine the effect of a longer period of sleep disruption on thermoregulation at rest and during exercise-heat stress in humans. In addition, it would be beneficial if skin blood flow could be measured by a method which provides valid and reliable measurements during ambulatory conditions.

9.3.2.3 Immune Function in Response to Exercise Stress

As mentioned previously, it would be beneficial to the current body of knowledge, to discover the relevant mechanism(s) behind modulation of the *in vivo* immune response, which,

unfortunately was not possible in this study. It is hoped that future studies will be able to examine the causal mechanisms via the methods listed above as well as other potential methodologies. It would also be of interest if future studies utilized this CHS methodology to examine the effects of different modes, intensities and durations of exercise.

9.3.2.2 Immune Function in Response to Sleep Disruption Stress

Again, it would be beneficial to the current body of knowledge, to discover the relevant mechanism(s) behind modulation of the *in vivo* immune response. It is hoped that future studies will be able to examine the causal mechanisms via the methods listed above as well as other potential methodologies. A limitation of the study was that it was not possible to take *in vivo* measures at 48 h post-challenge with DPCP, as the results would have been confounded by the exercise heat stress experienced by the participants in the study the day before. Furthermore, as the challenge was administered 48 h into trials and the responses measured 24 h later, it is hard to say with any certainty whether this in fact represents the response to two or three nights of sleep disruption. Although it could be argued that this is a picture of the response to the stress of two nights of disrupted sleep, the stressor continued for another night while the response developed, and as such, more likely truly represent the stress of three nights of sleep disruption. With ethical consent, it would also be of interest if future studies utilized this CHS methodology to examine the effects of longer durations of sleep disruption / sleep deprivation in an attempt to determine the threshold number of days when the stress response changes from being acute and adaptive to being chronic, mal-adaptive and detrimental. It would also be of benefit if *in vivo* immune responses were examined in participants with chronic sleep disorders such as chronic insomnia and sleep apnea.

9.3.3 The Mechanistic, Functional Link between Sleep, Thermoregulation and Immune Function

Although proximal and distal skin temperature as a surrogate measure of skin blood flow were used, it would be advantageous if measures of skin blood flow could be made during normal, ambulatory, free living conditions. However, current technologies for skin blood flow, such as Laser Doppler systems are very sensitive to physical movement and cannot be made accurately and reliably during ambulatory conditions. So, once the technology is available to make wireless measurements of skin blood flow, hopefully these methods will be employed in studies such as this one. Nevertheless, skin temperature is regulated by skin blood flow, so the measures used in the current study were the best available.

9.4 Conclusions

The major conclusions of this thesis are:

1. Wireless iButtons® provide a practical, valid and reliable method of measuring skin temperature.

2. Wireless iButtons® are of particular utility when skin temperature measurement using other currently available methods would prove problematic, such as free-living, ambulatory and clinical settings or in field studies.

3. A single bout of prolonged moderate intensity exercise-stress (2h treadmill running at 60% $\dot{V}O_{2peak}$) is enough to induce a reduction of both the induction as well as the elicitation of *in vivo* cell-mediated immunity in humans.

4. Contact sensitization with DPCP provides a simple, inexpensive, and robust method for research investigators to evaluate the effects of other stressors on both induction and elicitation of *in vivo* cell-mediated immunity.

5. A plateau in CHS response can be attained with repeated serial administration of the antigen, allowing utilization of the CHS method in repeated-measures, cross-over designed research investigations.

6. Two nights without sleep, with or without 90% restriction in energy intake, does not significantly impair thermoregulation responses at rest or during cold exposure of up to four hours.

7. Individuals wearing very little clothing, exposed to the cold (0°C) for relatively short periods of time (< 4h) when sleep deprived for two nights, even with a 90% reduction in energy intake, are at no greater risk of hypothermia than individuals that are well rested and well fed.

8. Three nights of severely disrupted sleep does not significantly impair thermoregulation at rest or during exercise-heat stress (45 min treadmill running at 60% $\dot{V}O_{2peak}$ in an environmental chamber set to 33°C and 40% RH with 0.2 $m \cdot s^{-1}$ wind velocity).

9. Three nights of sleep disruption causes alterations in the thermoregulatory pathways during exercise-heat stress, whereby, in order to maintain similar a) core temperature, b) heat balance and c) thermal strain to those experienced during conditions of normal sleep, upper body radiative / dry heat loss is increased to compensate and offset the decrease in regional evaporative / wet heat loss experienced during the conditions of sleep disruption.

10. Three nights of disrupted sleep significantly enhances *in vivo* T-cell-mediated immunity, but does not affect various individual *in vitro* immune indices.

11. The length of exposure required to evoke the characteristic symptoms of acute (immuno-enhancing, adaptive, beneficial and protective effects) versus chronic stress (immuno-suppressive, mal-adaptive, harmful and damaging effects) varies greatly between stressors. I.e. 2 h of exercise-induced stress displays chronic characteristics of exposure to stress, whereas three nights of sleep deprivation displays acute characteristics of exposure to stress.

12. Finally, alterations in distal skin temperature due to sleep disruption lend evidence to the functional, mechanistic coupling between circadian variations in skin temperature and skin

immune function, whereby prolonged periods of increased skin blood flow provides for the maintenance and enhancement of skin immunity.

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Appendix A

Bangor University
School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title: Validation of iButton wireless data loggers for skin temperature measurements during rest and exercise in cold, thermoneutral and warm environments.

Research Investigators: Adam Harper Smith, Daniel Crabtree, Jo Small.
Research Co-ordinator: Dr Neil Walsh
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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 treated confidentially**.

Background
The human skin forms the border between the human body and the outside environment. Therefore, skin temperature gives us a good idea of the amount of heat being lost or heat being gained by an individual. The 'real-time' measurement of skin temperature can be useful in numerous fields including studies of the human 'body-clock', sleep and occupational or recreational environmental exposure. Skin temperature measurements are of interest both at rest and during exercise.

Human skin temperature at rest and during exercise is usually measured using wired temperature 'sensors'. An advantage of using this method is that the sensors are relatively inexpensive. However, disadvantages of using wired skin temperature sensors are that they are connected to the skin via wires which can get tangled and are cumbersome, they can take a long time to put on, they can interfere with normal exercise and are impractical for use in real world situations. However, the new iButton devices, which we aim to evaluate in this study are small, wireless and may therefore provide a more practical alternative to the currently available methods.

Therefore the aim of this study is to evaluate the use of iButtons for measuring skin temperature, both at rest and during exercise in cold (10 degrees Celsius), room temperature (20 degrees Celsius) and warm (30 degrees Celsius) air temperature conditions.

What will be expected of you?

