DOCTOR OF PHILOSOPHY

The influence of anxiety, psychological stress and exercise on an In vivo measure of immune competence

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THE INFLUENCE OF ANXIETY, PSYCHOLOGICAL STRESS AND EXERCISE ON AN *IN VIVO* MEASURE OF IMMUNE COMPETENCE

By

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SUMMARY

Prolonged bouts of exercise and periods of chronic training induce transient immune perturbations, but the clinical relevance of these changes remain questionable with the majority of work relying on *in vitro* measures of immunity. As such, there is a need to utilise *in vivo* measures of immunity that likely relate better to infection than *in vitro* measures because they extend beyond isolated blood measures and involve whole-body integrated immune responses. Sensitisers such as diphenylcyclopropenone (DPCP) induce cutaneous responses by the activated chemical specific T-cells upon first exposure that require a period of time for the formation of 'immune memory' resulting in a boosting of responses upon re-exposure. As such, DPCP may provide an attractive tool for scientists to examine the influence of stressors on *in vivo* T-cell mediated immunity that allows control over the timing of initial exposure in relation to stressors. Indeed, decreases in contact hypersensitivity (CHS) responses to DPCP induction after prolonged exercise have previously been shown (Diment *et al.,* 2015; Harper Smith *et al.,* 2011). However, irritant properties of the antigen may influence the sensitising potential, and it remains unknown whether the influence of stressors on *in vivo* immunity to DPCP reflect localised responses at the skin mediated primarily by localised irritant properties or reflect a systemic *in vivo* immune response. In addition, there is strong supporting evidence that DPCP responses relate to clinical outcomes in immune deficient populations, but the association with regard to upper respiratory illness (URI) in otherwise healthy populations remains to be determined.

Psychoneuroimmunologists have long since acknowledged the role of psychological stress on immunity, with psychological factors likely playing a role in the decrease in immunity with prolonged heavy exercise and heavy training. However, empirical evidence supporting this notion is lacking and there is a need to bridge the gap between exercise immunology and psychoneuroimmunology by examining the role of psychological factors on exercise-immune modulation. With this information in mind, the broad aim of this thesis was to examine the role of anxiety and psychological stress on the *in vivo* immune response, and investigate their role in exercise-immune modulation, and examine the clinical relevance of DPCP with regard to URI.

Firstly, we demonstrated no influence of prolonged exercise stress on local cutaneous processes to a local irritant (croton oil), supporting the notion that the previously observed decrease in CHS responses to DPCP induction after prolonged exercise likely represents a systemic suppression of *in vivo* immunity (**Chapter 4**). We then re-interrogated our previous data which examined the influence of exercise intensity and duration on *in vivo* immunity, and found that low-moderate levels of state-anxiety and perceived psychological stress before exercise play an important role in determining the strength of the *in vivo* immune response after exercise, irrespective of the intensity and duration (**Chapter 5**). We next sought to determine the role of low, moderate and high levels of anxiety prior to an acute psychological stressor on *in vivo* immunity, and observed that the DPCP response was lower in those reporting low and high anxiety, supporting an inverted-U association between the a priori level of anxiety and the *in*

vivo immune response after an acute psychological stressor (**Chapter 6**). We then demonstrated lower DPCP responses during a common cold (**Chapter 7**), and prospectively showed an association between DPCP responses and URI, whereby, the CHS response to DPCP induction was a significant predictor of peak URI severity, and lower CHS responses were observed in those who reported more severe URI episodes compared with those reporting less severe URI episodes. Moreover, low DPCP responders reported more severe URI episodes, longer total URI duration and greater total URI symptom scores compared with high DPCP responders (**Chapter 8**). Taken together, these findings provide promising preliminary support for the clinical utility of experimental CHS using DPCP.

In conclusion, the findings of this thesis highlight the salient role of psychological factors on *in vivo* immunity, and their role on exercise-immune modulation, and provide promising initial support for the use of DPCP as a clinically relevant measure of *in vivo* immunity. The findings within this thesis support the recommendation that exercise scientists account for anxiety and psychological stress when examining the immune response to exercise, and that exercise immunologists and psychoneuroimmunologists are encouraged to utilise *in vivo* measures of immunity such as experimental CHS using DPCP.

DECLARATION AND CONSENT

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

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PUBLICATIONS

Publications arising from work presented within this thesis

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Chapter 5: Edwards J. P., Walsh N. P**.,** Diment B. C. and Roberts R. (2018). Anxiety and perceived psychological stress play an important role in the immune response after exercise. *Exerc Immunol Rev*, 24: 26-34.

Additional publications

Hanstock H. G., Edwards J. P. and Walsh N. P. (2019). Tear lactoferrin and lysozyme as clinically relevant biomarkers of mucosal immune competence. *Front Immunol,* 10: 1178.

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Conference proceedings

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Harrison S. E., Edwards J. P., Oliver S. J. and Walsh N. P. (2018). The effect of vitamin D supplementation by sunlight or oral D3 on mucosal immunity in military recruits. Verbal presentation at the inaugural United Kingdom Society of Exercise Immunology, Loughborough University, April 2018. Presented by S. Harrison.

TABLE OF CONTENTS

THESIS FORMAT

This thesis contains a critical literature review that outlines the main research aims (**Chapter 2**) and a general methods section (**Chapter 3**) that provides information on procedures and measures adopted in experimental chapters. Five experimental chapters form the main focus of this thesis.

The general discussion (**Chapter 9**) summarises the main findings of the thesis, recognising the limitations of the research and suggested potential areas for future research. Lists of abbreviations, tables and figures appear prior to **Chapter 1** and **bold type** is used to refer to chapters and sections within this thesis.

LIST OF TABLES

LIST OF FIGURES

hypersensitivity (CHS) assessed as the summed dermal thickening and erythema response to the full dose-series elicitation challenge with diphenylcyclopropenone (DPCP) 28 d after DPCP induction.

Figure 5.2 Effect of state-anxiety prior to exercise on the *in vivo* immune response after exercise. (A) Low (LOW) and moderate (MOD) levels of anxiety. Data are Mean ± SD. (B) Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. Dermal thickening response to the full dose-series challenge with DPCP is shown (30MI and 30HI). Data are Mean ± SEM for clarity. [1]Shown for comparison from Diment *et al.* (2015).

Figure 5.3 Effect of state-anxiety prior to exercise on the *in vivo* immune response after exercise of varying intensity and duration. (A−D) Summed increase in skinfold thickening response to DPCP challenge for each exercise group (30MI, 30HI and 120MI) and rested CON. Data are Mean ± SD.

Figure 6.1 Effect of state-anxiety prior to an acute psychological stressor on the subsequent *in vivo* immune response. Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. A. Level of state-anxiety (STAI-S). B. Summed increase in dermal thickening response to DPCP challenge. C. Summed increase in erythema response to DPCP challenge. Data are shown as Mean ± SD. # $P < 0.05$, ## $P <$

0.01 significant difference *vs.* MOD. [1]Shown for comparison from Diment *et al.* (2015).

Figure 6.2 Effect of state-anxiety prior to an acute psychological stressor on the subsequent *in vivo* immune response. Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. Dermal thickening response to the full dose-series challenge with DPCP is shown. Data are shown as Mean ± SEM for clarity. †† $P < 0.01$ LOW *vs.* MOD; ‡ $P < 0.05$ HIGH *vs.* MOD.

Figure 7.1 Comparison of common cold duration and severity between individuals with an unconfirmed upper respiratory illness (U-URI) and individuals with a pathogen confirmed upper respiratory tract infection (URTI). A. common cold duration. B. common cold peak severity. Data are shown as Mean ± SD.

Figure 7.2 Comparison of *in vivo* immunity in individuals reporting upper respiratory symptoms (URS) and individuals not reporting symptoms (HEALTHY). Contact hypersensitivity assessed as the summed response to elicitation challenge with diphenylcyclopropenone (DPCP) 28 days after DPCP induction. A. URS *vs.* HEALTHY for summed dermal thickening response. B. URS *vs.* HEALTHY for summed erythema response. Data are shown as Mean ± SD.

Figure 8.1 Flow chart depicting study protocol.

Figure 8.2 Comparison of *in vivo* immunity in those reporting low and high upper respiratory illness (URI) severity. Contact hypersensitivity (CHS) assessed as elicitation challenge 14 d after diphenylcyclopropenone (DPCP) induction. Dermal thickening response to the full dose-series challenge with DPCP is shown. URI symptom severity defined as the maximum reported URI symptom severity score. LOW URI severity and HIGH URI severity were determined by median split. Low URI severity had significantly lower summed increases in dermal thickness compared with High URI severity. Data are Mean ± SEM for clarity.

Figure 8.3 Comparison of upper respiratory illness (URI) peak symptom severity (A), total URI duration (B) and total URI symptom score (C) between low and high *in vivo* immune responders, using skin sensitisation to diphenylcyclopropenone (DPCP). Contact hypersensitivity (CHS) assessed as the summed increase in dermal thickening response to elicitation challenge with DPCP 14 d after DPCP induction. Peak URI symptom severity defined as the maximum reported URI symptom severity score. Total URI duration defined as the total number of days reporting URI. Total URI symptom score defined as the total number of days reporting URI x URI symptom severity score. An URI bout was defined as an URI score ≥ 6 on at least two consecutive days. LOW DPCP and HIGH DPCP were determined by median split based on the summed increase in dermal thickening response to DPCP challenge. Data presented are mean ± SD.

Figure 8.4 Comparison of *in vivo* immunity in Epstein-Barr virus seronegative and seropositive individuals. Contact hypersensitivity (CHS) assessed as the summed increase in dermal thickening response to elicitation challenge with diphenylcyclopropenone (DPCP) 14 d after DPCP induction. Data presented are mean ± SD.

LIST OF ABBREVIATIONS

°C	degrees celsius
120MI	prolonged, moderate intensity exercise trial
120MI-CO	prolonged, moderate intensity exercise and croton oil trial
30HI	short, high intensity exercise trial
30MI	short, moderate intensity exercise trial
ACTH	adrenocorticotropic hormones
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APC	antigen presenting cells
BML	body mass loss
bpm	beats per minute
BRUMS	brunel mood scale
CD	cluster of differentiation
CHS	contact hypersensitivity
CI	confidence interval
cm	centimetre
CMI	cell-mediated immunity
CMV	cytomegalovirus
CO	croton oil
CON	control trial
CON-CO	control and croton oil trial
CS	conditioned stimulus
CV	coefficient of variation
d	day

DC	dendritic cell
DNCB	dinitrochlorobenzene
DNFB	dinitrofluorobenzene
DPCP	diphenylcyclopropenone
DTH	delayed type hypersensitivity
EBV	epstein-barr virus
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ES	effect size
g	gravitational force
GET	gaseous exchange threshold
GH	growth hormone
GP	general practitioner
h	hour
HAART	highly active anti-retroviral treatment
HEALTHY	healthy
HIGH	high anxiety group
HIGH DPCP	high diphenylcyclopropenone responders
HIV	human immunodeficiency virus
HPA	hypothalamic-pituitary-adrenal axis
HR	heart rate
HRV	human rhinovirus
HSD	honest significant difference
ICAM	intacellular adhesion molecule
ICC	intra-class correlation coefficient

IL	interleukin
IP	illness-prone
IPAQ	international physical activity questionnaire
KC	keratinocyte
K_3EDTA	tripotassium ethylenediamin tetra-acetic acid
kg	kilogram
KLH	keyhole limpet hemocyanin
km	kilometre
LC	langerhans cell
LOW	low anxiety group
LOW DPCP	low diphenylcyclopropenone responders
µg	microgram
µL	microlitre
MHC	major histocompatibility complex
MJ	megajoule
MET	metabolic equivalent
mL	millilitre
mm	millimetre
min	minute
MOD	moderate anxiety group
NBM	nude body mass
NK	natural killer
nmol	nanomole
OR	odds ratio
PAR	primary allergic response

PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PSS	perceived stress scale
qPCR	quantitative real-time polymerase chain reaction
RER	respiratory exchange ratio
RI	rarely-ill
RPE	rating of perceived exertion
SAM	sympathetic-adrenal-medullary axis
SD	standard deviation
SEM	standard error of the mean
STAI	state-trait anxiety inventory
STAI-S	state aspect of the state-trait anxiety inventory
STAI-T	trait aspect of the state-trait anxiety inventory
Tcm	central memory t-cells
TCR	t-cell receptor
Treg	regulatory t-cells
Trm	resident memory t-cells
TNF	tumour necrosis factor
TRIMP	training impulse
UCS	unconditioned stimulus
U-URI	unconfirmed upper respiratory illness
URI	upper respiratory illness
URS	upper respiratory symptoms
URTI	upper respiratory tract infection
VCA	viral capsid antigen

$\dot{V}O_{2peak}$	peak oxygen uptake

CHAPTER ONE

General Introduction

CHAPTER ONE

General Introduction

Increased upper respiratory illness (URI) episodes have been reported in athletes during periods of heavy training and following prolonged exercise bouts (Peters and Bateman, 1983; Nieman *et al.*, 1990; Walsh *et al.*, 2011b). Decreases in immune function due to the influence of various stressors encountered by athletes are, at least in part, likely responsible for increased URI incidence (Shephard and Shek, 1995, 1997; Shephard, 1998; Walsh *et al.*, 2011b; Walsh, 2018). For instance, prolonged heavy exercise stress is widely acknowledged to transiently decrease *in vitro* measures of immune function in isolated blood samples (Shek *et al.*, 1995; Kakanis *et al.*, 2010; Walsh *et al.*, 2011b). Psychological stress is also widely acknowledged to impact infectious outcomes and host defence (Cohen *et al.*, 1991; Dhabhar, 2014), with athletes and military personnel often encountering situations that may increase psychological stress and anxiety e.g. athletes in competition, infantry training, parachute jumping etc. In addition, athletes and military personnel may experience psychological stress in their personal life related to relationship difficulties, financial hardship and bereavement (Walsh, 2018). There are common pathways by which psychological stress and exercise stress modulate immunity, principally via the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic-adrenal-medullary (SAM) axis that release stress hormones widely acknowledged to modulate immune function (Dhabhar, 2018). Moreover, there are parallels by which physical stress and psychological stress modulate immunity, whereby, moderate levels of physical and psychological stress upregulate aspects of immunity, whereas chronic periods of psychological stress and heavy periods of training downregulate immunity (Walsh *et al.*, 2011a; Dhabhar, 2018). The role of anxiety and psychological stress in the decrease in immunity associated with heavy exercise and training is widely speculated (Perna *et al.*, 1997; Clow and Hucklebridge,

2001; Walsh and Oliver, 2016), but unfortunately, little empirical evidence exists supporting this assertion as many exercise immunology studies fail to report measures of psychological stress. One such study reported exacerbated *in vitro* immune alterations around a marathon in those reporting higher levels of perceived psychological stress (Rehm *et al.*, 2013), but no evidence currently exists utilising *in vivo* measures of immunity.

The use of clinically relevant immune measures is of paramount importance within exercise immunology and psychoneuroimmunology to provide researchers with confidence about whether the influence of stressors on immune impairment increases susceptibility to infection. It is often not practical to follow-up investigations with infection incidence as a gauge of the clinical meaningfulness of changes in immunity, particularly in rigorously controlled laboratory studies with relatively small sample sizes (Campbell and Turner, 2018). Impairments in *in vitro* immune measures provide limited information about the clinical significance of the observed immune alterations as they may behave differently to those that are activated *in vivo* (Vedhara *et al.*, 1999; Akbar *et al.*, 2013). *In vivo* approaches to assessing immune function are considered more clinically relevant and likely relate better to infection than *in vitro* markers because they extend beyond blood measures and involve challenging individuals with antigenic stimuli and assessing relevant antigen-driven responses (Albers *et al.*, 2005; Albers *et al.*, 2013). The skin provides a particularly attractive location to assess *in vivo* immunity given that it represents the largest and most exposed interface with the external environment and contains a wide variety of immune cells to protect the host (Hickey, 2013). One approach involves using experimental contact hypersensitivity (CHS) using novel antigens such as diphenylcyclopropenone (DPCP) that induce cutaneous responses by the activated chemical specific T-cells upon first exposure, forming immune memory that results in a boosting of responses upon re-exposure. As such, the utility of novel antigens such as DPCP

allows researchers to rigorously control the timing of initial exposure in relation to stressors, the dose of exposure and the time elapsed since sensitisation, thus providing a valuable tool for scientists within the fields of stress and exercise immunology. The evidence that DPCP relates to clinical outcomes is promising in individuals with compromised immune function (Bathgate *et al.*, 2001; Levis *et al.*, 2006), but it remains to be determined whether experimental CHS to DPCP relates to URI in otherwise healthy populations.

