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Exercise stress, nutrition and novel measures of in vivo immunity

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EXERCISE STRESS, NUTRITION AND NOVEL MEASURES OF *IN VIVO* IMMUNITY

by

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Summary

In order to assess the influence of physical stress, such as exercise and nutritional restriction, on immunity, there is a need for well-controlled field studies and use of clinically relevant and practical *in vivo* measures of immune competence. The broad aim of this thesis was to examine the reductive effects of physical stress on immune responses in humans. Firstly, a daily mixed nutritional supplement prevented the decrease in circulating total leukocytes, lymphocytes and monocytes and increased saliva secretory immunoglobulin-A during 8 weeks of arduous military training, compared with a comparative control group (**Chapter 4**). A stronger approach to investigate the effects of physical stress includes utilising a measure of *in vivo* immunity, such as experimental contact hypersensitivity (CHS) with the novel antigen Diphenylcyclopropenone (DPCP). Prolonged, moderate intensity exercise, but not short, high or short, moderate intensity exercise, impaired induction to DPCP, despite elevated circulating catecholamines and greater circulating cortisol following short, high intensity exercise (**Chapter 5**). Experimental CHS was then coupled with a suction blister technique at the site of DPCP sensitisation to measure cutaneous cytokines thought to orchestrate dendritic cell migration and successful induction of new immune memory. After demonstrating this technique to be repeatable (**Chapter 6**) and identifying 6 h of patch exposure as a suitable time to initiate suction blisters (**Chapter 7**), results showed prolonged, moderate intensity exercise had no effect on local cutaneous interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-10 (**Chapter 8**), thought to be implicated in the initial events of immune induction. Therefore it is possible that prolonged, moderate intensity exercise suppresses further downstream events of immune induction, rather than local inflammatory processes at the skin. Topical application of DPCP provides an attractive tool for the assessment of *in vivo* immunity in future exercise stress and nutritional intervention studies. Further research is required to identify the mechanisms involved in the suppression of CHS responses to DPCP after prolonged, moderate intensity exercise.

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Publications

For all research chapters I was involved in the data collection, data analyses and preparation of material for publication. In addition, for **Chapters 5-8** I was also involved in all aspects of the study design. I gratefully acknowledge the input, advice and contribution from the other named authors for each publication. The following list shows the publications that have been produced from the material presented in this thesis.

Full papers

Chapter 4: Diment, BC., Fortes, MB., Greeves, JP., Casey, A., Costa, RJ., Walters, R., Walsh, NP., 2012. Effect of daily mixed nutritional supplementation on immune indices in soldiers undertaking an 8-week arduous training programme. Eur. J. Appl. Physiol. 112, 1411-1418.

Chapter 5: Diment, BC., Fortes, MB., Edwards, JP., Hanstock, HG., Ward, MD., Dunstall, HM., Friedmann, PS., Walsh, NP., 2015. Exercise intensity and duration effects on *in vivo* immunity. Med. Sci. Sports Exerc. 47, 1390-1398.

Abstracts

Chapter 4: Diment, BC., Fortes, MB., Casey, A., Greeves, J.P., Walsh, NP., 2011. Effect of nutritional supplementation on immune indices in soldiers undertaking arduous military training. Med. Sci. Sports Exerc. 43, S1:45.

The following list shows the publications that have been produced as a result of work undertaken for the thesis but are not presented here.

Full papers

Fortes, MB., Diment, BC., Greeves, JP., Casey, A., Izard, R., Walsh, NP., 2011. Effects of a daily mixed nutritional supplement on physical performance, body composition, and circulating anabolic hormones during 8 weeks military training. Appl. Physiol. Nutr. Metab. 36, 967-975.

Abstracts

Fortes, MB., Diment, BC., Casey, A., Greeves, JP., Walsh, NP., 2011. Effects of nutritional supplementation on the IGF axis in soldiers undertaking arduous military training. Med. Sci. Sports Exerc. 43, S1:44.

Greeves, JP., Casey, A., Izard, RM., Diment, BC., Fortes, MB., Walsh, NP., 2011. Effect of nutritional supplementation on physical performance and body composition during arduous military training. Med. Sci. Sports Exerc. 43, S1:44.

Table of Contents

Thesis Format

This thesis contains a literature review (**Chapter 2**) which starts by outlining the background and main research aims. The general methods section (**Chapter 3**) then provides information on procedures and measures that have been used in two or more of the subsequent experimental chapters. Four experimental studies form the main focus of the thesis. The first study investigates the effect of a daily mixed nutritional supplement during arduous training on selected *in vitro* immune indices (**Chapter 4**). The second study utilises experimental contact hypersensitivity as a tool to assess the effects of duration and intensity of exercise stress on *in vivo* immunity (**Chapter 5**). To better understand the underlying mechanisms associated with these responses, the following studies utilise a suction blister technique to examine the repeatability (**Chapter 6**), kinetics (**Chapter 7**) and effect of prolonged exercise stress (**Chapter 8**), on the cutaneous cytokine environment thought to orchestrate the contact hypersensitivity responses. Finally, the general discussion (**Chapter 9**) summarises the main findings and aims to critically analyse these with consideration of the recognised limitations and suggested areas for future research. A list of abbreviations, tables and figures appears prior to **Chapter 1,** for clarity. **Bold type** is used to refer to chapters or sections within this thesis.

List of Tables

List of Figures

List of Abbreviations

30HI	short, high intensity exercise trial
30MI	short, moderate intensity exercise trial
120MI	prolonged, moderate intensity exercise trial
120-SB	prolonged, moderate intensity exercise and suction blister trial
ACSM	American College of Sports Medicine
ANOVA	analysis of variance
CD	cluster of differentiation
CHS	contact hypersensitivity
cm	centimetre
CON	control trial
CON-SB	control and suction blister trial
CV_w%	within participant coefficient of variation
DC	dendritic cell
dDC	dermal dendritic cells
DEXA	dual energy x-ray absorptiometry
dLN	draining lymph node
DNCB	dinitrochlorobenzene
DNFB	dinitrofluorobenzene
DPCP	diphenylcyclopropenone
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
g	gram
GET	gas exchange threshold
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
HIV	human immunodeficiency virus
HPA	hypothalamic-pituitary-adrenal axis
HR	heart rate
HSD	honest significant difference
ICC	intraclass correlation coefficient
IL	interleukin
K_3EDTA	tripotassium ethylenediamin tetra-acetic acid
KC	keratinocyte

kg	kilogram
KLH	keyhole limpet hemocyanin
km	kilometre
L	litre
LC	Langerhans' cell
LF	lactoferrin
m	metre
min	minute
mg	milligram
MHC	major histocompatibility complex
MJ	megajoule
ml	millilitre
MMP	matrix metalloproteinases
NBM	nude body mass
NK	natural killer
nmol	nanomole
pg	picogram
RER	respiratory exchange ratio
RH	relative humidity
RPE	rating of perceived exertion
SB	suction blister
SD	standard deviation
SEE	standard error of the estimate
SEM	standard error of the mean
SIgA	secretory immunoglobulin-A
SUP	mixed supplement group
Tc	T cytotoxic cell
TCR	T cell receptor
Th	T helper cell
Treg	T regulatory cell
TNCB	trinitrochlorobenzene
TNF	tumour necrosis factor
URI	upper respiratory illness
URTI	upper respiratory tract infection
$\dot{V}O_{2peak}$	peak oxygen uptake

CHAPTER ONE

General Introduction

Athletes and military personnel frequently experience physical stressors, such as prolonged exercise and nutritional restriction, which are often shown to impair immune function. Early epidemiological survey-based studies suggest that athletes who perform prolonged and intensive exercise may experience increased susceptibility to infection (Nieman et al., 1990a; Peters and Bateman, 1983). Absence from training due to a greater number of sickness days may compromise performance during athletic competition and military fitness tests, which could also lead to back coursing of military personnel (Hanstad et al., 2011; Pyne et al., 2001). Given the ethical constraints of studying experimental infection in humans, further investigation of this survey-based data has been provided by laboratory and field-based stress research utilising *in vitro*, and to a lesser extent, *in vivo* measures of immune responses (Walsh et al., 2011b).

Regular moderate physical activity has been suggested to enhance immune function above sedentary levels (Nieman et al., 1993), but the effect of a single bout of short duration, moderate intensity exercise, in healthy humans, is associated with limited modulation in immune responses (Pascoe et al., 2014; Walsh et al., 2011b). On the other hand, it is well documented that prolonged moderate intensity exercise and high intensity training decreases many components of cellular and humoral immunity, and also increases various circulating metabolic and inflammatory indicators (Walsh et al., 2011b). Nutritional restriction can temporarily depress numerous aspects of immune function and the extent of this suppression has been associated with the severity of the energy deficit (Kramer et al., 1997). Raised stress hormones following nutrient restriction and heavy exercise/training can have immunosuppressive effects and may play a part in observed decreases in immune function (Gleeson, 2007; Pedersen and

Hoffman-Goetz, 2000). Therefore nutritional intervention studies typically aim to minimise nutrient deficits and stress hormone responses following exercise, thus attenuating immune depression and lowering the risk of opportunistic infections (Gleeson, 2006). Ethical and practical considerations limit the ability to study prolonged periods of intensive training and nutritional restriction within laboratory settings. Field studies with military personnel provide an attractive model to study the effects of these stressors (Hoyt and Friedl, 2006). However, many military field studies to date lack comparative control groups, making it difficult to determine the role of energy deficit on immune changes within the multi-stressor environment (Gomez-Merino et al., 2005; Tiollier et al., 2005).

Much of the research to support the effect of physical stressors on immune function has relied upon *in vitro* immune measures, which typically involves isolated analysis of immune cells in artificial environments (Akbar et al., 2013). The importance and need for utilising *in vivo* measures of immune function in further stress research has recently been highlighted because they overcome some of the limitations of *in vitro* measures by involving an integrated, whole-body, immune response (Albers et al., 2013). Given that the skin constitutes the body's largest immunological organ, providing the first line of defence against pathogenic and environmental assaults (Clark et al., 2007), *in vivo* measures of immunity at the skin are particularly attractive. One such measure includes experimental contact hypersensitivity (CHS) to a novel antigen, such as Diphenylcyclopropenone (DPCP), which provides a simple, practical and cheap method of assessing *in vivo* immune responses (Harper Smith et al., 2011). Although physical stressors such as high altitude exposure (Oliver et al., 2013) and prolonged, moderate intensity exercise (Harper Smith et al., 2011) have been shown to impair CHS responses to DPCP, the effect of intensity and duration of exercise or the mechanisms associated with the altered responses have not been examined. The broad aims of this thesis were to investigate the effect

of nutritional supplementation, in a well-controlled field study, on immune indices during training; to examine the effects of intensity and duration of exercise stress on *in vivo*, experimental CHS responses to DPCP; and to investigate the possible mechanisms associated with the exercise-induced inhibition of immune induction with DPCP.

CHAPTER TWO

Literature Review

2.1 Exercise and Infection

A large body of anecdotal and survey-based epidemiological research suggests that athletes undertaking intensive and prolonged exercise are at increased risk of minor illnesses, particularly upper respiratory tract infection (URTI) (Alonso et al., 2012; Gleeson, 2007; Nieman, 2000). In the weeks following a competitive ultra-endurance event, a 100-500% increase in the risk of URTI symptoms has been reported, compared with rested controls, which was also correlated with pre-race training distance and race finish times (Nieman et al., 1990a; Peters and Bateman, 1983). However, one study in a large cohort of marathon runners found that increased self-reported infection rates after a marathon, were only observed in participants who reported a pre-race infection (Ekblom et al., 2006). This suggests that recurrence of recent infection may be caused by completing strenuous exercise too soon after infection (Moreira et al., 2009), but it should be noted that lack of control over subsequent natural pathogen exposure may account, in part, for differences observed between individuals and/or studies.

Many studies that have explored the relationship between exercise and infection risk have relied upon evidence from self-reported surveys, rather than clinical diagnosis. It has now been questioned whether symptoms of upper respiratory illness (URI) may have a non-infectious cause in athletes, such as airway inflammation (Cox et al., 2008). Due to cost and technicality of pathology testing, few studies have identified whether the commonly reported upper respiratory symptoms are due to true infectious episodes (Cox et al., 2008; Spence et al., 2007). In a study looking at URI symptoms in elite athletes, a pathogen or laboratory parameter indicative of infection was identified in only 57% of cases (Cox et al., 2008), but it is possible that limitations of current diagnostic techniques may be partly responsible for unidentified

pathogens (Spence et al., 2007). Irrespective of the exact cause of the reported illnesses, the evidence does support the notion that exercise-induced immune suppression increases susceptibility to URI symptoms and that these may be detrimental to athletic performance (Gleeson and Walsh, 2012).

In contrast to the negative effects of prolonged, strenuous exertion, it is commonly believed that regular moderate exercise training is beneficial for health. A limited number of studies have provided support for an association between regular moderate exercise and a lower risk of URI compared with sedentary individuals, particularly in healthy populations (Matthews et al., 2002; Nieman et al., 2011). It is possible that this beneficial relationship is stronger in those with sub-optimal baseline immune responses such as the elderly (Nieman et al., 1990b), individuals affected by obesity (Nieman et al., 1993) or psychological stress (Fondell et al., 2011). Despite this, the relationship between physical activity and URTI is typically modelled as a J-shaped curve (Nieman, 1994). This model suggests that regular moderate physical activity decreases the risk of URTI compared with sedentary individuals, whereas prolonged, high intensity exercise or periods of strenuous exercise training increases the risk of URTI.

Given the restrictions associated with studying experimental infection in humans, animal models have provided valuable insight into the effects of exercise on clinically relevant *in vivo* outcome measures, such as infection responses to herpes simplex virus or influenza virus (Martin et al., 2009). The evidence from these murine studies indicates that prolonged and high intensity exercise is associated with higher mortality rates whereas short, moderate intensity exercise lowers mortality rates compared with controls (Martin et al., 2009). In a bid to explain the possibly altered susceptibility to infections in humans, many studies have investigated the effects of exercise on various *in vitro* and, to a lesser extent, *in vivo* measures of immune

function (Walsh et al., 2011b). Based on the findings of such studies, it has been proposed that intensive and prolonged exercise temporarily suppresses (~3-24 h) many components of the immune system, which may make the host more susceptible to opportunistic infections (Nieman, 2008; Walsh et al., 2011b). This concept is commonly referred to as the "Open Window Theory" (Kakanis et al., 2010).

2.2 Exercise and immune responses

The immune system involves multiple, functionally differing cell types, which act via a large variety of non-specific innate and adaptive responses in a bid to maintain homeostasis (Gleeson, 2006). Recently over 75 markers of immune function previously cited in the literature were reviewed for their suitability in assessment and interpretation of immune modulation (Albers et al., 2013). Therefore it is unsurprising that studies examining the effect of exercise on various aspects of immunity have adopted a range of immune system parameters, from the whole organism level, to the cellular, mechanistic level, which have shown different results (Albers et al., 2005; Walsh et al., 2011b). Evidence typically shows that a prolonged (> 1.5 h) bout of moderate to high intensity (55 - 75% $\dot{V}O_{2max}$) exercise results in transient changes in immunity and host defence. This includes, but is not limited to, decreased neutrophil responsiveness to bacterial stimulation (including reduced oxidative burst and decreased degranulation response on a per cell basis (Robson et al., 1999), natural killer (NK) cell count and cytotoxic activity (Kakanis et al., 2010; Shek et al., 1995), expression of toll-like receptors (Nickel et al., 2012), number of circulating lymphocytes, T lymphocyte subsets and mitogen stimulated T cell proliferation (Green and Rowbottom, 2003; Ronsen et al., 2001), cell-mediated delayed type hypersensitivity (DTH) responses to injected recall antigens (Bruunsgaard et al., 1997), experimental CHS responses to a novel antigen (Harper Smith et al., 2011) and mucosal immunoglobulin levels (Bishop and Gleeson, 2009). A shorter bout

(~30 - 45 min) of high intensity exercise (80% $\dot{V}O_{2max}$) can also suppress *in vitro* functional measures of neutrophils and lymphocytes (Nieman et al., 1994; Robson et al., 1999). Therefore it has been suggested that this immune function depression after both prolonged and high intensity exercise may make the host more susceptible to opportunistic infections (Walsh et al., 2011b).

Regular physical activity is generally thought to be beneficial to health, but a single short bout (< 60 min) of continuous, moderate intensity exercise (≤ 60% $\dot{V}O_{2max}$) has been associated with limited changes in the immune function of healthy humans. Based on evidence from animal studies, showing an enhanced effect of acute restraint stress or footshock on leukocyte mobilisation, antibody production and cell-mediated responses, it has been suggested that acute exercise may have a similar enhancing effect on immune function in humans (Dhabhar, 2009; Edwards et al., 2007). Research evidence in humans is not definitive, demonstrating for example, that acute bouts of continuous moderate intensity exercise (e.g. a 45 minute cycle or walk at 55% age predicted HR max) either induce an enhanced antibody response to influenza vaccine, or have no effect (Edwards et al., 2006; Long et al., 2012). Such work in humans tends to indicate that enhanced antibody responses to acute stress only occur in individuals with sub-optimal baseline immune status (Pascoe et al., 2014). This is further supported by reports of unchanged saliva secretory immunoglobulin-A (SIgA) responses after short, moderate intensity exercise in healthy humans (Bishop and Gleeson, 2009). It is noteworthy that for healthy, young individuals, elevating immunity above a level which is considered 'optimal' may in fact be just as detrimental as suppressed immunity by increasing disease susceptibility to conditions associated with allergy, hypersensitivity and autoimmunity (Walsh et al., 2011b).

2.2.1 Exercise intensity and duration

Studies directly comparing the effects of continuous exercise bouts of different intensities and/or durations in humans have predominantly relied upon circulating cell numbers or functional *in vitro* immunological assessments. Probably the most documented exercise-induced effect on the immune system is the redeployment of leukocytes and lymphocyte subsets into the circulation, followed by post exercise lymphocytopenia, which are influence by both the intensity and duration of exercise (McCarthy and Dale, 1988). For example, Robson et al. (1999) reported that leukocytosis was much larger after prolonged exercise (~160 minutes) at 55% $\dot{V}O_{2max}$ compared with short duration (~40 minutes) high intensity exercise (80% $\dot{V}O_{2max}$). This study also showed that reductions in the functional measure of neutrophil degranulation response to bacterial lipopolysaccharide during recovery was more pronounced following prolonged exercise (Robson et al., 1999). Given that both the intensity and duration were manipulated within the two exercise trials, it is difficult to conclude which parameter had the greatest influence. When exercise duration is short (~30 minutes) and kept constant, lymphocytopenia associated with exercise is more pronounced following high (80% $\dot{V}O_{2max}$) compared with moderate (60% $\dot{V}O_{2max}$) intensity exercise (Simpson et al., 2007). Similarly, 45 minutes of treadmill running at high (80% $\dot{V}O_{2max}$) intensity was associated with a greater fall (50%) in lymphocyte proliferation at one hour post exercise compared with a 25% decrease after moderate intensity (50% $\dot{V}O_{2max}$) exercise (Nieman et al., 1994). However, one study utilising the *in vivo* measure of antibody responses to influenza vaccine, identified no effect between different intensities of resistance exercise (Edwards et al., 2010). It is possible that differences in the mode of exercise (e.g. resistance *vs*. continuous) and the type of immune measure may be involved in these varied observations.

Stress hormones are commonly reported to orchestrate a large-scale redistribution of immune cells in the body, with adrenaline, noradrenaline and cortisol believed to be principally involved in such activities (Dhabhar et al., 2012). The actions of these stress hormones, which have been identified through observational associations, intravenous administration and *in vitro* studies, can have detrimental effects on immune function (Webster Marketon and Glaser, 2008). Moreover, glucocorticoid stress hormones are used clinically as anti-inflammatory agents and are widely regarded as being immunosuppressive following prolonged and intensive exercise (Dhabhar and McEwen, 1999; Pedersen and Hoffman-Goetz, 2000). Since concentrations of these stress hormones (particularly catecholamines and cortisol) have been related with the intensity and/or duration of exercise, they are often suggested to provide a mechanistic rationale for observed dose-dependent effects of exercise on immune responses (Pedersen and Hoffman-Goetz, 2000). Other physical stressors, such as nutritional restriction, are also capable of elevating stress hormones and may compound the negative effects of prolonged and/or intensive exercise on immune responses (Gleeson et al., 2004).

2.3 Nutritional restriction and immune responses

Inadequate nutrition due to dietary deficiencies of energy and macronutrients, and/or specific micronutrients has long been associated with suppressed immune functions that are fundamental to host defence (Gleeson et al., 2004; Walsh et al., 2011a). Nutrient availability is required for immune cell replication, protein synthesis, metabolism and antioxidant defences (Bishop et al., 1999). Therefore energy restriction may alter immune functions directly through reduced substrate availability. In addition, nutrient restriction may weaken many aspects of immune function indirectly through raised hypothalamic-pituitary-adrenal (HPA) activation and sympathetic-adrenal-medullary activation, resulting in increased stress hormones (e.g. cortisol and catecholamines), which can have immunosuppressive effects (Gleeson, 2007).

During a period of starvation, anorexia nervosa patients have been shown to present decreased circulating leukocytes (Marcos et al., 2003), T lymphocyte populations (Mustafa et al., 1997) and reduced DTH responses to recall antigens (Cason et al., 1986). Controlled laboratory studies with healthy participants have also shown that short periods of nutritional restriction can temporarily suppress various aspect of immune function. For example, fasting for 36-h decreased circulating lymphocyte counts and neutrophil chemotaxis (Walrand et al., 2001), whilst a 7-day fast decreased lymphocyte cytokine release in response to stimulation (Savendahl and Underwood, 1997). Furthermore, saliva SIgA has been shown to decrease during Ramadan fasting (Develioglu et al., 2013) but was unchanged after an 8-day fast in a similarly healthy population (Johansson et al., 1984). Differences in the reported immune responses are most likely due to differences in the duration and severity of the nutritional restriction (Marcos et al., 2003). In particular, for measures of saliva SIgA, differences in the collection technique, such as stimulated or unstimulated collection, and the way in which the data is presented, such as concentration or as a secretion rate, may also partly account for discrepant findings (Oliver et al., 2007). Due to the diluting and concentrating effects of saliva flow rate, associated with hydration status, reporting saliva SIgA as a secretion rate is recommended, which corrects for changes in hydration status and provides an indication of SIgA availability at the oral mucosa (Walsh et al., 1999).

When fluid restriction occurs in combination with energy deficit this can compound the negative influence of energy restriction on immunocompetence (Laing et al., 2008b; Oliver et al., 2007). A 48-h period of energy restriction (90% restriction per day) significantly decreased circulating leukocyte, lymphocyte, $CD3^+$ and $CD4^+$ counts by ~20% but when this was coupled with fluid restriction (75% restriction per day) a ~30% decrease in these measures was

observed. Using the same experimental model, such additive effects of nutritional restriction were also demonstrated on saliva SIgA secretion rate (Oliver et al., 2007). After just 24-h of combined fluid and energy restriction, saliva SIgA secretion rate was significantly reduced, however this was unaffected by fluid restriction alone or energy restriction alone (Oliver et al., 2007).

It is noteworthy that upon refeeding, immune disturbances have been shown to return to baseline values within 4 h (Walrand et al., 2001) or 6 h (Oliver et al., 2007) and this occurs without the need to replace the full deficit (Oliver et al., 2007). This is in line with other controlled laboratory studies of refeeding on immune suppression after prolonged exercise stress. One such study found that providing carbohydrate or carbohydrate with protein post exercise prevented the exercise-induced decrease in bacterially stimulated neutrophil degranulation (Costa et al., 2009). Given that inadequate nutrition and prolonged exercise, or heavy training, have the ability to negatively influence immunocompetence independently, it is expected that a combined effect of these stressors would result in greater suppression of immune functions (Gleeson et al., 2004).

2.3.1 Field studies

Due to practical and ethical reasons associated with investigating prolonged nutritional restriction and exercise stress, field studies in military personnel may provide a suitable model to study the effect of these factors (Hoyt and Friedl, 2006). Rather than imposing the stressors, such as in laboratory studies, these observational research designs allow investigators to study the consequences of the associated stressors and possible countermeasures to help guide recommendations, such as future training programmes and dietary intakes.

Arduous military training courses have been shown to decrease cellular, humoral and mucosal immunity and to increase circulating metabolic and inflammatory indicators (e.g. interleukin (IL-6), as well as symptoms of URI (Gomez-Merino et al., 2005; Tiollier et al., 2005; Whitham et al., 2006). Even short periods (5-7 days) of strenuous military training coupled with calorie restriction, has been associated with body mass losses of 4-5 kg and significant suppression of selective circulating immune markers (Boyum et al., 1996). During a 19-day Royal Australian Air Force survival course, the 29 participants experienced a decrease in saliva SIgA and 6.5 kg loss in body mass compared with day 0 baseline values (Carins and Booth, 2002). The authors suggested that the decrease in saliva SIgA concentration possibly contributed to increased incidence in URI symptoms. Although dietary restriction and body mass loss were negatively correlated with saliva SIgA, or SIgA to albumin ratio (SIgA:Alb), so were other stressors such as alcohol consumption and negative emotions (Carins and Booth, 2002). This highlights a limitation of field studies in that they typically incorporate a multi-stressor environment including heavy exertion, nutritional restriction, sleep deprivation and exposure to environmental extremes, all of which have been shown to independently alter immune responses (Castell et al., 2010; Walsh et al., 2011a). Therefore studies that only compare training-induced changes with baseline values make it difficult to understand the influence of a specific stressor, such as nutritional restriction, on immune responses.

A limited number of studies have investigated whether reducing the energy deficit prevents the decrease in immune function by including a control group, who completed the standard training course, compared with a group who completed the training course with additional energy intake. A recent study attempted to address this when investigating the effects of energy bar supplementation, in addition to traditional field rations, during an 8-day field training course (Tanskanen et al., 2012). Compared with participants who received the field rations alone, there

was no difference in self-reported symptoms of URI with the additional energy intake, but the energy deficit was also indifferent between groups, indicating that the supplementation protocol was unsuccessful. In a study of air field defence guards undergoing a 12-day training exercise, one group received half of their daily energy requirements (7.5 $MJ \cdot day^{-1}$) and subsequently experienced 1.9 kg body mass loss together with suppressed cellular and humoral immunity (Booth et al., 2003). When compared with a comparative control group who received sufficient dietary intake (15 $MJ \cdot day^{-1}$) to maintain energy balance, the decreases in markers of immunity, such as saliva SIgA:Alb, were attenuated. However, the results from this study should be interpreted with caution because the participants receiving half of their energy requirements were provided with combat ration packs whereas those receiving sufficient energy intake were provided with freshly prepared foods, thus highlighting a lack of experimental rigour.

Arguably one of the most demanding military training courses is the 62 day U.S. Army Ranger Training Couse (Korzeniewski et al., 2013). During this course trainees experience significant nutritional restriction and arduous combat training, resulting in mean body mass losses of 16% (Kramer et al., 1997). Additional feeding to reduce the energy deficit resulted in a less severe suppression of T-lymphocyte proliferative responses compared with trainees receiving the standard field rations (Kramer et al., 1997). This military field study also presented a number of limitations such as inconsistent timings of blood sampling after the last exercise bout, and trainees receiving the additional energy intake still experienced significant body mass losses (13%), which highlights the need for further well controlled nutritional supplementation studies.

Alterations in immune responses that occur when arduous training is combined with nutritional restriction are typically suggested to be driven by hormonal changes (Gomez-Merino et al., 2003; Makras et al., 2005). In particular, the immunosuppressive effects of stress hormones, such as raised circulating cortisol, is widely acknowledged to explain much of the immune suppression following prolonged exercise and intensive training (Pedersen and Hoffman-Goetz, 2000; Walsh et al., 2011b). Furthermore, elevated circulating cortisol was proposed to account for decreased T-lymphocyte populations during starvation in anorexia nervosa patients, in the absence of exercise stress (Mustafa et al., 1997). It has recently been shown that 7 days of military training, coupled with a large energy deficit (16 $MJ \cdot day^{-1}$), resulted in a 32% increase in resting circulating cortisol and these levels returned to baseline when the energy deficit was reduced (Kyrolainen et al., 2008). In light of this, it is logical to expect that nutritional supplementation, capable of reducing the energy deficit and stress hormone response, may also attenuate suppression of immune responses.

It is unsurprising that many of the field studies reported here have examined levels of SIgA, not only because of its relative ease of sample collection in a field environment, but because it is one of few measures that have actually been associated with URI (Gleeson and Bishop, 2013). Together with innate mucosal defences, SIgA acts to provide the 'first line of defence' against infectious agents presented at the mucosa and low levels of saliva SIgA has been related with increased risk of URI, in trained individuals (Gleeson et al., 2012). In addition to saliva SIgA, many of these field studies have reported circulating cell numbers and functional *in vitro* measures, such as neutrophil chemotaxis and lymphocyte proliferation, which are considered to have low to moderate suitability in assessing immunocompetence (Albers et al., 2005). A limitation of this work, and much of the exercise immunology literature to date, is that it rarely

adopts *in vivo* measures of immune function, which involve integrated, whole-body immune responses.

2.4 Physical stress and *in vivo* immune measures

The advantages of, and the need for further research utilising, *in vivo* measures to examine physical stress in humans has recently been highlighted (Akbar et al., 2013; Albers et al., 2013b; Walsh et al., 2011b). *In vivo* measures are considered more clinically relevant and informative than the commonly used *in vitro* measures where immune cells, typically from peripheral blood, are extracted from their normal environment and are analysed in artificial cultures (Albers et al., 2013). Isolated measures of immune function may react differently to a whole-body immune challenge because they lack the highly integrated neural and hormonal components within the specific tissue environment in which immune responses usually take place (Akbar et al., 2013).

At present there is no 'gold-standard' measure of *in vivo* immune competence. Therefore when selecting immunological assessment tools for research, it is advisable to give careful consideration to the aspect(s) of immunity of interest, clinical relevance, biological significance and feasibility (Albers et al., 2013). For example, elegant work performed by Cohen and colleagues has examined the effects of stress using the *in vivo* method of experimental infection with live pathogens, such as rhinovirus (Cohen et al., 2013). Although this method is highly clinically relevant, its use is somewhat restricted due to concerns associated with the risk to health of purposefully inducing symptoms of infection. An alternative approach is to use an *in vivo* vaccine challenge, such as an influenza vaccine, to determine the effects of stress on the antibody response. This method overcomes the risk associated with the above but it is noteworthy that the use of vaccine models as an *in vivo* measure of immunity also present a

number of limitations, including variable immunogenicity (e.g. hepatitis B (Hernandez-Bernal et al., 2011)), annual changes in vaccine (e.g. influenza (Burns, 2012)) and difficulty when comparing the circulating antibody results from different studies using in-house ELISAs.