Before starting experimental trials you will be required to:

- Perform a maximal aerobic exercise test on a stationary bicycle (VO_{2max} test)
- Weigh yourself nude (in privacy)
- Have your height and percentage body fat recorded (with skin fold callipers)

Prior to experimental trials you will be required to:

- Refrain from alcohol, caffeine and exercise for 24 h before trials
- Keep a record and try to consume the same foods in the day prior to each trial

During experimental trials you will be required to:

- Attend the lab on 3 separate occasions
  The visits will be divided into cold (10 degrees Celsius), room temperature (20 degrees Celsius) and warm (30 degrees Celsius) trials.
- Remain seated in the environmental chamber (a room which can regulate temperature and humidity) for 40 minutes at low wind speed and 40 minutes at higher wind speed at each of these temperatures (10, 20 and 30 degrees Celsius)
- Perform 1 bout of stationary bicycle exercise for 40 minutes at each of these temperatures (10, 20 and 30 degrees Celsius)
- Weigh yourself nude (in privacy)
- Use a small rectal temperature sensor to measure your core body temperature (for safety reasons)
- Have skin temperature sensors attached to your skin at 14 sites with tape
- Also have iButtons (wireless devices used to measure skin temperature) attached to your skin at the same sites with tape.

Summary of time commitment:

Briefing and Familiarization -	**3 hours maximum**
Preparation and set-up time -	**1 hour maximum**
Baseline measurements -	**4.5 hours**
Resting exposure component -	**4 hours**
Exercise component -	**2 hours**
In total, participation in this study will require you to commit	**~ 14.5 h of your time**

Summary of visits

Visit 1: 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 to allow you additional time to think of questions. Once you are satisfied that you understand what your involvement entails and you are happy to commit to participating, you will be asked to sign an informed consent form.

Visits 2: Familiarization (~2.5h)

During this visit, we will take various measurements such as your height, weight and percentage body fat (using skin fold callipers). We will then ask you to perform a maximal aerobic test (VO_{2max} test) on a stationary bicycle, to determine your aerobic fitness level. Following approximately 12-15 minutes where exercise intensity is increased every 3 minutes, the test will require you to cycle at your maximal aerobic capacity for approximately one minute at the end. You will also be shown all other equipment that will be used in the study, such as the skin temperature sensors and the iButtons, to show you what the procedures involve and how they feel. In order to complete the investigation it is a necessity to use these devices.

Visits 3, 4 and 5: Experimental trials (~4 h each)

You will be asked to take part in three experimental trials lasting ~4 hours each in a randomized order separated by at least 48 hours. Each session will involve both a resting and exercise component in either cold (10 degrees Celsius), room temperature (20 degrees Celsius) or warm (30 degrees Celsius) air temperature conditions.

The cold trial will involve resting in a seated position in the environmental chamber for 40 minutes at low wind speed and 40 minutes at a higher wind speed at an air temperature of 10 degrees Celsius. Following the resting portion you will be asked to complete a period of exercise which will involve exercising on a stationary bicycle at 50% (low-moderate intensity) of your maximal oxygen uptake for 40 minutes at 10 degrees Celsius. In addition, there will be 30 minutes of seated rest outside the environmental chamber before entering the chamber and 30 minutes between each of the periods spent in the chamber.

The room temperature and warm trials will be of the same format, but will take place at temperatures of 20 and 30 degrees Celsius respectively.

Respiratory, subjective, percentage body fat and thermoregulation measures
These measurements will take place during the familiarization and experimental trials.

1. Respiratory measures refer to the analysis of expired air (from breathing) which will involve wearing a face mask during the maximal aerobic exercise test (VO_{2max} test).

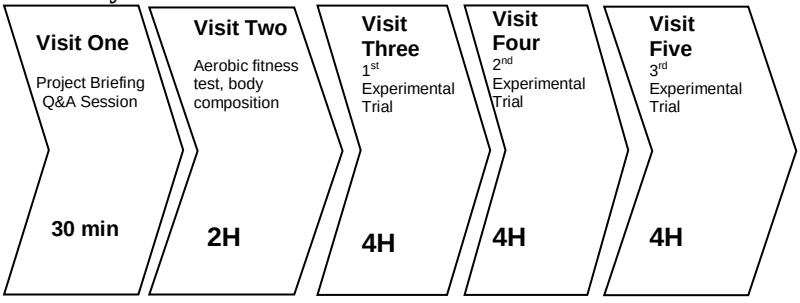
2. Subjective measures refer to perceived exertion ratings. This will be recorded after asking you to indicate a rating of how you feel from a scale during exercise.

3. Skin fold measurements using callipers to determine percentage body fat.

4. Thermoregulation measurements refer here to skin temperature which will be measured via wireless iButtons and wired electrode sensors placed on the skin sites, as well as core body temperature, which will require the use of a small rectal temperature sensor.

Please refer to the flow diagram below which provides an overview of the experimental trials.

Summary of visits



What are the possible risks of taking part?

Exercise, Cold and Heat exposure

- You will be asked to perform 3 bouts of exercise on each of the trials. This will be at low exercise intensity and for a short duration. Therefore, this should not be hazardous to your health if you are a recreationally active, young, healthy individual. The risk of collapse is very low in this population group.
- When exercising in the heat or during passive cold exposure, your core temperature (normally approximately 37 degrees Celsius) may be change by 1 or 2 degrees Celsius. Your core body temperature will be monitored throughout the experimental trials using a rectal temperature gauge to make sure that you stay within safe limits. Should your core body temperature rise or fall by 1.5 to 2°C you will be withdrawn from the environmental chamber.

Precautions:

- A researcher qualified in first aid and CPR will be on hand at all times.
- You will be re-warmed with blankets or cooled with fans, should your core body temperature fall or rise by these margins, in line with the SSHES departmental safety guidelines.

What are the possible benefits of taking part?

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, on your fitness level and body composition (e.g. body fat %). This feedback should help you with planning 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. In addition, participation in this project can cover both the open and personal performance skills units of the Undergraduate portfolio.

As a participant in this project you will also be helping to explore previously unanswered questions in this area of temperature measurement. The findings of this study will confirm the accuracy of the iButtons during rest and exercise. This has implications for future thermoregulation research, especially in the field where current methods are impractical.

Disadvantages of taking part

The disadvantages of taking part in this study, which you will probably be most concerned about, are: 1. Time commitment. 2. Temperature measurements 3. Environmental exposure. 4. Exercise.
At all times you will be closely supervised by an experimenter trained in First-Aid and CPR.

1. **Time commitment**: To complete all aspects of the study we will require you to visit the laboratory on five separate occasions for a total of approximately 14.5 hours. However, you are free to withdraw without reason from the study at anytime, should you wish.

2. **Temperature measurements**: For your safety, we will ask you to be fitted with a rectal core body temperature sensor. The sensor is small (~5mm diameter, 10cm long) and should not cause you discomfort. Wired skin temperature sensors and iButtons will be fitted with tape, but will not be uncomfortable.