The immunosuppressive influence of prolonged heavy exercise on both the primary and secondary *in vivo* immune response to DPCP has previously been reported (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). However, sensitising antigens such as DPCP also contain localised irritant properties at the skin, and it remains to be determined whether the influence of exercise stress on DPCP responses reflects localised processes at the skin or points towards a systemic immune response. One approach to answer this question is to examine the influence of heavy exercise on local cutaneous responses at the skin using an irritant such as croton oil that produces localised responses when epicutaneously applied to the skin. With this information in mind, the objectives of this thesis were to 1. determine whether stress-induced alterations to DPCP reflect localised processes at the skin or point towards systemic alterations in immunity 2. investigate the influence of psychological factors on the *in vivo* immune response and the role of psychological factors in exercise-immune modulation 3. examine the clinical relevance of *in vivo* immunity to DPCP with regard to URI.

CHAPTER TWO

Literature Review

CHAPTER TWO

Literature Review

2.1 Exercise stress and upper respiratory illness

2.1.1 Incidence of upper respiratory illness

Most adults experience 2-5 URI episodes per year, which are one of the most common reasons for General Practitioner (GP) visits (Monto and Sullivan, 1993; Heikkinen and Jarvinen, 2003; Eccles, 2005; Gulliford *et al.*, 2009). Upper respiratory illnesses are also of serious concern to athletes as they are the most common non-injury related medical complaint, accounting for 35-65% of reported visits to the medical team (Gleeson and Pyne, 2016). For example, approximately 5-8% of athletes reported illnesses at recent Winter and Summer Olympics, with 41-63% of these affecting the respiratory system (Engebretsen *et al.*, 2010; Engebretsen *et al.*, 2013; Soligard *et al.*, 2015; Soligard *et al.*, 2017). Indeed, there is a suggestion that athletes experience more URI episodes than the general population, likely due to the influence of numerous stressors on host defence, potentially resulting in an inability to compete, impaired performance during competition, and/or disruption to training. (Walsh *et al.*, 2011a; Walsh *et al.*, 2011b; Svendsen *et al.*, 2016).

The suggestion that athletes are at greater risk of URI was proposed in a landmark paper by Peters and Bateman (1983), who were the first to show that runners were twice as likely to experience URI in the 14 days following the Two Oceans Marathon in Cape Town than non-participating controls who shared the same living space (33% *vs.* 15%). Additionally, an inverse relationship between race time and URI, and between training load and URI was reported indicating that higher level athletes who were more likely to have heavier training loads were also more likely to experience URI. These findings were followed up in a large

cohort, whereby, 13% of marathon runners reported URI in the week following the event compared to 2% of trained runners who did not take part in the race (Nieman *et al.*, 1990). Furthermore, an association between training volume and URI was also reported, whereby, runners with the highest training volume (> 96 km/week) were twice as likely to experience a pre-race illness compared to those with the lowest training volume (< 32 km/week) (Nieman *et al.*, 1990). In contrast, the same author reported that moderate levels of physical activity reduces URI symptoms, albeit in small numbers of participants, and sedentary and elderly women (Nieman *et al.*, 1993). These findings led to the 'J-shaped' model, whereby, those undertaking moderate levels of exercise are at lower risk of experiencing URI, whereas sedentary individuals and those undergoing heavy exercise regimens have a greater risk of URI (Nieman, 1994). Studies have provided further support for the 'J-shaped' model whereby moderate physical activity was associated with reduced risk of URI in one study (Matthews *et al.*, 2002), and another study reported higher incidence rates of URI in both untrained sedentary control participants and highly trained athletes compared to recreational athletes (Spence *et al.*, 2007). However, not all studies report increased URI following prolonged exercise bouts and the view that elite athletes suffer frequent URI symptoms is paradoxical given the high volumes of training required (i.e. increased illness means the athlete can train less) (Ekblom *et al.*, 2006). As such, an 'S-shaped' association between exercise load and risk of infection has been proposed, indicating that elite athletes suffer fewer infections than non-elite athletes (Malm, 2006). There is some support for this notion, albeit in limited studies and a small number of athletes, where one study indicated an inverse association between training load and URI (Martensson *et al.*, 2014), and another demonstrated that international athletes suffer fewer URI episodes than national-level athletes (Hellard *et al.*, 2015). In addition, other factors have been shown to be associated with URI in elite athletes such as time of year (increased risk of URI during winter months) and a recent episode of illness (Hellard *et al.*, 2015). As such, the

causes of URI episodes in athletes are likely multifactorial, with behavioural and lifestyle factors (e.g. hygiene, infection avoidance, diet, sleep, and psychological stress) likely contributing to URI incidence.

2.1.2 Aetiology of upper respiratory illness in athletes

Undertaking a prolonged bout of exercise or intense periods of training are associated with increased URI incidence, but it remains unclear whether this increase in URI is due to infective causes as many studies have used self-report measures of URI (Cox *et al.*, 2008). Given the commonality between symptoms of URI, airway inflammation and reactivity to airborne allergens, it is possible that many self-reported cases of URI are non-infectious. An association between allergy and URI has been reported, with 58% of runners reporting URI following the London Marathon compared to 19% of non-runners, with 40% of these runners considered positive for allergy (Robson-Ansley *et al.*, 2012). Furthermore, only 30% of URI have been confirmed as upper respiratory tract infection (URTI) in elite athletes (Spence *et al.*, 2007). However, this study was conducted during the southern hemisphere summer when the average ambient temperature was approximately 28 °C and the common cold incidence is low (likely higher allergy in summer). Studies conducted during the peak common cold season of autumn and winter have shown much higher incidence of true infections (~70 - 80%) (Hanstock *et al.*, 2016; Valtonen *et al.*, 2019). Nevertheless, a study that was conducted year-round also reported a 30% URTI incidence rate, yet physician assessment documented a viral or bacterial aetiology in 90% of cases (Cox *et al.*, 2008). This discrepancy should be interpreted cautiously because despite advancements in molecular biology dramatically improving pathogen detection, there are over 200 serologically different viral types responsible for respiratory infections, thus limiting our ability to detect all pathogens (Eccles, 2005). Furthermore, newly identified viruses within the last 20 years highlight the potential for previously unknown pathogens to

cause common cold like illnesses, and thus it cannot be discounted that physicians are diagnosing respiratory infections that are not identified using the current pathogen detection methods (Van den Hoogen *et al*., 2001; Heikkinen and Jarvinen, 2003; Sloots *et al*., 2006). Nevertheless, there is a need for physician assessment to be used in conjunction with virology and bacteriology analysis to confirm the presence of infection where possible. However, virology and bacteriology analysis is both expensive and time consuming, limiting its applicability for athletes. In situations where these methods cannot be used, there is a need for validated self-report measures of assessing URI as a proxy for URTI. The Jackson Common Cold Scale has been widely used to subjectively identify the presence of URI, with the Wisconsin Upper Respiratory Symptom Survey (WURSS) more recently gaining attention as an alternative common cold questionnaire (Jackson *et al*., 1958; Barrett *et al*., 2002). The Jackson Common Cold Scale was chosen for use in this thesis for a number of reasons; firstly, the Jackson scale was validated by participants who were nasally inoculated with common cold pathogens, with the time-course and severity of symptoms subsequently assessed (Jackson *et al*., 1958). The WURSS validation used semi-structured interviews by members of the research team to determine the prevalence of symptoms, and thus has not been validated using nasal inoculation of respiratory pathogens, or even with pathology confirmation (Barrett *et al*., 2002). Secondly, the Jackson Common Cold Scale contains only 8 symptom items whereas the initial WURSS contains 44-items, thus increasing the burden for participants to complete on a daily basis, although 21 and 11-item scales have since been developed. Irrespective of the choice of self-report assessment method, the utility of biomarkers to determine infectious aetiology is required in the future, with some evidence supporting the use of novel biomarkers to determine virology and bacteriology for the purpose of reducing antibiotic prescriptions within GP surgeries. For instance, the assessment of C-reactive-protein to determine whether an infection is likely of bacterial origin has been used to determine whether antibiotics should be prescribed

(Cals *et al.,* 2007; Cals *et al.,* 2009; Cals *et al.,* 2010). However, the majority of infectious causes in athletes are of viral origin, with bacterial infections only accounting for approximately 5% of cases (Spence *et al.,* 2007; Cox *et al.,* 2008).

2.1.3 The impact of upper respiratory illness on training and performance

Irrespective of the cause of URI (infectious and non-infectious), the impact on athletic populations is especially important resulting in either an inability to compete, impaired performance during competition, and disruption to training (Reid *et al.*, 2004). For instance, illnesses caused over 30% of missed training sessions in elite British athletes from 30 different Olympic sports (EIS, 2012). Furthermore, high performance endurance athletes are at risk of experiencing recurring URI episodes, partly due to the high-volume and continued demands of training. For instance, 55% of athletes who reported an URI experienced recurring infections, a finding that was corroborated by the presence of Epstein-Barr virus reactivation in 22% of athletes (Reid *et al.*, 2004). This study suggests an association between recurrent infections and performance impairments given that only three participants (8%) reported no decrement in performance (Reid *et al.*, 2004).

Although it is generally agreed that URI disrupts high-volume athletic training, somewhat surprisingly, less is known about the influence of URI (infectious and non-infectious) on athletic performance (Martensson *et al.*, 2014; Walsh and Oliver, 2016). There have been numerous anecdotal accounts of athletes having impaired performance during competition, or even missing events due to URI, but empirical evidence supporting this notion is lacking. Accordingly, runners who were symptomatic when starting a race had a 2-fold increased risk of not finishing the race compared to asymptomatic control runners in a cohort of over 7000 participants (Van Tonder *et al.,* 2016). Furthermore, Pyne *et al.* (2001) conducted one of the

few studies examining an association between URI and impaired performance: although the authors showed no statistically significant findings, trends were observed for an association between URI and impaired competitive performance in elite swimmers. The authors then provided further support that URI can impair swimming performance in a larger sample, whereby, the association between URI and performance was trivial for female swimmers, but had a modest negative effect on male swimmers (Pyne *et al.*, 2005). However, evidence supporting this assertion in other sports is lacking and remains a topic ripe for investigation. Nevertheless, for elite athletes, even small decrements in performance may be vital to the outcome of competitive events, and evidence suggests that URI can impair alertness, reaction time, and information processing (Smith *et al.*, 1998; Smith, 2012; Smith and Jamson, 2012). As such, it is important to identify risk factors that contribute to increased URI susceptibility and consequently minimise the risk of URI in athletes.

2.2 Psychological stress and upper respiratory illness

Psychological stress is generally accepted to have a profound influence on multiple aspects of immunity and is widely considered to increase the risk of numerous clinical diseases including rheumatoid arthritis, cancer, and cardiovascular disease, and is associated with the progression from human immunodeficiency virus (HIV) to acquired immune deficiency syndrome (AIDS) (Leserman, 2000; Reiche *et al.*, 2004; Cohen *et al.*, 2007b; Dimsdale, 2008). Studies examining whether psychological stress confers increased susceptibility to infectious disease date back to the 1960s (Jensen and Rasmussen, 1963), with considerable evidence supporting an association between psychological stress and URI accumulating since then (Jacobs *et al.*, 1970; Graham *et al.*, 1986; Cohen *et al.*, 1991; Cobb and Steptoe, 1996; Mohren *et al.*, 2005; Pedersen *et al.*, 2010). One of the first studies to identify an association between psychological stress and URI prospectively monitored 235 adults for URI symptoms for a period of six months, and classified

into ‘low’ and ‘high’ stress based on a median split: those classified as ‘high stress’ had significantly more URI episodes and more days experiencing symptoms than those classified as ‘low stress’ (Graham *et al.*, 1986). These findings were subsequently investigated experimentally by one of the pioneers of psychoneuroimmunology, Dr. Sheldon Cohen, who performed a landmark study whereby participants were intentionally exposed to common cold pathogens, quarantined and monitored for the development of infection and symptoms: a dose-response association was observed between psychological stress and acute infectious respiratory illness (Cohen *et al.*, 1991). It is therefore somewhat surprising that the role of psychological stress in the aetiology of URI has largely been ignored in athletes. Recent work has identified psychological factors such as perceived psychological stress and depression as key predictors of illness in elite athletes (Drew *et al.*, 2017; Drew *et al.*, 2018). For instance, strong odds ratios (OR) of 8.4, 3.3 and 3.8 for depression, anxiety and psychological stress clearly highlight the influence of these factors on URI risk in athletes (Drew *et al.*, 2017).

High-level athletes experience intense physical and psychological demands that may affect their psychological well-being and mental health (Rice *et al.*, 2016). Sources of life stress experienced by athletes include, but are not limited to, injury or illness, team meetings, contract negotiations, player transfers, long-distance relocation, worries about performing poorly, interpersonal conflict and limited financial resources or possibility of losing funding (Noblet and Gifford, 2002). Psychological stress also likely contributes to other stressors such as impaired sleep that is associated with URI susceptibility (Cohen *et al.*, 2009; Prather *et al.*, 2015). Moreover, the psychological effects of chronic heavy training (e.g. overreaching) are generally accepted, as evidenced by reported mood disturbances being one of the key predictors to assess overreaching and overtraining (Meeusen *et al.*, 2013). Furthermore, the physical demands experienced by athletes and military personnel may compromise mental wellbeing,

increasing symptoms of anxiety and depression (Peluso and Guerra de Andrade, 2005). As such, it is likely that psychosocial stress at least partly accounts for, or modulates the influence of exercise on host defence and illness incidence (Walsh and Oliver, 2016). In support of this notion, one study has shown that regular physical activity had beneficial effects on URI incidence in those reporting high stress, but not low stress (Fondell *et al.*, 2011). In addition, stress management interventions can reduce the number of days competitive athletes are out due to illness and injury (Perna *et al.*, 2003), and improve antibody titres to vaccination (Davidson *et al.*, 2003). As such, studies are required to begin to disentangle the complex interactions between physical and psychological stress on URI susceptibility and immune modulation (Walsh, 2018).

2.3 Stress and immune function

The immune system is the collection of cells, tissues and molecules that play an integral role in providing defence against pathogenic microorganisms including viruses, bacteria, protozoa and fungi (Gleeson and Bosch, 2013; Peake *et al.*, 2017). The various components of the immune system can be partitioned into two general arms: innate (non-specific) and acquired (specific) immunity. Innate immunity is the first line of defence against infectious pathogens and comprises of physical and chemical barriers, phagocytes and non-specific natural killer cells. The acquired immune system primarily aims to combat infections by preventing colonisation of pathogens, keep them out of the body, and to specifically identify and destroy invading pathogens. This highly specialised system is considered the second line of defence, and comprises lymphocytes that proliferate and serve numerous functions including B cell antibody production, cytotoxic T cell killing and the development of memory T cells (Walsh, 2018). The major difference between innate and adaptive immune responses is that adaptive immunity generates immunological memory to specific pathogens that allows accelerated and

augmented responses upon subsequent exposure. The process of immunological memory will be discussed in further detail in **section 2.6**. While classifications such as 'innate' and 'adaptive' are useful to conceptualise different types of immune responses, *in vivo* immune responses consist of intricate and synchronous interactions among numerous proteins, cytokines, and cell types that include components of innate and adaptive immune systems (Vivier and Malissen, 2005).

Numerous definitions have been proposed for the concept of stress since the pioneering work of Walter B Cannon (who coined the 'fight or flight' response) and Hans Selye (who coined the term 'stress') in the 1930s. An integrated definition states that stress is a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight or flights systems in the body (stress response) (Dhabhar and McEwen, 1997). Although related, anxiety is a term often used interchangeably with 'stress', but there are differences between these terms: state anxiety is described as an unpleasant emotional state to demands or dangers, characterised by feelings of tension, apprehension, nervousness and worry, trait anxiety is a relatively stable personality characteristic, and psychological stress is the degree to which situations in one's life are appraised as stressful (Cohen *et al.*, 1983; Spielberger, 1983). The influence of physical and psychological stress on immune function will be discussed in the following sections.