Measures of *in vivo* immunity at the skin are particularly attractive because the skin constitutes the body's largest immunological organ, providing the first line of defence against pathogenic and environmental assaults (Clark et al., 2007). These measures include DTH responses to intradermal injection of antigens, or the less invasive CHS responses to epicutaneous application of antigens. *In vivo* cutaneous immune measures have long been used to assess immunocompetency in individuals with clinical conditions such as Hodgkin's disease (Brown et al., 1967), Crohn's disease (Jones et al., 1969) and HIV (Levis et al., 2006). The relevance of these measures has also been highlighted by studies reporting impaired responses in individuals during acute infectious illness e.g. Epstein-Barr virus (Bennett et al., 1998) and in diabetes and psoriasis patients (Bangsgaard et al., 2011). Furthermore, *in vivo* cutaneous immune measures have been shown to predict mortality in critically ill HIV patients (Dolan et al., 1995) and in patients with surgical infections (Poenaru and Christou, 1991).

A bout of prolonged, continuous exercise (half-ironman competition) has been shown to reduce DTH reactions to the Mérieux CMI Multitest® compared with rested controls (Bruunsgaard et al., 1997). However, this test is no longer commercially available and is subject to the same criticism as influenza vaccines in that the use of common recall antigens does not permit the assessment of the effects of stress on the induction of new immune memory. Alternatively, the use of novel antigens, such as keyhole limpet hemocyanin (KLH) (Smith et al., 2004c) or DPCP (Harper Smith et al., 2011), allows for the measurement of a primary induction response and allows rigorous control of both the sensitising dose and elapsed time since sensitisation.

However, it has been shown that some individuals demonstrate significant responses to KLH prior to immunisation, which questions whether this is a truly novel antigen (Smith et al., 2004a). Experimental CHS with DPCP overcomes this limitation and offers a simple, practical and safe immunological tool that can be administered without the need for expensive equipment, invasive injections or blood sampling, making it suitable to examine the effects of stress in both laboratory and field investigations (Harper Smith et al., 2011). Examples of the use of this measure within stress immunology are discussed further in **section 2.7**.

2.5 Diphenylcyclopropenone

DPCP is a specially synthesised chemical which was first developed by Breslow et al. (1959). It is considered to be a safe contact sensitizer after it was found not to be mutagenic in the Ames assay (Wilkerson et al., 1987). Deliberate contact sensitisation and repeated applications of DPCP has been used extensively for topical immunotherapy of conditions such as alopecia areata (Sotiriadis et al., 2007) and warts (Buckley and Du Vivier, 2001). Using lower concentrations than those applied in immunotherapy, DPCP can be utilised for experimental CHS to provide an attractive *in vivo* measure of immune function (Harper Smith et al., 2011). Given that cutaneous responses to DPCP have previously been shown to track immune status in patients with clinical conditions, it is considered that responses observed during experimental stress research could reflect the immune system's general ability to respond to an infectious challenge. Due to the fact that DPCP will not have previously been encountered in the natural environment, this permits the investigation of the influence of stress on both the sensitisation phase and the elicitation phase of the immune response.

2.6 Experimental contact hypersensitivity

Experimental CHS, with contact sensitizers such as DPCP, represents a primarily T cell-

mediated immune response, which is dictated by input from both the innate and adaptive immune systems (Friedmann, 2007). It is well acknowledged that CHS is characterised by two distinct phases: the sensitisation phase (termed induction) and the subsequent elicitation phase (termed recall) (Christensen and Haase, 2012). The sensitisation phase involves the very first application of the novel antigen and includes all the subsequent events that occur, up to the development of new immune memory. Figure 2.1 briefly outlines the key steps, which include the following:

- Step 1 - Upon contact, the foreign material is taken up by specialised antigen presenting dendritic cells (DC) within the skin, namely Langerhans' cells (LC) and dermal dendritic cells (dDC), which process and associate the material with major histocompatibility molecules class I and II (Friedmann, 2007).
- Step 2 - These cutaneous DC then mature and migrate towards the skin-draining lymph nodes (dLN), which is orchestrated by the local cytokine environment (Toebak et al., 2009). In particular, release of key cytokines, including IL-1β and tumour necrosis factor (TNF)-α, from cutaneous DC and the keratinocytes (KC), appear to be required for initiating migration of these cells (Christensen and Haase, 2012). In addition, DC expression of chemokine receptors, such as CCR7 and CXCR4, and release of chemokines, such as CCL19, CCL21 and CXCL12, are particularly important for migration of DC into the dLN (Kabashima et al., 2007).
- Step 3 - After migration and entry into the dLN, the DC present the major histocompatibility complex (MHC)-associated immunogen to naïve T cells, via the T-cell receptors (TCR).
- Step 4 - Cytokines, costimulatory molecules and adhesion molecules from the DC assist in the activation of naïve T cells to then differentiate, generating antigen-specific $CD4^+$ T helper 1-biased (Th1) and $CD8^+$ cytotoxic (Tc1) effector or memory T cells. In

addition, Th17 cells are also involved in the development of CHS (Honda et al., 2011). Following proliferation, the resultant memory T cells enter the blood stream, which marks the end of the sensitisation phase.

The elicitation phase follows after topical re-exposure to the same contact sensitizer, typically in a low concentration dose-series challenge 28 d after initial sensitisation (Harper Smith et al., 2011). Figure 2.1 highlights the following key events for the elicitation phase:

- Step 5 - Activated epidermal cells release inflammatory cytokines and chemokines (e.g. CXCL9, CXCL10 and CXCL11), which promote infiltration of antigen-specific T cells to the local area (Popov et al., 2011).
- Step 6 - The foreign material is taken up by cutaneous DC and other non-dedicated antigen presenting cells, such as KC and macrophages, which present the antigen to antigen-specific T cells.
- Step 7 - Additional inflammatory cytokines (e.g. IFN-γ and IL-4) and chemokines (e.g. CCL5) are released from infiltrating T cells, and the subsequently stimulated KC, further amplifying cellular infiltration of T cells, including some non-specific T cells as well as DC, monocytes and neutrophils (Toebak et al., 2009).

A recent study characterising the cellular and molecular responses during elicitation to DPCP in humans, albeit with high doses of DPCP, provides further support that the magnitude of elicitation responses are associated with the number of infiltrating T cells ($CD3^+$) and DC at the challenge site (Gulati et al., 2014). In practice, the strength of the *in vivo* immune response is commonly quantified by the vigour of the resultant oedema (thickening) and erythema (redness), typically 48 h after applying the challenge.

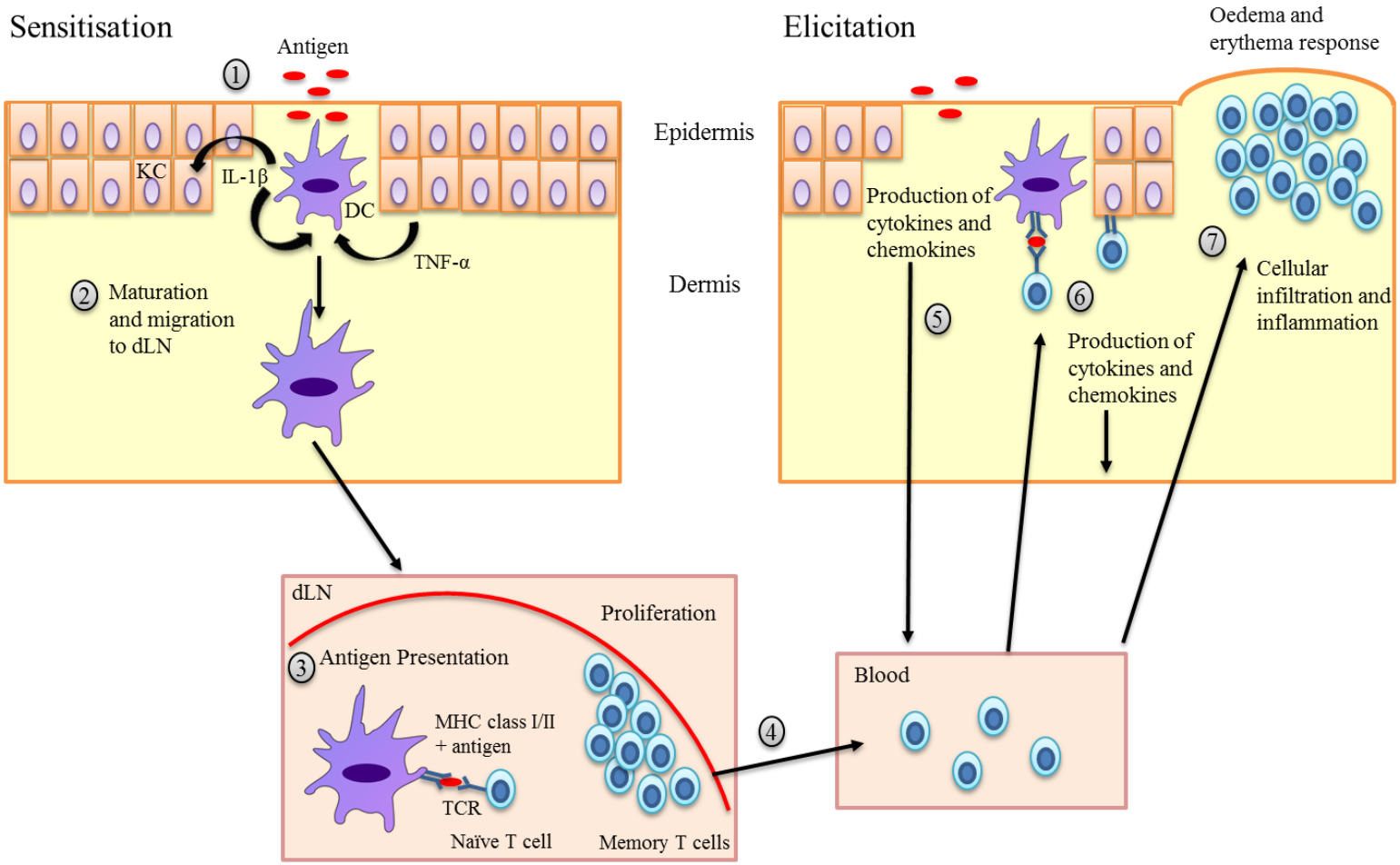


Figure 2.1 Key events in the sensitisation and elicitation phases of contact hypersensitivity (CHS). Adapted from Christensen and Haase (2012) and Toebak et al. (2009). Sensitisation phase. Step 1: uptake of foreign material; Step 2: maturation and migration of cutaneous DC; Step 3: presentation to antigen-specific naïve T cells; Step 4: differentiation and proliferation; Elicitation phase. Step 5: epidermal release of cytokines and chemokines; Step 6: presentation to antigen-specific T cells; Step 7: amplified cellular infiltration and resultant inflammation. DC, dendritic cells; KC, keratinocytes; MHC, major histocompatibility complex; TCR, T cell receptor.

2.7 Stress and experimental contact hypersensitivity with DPCP

Experimental CHS with DPCP has previously been used in human studies to investigate the effects of a number of stressors, such as psychological stress (Zachariae et al., 1997), high altitude exposure (Oliver et al., 2013) and prolonged exercise (Harper Smith et al., 2011), on *in vivo* immune responses. Although limited, one study has shown no effect of relaxation training, compared with a no training control group, when the relaxation techniques were implemented in the days after DPCP sensitisation or elicitation patch application (Zachariae et al., 1997). An alternative approach would be to examine the effects of an intervention or stressor prior to application of DPCP. Recently, Oliver et al. (2013) identified that 28 h of high altitude exposure, prior to initial sensitisation with DPCP, impaired the development of new immune memory measured 28 d later, compared with rested controls (skinfold thickness, -52%). The findings from this field-based study are consistent with those reported after 2 h prolonged, moderate intensity exercise (60% $\dot{V}O_{2max}$) within a controlled laboratory setting (skinfold thickness, -53%; Harper Smith et al., 2011). Interestingly, when participants who received several monthly challenges, to achieve a reproducible plateau in responses, performed the same bout of exercise stress prior to recall challenge, skinfold thickness was only suppressed by -19% (Harper Smith et al., 2011). This implies that the primary response to antigens is more susceptible to immune suppression and warrants further investigation.

Physical exercise provides a well-controlled model to further study the effects of prior stress on the induction of new immune memory. Although Harper Smith et al. (2011) identified that a single bout of prolonged exercise suppressed induction of new immune memory compared with rested controls, it is unclear how the intensity or duration of the exercise bout would influence these responses and possible mechanisms involved in the immune suppression were not explored.

2.8 Mechanisms of stress-induced alterations in experimental contact hypersensitivity

2.8.1 Stress hormones

Classically, stress hormones such as glucocorticoids and catecholamines have been implicated in altered cutaneous immune responses (Dhabhar, 2013; Flint et al., 2001; Seiffert et al., 2002). In mice, high dose corticosterone has been shown to suppress DTH responses (Dhabhar and McEwen, 1999). The authors also found that adrenalectomised mice, which are unable to produce a glucocorticoid and adrenaline stress response, failed to show stress induced alterations of skin DTH. Furthermore, to determine the involvement of serum corticosterone in CHS responses Flint et al. (2001) injected glucocorticoid receptor antagonist prior to restraint stress and sensitisation, which attenuated the stress induced suppression of elicitation responses in mice. However, some studies have failed to identify a role of corticosterone or cortisol in the stress-induced modulation of cutaneous immune responses (Blecha et al., 1982; Oliver et al., 2013). Instead, an involvement of alternative stress hormones, such as catecholamines, was suggested. Support for a role of catecholamines has been provided by studies showing that dermal injection of adrenaline both locally or distant from the sensitisation site inhibited CHS responses in mice (Seiffert et al., 2002) and levels of noradrenaline were correlated with DTH against KLH in humans (Smith et al., 2004a).

Given the critical role of cutaneous DC in the induction of new immune memory (Figure 2.1), an effect of stress hormones on either the number or function of these cells may dictate the CHS outcome (Toebak et al., 2009). In humans, studies indicate that cutaneous DC migration is inhibited by topical application of glucocorticoids (Burrows and Stoughton, 1976) and studies in mice have shown high dose intradermal injections of corticosterone or catecholamines, inhibit the antigen-presenting capability of cutaneous DC, reduce the number of T cells in draining lymph nodes and ultimately suppress DTH and CHS responses (Dhabhar and McEwen, 1999; Flint et al., 2001; Seiffert et al., 2002).

In summary, the previous research tends to suggest that stress capable of activating the HPA axis and in-turn increases glucocorticoids and catecholamines is likely to decrease the induction of CHS (Dhabhar, 2013; Flint et al., 2001; Seiffert et al., 2002). This may provide mechanistic insight into the previously reported reductive effects of prolonged exercise on immune induction to DPCP (Harper Smith et al., 2011). A stronger approach to examine the mechanisms involved would be to directly examine the events of the sensitisation phase of CHS. Given that the immunosuppressive effects of exercise are typically resolved within 24 h (Walsh et al., 2011b), the early events in response to an antigen challenge will be considered here.

2.8.2 Cutaneous cytokines

As outlined in **section 2.6** (Figure 2.1: Step 2), cutaneous DC migration is one of the first events in the initiation of immune induction and is believed to be pivotal to the development of new immune memory (Toebak et al., 2009). The maturation and migration of cutaneous DC has been shown to be orchestrated by the local cutaneous cytokine environment, which is stimulated upon contact with the antigen (Dearman et al., 2004). DC migration is also associated with the release of chemokines and expression of chemokine receptors on the surface of these cells (Toebak et al., 2009). In particular, chemokine receptors involved in homing to the dLN are upregulated, such as CCR7 and CXCR4, which directs the DC to the key chemokines produced within the dLN (i.e. CCL21, CCL19 and CXCL12) (Kabashima et al., 2007). It is acknowledge that interactions between these chemokines and their receptors is pivotal for the migration of DC into the dLN but the early events involving antigen-stimulated cytokine responses will be the focus here.

Various stimulated cytokines have been identified following epicutaneous antigen exposure, but IL-1β and TNF-α are the cytokines considered essential in facilitating cutaneous DC migration (Christensen and Haase, 2012). IL-1β derived from cutaneous DC is suggested to activate DC directly and also stimulate secretion of TNF-α from KC, which further facilitates DC migration (Toebak et al., 2009; Figure 2.1). In support of the importance of these cytokines, injection of anti-IL-1β antibody prior to initial epicutaneous antigen exposure, has been shown to completely prevent sensitisation in mice (Enk et al., 1993) and local intradermal injection of IL-1β, in both mice and humans, increased migration of cutaneous DC (Cumberbatch et al., 1997; Cumberbatch et al., 2003; Table 2.1). In a similar fashion, local intradermal injection of TNF-α, in mice and humans, increased migration of cutaneous DC (Bhushan et al., 2002; Cumberbatch et al., 1999; Cumberbatch et al., 2003; Nishibu et al., 2007) and administration of anti-TNF-α antibody or TNF-α-receptor antagonist, prior to skin sensitisation, inhibited the accumulation of DC in the dLN and impaired elicitation responses, in mice (Cumberbatch and Kimber, 1995; Nishibu et al., 2007). In addition, topical application of lactoferrin, which acts as an anti-inflammatory agent and has been associated with decreased IL-1β and TNF-α, impaired cutaneous DC migration at the site of DPCP sensitisation in humans, providing further support for the facilitative role of these cytokines (Cumberbatch et al., 2003; Griffiths et al., 2001).

IL-10 is also produced during the induction phase of CHS and since IL-10 down-regulates proinflammatory cytokine production, this cytokine has been implicated in the suppression of elicitation responses in mice (Enk et al., 1994; Toebak et al., 2009; Wang et al., 1999; Yoshiki et al., 2010). Intradermal injection of IL-10 prior to sensitisation resulted in suppressed elicitation responses, which was thought to be due to reduced production of cutaneous IL-1β and TNF-α observed at the mRNA level (Enk et al., 1994). However, Schwarz et al. (1994)

was unable to show an effect of IL-10 injection prior to sensitisation, which may be explained by the protocol timings of the study, given that there was a 24 h delay between IL-10 injection and application of the sensitiser. In contrast, further support for the influence of IL-10 has been presented in mice with disrupted IL-10 gene or IL-10 knockout, which resulted in a greater number of antigen-bearing cells in the draining lymph nodes and an exaggerated CHS response (Berg et al., 1995; Wang et al., 1999). Further research is required to determine if these findings are also relevant in humans.

Little is currently known about the kinetics of skin sensitisation, but in mice, a rapid increase in IL-1β mRNA expression has been identified at the site of sensitisation after only 15 minutes, with further increases of ~40-fold after 1 h (Enk and Katz, 1992). In humans cutaneous DC migration has been measured 6-24 h after DPCP application (Griffiths et al., 2001; Heffler et al., 2002), and in mice and humans, antigen-modified dendritic cells appeared in the regional lymph nodes within 24 h of epicutaneous antigen application (Hunger et al., 2001; Macatonia et al., 1987).

Table 2.1. A summary of investigations examining the effect of IL-1β, TNF-α and IL-10 on cutaneous DC migration and elicitation responses.

Study	Human or mouse model	Cytokine of interest	Protocol	Outcome measure	Results
Enk et al. (1993)	Mice: n = 5 per group.	IL-1β	Injection of anti-IL-1β or PBS control immediately prior to sensitisation with TNCB.	Ear thickness elicitation responses.	Anti-IL-1β ↓ elicitation skin thickness 75% and was ND to non-sensitised skin.[b]
Cumberbatch et al. (1997)	Mice. Part 1: n = 10 per group.	IL-1β and TNF-α	Injection of anti-IL-1β, anti-TNF-α or serum control 2 h prior to sensitisation with oxazolone.	DC accumulation in dLN.	DC accumulation in dLN ↓ 45% by anti-IL-1β and 68% by anti-TNF-α.[b]
	Mice. Part 2: n = 10 per group.	IL-1β and TNF-α	Injection of anti-IL-1β or serum control 2 h prior to TNF-α injection.	DC accumulation in dLN & cutaneous DC frequency.	Anti-IL-1β ↓ DC accumulation in dLN by 59% and prevented the TNF-α-induced decrease in cutaneous DC frequency (ND).
Cumberbatch et al. (2003)	Humans. Part 1: n = 7.	IL-1β	Injection IL-1β or saline 2 h or 4 h prior to local punch biopsies.	Cutaneous DC frequency.	IL-1β ↓ cutaneous DC frequency by 20%.
	Humans. Part 2: n = 5.	IL-1β	Topical LF or aqueous cream control 2 h prior to injection of IL-1β or saline control and local punch biopsies.	Cutaneous DC frequency.	LF prevented the IL-1β induced ↓ in DC frequency by 23% and was ND to aqueous cream and saline.

Cumberbatch and Kimber (1995)	Mice. Part 1: n = 10 per group.	TNF-α	Injection of anti-TNF-α or saline control 2 h prior to sensitisation with oxazolone.	DC accumulation in dLN.	Anti-TNF-α ↓ accumulation of DC in dLN by 68%.
	Mice. Part 2: n = 15 per group.	TNF-α	As above.	Ear thickness elicitation responses.	Anti-TNF-α ↓ elicitation responses by 31% to 48%.
Nishibu et al. (2007)	Mice[c]	TNF-α	Injection of TNFR2 30 min prior to sensitisation with DNFB and time-lapse confocal imaging.	Cutaneous DC migration.	TNF-α significantly ↓ DC movement.[a]
Bhushan et al. (2002)	Humans: n = 10.	TNF-α	Injection of TNF-α or saline control 2 h prior to local punch biopsies.	Cutaneous DC frequency.	TNF-α ↓ cutaneous DC frequency by 23%.
Cumberbatch et al. (1999)	Humans: n = 17.	TNF-α	Injection of TNF-α or saline control 2 h prior to local punch biopsies.	Cutaneous DC frequency.	TNF-α ↓ cutaneous DC frequency by 24%.
Enk et al. (1994)	Mice[c]	IL-10	Injection of IL-10, saline or IL-10 with anti-IL-10 prior to sensitisation with TNCB.	Ear thickness elicitation responses.	IL-10 ↓ elicitation responses by 87% compared with saline. Anti-IL-10 was ND to saline control.

Schwarz et al. (1994)	Mice[c]	IL-10	Intradermal injection of IL-10 or saline 24 h prior to sensitisation with DNFB.	Ear thickness elicitation responses.	ND.
Berg et al. (1995)	Mice: n = 6 per group.	IL-10	IL-10 gene disrupted mice or wild-type mice sensitised with oxazolone.	Ear thickness elicitation responses.	IL-10 gene disrupted mice ↑ elicitation responses by 120%[b].
Wang et al. (1999)	Mice: n = 10 per group.	IL-10	IL-10 knockout mice or wild-type mice sensitised with FITC.	DC cells in dLN and ear thickness elicitation.	IL-10 knockout mice ↑ accumulation of DC in dLN by 73% and ↑ elicitation responses by 75%.

Abbreviations = DC, dendritic cell; IL, interleukin; LF, lactoferrin; TNF-α, tumour necrosis factor; R2, receptor 2; ND, no significant difference; dLN, draining lymph node; TNCB, trinitrochlorobenzene; DNFB, dinitrofluorobenzene; FITC, fluorescein isothiocyanate; ↑ increase; ↓ decrease; [a] comparative control value not available for percentage difference calculation; [b] estimated from figures; [c] n not specified.

In humans, the direct measurement of cutaneous DC migration is typically assessed by examining cell frequencies within a punch biopsy of the skin. Punch biopsies are considered to be an invasive procedure that requires local anaesthesia and typically result in a small scar (Kiistala, 1968). The suction blister technique offers a less invasive, means of providing a window into the events occurring locally within the skin. Some studies have examined the frequencies of cutaneous DC in suction blister roofs (Dam et al., 1996), but this is not recommended by others because the blister technique stretches the epidermis and yields much lower values than those observed in punch biopsies, thus providing less scope to study the effects of an intervention (Dearman et al., 2004). Alternatively, the examination of the suction blister fluid provides an attractive means of assessing changes in the local cytokine environment, thought to orchestrate cutaneous DC migration (Dearman et al., 2004).

2.8.3 Suction blister technique

Skin suction blisters can be induced using the method originally developed by (Kiistala, 1968). This involves separation of the epidermis from the dermis using a small suction cup attached to a negative pressure device. The application of prolonged, gentle, negative pressure results in a small suction blister from which the blister fluid can then be aspirated for analysis. The Dermovac® suction blister device, which is widely cited within the literature, is no longer commercially available. Therefore many researchers use in-house built devices based on the original Dermovac® instrument and recommendations made by Kiistala, (1968) (Akbar et al., 2013; Janssens et al., 2009; Nicolaou et al., 2012). A transparent Perspex suction cup allows for the visual determination of blister formation and rounded edges minimises trauma from impression of the cup edges (Kiistala, 1968). Factors known to influence the formation of suction blisters, and thus are important to control, include the temperature, with warmer environments reducing the formation time (Hatje et al., 2015), and the extent of the vacuum

applied to the skin, with pressures greater than 300 mmHg reducing the formation time but increasing the risk of trauma to the site (Gupta and Kumar, 2000). In addition, as to be expected, the larger the diameter of the inner part of the suction cup, the longer the formation time (Gupta and Kumar, 2000). Therefore negative pressures of 200-300 mmHg and an inner cup diameter of 10 mm are commonly used in previous research, which provides sufficient volume of blister fluid for subsequent analysis, within a reasonable amount of time (typically ~1-2 h) (Akbar et al., 2013; Janssens et al., 2009; Nicolaou et al., 2012).

The suction blister technique has been used in a variety of disciplines such as the study of drug pharmacokinetics (Mazzei et al., 2000), UV exposure (Skov et al., 1998), inflammatory markers and biomarker proteins (Muller et al., 2012). Blister fluid that is harvested immediately from freshly formed blisters corresponds to the interstitial tissue fluid of the epidermis and dermis, thus providing information about the activities within the local cutaneous environment (Kool et al., 2007; Rhodes et al., 1999). After 18-24 h of blister formation the fluid turns amber colour and contains cells (Agius et al., 2009; Kiistala, 1968). This is of interest to answer alternative research questions such as the infiltration of leukocytes during recall responses to a DTH skin antigen test (Agius et al., 2009; Akbar et al., 2013). A variation of this suction blister approach has also been used to investigate the effect of stress on wound healing (Kiecolt-Glaser et al., 2005). To investigate the induction phase of experimental CHS with DPCP, the cytokine content of freshly formed blisters, induced directly over the site of DPCP sensitisation, is of interest here.

The suction blister technique can be used to examine the local cutaneous environment of cytokines thought to orchestrate DC migration. Blisters raised on normal, untreated skin yield low but detectable levels of IL-1β, TNF-α and IL-10 (Dearman et al., 2004; Janssens et al.,

2009; Skov et al., 1998), but there is some variation in results from different studies. For example, blister fluid TNF-α concentration values of 44 to 224 $pg \cdot ml^{-1}$, from normal, untreated skin, have been reported by different studies (Janssens et al., 2009; Skov et al., 1998). These differences are likely due to differences in the suction blister procedure used and the means of blister fluid analysis, such as ELISA or multiplex assays, with the later associated with the greatest variation (Dearman et al., 2004; Janssens et al., 2009). Stimulated changes in the local cutaneous environment have also been successfully identified in suction blister fluid. Prior local injection of IL-β induced a 3- to 6-fold increase in TNF-α production detected in suction blister fluid and application of lactoferrin, known to inhibit antigen-induced cutaneous DC migration, decreased blister fluid TNF-α content (Cumberbatch et al., 2003; Dearman et al., 2004).

Although the suction blister technique appears to be a suitable tool for the measurement of the cutaneous cytokine environment, there is a lack of standardisation in the way of reporting suction blister cytokine content. Some authors express the blister fluid cytokines as an absolute concentration ($pg \cdot ml^{-1}$) (Janssens et al., 2009; Shahbakhti et al., 2004; Skov et al., 1998), whereas others choose to report these relative to total protein ($pg \cdot mg^{-1}$ protein) (Cumberbatch et al., 2006; Dearman et al., 2004). Dearman et al. (2004) suggests that expressing blister fluid cytokines as a function of total protein is the preferred option because it was shown to reduce the inter-individual variation of the results compared with the absolute concentration. Irrespective of the method of results presentation, it is recommended to examine the repeatability, where possible, of measurement techniques prior to use in research to ensure that the results presented are adequately reliable and valid (Atkinson and Nevill, 1998; Hopkins, 2000). A recent study (article in press) has shown a suction blister method to be reliable for assessing wound healing (Smith et al., 2015). However, this method involves removing the

blister roofs of eight small blisters, placing a chamber over these sites and adding 1 ml of 30% autologous serum to each. Comparisons of cytokine concentrations were then made by collection of this fluid over a 24 h period and reporting as area-under-the-curve values. Therefore, to the best of our knowledge, the repeatability of the suction blister technique to measure the cutaneous cytokine content of freshly formed blisters has not previously been reported.

Raising suction blisters directly over the site of an *in vivo* immune response has previously been recommended, to provide an insight into the local cutaneous responses (Agius et al., 2009; Akbar et al., 2013). These authors were interested in the secondary, recall responses to intradermal injection of tuberculin purified protein derivative. Therefore, although coupling the suction blister technique with a measure of *in vivo* immunity provides an attractive approach, a specific understanding of the local blister fluid cytokine responses to DPCP induction remains to be determined. As discussed in **section 2.8**, after the first application of a sensitising antigen, cutaneous DC migration and arrival of antigen-bearing cells in the draining lymph typically commences within 24 h (Griffiths et al., 2001; Heffler et al., 2002; Hunger et al., 2001). However, information regarding the kinetics of the specific DPCP-stimulated cutaneous cytokine responses is required before this approach can be adopted to study the exercise effects on DPCP induction.

2.4 Thesis objectives

With this information in mind, the objectives of this thesis were to investigate: 1. the effect of daily mixed nutritional supplementation, in a well-controlled field study, on immune indices during an 8-week arduous training programme; 2. the use of DPCP as a tool to assess *in vivo* immunity and the effects of intensity and duration of exercise stress; 3. the repeatability of

raising controlled suction blisters for the measurement of cutaneous cytokines; 4. the kinetics of local cytokine release in response to DPCP sensitisation; and 5. the effect of prolonged, moderate intensity exercise on DPCP-induced changes in the local cutaneous cytokine environment, to provide an insight into the possible mechanisms involved in the suppression of these CHS responses.

CHAPTER THREE

General Methods

3.1 Ethical approval. Approval was obtained from the Ministry of Defence (UK) Research Ethics Committee (**Chapter 4**) or from the local Ethics Committee (School of Sport, Health and Exercise Sciences, Bangor University; **Chapters 5-8**). The nature and purpose of each study was fully explained in writing and verbally to each participant. All participants gave written informed consent (Appendix A) and completed a medical health questionnaire (Appendix B).

Participants had no current injury or illness and 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; **Chapters 5-8**). In addition, participants in **Chapters 5-8** experienced no prolonged sun exposure (e.g. sunbathing) in the two weeks prior to testing and had Caucasian skin-type, because one of our measures is skin redness, which cannot be measured with darker skin tones. These participants were non-smokers, free from medication or dietary supplements, and were required to abstain from caffeine, alcohol, and exercise for 24 h before the experimental trials. Participants in **Chapters 5, 7 and 8** had no previous history of exposure to DPCP.