3. **Environmental Exposure**: Numerous scientific experiments have exposed individuals to cold and/or warm conditions and reported no medical complications. In this experiment your core body temperature could decrease to 36°C or rise to 39.5°C; this is within the SSHES Health and Safety guidelines. For your safety, your core temperature will be monitored continuously throughout the environmental exposure and in recovery. Should your core body temperature fall to 36°C or rise to 39.5°C during the environmental exposure you shall be withdrawn from the

chamber and then either warmed with blankets or cooled with fans, as appropriate, to restore normal core temperature.

4. **Exercise Component**: In this experiment you will be required to exercise in both cold and warm environments. However, this will be at low exercise intensity and for short durations; this should not be hazardous to your health if you are a recreationally active, healthy individual. Throughout exercise your core body temperature will be monitored for your safety, using a rectal temperature sensor. Again, should your core body temperature fall to 36°C or rise to 39.5°C during the environmental exposure you shall be withdrawn from the chamber and then either warmed with blankets or cooled with fans, respectively, to restore normal core temperature.

Any further questions will be happily answered by Adam Harper Smith, Daniel Crabtree or Dr. Neil Walsh.

Participant Information Sheet – Investigation One.

Bangor University

School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title:

The effect of prolonged exercise prior to sensitization on contact hypersensitivity (CHS) elicitation responses to diphenylcyclopropenone (DPCP) in active human males.

Research Co-ordinator: Dr. Neil Walsh **Tel:** 01248 383480

Email: n.walsh@bangor.ac.uk

Additional Investigators: Adam Harper Smith, Dr. Andrew Macfarlane, Prof. Peter Friedmann, Alison Owen and Sarah Coakley

Invitation to take part

We invite you 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 you 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 will 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 Coordinator's computer for 5 years before being confidentially destroyed.

Background

Elite athletes and the recreationally active may be more susceptible to infection after they exercise at high intensity or exercise for long durations. Previous research has suggested that your immune system may be depressed or compromised by very long or very hard exercise, but it is not known whether this is actually clinically significant. Most tests that currently exist only look at markers of immune function in the blood or saliva. For example, these tests can see whether you have a normal number of white blood cells. However, as these are just markers, they cannot really be used to predict whether you may be more likely to get, say, a cold or the flu. It is currently not possible to predict an outcome of clinical significance based on these tests alone. Contact hypersensitivity tests like the one we will use in this study, may be able to give us a more accurate picture of your immune status. These tests use very low concentrations of an allergic compound (allergen) placed on an adhesive patch and left on the skin for up to two days.

Your body's responses to the allergen can be used as a measure of your immune function. The patches are very similar to those used by doctors (such as the dermatology specialist consultants in our team: Dr. Andrew Mcfarlane and Prof. Peter Friedmann) to see what an individual may be allergic to (e.g. nickel, rubber). What we would like to find out is whether prolonged exercise will significantly affect the immune system responses of those participants performing the requisite exercise, when compared with the participants performing the condition without exercise.

What will be expected of you?

If you decide to take part in this study you will make 7 visits to the laboratory and there will be a few constraints placed upon your normal everyday life and activities.

In the days(s) before Visit 3 and Visit 5 (see below) you will be expected to:

- Avoid drinking alcohol and caffeinated drinks (i.e. coffee, tea, cola and diet cola) for 48 hours before arriving at the laboratory and for 48 hours after leaving the lab on these occasions.
- Avoid participating in any exercise for 24 hours before you arrive at the laboratory;

Whilst participating in the study, (exercise OR seated rest – randomly assigned) you will be expected to:

- Weigh yourself nude (behind screens to maintain privacy)
- Perform a maximal aerobic fitness running test (details below).
- You will be randomly assigned to perform **EITHER** the prescribed physical activity (120 min of moderate paced running on a treadmill) **OR** seated rest (Control), immediately before the application of the first patch (please be prepared to perform either condition).
- Straight after the period of exercise OR seated rest, all participants will have a single, small allergen (DPCP) skin patch applied to your mid-lower back that will remain in place for 48 hours.
- Return to the laboratory 14 days later to determine whether you have reacted to the DPCP.
- Four weeks later, have a set of 7 small skin patches with very low concentrations of DPCP and an acetone control patch applied to your inner, upper arm that will remain in place for 6 hours.
- Have measurements of skin redness and thickness taken at the skin patch sites at 6, 24 and 48 hours after the set of skin patches are applied (all measurements are non-invasive and painless).
- Have the set of skin patches visually assessed by a dermatologist 48 hours after they are applied.

You will be excluded if you are a smoker or if you are on specific types of medication or supplements (i.e. Creatine), have any immune disorders, tendencies to abnormal scarring (e.g. keloid), have eczema or dermatitis on the areas to be skin patch tested, a history of atopic eczema or have been a participant in other studies involving skin patch testing. Asthmatics and Diabetics will also be excluded.

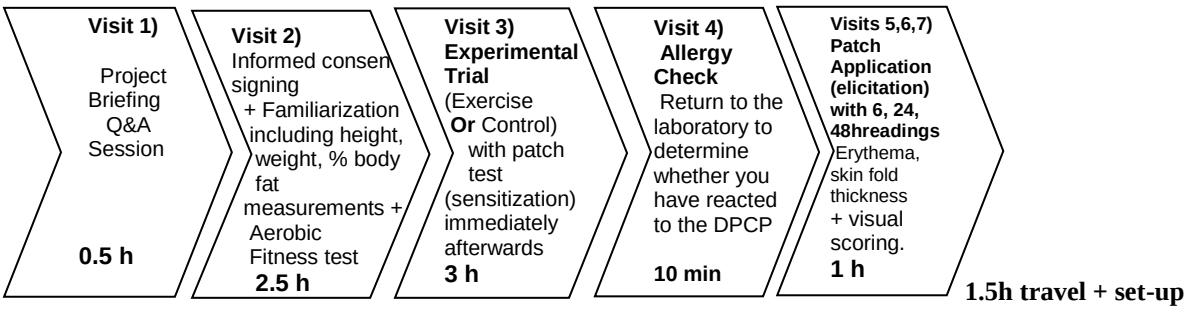
Summary of time commitment:

Patch applications -	0.5 hours maximum
Skin redness and thickness measures -	1 hour maximum
Exercise, with warm up and preparation -	2.5 hours
Allergy Reaction check	10 min
Travel and set up time -	1.5 hours maximum
Briefing and Familiarization -	3 hours maximum

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~ 8 h and 40 min OF YOUR TIME.

Summary of the study

Please refer to the flow diagram below that provides an overview of the study.