2.3.1 Exercise stress and immune function

The most widely documented exercise-induced effect on immunity is an increase in the number of leukocytes into the circulation, which was first documented by Larrabee (1902) over 100 years ago. This mobilisation of leukocytes into circulation is driven by the demargination of leukocyte reservoirs as a result of increased blood flow (shear stress) and the actions of

catecholamines reducing adhesion molecule expression (Peake *et al.*, 2017). Moreover, an influx of immature neutrophils from the bone marrow also contributes to this leukocytosis following prolonged exercise bouts which consists primarily of neutrophils and lymphocytes (Shek *et al.*, 1995). The lymphocytosis observed during and immediately after exercise is dependent on the intensity and duration of the exercise, and is followed by the numbers of these circulating cells falling below pre-exercise levels during the early stages of recovery, before steadily returning to resting values (Robson *et al.*, 1999; Walsh *et al.*, 2011b). This post-exercise lymphocytopenia has previously been thought to indicate immunosuppression (Shek *et al.*, 1995; Kakanis *et al.*, 2010). However, this phenomenon has been challenged, whereby, the reductions in blood lymphocyte counts following exercise reflects a transient redistribution of immune cells to peripheral tissues, resulting in a heightened state of immune surveillance and immune regulation (Kruger *et al.*, 2008; Campbell and Turner, 2018). Nevertheless, a multitude of immune measures are transiently decreased following acute bouts of heavy exercise (> 90 min duration) and periods of chronic intensive exercise training which are thought to provide an 'open window' for opportunistic infections (Table 2.1). A recent meta-analysis of 24 studies supported this notion by concluding that mitogen stimulated lymphocyte proliferation is impaired following acute prolonged bouts of exercise (Siedlik *et al.*, 2016). Interestingly, this effect was only observed outside of competitive events with enhanced lymphocyte proliferation observed following competitive events, inferring that other behavioural and psychological factors are potentially involved in the observed decrease in immune function in athletes and military personnel. For example, one study surprisingly reported an increase in lymphocyte proliferation following an adventure cycling race (Tossige-Gomes *et al.*, 2014) and another study observed an increase in lymphocyte proliferation immediately after an Olympic distance triathlon (Bassit *et al.*, 2000). However, only a small subset of studies mention a putative role of psychological stress or anxiety in exercise-immune

modulation, with only a handful of original investigations attempting to either manipulate psychological stress or include objective measures of psychological stress (Huang *et al.*, 2010; Moreira *et al.*, 2011; Rehm *et al.*, 2013; Rehm *et al.*, 2016).

Table 2.1 Overview of the literature showing decreased immunity following prolonged heavy exercise (> 90 mins) and intense training periods.

Reference	Exercise bout(s)	Findings
Bruunsgaard *et al.* (1997)	Half-ironman	↓ *in vivo* cell-mediated immunity
Diment *et al.* (2015)	2 h running at 60% $\dot{V}O_{2max}$	↓ *in vivo* response to DPCP induction
Gill *et al.* (2013)	230 km multi-stage ultramarathon	↓ saliva SIgA secretion rate
Gillum *et al.* (2013)	50 km trail race	↓ saliva SIgA concentration and secretion rate
Hanstock *et al.* (2016)	2 h running at 60% $\dot{V}O_{2max}$	↓ tear SIgA concentration
Hanstock *et al.* (2019)	2 h running at 60% $\dot{V}O_{2max}$	↓ tear lactoferrin and lysozyme secretion rates
Harper Smith *et al.* (2011)	2 h running at 60% $\dot{V}O_{2max}$	↓ *in vivo* response to DPCP induction and elicitation

Kakanis *et al.* (2010)	2 h cycling at 90% VT2	↓ NK cell counts and neutrophil phagocytic function
Lancaster *et al.* (2004)	73% increase in training volume during 6-day ITP	↓ percentage and number of IFN-γ T lymphocytes
Nickel *et al.* (2012)	Marathon	↓ plasmacytoid DCs and ↓ TLR7 mRNA expression
Nieman *et al.* (2006)	160 km endurance race	↓ saliva SIgA secretion rate
Nieman *et al.* (2014)	3 days of 2.5 h running or cycling at 70% $\dot{V}O_{2max}$	↓ granulocyte and monocyte phagocytosis and oxidative burst
Oliveira and Gleeson (2010)	1.5 h cycling at 75% $\dot{V}O_{2max}$	↓ monocyte TLR4 expression
Robson *et al.* (1999)	3 h cycling at 55% $\dot{V}O_{2max}$	↓ neutrophil function
Ronsen *et al.* (2001)	2 x 75 min cycling at 70% $\dot{V}O_{2max}$	↓ $CD56^{+}$ percentage expressing CD69 after mitogen stimulation
Shek *et al.* (1995)	2 h cycling at 65% $\dot{V}O_{2max}$	↓ lymphocyte counts, ↑ CD4/CD8 ratio, ↓ NK cell counts

Starkie *et al.* (2000)	2 h cycling at 70% $\dot{V}O_{2peak}$	↓ LPS-induced cytokine secretion by monocytes
Steensberg *et al.* (2001)	2.5 h running at 75% $\dot{V}O_{2max}$	↓ T cell counts with % of type 1 T cells accounting for this change
Suzuki *et al.* (2003)	Competitive marathon race	↑ presence of immature, less differentiated, precursor neutrophils
Tomasi *et al.* (1982)	Cross-country skiing race	↓ saliva SIgA concentration
Verde *et al.* (1992)	38% increase in training load over 3 weeks	↓ T cell proliferation (mitogen induced)
Witard *et al.* (2012)	70% increase in training load for 1 week	↓ egress of naïve $CD8^+$ T lymphocytes and cytotoxic T lymphocytes

CD: cluster of differentiation; DC: dendritic cells; DPCP: diphenylcyclopropenone; IFN: interferon; ITP: intensified training period; LPS: Lipopolysaccharide; NK: natural killer; SIgA: secretory immunoglobulin A; TLR: toll like receptor; $\dot{V}O_2$: oxygen uptake; VT2: second ventilatory threshold. The references presented were chosen to provide an overview of different aspects of immune function that are impaired following prolonged exercise bouts and intense periods of training, and are not an exhaustive list of the literature.

In contrast to the immune lowering influence of prolonged bouts of exercise, regular moderate exercise is widely acknowledged to have prophylactic effects (Walsh *et al.*, 2011b). In fact, some aspects of immunity are enhanced following regular physical activity or short bouts of exercise that may translate into better protection from infectious agents and greater immune surveillance (Campbell and Turner, 2018). For instance, acute short bouts of exercise increase vaccine efficacy in accordance with the acute-stress induced immune enhancement theory of stress (Pascoe *et al.*, 2014). Furthermore, enhanced pneumococcal vaccination responses were observed following a 15 minute resistance exercise task compared to a control group, albeit this was only observed when a half-dose-vaccine was administered (Edwards *et al.*, 2012). Moreover, studies have generally only shown immune enhancement in populations with sub-optimal immune status (e.g. elderly), using half-dose vaccines or with low immunogenicity antigens. No studies have showed enhanced immune responses following prolonged aerobic exercise bouts (e.g. ≥ 1 h duration) or in healthy populations undertaking heavy periods of training and experiencing severe psychological stress (e.g. athletes, military personnel). Other studies have also observed the benefit of acute exercise (both resistance and aerobic) on vaccine responses (Edwards *et al.*, 2006; Edwards *et al.*, 2008; Edwards *et al.*, 2010; Ranadive *et al.*, 2014), but, only certain types of vaccine strains appear to be influenced by exercise stress, with most studies showing sex effects. There are widely documented sex effects on immune responses, whereby, females typically mount stronger innate and adaptive immune responses than males (Klein and Flanagan, 2016). These sex-based immunological differences typically contribute to faster clearance of pathogens and greater *in vivo* immune responses in females than males (Edwards *et al.*, 2006), but also contribute to their increased susceptibility to inflammatory and autoimmune diseases (Klein *et al.*, 2010). Although the mechanisms by which sex differences influence immune responses are poorly understood, sex hormones (e.g. oestradiol, progesterone) are widely speculated to play an important modulating role.

Irrespective of the mechanisms, it is important to take account of sex differences, contraception and different menstrual cycle phases when including females in studies examining immunity.

As with acute bouts of exercise, the majority of literature showing beneficial effects of regular exercise on immunity has typically has focused on older adults (Kohut *et al.*, 2002; Kohut *et al.*, 2004; Smith *et al.*, 2004b; Kohut *et al.*, 2005a). For example, a 10-month exercise intervention increased antiviral defence in the form of IFN-γ production and antibody titres to influenza vaccination in older adults (Kohut *et al.*, 2005a). Interestingly, the exercise intervention improved self-reported depression scores and the increase in IFN-γ was partly mediated by psychosocial factors, indicating that the improvement in immune competence involves both physiological and psychological pathways (Kohut *et al.*, 2005a).

2.3.2 Psychological stress and immune function

The interactions among behavioural, neural, endocrine, and immune processes was coined 'psychoneuroimmunology' by Professors Robert Ader and Nicholas Cohen in the late 1970s (Ader, 2000). Professor Ader first hypothesised a relationship between the brain and the immune system during a taste aversion conditioning study in rats, whereby using a variant of Pavlovian conditioning, different volumes of saccharin were given as a conditioned stimulus (CS), followed by a noxious agent that elicited temporary gastrointestinal upset, an unconditioned stimulus (UCS). Under these circumstances, the animal learns, after a single CS–UCS pairing, to avoid consumption of the CS solution. As expected, the magnitude of the conditioned response was directly related to the volume of saccharin consumed on the single conditioning trial. Repeated conditioning without the drug then removed the avoidance behaviour (meaning that the rats re-started to drink the saccharin solution), with avoidance removal being inversely related to the magnitude of the CS (i.e. rats that consumed the greatest

fluid volume were slowest to reverse behaviour). Unexpectedly, animals began to die in the course of these trials, and it soon became evident that the mortality rate varied directly with the amount of saccharin consumed on the single-conditioning trial, prompting the hypothesis that immune responses could be modified by classical conditioning (Ader, 1974, 2000). This hypothesis was then confirmed by demonstrating attenuated antibody responses in conditioned animals that were re-exposed to saccharin, previously paired with the immunosuppressive effects of noxious agent, compared with conditioned animals that were not re-exposed to the CS, non-conditioned animals that were exposed to saccharin and a placebo-treated control group (Ader and Cohen, 1975). These findings demonstrated that the immune system was subject to classical conditioning, thereby documenting a functional relationship between the brain and the immune system.

At a similar time to the work of Ader and Cohen, studies were also starting to associate altered immune function with emotional states and stressful life experiences (Solomon *et al.*, 1974; Bartrop *et al.*, 1977). For instance, depressed lymphocyte function was observed in individuals following recent bereavement of a spouse (Bartrop *et al.*, 1977). Since then, substantial literature has demonstrated that chronic psychological stress impairs numerous aspects of immunity with a highly cited meta-analysis supporting this assertion (Segerstrom and Miller, 2004). In contrast to the effects of chronic psychological stress, acute stress is considered one of nature's fundamental survival mechanisms that can prepare the immune system and the brain for challenges imposed by a stressor (e.g. surgery, job opportunity, athletic competition etc.) and has thus been associated with adaptive upregulation of some aspects of immunity in both humans and rodents (Altemus *et al.*, 2003; Segerstrom and Miller, 2004; Altemus *et al.*, 2006; Dhabhar, 2014; Breen *et al.*, 2016; Dhabhar, 2018). Acute stress induces changes in leukocyte distribution that is considered a defence mechanism to challenge, with the initial increase in

leukocyte number in the blood likened to soldiers exiting the ‘‘barracks’’ (spleen and connective tissue), where they normally reside during inactive periods, and entering the ‘‘boulevards’’ (circulating blood) to patrol, ready for action. Moreover, the reduction below baseline levels in leukocyte number after acute stress exposure is suggested to be caused by recruitment of leukocytes to potential ‘‘battle sites (skin and lymph nodes) in preparation for immune challenge (Dhabhar *et al.*, 2012).

Despite differing immune responses to the type of stress, there are individual differences in how individuals respond to the same stressor. For example, attenuated T-cell responses to phytohaemagglutinin (PHA) only occurred in individuals who exhibited heightened cardiovascular and catecholamine responses following an acute psychological stressor (Manuck *et al.*, 1991). Similarly, another study found that individuals who showed the greatest stress-related changes in plasma cortisol also exhibited the greatest diminution in cellular immunity (Cacioppo *et al.*, 1998). However, the biological responses to stressful situations are heavily dependent on the individuals' appraisal of the situation and cognitive and emotional responses (Frankenhauser, 1986; Tomaka *et al.*, 1997; Segerstrom and Miller, 2004), with few studies focussing on the individuals' perception of the upcoming stressor (Feldman *et al.*, 2004). Given that there are individual differences in how a stressor is perceived, and that different individuals report varying levels of anxiety and psychological stress prior to undertaking the same stressor, it is likely that the level of anxiety or perceived psychological stress prior to challenge is involved in modulating the subsequent immune response.

2.3.3 Shared pathways between exercise stress and psychological stress on immune function

There are common pathways by how stress modulates immunity, irrespective of the type of stressor (Selye, 1998) (Figure 2.1). The stress response activates physiological systems to

respond to stressors such as the HPA axis and SAM axis that release the stress hormones cortisol, epinephrine and norepinephrine (Figure 2.1). These hormones are widely acknowledged to modulate immune function with virtually every cell in the body expressing receptors for one or more of these hormones (Dhabhar, 2018). Similarly, there are commonalities by which physical stress and psychological stress modulate immunity: moderate levels of physical and psychological stress are potentially adaptive and upregulate aspects of immunity, whereas chronic periods of psychological stress and heavy periods of training downregulate immunity (Walsh *et al.*, 2011a; Dhabhar, 2018). In line with this notion, an inverted-U association has been proposed to describe the relationship between exercise stress and immune function (Nieman, 1994), and stress reactivity and health; whereby, both diminished and exaggerated responses to acute stress have been associated with negative health outcomes (Carroll *et al.*, 2003; Phillips *et al.*, 2005; Carroll *et al.*, 2011; Lovallo, 2011; Phillips *et al.*, 2011),

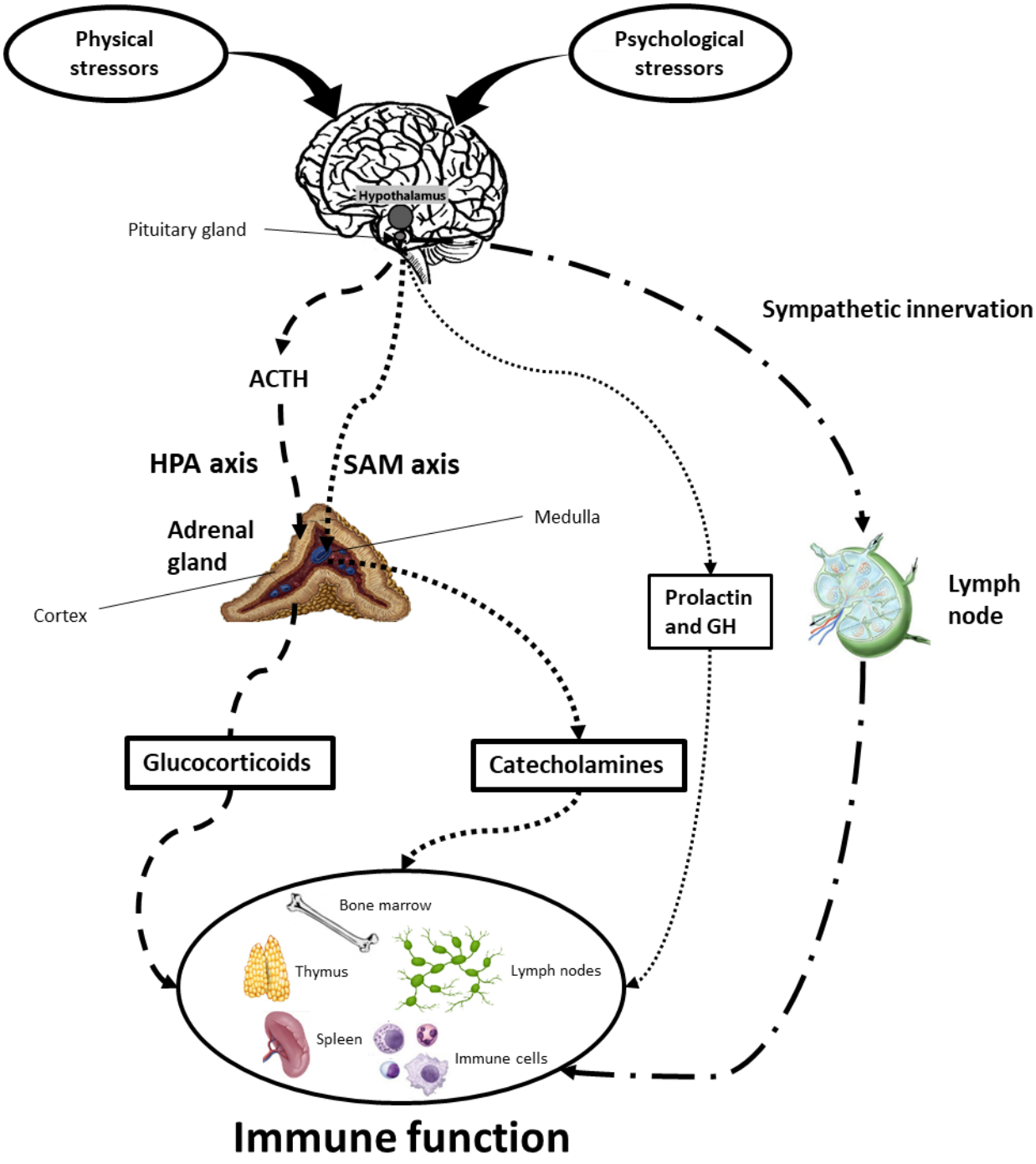

Figure 2.1 Common pathways for the immune response to physical and psychological stressors. The brain influences the body's response to challenge by controlling the production of adrenal hormones known to modulate immune function. Physical and psychological stressors initiate a series of co-ordinated neuroendocrine responses resulting in the stimulation of the hypothalamic-pituitary-adrenal (HPA) and the sympathetic-adrenal-medullary (SAM)

axis. The hypothalamus is the central control station linking the endocrine system and the nervous system via the pituitary gland. The production of adrenocorticotropic hormone (ACTH) from the pituitary gland results in the production of glucocorticoid hormones (e.g. cortisol) by the adrenal cortex. The SAM axis can be activated by stimulation of the adrenal medulla to produce catecholamines. Sympathetic innervation of lymphoid organs indicate a direct connection between the sympathetic nervous system and lymphoid organs. Other hormones such as prolactin and growth hormone (GH) may also be released in response to stressors. Figure adapted from Glaser and Kiecolt-Glaser (2005) and Walsh (2018).