3.2 Anthropometry and body composition. Prior to commencement of the first experimental trial, body mass was determined using a digital platform scale to the nearest 50 g (Model 705, Seca, Hamburg, Germany), and height, using a wall stadio-meter (Bodycare Ltd, Warwickshire, UK). Body composition was assessed (**Chapters 4 and 5**) using whole body Dual Energy X-ray Absorptiometry (DEXA; Hologic 4500A, Bedford, USA) and body fat mass, lean body mass, and percent body fat were calculated.

3.3 Peak oxygen uptake. Peak oxygen uptake ($\dot{V}O_{2peak}$) was estimated (**Chapters 5 and 8**) by means of a ramped exercise test on a treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany). Following 3-minutes of walking at 5 $km \cdot h^{-1}$ with an incline of 1%, speed increased at a rate of 1 $km \cdot h^{-1} \cdot min^{-1}$ to a maximum of 18 $km \cdot h^{-1}$, after which the incline increased at a rate of 1%$\cdot min^{-1}$ until volitional exhaustion. Pulmonary gas exchange was measured breath-by-breath for the duration of the test (Cortex Metalyser 3B, Biophysik, Leipzig, Germany). The $\dot{V}O_{2peak}$ was taken as the highest 30-s average value before the participant's volitional exhaustion. Additional criteria for attaining $\dot{V}O_{2peak}$ included a heart rate within 10 beats$\cdot min^{-1}$ of age predicted heart rate maximum and a respiratory exchange ratio greater than or equal to 1.15 (Bird and Davison, 1997).

3.4 Induction of contact sensitivity. The sensitizing exposure to the novel antigen DPCP involved application of an occluded patch, constituting a 12 mm aluminium Finn chamber (Epitest Oy, Tuusula, Finland) on Scanpor hypoallergenic tape containing an 11 mm filter paper disc. The paper disc was soaked in 22.8 µl of 0.125% DPCP in acetone (patch = 30 $\mu g \cdot cm^{-2}$ DPCP) and allowed to dry for 5 minutes before being applied to the skin (Harper Smith et al., 2011; **Chapters 5, 7 and 8**).

3.5 Suction blister formation. The suction blisters were induced (**Chapters 6-8**) using a Perspex suction cup with a 10 mm diameter opening (Akbar et al., 2013; Janssens et al., 2009; Nicolaou et al., 2012; Figure 3.1a), which was attached to a negative pressure device. The negative pressure was generated using a 50 ml syringe (Gupta et al., 2005; Figure 3.1d) which was attached to a three-way tap to lock and retain the negative pressure inside the suction cup. Seated in a phlebotomy chair, participants placed their arm on the arm rest and maintained that

position for the duration of the trial. A small amount of Vaseline was added to the edge of the suction cup, to improve adherence to the skin, and secured in place on the inner aspect of the upper arm. The negative pressure was slowly induced, over a minute, to the maintenance level of 250 mmHg below atmospheric pressure (Dearman et al., 2004; Rhodes et al., 2001). Throughout the trial, the pressure was recorded at 10 minute intervals using a digital manometer (Dwyer Series 475 Mark III, Hanwell, London; Figure 3.1c). After the first 10 minutes the pressure was readjusted, to ensure a good seal against the skin, and again after this, if there was a drop by more than 10% of the starting pressure. The negative pressure was maintained until the experimenter could visually determine a single blister measuring ~10 mm in diameter, by looking through the transparent suction cup, but not longer than 3h. Suction blister fluid, of visually blood uncontaminated blisters, was aspirated using a 23-gauge needle and syringe, and collected into an Eppendorf tube.

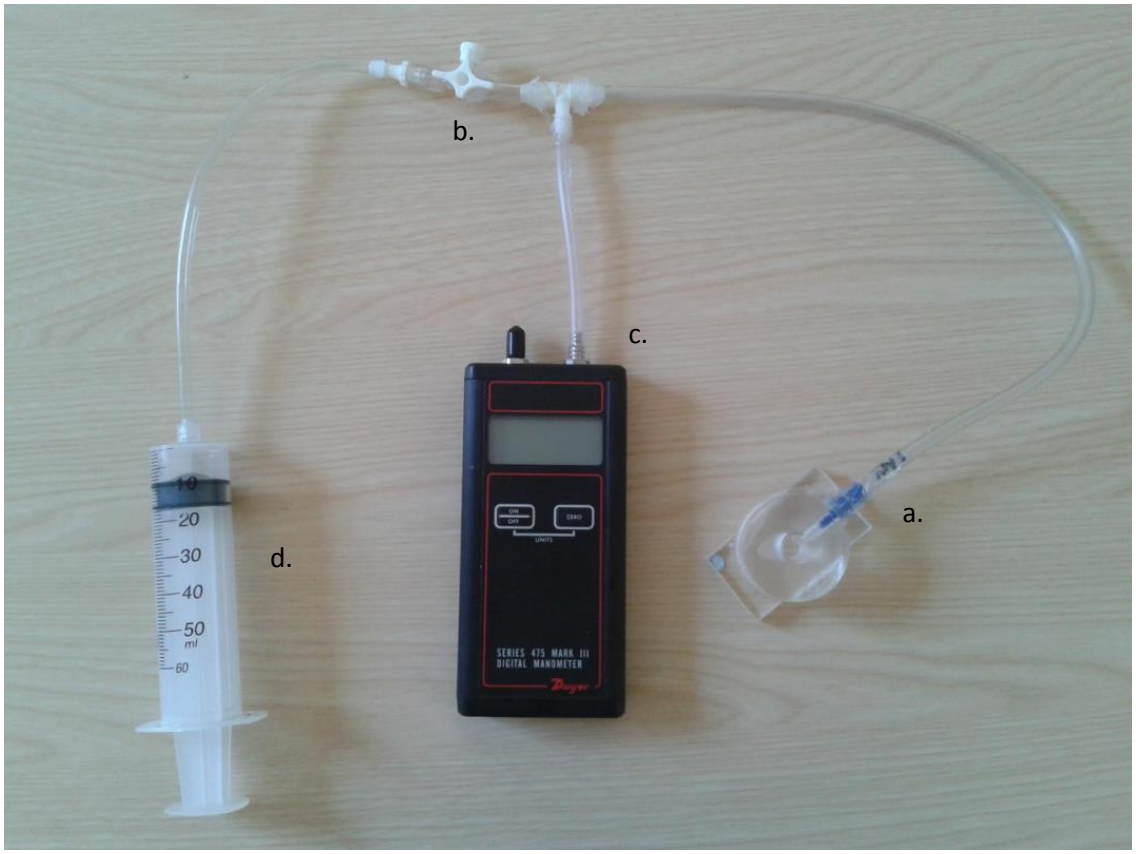


Figure 3.1. In-house built suction blister device. Suction cup (a); tap and tubing (b); digital manometer (c); syringe to generate negative pressure (d).

3.6 Suction blister analysis. Suction blister samples were analysed for cytokine concentration and total protein content (**Chapters 6-8**). All samples were centrifuged for 5 minutes at 1500 *g* and the supernatant was stored at –80 ºC for later analysis. Suction blister IL-1β, TNF-α and IL-10 concentration was determined using human high sensitivity ELISA kits (eBioscience, Hatfield, UK). Protein content was determined using a modification of the Lowry assay (Lowry et al., 1951); the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hertfordshire, UK). Initial pilot assays were performed to determine appropriate sample dilutions. Blister fluid samples were diluted 1:4 in PBS for the IL-1β and TNF-α measurement, 1:8 for IL-10 measurement and 1:41 for total protein.

3.7 Statistical analysis. Data in the text and tables are presented as mean (SD), unless otherwise stated, and statistical significance was accepted at $P < 0.05$. Data were checked for normality and sphericity. Greenhouse-Geisser adjustments to the degrees of freedom were applied where necessary. When significant differences were detected, a one-way ANOVA was performed to identify between group effects and *post hoc* Tukeys honestly significant difference (HSD) or Bonferroni corrected t-tests were used, where appropriate. All statistical analysis was conducted using SPSS software.

CHAPTER FOUR

Effect of daily mixed nutritional supplementation on immune indices in soldiers undertaking an 8-week arduous training programme

4.1 SUMMARY

The aim was to investigate the influence of a daily mixed nutritional supplement during an 8-week arduous training programme on immune indices and mediators including circulating leukocyte counts; bacterially stimulated neutrophil degranulation; IL-6, cortisol and saliva SIgA. Thirty healthy men received a habitual diet (CON, n = 15) or received a habitual diet plus an additional food supplement (SUP, n = 15). From weeks 0–6, CON received 14.0 MJ·day^{-1} and SUP received 19.7 MJ·day^{-1}, and during a final 2-week field exercise in weeks 7 and 8, CON received 17.7 MJ·day^{-1} and SUP received 21.3 MJ·day^{-1}. Blood and saliva were taken at rest after an overnight fast at weeks 0, 6 and 8. Body mass loss over the 8 weeks was greater in CON (CON, 5.0 (2.3); SUP, 1.6 (1.5) kg: $P < 0.01$). Training induced decreases in circulating total leukocytes (CON: weeks 0, 8.0 (2.1); weeks 8, 6.5 (1.6) 10^9 l^{-1}, $P < 0.01$), lymphocytes (21%, $P < 0.01$) and monocytes (20%, $P < 0.01$) were prevented by the nutritional supplement. Saliva SIgA secretion rate increased approximately 2-fold by week 8 in SUP ($P < 0.01$) and was greater at week 8 compared with CON ($P < 0.01$). Circulating neutrophils, bacterially stimulated neutrophil degranulation, IL-6 and cortisol were similar in CON and SUP at week 8. In conclusion, a daily mixed nutritional supplement prevented the decrease in circulating total leukocytes, lymphocytes and monocytes and increased saliva SIgA output during an 8-week arduous training programme. The increase in saliva SIgA with nutritional supplementation during training may reduce susceptibility to upper respiratory infection.

4.2 INTRODUCTION

Military personnel and athletes often experience prolonged periods of nutritional insufficiency, coupled with the additional stress of demanding training regimens. It is widely acknowledged that nutritional restriction can impair immune function, which in-turn may increase an individual's susceptibility to infection (Walsh et al., 2011b). Laboratory studies show that nutritional restriction for periods of 2-7 days temporarily decrease various aspects of immune function including: circulating leukocyte and lymphocyte subset number (Laing et al., 2008b); lymphocyte cytokine release in response to stimulation (Savendahl and Underwood, 1997); saliva SIgA responses (Oliver et al., 2007) and neutrophil chemotaxis (Walrand et al., 2001). These findings are unsurprising given that nutrient availability is required for immune cell replication, metabolism and antioxidant defences (Bishop et al., 1999). In addition, nutrient restriction may weaken immune function through raised HPA activation and sympathetic-adrenal-medullary activation, resulting in increased stress hormones, which can have immunosuppressive effects (Gleeson, 2007).

Intensive periods of training in soldiers and athletes have been shown to decrease cellular, humoral and mucosal immunity and to increase circulating metabolic and inflammatory indicators (e.g. IL-6) and symptoms of URI (Baj et al., 1994; Gomez-Merino et al., 2005; Tiollier et al., 2005; Whitham et al., 2006). The multi-stressor nature and the lack of experimental control in many military field studies, makes it difficult to identify the role that energy deficit plays in the observed decrease in immune health. Soldiers simultaneously experience stressors including heavy exertion, energy restriction, sleep deprivation and exposure to environmental extremes: all of these stressors have been shown to modulate immune function independently (Walsh et al., 2011a; Walsh and Whitham, 2006). One recent study showed that soldiers training for 7 days with an estimated 16 $MJ \cdot d^{-1}$ energy deficit

experienced a 32% increase in resting circulating cortisol (Kyrolainen et al., 2008). Given the likely role for stress hormones in the modulation of immune function with heavy training (Pedersen and Hoffman-Goetz, 2000) we might expect that the energy-deficit-evoked increase in circulating cortisol during arduous training accounts, at least in part, for the observed decrease in immune function in military training studies; however, this remains unsubstantiated. Unfortunately, well-controlled field studies have not been conducted to show whether providing additional energy intake to reduce the energy deficit prevents the decrease in immune function during military training. Blunting the energy deficit with additional feeding during a 2-month army ranger course (4.0 *vs*. 4.9 $MJ \cdot d^{-1}$) induced a less-severe suppression of *in vitro* T-lymphocyte responses (Kramer et al., 1997). Study limitations include the variable timing of blood sampling after the last bout of exercise, some problems with blood sample viability and that soldiers receiving the additional supplementation experienced considerable body mass losses by the end of the course (13%).

Therefore, the aim of this study was to investigate the influence of daily mixed nutritional supplementation on resting circulating leukocyte counts, bacterially-stimulated neutrophil degranulation, saliva SIgA and on circulating cortisol and IL-6 responses during an 8-week arduous training programme. It was hypothesised that reducing the daily energy deficit with nutritional supplementation would attenuate the immune modulation during arduous training.

4.3 METHODS

Participants. Thirty healthy males were recruited from two platoons of soldiers undertaking the 8-week section commanders' battle course at the Infantry Battle School, Brecon, UK. This training course is a promotion-qualifying course with successful soldiers promoted to full corporal. One platoon formed the control group (CON, *n* = 15, mean (SD): age 25 (3) years; height 179 (6) cm; body mass 81.6 (6.6) kg; body fat 16 (4)%), and the other platoon formed the supplemented group (SUP, n = 15, mean (SD): age 25 (3) years; height 177 (4) cm; body mass 80.2 (8.8) kg; body fat 15 (3)%). The groups were matched, with no significant differences between CON and SUP for the above physical characteristics.

Experimental design and procedures. A mixed model, repeated measures design was used to monitor two platoons at three time points during the 8-week training programme: at baseline (week 0), pre-field exercise (end of week 6) and post-field exercise (the day following completion of field exercise, week 8). During the first 6 weeks, soldiers resided in barracks and completed daily training excursions. This was followed by a 2-week field exercise where soldiers resided in the field and underwent more rigorous training. Soldiers completing this training programme have previously been shown to be in energy deficit resulting in body mass loss of 5.1 (2.6) kg. The estimated energy expenditure using doubly labelled water was 19.7 $MJ \cdot d^{-1}$ during weeks 0-6 and 21.3 $MJ \cdot d^{-1}$ during the field exercise (Richmond et al., 2010). In the present study, CON consumed a habitual diet alone and SUP received a habitual diet plus an additional daily mixed nutritional supplement. All soldiers were given the habitual diet of ~14.0 $MJ \cdot d^{-1}$ during in-barrack feeding between week 0 and 6 (estimated energy shortfall of 5.7 $MJ \cdot d^{-1}$) and 17.7 $MJ \cdot d^{-1}$ during the final 2-week field exercise (estimated energy shortfall of 3.6 $MJ \cdot d^{-1}$). The SUP group received an additional 5.7 $MJ \cdot d^{-1}$ during week 0-6 and 3.6 $MJ \cdot d^{-1}$ during the field exercise. These values were derived from a previous data taken during this

battle course where soldiers experienced an estimated energy deficit of 2.7 $MJ \cdot d^{-1}$ for the 8-week training programme despite self-supplementing their diet by ~2.9 $MJ \cdot d^{-1}$ (Richmond et al., 2010). Since we did not want soldiers in SUP using the nutritional supplement as an alternative to food they might normally purchase, and accounting for a small increase in food provision (0.5 $MJ \cdot d^{-1}$) we provided a nutritional supplement to cover both the estimated energy deficit (2.7 $MJ \cdot d^{-1}$) and self-supplementation (2.9 $MJ \cdot d^{-1}$), thus providing 5.1 $MJ \cdot d^{-1}$ for the 8-week training programme. To assess supplement adherence, all food wrappers and packaging that the supplement was provided in were collected daily, with any uneaten foods accurately weighed. It was assumed that CON would self-supplement their diet as previously shown. The macronutrient breakdown of the supplement was ~45% carbohydrate, ~40% fat and ~15% protein in order to match the composition of the habitual diet.

Body composition, estimation of energy deficit and physical activity. Body composition was assessed at baseline and post-field exercise using DEXA (for details see **Chapter 3, section 3.2**) and body fat mass, lean body mass, and percent body fat were calculated. Estimations of energy deficit over the 8-week training period were calculated based upon DEXA estimated changes in fat mass and lean mass as previously described (Westerterp et al., 1995). The energy densities of fat mass and lean mass were assumed to be 38 $MJ \cdot kg^{-1}$ and 6 $MJ \cdot kg^{-1}$ respectively (with lean mass comprising 73% water and 27% protein). Physical activity was monitored continuously throughout the field exercise by a tri-axial accelerometer (3DNX, BioTel, Bristol, UK) which is sensitive to body accelerations in the vertical, anterior-posterior and medial lateral directions. The accelerometer was worn around the waist and positioned at the base of the spine, as previously described (Carter et al., 2008). Body movement was recorded in one minute intervals and the data output was in the form of individual axis counts per minute, where a count of 1000 equated to an acceleration of 1 *g*. The counts from the x-, y- and z-axes were summed to produce an arbitrary 'physical activity count' value.

Blood collection and analysis. After an overnight fast, and 12 h since the last exercise bout, samples were collected between 0530 h and 0600 h at baseline, pre-field exercise and post-field exercise. Blood samples were collected by venepuncture from an antecubital vein into one K_3EDTA coated vacutainer, and one lithium heparin coated vacutainer (Becton Dickinson, Oxford, UK). For each sample taken, a 2 ml aliquot of K_3EDTA whole blood was kept at room temperature prior to haematological analysis. Haematological analysis of circulating total and differential leukocyte counts was performed using an automated cell counter (Gen-S, Beckman Coulter, High Wycombe, UK). A 1 ml aliquot of lithium heparin whole blood was used to determine bacterially-stimulated neutrophil release. The remaining K_3EDTA and lithium heparin treated blood was centrifuged at 1500 *g* for 10 minutes in a refrigerated centrifuge. Plasma was aliquoted into Eppendorf tubes, and immediately frozen at -40°C for later analysis.

Bacterially-stimulated neutrophil degranulation. A 1 ml aliquot of lithium heparin whole blood was added to 50 µl of bacterial stimulant solution (Stimulant, Sigma, Poole, UK) and gently vortex-mixed. Samples were immediately incubated for 60 minutes at 37°C, with gentle inversion at 30 minutes, as previously described (Laing et al., 2008a; Robson et al., 1999). After incubation, samples were centrifuged at 5000 *g* for 2 minutes and the supernatant was frozen at -40°C for later analysis. Bacterially-stimulated and unstimulated lithium heparin plasma samples were analysed for concentrations of elastase using a high sensitivity ELISA kit (Biovendor, Heidelberg, Germany). The unstimulated elastase concentration was subtracted from the bacterially-stimulated elastase concentration and corrected for the individual neutrophil count to determine neutrophil degranulation (elastase release per neutrophil). The intra-assay coefficient of variation for elastase concentration was 5.0%.

Plasma IL-6 and cortisol concentration. Plasma IL-6 concentration was determined on K_3EDTA plasma using a commercially available high sensitivity IL-6 ELISA (RnD systems,

Minneapolis, USA). Aliquots of lithium heparin plasma were used to determine cortisol concentration by ELISA according to the manufacturer's instructions (DRG Instruments, Marburg, Germany). The intra-assay coefficient of variation for plasma IL-6 and cortisol was 5.1% and 1.9%, respectively.

Saliva collection and analysis. Unstimulated whole saliva samples were collected from participants using a passive dribble for 5 minutes into a pre-weighed universal tube (HR 120-EC, A & D instruments, Tokyo, Japan) between 0530 h and 0600 h at baseline, pre-field exercise and post-field exercise (Costa et al., 2010). All saliva samples were collected while the participant sat quietly after first rinsing their mouth with water and swallowing. Saliva collections were performed with minimal orofacial movements. Saliva volume was measured by weighing the samples to the nearest milligram immediately after collection. Saliva flow rate was determined by dividing the volume collected, by the time of collection, and assuming the density of saliva to be 1.00 $g \cdot ml^{-1}$ (Cole and Eastoe, 1988). The samples were frozen immediately at -40°C. Saliva samples were analysed for SIgA concentration using a commercially available competitive ELISA (Salimetrics, Pennsylvania, USA). Salivary SIgA secretion rate was calculated by multiplying the SIgA concentration by the saliva flow rate. The intra-assay coefficient of variation was 3.0%.

Statistical analysis. The required sample size was estimated to be 14 participants per group using data for total leukocyte counts from a previous military training study (Whitham et al., 2006) and alpha (Type I error rate) set at 0.05, and power at 0.85 (1 - Type II error rate) (G*Power software, version 3.1.2). Independent sample *t*-tests were used to assess differences between the two groups in: demographic data; physical activity; body mass loss; lean body mass loss; fat mass loss and estimated energy deficit. A two-way mixed model ANOVA (group × time) was used to compare all other data.

4.4 RESULTS

Physical activity, body composition and estimated energy deficit

There was no difference between CON and SUP for physical activity counts during the field exercise (CON 130 (18); SUP 133 (19) 10^3). Body mass decreased significantly in both CON and SUP throughout the 8-week training programme (interaction $F(1,28) = 21.3$, $P < 0.01$). However, body mass loss (t (28) 4.6, $P < 0.01$), lean mass loss (t (28) 2.6, $P < 0.05$) and fat mass loss (t (28) 3.4, $P < 0.01$) were all significantly greater in the CON group over the 8-week training period (Table 4.1). By collecting all food wrappers and weighing uneaten supplement product, it was estimated that the supplement group consumed on average 66 (13)% of the daily mixed supplement (64% during weeks 0-6, and 77% during weeks 6-8). Estimated daily energy deficit during the training programme was significantly greater in the CON group compared with the SUP group (t (28) 3.8, $P < 0.01$).

Table 4.1. Body mass loss, lean mass loss, fat mass loss and estimated daily energy deficit response to an 8-week military training programme where soldiers consumed either a habitual diet alone (CON) or a habitual diet plus a daily mixed nutritional supplement (SUP).

	CON	SUP
Body mass loss (kg)	5.0 (2.3)	1.6 (1.5) $^{\#\#}$
Lean mass loss (kg)	2.0 (1.5)	0.7 (1.5)$^{\#}$
Fat mass loss (kg)	3.0 (1.6)	0.9 (1.8)$^{\#\#}$
Estimated energy deficit ($MJ \cdot d^{-1}$)[a]	2.2 (1.1)	0.7 (1.1)$^{\#\#}$

Data are mean (SD). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus CON.

[a] Based upon changes in fat mass and lean mass as described (Westerterp et al., 1995).

Circulating leukocyte, lymphocyte and monocyte counts

A significant trial x time interaction was observed for circulating leukocyte ($F(1.6,43.5) = 3.7$, $P < 0.05$; Figure 4.1a), lymphocyte ($F(2,56) = 3.4$, $P < 0.05$; Figure 4.1c) and monocyte counts ($F(2,56) = 4.9$, $P < 0.05$; Figure 4.1d), but no significant interaction or main effects were observed for neutrophil count (Figure 4.1b). In the CON group, circulating leukocyte, lymphocyte and monocyte counts decreased significantly post-field exercise compared with baseline ($P < 0.01$) and lymphocyte counts were also significantly lower at post-field exercise compared with pre-field exercise ($P < 0.01$) and monocyte counts were lower at pre-field exercise compared with baseline ($P < 0.05$) The addition of a daily mixed nutritional supplement prevented the decrease in circulating leukocyte ($P < 0.01$), lymphocyte ($P < 0.01$) and monocyte ($P < 0.05$) counts at post-field exercise compared with CON.

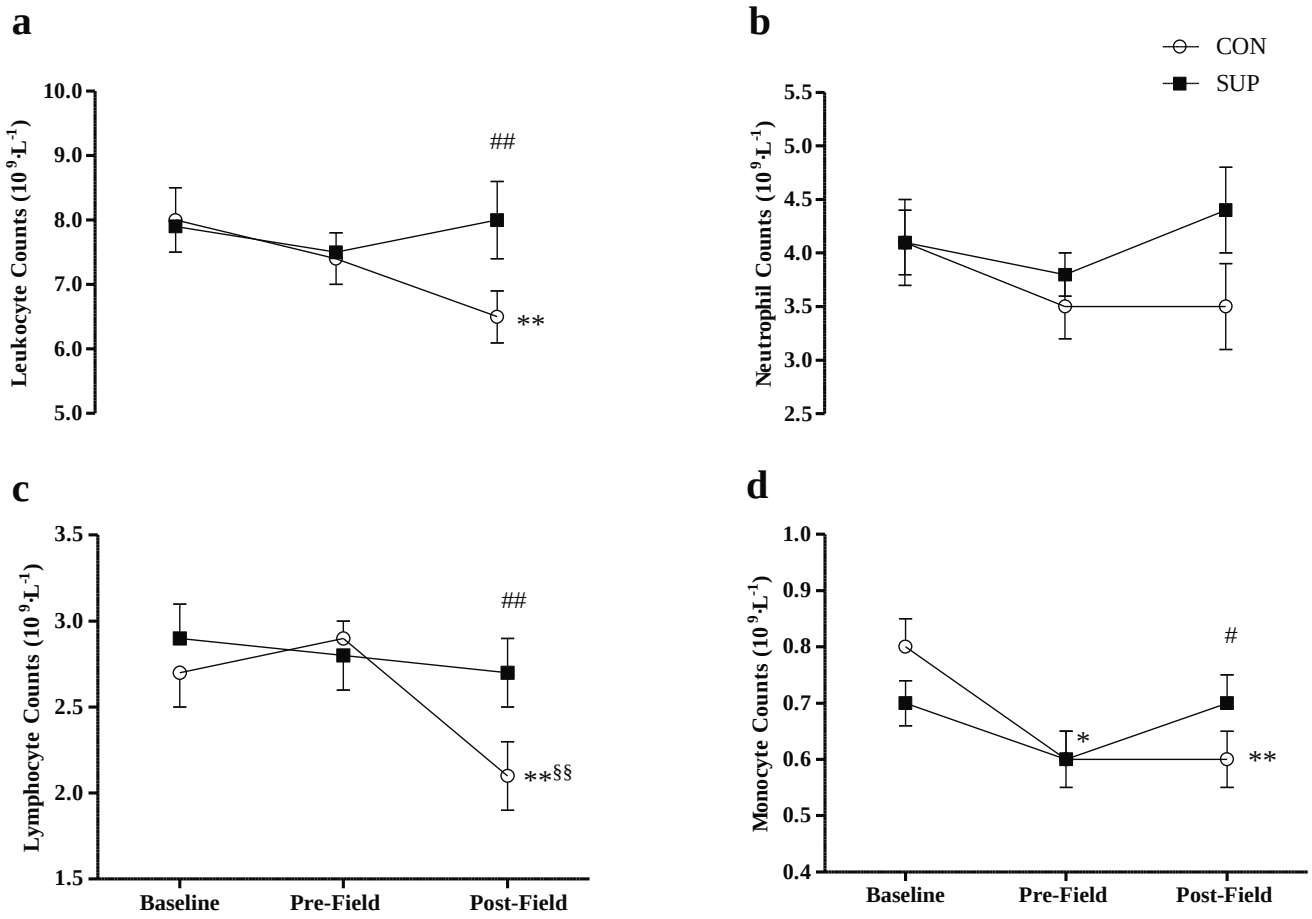


Figure 4.1. Circulating total leukocyte (a), neutrophil (b), lymphocyte (c) and monocyte (d) count response to an 8 week military training programme where soldiers consumed either a habitual diet alone (CON), or a habitual diet plus a daily mixed nutritional supplement (SUP). Data are mean (SEM): * $P < 0.05$ and ** $P < 0.01$ *vs*. Baseline, §§ $P < 0.01$ *vs*. Pre-field exercise; # $P < 0.05$ and ## $P < 0.01$ *vs*. CON.

Bacterially stimulated neutrophil degranulation

There was no significant trial × time interaction or main effect for time or trial for unstimulated plasma elastase concentration. A significant trial x time interaction was observed for bacterially-stimulated neutrophil degranulation (elastase release per neutrophil; $F(1.6,56) = 5.8$, $P < 0.01$). Neutrophil degranulation was not significantly different between CON and SUP at baseline or post-field exercise. However, at pre-field exercise, neutrophil degranulation increased significantly on CON (*vs*. baseline: $P < 0.01$) and was greater than SUP ($P < 0.01$)

at this time (baseline, 529 (112); pre-field exercise, 675 (175); post-field exercise, 557 (134) fg·cell^{-1}). Neutrophil degranulation remained unchanged in the SUP group (baseline, 553 (128); pre-field exercise, 531 (130); and post-field exercise, 534 (156) fg·cell^{-1}).

Plasma IL-6 and cortisol concentration

There was no trial × time interaction for plasma IL-6 concentration. However, a significant main effect of time was observed ($F(2,52) = 5.9$, $P < 0.01$). Plasma IL-6 concentration for both groups pooled was significantly greater at post-field exercise (0.97 (0.59) pg·ml^{-1}) than at baseline (0.68 (0.35) pg·ml^{-1}) and pre-field exercise (0.60 (0.58) pg·ml^{-1}) ($P < 0.05$). No trial x time interaction was observed for plasma cortisol concentration. However, there was a main effect of time ($F(2,56) = 5.1$, $P < 0.01$). Plasma cortisol concentration was 519 (104) nmol·l^{-1} at baseline and increased from pre-field exercise (494 (85) nmol·l^{-1}) to post-field exercise (548 (83) nmol·l^{-1}; $P < 0.01$) in the whole cohort (n = 30).

Saliva SIgA responses

A main effect of time was observed for saliva flow rate ($F(1.2,31.8) = 5.0$, $P < 0.05$; Figure 4.2a) and saliva SIgA concentration ($F(2,54) = 8.46$, $P < 0.01$; Figure 4.2b). Saliva flow rate was significantly lower at post-field exercise than at baseline ($P < 0.05$), and saliva SIgA concentration was significantly greater at pre-field exercise and post-field exercise than at baseline ($P < 0.01$). A trial × time interaction was observed for saliva SIgA secretion rate ($F(2,54) = 3.2$, $P < 0.05$; Figure 4.2c), which was significantly greater at post-field exercise in the SUP group compared with the CON group ($P < 0.01$). Furthermore, within the SUP group saliva SIgA secretion rate significantly increased from baseline to pre-field exercise ($P < 0.05$) and from baseline to post-field exercise ($P < 0.05$).