Visit 1) Project briefing & question and answer session (~1/2 hour)

At this meeting, you will come to the Bangor University, School of Sport, Health and Exercise Sciences (SSHES) physiology laboratory and we will fully brief you concerning 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 to allow you at least 24 hours to discuss your involvement in the study with your significant other(s).

Visit 2) Aerobic fitness test and Familiarization (~2.5 hours)

At any time in the few days following the briefing, 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 arrange for further visits. After you have signed the informed consent form, you will have measurements of height, weight and body composition taken and will then will perform an aerobic fitness and we will also familiarize you with the measurement devices.

Aerobic Fitness test: An incremental treadmill test will be used to measure your aerobic fitness (maximal oxygen uptake). Following approximately 12 minutes of exercise on a treadmill where the intensity is increased every 3 minutes, the test will require you to run at your maximal aerobic capacity for approximately one minute at the end. We will require you to wear a facemask during this fitness test so that we can make measurements of your expired air: the facemask is not uncomfortable and will not impede your breathing.

Measurement familiarization: Following the fitness test, you will be shown the devices that will be used to measure the various skin response parameters (skin redness and thickness). Measurements will be taken by each of the devices to show you what the procedures involve and how they feel. You will not feel anything but the contact of the probe/callipers on your skin. In order to complete the investigation these measurements are a necessity. Both the fitness test and the measurement familiarization will again take place in the SSHES physiology laboratory.

Visit 3) Experimental trial (3 hours)

Following the briefing and familiarization, you will return to the SSHES physiology laboratory 2-7 days later and perform **EITHER** a 2 hour exercise bout of treadmill running exercise (60% VO_{2max}) **OR** 2 hours of seated rest prior to the first application of the allergen skin patch containing diphenylcyclopropenone (DPCP) to the mid-lower back (the patch is very small; approximately 1cm x 1cm). This first skin test application will be used to sensitize you to the allergen so that we can determine whether there is an allergic reaction.

48 hours prior to the experimental trial, you will be asked to avoid alcohol, caffeinated beverages, refrain from exercise 24 hours before arrival at the laboratory and only consume the food and drink that we provide you.

DPCP itself is a non-irritating and non-mutagenic (not cancer causing) substance with only possible mild side-effects of itching at the site of the skin patch. DPCP is used safely in clinical settings as a therapeutic treatment for alopecia (hair loss) in much higher concentrations than those proposed here.

Visit 4) Allergy Reaction Check (10 min)

You will be asked to return to the laboratory 14 days after the experimental trial (visit 3) to determine whether you have reacted to the allergen DPCP, so that we know whether or not you have been sensitized. This will take 10 minutes or less.

Visit 5, 6 and 7) Patch Test Application and Readings (1 hour)

Four weeks after the experimental trial (visit 3), a set of seven small patches containing very low concentrations of DPCP and an acetone control patch will be applied (surface area of patches = 8 cm^2; the size of a small gauze wound dressing) to the upper arm (visit 5).

Measurements of skin redness and skin thickness will be made at 6, 24 (visit 6) and 48 hours (visit 7). Again, these measurements are painless and all you will feel is the touch of the probe/callipers on your skin. The skin patches will be visually assessed by a dermatologist at the 48 hour visit (visit 7).

48 hours prior to visit 5, you will be asked to avoid alcohol, caffeinated beverages, and refrain from exercise 24 hours before arrival at the laboratory and 48 hours after departure from the laboratory (visit 5 until visit 7) and only consume the food and drink that we provide you.

You will be given and asked to complete a simple health and illness log for the duration of the study where for each day you will be asked to record the occurrence of any health problems (e.g. cold symptoms). We will ask that you bring the health and illness log to the laboratory on every occasion.

Advantages of taking part

- By taking part in this study you will be contributing to our knowledge about the effects of exercise on immune function. Your participation will help us determine whether a simple skin patch test can be used to assess changes in immune function brought about by exercise.

- You will receive comprehensive feedback, with full explanations, on your fitness level, and body composition (e.g. body fat percentage). 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.

- Undergraduates in SSHES will gain valuable insight into the procedures and work involved in a 3rd year project. In addition, 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. Skin reactions, 2. Skin response measurements 3. Time commitment. 4. Exercise.

1. **Skin reactions**: Responses to the allergen skin patch test are normally mild. Minimal side effects of itching and reddening at the site of contact are possible. In previous research by our group, we have shown the use of DPCP to be safe. In a previous study, those individuals who were the highest responders presented with small localized blisters (less than 1cm x 1cm) and itching at the sites of highest concentration. The lowest responders either had no reaction or small areas of redness. Future concentrations of allergen on the patch will be adjusted and decreased if the higher reactions occur. Dr. Andy Macfarlane, our consultant dermatologist, has

frequently used DPCP at the dermatology clinic at Ysbyty Gwynedd (hospital) and will be on hand during patch readings should you wish to discuss any concerns.

2. **Skin response measurements**: Only trained experimenters will perform these procedures (application as well as measurement). To ensure that you are completely happy with these measurements we will familiarize you with the procedures on your second visit to the laboratory (skin redness and skin thickness). These measurements are painless and all you will feel is the contact of the probe/callipers on your skin.

3. **Time commitment**: To complete all aspects of the study we will require you to visit the laboratory on seven for a total of **approximately 8 hours and 40 minutes**. 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 **£30 compensation** upon completion in participating in the study. This incentive does not affect your freedom to withdraw without reason from the study at anytime in anyway.

4. **Exercise Component**: In this experiment, you **MAY** be assigned to the group that will be required to exercise for a prolonged period. Although you may be assigned to the resting group, you should be prepared for being assigned to the group that will perform 2h of running exercise on a treadmill. Although this exercise is for a prolonged period, this will be at moderate intensity and should not be hazardous to your health if you are a recreationally active, healthy individual. At all times you will be closely supervised by an experimenter trained in First Aid and CPR.

Dr. Neil Walsh, Adam Harper Smith or any of the additional investigators, will happily answer any further questions.

Dr Neil Walsh (Reader Physiology): **n.walsh@bangor.ac.uk**; telephone 01248 383480

Adam Harper Smith (PhD Student): **pep62b@bangor.ac.uk**; telephone 01248 388288

Participant information sheet: Investigation Two

Bangor University

School of Sport, Health and Exercise Sciences

Participant Information Sheet

Research Title:

The repeatability of a contact hypersensitivity skin test, and the effect of prolonged strenuous exercise on this response in active males.

Research Co-ordinator: Dr. Neil Walsh **Tel:** 01248 383480

Email: n.walsh@bangor.ac.uk

Additional Investigators: Adam Harper Smith, Dr. Andrew Macfarlane, Prof. Peter Friedmann and Alison Owen

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 Coordinator's computer for 5 years before being confidentially destroyed.