Given that athletes experience heavy physical stress, but also lesser highlighted psychological stress (e.g. related to life stress, contractual issues, team selection, injury, travel, sleep disruption, jetlag etc.), it is reasonable to assume that psychological factors moderate the influence of exercise on immunity. There is some support for this notion, whereby, cortisol responses were higher in those reporting high life stress compared with those reporting low life stress following an intense acute exercise bout in elite athletes (Perna and McDowell, 1995). It is possible that concomitant behavioural disruptions and intense physical stress act in synergy to modulate immunity, and potentially contribute to augmenting other stressors such as sleep disruption. Furthermore, the salient influence of psychological stress on immunity was clearly highlighted in military recruits experiencing severe physical and psychological stress, whereby higher herpesvirus antibody responses were observed during final examinations, but not during physical training (Glaser *et al.*, 1999): activation of latent viruses provide a measure of CMI because CMI plays an important role in controlling the steady-state expression/replication of latent herpesviruses, with increases in antibody titres reflecting the loss of immunological control over the virus (Morey *et al.*, 2015). Although it has long been acknowledged that psychological stress plays a role in the decrease in immunity associated with heavy exercise

and training, there is little empirical research to support this notion (Perna *et al.*, 1997; Clow and Hucklebridge, 2001). One such study reported exacerbated *in vitro* immune alterations (e.g. regulatory T cell counts) in those reporting higher levels of perceived psychological stress, albeit in small participant numbers (Rehm *et al.*, 2013). As such, there is a need to bridge the gap between exercise immunology and psychoneuroimmunology by examining the role of psychological factors in exercise physiology and immunology studies, particularly focussing on clinically relevant *in vivo* immune measures (Wehrwein and Carter, 2016).

2.4 *In vivo* immunity

The influence of acute prolonged exercise bouts or chronic training on immunity in humans has mostly relied on *in vitro* measures removed from peripheral blood (Walsh *et al.*, 2011b). Investigating the function of isolated cells *in vitro* loses the tissue-specific context within which immune responses operate and may behave differently to those that are activated *in vivo* which questions the clinical relevance of these type of measures (Akbar *et al.*, 2013). *In vivo* approaches to assessing immune function are considered more clinically relevant and likely relate better to infection than *in vitro* markers because they extend beyond blood measures and involve challenging individuals with antigenic stimuli and assessing relevant antigen-driven responses including antigen specific cell-mediated delayed type hypersensitivity (DTH) responses, circulating antibody responses and vaccination (Albers *et al.*, 2005; Albers *et al.*, 2013). There is limited information about the impact of acute prolonged exercise bouts or chronic training on cutaneous measures of *in vivo* immunity in humans. One such study showed reduced skin reactions to the Mérieux CMI Multitest in endurance athletes after prolonged exercise in comparison with rested controls (Bruunsgaard *et al.*, 1997). In contrast, a 5-month training season had no influence on skin reactions to the Mérieux CMI Multitest in 11 elite swimmers compared to a recreationally active population (Gleeson *et al.*, 2004). The Mérieux

CMI Multitest involves the intra-dermal injection of seven previously encountered antigens and measurement of the skin reaction 48 h later. Unfortunately, this test is no longer commercially available and injecting recall antigens in this way only permits the assessment of pre-existing immunological memory and not the primary induction response (Harper Smith *et al.*, 2011). As such, even *in vivo* immune measures such as vaccinations have limitations including variable immunogenicity (e.g., hepatitis B), annual changes in vaccines (e.g., influenza) and difficulty when comparing the circulating antibody results from different studies using in-house ELISA (Burns, 2012). Furthermore, there is wide variation in vaccination history of individuals in many studies, and controlling prior antigen exposure is critical because the T-cell dependent primary *vs.* secondary or tertiary responses differ (Fleshner, 2005). One approach often used to assess primary immune responses is the novel antigen keyhole limpet hemocyanin (KLH) because it is suggested to be novel to all participants (Fleshner, 2005). However, KLH is a derivative of a sea mollusc native to a limited stretch of Pacific Ocean coastline, meaning availability is limited, and studies often exclude individuals with shellfish allergies (Smith *et al.*, 2004b). Past research suggests that some individuals demonstrate responses to KLH prior to immunization with the antigen from individuals with no known history of exposure to KLH, which questions whether this is a 'truly' novel antigen (Smith *et al.*, 2004a). The utility of alternative novel antigens such as DPCP overcomes this limitation and allows researchers to rigorously control the timing of initial exposure in relation to stressors, the dose of exposure and the time elapsed since sensitisation providing a valuable tool for scientists within the fields of stress and exercise immunology.

2.4.1 Clinical relevance of in vivo immunity

For exercise immunologists, it is often not practical to follow-up investigations with infection incidence as a gauge of the clinical meaningfulness of changes in immunity (Campbell and

Turner, 2018). As such, determining the clinical relevance of the chosen immune measures is of paramount importance. As previously highlighted, substantial literature outlines decreases in numerous aspects of immunity following prolonged exercise bouts and heavy training (Table 2.1), but the clinical meaningfulness of these changes remains unknown given that the clinical relevance of many of these immune measures has failed to be convincingly demonstrated. As such, stress and exercise immunologists should focus on more clinically relevant *in vivo* measures of immunity where possible. There is substantial supporting evidence that intradermal *in vivo* immune measures are clinically relevant, whereby, impaired T-cell-mediated immunity has been demonstrated in immune deficient populations (Haider *et al.*, 1973; Lloyd *et al.*, 1992; Blatt *et al.*, 1993; Gordin *et al.*, 1994; Dolan *et al.*, 1995; Zaman *et al.*, 1997). For instance, *in vivo* immunity relates to the time of progression to AIDS (Blatt *et al.*, 1993; Gordin *et al.*, 1994) and to survival time in HIV-infected patients (Dolan *et al.*, 1995). Moreover, *in vivo* immunity is decreased during various viral illnesses (Reed *et al.*, 1972; Kauffman *et al.*, 1974b; Kauffman *et al.*, 1976), albeit one study reported no effect of mild viral infection on cell-mediated immunity when four participants were experimentally infected with human rhinovirus (Kauffman *et al.*, 1974a). However, these studies have assessed *in vivo* immunity using DTH which involves the intradermal injection of common recall antigens. Epicutaneous application of novel antigens using experimental contact hypersensitivity (CHS) provides a less invasive alternative than intradermal DTH to assess *in vivo* immunity and there is strong supporting evidence that experimental CHS using the novel antigen DPCP relates to clinical outcomes. One such study found that HIV seropositive patients who responded to DPCP had greater $CD4^+$ counts; the primary measure used to determine when to start HAART (highly active anti-retroviral treatment) (Levis *et al.*, 2006). Additionally, *in vivo* DPCP responses successfully predicted allograft rejection in liver transplant patients, whereby those with lower (or non-detectable) immune responses were less likely to reject the transplant. These

findings indicate that those with an immune system unable to respond to a challenge did not reject the transplant whereas those able to mount an immune response to DPCP were more likely to reject the liver transplant (Bathgate *et al.*, 2001). However, studies demonstrating the clinical relevance of DPCP as an *in vivo* measure of immune competence have done so in clinical populations with compromised immune function. As such, it remains to be determined whether experimental CHS using DPCP relates to URI in otherwise healthy populations.

2.5 Diphenylcyclopropenone and Croton oil

The skin represents the largest and most exposed interface with the external environment, and in addition to providing a physical and chemical barrier, is equipped with a diverse armoury of immune cells to protect the host (Hickey, 2013). As such, topical sensitisers are used to induce experimental CHS for the investigation of human immune responses (Friedmann, 2007). For instance, during the 1960s and 1970s, the sensitiser, dinitrochlorobenzene (DNCB) was widely used to assess immune competence, but its use in humans has since diminished after it was found to be mutagenic in the Ames assay, which is widely used to assess the potential carcinogenic effect of chemicals (Wilkerson *et al.*, 1988; Levis *et al.*, 2006). An alternative approach involves using the specially synthesised non-mutagenic sensitiser, DPCP, which is used in clinical settings for topical immunotherapy of various conditions including viral warts and alopecia areata, with humans unlikely to have had previous DPCP exposure unless treated for these conditions (Wilkerson *et al.*, 1984). Sensitisers such as DPCP induce cutaneous responses by the activated chemical specific T-cells upon first exposure that require a period of time for the formation of 'immune memory' resulting in a boosting of responses upon re-exposure (mechanisms are explained in further detail in **section 2.6**). For instance, our research group and others have previously demonstrated the 'boosting' effect of repeated recalls to DPCP up to a plateau (Harper Smith *et al.*, 2011; Mose *et al.*, 2017). As such, DPCP may

provide an attractive tool for scientists to examine the influence of stress on T-cell mediated immunity that allows control over the timing of initial exposure (sensitisation) or recall (elicitation).

Sensitising antigens such as DPCP contain irritant components, and it has been suggested that these properties influence the sensitising potential (Pickard *et al.*, 2009). As such, it remains unknown whether the influence of stressors on *in vivo* immunity to DPCP reflect localised responses at the skin mediated primarily by localised irritant properties or reflect a systemic *in vivo* immune response. One approach to answer this question is to examine the influence of stressors on local cutaneous responses at the skin using an irritant. Croton oil (CO) is an irritant (does not contain sensitising properties) that produces local mild irritant and erythematous responses when applied to human skin (Hecker, 1968). Irritants such as CO predominantly induce cutaneous responses due to the direct pro-inflammatory properties of these types of chemicals, which stimulate a localised T-cell-independent response primarily mediated by local cutaneous innate processes after a single exposure (Berg *et al.*, 1995). The primary difference between sensitisers (DPCP) and irritants (CO) is that sensitisers induce cutaneous responses by the activated chemical specific T-cells and induce immunological memory (and boosting with multiple exposures) whereas irritants induce cutaneous responses due to the direct effects of the chemical which do not induce immune memory and no boosting with subsequent exposures. This may partly explain why responses to sensitisers are characterised by oedema whereas irritants are typically characterised by erythematous responses (Memon and Friedmann, 1996). Nevertheless, there are similarities in the pathways by which sensitisers and irritants produce cutaneous responses, partly because sensitisers such as DPCP also contain irritant properties that influence sensitisation. For example, cytokines such as TNF-α and IL-1β are key inflammatory mediators during the initial stages in cutaneous immune responses,

and are upregulated in the skin with both sensitisers and irritants, which act directly to promote increased blood flow, vascular permeability and leukocyte infiltration into contact sites (Piguet *et al.*, 1991; Flint *et al.*, 2000). Moreover, an elegant study demonstrated that the irritant properties of CO allowed increased penetration of a weak sensitiser into the epidermis that subsequently increased the sensitising potency, demonstrating that localised cutaneous innate defence mechanisms at the skin are important for sensitising potential (Pickard *et al.*, 2009). As such, it is important to investigate the effects of stress on local processes at the skin and to establish whether the effects of stress on DPCP responses reflect local irritant responses at the skin or reflect a measure of systemic immune status.

2.6 Mechanisms of experimental CHS

Cutaneous DTH and experimental CHS are generally accepted methods of assessing CMI that assess the response to cutaneous administration or application of antigens (Kniker *et al.*, 1984; Friedmann, 2007). Experimental CHS is a less invasive form of DTH that provides a useful tool to investigate *in vivo* immune responses.

2.6.1 Classical viewpoint of experimental CHS mechanisms

Classically, CHS is divided into two distinct phases: the sensitisation phase and the elicitation phase with the key stages outlined in Figure 2.2. In the sensitisation phase, a sensitiser is applied to the skin and is taken up locally by Langerhans cells (LC). The LC are stimulated to migrate to the draining lymph nodes where they present the sensitising antigen to appropriate T lymphocytes. In the elicitation phase, the sensitiser is reapplied to the skin resulting in the generation of a delayed T cell response. In humans, the sensitiser is often applied to the lower back, and a primary allergic response (PAR) typically develops 7-10 days later in participants who have not previously come into contact with the sensitiser (Friedmann, 2007). Some weeks

later, the individual is challenged by a second exposure to a range of concentrations of the sensitiser to another skin site, typically the upper inner arm. The CHS response peaks after 48-72 hours, with the magnitude of the elicitation responses providing an *in vivo* measure of CMI (Gulati *et al.*, 2014).

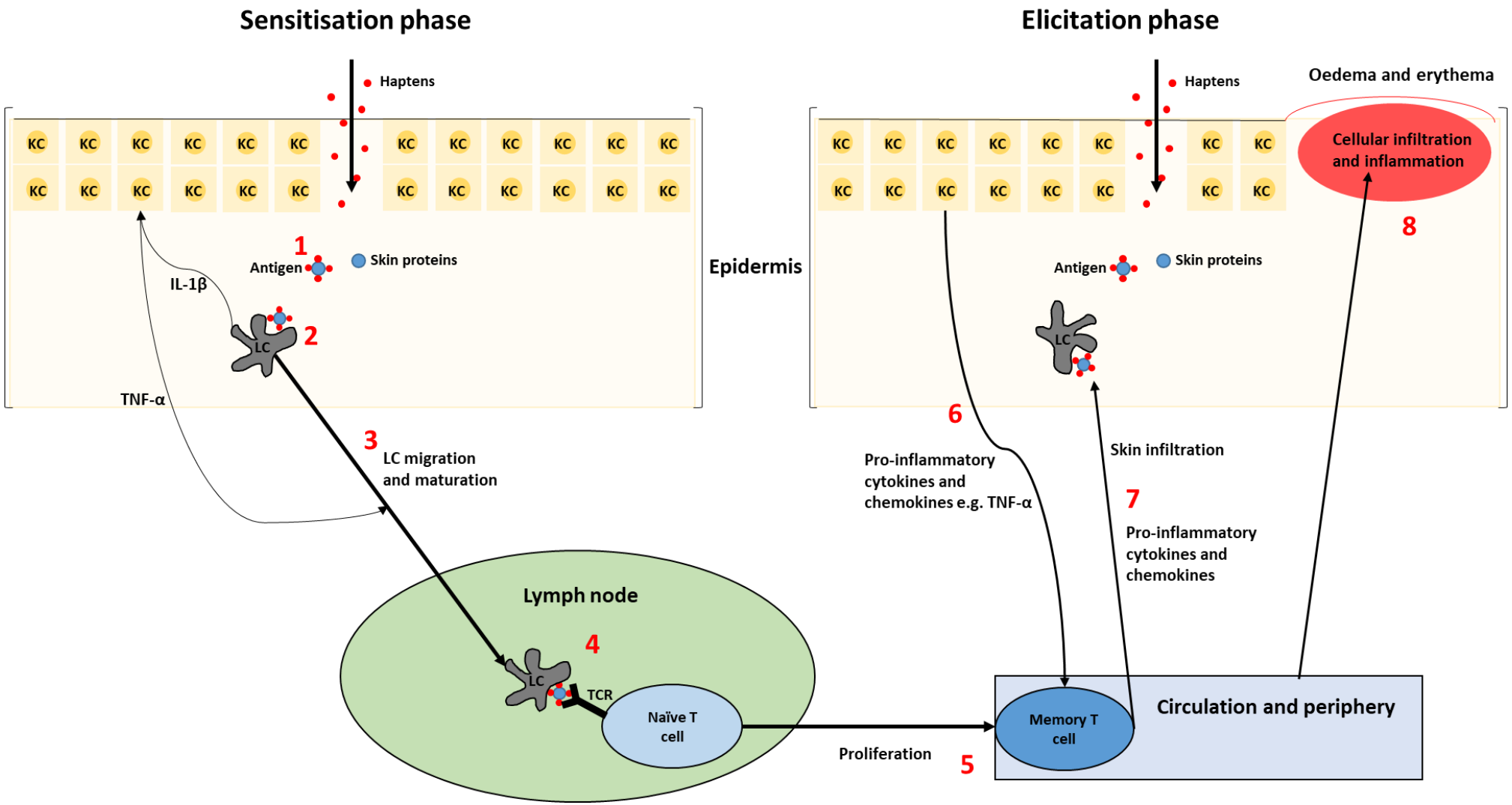
Sensitisation phase
Elicitation phase
Haptens
Oedema and erythema
KC
Cellular infiltration and inflammation
8
1
Antigen
Skin proteins
Epidermis
IL-1β
LC
2
TNF-α
3
LC migration and maturation
6
Pro-inflammatory cytokines and chemokines e.g. TNF-α
Skin infiltration
7
Pro-inflammatory cytokines and chemokines
Lymph node
4
TCR
Naïve T cell
Memory T cell
Circulation and periphery
Proliferation
5