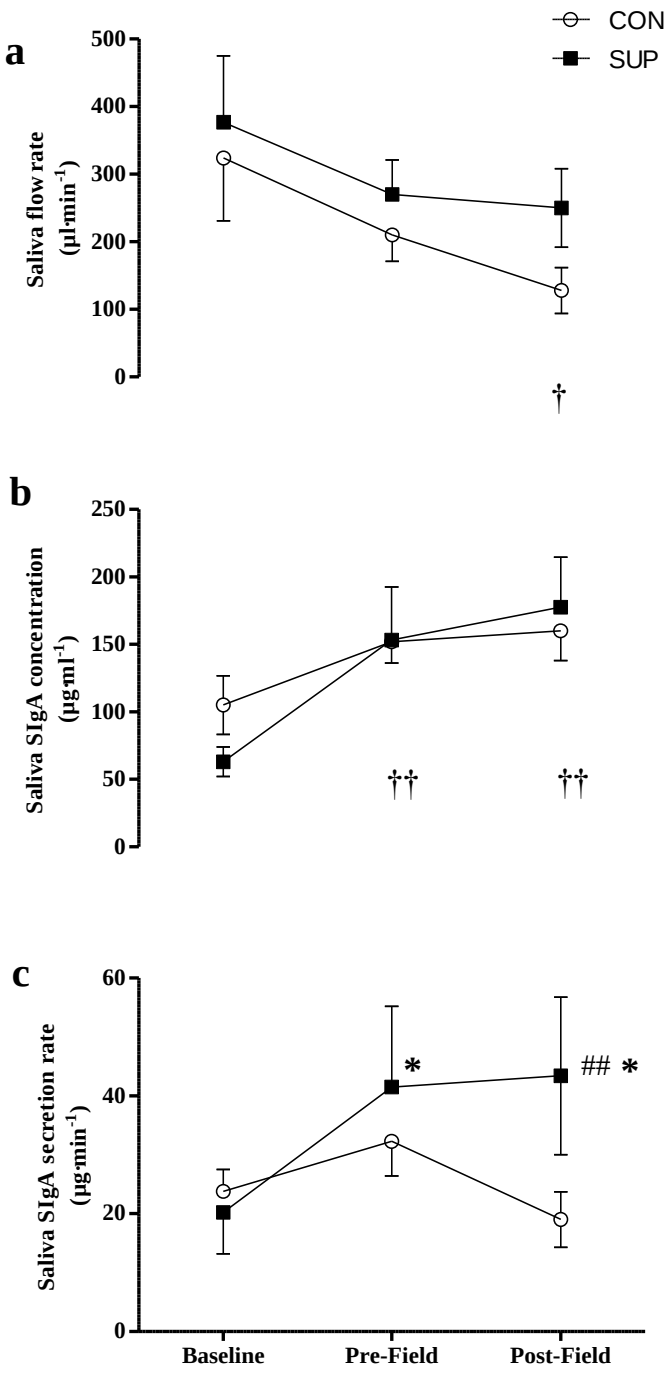


Figure 4.2. Saliva flow rate (a), saliva SIgA concentration (b) and saliva SIgA secretion rate (c) responses to an 8 week military training programme where soldiers consumed either a habitual diet alone (CON), or a habitual diet plus a daily mixed nutritional supplement (SUP). Data are mean (SEM): † $P < 0.05$ and †† $P < 0.01$ *vs*. Baseline (main effect of time); * $P < 0.05$ *vs*. Baseline; ## $P < 0.01$ *vs*. CON.

4.5 DISCUSSION

The aim of the present study was to determine the effect of daily mixed nutritional supplementation on resting immune indices during an 8-week arduous training programme. Compared with many previous field studies, particular strengths of the present study were the inclusion of a comparative control group and standardisation of both the time of day that samples were collected and the time between the last exercise bout and sample collection. In line with our hypothesis, daily mixed nutritional supplementation prevented the training-induced decrease in circulating leukocyte, lymphocyte and monocyte counts. Particularly novel, was our finding that nutritional supplementation increased saliva SIgA secretion rate during training: this finding can be considered favourable for host defence. However, nutritional supplementation did not significantly alter circulating neutrophil counts, plasma IL-6 or cortisol concentration. These data indicate that daily mixed nutritional supplementation can modulate selected immune indices during training in the absence of a significant treatment effect on circulating cortisol.

Soldiers completing the 8-week training course experienced an average energy deficit in the CON group of 2.2 $MJ \cdot d^{-1}$ which was smaller in the SUP group (0.7 $MJ \cdot d^{-1}$). Soldiers in SUP were provided with the same basal diet as CON, with, on average, an additional 5.1 $MJ \cdot d^{-1}$ provided as a daily supplement during training. We assumed, based upon previous findings (Richmond et al., 2010), that self-supplementation by CON (~2.9 $MJ \cdot d^{-1}$) would lead to a theoretical difference in energy deficit between the two groups of ~2.2 $MJ \cdot d^{-1}$. The difference in estimated energy deficit between CON and SUP was 1.5 $MJ \cdot d^{-1}$: this is likely explained by the finding that SUP consumed on average only 66% of the supplement. The resulting body mass losses (6% for CON and 2% for SUP) are relatively modest compared with those reported in other military field studies (Friedl et al., 1994; Kramer et al., 1997; Nindl et al., 2007). For

example, soldiers experienced ~16% body mass losses during a 2-month army ranger course where the energy deficit was 4.9 $MJ \cdot d^{-1}$ (Kramer et al., 1997). The modest energy restriction experienced by soldiers in this study likely explains the limited effect observed on resting circulating cortisol and IL-6 concentration. It is likely that a more severe energy deficit is required to substantially increase circulating cortisol and IL-6. In support of this contention is the finding of one study that showed a 32% increase in circulating cortisol when soldiers trained with a 16 $MJ \cdot d^{-1}$ energy deficit but a return to baseline levels when soldiers subsequently trained with a similar energy deficit to the present study (~2 $MJ \cdot d^{-1}$) (Kyrolainen et al., 2008). Given the widely accepted role for stress hormones in mediating immune alterations with heavy training (Pedersen and Hoffman-Goetz, 2000) and the absence of a significant effect of nutritional supplementation on circulating cortisol, we might anticipate no significant effect of nutritional supplementation on the various immune indices measured here. In contrast, significant training-induced decreases in circulating total leukocyte, lymphocyte and monocyte counts were observed and this was prevented with nutritional supplementation sufficient to offset two-thirds of the estimated energy deficit. The mechanism(s) responsible for altered leukocyte trafficking with heavy training, and the modulatory effect of nutritional supplementation remain unclear. Possible explanations for training-induced decreases in circulating leukocytes include, but are not limited to, decreased bone marrow production, increased apoptosis and plasma volume expansion. That offsetting some of the energy deficit prevented the training-induced decrease in circulating leukocytes points to the interesting possibility that a modest energy deficit, as commonly experienced by an athlete or soldier, may account, at least in part, for the lower circulating leukocyte counts in those under heavy training (Horn et al., 2010). The mechanism(s) responsible for this observation (e.g. energy deficit *per se* or specific macronutrient or micronutrient deficit) remains unclear. Nevertheless, current thinking indicates that the modest influence of training on circulating leukocyte numbers that we, and others, have observed (i.e. decrease within the normal clinical range: 4.5-11 x $10^{9} l^{-1}$),

reflects an adaptive response that is not pathological (Horn et al., 2010). Athletes engaged in heavy training typically have lower circulating and differential leukocyte counts than their sedentary counterparts when blood samples are collected in the morning following an overnight rest (Horn et al., 2010). In the present study, soldiers underwent 6 weeks of light skill acquisition training prior to baseline sampling. This may account for the higher baseline circulating total leukocyte count in soldiers undertaking light training (7.9 x $10^{9}l^{-1}$) than previously reported for endurance athletes undertaking heavy training (e.g. Triathletes: 5.9 x $10^{9}l^{-1}$) (Horn et al., 2010). In addition, its noteworthy that the mean baseline circulating total leukocyte count of 7.9 x $10^{9}l^{-1}$ falls in the middle of the normal clinical range and is comparable with rested soldiers reported elsewhere (Brenner et al., 2000; Whitham et al., 2006). Although similar bacterially-stimulated neutrophil degranulation before and after training in CON and at post-field exercise in CON and SUP was observed, it remains unclear why an increase in neutrophil degranulation at pre-field exercise was shown in the CON group.

Some studies have observed decreased saliva SIgA concentration (Gomez-Merino et al., 2003; Tiollier et al., 2005) and increased URI symptoms (Tiollier et al., 2005) during periods of heavy training in soldiers experiencing a range of stressors including, amongst others, notable energy deficit. Potential explanations for lower saliva SIgA during energy deficit include decreased plasma cell IgA synthesis (Revillard and Cozon, 1990) and decreased availability of polymeric Ig receptor (Ha and Woodward, 1998) albeit the latter was shown in intestinal mucous secretions in a mouse model of protein-energy malnutrition. Nevertheless, there is some evidence in malnourished children that free secretory component is decreased in secretions such as tear fluid: secretory component is the extracellular fragment of the polymeric Ig receptor responsible for transport of dimeric IgA into saliva (Watson et al., 1986; Woof and Kerr, 2006). A strength of the present study is that saliva SIgA is presented as both

concentration and secretion rate: by considering flow rate and SIgA concentration, saliva SIgA secretion rate reflects the total availability of SIgA at the oral surface (Walsh et al., 1999). As a significant decrease in either saliva SIgA concentration or secretion rate was not observed, these results indicate that mucosal immunity was preserved despite the modest energy deficit during training (~2.2 $MJ \cdot d^{-1}$). Previous work indicates that saliva SIgA availability may have been compromised in the present study had the soldiers trained with a more severe energy deficit (~11 $MJ \cdot d^{-1}$ in the previous study) particularly when accompanied by fluid restriction (Oliver et al., 2007). The particularly novel finding in the present study is the increase in saliva SIgA secretion rate during training in soldiers who received daily mixed nutritional supplements (Figure 4.2c). The observed increase in saliva SIgA output in the SUP group might, in small part, be explained by the trend for a smaller decrease in saliva flow rate during training in SUP than CON (-31% *vs*. -61% for post-field exercise *vs*. baseline). We speculate that hydration status was better maintained in the SUP group and that this accounts for the smaller decrease in saliva flow rate compared with CON (Walsh et al., 2004a; Walsh et al., 2004b). This contention is supported by lower plasma osmolality (SUP: 289 (3) and CON: 293 (4) $mOsmol \cdot kg^{-1}$) and urine colour (SUP: 3 (1) and CON: 5 (1) using 1-8 scale (Armstrong et al., 1994)) at post-field exercise in CON: these hydration assessments were made to achieve other study objectives. Alternative reasons for the increase in saliva SIgA output from baseline to post-field exercise in the SUP group, such as increased IgA synthesis and transport into the saliva, are difficult to explain, particularly given that this group experienced small, but significant, energy deficit and a similar cortisol response to the CON group during training. To summarise, the novel finding that saliva SIgA output increased during training in soldiers who received daily mixed nutritional supplementation, sufficient to offset two-thirds of the estimated energy deficit, might be favourable for immune health, particularly, when the soldier is exposed to more severe stressors known to compromise host defence.

It is recognised that a limitation of the current study is that we were unable to record daily URI symptoms during or shortly after the training programme: however, the validity of self-reporting URI has recently come under scrutiny (Cox et al., 2008; Walsh et al., 2011b). It is also acknowledged that the collection of blood samples 12 h after the last exercise bout in the current study, may not represent a truly rested sample, given that previous research has shown intensive and prolonged exercise can temporarily suppress many components of the immune system for ~3-24 h later (Walsh et al., 2011b). Nevertheless, both groups experienced the same experimental conditions and timings, which were dictated by the military training programme. Future training studies should assess the influence of similar feeding regimens on the responses to a clinically relevant *in vivo* immune challenge e.g. using DTH (Bruunsgaard et al., 1997) or experimental CHS tests (Harper Smith et al., 2011). These *in vivo* measures are considered more informative than commonly used *in vitro* measure of immunity because they assess an orchestrated response, within the tissue specific environment that immune responses usually take place (Akbar et al., 2013). It was our original intention to include the measure of experimental CHS with DPCP in this study, but unfortunately this was not possible at the time because the work from our group, which provides further support for the use of this measure in experimental stress immunology, had not been published yet (Harper Smith et al., 2011; Oliver et al., 2013). In conclusion, daily mixed nutritional supplementation during an 8-week arduous training programme prevented the decrease in circulating leukocyte, lymphocyte and monocyte counts and increased saliva SIgA secretion rate. Alterations in these immune indices occurred despite no effect of daily mixed nutritional supplementation on circulating cortisol concentration.

CHAPTER FIVE

Using Diphenylcyclopropenone as a tool to assess *in vivo* immunity: influence of exercise intensity and duration

5.1 SUMMARY

In vivo immune measures such as experimental CHS with the novel antigen DPCP, provide a robust, non-invasive means to assess a highly orchestrated, multi-cellular response, within the tissue specific environment that immune responses usually occur. It has recently been shown that 2 h of exercise significantly reduces the *in vivo* immune responses to DPCP but the impact of the intensity and duration of exercise stress on this measure and the underlying mechanisms for the exercise-induced alterations remains unknown. The purpose of the present study was to examine the effects of exercise intensity and duration on induction of *in vivo* immunity using DPCP. Sixty four healthy males completed either 120 minutes of moderate intensity (60% $\dot{V}O_{2peak}$) exercise (120MI, n = 16), 30 minutes of moderate intensity exercise (30MI, n = 16), 30 minutes of high intensity (80% $\dot{V}O_{2peak}$) exercise (30HI, n = 16), or 120 minutes of seated rest (CON, n = 16). Twenty minutes later, participants received a sensitising dose of DPCP and 4 weeks later they received a low, dose-series challenge with DPCP on the inner aspect of their upper arm. This was read at 48 h as oedema (skinfold and dermal thickness) and redness (erythema). Circulating adrenaline, noradrenaline and cortisol were measured pre, post and 1 h post-exercise or CON. Results show that 120MI impaired immune induction by DPCP (skinfold thickness -67% *vs.* CON; $P < 0.05$). However, immune induction was unaffected by 30MI and 30HI despite elevated circulating catecholamines (30HI *vs.* pre: $P < 0.01$) and greater circulating cortisol post 30HI (*vs.* CON: $P < 0.01$). Prolonged, moderate intensity exercise, but not short-lasting high or short-lasting moderate intensity exercise, deceases the induction of *in vivo* immunity in healthy humans. Further research is required to better understand the potential mechanisms involved in the reductive effect of 120MI on *in vivo* immunity.

5.2 INTRODUCTION

It has recently been highlighted that there is a need to better understand clinically relevant, biologically sensitive and practical *in vivo* immune measures to assess the influence of stress on immunity in humans (Albers et al., 2013; Calder et al., 2013). *In vivo* measures which challenge participants with an antigenic or pathogenic challenge are considered to be more informative than the commonly used *in vitro* measures where immune cells are extracted from their normal *in vivo* environment and analysed in artificial cultures, without the highly integrated neural and hormonal factors within the tissue specific environment that immune responses usually take place (Akbar et al., 2013; Albers et al., 2013).

Physical exercise provides a well-controlled model to study the effects of stress on immune responses. Given the obvious concerns associated with purposefully inducing infections in humans, animal models have provided valuable insight into the effects of exercise on clinically relevant responses to viral infection. The work in animals indicates that prolonged and high intensity exercise is associated with higher mortality rates whereas short, moderate intensity exercise lowers mortality rates, compared with controls (Martin et al., 2009). The research evidence on immune responses after short, moderate intensity exercise in humans is not definitive and tends to indicate immune enhancement only in individuals with sub-optimal immune status (Edwards et al., 2012; Pascoe et al., 2014). Work in humans indicating that a single bout of short duration, high intensity exercise and prolonged duration, moderate intensity exercise decreases immunity, is largely based upon results of studies examining *in vitro* immune measures (Nieman et al., 1994; Robson et al., 1999). A limited number of studies have investigated the impact of a single bout of exercise on cutaneous *in vivo* measures of immunity in humans. One such study showed that after an acute bout of prolonged, continuous exercise (lasting 6.5 h), DTH reactions to common recall antigens in the Mérieux CMI Multitest® were reduced but this test is no longer commercially available (Bruunsgaard et al., 1997). Moreover,

the use of previously encountered antigens does not permit the assessment of the effect of stress on the induction of new immune memory and findings may be confounded by the lack of control over the prior immunological memory, thus inducing secondary or tertiary responses. Alternatively, the use of novel antigens such as KLH or DPCP, allows for the measurement of a primary induction response and allows rigorous control of both the dose and timing of sensitisation (Harper Smith et al., 2011; Smith et al., 2004c). It has recently been shown that 2 h of moderate intensity exercise decreases *in vivo* immune function when assessed by the less invasive, epicutaneous application of DPCP (Harper Smith et al., 2011). However, the impact of the intensity and duration of continuous exercise stress on *in vivo* immunity in humans and the underlying mechanisms for exercise-induced immune alterations remains unknown.

Alterations in the HPA axis and sympatho-adrenomedullary system have been implicated in the modulation of immune function (Dhabhar, 2013). Prolonged stress or high intensity stress that activates the HPA axis may in-turn increase glucocorticoids and catecholamines previously shown to decrease the induction of CHS in mice (Dhabhar, 2013; Flint et al., 2001; Seiffert et al., 2002). However, the role of stress hormones in the exercise-induced changes in CHS responses to DPCP in humans has not been investigated (Harper Smith et al., 2011).

At present, further investigation is required to compare the impact of the intensity and duration of continuous exercise stress on the induction of *in vivo* immunity in humans. In addition, it remains unclear whether stress hormone responses may provide a physiological rationale for exercise-induced alterations in experimental CHS. Therefore the aim of the present study was to examine the effect of exercise intensity (30 minutes at 80% $\dot{V}O_{2peak}$ or 30 minutes at 60% $\dot{V}O_{2peak}$) and duration (120 minutes at 60% $\dot{V}O_{2peak}$) on the induction of *in vivo* immune responses to a novel antigen DPCP, using an experimental CHS method. It was hypothesised

that prolonged, moderate intensity exercise and short, high intensity exercise, which alters circulating glucocorticoids and catecholamines, would suppress the CHS responses to DPCP compared with short, moderate intensity exercise and seated rest.

5.3 METHODS

Participants. Sixty four healthy recreationally active males agreed to participate in the study. Participants were required to abstain from caffeine, alcohol, and unaccustomed exercise for 24 h before and 48 h after the experimental trials.

Preliminary measures and familiarization. Anthropometric measures were recorded on arrival to the laboratory and $\dot{V}O_{2peak}$ was estimated by means of a ramped exercise test on a treadmill (see **Chapter 3, section 3.3** for details). A speed equivalent to 60% or 80% of the $\dot{V}O_{2peak}$ was calculated, with account taken for the mean response time for $\dot{V}O_2$ during ramp exercise (Whipp et al., 1981). The gas exchange threshold (GET) was also determined from the ramped exercise test to ensure the exercise intensity domain was standardised between participants. This was deemed appropriate for a cross-sectional study of exercise intensity and duration because it has been shown that participants with similar $\dot{V}O_{2peak}$ values, exercising at a set percentage of their $\dot{V}O_{2peak,}$ reach fatigue at significantly different times, which was related to their individual lactate threshold or GET (Coyle et al., 1988). The GET in the current study was estimated using the V-slope method (Beaver et al., 1986).

At least 24 h after the preliminary test each participant's calculated exercise intensity was verified by running for 50% of their allocated exercise duration and all participants were familiarised with laboratory equipment. This was undertaken to ensure that the participants were accustomed to the procedures used during the investigation.

Study design. In a cross-sectional design, participants were matched for age and aerobic fitness (GET and $\dot{V}O_{2peak}$) before being randomly assigned to one of four experimental groups: *1*) 120 minutes moderate intensity (60% $\dot{V}O_{2peak}$) exercise (120MI); *2*) 30 minutes moderate intensity

(60% $\dot{V}O_{2peak}$) exercise (30MI); *3*) 30 minutes high intensity (80% $\dot{V}O_{2peak}$) exercise (30HI); or *4*) 120 minutes of seated rest (CON). These exercise intensities and durations were chosen to allow comparison with the relevant literature (Harper Smith et al., 2011), to assess the *in vivo* immune response to exercise recommended to healthy adults for fitness and health (e.g. the ACSM recommends 30 minutes, moderate intensity exercise on most days), to best separate intensity and duration effects on *in vivo* immunity; and finally, with feasibility in mind (e.g. our participants could complete 30 minutes at 80% $\dot{V}O_{2peak}$). There were no significant differences between groups for selected physical and anthropometric characteristics (Table 5.1). After the main experimental trial all participants received the same sensitizing dose of DPCP and four weeks later the strength of immune memory induction was quantified by measuring the immune responses elicited by simultaneous topical challenge with a low concentration, dose-series of DPCP (Figure 5.1).

Table 5.1. Participant characteristics. Data are mean (SD).

	CON	120MI	30MI	30HI
Age (years)	23 (4)	22 (4)	20 (2)	22 (4)
Height (cm)	180 (7)	180 (7)	180 (5)	179 (7)
Body mass (kg)	77.3 (11.3)	78.8 (12.1)	74.5 (10.1)	76.3 (12.8)
Body fat (%)	15 (3)	16 (4)	15 (5)	15 (5)
$\dot{V}O_{2peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	57 (7)	56 (5)	58 (5)	58 (6)
GET ($L \cdot min^{-1}$)	3.04 (0.31)	3.11 (0.51)	3.09 (0.59)	3.08 (0.60)
Weekly exercise (h)	6 (4)	6 (3)	6 (2)	5 (2)

GET, gas exchange threshold

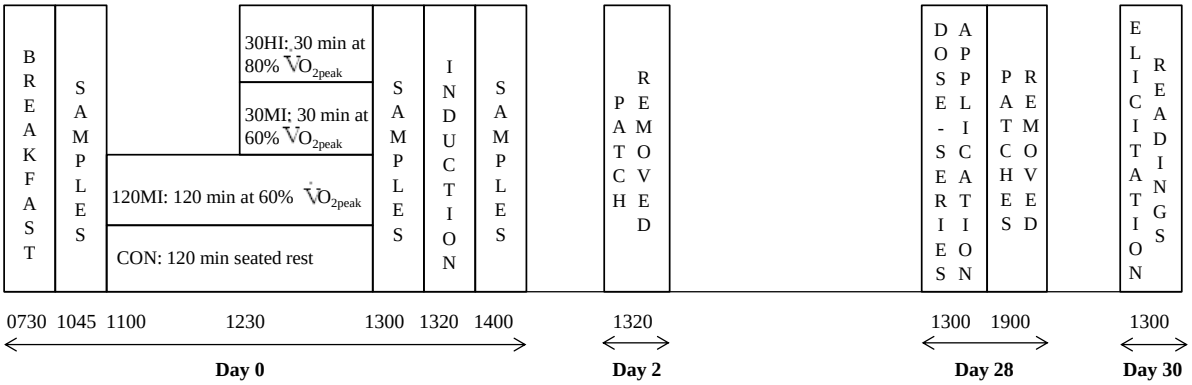


Figure 5.1. Schematic of experimental trial events. Samples: venepuncture blood.

Experimental procedures. Dietary intake was controlled for during the 24 h before the main experimental trial by providing participants with their estimated daily energy requirement using DEXA determined fat free mass as described (mean (SD): 11.2 (1.1) MJ·day^{-1}) (Cunningham, 1991) multiplied by a physical activity factor (World Health Organization, 1985). Fluid was provided proportional to 35 ml·kg^{-1}·d^{-1} body mass (Todorovic and Micklewright, 2004).

Within 3 weeks of the preliminary testing, on the day of the experimental trial, all participants were transported to the laboratory at 0730 h and provided with a standard breakfast (0.03 MJ·kg^{-1}, which was comprised of 11%, 46% and 43% of energy from protein, carbohydrate and fat respectively). Participants were permitted to perform light activity before commencing the intervention. Nude body mass (NBM) was recorded before and after exercise on a digital platform scale to determine water allowance (Model 705; Seca, Hamburg, Germany). Exercising participants received 5 ml·kg^{-1}NBM of water immediately before and after the exercise, and 2 ml·kg^{-1}NBM at 15 minute intervals throughout, and any additional exercise fluid loss was replaced following exercise. Participants assigned to the 120MI began running on a treadmill at 1100 h and those assigned to 30HI and 30MI began at 1230 h, so that all participants completed the exercise at the same time of day (1300 h; Figure 5.1). Immediately after the exercise, participants showered and returned to the laboratory within 15 minutes 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 fluid intake proportional to 35 $ml \cdot kg^{-1} \cdot d^{-1}$ body mass. The CON participants did not shower after completion on the seated rest.

Induction of Contact Sensitivity. Participants were sensitised to DPCP at 1320 h, exactly 20 minutes after exercise cessation or equivalent seated rest, as described previously (Harper Smith et al., 2011; **Chapter 3, section 3.4**). For this study, the sensitising patch was placed on the lower back and left in place for exactly 48h.

Elicitation. The magnitude of *in vivo* antigen-specific immune responsiveness was quantified by measuring the responses elicited by secondary exposure to the same antigen (Figure 5.1). Exactly four weeks after the initial sensitisation to DPCP, all participants received a challenge with a low concentration, 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 inner aspect of the upper arm in the following concentrations: 10 µl of DPCP: 0.0048%, 1.24 $\mu g \cdot cm^{-2}$; 0.0076%, 1.98 $\mu g \cdot cm^{-2}$; 0.012%, 3.17 $\mu g \cdot cm^{-2}$; 0.0195%, 5.08 $\mu g \cdot cm^{-2}$; 0.0313%, 8.12 $\mu g \cdot cm^{-2}$ and 10 µl 100% acetone control patch for background subtraction. Patches were applied in a randomly allocated order at the local site to minimise any anatomical variability in responses. Elicitation patches were removed after exactly 6 h and the strength of immune reactivity was measured as oedema (skinfold thickness and dermal thickness) and redness (skin erythema) at 48 h post-application.

Assessment of CHS responses.

Skinfold thickness. Skin oedema (inflammatory swelling) is considered the key measure of

CHS elicitation responses (Harper Smith et al., 2011). This was assessed as mean skinfold thickness from triplicate measurements at each elicitation site using modified spring-loaded skin callipers (Harpenden Skinfold Calliper, British Indicators, England), as described (Harper Smith et al., 2011). Skinfold 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).

Dermal thickness. Dermal thickness was determined at each elicitation site using a high-frequency ultrasound scanner (Episcan, Longport Inc., Reading, UK). The ultrasound probe was placed over the centre of each elicitation site together with ultrasound gel. The mean of three measurements was taken from each 12 mm scan image by an independent investigator, who was blinded to the trial assignment. Due to a delay in the availability of this equipment, dermal thickness was assessed in a subpopulation of 50 participants (CON = 13, 120MI = 11, 30MI = 14, 30HI = 12). An example of typical scan images from a CON participant is shown in Figure 5.2.

Skin erythema. Skin erythema, an objective measure of skin redness, was determined from triplicate measurement at each elicitation site using an erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark) as previously described (Harper Smith et al., 2011). Mean background values were determined from triplicate measurements at the acetone patch site for thickness and redness. In order to determine the increase in thickness and redness in response to DPCP, the value from the acetone-only site was subtracted from each elicitation site value. The values for increase in skinfold thickness, dermal thickness and erythema 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 (Harper Smith et al., 2011; Palmer and Friedmann, 2004).

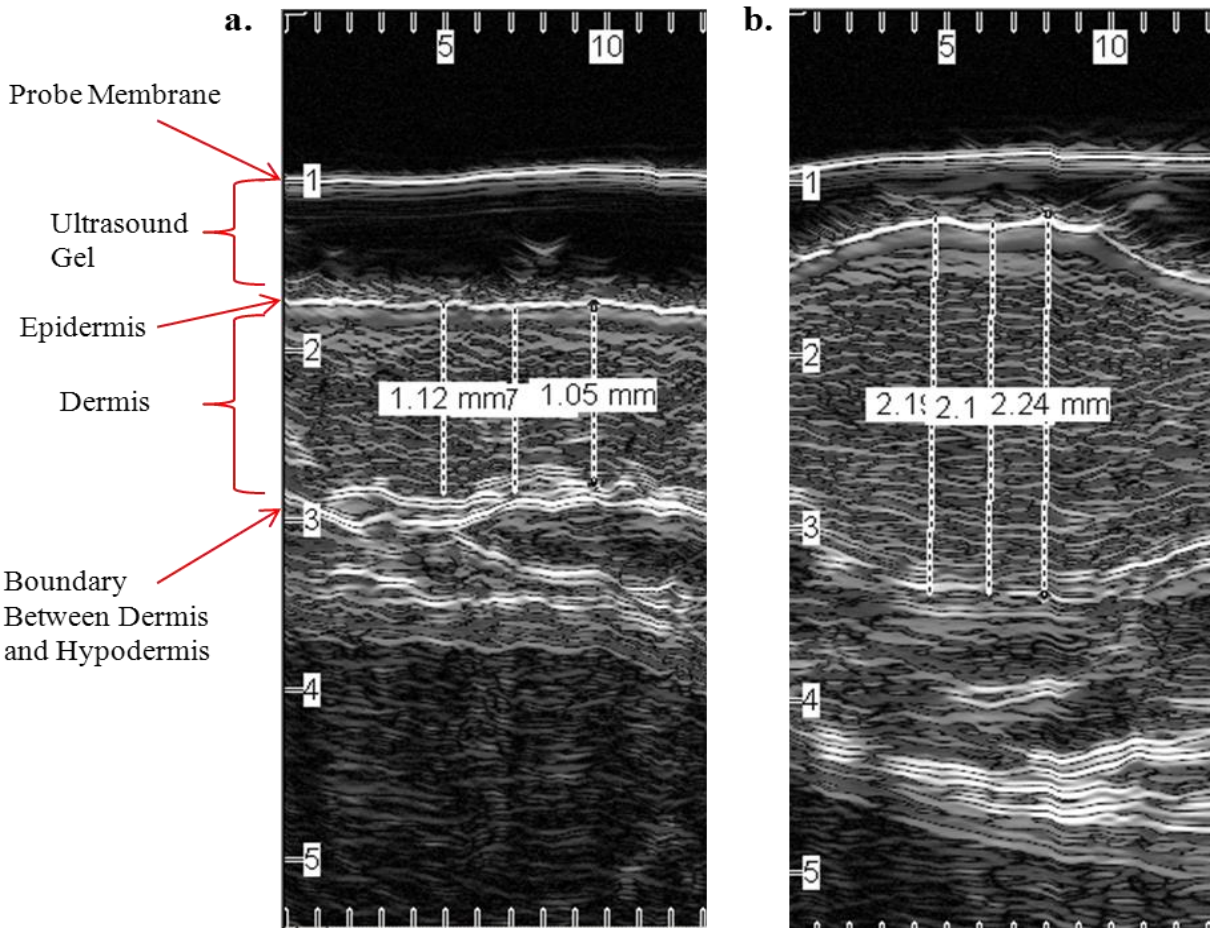


Figure 5.2. Typical high-frequency ultrasound scan images 48 h after elicitation at an acetone site (a) and a 0.0313% DPCP site (b) in a CON participant. The numbers across the horizontal axis are the distance across the skin surface (mm). The vertical lines indicate dermal thickness (mm).

Blood collection and analysis. Blood samples were collected at pre, immediately post exercise and 1 h post exercise by venepuncture of an antecubital vein into one K_3EDTA coated vacutainer, and one lithium heparin coated vacutainer (Becton Dickinson, Oxford, UK). The samples were spun at 1500 *g* for 10 minutes in a refrigerated centrifuge. Plasma was aliquoted into Eppendorf tubes, and immediately frozen at -80°C for later analysis.

Plasma adrenaline and noradrenaline concentrations were determined on K_3EDTA plasma using a commercially available CatCombi ELISA (IBL International, Hamburg, Germany).

Aliquots of lithium heparin plasma were used to determine cortisol concentration by ELISA, performed according to the manufacturer's instructions (DRG Instruments, Marburg, Germany). The intra-assay coefficient of variation for plasma adrenaline, noradrenaline and cortisol was 4.1%, 4.1% and 4.4%, respectively.