Background

Elite athletes and the recreationally active may be more susceptible to infection after they exercise at high intensity or exercise for long durations. Previous research has suggested that your immune system may be depressed or compromised by very long or very hard exercise, but it is not known whether this is actually clinically significant. Most tests that currently exist only look at markers of immune function in the blood or saliva. For example these tests can see whether you have a normal number of white blood cells. However, as these are just markers, they cannot really be used to predict whether you may be more likely to get, say, a cold or the flu. It is currently not possible to predict an outcome of clinical significance based on these tests alone. Contact hypersensitivity tests like the one we will use in this study, may be able to give us a more accurate picture of your immune status. These tests use very low concentrations of an allergic compound (allergen) placed on an adhesive patch and left on the skin for up to two days. Your body's responses to the allergen can be used as a measure of your immune function. The patches are very similar to those used by doctors to see what an individual may be allergic to (e.g. nickel, rubber). What we would like to find out is whether these skin patch tests can be used repeatedly on an individual in order to give us an idea of their current immune status and whether prolonged exercise influences this response.

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.

<u>Prior to commencing a trial</u> you will be expected to:

- Avoid drinking alcohol and caffeinated drinks (i.e. coffee, tea, cola and diet cola) for 48 hours
- Avoid participating in any exercise for 24 hours before you arrive at the laboratory;

Whilst completing the experimental trials you will be expected to:

- Weigh yourself nude (behind screens to maintain privacy)
- Perform a maximal aerobic test.
- Come to the laboratory to have an allergen skin patch applied to either your upper back or upper inner arm 6 times at intervals of 1 month.
- Have the 2nd through 6th skin patches visually assessed by a dermatologist 48 hours after they are applied.
- Have measurements of skin redness and thickness taken at the skin patch site on the same day and time as the above readings (all measurements are non-invasive and painless).
- Perform prescribed physical activity (120 min moderate paced run on a treadmill) immediately before **either** the application of skin patch 5 **or** skin patch test 6 (randomly assigned).

You will be excluded if you are a smoker or if you are on specific types of medication or supplements (i.e. Creatine), have any immune deficiencies, tendencies to abnormal scarring (e.g. keloid), have eczema or dermatitis on the areas to be skin patch tested or have been a participant in other studies involving skin patch testing. Asthmatics and Diabetics are also excluded.

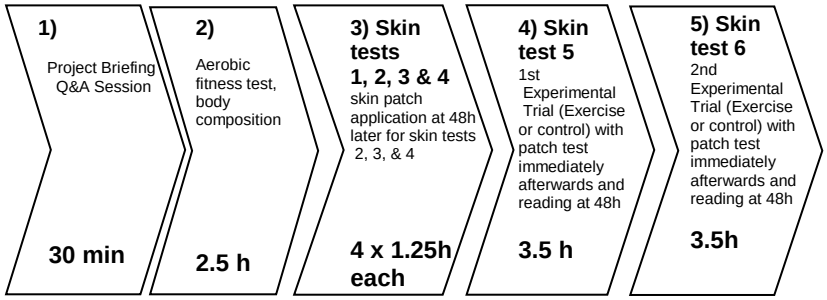
Summary of time commitment:

Patch application and reading -	6 x 1 hour = 6 hours
Skin redness and thickness measures -	2 hours maximum
Exercise, with warm up and preparation -	2.5 hours
Travel and set up time -	1.5 hours maximum
Briefing and Familiarization -	3 hours maximum

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~15 HOURS OF YOUR TIME.

Summary of the study

Please refer to the flow diagram below which provides an overview of the study.



1) Project briefing & question and answer session (~1/2 hour)

At this meeting you will come to Bangor University, School of Sport, Health and Exercise Sciences (SSHES) physiology laboratory and 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 your significant other(s). At any time in the few days following the briefing, 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.

2) Familiarization and aerobic fitness tests (~2.5 hours)

Fitness test*:* An incremental treadmill test will be used to measure your aerobic fitness (maximal oxygen uptake). Following approximately 12 minutes of exercise on a treadmill where the intensity is increased every 3 minutes, the test will require you to run at your maximal aerobic capacity for approximately one minute at the end. We will require you to wear a face mask during this fitness test so that we can make measurements of your expired air: the face mask is not uncomfortable and will not impede your breathing.

Measurement familiarization: Following the fitness test, you will be shown the devices that will be used to measure the various skin response parameters (skin redness and thickness). Measurements will be taken by each of the devices to show you what the procedures involve and how they feel. You will not feel anything but the contact of the probe/callipers on your skin. In order to complete the investigation these measurements are a necessity. Both the fitness test and the measurement familiarization will again take place in the SSHES physiology laboratory.

3) Skin patch tests one, two, three and four (~ 1.25 hours each)

Following the briefing and familiarization, you will return to the SSHES physiology laboratory 2 to 7 days later, where an allergen patch containing diphenylcyclopropenone (DPCP) will be applied to the skin of the upper back. DPCP itself is a non-irritating and non-mutagenic (not cancer causing) substance with only possible mild side-effects of itching at the site of the skin

patch. (DPCP is used safely in clinical settings as a therapeutic treatment for alopecia (hair loss) in much higher concentrations than those proposed here). These skin patch tests will be separated by one month. On each of these occasions, a patch with five low concentrations of DPCP and an acetone control will be applied (patch = **6.5 cm^2 surface area patch; the size of a small gauze wound dressing**). The first skin test application will be to sensitize you to the allergen, so that we can determine whether there is an allergic reaction. Subsequent skin tests (2 through 6) will be read and scored at 48 hours after each patch is applied by a dermatologist. Measurements of skin redness and skin thickness will also be made at these times. Again, these measurements are painless and all you will feel is the touch of the probe/callipers on your skin.

4) & 5) Exercise / Control trials plus skin tests five and six (~3.5 hours each)

Skin tests five and six will also be one month apart and will be of the same format as skin tests one, two, three and four. The only difference is that one of these visits will involve you performing 2 hours of treadmill running exercise (60% VO_{2max}) immediately prior to the application of the allergen skin patch. On the day prior to each experimental trial you will be asked to refrain from exercise and only consume the food and drink that we provide you. You will also be asked to refrain from exercise in the 2 days (48 hours) after each of these trials.

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 on every occasion.

Advantages of taking part

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, on your fitness level, body composition (e.g. body fat %) and immune function measures. 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 valuable insight into the procedures and work involved in a 3rd year project. In addition, 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. Skin reactions; 2. Skin response measurements 3. Time commitment. 4. Exercise.