Figure 2.2 Overview of sensitisation and elicitation phases of experimental contact hypersensitivity (CHS). **Sensitisation phase:** 1. Low molecular weight haptens (< 1000 Da) bind to proteins within the epidermis, forming strong immunogenic antigens (a hapten is a small molecule that is only immunogenic when attached to a large carrier such as a protein) 2. Antigen is taken up by antigen presenting cells, primarily Langerhans cells (LC), which are processed and expressed in the antigen-binding groove of major histocompatibility complex (MHC) class II molecules on the cell surface 3. LC migrate from the epidermis to the draining lymph node which is facilitated by cross-talk between LC and Keratinocytes (KC), stimulating the production of cytokines, namely TNF-α and IL1-β 4. Antigen presenting cells (APC) complete maturation into professional APC and migration to the draining lymph node, and the hapten-protein-MHC complex is presented to naïve T cells in the paracortical region of lymph node 5. Antigen specific T cells are activated, which then proliferate and migrate out of the lymph node and into the bloodstream and periphery (i.e. skin) with generation of antigen specific memory T-cells formed after 1-2 weeks, and are present in circulation. **Elicitation phase:** 6. Subsequent re-exposure to the sensitiser stimulates the production of pro-inflammatory cytokines and chemokines activated by epidermal cells (e.g. TNF-α) 7. Antigen specific T-cells are recruited to site of exposure by inflammatory cytokines and chemokines 8. Infiltrating antigen specific T cells, and some monocytes, LC and non-antigen specific T cells migrate into dermal and epidermal compartments of the skin, inducing an inflammatory response that peaks at 48-72 hours. IL: interleukin; TCR: T-cell receptor; TNF: tumour necrosis factor. Figure adapted from Christensen and Haase (2012) and Toebak *et al.* (2009).

2.6.2 Recent insights

Recent evidence in mice indicates that the immune system generates and distributes antigen-specific T cells to both lymph nodes and peripheral tissues derived from a common naïve T-cell precursor, thus generating two compartments of memory T cells with identical antigen specificity but different effector responses (Gaide *et al.*, 2015). Tissue resident memory T (Trm) cells mediate rapid CHS responses whereas circulating central memory T (Tcm) cells mediate delayed and attenuated responses (Gaide *et al.*, 2015). In support of these findings, recent *in vitro* work indicates that reactivity to DPCP in peripheral blood is slow to develop (~20 weeks), which likely reflects that hapten-specific effector/memory T cells are initially recruited to the skin, where they take up residence as memory T cells. This may partly explain why recall responses are demonstrable only 2-4 weeks after initial contact with DPCP at the skin (Friedmann *et al.*, 2017). However, the identification of Trm and Tcm cells are a relatively recent phenomenon and more research is required to delve into elucidating the roles of these different cellular compartments.

Different molecular characteristics of the CHS response have recently been identified when examining the 'peak' (3 days) and 'resolution' (14 days) phases of elicitation to DPCP (Gulati *et al.*, 2014). As expected, the peak phase was accompanied by large increases in infiltrating T cells and DCs, which evolved from an inflammatory 'peak' after 3 days to a regulated immune response after 14 days, indicated by higher levels of gene transcripts associated with regulatory pathways of immune responses and decreased expression of T cell effector cytokines associated with the activation phase of immune responses. These findings highlight the complex nature of CHS responses and implicate a role for regulatory immune mechanisms in the CHS response to DPCP.

2.6.3 Potential mechanisms by which stress modulates CHS

Experimental CHS involves many different pathways and mechanisms of the immune system, and comprises a wealth of different cell types, cytokines, chemokines and receptors, which make identifying the exact mechanisms by which stress modulates the CHS response a difficult task (Christensen and Haase, 2012). However, the role of stress hormones on immune responses are widely acknowledged with virtually all cells in the body expressing receptors for the primary stress hormones epinephrine, norepinephrine and cortisol (Dhabhar, 2018). Stress hormones are widely acknowledged to be immunosuppressive and play a role in the decrease in immune function, but are also considered to play important roles in preparing the immune system for challenge (Dhabhar and McEwen, 1999; Walsh *et al.*, 2011a; Dhabhar, 2014). For example, administration of physiological doses of corticosterone and epinephrine increased T-cell drainage away from the site of DTH challenge to lymph nodes, which in-turn enhanced the DTH response in rats (Dhabhar and McEwen, 1999). In addition, adrenalectomy has been shown to eliminate stress-induced immune-enhancement in rats, likely by reducing the glucocorticoid and epinephrine response (Dhabhar and McEwen, 1999). Moreover, the release of norepinephrine during acute psychological stress exerts an adjuvant effect on DCs by promoting enhanced migration to lymph nodes, resulting in enhanced DTH responses (Saint-Mezard *et al.*, 2003). As such, altered LC maturation or migration is one potential mechanism by which stress modulates CHS responses in humans. Furthermore, stress-induced modulation of pro-inflammatory cytokines are another possibility given that secretion of these cytokines at the skin, namely IL-1β and TNF-α are important aspects of LC migration and are critical mediators of CHS responses (Piguet *et al.*, 1991). Given that LC residing in the skin are a type of dendritic cell, it is plausible that CHS measures at the skin provide a surrogate marker of the immune response and reflect antigen presenting processes in other parts of the body (e.g. upper respiratory tract).

Regulatory T (Treg) cells play a central role in maintaining self-tolerance by regulating cells in the immune system, and it is generally accepted that defects in Treg-mediated suppression are present in autoimmune diseases (Long and Buckner, 2011). For instance, a lack of $CD4^+FOXP3^+$ Treg cells results in increased autoimmunity, with adoptive transfer of Treg cells preventing and reversing autoimmunity in mice (Sakaguchi *et al.*, 2006). It is possible that these regulatory cells are involved in the stress-induced modulation of experimental CHS as Treg cells have recently been discovered to populate specific tissues (e.g. skin) during stress conditions (Dominguez-Villar and Hafler, 2018). For instance, heavy exercise and psychological stress have been shown to alter the proportion of Treg cells in peripheral circulation, whereby, athletes undertaking heavy training reported higher Treg cell counts than healthy control participants, and athletes with higher aerobic fitness, as assessed by $\dot{V}O_{2peak}$, also reported increased Treg cells than those with lower aerobic fitness (Weinhold *et al.*, 2016). In contrast, a 21% decrease in the absolute number of Treg cells was observed 1 h following a marathon, with a 21% increase reported 1 day following the marathon compared to baseline (Clifford *et al.*, 2017). Acute psychological stress also decreased $CD4^+FOXP3^+$ Treg cells in peripheral circulation (Freier *et al.*, 2010). These findings potentially implicate a role for Treg cells in stress-induced immune modulation. Finally, given the recent novel work of Gaide *et al.* (2015), whereby, two compartments of memory T cells were generated from a common naïve T-cell precursor, it is possible that the distribution and/or generation of resident memory T cells are impaired or altered when first exposed to a sensitiser under stress conditions.

2.7 Summary and thesis objectives

Acute bouts of prolonged exercise and chronic training induce transient immune perturbations (Verde *et al.*, 1992; Kakanis *et al.*, 2010), but the clinical relevance of these changes remain unclear with the majority of work relying on *in vitro* measures of immunity. As such, there is

a need to utilise clinically relevant *in vivo* measures of immunity that extend beyond blood measures and involve whole-body integrated immune responses. One such model is the utility of experimental CHS using DPCP, however, given that sensitisers such as DPCP also contain localised irritant properties, it remains to be determined whether the influence of stressors on *in vivo* immunity to DPCP reflects a systemic immune response or a localised response at the skin. One approach to investigate this question is to examine the influence of prolonged exercise on local inflammatory processes at the skin using the irritant, CO. Nevertheless, there is supporting evidence that DPCP provides a clinically relevant marker of *in vivo* immunity in individuals with sub-optimal immune status, however, the clinical relevance of DPCP with regards to URI in otherwise healthy populations remains to be determined.

Psychoneuroimmunologists have long since acknowledged the role of psychological stress on immunity, yet exercise immunologists have largely ignored the role of psychological factors on exercise-immune modulation. Moreover, there are commonalities by which psychological stress and physical stress alter immunity, with psychological factors likely playing a role in the decrease in immunity with prolonged heavy exercise and heavy training. However, empirical evidence supporting this notion is lacking. As such, there is a need bridge the gap between exercise immunology and psychoneuroimmunology by examining the role of psychological factors on exercise-immune modulation.

With this information in mind, the objectives of this thesis were to investigate: 1. the influence of prolonged exercise on local cutaneous processes; 2. the role of anxiety and perceived psychological stress on the *in vivo* immune response after exercise; 3. the role of anxiety prior to acute psychological stress on *in vivo* immunity; 4. the influence of a common cold on *in vivo*

immunity; 5. the clinical relevance of DPCP with regards to URI as an *in vivo* marker of immune competence.

CHAPTER THREE

General Methods

CHAPTER THREE

General Methods

3.1 Ethical approval

Approval was obtained for all studies from the local Ethics Committee (School of Sport, Health and Exercise Sciences, Bangor University, Bangor) and all laboratory protocols were conducted in accordance with the Declaration of Helsinki. Following a full briefing of experimental procedures, participants provided written consent (Appendix A) and completed a medical health questionnaire (Appendix B).

All participants were recreationally active adult volunteers aged 18-40 years, and were free from any known cardiovascular or metabolic diseases. Participants with a history of atopy or any other immune-related or inflammatory dermatological condition, or taking prescription medication were excluded except for female participants using contraceptives. In addition, participants experienced no prolonged sun exposure (e.g. sunbathing) in the two weeks prior to any skin patch applications and had Caucasian skin-type, because one of the measures is skin redness, which cannot be measured with darker skin tones. Participants abstained from alcohol and strenuous exercise for 24 h before and 48 h after skin patch applications. Participants in **Chapters 5-8** had no previous history of exposure to DPCP.

3.2 Peak oxygen uptake

Peak oxygen uptake ($\dot{V}O_{2peak}$) was estimated (**Chapters 4 and 5**) 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 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 bpm of age predicted heart rate maximum and a respiratory exchange ratio greater than or equal to 1.15.

3.3 Assessment of physical activity

A standard short-form International Physical Activity Questionnaire (IPAQ) was used (http://www.ipaq.ki.se/downloads.htm) to provide quantitative data of physical activity at moderate-vigorous intensity in metabolic-equivalent (MET) $h \cdot wk^{-1}$ (Craig *et al.*, 2003) (**Chapters 7 and 8**).

3.4 State anxiety, perceived psychological stress and mood

Measurements of state-anxiety, perceived psychological stress and mood were made using validated psychological instruments. State-anxiety was assessed using the state aspect of the State Trait Anxiety Inventory (STAI-S); the STAI-S is one of the most commonly used scales to measure anxiety, which has been defined as an unpleasant emotional state that exists at a given moment in time and at a particular level of intensity, and is characterised by subjective feelings of tension, apprehension, nervousness, and worry (Spielberger, 1983). The STAI-S consists of 20-items, with responses being measured on a four-point Likert scale (from 1 'not at all' to 4 'very much so') and a range of scores from 20−80 (**Chapters 4, 5, 6, and 8**). Perceived psychological stress was assessed using the 10-item (**Chapter 4, 7 and 8**) or 14-item (**Chapter 5**) Perceived Stress Scale (PSS). The PSS is a widely used psychological instrument for measuring the perception of stress, and measures the degree to which life situations are considered stressful by the individual during the previous month (Cohen and Williamson, 1988). The PSS responses are measured on a five-point Likert scale (from 0 'never' to 4 'very

often') with a range of scores from 0-40 and 0−56 for the 10-item and 14-item inventories, respectively. Mood was assessed using the Brunel Mood Scale (BRUMS), which contains 24 items divided into six respective subscales: anger, confusion, depression, fatigue, tension, and vigour. The items are answered on a 5-point Likert scale (0; not at all, 1; a little, 2; moderately, 3; quite a bit, 4; extremely), and each subscale, with four relevant items, can achieve a raw score in the range of 0 to 16 (Terry *et al.*, 1999; Terry *et al.*, 2003) (**Chapters 7 and 8**).

3.5 Induction of contact sensitivity

The sensitising 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 (Harper Smith *et al.*, 2011). 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 min before being applied to the skin on the lower back for exactly 48 h (Harper Smith *et al.*, 2011) (**Chapters 5-8**).

3.6 Elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP. At least 14 days 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 volar aspect of the upper arm in the following concentrations unless otherwise stated: 10 μL of DPCP: 0.0048%, 1.24 $\mu g \cdot cm^{-2}$; 0.0076%, 1.98 $\mu g \cdot cm^{-2}$; 0.0122%, 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 of 100% acetone served as a control patch for background subtraction. Patches were applied in randomly allocated order at the local site to

minimise any anatomical variability in responses. Elicitation patches were removed after 6 h, and the strength of immune reactivity was assessed as cutaneous responses 48 h after application (Diment *et al.*, 2015) (**Chapters 5-8**).

3.7 Assessment of cutaneous responses

Dermal thickness was determined at each elicitation site using a high-frequency ultrasound scanner (Episcan, Longport Inc, Reading, UK; **Chapters 4-8**). The ultrasound probe was placed over the centre of each patch site together with ultrasound gel. The mean of three measurements was taken from each 12-mm scan image assessed at a later time by a blinded investigator. Mean skinfold thickness was determined from triplicate measurements at each elicitation site using modified spring-loaded skin callipers (Harpenden Skinfold Calliper, British Indicators, England, UK; **Chapters 5-7**). As previously described, this method provides an objective measure of skin oedema (Harper Smith *et al.*, 2011). Skinfold thickness was recorded to the nearest 0.1 mm by placing the jaws of the calliper at the outer diameter of the response site and measuring skin thickness only (no subcutaneous fat). Skinfold thickness assessed using skinfold callipers has previously been shown to be strongly related ($r = 0.93$) with high-frequency ultrasound readings of dermal thickness (Diment *et al.*, 2015). Mean skin erythema was determined from triplicate measurements at each elicitation site using an erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark) which provides an objective measure of skin redness (Harper Smith *et al.*, 2011) (**Chapters 4-8**). Mean background values were determined from triplicate measurements at the acetone patch site for both thickness and redness. To determine the increase in thickness and redness, the value from the acetone site was subtracted from each elicitation site value. The values for the increase in dermal thickness, skinfold thickness and erythema over all the doses were summed to give an

approximation of the area under the dose–response curve, representative of the overall reactivity of each participant (Harper Smith *et al.*, 2011) (**Chapters 4-8**).

3.8 Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, IBM, Chicago, IL, USA) and GraphPad Prism (GraphPad Inc, San Diego, CA, USA). All data are presented as mean ± SD unless otherwise stated and statistical significance was accepted at $P < 0.05$. All data were checked for normality and sphericity using the Shapiro-Wilk test and Mauchly's test of sphericity, respectively. In cases where these assumptions were violated, data were natural log transformed and Greenhouse-Geisser adjustments were applied to the degrees of freedom where necessary. Independent t-tests or one-way ANOVAs were used to compare summed DPCP responses between groups, and mixed-model ANOVAs were used to analyse DPCP responses across the full dose-series challenge, with significant differences identified using *post hoc* Tukey honestly significant difference (HSD) or Bonferroni corrected t-tests, where appropriate. Effect sizes (ES) were calculated (Cohen's *d*) for the difference between two means for primary outcome variables. Cohen's *d* effect sizes greater than 0.2, 0.5 and 0.8 represent small, medium, and large effects respectively (Cohen, 1988). Pearson correlation coefficients were used to examine bivariate relationships between variables unless otherwise stated. Pearson correlation coefficients of 0.10, 0.30 and 0.50 are considered to represent small, moderate and large effect sizes, respectively (Cohen, 1988).