Statistical analysis. The required sample size was estimated to be 15 participants per group using data from a previous study examining the effect of prior exercise stress on CHS responses to DPCP (Harper Smith et al., 2011). The alpha (Type I error rate) was set at 0.05, and power at 0.95 (1 - Type II error rate) (G*Power software, version 3.1.2). A one-way ANOVA was used to assess differences between the groups in physical characteristics. The effect of exercise intensity and duration was analysed using a one-way ANOVA to determine differences in the summed increase in responses to DPCP between the CON, 120MI, 30MI and 30HI trials. Simple linear regression and a calculation of the standard error of the estimate (SEE) were performed to assess the validity of skinfold measurement, using skinfold callipers, as a practical method to determine dermal thickening compared with the objective criterion, high frequency ultrasound. This was performed on the sum of the 5 elicitation sites for a sub-population with complete data sets (n=50). To further investigate the differences between CON and 120MI an independent t-test was used to assess the summed increase in erythema and a two-way mixed model ANOVA was used to analyse the skinfold thickness response across the full dose-series challenge (trial × dose). Logarithmic transformation was performed on the data to allow for the calculation of the *x*-intercept when $y = 0$, utilising linear regression on the linear portion of the dose response curve. A threshold dose for a response to DPCP was then calculated for the CON and 120MI groups by back transformation (anti-log). A two-way mixed model ANOVA (trial × time) was used to compare the circulating stress hormone data. The mean difference with 95% confidence intervals, are presented for the main outcome measures.

5.4 RESULTS

Assessment of CHS responses

The skinfold response, summed from five challenge doses, was significantly different between groups ($F(3,60) = 3.6$, $P < 0.05$). Post hoc analysis revealed that skinfold thickness was reduced (67%) by 120MI compared with CON ($P < 0.05$; Figure 5.3a). The mean difference was 3.17 mm (95% confidence intervals 0.31 to 6.03 mm). There was no significant difference between the short duration 30MI or 30HI exercise groups compared with CON. Skinfold thickness, assessed using skinfold callipers, was strongly related with high frequency ultrasound readings of dermal thickness ($r = 0.93$, $r^2 = 0.86$, SEE = 1.3 mm; $P < 0.05$). A one-way ANOVA also revealed differences between groups for dermal thickness assessed by ultrasound ($F(3,46) = 2.8$, $P = 0.05$). Post hoc analysis revealed that dermal thickness was reduced (71%) by 120MI compared with CON ($P < 0.05$; Figure 5.3b). The mean difference was 3.17 mm (95% confidence intervals 0.11 to 6.23 mm). There was no significant difference between the short duration 30MI or 30HI exercise groups compared with CON. There were no significant differences for summed erythema responses between groups ($F(3,59) = 2.0$, $P = 0.128$; Figure 5.3b). Interestingly, if the erythema was compared between the CON and 120MI groups, as in (Harper Smith et al., 2011), 120MI significantly decreased these responses ($t(30) = 2.1$, $P < 0.05$).

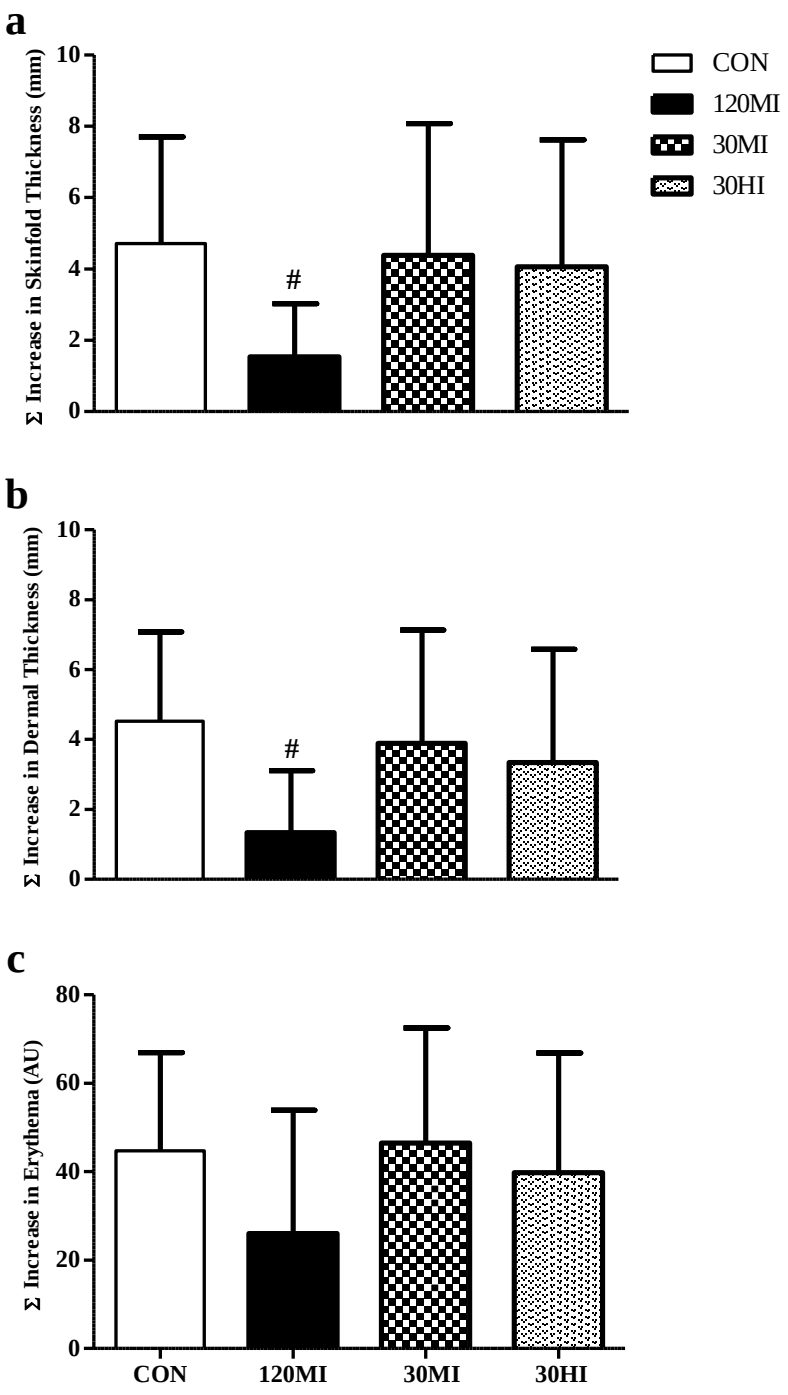


Figure 5.3. Effect of exercise stress prior to induction of contact hypersensitivity with DPCP on responses to elicitation challenge 28 d later measured as (a) skinfold thickness (callipers), (b) dermal thickness (ultrasound), and (c) erythema. Data are mean (SD). # significant difference *vs.* CON ($P < 0.05$).

To further examine the differences between CON and 120MI, the immune reactivity of these participants was assessed over the full dose range of DPCP. The dose response curve for these groups was determined for the increase in skinfold thickness (Figure 5.4). The skinfold thickness responses from the five individual doses revealed a significant trial × dose interaction ($F(2.0,61.0) = 6.3$ $P < 0.01$). Post hoc analysis revealed that skinfold thickness was significantly lower in 120MI compared with CON at all but the 1.24 μg·cm^{-2} dose ($P < 0.01$).

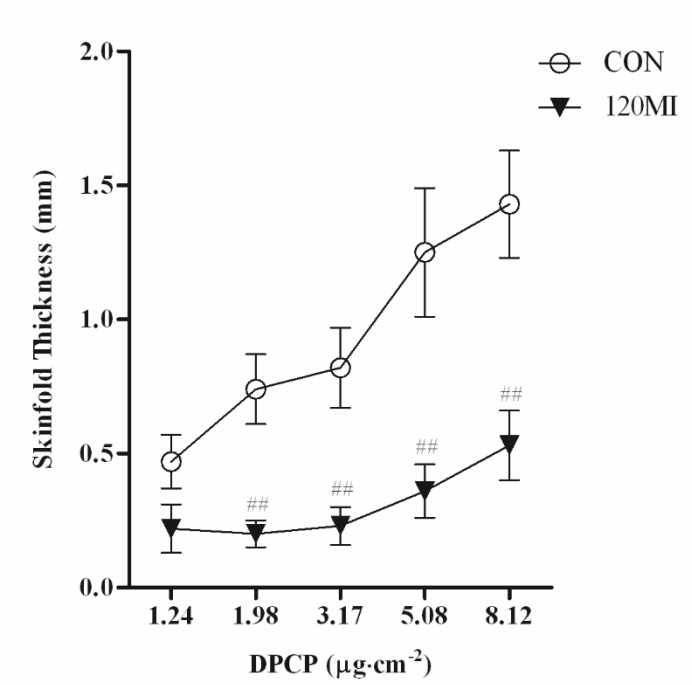


Figure 5.4. Effect of prolonged exercise stress (120MI) or seated rest (CON) prior to induction of contact sensitivity with DPCP. Shown here is the skinfold thickness responses to the full dose-series challenge with DPCP 28 d later. Data are mean (SEM). ## Significant difference *vs*. CON ($P < 0.01$).

The threshold dose for a positive response to DPCP was calculated, using the linear part of the dose response curve, as 0.48 and 2.09 μg·cm^{-2} for the CON and 120MI groups, respectively. This suggests that to elicit a positive response 120MI required a 4.4 times greater DPCP dose in comparison with CON (Figure 5.5).

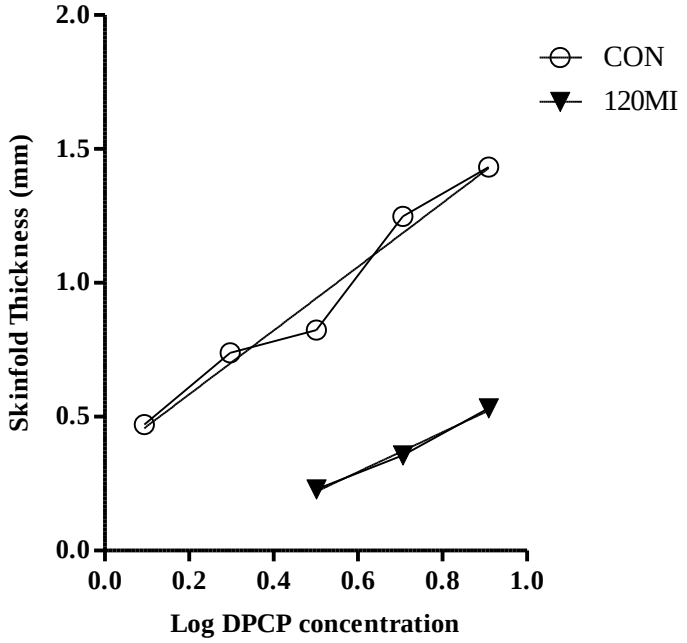


Figure 5.5. Effect of prolonged exercise stress (120MI) or seated rest (CON) prior to induction of contact sensitivity with DPCP. Shown here is the skinfold thickness responses to the dose-series challenge with DPCP 28 d later. Data are log transformed and the solid lines represent the linear portion of the dose-response curve.

Circulating stress hormones

At baseline, pre-exercise, there were no significant differences between groups for circulating adrenaline, noradrenaline or cortisol concentration. A significant trial × time interaction was observed for circulating adrenaline ($F(4.6,88.5) = 7.0$, $P < 0.01$; Figure 5.6a), noradrenaline ($F(3.4,67.1) = 24.0$, $P < 0.01$; Figure 5.6b) and cortisol concentration ($F(4.6,90.6) = 7.0$, $P < 0.01$; Figure 5.6c). The raised circulating adrenaline and noradrenaline concentration observed immediately post on both 120MI and 30HI ($P < 0.01$) had returned to pre-exercise levels by 1 h post exercise. Circulating adrenaline concentration was greater at post on 120MI compared with CON ($P < 0.01$) and circulating noradrenaline concentration was greater at post on 30HI compared with CON ($P < 0.01$). Circulating cortisol concentration was greater at post and 1 h post on 120MI and at post on 30HI compared with CON ($P < 0.01$). The typical diurnal response in circulating cortisol concentration is shown, whereby levels were lower at post

(1300) and 1 h post (1400) compared with pre-exercise (1100) on both 30MI and CON (P < 0.01).

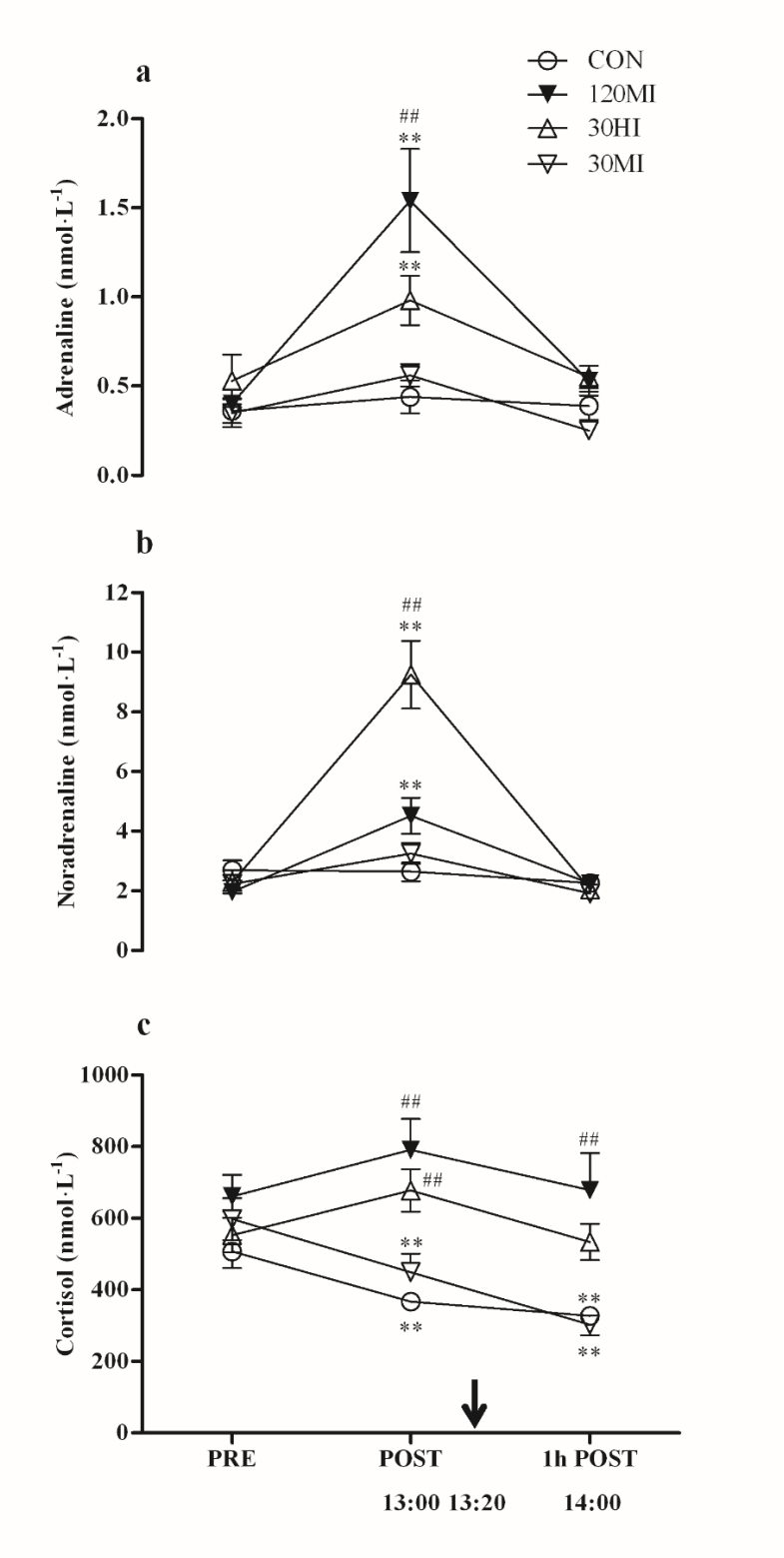


Figure 5.6. Circulating adrenaline (a), noradrenaline (b) and cortisol (c) response to exercise or seated rest. ↓= induction of contact sensitivity by DPCP application. ** P < 0.01 *vs*. pre-exercise; ## P < 0.01 *vs*. CON. Data are mean (SEM).

5.5 DISCUSSION

The purpose of this study was to investigate the unknown effects of the intensity and duration of continuous exercise stress on the induction of *in vivo* immunity to the novel antigen DPCP in humans. In line with the hypothesis, prolonged, moderate intensity exercise decreased the induction of *in vivo* immunity; however short lasting moderate intensity or high intensity exercise did not impair this response. This result was somewhat surprising for the 30HI group given the elevated circulating catecholamines and greater circulating cortisol on 30HI compared with CON. Collectively these findings provide little support for a role of circulating catecholamines and cortisol in the exercise-induced modulation of CHS responses at the skin.

This is the first study to compare the effects of intensity and duration of continuous exercise stress on *in vivo* immunity assessed by use of an experimental CHS model, in humans. In keeping with previous findings, the current study observed that 120MI had a significant inhibitory effect (-67% for skinfold thickness) on the induction of new immunity via the skin (Harper Smith et al., 2011). It was also shown that in the 120MI group a threshold DPCP dose of 4.4 times greater was required to induce a similar elicitation response to that of the CON group, which highlights the magnitude of this reductive effect. The finding that short duration, moderate intensity exercise had no effect on *in vivo* immune induction is at odds with one hypothesis underpinning the J-shaped model (Nieman, 1994), whereby a moderate dose of exercise is proposed to be immune-enhancing, but in accordance with recent research showing no effect of a moderate dose of exercise on the response to vaccination in young, healthy adults (Long et al., 2012). While other studies have shown that short duration, moderate intensity exercise can enhance antibody responses to vaccination, this typically occurs in individuals with sub-optimal immune status or when a half dose of vaccine is administered (Edwards et

al., 2012; Grant et al., 2008; Pascoe et al., 2014). It is acknowledged that exercise might have differential effects on CHS, a cutaneous T-cell mediated response, and the vaccine-induced antibody response, a systemic B-cell mediated response. Based upon rather limited evidence from *in vitro* work (Nieman et al., 1994; Robson et al., 1999) it was hypothesised that 30HI would decrease *in vivo* immune induction to DPCP. However, our results do not support this because the DPCP responses to 30HI were not significantly different to CON. This indicates that the duration of exercise stress, rather than the intensity of a short exercise bout, appears to be key in the impairment of *in vivo* immune induction to DPCP.

Previous research has implicated a role for adrenal stress hormones in the modulation of antigen specific cell mediated immune responses (Dhabhar and McEwen, 1999; Seiffert and Granstein, 2006; Webster Marketon and Glaser, 2008). Despite this, the findings from the current study provide limited support for an involvement of circulating stress hormones in the mechanisms associated with altered *in vivo* immune responses to DPCP at the skin. For example, elevated circulating noradrenaline was highest after 30HI when there was no immunosuppression, suggesting that circulating noradrenaline has little immunosuppressive effect on the CHS system. Although circulating cortisol tended to be higher on 120MI compared with 30HI, this did not reach statistical significance. In addition, circulating cortisol exceeded the purported binding capacity (~552 nmol·L) (McCarthy and Dale, 1988) at post-exercise, before DPCP application, in a similar proportion of participants on 30HI (11 of 16) and 120MI (12 of 16) yet 30HI did not decrease immune induction by DPCP. In murine models, high dose intradermal injections of corticosterone or catecholamines, both locally or distant from the sensitisation site, inhibit the antigen-presenting capability of cutaneous DC, reduce the number of T cells in draining lymph nodes and ultimately suppress DTH and CHS responses (Dhabhar and McEwen, 1999; Flint et al., 2001; Seiffert et al., 2002). In addition,

glucocorticoid stress hormones have long been regarded as being immunosuppressive and the anti-inflammatory use of corticosteroid therapy is well known. Results from human studies are less consistent than the murine models with some authors reporting a lack of association between circulating stress hormones and *in vivo* immune responses (Altemus et al., 2006; Edwards et al., 2006; Oliver et al., 2013). One frequently proposed explanation is that human studies typically rely on individual snapshot assessments of circulating stress hormones, which may miss important information regarding the peaks and recovery of these responses. One might argue therefore that a limitation of the current study is that blood samples were only taken for stress hormone assessment at immediately post and 1 h post exercise, but the DPCP sensitising patch was applied 20 minutes after exercise. It is acknowledged that at 20 minutes after exercise, circulating cortisol is likely to reach a peak but circulating catecholamines would likely have returned to pre-exercise values. Nevertheless, this time was selected to avoid possible confounding due to raised skin blood flow and sweating. It may be considered another limitation that blood samples were only taken at post and 1 h post exercise yet the DPCP sensitising patch remained in place for 48h. However, work in young adults showed the inhibitory effect of stress on the development of immune memory is particularly evident when stress is experienced at the time of sensitisation, supporting our choice of sample timing (Smith et al., 2004b).

An alternative explanation for the findings of the current study may be that the circulating stress hormones do not necessarily reflect the conditions within other body compartments, such as the skin. Using microdialysis, Cohen et al. (2009) demonstrated that interstitial cortisol concentrations did not correlate with plasma concentrations in humans and others have shown that stress-induced suppression of antibody responses to KLH correlated with splenic noradrenaline content but not plasma concentration in mice (Kennedy et al., 2005). Although the majority of cortisol and catecholamines are likely to arrive at the skin tissue as circulating

hormones, many nerves innervate the skin which also release catecholamines and recently it has been shown that human skin cells can produce cortisol and adrenaline locally (Altemus et al., 2006; Arck et al., 2006; Cirillo and Prime, 2011; Madva and Granstein, 2013; Schallreuter et al., 1992).

The use of experimental CHS in human stress research provides an attractive measure of *in vivo* immunity, not only because the skin is immediately accessible but because it overcomes many of the shortcomings of the commonly used *in vitro* approaches. However, it should be recognised that a standard protocol for measuring CHS elicitation responses in humans has yet to be established. The use of erythema to quantify CHS elicitation has previously been questioned, particularly at sites of stronger responses, where yellow vesicles can interfere with the erythema (redness) readings (Harper Smith et al., 2011; Narbutt et al., 2005). This may go some way to explaining why the erythema responses in the current study did not reach significance when all four groups were compared, but it is noteworthy that these responses were still similar to those of Harper Smith et al. (2011) when only the 120MI and CON groups were compared. Skin oedema is considered the key measure of CHS elicitation responses and is commonly assessed using the simple and cost-effect method of skinfold callipers (Harper Smith et al., 2011). Although this measure is accompanied by some degree of subjectivity, it provides a much stronger approach than the visual scoring system, which is often utilised in clinical dermatology (Lesiak et al., 2007; Sleijffers et al., 2001). A particular strength of the current findings is that skinfold thickness was strongly related with dermal thickness, measured by a high-frequency ultrasound scanner and read by a blinded investigator ($r = 0.93$).

A limitation of the current study is that it is unclear which aspects of the induction phase to DPCP are being modulated by the exercise stress. Given that exercise induced changes in immune indices are typically resolved within 24 h (Walsh et al., 2011b), it could be speculated

that the early events in response to an antigen challenge would be affected by prolonged exercise. One approach would be to adopt measures commonly used in dermatological research, such as the suction blister technique, to investigate the local cutaneous events at the site of antigen exposure. Sampling suction blister fluid allows for the assessment of the key cytokines, such as IL-1β, TNF-α and IL-10, thought to orchestrate the activation and migration of cutaneous DC, which is considered instrumental in the initial events of successful development of new immune memory (Figure 2.1; Dearman et al., 2004; Toebak et al., 2009).

In summary, this is the first study to compare the effects of intensity and duration of continuous exercise stress on *in vivo* immunity in humans. This model of experimental CHS with DPCP demonstrated that prolonged, moderate intensity exercise, but not short-lasting high or short-lasting moderate intensity exercise, significantly decreases the induction of *in vivo* immunity in healthy humans. These results provide little support for a role of circulating catecholamines and cortisol in the exercise-induced modulation of CHS responses at the skin. The topical application of DPCP is an attractive immunological tool for the assessment of stress on *in vivo* immunity in humans, however further research is required to gain a better understanding of the underlying mechanisms for the observed reductive effect of prolonged, moderate intensity exercise on the development of new immune memory.

CHAPTER SIX

The repeatability of raising controlled suction blisters on the inner aspect of the upper arm

6.1 SUMMARY

To better understand the reductive effects of prolonged, moderate intensity exercise on the induction of *in vivo* immunity, a suction blister technique was adopted to assess key cytokines (specifically IL-1β, TNF-α, and IL-10), thought to be instrumental in cutaneous DC migration. Before utilising this technique in an exercise study, it was necessary to identify the repeatability of a suction blister model to assess the cutaneous cytokine environment. Twelve rested male participants (mean (SD): age 23 (6) years, height 180 (4) cm, body mass 73.4 (8.7) kg) completed three repeated trials, separated by ≥ 48 h, to induce a suction blister ~10 mm in diameter on the inner aspect of alternate upper arms. Blister fluid was aspirated immediately and assessed for IL-1β, TNF-α, IL-10, and total protein concentration. A one-way ANOVA showed no difference in blister fluid IL-1β (P = 0.28), TNF-α (P = 0.94) or IL-10 (P = 0.69) concentration ($pg \cdot ml^{-1}$) between trials, or when these cytokines were expressed relative to total protein ($pg \cdot mg^{-1}$ protein: IL-1β, P = 0.26; TNF-α, P = 0.96; or IL-10, P = 0.78). The intraclass correlation coefficients (ICCs) indicated excellent relative reliability for IL-1β and TNF-α measures (0.80-0.92), and fair relative reliability for IL-10 concentration (0.48) and IL-10 relative to total protein (0.42). Mean within-participant coefficient of variation (CV_w%) for IL-1β, TNF-α and IL-10 cytokine measures ranged from 25% to 41%. The findings of this study suggest that the suction blister technique is a repeatable tool for the measurement of cutaneous cytokines involved in DC migration.

6.2 INTRODUCTION

Prolonged, moderate intensity exercise has been shown to suppress *in vivo* immune induction when assessed by the epicutaneous application of DPCP (**Chapter 5**; Harper Smith et al., 2011). However, the potential mechanisms involved remain unclear. As raised in **Chapter 2**, these mechanisms may include reduced migration of the DC towards the dLN, where development of immune memory occurs (Figure 2.1). Direct measurement of cutaneous DC migration in humans typically involves punch biopsies or extraction of a suction blister roof, which are invasive procedures that require anaesthesia, specialist laboratory expertise, and punch biopsies also result in a small scar (Kiistala, 1968). For ethical reasons, a suction blister model was adopted, which provides a window into the events that are occurring locally within the skin, such as changes in the cutaneous cytokine environment (Dearman et al., 2004). The levels of cutaneous cytokines known to facilitate DC migration in humans and mice (specifically IL-1β and TNF-α) and purported to inhibit DC migration in mice (e.g. IL-10) are of particular interest here given the importance of this process in successful immune induction (Cumberbatch et al., 1999; Cumberbatch et al., 2003; Enk et al., 1994; Toebak et al., 2009).

The method to induce suction blisters on the skin has been used extensively within the study of drug pharmacokinetics (Mazzei et al., 2000), UV exposure (Skov et al., 1998), inflammatory markers and biomarker proteins (Muller et al., 2012). Skin suction blisters can be induced using the method developed by (Kiistala, 1968) involving the application of a gentle vacuum to the skin, typically using a small suction cup, attached to a negative pressure device. Applying a low negative pressure to the skin's surface induces the separation of the epidermis from the dermis, resulting in a small suction blister. The blister fluid from the freshly formed blister can then be harvested and analysed. This corresponds to the interstitial tissue fluid of the epidermis

and dermis, thus providing information about the activities within the local cutaneous environment (Kool et al., 2007; Rhodes et al., 1999).

Unfortunately, the Dermovac® suction blister device, which is widely cited within the dermatology literature, is no longer commercially available. Therefore, following discussions with our collaborator, Professor Peter Friedmann from Southampton University, we produced an in-house, purpose built suction blister device, based on the original Dermovac® instrument and similar to those used by Akbar et al. (2013), Janssens et al. (2009) and Nicolaou et al. (2012). During the design process it was necessary to consider ways to minimise trauma to the site, which could potentially contaminate our blister sample (Kiistala, 1968). The width of the Perspex suction cup was selected to ensure the pressure on the skin was spread out, which minimises impression of the cup edge. In addition, all edges were rounded and polished to minimise trauma and allow clear view of the blister formation.

Many studies utilising the suction blister technique rely on a single blister fluid sample, therefore assuming that the measurement provides an accurate and reproducible representative of the local cutaneous environment. To the best of our knowledge, the repeatability of the suction blister technique to measure the cytokine content of freshly formed blisters has not previously been reported. While the thesis was being prepared, one recent study (article in press) reported a suction blister method to be reliable for assessing wound healing (Smith et al., 2015). However, this method involves a number of differences compared with the method required here. For example, the wound healing model involves removing the blister roofs of eight small blisters, placing a chamber over these sites and adding 1 ml of 30% autologous serum to each wound. Comparisons of cytokine concentrations were then made by collection of this fluid over a 24 h period and reporting as area-under-the-curve values. Therefore, before

utilising our suction blister device in the study of DPCP induction after prolonged, moderate intensity exercise, it was necessary to determine the repeatability of baseline cutaneous cytokines in rested participants. It was hypothesised that the suction blister device would produce repeatable suction blister fluid measures of IL-1β, TNF-α and IL-10 concentrations and total protein content.

6.3 METHODS

Study design. Twelve healthy recreationally active males (mean (SD): age 23 (6) years, height 180 (4) cm, body mass 73.4 (8.7) kg) volunteered to participate in this study. In a repeated measures design, participants reported to the laboratory for three experimental trials to investigate the repeatability of suction blister fluid cytokines. All trials were repeated at the same time of day and were separated by ≥ 48h.

Experimental procedures. Prior to commencement of the first experimental trial, anthropometric measurements of body mass and height were collected. During the experimental trials, suction blisters were induced alternately on the inner aspect of the left and right upper arm (see **Chapter 3, section 3.5** for details). Room temperature was (mean (SD)) 22 (1.2)°C and relative humidity was 40 (6.8)%. The suction blister fluid was analysed for IL-1β, TNF-α, IL-10 concentration and total protein content (as described in **Chapter 3, section 3.6**). The intra-assay coefficient of variation for suction blister IL-1β, TNF-α, IL-10 concentration and total protein content was 2.3%, 3.1%, 4.7% and 3.6% respectively.

Statistical analysis. The required sample size was estimated to be 10 participants using mean and standard deviation data taken from a previous study within our laboratory which examined serum concentration of IL-1β, on three separate occasions, in 12 rested participants (data unpublished). An intraclass correlation among repeated measures was calculated based upon the results from this study and used within a bivariate correlation, with alpha level (Type I error rate) set at 0.05, and power set at 0.85 (1 - Type II error rate) (G*Power software, version 3.1.2).