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

1. **Skin reactions**: Responses to the allergen skin patch test are normally mild. Minimal side effects of itching and reddening at the site of contact are possible. Future concentrations of allergen on the patch will be adjusted and decreased if these reactions occur. A consultant dermatologist will be on hand during patch readings should you wish to discuss these concerns.
2. **Skin response measurements**: Only trained experimenters will perform these procedures. To ensure that you are completely happy with these measurements we will familiarize you with the procedures on your second visit to the laboratory (skin redness

and skin thickness). These measurements are painless and all you will feel is the contact of the probe/callipers on your skin.

3. **Time commitment**: To complete all aspects of the study we will require you to visit the laboratory on eight occasions for a total of approximately 15 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 £50 compensation for completion up to the first experimental trial (skin patch 5) and on the completion of both experimental trials (skin patch 6), a further £50 (total £100). This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime.

4. **Exercise Component**: In this experiment you will be required to exercise for a prolonged period of time. However, this will be at moderate intensity; this should not be hazardous to your health if you are a recreationally active, healthy individual.

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: Thermoregulation and immune responses to cold exposure after two days of sleep deprivation with or without energy restriction in active males.

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

Additional Investigators: Dr. Sam Oliver**,** Ricardo Costa, Adam Harper Smith.

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.

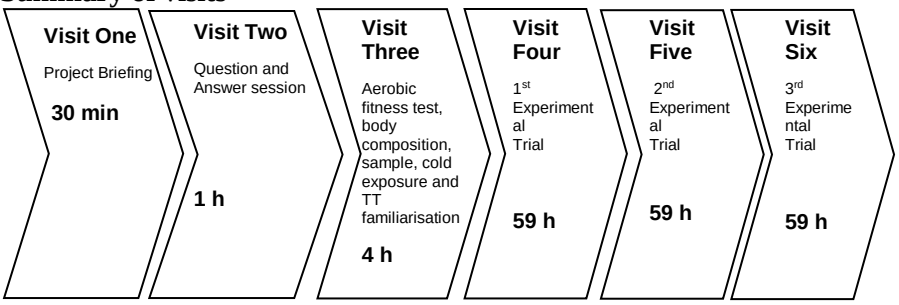
Whilst completing the experimental trials you will be expected to:

- 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 SSHES Health and Safety 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.

PARTICIPANT INFORMATION SHEET

Bangor University
School of Sport, Health and Exercise Sciences

Research Title: The effects of three nights of sleep disruption on thermoregulation and immune function at rest and with additional exercise-heat stress

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

Additional Investigators: Adam Harper Smith, Dr. Jonathan Moore, Umberto di Felice, Dr. Peter Friedmann, Dr. Andrew Macfarlane, Katharine Richardson

Invitation to take part

You are 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 coordinator's computer for 5 years before being confidentially destroyed.

Background

Shift workers, athletes, emergency and armed forces personnel frequently experience sleep loss whilst still being expected to perform in potentially extreme environments (e.g. hot/humid conditions). There have been relatively few controlled laboratory studies that have investigated the effects of three nights of sleep disruption on thermoregulation (the body's ability to regulate its temperature) and immune function at rest or with additional exercise-heat stress. It has been suggested that sleep disruption may have detrimental effects on both thermoregulation and immune function, but this has not been fully explored. Therefore, the main aim of this study is to identify the effect of three nights of sleep disruption and subsequent exercise in the heat, in a controlled laboratory setting, on thermoregulation and immune function.

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. You will be asked to schedule ten visits to the laboratory as outlined below.

In the eight weeks before trials begin, you will be expected to:

- come to the laboratory to have an allergen skin patch (containing DPCP, a chemical which is safely used to assess skin immune function, and causes a mild allergic reaction) applied to your lower back 5 times. After the first patch is applied, four weeks later another patch will be administered, followed by an additional 4 patches at one week intervals.
- have your height, weight, body mass and body composition recorded
- perform a maximal aerobic capacity (VO_{2max}) test.

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

- avoid drinking alcohol, caffeinated drinks (i.e. coffee, tea, cola) 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.
- eat and drink only what is given to you the day before trials.

Whilst completing the two experimental trials you will be expected to:

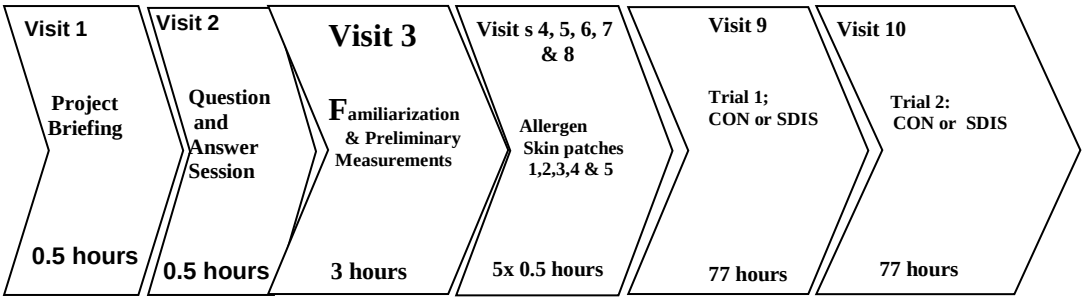
- spend four consecutive days in the laboratory on two separate occasions.
- live and sleep in the laboratory during each four day trial (soft furnishings, bedding, computers, TV, video, DVD and games will be provided and living temperature will be between 18 to 22°C).
- stay awake for most of the night (2 x 1 hour blocks of sleep will be permitted), for three nights, in a living quarters in the SSHES department, on ONE of these occasions (sleep disruption trial) and sleep in a bed in the living quarters on another occasion (control trial).
- eat and drink only what is given to you during the trials.
- follow the study's daily timetable, including going to bed and waking from sleep when asked.
- perform prescribed physical activity: 1) 60 minutes at 50%VO_{2max} (walk) on day 1, 2 and 3 in a temperate environment (20 to 22 ^{o}C) on a treadmill in a laboratory. 2) perform exercise in the heat (33^{o}C/ and 40% humidity) on day 4, consisting of a 45 min run at 60% of your maximal aerobic capacity (VO_{2max}), which is a moderate paced run. 3) Then, 45 minutes later a time to exhaustion (TTE) exercise bout at 70%VO_{2max} (moderate-high intensity) or until your core temperature reaches 39.9^{o}C, all of which will be performed on a treadmill. Any fluid loss during exercise will be added to your daily total water consumption.
- wear skin temperature sensors and a rectal temperature probe (during exercise only) for the measurement of body temperature (i.e. skin and core) during trials.
- weigh yourself nude (behind screens to maintain privacy).
- have nine blood samples taken during each trial (~180 ml during each trial) totalling 18 blood samples during the study (total blood ~360 ml)
- have nine saliva and urine samples collected during each trial totalling 18 samples.
- have measurements of skin blood flow taken (non-invasive laser Doppler) at 12 time points during trials.
- during trials, have skin patches applied on day 3 at approximately 8:30am and have them assessed 6, 12, 24 and 48 hours later (assessments are non-invasive, involving a visual assessment, skin-fold callipers – the same used for body composition and a colour-meter, which sends out a beam of light to measure redness of the skin)

You will be excluded if you are a smoker or if you are on specific types of medication or supplements (i.e. Creatine), have any immune disorders, tendencies to abnormal scarring (e.g. keloid), have skin disorders such as eczema or dermatitis or have been a participant in other studies involving allergen skin patch testing. Asthmatics and Diabetics are also excluded.