CHAPTER FOUR

No influence of prolonged exercise on local cutaneous inflammatory processes points towards a systemic suppression of *in vivo* immunity

These data formed part two of a multi-study manuscript that was published in Medicine and Science in Sports and Exercise in 2015

Diment B. C., Fortes M. B., Edwards J. P., Hanstock H. G., Ward M. D., Dunstall H. M., Friedmann P. S. and Walsh N. P. (2015). Exercise intensity and duration effects on *in vivo* immunity. *Med Sci Sports Exerc*, 47: 1390-1398.

CHAPTER FOUR

No influence of prolonged exercise on local cutaneous inflammatory processes points towards a systemic suppression of *in vivo* immunity

4.1 SUMMARY

Prolonged exercise is widely accepted to suppress various aspects of immune function in humans. We have previously demonstrated that prolonged exercise reduces *in vivo* immunity using skin sensitisation with DPCP. However, it is unknown whether the inhibitory effects of prolonged exercise on the immune response to DPCP are due to systemic effects on the dendritic cell/T-cell axis between the skin and lymph nodes or whether they involve local effects on cutaneous inflammatory processes mediated principally via innate immune mechanisms. With this in mind, the aim of this study was to determine the influence of prolonged exercise stress on local inflammatory processes using an *in vivo* challenge model. Eleven healthy males completed 120 min running at 60% $\dot{V}O_{2peak}$ (120MI-CO) and 2 h seated rest (CON-CO) in a randomised, repeated-measures crossover design. Twenty min after exercise or seated rest, a low dose-series (0.3%, 0.55%, 1% and 3%) challenge of the irritant, CO, was applied to the upper inner arm for 24 h. Cutaneous responses were assessed by skin erythema and dermal thickness. No significant differences were observed between 120MI-CO and CON-CO for summed erythema (25.6 ± 13.1 *vs.* 26.3 ± 10.3 AU; $P = 0.82$) or dermal thickness responses (1.02 ± 0.54 *vs.* 0.87 ± 0.38 mm; $P = 0.25$), respectively. In conclusion, prolonged exercise does not influence local cutaneous inflammatory processes *in vivo*, supporting that the previously observed decrease in CHS responses following prolonged exercise likely represents a systemic suppression of *in vivo* immunity.

4.2 INTRODUCTION

The skin represents the largest and most exposed interface with the external environment, and in addition to providing a physical and chemical barrier, the skin is equipped with a diverse armoury of immune cells to protect the host (Hickey, 2013). Measures of *in vivo* immunity at the skin include DTH responses to intradermal injection of antigens, or the less invasive CHS responses to cutaneous application of antigens. *In vivo* measures are considered more informative than commonly used *in vitro* measures where immune cells, typically from peripheral blood, are extracted from their normal environment and analysed in artificial cultures (Albers *et al.*, 2013). Although cutaneous *in vivo* immune measures have shown impaired responses in individuals exposed to physical stress, psychological stress and during acute infectious illness, e.g., Epstein-Barr virus (Bennett *et al.*, 1998; Altemus *et al.*, 2006; Harper Smith *et al.*, 2011), it remains to be determined whether cutaneous measures of *in vivo* immunity extend beyond localised effects at the skin.

T-cell-mediated immune responses to cutaneous application of sensitisers such as DPCP are initiated by inflammatory mediators (e.g. cytokines) at the skin that facilitate the migration of antigen-presenting LCs to the T cell rich lymph nodes. Upon presentation, naïve T cells specifically recognise the antigen, and are activated to proliferate, generating effector and memory T cells, which are released by the efferent lymphatics into the circulation. As such, repeated recall challenges of sensitisers activate these memory T cells and result in an initial 'boosting' of responses (Harper Smith *et al.*, 2011). Conversely, irritants such as CO do not exhibit immune memory and repeated exposure does not result in an amplification of responses because they do not contain sensitising properties. Cutaneous application of CO triggers a localised, innate, T-cell-independent inflammatory response that is also initiated by the release of inflammatory mediators (e.g. cytokines) which then activate leukocytes in a non-specific

manner (Baadsgaard and Wang, 1991; Berg *et al.*, 1995; Dhabhar and McEwen, 1996; Nosbaum *et al.*, 2009). For instance, increases in the number of neutrophils (innate cells) have been observed in both the dermis and epidermis of human skin in response to CO application (Willis *et al.*, 1993) and it has been shown that interleukin (IL) 8, a neutrophil chemotactic cytokine, was increased 112-fold when CO was added to cell cultures (Wilmer *et al.*, 1994). Taken together, these findings support that assessing the inflammatory response to CO provides an *in vivo* challenge model to investigate the influence of stressors on local inflammatory responses at the skin mediated principally by innate processes.

Physical exercise provides a well-controlled model to investigate the effects of stress on immune responses (Harper Smith *et al.*, 2011). For example, the intensity and duration of the stressor, and the timing of immune challenge following stress can be controlled. Acute bouts of prolonged exercise (≥ 90 min) have been shown to temporarily compromise various aspects of immune function in humans including neutrophil function, NK cell function, mucosal immunity and *in vivo* measures of DTH and CHS (Tomasi *et al.*, 1982; Bruunsgaard *et al.*, 1997; Robson *et al.*, 1999; Kakanis *et al.*, 2010; Harper Smith *et al.*, 2011). Possible mechanisms include activation of the HPA axis and SAM axes, which are widely acknowledged to occur after prolonged stress (typically lasting hours), and in turn increase glucocorticoids and catecholamines, previously shown to decrease the induction of CHS in mice and delay recovery of skin barrier function in humans (Altemus *et al.*, 2001; Seiffert *et al.*, 2002; Dhabhar, 2013).

Prolonged exercise has previously been shown to reduce the induction of immune memory to DPCP (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). However, it remains unknown whether the inhibitory effects of prolonged exercise on *in vivo* immunity to DPCP are due to systemic

effects on the dendritic cell/T-cell axis between the skin and lymph nodes or whether they involve local effects on cutaneous inflammatory processes mediated principally via innate immune mechanisms. Therefore, the aim of this study was to determine the influence of prolonged exercise stress on local inflammatory processes. In line with our hypothesis, we observed no influence of prolonged, moderate intensity exercise on CO responses, indicating that the previously observed decrease in CHS responses to DPCP induction likely represents a systemic suppression of *in vivo* immunity.

4.3 METHODS

4.3.1 Participants

Eleven recreationally active males (mean ± SD: age 24 ± 5 years; body mass 79 ± 9 kg; height 179 ± 8 cm; $\dot{V}O_{2peak}$ 53 ± 6 ml·kg^{-1}·min^{-1}) participated in the study. Participants were required to abstain from alcohol and strenuous exercise for 24 h preceding all experimental trials and until cutaneous responses were assessed.

4.3.2 Preliminary measures

$\dot{V}O_{2peak}$ was estimated by means of a ramped exercise test on a treadmill (see **Chapter 3, section 3.2** for details). A verification protocol to determine the treadmill speed to elicit 60% $\dot{V}O_{2peak}$ took place after a 30-minute recovery period. Verification was performed by recording steady-state $\dot{V}O_2$ during the last minute of a 5 min exercise bout, with treadmill speed adjusted where necessary.

4.3.3 Experimental procedures

In a randomised, crossover design, participants performed 2 h of moderate intensity exercise at 60% $\dot{V}O_{2peak}$ (120MI-CO) or 2 h of seated rest (CON-CO) with each trial separated by at least 7 days. At least 5 days after determination of $\dot{V}O_{2peak}$, participants reported to the laboratory at 0730 h on both trials, where a standardised breakfast and fluid allowance of 35 mL·kg^{-1}·day^{-1} *pro rata* for the pre-exercise period was provided. Participants then completed STAI-S and PSS (10-item) questionnaires to assess state-anxiety and perceived psychological stress (see **Chapter 3, section 3.3** for details). Participants remained in the laboratory prior to commencing the exercise or seated rest at 1100 h, and were permitted to perform light activities (e.g. reading, watching television, browsing the internet) during this period. Nude body mass (NBM) was recorded pre- and post- exercise to estimate fluid losses (Model 705; Seca,

Hamburg, Germany). Exercising participants received 3 $mL \cdot kg^{-1} \cdot h^{-1}$ water to partially offset fluid losses through sweating, and any additional fluid loss was replaced following exercise; during CON-CO, fluid intake remained at 35 $mL \cdot kg^{-1} \cdot d^{-1}$ pro *rata*. Heart rate (Polar FT1, Polar, Kempele, Finland) was monitored continuously throughout the 2 h exercise or seated rest period. Following exercise completion, participants showered and returned to the laboratory within 15 min, and patches were applied exactly 20 min after exercise. This was to allow cutaneous blood flow to return to baseline and to match the timing of CO application following exercise with that of DPCP sensitisation in a previous study (Diment *et al.*, 2015). Participants returned to the laboratory the following day at 1120 h for removal of patches with responses assessed at 1320 h. This 2 h delay in assessment of cutaneous responses was chosen to eliminate effects of occlusion confounding the cutaneous responses. A pilot study within our laboratory revealed that the erythema responses were 23% greater 2 h after patch removal compared to readings taken immediately after patch removal (data not shown). Participants recorded a diet diary and replicated on both trials until assessments of cutaneous responses were completed.

4.3.4 Cutaneous application of croton oil

Participants received a CO challenge at 1320 h, exactly 20 minutes after exercise cessation or seated rest. This involved the topical application of a dose-series of CO on individual patches comprising 8 mm aluminium Finn chambers mounted on Scanpor hypoallergenic tape and 7 mm filter paper discs. Patches were applied in duplicate to the inner aspect of the upper arm in the following concentrations: 10 μL of CO in ethanol: 0.3%, 0.55%, 1%, 3% and 10 μL 100% ethanol control patch (Figure 4.1). To account for local anatomical variability, the location of each concentration was randomised. Patches remained in place for exactly 24 h and the assessment of cutaneous responses was performed 2 h after removal of CO patches.

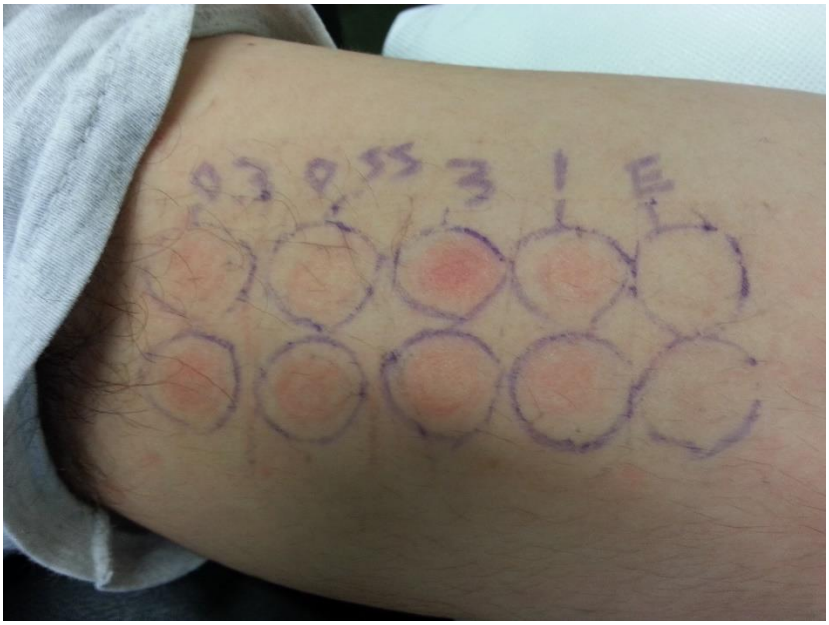

Figure 4.1 Image of croton oil (CO) dose-series patch layout in duplicate, and cutaneous responses to CO application. CO patches were applied for 24 h following prolonged exercise stress (120MI-CO) or seated rest (CON-CO), with responses assessed 2 h after patch removal.

4.3.5 Assessment of cutaneous responses

The magnitude of the irritant response was quantified by assessing skin erythema and dermal thickness (see **Chapter 3, section 3.6** for details). Skin erythema is an objective measure of skin redness, considered the key measure of irritant responses (Parslew and Friedmann, 1999).

4.3.6 Statistical analysis

A sample size of 10 participants was estimated by calculating a minimum important difference using data from the biological variability of this model (**Appendix C**). This involved calculating the coefficient of variation (CV) for the summed erythema responses across 3 repeat trials, determining a difference greater than this variability, and estimating an effect size based on this difference (ES 0.91). Paired t-tests were used to investigate differences between CON-CO and 120MI-CO for STAI-S and summed CO responses (erythema and dermal thickness).

A repeated measures ANOVA was used to analyse CO responses across the full dose series (trial x dose).

4.4 RESULTS

4.4.1 STAI-S and PSS

Participants reported increased levels of state-anxiety prior to exercise (120MI-CO) compared with CON-CO (29.8 ± 8.1 *vs.* 26.8 ± 8.3 AU; $t_{(10)} = 1.90$, $P < 0.05$), indicating a modest anticipatory effect of exercise stress. The reported levels of state-anxiety were low-to-moderate, in line with previous literature (Spielberger, 1983; Julian, 2011). Participants also reported low-to-moderate PSS scores (9.1 ± 6.7 AU) in accordance with previous literature (Cohen and Williamson, 1988).

4.4.2 Assessment of cutaneous responses

Results are presented as erythema, considered a key measure of irritant responses, and dermal thickness, considered a key measure of CHS responses (Parslew and Friedmann, 1999; Harper Smith *et al.*, 2011). No difference was observed between 120MI-CO and CON-CO for summed erythema ($t_{(10)} = 0.23$, $P = 0.83$; Figure 4.2A) or dermal thickening responses ($t_{(10)} = 1.21$, $P = 0.25$; Figure 4.2B) indicating that prolonged exercise does not inhibit local inflammatory processes at the skin. For visual comparison, the increase in erythema responses to the full dose series of CO is presented (Figure 4.3). No trial x dose interaction was observed for erythema responses ($F_{(3,30)} = 1.38$, $P = 0.27$).

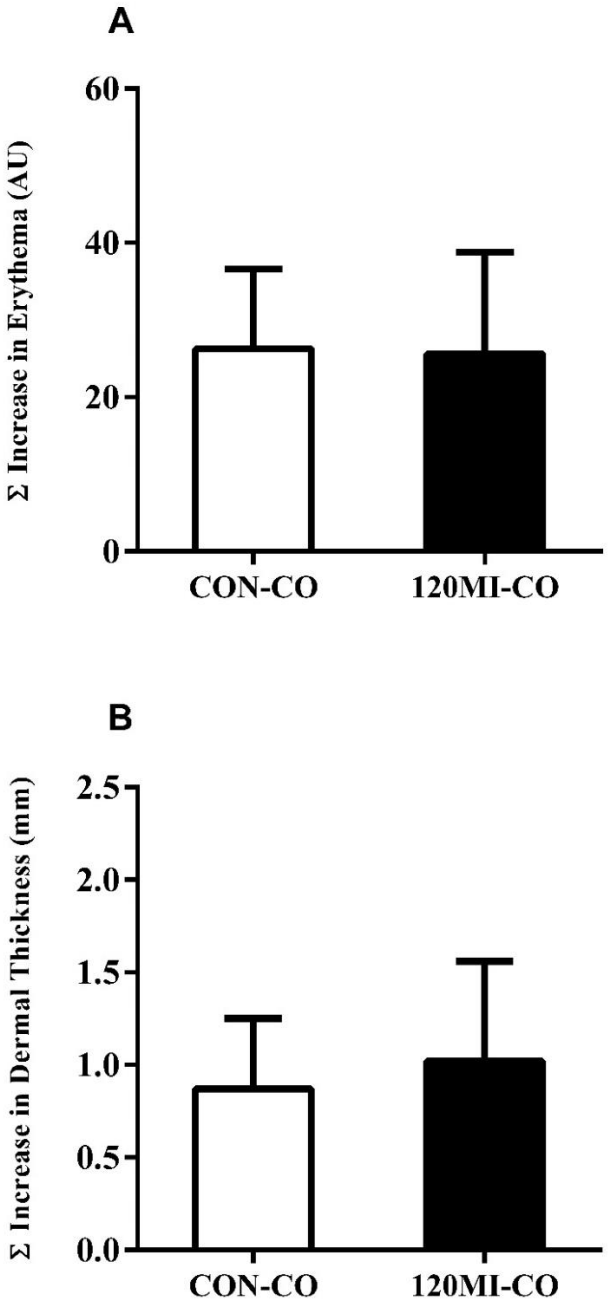

Figure 4.2 Effect of prolonged exercise stress (120MI-CO) or seated rest (CON-CO) on summed responses to croton oil (CO) challenge, measured as erythema (A) and dermal thickness (B). Data are mean ± SD.