All variables were compared over the three repeated trials using a simple one-way repeated measures ANOVA. The intraclass correlation coefficients ($ICC_{3,k}$: two-way mixed effect model) were used to estimate relative reliability for all data. According to (Rosner, 2000), values of ICC $\geq$ 0.75 indicates excellent reproducibility, 0.4 $\geq$ to < 0.75 indicates fair to good reproducibility, and < 0.4 indicates poor reproducibility. Absolute reliability was explored using the mean within participant coefficient of variation (CV_w%), calculated as (SD/mean) x 100. Pearson's correlations were calculated for all available data points, to assess relationships between suction blister formation time and cutaneous cytokine responses.

6.4 RESULTS

Suction blister formation

Suction blisters were successfully raised on all participants (n=12) with a blister fluid grand mean (SD) volume of 140 (43) µl. This was the first time that we have utilised this technique and attempted to measure blister fluid cytokine concentrations within our laboratory. Therefore blister fluid samples from this study were also used in pilot ELISA assays to determine the suitable sample dilutions for future analysis (as outlined in **Chapter 3, section 3.6**). For this reason, unfortunately there was insufficient sample volume left to examine all three cytokines of interest, in all participants. Here blister fluid data are presented for IL-1β (n=12), TNF-α (n=11), and IL-10 (n=8).

There were no significant differences between the three repeated trials for time taken to raise a blister ($F(2,22) = 0.9$, $P = 0.42$). Individual participant data showed that all suction blisters were formed within 145 min (Table 6.1). The ICC for suction blister formation time indicated excellent reliability (0.80) and for absolute reliability, the mean within participant CVw% was 10%.

Table 6.1. Individual suction blister formation times and mean (SD) values for each trial.

Participant	Formation Time (min)		
	Trial 1	Trial 2	Trial 3
1	130	132	120
2	115	145	140
3	134	115	135
4	110	110	120
5	105	100	110
6	130	106	117
7	140	110	130
8	108	143	125
9	80	95	95
10	100	90	120
11	100	70	87
12	120	125	120
Mean (SD)	114 (17)	112 (22)	118 (15)

Suction blister cytokines and total protein

There were no significant differences between the three repeated trials for resting levels of suction blister IL-1β, TNF-α, or IL-10 concentrations (Table 6.2). Similarly, when these cytokines were expressed relative to total protein content of the blister fluid samples, there were no significant differences (Figure 6.1). The grand mean (SD) of total protein content over the three trials was 24.0 (3.5) $mg \cdot ml^{-1}$ and there were no significant differences observed $F(2,22) = 0.07$, $P = 0.93$.

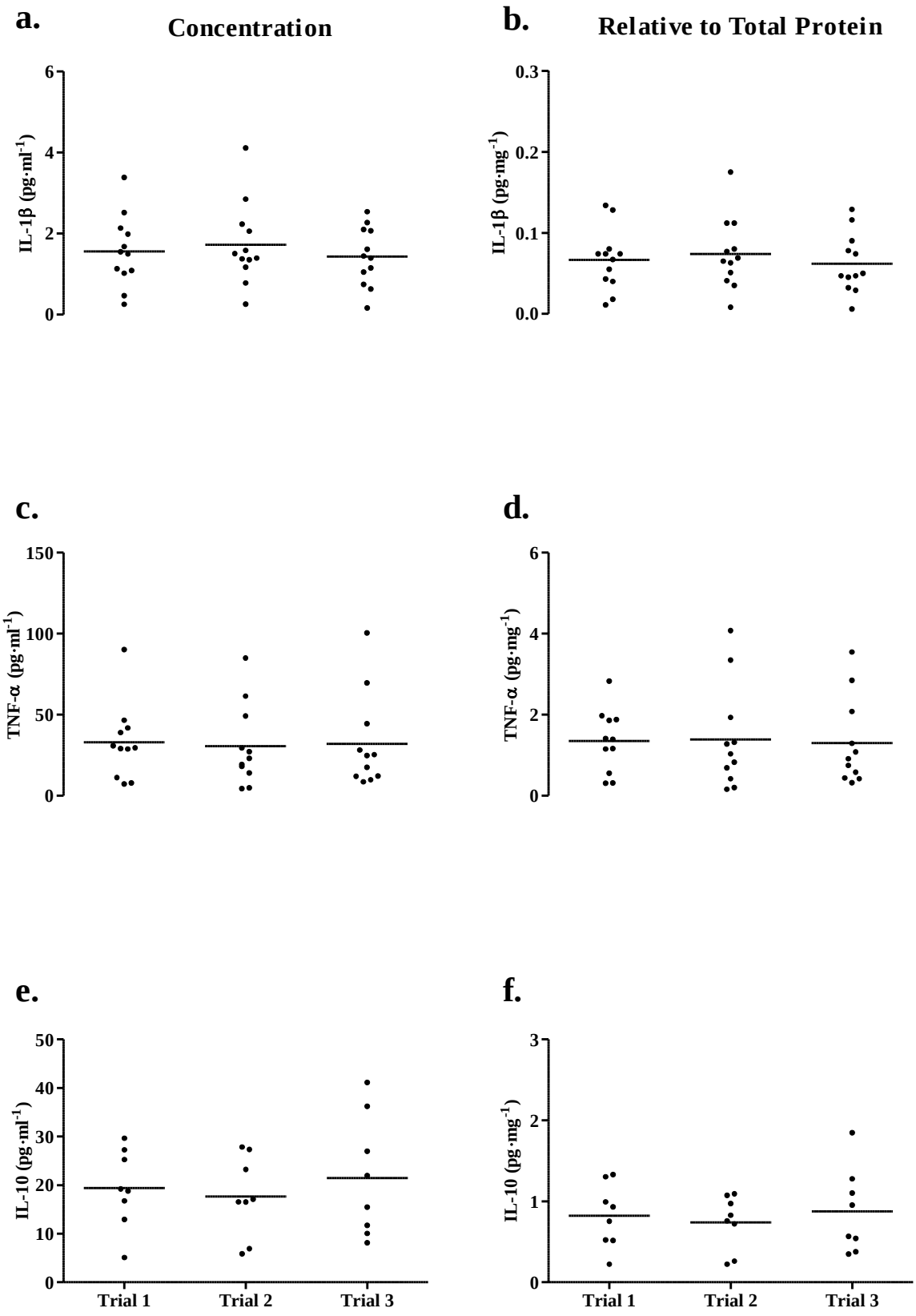


Figure 6.1. The repeatability of suction blister cytokine content. Results are expressed as cytokine concentration pg·ml^{-1} and relative to total protein pg·mg^{-1} for IL-1β (a) and (b), TNF-α (c) and (d), and IL-10 (e) and (f), respectively.

For relative reliability, the ICCs for blister fluid IL-1β and TNF-α, when expressed as a concentration or relative to total protein, were all higher than 0.75, indicating excellent reliability (Table 6.2). The ICCs for IL-10 concentration and IL-10 relative to protein were greater than 0.4, indicating fair reliability. For absolute reliability, the mean within participant CV_w% were very similar when a blister fluid cytokine was expressed as a concentration or relative to total protein. Suction blister IL-1β concentration had the lowest CV_w% and IL-10 relative to total protein had the greatest CV_w% (Table 6.2).

Table 6.2. The relative and absolute reliability of suction blister fluid cytokines.

Measurement	ICC	CV_w	One-way ANOVA main effects
IL-1β concentration (pg· ml^{-1})	0.90	25%	$F(2,22) = 1.4$, $P = 0.28$
IL-1β relative to total protein (pg· mg^{-1})	0.92	27%	$F(2,22) = 1.4$, $P = 0.26$
TNF-α concentration (pg· ml^{-1})	0.82	37%	$F(2,20) = 0.06$, $P = 0.94$
TNF-α relative to total protein (pg· mg^{-1})	0.80	39%	$F(2,20) = 0.05$, $P = 0.96$
IL-10 concentration (pg· ml^{-1})	0.48	40%	$F(2,14) = 0.4$, $P = 0.69$
IL-10 relative to total protein (pg· mg^{-1})	0.42	41%	$F(2,14) = 0.3$, $P = 0.78$

ICC = Intraclass correlation coefficient; CV_w% = Mean within-participant coefficient of variation.

Correlational analysis indicated that suction blister formation time was not related to suction blister cytokine concentration for IL-1β (r = 0.23, P = 0.18; Figure 6.2a) or IL-10 (r = -0.19, P = 0.37; Figure 6.2e), or when these cytokines were expressed relative to total protein content (IL-1β: r = 0.31, P = 0.06; Figure 6.2b; and IL-10: r = -0.22, P = 0.29; Figure 6.2f). However suction blister formation time correlated modestly, but significantly, with TNF-α concentration (r = 0.36, $P < 0.05$; Figure 6.2c), and TNF-α expressed relative to total protein content (r = 0.41, $P < 0.05$; Figure 6.2d).

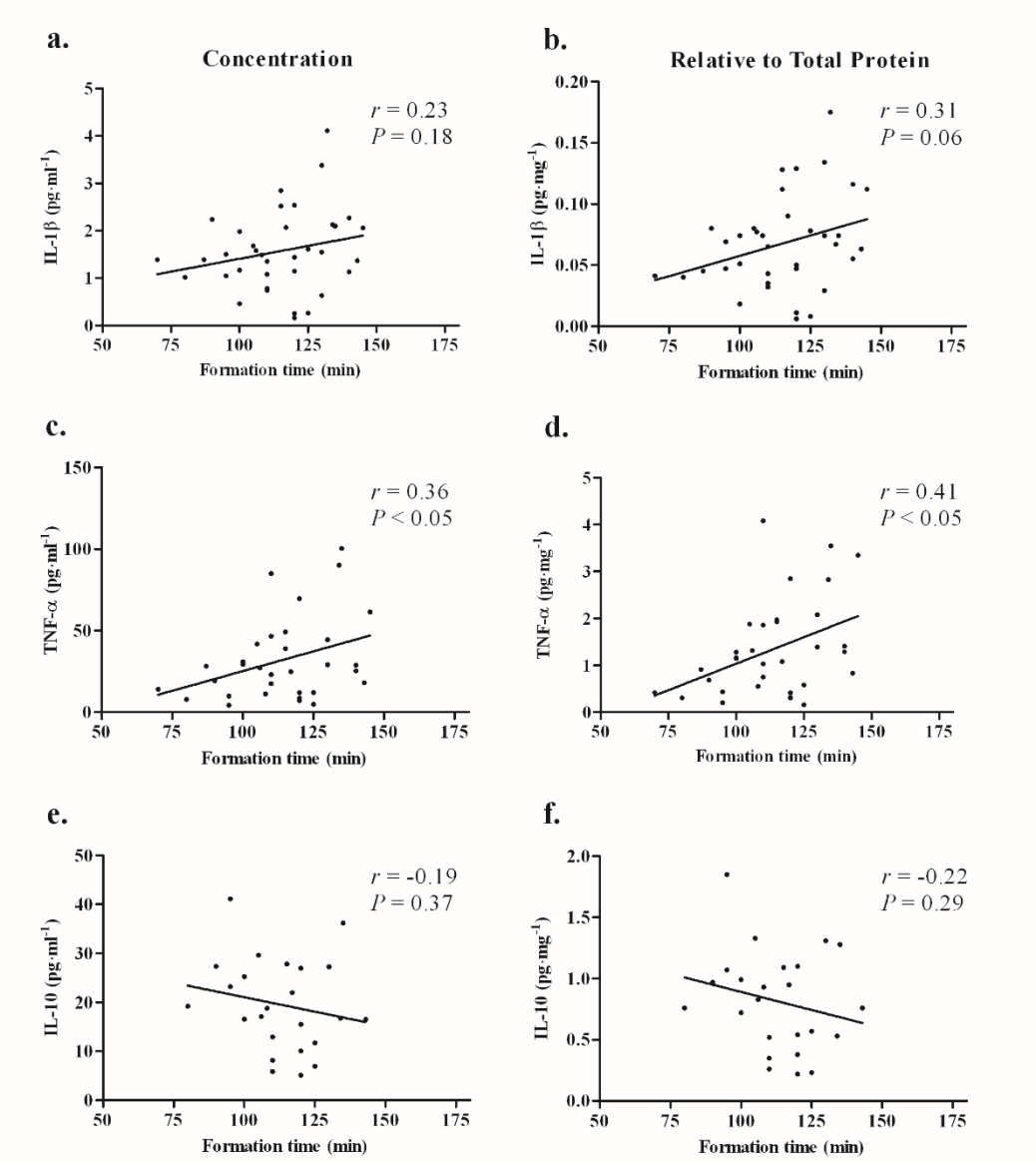


Figure 6.2. The relationship between suction blister formation time and cutaneous cytokine responses. Results are expressed as cytokine concentration pg·ml^{-1} and relative to total protein pg·mg^{-1} for IL-1β (a) and (b), TNF-α (c) and (d), and IL-10 (e) and (f), respectively.

6.5 DISCUSSION

The purpose of this study was to determine the repeatability of a suction blister device to examine the cutaneous environment of cytokines thought to orchestrate the migration of cutaneous DC, namely IL-1β, TNF-α and IL-10. To the best of our knowledge, this is the first study to examine the repeatability of a suction blister technique to measure blister fluid cytokines from freshly formed blisters. In line with the hypothesis, the results demonstrated that blister fluid concentrations of IL-1β, TNF-α IL-10, and total protein were not significantly different between trials. The relative reliability of suction blister cytokine responses, presented here as ICCs, was fair to excellent, and the absolute reliability, presented here as CV_w% was similar to those previously reported for serum cytokines (range: 25%-41%; Gonzalez et al., 2001). These findings suggest that the suction blister technique is a repeatable tool for measuring IL-1β, TNF-α and IL-10 blister fluid content, making it suitable for future investigations of the local cutaneous cytokine environment.

In the current study there were no significant differences between trials for any of the suction blister cytokines examined. This is similar to the findings from previous research, indicating no differences in plasma cytokine concentrations over three repeated trials (Picotte et al., 2009). However, it is widely thought that the use of repeated measures ANOVA alone is insufficient to quantify reliability (Atkinson and Nevill, 1998; Hopkins, 2000; Weir, 2005). In particular, using this test of mean differences with small sample sizes can decrease power and potentially hide systematic error. Therefore a particular strength of the current study is that a battery of recommended reliability statistics, which cover both the relative reliability and absolute reliability, have been presented (Atkinson and Nevill, 1998).

The selected measure of relative reliability (ICC), revealed similar blister fluid IL-1β and TNF-α concentration results (ICCs = 0.90 and 0.82, respectively) to studies reporting ICCs for serum

IL-1β (0.86) and TNF-α (0.88) concentration (Gu et al., 2009). Recently, Navarro et al. (2012) reported a slightly higher ICC of 0.92 for serum TNF-α concentration. This may be explained by the greater number of samples measured per participant in their study, which can reduce the impact of the within-subject variance (Francis et al., 2005). The ICC for IL-10 concentration in this study was fair (0.48). This is lower than other reports of serum IL-10 concentration (0.75) in a large group of healthy women (n=65). It is not clear why this marker performed poorly relative to other blister fluid cytokines in the current study. All samples for the same individual were run on the same assay plate, however due to low sample volumes associated with suction blisters compared with blood samples, this could only be performed in single analysis. Therefore assay variability may have had an effect on these results, although this is unlikely given the low intraassay CV% for IL-10 concentration shown in pilot work within our laboratory (4.8%). It is speculated that the small sample size (n=8) available for this measure may provide some explanation for the current findings.

The selected measure of absolute reliability (CV_w%), showed the blister fluid cytokine concentration results for IL-1β (25%) and TNF-α (37%) were comparable to previous work reporting CV_w% for serum cytokine repeatability (IL-1β = 30%; TNF-α = 43%) (Gonzalez et al., 2001). The CV_w% for IL-10 concentration in the present study (40%) was also within a similar range to this previous work. It is recognised that all suction blister CV_w% values in the current study may be considered quite high, but it should be stressed that they are similar to other indices commonly used with single, one-off measurements to study inflammatory markers, such as serum cytokines (Gonzalez et al., 2001), or various markers of mucosal immunity (Dwyer et al., 2010).

Within the existing literature, there is currently variation in the way of reporting suction blister cytokine responses, with some authors choosing to express these as an absolute concentration

($pg \cdot ml^{-1}$) (Janssens et al., 2009; Shahbakhti et al., 2004; Skov et al., 1998), whereas others report these relative to total protein ($pg \cdot mg^{-1}$ protein) (Cumberbatch et al., 2006; Dearman et al., 2004). The suction blister findings for IL-1β, TNF-α and IL-10 in this study were presented together as a concentration and relative to total protein, which both produced very similar results. Dearman et al. (2004) recommend expressing blister fluid cytokines as a function of total protein to reduce the variation in responses compared with the absolute concentration. However, in their study, suction blisters were raised on areas of skin that had been stimulated with IL-1β injection, rather than untreated, normal skin, as was used here. Given these findings, it would be advisable for future studies utilising the suction blister device at the site of DPCP stimulated skin, to use this preferred method of expressing the results relative to total protein.

In the current study suction blister formation time was shown to be repeatable, with no significant differences between trials, excellent relative reliability (ICC = 0.80), and a low CV_w% (10%). In addition, the time taken to raise suction blisters was not correlated with suction blister IL-1β, or IL-10 content and therefore suggests that blister formation time is unlikely to play a role in these cutaneous responses. However, a modest correlation was observed between suction blister formation time and blister fluid TNF-α content. Although this is recognised as a limitation in the interpretation of the suction blister TNF-α data, the cutaneous responses at the site of DPCP sensitisation were of greatest interest here. Therefore it was deemed suitable to proceed to use this measure at the site of DPCP sensitisation.

A recognised limitation of the current study is that it does not provide information on the stimulated cutaneous cytokine environment following DPCP patch exposure. Given that DPCP induction can only be performed once in an individual, it was not possible to investigate the suction blister repeatability at the site of DPCP patch application, thus the approach adopted in

the current study was deemed most appropriate. Next it was necessary to investigate the stimulated cutaneous cytokine kinetics at the site of a DPCP sensitising patch, to determine an appropriate time to raise suction blisters in a future study of the mechanisms associated with the inhibitory effects of prolonged exercise on immune induction with DPCP.

In conclusion, the suction blister device has been shown to be a reliable and repeatable tool for measuring IL-1β, TNF-α and IL-10 blister fluid content. The relative reliability results for IL-10 content were not as strong, however given the sample size limitation of the current study, we felt this should not preclude the use of this tool to measure cutaneous cytokines in future studies. A follow-up study should now aim to utilise this suction blister tool to examine the stimulated cytokine responses, locally at the site of a DPCP sensitisation patch.

CHAPTER SEVEN

The kinetics of cutaneous cytokine changes in response to Diphenylcyclopropenone induction

7.1 SUMMARY

Prior to utilising a suction blister technique to examine the potential mechanisms involved in exercise induced suppression of DPCP induction, it is necessary to better understand the kinetics of cutaneous cytokine responses, at the site of DPCP sensitisation, in rested individuals. The aim of this study was to determine an appropriate time to remove a DPCP sensitisation patch and commence a suction blister procedure, for measurement of the stimulated cutaneous cytokines thought to be involved in DC migration. Twenty rested men received a sensitising DPCP patch on the inner aspect of their upper arm, which was removed after either 6 h (PATCH6, n = 10; mean (SD): age 22 (3) years, height 182 (6) cm, body mass 75.9 (11.3) kg) or 24 h (PATCH24, n = 10; mean (SD): age 22 (3) years, height 183 (6) cm, body mass 81.8 (14.5) kg) of patch exposure. Immediately following patch removal, suction blisters were induced directly over the patch sites for measurement of blister fluid IL-1β, TNF-α, IL-10, and total protein content. The pooled means from the untreated bare skin (BSk) blisters in **Chapter 6** were used as a comparative control. There were no significant differences observed between groups for suction blister IL-1β content. The PATCH6 group demonstrated significantly higher levels of blister fluid TNF-α ($P < 0.01$) and IL-10 ($P < 0.01$) than the PATCH24 and the BSk groups. In conclusion, 6 h of DPCP patch application appears to be suitable for subsequent measurement of DPCP induced cytokine responses within suction blister fluid.

7.2 INTRODUCTION

The suction blister technique is considered to be a powerful tool for the measurement of the cutaneous environment (Akbar et al., 2013; Dearman et al., 2004; Muller et al., 2012) and it has recently been shown to be a repeatable method for the determination of blister fluid cytokines (**Chapter 6**). The next step, prior to using this technique to investigate the exercise-induced suppression of DPCP induction, was to examine the stimulated cutaneous cytokine responses, of those thought to orchestrate cutaneous DC migration (i.e. IL-1β, TNF-α and IL-10), at the site of a DPCP sensitising patch.

Previous research utilising experimental CHS with DPCP has resulted in successful sensitisation of participants after 48 h of patch exposure (**Chapter 5**; Harper Smith et al., 2011). However the time course of events that lead to this end result are poorly understood. Given that individuals can only be sensitised to a novel antigen once, it is unsurprising that the current understanding on the kinetics of this process is largely provided by murine models (Enk et al., 1993; Enk and Katz, 1992; Hope et al., 1994). In mice, a rapid increase in IL-1β mRNA expression has been identified at the site of antigen painting after only 15 minutes (Enk and Katz, 1992) and the number of antigen-modified DC in the regional lymph nodes increases 30 minutes to 4 h later (Macatonia et al., 1987). However, the time course of responses in animals may not be relevant in humans due to species specific differences (Davis, 2008). In humans, cutaneous TNF-α levels in suction blister fluid has been shown to be significantly increased 2 h after intradermal injection of IL-1β (Cumberbatch et al., 2003), but it is unclear if this concentration of IL-1β reflects the levels stimulated during DPCP sensitisation. Using the highly invasive technique of lymph node cannulation, antigen-containing DC have been measured in the afferent lymphatic vessels of humans within 24 h after dinitrochlorobenzene (DNCB) application (Hunger et al., 2001). A limited number of studies have examined the site

of DPCP sensitisation, by adopting the more commonly used, but still invasive, punch biopsies to directly measure cutaneous DC frequency (Griffiths et al., 2001; Heffler et al., 2002). These measures were taken between 6 h and 24 h after initial application of 2% DPCP. Griffiths et al. (2001) identified significantly reduced cutaneous DC frequency 17 h after DPCP application, so it may be logical to assume that changes in the cutaneous cytokines associated with orchestrating DC migration would occur before this. Preliminary work by this group also showed no difference in DC migration 4 h after exposure to DPCP, which is why time points to commence the suction blister procedure before 6 h were not included in the current study. It is recognised that variables such as the potency and dose of the sensitising agent, as well as the population of participants studied, have been suggested to influence the kinetics of cytokine expression and cutaneous DC migration following antigen exposure (Cumberbatch et al., 2003; Heffler et al., 2002; Pickard et al., 2009). Therefore it is necessary to examine the kinetics of suction blister cytokine responses following the specific DPCP sensitisation protocol of interest (i.e. a single occluded patch of 0.125% DPCP; **Chapter 3, section 3.4**; Harper Smith et al., 2011).

The aim of the present study was to determine an appropriate time to remove a DPCP sensitisation patch and commence a suction blister procedure at the patch site, for measurement of cutaneous cytokines thought to orchestrate DC migration (i.e. IL-1β, TNF-α and IL-10). Based on the timings used in similar studies and recommendations from our collaborators, DPCP patch applications of 6 h and 24 h were selected for the subsequent analysis of suction blister fluid cytokine responses. It was hypothesised that cytokine responses would be significantly greater after 6 h of DPCP patch application compared with 24 h of DPCP patch application and untreated control skin.

7.3 METHODS

Study design. In a cross-sectional design, twenty healthy, recreationally active male participants were randomly allocated to one of two groups: 1) 6 h of DPCP sensitising patch exposure (PATCH6: mean (SD): age 22 (3) years, height 182 (6) cm, body mass 75.9 (11.3) kg); or 2) 24 h of DPCP sensitising patch exposure (PATCH24: mean (SD): age 22 (3) years, height 183 (6) cm, body mass 81.8 (14.5) kg), to investigate blister cytokine responses to DPCP. The results from the twelve recreationally active males in **Chapter 6**, who had blisters raised on untreated bare skin (BSk), acted as a comparative control group. There were no significant differences between groups for anthropometric and physical characteristics.

Experimental procedures. Prior to commencement of the experimental trial, anthropometric measurements of body mass and height were collected. On the day of the experimental trial, rested participants were transported to the laboratory at 0930h. A single patch containing 22.8 µl of the DPCP sensitizer (as described in **Chapter 3, section 3.4**) was applied to the inner aspect of the participant's upper arm because suction blisters cannot be raised on the lower back. All participants were sensitised to DPCP at the same time of day (1000 h) and patches remained in place for exactly 6 h or 24 h, during which participants were allowed to perform light activity only (Figure 7.1). Immediately following patch removal, suction blisters were induced directly over the patch site (as described in **Chapter 3, section 3.5**). Room temperature was (mean (SD)) 21 (0.9)°C and relative humidity was 39 (5.9)%. The suction blister fluid was analysed for IL-1β, TNF-α, IL-10 concentration and total protein content (as described in **Chapter 3, section 3.6**). The intra-assay coefficient of variation for suction blister IL-1β, TNF-α, IL-10 concentration and total protein content was 2.6%, 2.6%, 3.3% and 3.9%, respectively. All kits were performed according to the manufacturer's instructions.

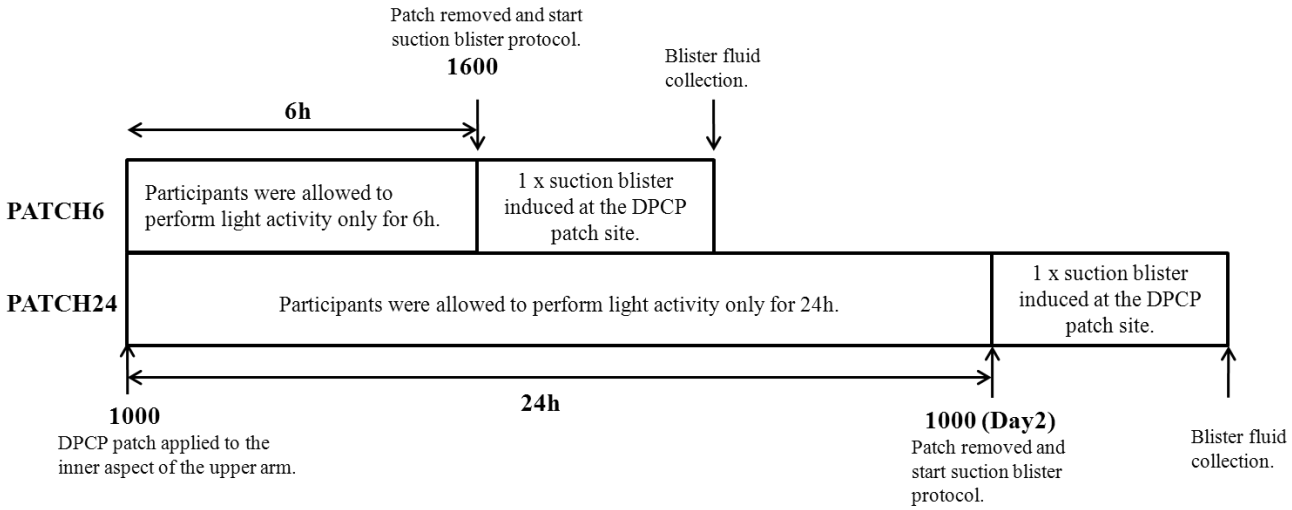


Figure 7.1. Study design schematic.

Statistical analysis. The required sample size was estimated to be 10 participants per group by calculating a minimum important difference using the blister fluid IL-1β repeatability data from **Chapter 6**. The alpha (Type I error rate) was set at 0.05, and power at 0.85 (1 - Type II error rate) (G*Power software, version 3.1.2). A one-way ANOVA was used to assess differences between the three groups for all data. The mean difference with 95% confidence intervals, are presented for the main outcome measures. Pearson's correlations were calculated for all participants to assess relationships between suction blister formation time and cutaneous cytokine responses.

7.4 RESULTS

Suction blister formation

All suction blisters were successfully raised within 145 min. The suction blister formation time was longest for the BSk group (mean (SD): 115 (16) min), followed by the PATCH6 group (84 (17) min), and the shortest formation time was observed for the PATCH24 group (49 (11) min).

Suction blister response to DPCP induction

There was no significant difference observed between groups for suction blister IL-1β content ($F(2,29) = 2.1$, $P = 0.143$; Figure 7.2a). The suction blister TNF-α content was significantly different between groups ($F(2,28) = 9.9$, $P < 0.01$). Post hoc analysis revealed that TNF-α content was increased by PATCH6 compared with BSk and PATCH24 ($P < 0.01$; Figure 7.2b). The mean difference between PATCH6 and BSk was 2.1 $pg \cdot mg^{-1}$ (95% confidence intervals 0.63 to 3.56) and between PATCH6 and PATCH24 was 2.5 $pg \cdot mg^{-1}$ (95% confidence intervals 1.00 to 4.01). There was no significant difference between BSk and PATCH24 groups. The suction blister IL-10 content was significantly different between groups ($F(2,25) = 27.2$, $P < 0.01$). Post hoc analysis revealed that IL-10 content was increased by PATCH6 compared with BSk and PATCH24 ($P < 0.01$; Figure 7.2c). The mean difference between PATCH6 and BSk was 2.7 $pg \cdot mg^{-1}$ (95% confidence intervals 1.50 to 3.82) and between PATCH6 and PATCH24 was 3.00 $pg \cdot mg^{-1}$ (95% confidence intervals 1.90 to 4.09). There was no significant difference between BSk and PATCH24 groups. Total protein was not significantly different between the BSk (mean (SD): 24.0 (3.5) $mg \cdot ml^{-1}$), PATCH6 (27.7 (5.7) $mg \cdot ml^{-1}$) and PATCH24 (28.3 (4.9) $mg \cdot ml^{-1}$) groups. There were no significant correlations between suction blister formation time and suction blister cytokine responses for IL-1β ($r = -0.30$, $P = 0.10$), TNF-α ($r = 0.29$, $P = 0.10$), or IL-10 ($r = 0.22$, $P = 0.32$).