Summary of time commitment:

Skin patch application and reading -	5 x 0.5 hour = 2.5 hours
Travel and set up time -	1.5 hour maximum
Briefing, Familiarization & Preliminary measurements-	3 hours maximum
Control trial	77 hours
Sleep disruption trial	77 hours
Total time commitment	161 hours

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 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 regarding the study.

Visit two: Question and answer session (~30 minutes)
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 arrange for further visits.

Visit three: Familiarization, body composition and aerobic fitness tests (~3hours)

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 a specialized 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 or $V0_{2max}$). This test will require you to run for approximately fifteen minutes,

culminating in approximately one minute at your maximal aerobic capacity. 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 get back on the treadmill to allow us to calculate your running speed for exercise bouts during the experimental trials.

Sample familiarization: Following the fitness test a venous blood sample, a saliva sample and a urine sample will be collected to ensure you are fully familiar with these collection methods. A qualified member of staff will collect all blood samples 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.

Environmental chamber and exercise in the heat familiarization: Following the incremental test and after a short recovery period you will be asked to run at 60%$V0_{2max}$ for 45 minutes in the climate chamber set at 33°C and 40% humidity wearing athletic shorts, socks and shoes (no T-shirt). During this time you will be required to practice breathing through a mouth piece, while having rectal core and skin temperature measured. After twenty minutes rest, you will be asked to run in the same conditions for at 70%$V0_{2max}$ to exhaustion (until you decide you want to stop or cannot run any further). Completing a familiarization exercise session in the heat will help you to prepare yourself for the main experimental trials. You may stop the exercise in the heat familiarization at any time should you feel the need to.

Visits four, five, six, seven and eight: allergen patches (5 x 0.5 hours)
Before the experimental trials begin, you will be required to have five allergen patches applied to your lower back. The first will be a sensitization patch, followed four weeks later by the first of four recall patches, administered at one-week intervals.

Visit nine and ten: Experimental trials (2 x 77 hours)
You will be required to complete two experimental trials in a random order, each separated by ten days. Each experimental trial will consist of a 77 hour period (4.5 days) where you will reside in the laboratory during each of the experimental trials, prior to completing running exercise in a warm environment. The trials are as follows: control trial and sleep disruption trial. The control trial will involve you sleeping for approximately eight hours per night. The sleep disruption trial will involve sleep being restricted to 2 x 1hour blocks of sleep per night.

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. The food and water we provide you, will match your daily energy intake requirements, based on information we obtained during the whole body scan. 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 from your home at around 7 am and driven to SSHES by one of the investigators. At 8am on days 1, 2, 3 and 4 of trials, blood, urine and saliva samples will be collected, followed by assessment of skin blood flow on the forearm (using non-invasive laser Doppler Flowmetry). On each trial on days 1, 2 and 3 you will be asked to walk for 60 minutes at 50%VO_{2max} on a treadmill in a temperate laboratory (20 to 22 ^{o}C) For the remainder of each day you will be free to complete normal light habitual activities (e.g. office work, watching TV, etcetera). We will provide you with all your water and food (breakfast ~9 am, lunch ~2 pm and dinner ~7 pm). Again, the food and water we provide you, will match your daily energy intake requirements, based on information we obtained during the whole body scan, plus additional water to replace any body mass loss during exercise. On the control trial you will sleep in comfortable beds in a quiet room in SSHES for approximately eight hours each night (11:45 pm to 7:45 am). On the sleep

disruption trial, you will be allowed to sleep for 2 hours between 11:45 pm and 12:45 am and for another hour between 4:45 and 5:45 am each day. We will keep you entertained throughout the wakeful period with board games, TV, DVDs and a games console. You will be supervised at all times.

During the sleep disruption and control trials the allergen skin patches will be applied on day 3 at approximately 8:30 am and assessed 6, 12, 24 and 48 hours later (again by non-invasive visual, skin-fold calliper and colour-meter assessments).

On day 4 at 10:30 am, 74.5 hours into experimental trials, you will be required to perform exercise in the heat in an environmental chamber (33ºC and 40% humidity). After 45 minutes of passive heat exposure (seated on a chair), you will run for 45 minutes at 60% of your maximal aerobic capacity, wearing athletic shorts, socks and shoes. After 45 minutes of seated rest, you will be asked to run to exhaustion at 70% of your maximal aerobic capacity, which will stop when you feel that you cannot run any further, or if your core body temperature reaches 39.9ºC. For your safety throughout exercise test and during a recovery period, your core temperature will be monitored continuously. You may also stop exercise in the heat at any time should you feel the need to. During the first hour after exercise, you will be provided, upon request, with cold water, fan cooling. An ice bath will also be on hand. After 1 hour you will be provided with a recovery meal (~1200 kcal). After one and two hours of recovery (~1:30pm and 2:30pm) you will be asked to provide a blood, urine and saliva sample, followed by an assessment of forearm skin blood flow. An investigator will then transport you back to your home.

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 on each of your visits

It is very important that you stay at home and rest during the overnight recovery after each of the trials. **Sleep disruption 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.

Ten days after visit nine, you will be required to return to complete the remaining experimental trial**.**

Advantages of taking part

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, on your fitness level, body composition (e.g. body fat %) and blood measures (e.g. immune status). 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 disruption; 2. exercise in the heat 3. blood samples 4. time commitment and 5. skin patches.

1. **Sleep disruption**: Many scientific investigations, including those completed in our laboratory, show that 1 to 3 nights of sleep disruption have only very modest effects on physical and psychological function. Indeed, military personnel regularly endure 4 to 5 day operations that involve constant activity, psychological stress and sleep loss. Field studies do not report medical complications even in military personnel on these operations.

2. **Exercise in the heat**: Numerous scientific experiments have exposed individuals to hot/humid conditions and reported no medical complications when individuals' core temperatures are raised to 39.9°C. Core temperatures of 39.9°C and above are frequently recorded in athletes exercising in warm environments, with temperatures up ~ 41.5°C recorded after marathon events with no complication. It is important to note that you may stop exercise at any time should you feel the need to and that it is your decision when to stop the time to exhaustion bout, unless your core temperature reaches 39.9°C, in which case we will ask you to stop. After exiting the environmental chamber you can be cooled with a cold water spray and fans, and if necessary you will be placed in an ice bath. For your safety, your core temperature will be monitored continuously throughout exercise and recovery.