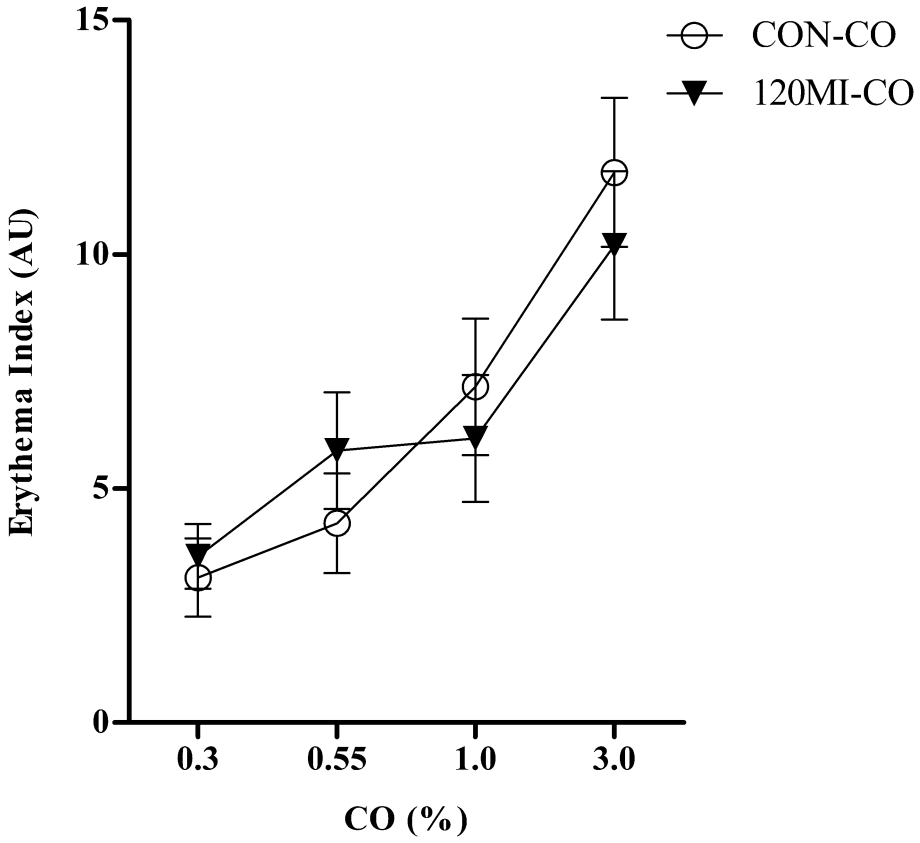

Figure 4.3 Effect of prolonged exercise stress (120MI-CO) or seated rest (CON-CO) on erythema responses to croton oil (CO) challenge. Shown are the erythema responses to the full dose-series challenge. Data are mean ± SEM for clarity.

4.5 DISCUSSION

It has previously been shown that prolonged exercise impairs the induction of immune memory using experimental CHS to DPCP (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). However, it remains to be determined whether *in vivo* immunity to DPCP extends beyond localised effects at the skin. With this in mind, the aim of this study was to investigate the influence of prolonged exercise on local cutaneous inflammatory processes using an *in vivo* challenge model with the irritant CO. The key finding of the current study was that prolonged exercise had no influence on cutaneous responses to CO, as assessed by erythema and dermal thickness. These findings suggest that local effects on cutaneous inflammatory processes are not inhibited by exercise stress, supporting that the previously observed decrease in *in vivo* immunity to DPCP following prolonged exercise stress likely represents a systemic suppression of cell-mediated immunity rather than exercise effects on local inflammatory processes at the skin (Harper Smith *et al.*, 2011; Diment *et al.*, 2015).

This is the first study to investigate the effects of prolonged exercise stress on local cutaneous inflammatory responses in humans. The current findings support previous literature in a murine model, whereby, 2 h restraint stress modulated cell-mediated immune responses using a CHS model, but did not alter local inflammatory responses to CO (Flint *et al.*, 2000). Upon initial contact with sensitisers such as DPCP, inflammatory mediators are upregulated in the skin, and facilitate the migration and mobilisation of LCs to T-cell rich lymph nodes where the specific antigen is presented to naïve T cells that specifically recognise the antigen–MHC molecule complexes. The naïve T cells are activated to proliferate, generating effector and memory T cells specifically able to recognise and react to the antigen, which are released by the efferent lymphatics into the circulation (Friedmann, 2006; Toebak *et al.*, 2009). TNF-α and IL-1β are key inflammatory mediators during the initial stages in cutaneous immune responses, and are

upregulated in the skin in both cell mediated immunity (DPCP model) and local cutaneous responses (CO model). These cytokines act directly to promote increased blood flow, vascular permeability and leukocyte infiltration into contact sites, and are critical steps in the development of contact hypersensitivity (DPCP model) and local cutaneous responses (CO model) (Piguet *et al.*, 1991; Flint *et al.*, 2000). Given that no differences were observed in CO responses with prolonged exercise in the present study (*vs.* CON-CO), these findings indicate that inflammatory cytokines such as TNF-α and IL-1β are not key mediators in the exercise induced immune suppression to DPCP. However, we recognise that in both the current study, and our previous studies investigating the influence of prolonged exercise on DPCP responses (Harper Smith *et al.*, 2011; Diment *et al.*, 2015), we did not assess concentrations of cytokines in either plasma, or more importantly, blister fluid at the skin. Consequently, further research is required to delve into the mechanistic processes of impaired *in vivo* immunity to DPCP induction following prolonged exercise. Furthermore, the duration of the inhibitory effect of prolonged heavy exercise on CHS induction in humans remains unknown and could be determined in a study that manipulates the timing of DPCP sensitisation after prolonged exercise. Nevertheless, the findings in the current study provide support that the previously observed exercise induced immune suppression to DPCP is not inhibited during the initial stages of CHS at the skin, and likely occurs further downstream (Harper Smith *et al.*, 2011; Diment *et al.*, 2015).

Stress hormones such as glucocorticoids and catecholamines have been implicated in altered cutaneous immune responses (Flint *et al.*, 2001). For instance, CO responses have been shown to be inhibited in response to pharamacological doses of corticosteroids (Towbin *et al.*, 1995). In addition, intradermal injections of high-dose corticosterone or catecholamines have been shown to suppress DTH and CHS responses, inhibit the antigen presenting capability of

cutaneous DCs, and reduce the number of T cells in draining lymph nodes (Dhabhar and McEwen; Flint *et al.*, 2001; Seiffert *et al.*, 2002). Although it is recognised that the lack of stress hormone assessment in the current study is a limitation, we have previously demonstrated significant increases in stress hormones using the same exercise model (Diment *et al.*, 2015). Stress hormones are also widely accepted to play a key role in modulating immune function in response to psychological stress (Dhabhar, 2014). Given that we observed a modest increase in anxiety prior to exercise (*vs.* CON-CO), it is possible that psychological measures such as anxiety and psychological stress are important factors in modulating *in vivo* immunity, and requires further investigation.

Cutaneous measures of *in vivo* immunity are practical, safe, and can be administered without the need for expensive equipment, invasive injections, or blood sampling, making them attractive tools to assess immune function in both laboratory and field investigations. However, a standard protocol for measuring CHS responses in humans has yet to be established. For instance, 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 (Narbutt *et al.*, 2005; Harper Smith *et al.*, 2011). Erythema is typically the preferred measure of irritant responses which induce less edema than CHS responses (Parslew and Friedmann, 1999). Notwithstanding, the use of sensitisers 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. Furthermore, the standardised CHS model using DPCP overcomes some of the limitations of vaccine models of *in vivo* immunity including variable immunogenicity, annual changes in vaccines, and difficulty when comparing the circulating antibody results from different studies using in-house ELISA (Hernandez-Bernal *et al.*, 2011; Burns, 2012).

In summary, prolonged, moderate intensity exercise does not influence local cutaneous inflammatory processes mediated principally via innate immune mechanisms. These findings support that the previously observed decrease in *in vivo* immunity to DPCP following prolonged exercise stress represents a systemic suppression of cell-mediated immunity rather than exercise effects on local inflammatory processes at the skin.

CHAPTER FIVE

Anxiety and perceived psychological stress play an important role in the immune response after exercise

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CHAPTER FIVE

Anxiety and perceived psychological stress play an important role in the immune response after exercise

5.1 SUMMARY

There are common pathways by which psychological stress and exercise stress alter immunity. However, it remains unknown whether psychological stress plays a role in the *in vivo* immune response to exercise. We examined the relationship between anxiety and perceived psychological stress reported before exercise and *in vivo* immunity after exercise using skin sensitisation with DPCP. In a randomised design, sixty-four, thoroughly familiarised, males completed widely used psychological instruments to assess state-anxiety and perceived psychological stress before exercise, and ran either 30 minutes at 60% (30MI) or 80% (30HI) $\dot{V}O_{2peak}$, 120 minutes at 60% (120MI) $\dot{V}O_{2peak}$ or rested (CON) before DPCP sensitisation. Cutaneous recall to DPCP was measured as the dermal thickening response to a low-dose series DPCP challenge 4-weeks after sensitisation. After accounting for exercise ($R^2 = 0.20$; $P < 0.01$), multiple-regression showed that pre-exercise state-anxiety (STAI-S; $\Delta R^2 = 0.19$; $P < 0.01$) and perceived psychological stress ($\Delta R^2 = 0.13$; $P < 0.05$) were moderately associated with the DPCP response after exercise. The STAI-S scores before exercise were considered low-to-moderate in these familiarised individuals (median split; mean STAI-S of low 25 and moderate 34). Further examination showed that the DPCP response after exercise (30MI, 30HI or 120MI) was 62% lower in those reporting low *vs*. moderate state-anxiety before exercise (mean difference in dermal thickening: -2.6 mm; 95% CI: -0.8 to -4.4 mm; $P < 0.01$). As such, the results indicate a beneficial effect of moderate (*vs*. low) state-anxiety and perceived psychological stress on *in vivo* immunity after exercise. Moreover, correlations were of comparable strength for the relationship between physiological stress (heart rate training

impulse) and the summed dermal response to DPCP (r = -0.37; 95% CI: -0.05 to -0.62; P = 0.01), and state-anxiety and the summed dermal response to DPCP (r = 0.39; 95% CI: 0.08 to 0.63; P < 0.01). In conclusion, state-anxiety and perceived psychological stress levels before exercise play an important role in determining the strength of the *in vivo* immune response after exercise. These findings indicate a similar strength relationship for the level of state-anxiety prior to exercise and the level of physiological stress during exercise with the *in vivo* immune response after exercise. Future research is required to investigate exercise-immune responses in athletes, military personnel and others in physically demanding occupations experiencing higher levels of psychological stress than those reported in this study e.g. related to important competition, military operations and major life events. Nevertheless, the present findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

5.2 INTRODUCTION

Numerous studies report an increase in URI symptoms following a bout of strenuous exercise and during periods of heavy training in athletes (Peters and Bateman, 1983; Nieman *et al.*, 1990; Hellard *et al.*, 2015), and there is widespread agreement that a transient suppression of immune function is at least partly responsible (Walsh *et al.*, 2011b). A multitude of training and lifestyle stressors are thought to be involved in the observed decrease in immune function in athletes and military personnel; including, prolonged training sessions, exposure to environmental extremes (e.g. heat, cold and high altitude), poor nutrition and poor sleep (Shephard and Shek, 1995, 1997; Shephard, 1998; Walsh *et al.*, 2011a; Walsh *et al.*, 2011b). For example, prolonged heavy exercise (≥ 2 h) transiently decreases *in vitro* measures of immunity in isolated blood samples (Walsh *et al.*, 2011b) and more clinically meaningful *in vivo* measures of immunity instigated at the skin, including DTH and CHS (Bruunsgaard *et al.*, 1997; Harper Smith *et al.*, 2011; Diment *et al.*, 2015). Indeed, recent work highlights the immunosuppressive effect of prolonged exercise (2 h) on the induction of CHS using DPCP (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). Besides the immunosuppressive effects of prolonged heavy training sessions, the training environment and lifestyle stressors such as nutritional deficits (e.g. energy, macro- and micro- nutrients) and poor sleep (e.g. total deprivation and disruption) have long been implicated in the decrease in immune function in athletes and military personnel (Shephard and Shek, 1995, 1997; Shephard, 1998). Somewhat surprisingly, field studies (multi-stressor environment) and laboratory studies mimicking real-world athletic and military scenarios by exposing participants to these stressors, either separately or combined, demonstrate only subtle and short-lived modulation of immunity at rest and in response to exercise (Severs *et al.*, 1996; Booth *et al.*, 2003; Laing *et al.*, 2008; Ingram *et al.*, 2015). Rather than decrease immunity, some studies actually show a beneficial 'priming' effect of stressors such as short-term sleep disruption (1 night) (Ingram *et al.*, 2015),

intermittent cold exposure (Jansky *et al.*, 1996) and intermittent hypoxic exposure on immunity (Wang *et al.*, 2011). As such, there is a pressing need for research investigating other likely behavioural, environmental and lifestyle candidates involved in the observed decrease in immune function in athletes and military personnel.

Given the well-known and marked influence of psychological stress on immunity and infection resistance (Cohen *et al.*, 1991; Dhabhar, 2014), and the likely shared mechanisms by which psychological stress and exercise stress alter immunity (Perna *et al.*, 1997); i.e. principally through activation of the HPA axis and SAM axis and subsequent immunomodulatory hormones, it has been hypothesised that psychological stress can play a role in the decrease in immunity with prolonged heavy exercise and heavy training (Perna *et al.*, 1997; Clow and Hucklebridge, 2001; Walsh and Oliver, 2016). Unfortunately, exercise immunologists rarely report measures of psychological stress in their studies and so there is little by way of empirical evidence to support this hypothesis (Rehm *et al.*, 2013). That there are striking similarities in the way acute and chronic psychological stress and acute and chronic exercise stress influence immunity provides indirect support for this hypothesis. For example, although chronic psychological stress is widely accepted to decrease immunity and increase infection risk (Cohen *et al.*, 1991; Dhabhar, 2014), short-lasting, moderate-intensity psychological stress can enhance *in vivo* immunity (Edwards *et al.*, 2006) and is considered a fundamental adaptive response to help us survive (Dhabhar, 2014). Similarly, prolonged heavy exercise and heavy training are widely accepted to decrease immunity and increase infection risk (Walsh *et al.*, 2011b), but short-lasting, moderate-intensity exercise stress can enhance *in vivo* immunity (Pascoe *et al.*, 2014).

With this information in mind, using a multiple linear regression model, the present study tested, and provides evidence supporting the hypothesis that the level of anxiety and perceived psychological stress reported by an individual prior to exercise play an important role in determining the strength of the *in vivo* immune response to DPCP after exercise.

5.3 METHODS

Using the CHS responses to exercise from a previous study (Diment *et al.*, 2015), this study provides novel insights regarding the influence of anxiety and perceived psychological stress on *in vivo* immunity after exercise.

5.3.1 Participants

Sixty-four healthy, recreationally active males (age 22 ± 3 years; height 180 ± 6 cm; body mass 76.7 ± 11.5 kg; $\dot{V}O_{2peak}$ 57 ± 6 mL·kg·min^{-1}) participated in the study. Participants were required to abstain from alcohol and strenuous exercise for 24 h before and 48 h after the experimental trials.

Participants were matched for age and aerobic fitness (gas exchange threshold and $\dot{V}O_{2peak}$) before being randomly assigned to one of four groups. Groups were 1) 120 min of seated rest (CON); 2) 30 min of moderate-intensity (60% $\dot{V}O_{2peak}$) exercise (30MI); 3) 30 min of high-intensity (80% $\dot{V}O_{2peak}$) exercise (30HI); or 4) 120 min of moderate-intensity (60% $\dot{V}O_{2peak}$) exercise (120MI).

5.3.2 Preliminary measures and familiarisation

$\dot{V}O_{2peak}$ was estimated by means of a ramped exercise test on a treadmill (see **Chapter 3, section 3.2** for details). At least 24 h after the preliminary measures and approximately 7 days before the experimental trial, participants were informed of their group allocation and attended the laboratory for familiarisation. For exercising participants, the calculated exercise intensity was verified, and the participant was familiarised by running for 50% of their allocated exercise duration. During this visit, all participants were familiarised with blood sampling and other relevant procedures.

5.3.3 Experimental procedures

On the day of the experimental trial, participants were transported to the laboratory at 0730 h and provided with a standard breakfast (0.03 $MJ \cdot kg^{-1}$). Participants then completed STAI-S and PSS (14-item) questionnaires to assess state-anxiety and perceived psychological stress (see **Chapter 3, section 3.3** for details). Participants remained in the laboratory prior to commencing the exercise or seated rest, and were permitted to perform light activities (e.g. reading, watching television, browsing the internet) during this period. Participants assigned to 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). Heart rate was monitored continuously during the experimental trials (Polar FT1, Polar Electro, Kempele, Finland) (**Figure 5.1**).