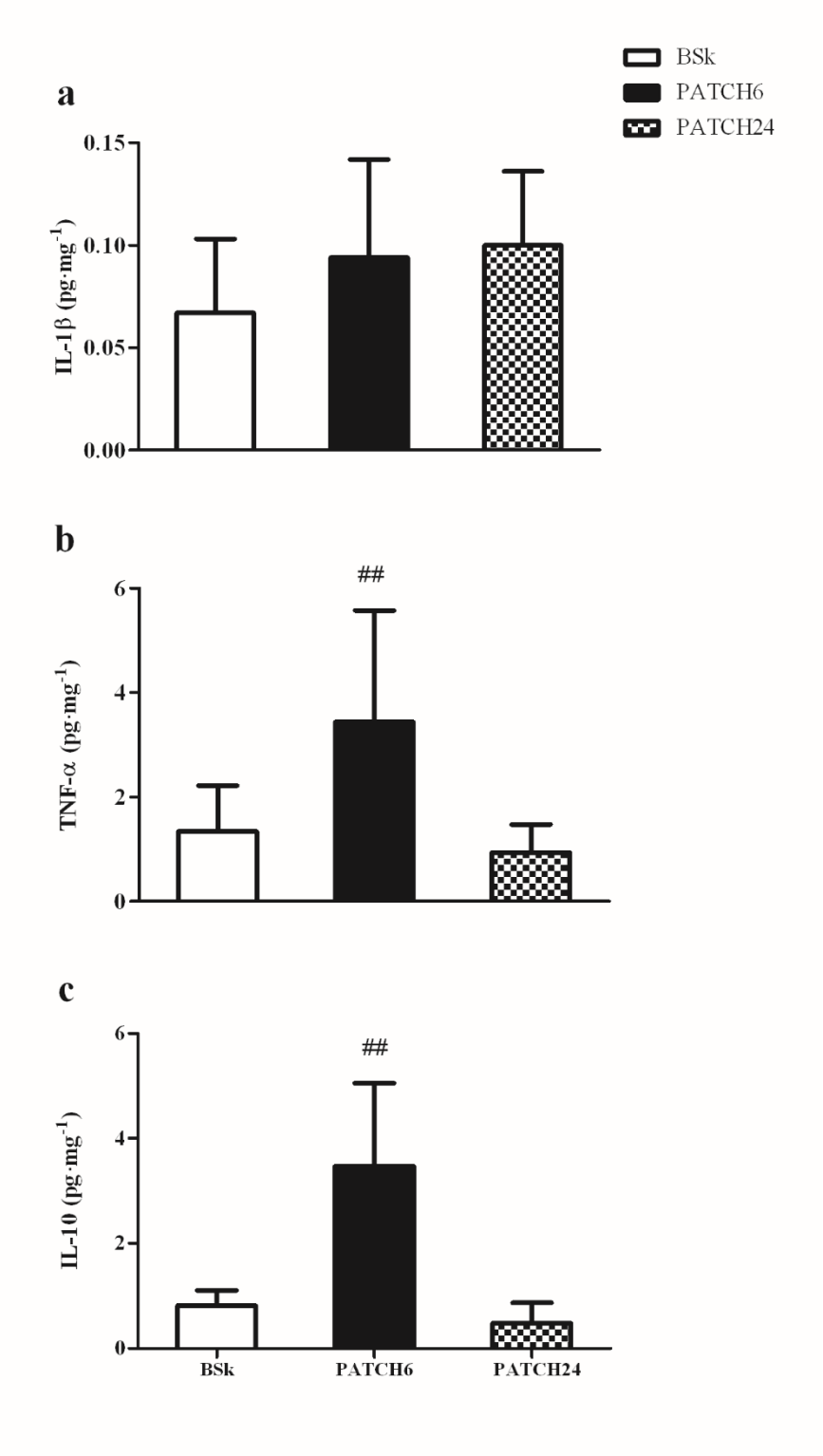


Figure 7.2. Suction blister IL-1β (a), TNF-α (b) and IL-10 (c) content from untreated bare skin (BSk), or at the site of DPCP sensitisation after 6 h (PATCH6) and 24 h (PATCH24) patch applications. Data are mean (SD): ## $P < 0.01$ *vs*. BSk and PATCH24.

7.5 DISCUSSION

The aim of the present study was to determine an appropriate time to remove a DPCP sensitisation patch, and commence a suction blister procedure at the patch site, for measurement of key cutaneous cytokines thought to orchestrate DC migration. To the best of our knowledge, this is the first study to examine suction blister cytokine responses during the sensitisation phase to the novel antigen, DPCP. Significantly higher levels of blister fluid TNF-α and IL-10 were shown in the PATCH6 group compared with the BSk and PATCH24 groups. However, there were no significant differences between groups for blister fluid IL-1β content. These data indicate that 6 h after the very first exposure to DPCP is an appropriate time to remove the sensitisation patch and raise suction blisters for examination of local cutaneous cytokines.

Little is currently known about the kinetics or relative release of CHS induced cytokines in human skin. In line with our hypothesis, the PATCH6 group demonstrated significantly greater levels of blister fluid TNF-α and IL-10 compared with both the BSk and PATCH24 groups. This suggests that these stimulated cutaneous cytokine responses had returned to baseline levels after 24 h of patch exposure. These findings are consistent with previous work in mice showing that increased mRNA expression for TNF-α, at the site of sensitisation, began to decrease after 12 h (Enk and Katz, 1992). Unexpectedly, the blister fluid IL-1β content was not significantly different between the groups despite the grand mean of the PATCH6 and PATCH24 groups being marginally higher (+45%) than the mean unstimulated BSk IL-1β levels. This lack of significant difference is not in accordance with observations in mice, showing that IL-1β mRNA expression at the site of sensitisation increases after 15 minutes and persisted at peak levels 24 h later (Enk and Katz, 1992).

The lack of observed DPCP-induced increase in suction blister IL-1β levels may be somewhat surprising given the widely acknowledge pivotal role of IL-1β in the orchestration of cutaneous DC migration (Cumberbatch et al., 2003; Enk et al., 1993; Toebak et al., 2009). Although the precise source of blister fluid cytokines is a recognised topic of debate, in rested individuals it is believed that these are predominantly released locally within the cutaneous environment (Dearman et al., 2004). This is supported, in part, by observations of significantly different cytokine concentrations detected in suction blister fluid compared with plasma (Rhodes et al., 1999). Furthermore, *in vitro* studies have identified that epidermal KC are the main source of cutaneous TNF-α and IL-10, and that DC are also capable of releasing these cytokines upon antigen stimulation, whereas IL-1β is produced in comparatively smaller amounts by the cutaneous DC (Cumberbatch et al., 2003; Ferguson et al., 1994; Toebak et al., 2009; Uchi et al., 2000). As such, the lower levels of IL-1β following epicutaneous antigen exposure, may be more difficult to detect in human suction blister fluid compared with previous reports at the mRNA level in mice. Nevertheless, all our values were within range on the ELISA assay and even though the results were not of the magnitude that we would have expected, the TNF-α and IL-10 findings were particularly encouraging for the future use of this model to examine the effects of 120MI on these responses.

Interestingly, suction blister formation time was longest for the BSk group and shortest for the PATCH24 group, but the blister fluid cytokine content was not different between these groups. Therefore it is unsurprising that time taken to raise the suction blisters was not found to be correlated with suction blister cytokine content, suggesting that blister formation time is unlikely to play a role in these cutaneous responses. This finding for suction blister TNF-α content is different to the significant correlation observed in **Chapter 6**, indicating that

cytokine responses in suction blisters raised at the site of a DPCP sensitisation patch are not influenced by formation time.

It is recognised that a limitation of this study is that suction blisters were only examined at two time points. Therefore important information regarding the peaks and recovery of these responses may have been missed. With the original aim in mind, we also had to select time points that would work, logistically, in future studies incorporating an exercise protocol. A stronger approach would have been to raise multiple suction blisters at various time points after sensitisation, within a single group of participants. However, this would require multiple sensitisation patches and the obvious ethical issue of repeated blistering in the same individual.

In summary, the results from the present study suggest that 6 h of DPCP patch application is suitable for subsequent measurement of DPCP induced cytokine responses within suction blister fluid. The next step in this series of experiments was to utilise the suction blister technique to examine the local cytokine release at the site of DPCP sensitisation following prolonged, moderate intensity exercise.

CHAPTER EIGHT

The effect of prolonged moderate intensity exercise on the Diphenylcyclopropenone-induced changes in the cutaneous cytokine environment

8.1 SUMMARY

Prolonged, moderate intensity exercise significantly reduces the *in vivo* immune responses to DPCP (**Chapter 5**) but the underlying mechanisms for the exercise-induced alterations remains unknown. After showing the suction blister technique to be repeatable (**Chapter 6**), and after identifying an appropriate time to raise suction blisters at the DPCP sensitisation site (**Chapter 7**), the aim of this study was to investigate the effect of prolonged, moderate intensity exercise on cutaneous cytokines thought to facilitate (IL-1β and TNF-α) and inhibit (IL-10) cutaneous DC migration, at a site of DPCP exposure. Twenty one healthy males completed 120 minutes running at 60% $\dot{V}O_{2peak}$ with suction blister sampling (120-SB: mean (SD): age 25 (6) years; height 180 (8) cm; body mass 79.0 (10.8) kg) or 120 minutes of seated rest with suction blister sampling (CON-SB: mean (SD): age 23 (6) years; height 181 (9) cm; body mass 77.2 (9.5) kg). Each participant had one suction blister raised at the DPCP sensitisation site, 6 h after patch application, and one suction blister raised over untreated bare skin (BSk), on the adjacent upper inner arm, for assessment of IL-1β, TNF-α, IL-10 and total protein. Prolonged, moderate intensity exercise had no effect on cutaneous blister fluid IL-1β, TNF-α or IL-10 responses to DPCP or BSk compared with CON-SB. DPCP induced greater TNF-α and lower IL-1β levels compared with BSk but there were no differences between 120-SB and CON-SB groups. It is speculated that prolonged, moderate intensity exercise may suppress further downstream events during CHS induction, such as interactions between DC and T cells, rather than local cutaneous inflammatory processes.

8.2 INTRODUCTION

The findings reported in **Chapter 6** of this thesis showed the suction blister technique to be a repeatable tool for the measurement of cutaneous cytokines. After identifying 6 h of DPCP patch application to be a suitable time to raise suction blisters at the site of sensitisation (**Chapter 7**), the next step in this sequence of studies was to couple experimental CHS with the suction blister technique to investigate the mechanisms associated with the reductive effects of prolonged, moderate intensity exercise on induction of *in vivo* immunity.

The cutaneous cytokine environment is considered essential for local DC migration and the successful induction of CHS (Toebak et al., 2009). In support of this, local intradermal injection of IL-1β or TNF-α in humans increased migration of cutaneous DC (Bhushan et al., 2002; Cumberbatch et al., 2003) and administration of anti-TNF-α antibody prior to skin sensitisation in mice inhibited the frequency of DC in the draining lymph nodes and impaired the elicitation responses (Cumberbatch and Kimber, 1995). Furthermore, topical application of lactoferrin, which has anti-inflammatory properties, has been associated with decreased cutaneous IL-1β and TNF-α, and impaired cutaneous DC migration at the site of DPCP sensitisation in humans (Cumberbatch et al., 2003; Griffiths et al., 2001). In addition, IL-10 has been shown to suppress CHS responses in mice (Enk et al., 1994; Toebak et al., 2009; Wang et al., 1999; Yoshiki et al., 2010) and prolonged exercise has been shown to increase the production of IL-10 in response to an *in vitro* antigen challenge in humans (Svendsen et al., 2014). If prolonged, moderate intensity exercise alters the DPCP-induced changes in the cutaneous cytokine environment, this may provide some insight into the mechanisms associated with the inhibitory effect on the induction phase of CHS.

The aim of this study was to investigate the influence of prolonged, moderate intensity exercise on the cutaneous cytokines thought to be key in DC migration. It was hypothesised that, compared with rested control, prolonged, moderate intensity exercise would decrease local suction blister levels of IL-1β and TNF-α, known to facilitate cutaneous DC migration, and increase the level of IL-10, purported to inhibit cutaneous DC migration, at the site of DPCP sensitisation.

8.3 METHODS

Study design. Twenty one healthy, recreationally active males performed either 120 minutes of moderate intensity (60% $\dot{V}O_{2peak}$) exercise with suction blister sampling (120-SB (*n* = 10): mean (SD): age 25 (6) years; height 180 (8) cm; body mass 79.0 (10.8) kg; $\dot{V}O_{2peak}$ 54 (7) $ml \cdot kg^{-1} \cdot min^{-1}$)) or 120 minutes of seated rest with suction blister sampling (CON-SB (*n* = 11): mean (SD): age 23 (6) years; height 181 (9) cm; body mass 77.2 (9.5) kg; $\dot{V}O_{2peak}$ 54 (4) $ml \cdot kg^{-1} \cdot min^{-1}$)), to investigate blister cytokine responses to DPCP. There were no significant differences between groups for physical characteristics.

Experimental procedures. Anthropometric measures were recorded on arrival to the laboratory and $\dot{V}O_{2peak}$ was estimated as outlined in **Chapter 3, section 3.3**. A speed equivalent to 60% of the $\dot{V}O_{2peak}$ was calculated (see **Chapter 5, section 5.3** for details) and verified 30 minutes after the maximal exercise test. All participants were thoroughly familiarised with the laboratory equipment and experimental trial procedures. Within 3 weeks of preliminary testing, participants completed the experimental trial. On the day of the experimental trial, participants were transported to the laboratory at 0700 h and commenced exercise or seated rest at 0730 h, in a fasted state. Fluid allowance was provided as outlined in **Chapter 5, section 5.3**. A single patch containing 22.8 µl of the DPCP sensitizer (as described in **Chapter 3, section 3.4**) was applied to the inner aspect of the participant's upper arm. All participants were sensitised to DPCP 20 minutes after completing the exercise or rest, as in **Chapter 5, section 5.3**. Patches remained in place for exactly 6 h, during which participants were allowed to perform light activity under supervision and were provided with a small standardised breakfast and lunch (4.96 MJ, which was comprised of 14%, 54% and 32% of energy from protein, carbohydrate and fat respectively). Immediately following patch removal, each participant had one suction

blister raised directly over the DPCP patch site and one suction blister raised over untreated bare skin (BSk) on the adjacent upper inner arm (see **Chapter 3, section 3.5** for details). Room temperature was (mean (SD)) 22 (0.8)°C and relative humidity was 54 (5.6)%. The suction blister fluid was analysed for IL-1β, TNF-α, IL-10 concentration and total protein content (as described in **Chapter 3, section 3.6**). The intra-assay coefficient of variation for suction blister IL-1β, TNF-α, IL-10 concentrations and total protein content was 2.6%, 2.8%, 4.6% and 2.6%, respectively. All kits were performed according to the manufacturer's instructions.

Statistical analysis. Sample size was estimated to be 10 participants per group by calculating a minimum important difference using the blister fluid IL-1β repeatability data from **Chapter 6**. The alpha (Type I error rate) was set at 0.05, and power at 0.85 (1 - Type II error rate) (G*Power software, version 3.1.2). Independent *t*-tests were used to compare all physical characteristics between the CON-SB and 120-SB groups. Suction blister IL-1β cytokine data was not normally distributed but was normalised by log transformation before analysis. A two-way mixed model ANOVA was used to analyse differences in blister fluid cytokine responses between groups. All data is presented prior to transformation.

8.4 RESULTS

Suction blister formation

All suction blisters were successfully raised within 140 min. The suction blister formation time was longest at the BSk site for both the CON-SB (mean (SD): 106 (15) min) and 120-SB (112 (18) min) groups. The shortest suction blister formation time was observed at the DPCP site for the 120-SB group (70 (10) min) followed by the CON-SB group (90 (26) min).

Suction blister cytokine response to DPCP induction after prolonged, moderate intensity exercise

Prolonged, moderate intensity exercise appeared to have no effect on suction blister cytokine responses. There was no significant interaction for IL-1β ($F(1,19) = 1.2$, $P = 0.29$; Figure 8.1a), TNF-α ($F(1,19) = 0.08$, $P = 0.78$; Figure 8.1b) or IL-10 ($F(1,19) = 0.09$, $P = 0.77$; Figure 8.1c). However, a significant effect of suction blister site was identified for IL-1β ($F(1,19) = 9.1$, $P < 0.05$; Figure 8.1a) and TNF-α ($F(1,19) = 4.6$, $P < 0.05$; Figure 8.1b). This showed that IL-1β was greater at the BSk site compared with the DPCP site, whereas TNF-α levels were greater at the DPCP site compared with the BSk site ($F(1,19) = 4.6$, $P < 0.05$). Total protein was not significantly different (grand mean (SD): 30.8 (5.3) $mg \cdot ml^{-1}$).

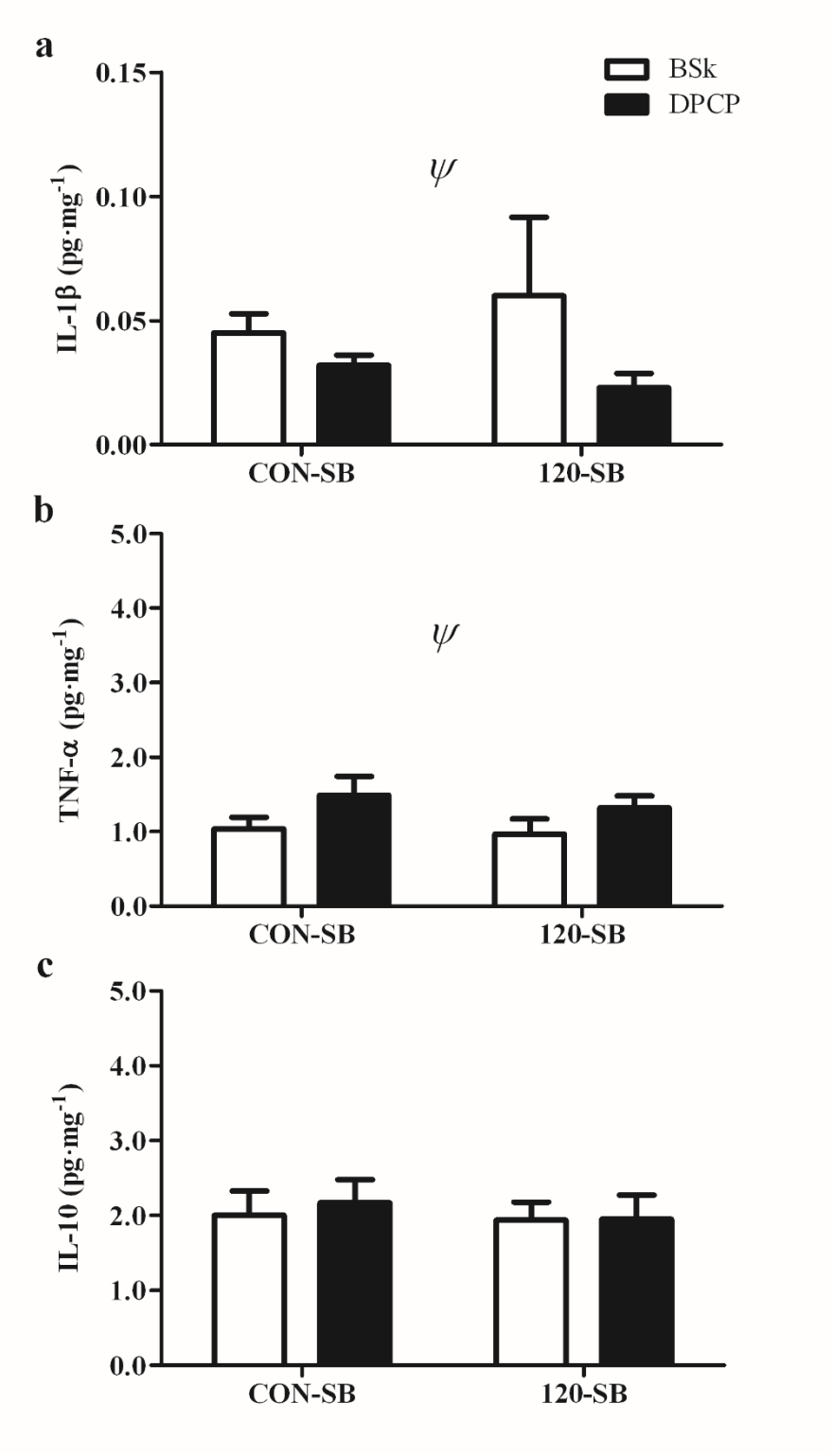


Figure 8.1. Suction blister IL-1β (a), TNF-α (b) and IL-10 (c) from untreated, bare skin (BSk) and the site of DPCP sensitisation (DPCP) after 120 minutes of seated rest (CON-SB) or 120 minutes of prolonged, moderate intensity exercise (120-SB). Data are mean (SEM). Main effect of blister site: ψ $P < 0.05$ *vs.* BSk.

8.5 DISCUSSION

The aim of this study was to investigate the possible local cutaneous mechanisms associated with the inhibitory effect of prolonged, moderate intensity exercise on the induction phase of CHS. The cutaneous cytokines of interest were IL-1β and TNF-α, thought to facilitate cutaneous DC migration, and the level of IL-10, purported to inhibit cutaneous DC migration. However, the findings from the current study did not agree with the hypothesis and instead showed that prolonged, moderate intensity exercise had no effect on blister fluid IL-1β, TNF-α or IL-10, at the site of DPCP induction or at a BSk site. These findings do not support a role for these cutaneous suction blister cytokines in the observed decrease in *in vivo* immune induction after prolonged, moderate intensity exercise.

The induction phase of experimental CHS with a novel antigen such as DPCP is considered a complex process, which is influenced by successful activation and migration of cutaneous DC towards the draining lymph nodes (Christensen and Haase, 2012). To investigate the possible inhibitory effect of prolonged, moderate intensity exercise on this process, the current study measured the cutaneous cytokines in suction blisters taken from the site of DPCP sensitisation. Unexpectedly, after prolonged, moderate intensity exercise, no significant difference in blister levels of IL-1β, TNF-α or IL-10 was observed compared with rested controls, either at the site of DPCP induction or BSk. These cytokines are considered to be important for cutaneous DC migration and the successful induction of CHS (Cumberbatch et al., 2003; Toebak et al., 2009). The current findings differ from previous research in humans where topical application of lactoferrin, which has been associated with decreased IL-1β and TNF-α, impaired cutaneous DC migration at the site of DPCP sensitisation (Griffiths et al., 2001). Also, studies in mice have shown that intradermal injection of anti-IL-1β, anti-TNF-α, TNF-α receptor antagonist

or IL-10 prior to skin sensitisation inhibited DC migration to the draining lymph nodes and impaired the later elicitation responses (Cumberbatch et al., 1997; Cumberbatch and Kimber, 1995; Enk et al., 1993; Enk et al., 1994; Nishibu et al., 2007).

The current findings are, however, in line with recent work in humans showing that prolonged, moderate intensity exercise had no effect on cutaneous inflammatory responses (measured as erythema) to the irritant croton oil (CO) (Diment et al., 2015). CO stimulates a local, non-T-cell mediated, inflammatory response after a single exposure (Nosbaum et al., 2009). In addition, work in mice indicates that after UVB irradiation, IL-10 induced suppression of CHS occurs further downstream, not at the skin but at the site of the draining lymph nodes and the authors suggest that this suppressive signal is transmitted by epidermal DC (Yoshiki et al., 2010). Considered together with the unaltered response to irritant challenge, it is possible that prolonged, moderate intensity exercise influences activities further downstream, which cannot be detected in suction blister fluid. This may include interactions between DC and T cells in terms of antigen processing and presentation, and activation of T cells, and the subsequent balance between effector and regulatory T cells, considered central to the successful induction of CHS (Toebak et al., 2009). In addition, it is acknowledged that prolonged, moderate intensity exercise may suppress production of key chemokines, such as CCL19, CCL21 and CXCL12, which are important for migration of cutaneous DC into the dLN (Kabashima et al., 2007). This migration is mediated by expression of the corresponding chemokine receptor (i.e. CCR7 and CXCR4) on the surface of cutaneous DC (Toebak et al., 2009). Exercise has previously been associated with reduced chemokine expression on monocytes (Hong and Mills, 2008) and T cells (Bishop et al., 2009). Therefore, it is possible that downregulation or inhibition of the expression of chemokine receptors on DC may also be involved in the suppression of *in vivo* immune induction after prolonged, moderate intensity exercise.

Given that we were only able to raise suction blisters at a single time point, it is possible that an effect of exercise on DPCP-induced changes in cutaneous cytokines was missed. However, a further observation from the current study was that the CON-SB cytokine values showed no effect of DPCP induction compared with the CON-SB values at the BSk site. This finding is not in accordance with the results from rested participants in **Chapter 7**, which demonstrated increased TNF-α and IL-10 in blisters raised 6 h after DPCP induction compared with BSk (albeit in different participants). The main effect of patch site, observed for TNF-α in the current study, partially supports the previous findings of **Chapter 7,** but rather disappointingly the CON-SB mean values for IL-1β, TNF-α and IL-10 at the DPCP site were all much lower than the PATCH6 mean values from **Chapter 7**. The reason for this remains unclear given that the groups underwent the same experimental protocol, such as the same DPCP sensitisation and suction blister procedure, at the same time of day, within the same environmental conditions. This could not be explained by differences in the assay because the standard curves were the same for the different studies and the values were within the same range. One possible explanation is that seasonal variation may have influenced the differences in results found between these studies. The data for **Chapter 7** was collected between the months of February to June and for **Chapter 8**, this was collected between August and November. The prevalence of self-reported URI symptoms has been found to be four times greater during the winter months compared with the summer months (Matthews et al., 2002) but further research is required to determine the influence of seasonal variation on cutaneous responses to DPCP.

A limitation of the approach adopted in the current study is that we did not actually assess cutaneous DC migration per se, but instead measured the levels of key mediators of this process, namely IL-1β, TNF-α and IL-10 (Christensen and Haase, 2012; Toebak et al., 2009).

It is recognised that the modest correlation previously observed between suction blister formation time and TNF content of blisters raised at BSk sites (Chapter 6), is a limitation for the interpretation of this measure in the current study. However, differences in cutaneous responses between CON-SB and 120-SB, at the DPCP patch site, were of greatest interest here, which were shown not to be related to formation time (Chapter 7). Nevertheless, the adoption of punch biopsies, which are not subject to differences in sample timings, may provide a more suitable comparative control for measures taken from untreated, BSk, in future research.

We cannot rule out the possibility that prolonged exercise may evoke changes in other cutaneous cytokines, epidermal basement membrane degrading enzymes, chemokines and expression of chemokine receptors that have also been implicated in the maturation and migration of antigen-bearing DC towards the draining lymph nodes, albeit to a lesser extent than the cytokines examined here (Toebak et al., 2009). Alternative factors of interest that have received attention for their involvement in DC migration include, but are not limited to, IL-1α (Wang et al., 1999), IL-18 (Antonopoulos et al., 2008), granulocyte macrophage colony-stimulating factor (GM-CSF) (Kremer et al., 2000), matrix metalloproteinase (MMP) activity (Kobayashi et al., 1999), CCL21/CCL19-CCR7 and CXCL12-CXCR4 interactions (Kabashima et al., 2007), which warrant further investigation. Future human research should now consider utilising skin biopsies as a direct measure of DC migration at the sensitisation site, to confirm the conclusions of the current study and those of Diment et al. (2015). Additional animal research might also unravel reductive effects of prolonged, moderate intensity exercise on events further downstream in CHS induction.

In conclusion, this is the first study to explore cutaneous cytokine responses as a possible mechanism associated with the reductive effects of prolonged, moderate intensity exercise on induction of *in vivo* immunity with DPCP. Here this was investigated using a suction blister technique to examine the cutaneous cytokine responses at the site of DPCP sensitisation and BSk. The lack of observed differences following prolonged, moderate intensity exercise may indicate that suppression of CHS occurs on activities further downstream during induction, rather than on local inflammatory processes. However, given that the blister cytokine responses for the current rested control participants were unable to replicate the results in **Chapter 7**, it is recommended that a direct measure of cutaneous DC migration is examined within this exercise and experimental CHS model, to support these initial conclusions.

CHAPTER NINE

General Discussion

9.1 General Discussion

Athletes and military personnel often experience physical stressors, such as prolonged exercise and periods of nutritional insufficiency, which can impair immune function and subsequently may increase an individual's susceptibility to infection (Walsh et al., 2011b). Ethical and practical considerations naturally limit the ability to study prolonged periods of intensive training and nutritional restriction within laboratory settings. Alternatively, field studies with military personnel provide an attractive model to study the effects of these stressors (Hoyt and Friedl, 2006). However, well-controlled field studies have not been conducted to show whether providing additional energy intake to reduce the energy deficit prevents the decrease in immune function during military training.

To date, the effect of physical stressors on immune function in humans has typically relied upon *in vitro* measures of immunity, which involves isolated analysis of immune cells in artificial environments (Akbar et al., 2013). The importance of utilising *in vivo* measures of immune function, such as experimental CHS to the novel antigen DPCP, has recently been highlighted (Albers et al., 2013). Work by our group has shown that physical stressors such as high altitude exposure (Oliver et al., 2013) and prolonged, moderate intensity exercise (Harper Smith et al., 2011) impair CHS responses to DPCP, but the effect of intensity and duration of exercise or the mechanisms associated with the altered responses have not been examined. In particular, exercise-induced changes in the cutaneous cytokine environment, at the site of antigen exposure, was of interest here because this initial event is believed to be pivotal for successful immune induction (Toebak et al., 2009). A suction blister technique can be used to provide information about the local cutaneous environment (Akbar et al., 2013).

Therefore the main objectives of this thesis were to investigate: 1. the effect of daily mixed nutritional supplementation, in a well-controlled field study, on immune indices during an 8-week arduous training programme; 2. the use of DPCP as a tool to assess *in vivo* immunity and the effects of intensity and duration of exercise stress; 3. the repeatability of raising controlled suction blisters for the measurement of cutaneous cytokines; 4. the kinetics of local cytokine release in response to DPCP sensitisation; and 5. the effect of prolonged, moderate intensity exercise on DPCP-induced changes in the local cutaneous cytokine environment, to provide an insight into the possible mechanisms involved in the suppression of these CHS responses.

Firstly, **Chapter 4** sought to address some of the limitations of previous field studies examining nutritional interventions by including a comparative control group and by standardising sample collection timings. The results indicated that daily mixed nutritional supplementation can modulate selected resting immune indices during an 8-week arduous training programme, in the absence of a treatment effect on circulating cortisol. As expected, findings showed that 8 weeks of daily mixed nutritional supplementation prevented the training-induced decrease in circulating total leukocyte, lymphocyte and monocyte counts. This finding is in line with previous work on resting immune indices, indicating that energy restriction (Laing et al., 2008b) and heavy training schedules (Horn et al., 2010) can both lead to lower circulating total and differential leukocyte counts. The observation of decreased leukocyte counts may be of interest because this finding was shown to coincide with peak URI incidence during a Parachute Regiment training course (Whitham et al., 2006). An alternative line of thinking is that modest training-induced decreases in circulating leukocyte numbers, that remain within the clinical range of normal values, reflects an adaptive response that is not in fact pathological (Horn et al., 2010). Although the responsible mechanisms remain unclear, possible

explanations for the training-induced decrease in circulating leukocyte counts may include decreased bone marrow production, increased apoptosis, plasma volume expansion, redistribution of cells to other compartments, or a combination of these factors. Slightly disappointingly, **Chapter 4** reported no differences at post-field exercise, for circulating neutrophil count or the functional measure of bacterially stimulated neutrophil degranulation, between treatment groups. However, a particularly novel finding of this study was that nutritional supplementation increased saliva SIgA secretion rate during training, which could be considered beneficial to host defence. Levels of saliva SIgA are of great interest to immunology research because they are one of few measures that have repeatedly been associated with the risk of developing URI in physically active individuals (Gleeson and Bishop, 2013). For example, when compared with symptom free participants, athletes with recurrent self-reported symptoms of URI had lower saliva SIgA secretion rates (Gleeson et al., 2012) and soldiers experiencing a prolonged period of training, within a multi-stressor environment, experienced decreased saliva SIgA concentration and increased URI symptoms (Tiollier et al., 2005). A further strength of **Chapter 4** was that saliva SIgA was presented as a concentration and as a secretion rate, which reflects the total availability of SIgA at the oral surface (Walsh et al., 1999). Although a decrease in SIgA concentration or secretion rate was not observed with the modest energy deficit reported (2.2 $MJ \cdot day^{-1}$; **Chapter 4**), it is anticipated that training with a more severe energy deficit (~11 $MJ \cdot day^{-1}$) may have compromised saliva SIgA availability (Oliver et al., 2007). Decreased plasma cell IgA synthesis (Revillard and Cozon, 1990) and decreased availability of polymeric Ig receptor (Ha and Woodward, 1998) provide possible explanations for lower saliva SIgA during energy deficit but further studies are required to determine potential roles for an increase in these factors during increased nutritional supplementation.