3. **Blood samples**: Only investigators fully trained in taking blood will collect blood samples. To ensure you are completely happy with giving a blood sample we will familiarize you with the blood sample collection procedure on your first visit to the laboratory. The blood samples will be taken using smaller needles than are typically used by doctors in your local surgery or hospital.

4. **Allergen skin patches:** Skin patches containing allergen will be applied to the lower back on 5 separate occasions prior to the experimental trials and once during each trial (upper, inner arm). These patches are non-irritating and the chemical allergen (DPCP) is safe for use in humans. In fact it is used for therapeutic treatment by dermatologists of alopecia, warts, dermatitis and melanomas (skin cancer). The only possible mild side-effects are itching and potential small blisters at the site of the skin patch. We will provide you with first-aid ointments and dressings if requested.

5. **Time commitment**: To complete all aspects of the study we will require you to visit the laboratory on ten occasions for a total of approximately 161 hours. We understand that this study will ask a lot of you as a participant and so we are offering monetary compensation. As a participant, you will be eligible for £20 for completing the first trial and on the completion of the second experimental trial a further £80 (total £100). This monetary compensation does not affect in any way your freedom to withdraw without reason from the study at anytime.

Dr Neil Walsh or any of the additional investigators will happily answer any further questions.

Appendix B

INFORMED CONSENT TO PARTICIPATE
IN A RESEARCH PROJECT OR EXPERIMENT

Title of Research Project: _____

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

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

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

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

I also understand that I may register any complaint I might have about this experiment to Professor Mike Khan, Head of the School of Sport Health and Exercise Sciences, and that I will be offered the opportunity of providing feedback on the experiment using standard report forms.

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

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

I have been informed that the research material will/will not **[SELECT ONE]** be held confidential by the researcher.

I agree to participate in the study

Signature:_____

NAME (please type or print legibly): _____

ADDRESS:(Optional)_____

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

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

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

Appendix C

PHYSIOLOGY INFORMED CONSENT & MEDICAL QUESTIONNAIRE

Name: …….

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 approx. duration.

vigorous moderate low intensity

Duration (Min).

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

Asthma	Yes	No
Diabetes	Yes	No
Bronchitis	Yes	No
Epilepsy	Yes	No
High blood pressure	Yes	No

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, 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, hemorrhoids, 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 of Participant:……..

Date:……..

Signature of Experimenter:……

Date: …….

Appendix D

Perceived Stress Scale Questionnaire

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, you will be asked to indicate *how often* you felt or thought a certain way. Although some of the questions are similar, there are differences between them and you should treat each one as a separate question. The best approach is to answer each question fairly quickly. That is, don't try to count up the number of times you felt a particular way, but rather indicate the alternative that seems like a reasonable estimate.

For each question choose from the following alternatives:

0. never
1. almost never
2. sometimes
3. fairly often
4. very often

1. In the last month, how often have you been upset because of something that happened unexpectedly?
2. In the last month, how often have you felt that you were unable to control the important things in your life?
3. In the last month, how often have you felt nervous and "stressed"?
4. [a] In the last month, how often have you dealt successfully with irritating life hassles?
5. [a] In the last month, how often have you felt that you were effectively coping with important changes that were occurring in your life?
6. [a] In the last month, how often have you felt confident about your ability to handle your personal problems?
7. [a] In the last month, how often have you felt that things were going your way?
8. In the last month, how often have you found that you could not cope with all the things that you had to do?
9. [a] In the last month, how often have you been able to control irritations in your life?
10. [a] In the last month, how often have you felt that you were on top of things?

11. In the last month, how often have you been angered because of things that happened that were outside of your control?
12. In the last month, how often have you found yourself thinking about things that you have to accomplish?
13. [a] In the last month, how often have you been able to control the way you spend your time?
14. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?

Appendix E

Daily Habits

In the last 48h have you:

1) Performed any form of relaxation technique, mediation or similar activity?

2) Per formed any physical activity?
    If so, for how long and of what intensity?

3) Taken any medication? If so, please describe:

4) Consumed any caffeinated beverages or food items?
    If so, what and of what quantity?

5) Consumed any alcohol?
    If so, what and of what quantity?

6) Used any products containing nicotine?
    If so, what and of what quantity?

7) Used any other stimulants?
    If so, please describe:

Please estimate the number of hours of sleep you think you had last night and the night before last:

In the last two weeks, have you had any physical symptoms, including fever, pain, gastro-intestinal symptoms or yellow/green mucous when you blow your nose?
    If so, please describe:

If there are any other symptoms or significant changes in your life that have taken place over the last month, please describe them below:

Appendix F

Name:_____

Date:_____

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday

Instructions:

A. Please fill in the **health code(s)** (as many as apply) for the previous week

1. No health problems today
2. COLD SYMPTOMS (runny, stuffy nose, sore throat, coughing, sneezing, coloured discharge)
3. FLU SYMPTOMS (fever, headache, general aches and pains, fatigue and weakness, chest discomfort, cough)
4. Nausea, vomiting, and/or diarrhoea
5. Muscle, joint, or bone problems/injury
6. Other health problems (describe)

Please rate **severity** of symptoms:

A = Mild, B = Moderate, C = Severe

For example, for a moderate cold, write "2-B" in blank for the specific days.

Extra Questions

1a Have you been on any medication over the last 4 weeks? _____

b If yes, what was your medication, why were you taking it, and when did you start and finish taking it?

_____
_____
_____
_____

2a Have you been taking any take any dietary supplements? _____

b If so, what are they?

_____
_____

3a Do you suffer from any allergies that produce symptoms noted under cold or flu symptoms (number 2 or 3 in the health log)? _____

b What are your allergies to? _____
_____

c How often are you affected by them?

_____

If you suffer any allergies that cause you to record symptoms in the health logs please note that in the box.

Appendix G

3-day diet diary

Name: _____

Beginning on the day of EX / CON trial (2h run or 2h seated in lab) please **record all food and drink consumed**. Add snacks in between meals to the end of the list for the previous meal (ie. Mid morning snack, add to breakfast list)

Please try to be as specific as possible with weight, volume and food descriptions

Also, please bring this form with you to the 48h reading session and we will give you a copy so that you are able to copy the diet in the same 3 day period next month.

Day 1: Date _____

	Item Descriptions	Estimated Weight/Volume/Potion Size
Breakfast		
Lunch		
Dinner		

Day 2: Date _____

	Item Descriptions	Estimated Weight/Volume/Potion Size
Breakfast		
Lunch		

Dinner

Day 3: Date _____

Item Descriptions	Estimated Weight/Volume/Potion Size

Breakfast

Lunch

Dinner