Chapter 4 highlighted that daily mixed nutritional supplementation can modulate selected immune indices during training despite relatively modest body mass losses (6% for CON and 2% for SUP) compared with other military studies (~16% during a 2-month army ranger course). These findings are likely to be relevant to a wider population of individuals, who frequently experience similar body mass losses, such as athletes undergoing heavy training and those making weight for competition. It should also be considered that a modest loss in body mass (mainly fat mass) may not be detrimental to all military personnel and athletes, such as those with a high starting fat mass, and could be interpreted as a positive training adaptation in these individuals (Friedl et al., 1994). At this point, it is unclear whether the magnitude of immune modulation reported in **Chapter 4** translates into altered risk of illness, and it is acknowledged that this presents a major challenge for all future stress immunology research. It is also recognised that a stronger approach to utilising circulating cell numbers and *in vitro* immune measures is to adopt an *in vivo* measure of immune function, which represents an integrated, whole-body immune response. It was our original intention to also examine experimental CHS responses to DPCP in **Chapter 4**, as was adopted in **Chapters 5, 7** and **8**, but unfortunately this was not possible at the time because the work from our group, which provides preliminary support for the use of this measure in experimental stress immunology, had not yet been published (Harper Smith et al., 2011; Oliver et al., 2013). The use of this *in vivo* measure of immunity in training studies, to assess the influence of similar feeding regimes, presents an interesting avenue for future research (Figure 9.1).

To the best of our knowledge **Chapter 5** presented the first study to compare the effects of intensity and duration of continuous exercise stress on *in vivo* immunity, assessed by use of an experimental CHS model, in humans. In accordance with previous findings, 120MI significantly inhibited the induction of new immunity via the skin (Harper Smith et al., 2011),

however 30MI or 30HI did not effect this response. This finding for 30MI is in line with recent research showing no effect of short, moderate exercise, when examining *in vivo* vaccination responses, in young, healthy adults (Long et al., 2012), but is at odds with one suggestion of the J-shaped model, that moderate exercise is immune-enhancing (Nieman, 1994). To date, this hypothesis of the J-shaped model has largely been supported by observations that regular, moderate exercise training may lower the risk of URI in individuals with sub-optimal baseline immune responses or when compared with sedentary individuals (Fondell et al., 2011; Nieman et al., 1990b; Nieman et al., 1993). Therefore our finding of no effect of a single bout of short, moderate intensity exercise on *in vivo* immunity may be unsurprising, particularly when considering the point that for healthy, young individuals, elevating immunity above a level which is considered 'optimal' would be undesirable and may in fact be just as detrimental as suppressed immunity, by increasing disease susceptibility to conditions associated with allergy, hypersensitivity and autoimmunity (Walsh et al., 2011b). Based on limited evidence from *in vitro* work, showing that short lasting, high intensity exercise decreases measures of lymphocyte and neutrophil function (Nieman et al., 1994; Robson et al., 1999), it was hypothesised that 30HI would decrease *in vivo* immune induction to DPCP. The results of **Chapter 5** do not support this despite identifying significant differences in circulating stress hormones following 30HI. However, this does support recent recommendations for athletes to employ shorter spike sessions within their training, to limit the risk of training-induced impairments in immune health (Walsh et al., 2011a). **Chapter 5** also showed that in the 120MI group, a threshold DPCP dose of 4.4 times greater was required to induce a similar elicitation response to that of the CON group, which highlights the magnitude of this reductive effect. The clinical implications of this finding include a potential increased risk of infection, particularly in the endurance athlete who may perform long duration exercise several times a week. Interestingly, research in HIV patients typically reports that opportunistic infections

become more frequent when total circulating $CD4^+$ T cells fall by at least ~55% (Janeway et al., 2001), and in healthy individuals, circulating cell numbers are often modulated by 15-25% in response to exercise, but it is unclear whether this, or the magnitude of 120MI-induced suppression of DPCP responses, is great enough to increase susceptibility to infection (Walsh et al., 2011b). Although *in vivo* measures of immunity are considered more informative than the commonly used *in vitro* measures, it is recognised that investigating the clinical significance of experimental CHS responses in athletes presents an important avenue for future research (as discussed further in **section 9.1.1**).

Chapters 4 and **5** provide limited support for a role of circulating stress hormones in the modulation of immune responses. A role for stress hormones in mediating immune alterations following prolonged exercise or heavy training is widely accepted (Pedersen and Hoffman-Goetz, 2000), and nutritional restrictions, capable of elevating stress hormones are suggested to further compound the negative effects of such exercise (Gleeson et al., 2004; Makras et al., 2005). Despite this, daily nutritional supplementation modulated selected resting immune indices during an 8-week training programme, even though a treatment effect for circulating cortisol was not observed (**Chapter 4**), and a single bout of short duration, high intensity exercise, which elevated circulating catecholamines and resulted in greater circulating cortisol, did not impair *in vivo* immunity (**Chapter 5**). The modest energy restriction experienced by soldiers in **Chapter 4** likely explains the limited effect observed on resting circulating cortisol concentration. For example, it has previously been shown that when soldiers trained with a 16 $MJ \cdot d^{-1}$ energy deficit, circulating cortisol increased 32% but these level returned to baseline when the energy deficit was reduced (Kyrolainen et al., 2008). The findings from **Chapter 5** are not in accordance with previous murine models showing that intradermal injections of corticosterone or catecholamines, both locally or distant from the sensitisation site, suppress

DTH and CHS responses (Dhabhar and McEwen, 1999; Flint et al., 2001; Seiffert et al., 2002). However, this is not the first study to report a lack of association between circulating stress hormones and *in vivo* immune responses, particularly in human studies, which present less consistent results than the murine models (Altemus et al., 2006; Edwards et al., 2006; Oliver et al., 2013). One possible explanation is that human studies rely on individual snapshot assessments of circulating stress hormones, which may miss important information regarding the peaks and recovery of these responses. In addition to the classical stress-related hormones, such as cortisol and catecholamines, alternative factors that may mediate the effects of stress on CHS responses include calcitonin gene-related peptide (Asahina et al., 1995; Niizeki et al., 1997), pituitary adenylate cyclase-activating peptide (Kodali et al., 2003), substance P (Niizeki et al., 1997) and vasoactive intestinal polypeptide (Ding et al., 2012), which require further research to investigate the potential role of these in exercise-induced reduction of CHS responses. Furthermore, examination of long term training and nutritional interventions may benefit from more long term assessments of stress hormones, such as hair cortisol levels, which provides a retrospective reflection of integrated cortisol secretion over a period of several months (Stalder and Kirschbaum, 2012).

To better understand the possible mechanisms associated with the inhibitory effect of 120MI on immune induction to DPCP, **Chapters 6, 7** and **8** examined the local cutaneous cytokine environment of cytokines previously identified to play key roles in the activation and migration of cutaneous DC (e.g. IL-1β, TNF-α and IL-10), which is considered instrumental in the initial events of immune induction (Dearman et al., 2004; Toebak et al., 2009). As a starting point, a suction blister technique was utilised to examine these cytokines in blister fluid because this is less invasive than removing the blister roof or performing punch biopsies, which requires local anaesthesia, particular laboratory expertise and biopsies can also result in a small scar (Kiistala,

1968). Firstly, **Chapter 6** examined the repeatability of the suction blister technique for measuring IL-1β, TNF-α and IL-10 in blister fluid content of non-sensitised BSk in rested individuals. A particular strength of this study was that a battery of recommended statistical techniques, which cover both the relative reliability and absolute reliability, were examined (Atkinson and Nevill, 1998). The reliability statistics were deemed acceptable for all cytokines of interest, although it was recognised that all suction blister CV_w% values may be considered quite high (range: 25%-41%). Nevertheless, these findings were similar to other indices commonly used with single, one-off measurements to study inflammatory markers, such as serum cytokines (Gonzalez et al., 2001), or various markers of mucosal immunity (Dwyer et al., 2010). Given that immune induction with DPCP can only be performed once in an individual, it was not possible to investigate the suction blister repeatability at the site of DPCP patch application, thus the approach adopted in **Chapter 6** was considered most appropriate.

Chapter 7 identified 6 h as a suitable time to remove a DPCP sensitising patch and commence a suction blister technique at the patch site, for measurement of stimulated cutaneous cytokine responses. This represented the first study to examine suction blister cytokine responses during sensitisation with experimental CHS, in rested humans. Suction blister levels of TNF-α and IL-10 showed significant increases after 6 h compared with 24 h of DPCP patch application and BSk. This was in line with previous work in mice, examining cytokine responses at the site of antigen exposure (Enk and Katz, 1992) but given the widely acknowledged pivotal role for IL-1β in the facilitation of cutaneous DC migration, the lack of a significant increase for this cytokine was unexpected (Cumberbatch et al., 2003; Enk et al., 1993; Toebak et al., 2009). Still, the potential effect of prolonged exercise on DPCP-induced changes in cutaneous IL-1β responses was unknown. Therefore, even though it is recognised that the findings from **Chapter 7** were not of the magnitude that we would have expected, we proceeded with some

optimism to utilise this measure to examine responses following 120MI. In particular, this decision was encouraged by the DPCP stimulated IL-10 responses, which may be increased further by exercise, given the recent report that prolonged exercise increased the production of IL-10 in response to an *in vitro* antigen challenge (Svendsen et al., 2014).

Ideally the work in **Chapter 7** may have examined more than two time points, within a single group of participants, to determine further information regarding the peaks and recovery of the cytokine responses. However, time points had to be selected that would work logistically in future studies of 120MI, and multiple sensitisation patches and suction blistering within the same individual may not be deemed the most ethical approach.

Chapter 8 did not support a role for cutaneous measures of IL-1β, TNF-α or IL-10 in the inhibitory effect of 120MI on *in vivo* immune induction with DPCP. This differs from one line of research where topical application of lactoferrin, which acts as an anti-inflammatory agent and has been associated with decreased IL-1β and TNF-α, impaired cutaneous DC migration at the site of DPCP sensitisation (Cumberbatch et al., 2003; Griffiths et al., 2001). The findings were, however, in keeping with an alternative line of research showing that 120MI had no effect on cutaneous inflammatory responses to an irritant (Diment et al., 2015), which, taken together, may suggest that 120MI suppresses further downstream activities in the induction of new immune memory, as discussed in **section 9.1.1**. Somewhat disappointingly, the rested control participants in **Chapter 8** did not experience the same DPCP-induced increase in TNF-α or IL-10 blister fluid content compared with BSk that was observed in **Chapter 7**. These results are difficult to explain because the groups experienced the same experimental protocol, within the same environmental conditions and there were no differences in the standard curves of the assays. One possible explanation is that seasonal variation may have influenced the

differences in results found between these studies. The data for **Chapter 7** was collected between the months of February to June and for **Chapter 8**, this was collected between August and November. It is widely acknowledged that the incidence of URI is influenced by seasonal variation, with the prevalence of self-reported URI symptoms four times greater during the winter months compared with the summer months (Matthews et al., 2002) but it is currently unknown how this may effect cutaneous responses to DPCP. Ideally seasonal variation would have been controlled for in these studies but unfortunately this was not possible due to the time constraints of a PhD. It is acknowledged that investigation of the effect of seasonal variation on both cutaneous cytokine responses and CHS responses (i.e. skinfold and erythema) presents an interesting avenue for future research.

It would have been of interest to utilise the suction blister technique with all exercise groups studied in **Chapter 5**, however the time restraints of this PhD would not have allowed for this and it was deemed most appropriate to further investigate 120MI, which demonstrated a significant inhibitory effect on the CHS responses. **Chapters 6, 7** and **8** did not measure cutaneous DC migration, but instead utilised the suction blister technique as a less invasive measure of the mediators of this process, namely IL-1β, TNF-α and IL-10. Future approaches to investigate the mechanisms associated with the inhibitory effect of 120MI on CHS responses to DPCP are discussed in the following section and illustrated in Figure 9.1.

9.1.1 Perspectives and future directions for experimental CHS in stress-immunology research

Experimental CHS provides an attractive measure of *in vivo* immunity, not only because the skin is immediately accessible but because it overcomes many of the limitations of commonly used *in vitro* measures, which are lacking in terms of clinical significance and practicality. It is recognised that there are limitations with using DPCP in the CHS model described. Given that

DPCP is benign, determining the clinical significance of the response, with specific regard to infection (skin and other) is an important avenue for future research. Preferably, the strength of the cutaneous recall response to DPCP could be generalised beyond skin immunity to indicate the immune system's general ability to respond to an infectious challenge. The available evidence is encouraging in this regard as cutaneous immune measures have been used to assess immunocompetency in individuals with Hodgkin's disease (Brown et al., 1967), Crohn's disease (Jones et al., 1969) and HIV (Levis et al., 2006) and have also been shown to be impaired during acute infectious illness (Bennett et al., 1998). As previously mentioned, it would be beneficial for a future study to determine if DPCP responses are impaired in athletes during a period of URTI (Figure 9.1). One viewpoint is that the benign characteristic of DPCP actually overcomes the ethical constraints associated with using live pathogens, such as rhinovirus to assess *in vivo* immunity. It is also recognised that experimental CHS requires purposefully inducing CHS; nevertheless, the selected doses we use are low and the mild elicitation responses are temporary (these typically peak at 48 h).

Experimental CHS with DPCP is practical, safe, and can be administered without the need for expensive equipment, invasive injections or blood sampling, making it a suitable immunological tool for both laboratory and field investigations. Moreover, the use of a novel antigen such as DPCP provides investigators with rigorous control over the timing and dose of sensitising exposure, enabling the effects of various stressors on the primary immune response to be studied. The measurement of DTH responses to KLH is an alternative per-cutaneous *in vivo* method, also reported to represent a primary immune response (Smith et al., 2004c). However, since KLH is derived from a shellfish this may explain why some individuals exhibit significant responses to KLH prior to immunisation (Smith et al., 2004a). Experimental CHS with DPCP is not restricted to examining the effects of stress on the induction phase. Recently

work from our laboratories has shown that this approach can be used to assess the effect of stress on the elicitation phase in participants who, following repeated monthly DPCP skin challenges, achieved a reproducible plateau in responses (Harper Smith et al., 2011). This method may be of interest for examining changes in immune status of athletes or military personnel during training, alternatively, providing that there is a comparative control group, in some experimental models, it may not be necessary for participants to achieve a plateau prior to starting an intervention. Furthermore, the standardised CHS model overcomes some of the limitations of vaccine models of *in vivo* immunity including variable immunogenicity, annual changes in vaccine and difficulty when comparing the circulating antibody results from different studies using in-house ELISAs (Burns, 2012; Hernandez-Bernal et al., 2011). Nevertheless, a standard protocol for measuring CHS elicitation responses in humans has yet to be established. The use of erythema to quantify CHS elicitation has been questioned, particularly at sites of stronger responses, where yellow vesicles can interfere with the erythema (redness) readings (Harper Smith et al., 2011; Narbutt et al., 2005). This may provide some explanation as to why the erythema responses in **Chapter 5** did not reach significance following 120MI when all groups were compared, despite the significantly reduced skinfold thickness. Erythema measurement is also confounded by dark skin colour which further adds to the support for why skin oedema (inflammatory swelling) is considered the key measure of CHS responses (Harper Smith et al., 2011). The participants in **Chapters 5, 6, 7** and **8** had Caucasian skin-type to allow for the measurement of both skin oedema and erythema. However, it is unclear whether racial differences would affect skin oedema responses to DPCP, should future research select to use this measure in isolation (Friedmann and Pickard, 2010). Notwithstanding the degree of subjectivity, a particular strength of the **Chapter 5** findings is that skinfold thickness was strongly related with dermal thickness measured by a high-frequency ultrasound scanner and read by a blinded investigator ($r = 0.93$). As such, this agrees

with the recommendation of others that, skinfold callipers present a simple and cost-effective measure of CHS oedema (Cooper et al., 1992; Narbutt et al., 2005). It is noteworthy that a possible effect of factors such as altered cutaneous temperature and cutaneous blood flow on induction of experimental CHS with DPCP is unknown. Therefore, the studies to date that have coupled experimental CHS with exercise (Harper Smith et al., 2001; **Chapter 5** and **Chapter 8**) have selected to sensitise participants to DPCP 20 minutes after exercise cessation to allow cutaneous blood flow to return to baseline (Kenny et al., 2008). Future research may want to examine these factors further.

When selecting immunological assessment tools for their studies, investigators need to give careful consideration to the aspect(s) of immunity of interest, clinical relevance, biological significance and feasibility (Albers et al., 2013). With this in mind, and after carefully considering the strengths and limitations, it is suggested that adopting the CHS model described herein will facilitate our understanding of the influence of stress on *in vivo* immunity in humans.

Figure 9.1 summarises suggested areas for future research utilising experimental CHS with DPCP and highlights potential approaches to investigate the mechanisms associated with the inhibitory effect of 120MI. Firstly, in addition to utilising experimental CHS to examine nutritional and training interventions, as was our original aim for **Chapter 4**, the duration of the inhibitory effect of 120MI on CHS induction remains unknown. This could be determined in a follow-up study that delays the timing of sensitisation after 120MI to enable a more complete understanding of the time course involved in the restoration of immunity after heavy exercise. In addition, a limitation of **Chapters 4, 5, 6, 7** and **8** was that only young, healthy males were included. Sex differences in immune responses to exercise have previously been

reported (Gillum et al., 2011), but this has not been well studied in relation to experimental CHS. A limited number of studies have investigated the effect of sex differences on CHS responses in rested individuals, which has shown mixed results of either greater responses in males or greater responses in females (Friedmann and Pickard, 2010). Therefore investigating sex differences in CHS responses to exercise is encouraged. Although the sensitising dose of DPCP used in our model of experimental CHS was chosen to be on the linear part of the sensitising dose-response curve (Friedmann et al., 1983; Harper Smith et al., 2011), another follow-up study might investigate the potentially immune-enhancing effects of short duration, moderate intensity exercise on the induction to DPCP, using half the sensitising dose in elderly individuals or those with sub-optimal immunity, as previously shown with an influenza vaccine model (Edwards et al., 2012). Furthermore, it is acknowledged that the immune system incorporates a large variety of defence mechanisms and it is possible that exercise may have differential effects on CHS, a cutaneous T-cell mediated response, and other *in vitro* and *in vivo* immune measures. An experimental approach that compares the effect of stress on the development DPCP responses with alternative measures such as the *in vivo* vaccine-induced antibody response, a systemic B-cell mediated response, would be of interest. It is our hope that experimental CHS with DPCP will continue to be used as a measure of *in vivo* immunity, not only to study exercise stress, but also to investigate the effect of alternative stressors such as environmental extremes, psychological stress and nutritional interventions, on immune modulation.

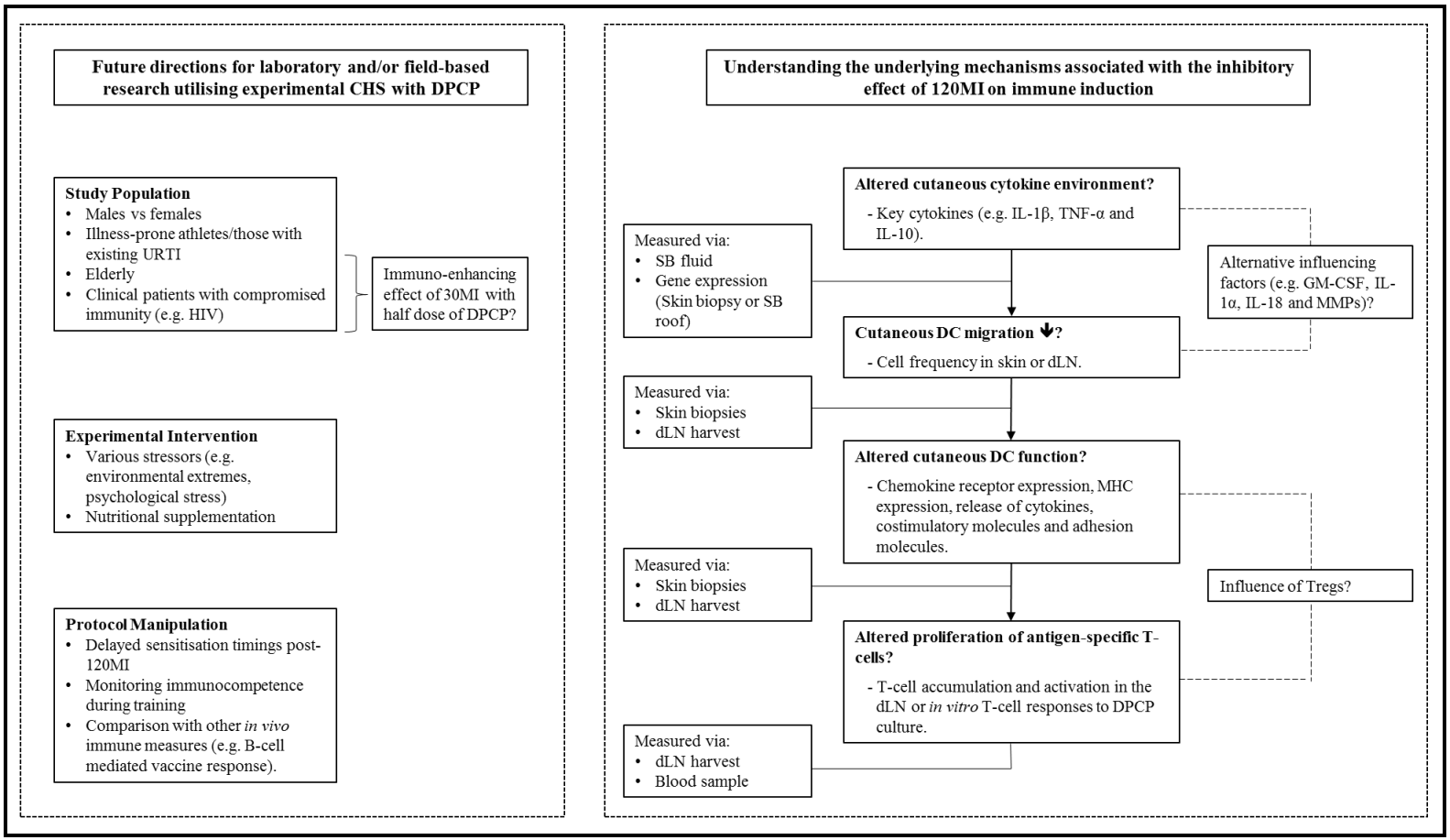
With consideration of the key events involved in the sensitisation phase of CHS (Figure 2.1), it is recognised that there are a number of possible approaches to examine the mechanisms associated with the inhibitory effect of 120MI on immune induction to DPCP, which present exciting avenues for future research (Figure 9.1). In order to further the work presented in this

thesis, the next step to follow on from examination of key suction blister fluid cytokines (**Chapters 6, 7** and **8**) may involve punch biopsies to directly measure cutaneous DC migration and/or to examine mRNA expression of cytokines, as has previously been adopted in human dermatological research of CHS (Dearman et al., 2004; Griffiths et al., 2001). It is also recognised that prolonged exercise may influence alternative mediators of the migration of antigen-bearing DCs towards the dLN, such as other cutaneous cytokines (e.g. IL-1α, IL-18, GM-CSF), epidermal basement membrane degrading enzymes (e.g. MMPs) and expression chemokine receptors (e.g. CCR7 and CXCR4) on the surface of cutaneous DC, which could be investigated in future human research (Toebak et al., 2009). In addition, it cannot be ruled out that such exercise may have a suppressive effect on the maturation and ability of these cells to present the immunogen to naïve T cells within the dLN. The maturation of DC is closely linked to the migration, and both processes are influenced by many of the same factors (Christensen and Haase, 2012). DC function has previously been shown to be influenced by factors such as MHC expression and the release of cytokines, costimulatory molecules (e.g. CD80 and CD86) and adhesion molecules (e.g. ICAM-1) (Christensen and Haase, 2012; Toebak et al., 2009). Detection of these factors is often limited in cutaneous samples compared with examinations of DC in the dLN, thus their measurement in humans may favour *in vitro* stimulation experiments with isolated skin cells (Nuriya et al., 1996; Yokozeki et al., 1998). Very few human studies have attempted to use an *in vivo* system at the dLN, to investigate DC or other lymph node cells during sensitisation to a contact antigen (Hunger et al., 2001; Yawalkar et al., 1998). This is unsurprising given that it requires an invasive microsurgical technique for cannulation of a peripheral lymph vessel. Therefore, to complement the human *in vitro* cutaneous DC investigations, studies using animal models may be required to examine the effects of prolonged exercise on events further downstream in CHS induction, such as the number and function of DC within the dLN and their ability to activate T cell responses (Figure

9.1). In particular, this animal work would also allow further investigation of the effect of both endogenous and exogenous regulatory T (Tregs) cells in the exercise-induced suppression of CHS. Recently the role of Tregs in CHS has received increasing interest and findings indicate that Tregs can influence the functional capabilities of DC, and the subsequent priming of naïve t cells, within the dLN (Honda et al., 2011; Ring et al., 2010).

An alternative approach to further investigation in humans, would be to quantitatively demonstrate differences in an *in vitro* assay of DPCP-specific T cell proliferation, between 120MI and CON participants. This could be achieved with blood samples taken several weeks after sensitisation and using similar methods to those adopted to investigate the mechanisms underlying CHS responses to the antigen, DNCB (Pickard et al., 2007). From this point it may then be possible to further characterise the responses in terms of T cell subtype numbers and stimulated cytokine production to identify which elements of the cellular immunological response have been affected by 120MI.

Figure 9.1. Overview of possible future research approaches to further our understanding of the influence of stress on *in vivo* CHS responses to DPCP.



Abbreviations = CHS, contact hypersensitivity; DC, dendritic cell; DPCP, diphenylcyclopropenone; IL, interleukin; TNF-α, tumour necrosis factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; MMPs, matrix metalloproteinases; SB, suction blister; dLN, draining lymph node; MHC, major histocompatibility complex; Tregs, T regulatory cells; HIV, human immunodeficiency virus; URTI, upper respiratory tract infection; 120MI, prolonged, moderate intensity exercise; 30MI, short, moderate intensity exercise; ↓ decrease.

9.2 Conclusions

The major conclusions from this thesis are:

1. A daily mixed nutritional supplement, which reduced the energy deficit during an 8-week arduous military training programme, prevented the training-induced decreases in rested circulating leukocyte, lymphocyte and monocyte counts and increased saliva SIgA. Differences in these immune indices occurred despite no effect of daily mixed nutritional supplementation on circulating cortisol concentration. The increase in saliva SIgA with nutritional supplementation may reduce susceptibility to URI.

2. Using experimental CHS with DPCP as a measure of *in vivo* immunity, results demonstrate that prolonged, moderate intensity exercise decreases the induction of DPCP responses measured 28 d later. Short-lasting moderate intensity or short-lasting high intensity exercise had no effect on these *in vivo* immune responses.

3. Little support was provided for an involvement of circulating cortisol or catecholamines in the mechanisms associated with altered *in vivo* immune response to DPCP. This was shown by significantly elevated circulating catecholamines (*vs.* pre-exercise) and greater circulating cortisol (*vs.* rested controls) after short-lasting, high intensity exercise but immune induction was unaffected.

4. The suction blister technique is a repeatable tool for measurement of the cutaneous cytokine environment on untreated bare skin. Blister fluid IL-1β, TNF-α and IL-10 levels produced similar relative and absolute reliability results to those previously reported for serum cytokines. Presenting blister fluid cytokines as a concentration or relative to total protein did not affect the outcome of the results.

5. Six hours after the very first exposure to DPCP provided an appropriate time for subsequent measurement of local DPCP-induced cutaneous cytokine responses within suction blister fluid.

6. Prolonged, moderate intensity exercise had no effect on blister fluid IL-1β, TNF-α or IL-10, at the site of DPCP induction or at an untreated bare skin site. These findings do not support a role for these cutaneous suction blister cytokines in the observed decrease in *in vivo* immune induction after prolonged, moderate intensity exercise. Therefore, it is possible that prolonged, moderate intensity exercise may suppress further downstream events of immune induction, rather than local inflammatory processes at the skin.

7. After carefully considering the strengths and limitations, the topical application of DPCP appears to provide an attractive tool for the assessment of exercise stress on *in vivo* immunity.

8. A main focus for future research should be to investigate the clinical significance of experimental CHS responses in athletes, preferably by taking measurements during a period of URTI.

9. To better understand the mechanisms associated with the inhibitory effect of prolonged exercise on CHS induction with DPCP, examination of cutaneous DC activation and migration in human punch biopsies should be complemented by animal studies that are able to investigate further downstream events of immune induction, within the dLN.

References

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Appendix A: Example Informed Consent Form

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.

Please tick boxes

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

2 I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights being affected. ☐

3 I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason. If I do decide to withdraw I understand that it will have no influence on the marks I receive, the outcome of my period of study, or my standing with my supervisor, other staff members of with the School. ☐

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

5 I agree to take part in the above study. ☐

Name of Participant …….

Signature ……. Date ……..

Name of Person taking consent…….

Signature ……. Date ……..

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

Appendix B: Example Medical Questionnaire

Bangor University
SCHOOL OF SPORT, HEALTH AND EXERCISE SCIENCES

Name of Participant ……..……..

Age …… Height ..... Body Mass .....

Are you in good health? ☐ YES ☐ NO

If no, please explain

How would you describe your present level of activity?
Tick intensity level and indicate approximate duration.

Vigorous		Moderate		Low intensity	

Duration (minutes)……

How often?

< Once per month		2-3 times per week	
Once per month		4-5 times per week	
Once 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 from allergies? ☐ YES ☐ NO

If yes, please give particulars:

Do you suffer, or have you ever suffered from:

	YES	NO		YES	NO
Asthma			Epilepsy		
Diabetes			High blood pressure		
Bronchitis			Eczema or dermatitis		
Immune deficiency			Tendencies to abnormal scarring (e.g. keloid)		

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:

Do you smoke? ☐ YES ☐ NO

Have you, or are you presently taking part in any other laboratory experiment?
☐ YES ☐ NO

PLEASE READ THE FOLLOWING CAREFULLY

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

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

PLEASE COMPLETE AND SIGN THE DECLARATION BELOW

DECLARATION

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

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

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

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

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

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

Print name ……..

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

Print name ……..…...