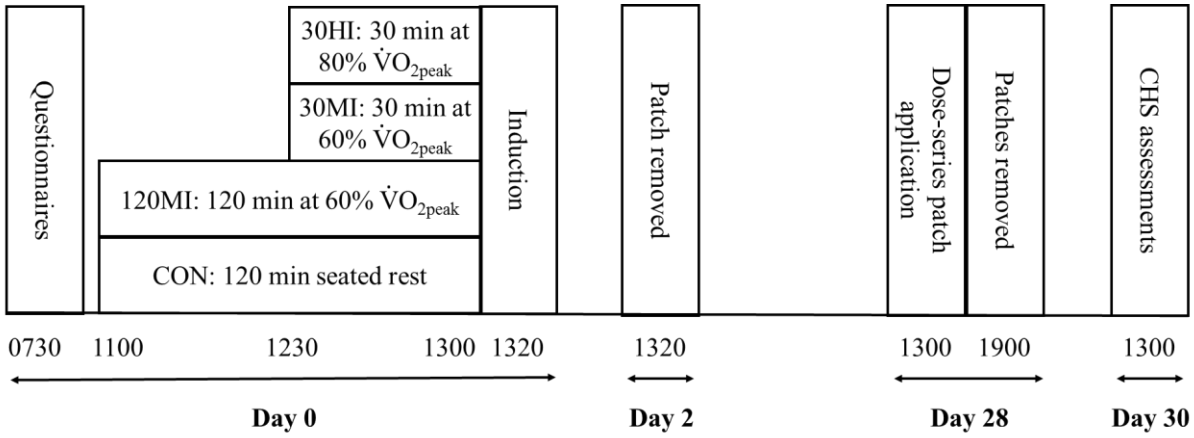

Figure 5.1 Schematic of experimental protocol examining the influence of state-anxiety and perceived psychological stress level prior to exercise on the subsequent *in vivo* immune response after exercise. Contact hypersensitivity (CHS) assessed as the summed dermal thickening and erythema response to the full dose-series elicitation challenge with diphenylcyclopropenone (DPCP) 28 d after DPCP induction.

5.3.4 Blood collection and analysis

Blood samples were collected before, immediately after, and 1 h after exercise or seated rest by venepuncture into two separate vacutainer tubes (Becton Dickinson, Oxford, UK), one containing K_3EDTA, and one containing lithium heparin. 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 epinephrine and norepinephrine were determined on K_3EDTA plasma, and plasma cortisol was determined on lithium heparin plasma using commercially available ELISA kits (CatCombi, IBL International, Hamburg, Germany and DRG Instruments, Marburg, Germany, respectively). The intra-assay coefficient of variation (CV) for plasma epinephrine, norepinephrine and cortisol was 4.1%, 4.1% and 4.4%, respectively.

5.3.5 Induction of contact sensitivity

Immediately after exercise, participants showered and returned to the laboratory within 15 min of completion before being sensitised to DPCP at 1320 h, exactly 20 min after exercise cessation. This short standardised delay in sensitisation allowed cutaneous blood flow to return to baseline (Diment *et al.,* 2015). Participants were sensitised to DPCP as described in **Chapter 3, section 3.4**.

5.3.6 Elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP. Twenty-eight days after the initial sensitisation to DPCP, participants received a challenge with a low-concentration dose-series of DPCP on individual patches, as described in **Chapter 3, section 3.6**.

5.3.7 Assessment of CHS responses

Dermal thickness, skinfold thickness and skin erythema were determined as described in **Chapter 3, section 3.7**.

5.3.8 Statistical analyses

Hierarchical linear regression analysis was used to examine the relationship between STAI-S and PSS (in 2 separate models) and *in vivo* immunity after exercise. In step 1 of each model, the influence of exercise on the summed dermal thickening response to DPCP was accounted for by calculating the training impulse (TRIMP) to reflect the level of physiological stress, as described (Banister, 1991). In step 2, the influence of each psychological measure on the summed dermal thickening response to DPCP was assessed. Sample size was deemed appropriate for the multiple linear regression analysis with 2 steps, in line with recommendations (Tabachnick and Fidell, 2013). To further illustrate the influence of anxiety on *in vivo* immunity after exercise, additional analyses were performed by categorising the population based on STAI-S scores using a median split; whereby, the levels before exercise were defined as low anxiety (LOW: STAI-S $\leq$ 29; mean 25) and moderate anxiety (MOD: STAI-S $\geq$ 30; mean 34): the STAI-S ranges for LOW and MOD are in line with those reported in the literature (Spielberger, 1983; Julian, 2011). Independent *t*-tests were used to compare the summed dermal responses to DPCP in LOW and MOD in each group (30MI, 30HI, 120MI and CON). Comparisons of psychological measures between groups (30MI, 30HI, 120MI and CON) were made using one-way ANOVA. A two-way, mixed-model ANOVA was used to analyse DPCP responses across the full dose-series challenge (anxiety level x dose) and circulating stress hormones (anxiety level x time) with significant differences identified using *post hoc* Tukey HSD, where appropriate. Pearson correlation coefficients were also calculated between physiological stress (TRIMP) and the DPCP response, anxiety and the DPCP

response, and circulating stress hormones and the DPCP response. To determine the influence of anxiety on the threshold DPCP dose that elicits a response, logarithmic transformation was performed on the DPCP data (LOW *vs*. MOD). This enabled the calculation of the *x*-intercept when $y = 0$, using linear regression on the linear portion of the dose-response curve. A threshold dose for a response to DPCP was then calculated by back transformation (antilog).

5.4 RESULTS

5.4.1 STAI-S Anxiety

Prior to exercise, there were no differences in psychological measures between groups (e.g. STAI-S scores for 30MI, 30HI, 120MI and CON) and participants reported low-to-moderate STAI-S scores (Figure 5.2A). In step 1 of the regression model (Table 5.1), exercise (TRIMP; 78 ± 60 AU) was a significant predictor accounting for 20% of the variance in the summed dermal thickening response to DPCP ($P < 0.01$); whereby, greater physiological stress was associated with a lower DPCP response following exercise. In step 2, STAI-S score was a significant predictor over and above exercise, accounting for an additional 19% of the variance in DPCP response ($P < 0.01$); together, exercise and anxiety accounted for 39% of the variance in the dermal thickening response to DPCP (Table 5.1). Pearson correlation coefficients were of comparable, moderate strength for the relationship between physiological stress and the summed dermal response to DPCP (TRIMP; $r = -0.37$, $R^2 = 0.13$, $P = 0.01$), and anxiety and the summed dermal response to DPCP (STAI-S score; $r = 0.39$, $R^2 = 0.15$, $P < 0.01$). This association between anxiety before exercise and *in vivo* immunity after an exercise challenge indicates that LOW were more likely to have a lower DPCP response following exercise stress than MOD (Figure 5.2B). When reported as the summed response to the five DPCP challenge doses, dermal thickening response was 62% lower in LOW than MOD (LOW 1.6 ± 2.3 and MOD 4.2 ± 3.1 mm; $P < 0.01$; $d = 1.0$).

Table 5.1 Multiple linear regression analysis examining the influence of state-anxiety and perceived psychological stress level prior to exercise on the subsequent *in vivo* immune response after exercise. Contact hypersensitivity (CHS) assessed as the summed dermal thickening response to the full dose-series elicitation challenge with DPCP 28 d after DPCP induction. After accounting for the negative influence of exercise in step 1, separate models show the positive influence of anxiety (from low to moderate levels), assessed using STAI-S in step 2 (A) and perceived psychological stress (from low to moderate levels) over the last month, assessed using PSS in step 2 (B), respectively.

Dependent variable: CHS	**B**	**SE**	***β***	***t***	**Δ*F***	**R^2**	**ΔR^2**
A. Step 1							
Exercise (TRIMP)	-0.005	0.002	-0.44	-2.93	8.56	0.20**	0.20**
Step 2							
STAI-S	0.06	0.02	0.44	3.24	10.50	0.39**	0.19**
B. Step 1							
Exercise (TRIMP)	-0.005	0.002	-0.44	-2.93	8.56	0.20**	0.20**
Step 2							
PSS	0.06	0.02	0.36	2.54	6.45	0.33**	0.13*

TRIMP = training impulse; STAI-S = State Trait Anxiety Inventory; PSS = Perceived Stress Scale;* $P < 0.05$; ** $P < 0.01$.

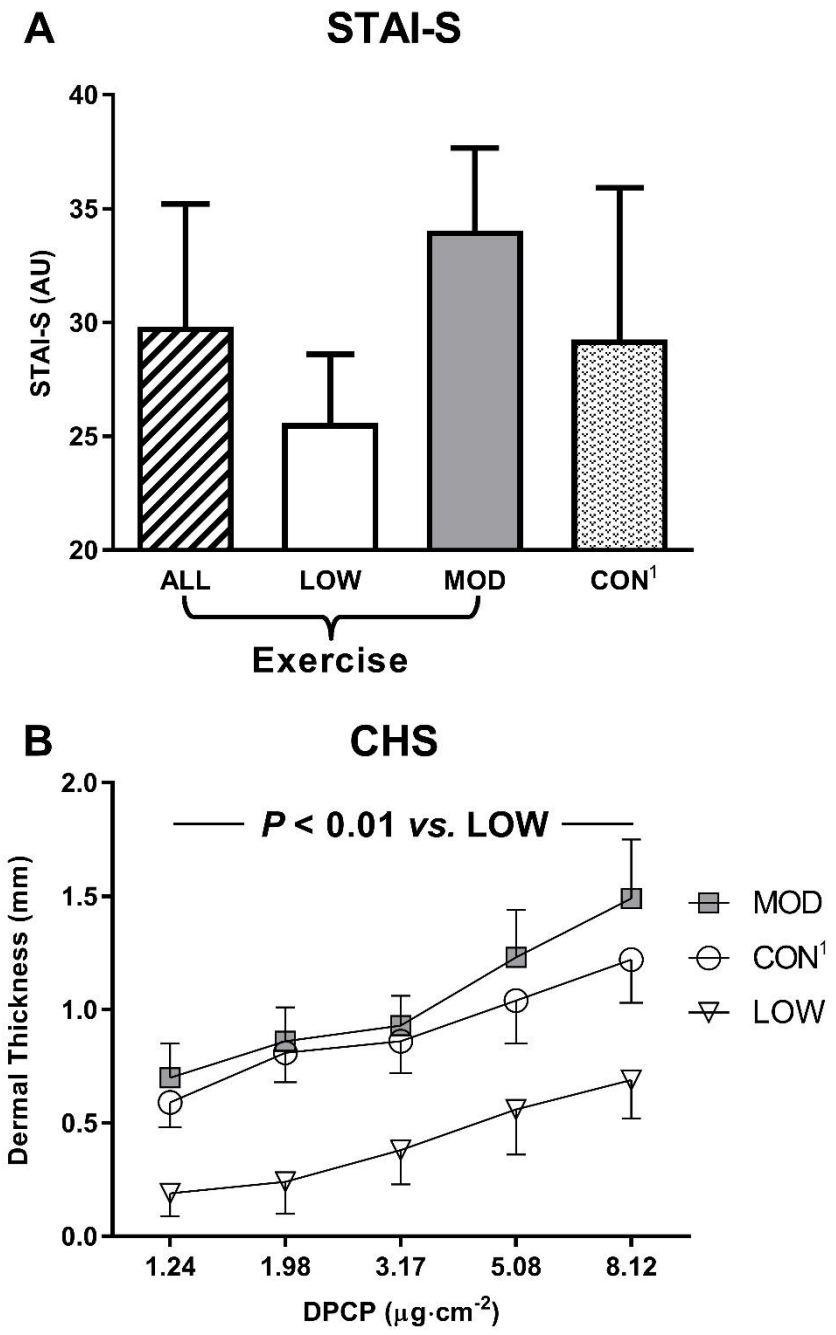

Figure 5.2 Effect of state-anxiety prior to exercise on the *in vivo* immune response after exercise. (A) Low (LOW) and moderate (MOD) levels of anxiety. Data are Mean ± SD. (B) Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. Dermal thickening response to the full dose-series challenge with DPCP is shown (30MI and 30HI). Data are Mean ± SEM for clarity. [1]Shown for comparison from Diment *et al.* (2015).

The ubiquitous influence of anxiety on *in vivo* immunity after exercise challenge (but not rested CON) is further illustrated in the comparisons between LOW and MOD in each group (30MI, 30HI, 120MI and CON; Figure 5.3A-D). Responses to DPCP assessed as skinfold thickness and erythema (data not shown for brevity), were smaller in LOW *vs*. MOD for 30MI ($P < 0.01$) and 30HI ($P < 0.05$; Figure 5.3A-B), but not CON. The suppressive effect of LOW *vs*. MOD was also apparent in 120MI ($P = 0.05$; $d = 0.9$; Figure 5.3C) which is particularly striking given that the suppressive effect of prolonged exercise on the induction of DPCP immune memory has been reported (Diment *et al.*, 2015). The lower CHS response to exercise in LOW *vs*. MOD is also illustrated in the smaller dermal thickening response across the full dose-series of DPCP in LOW *vs*. MOD ($F_{(1, 35)} = 11.1$, $P < 0.01$; Figure 5.2B for 30MI and 30HI). Furthermore, the threshold dose for a positive response to DPCP was calculated using the linear part of the dose-response curves. Compared with MOD, LOW required a 4-times greater DPCP dose (1.5 $\mu g \cdot cm^{-2}$) to elicit a positive response.

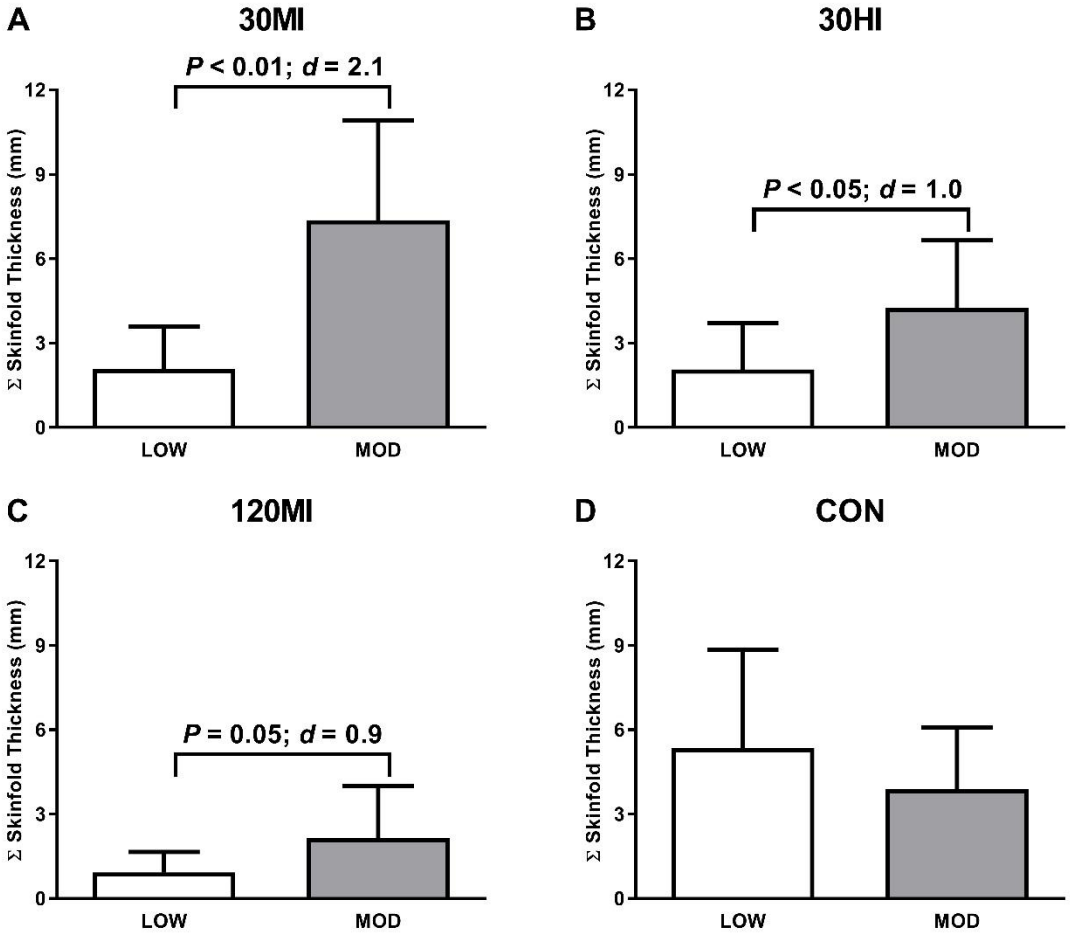

Figure 5.3 Effect of state-anxiety prior to exercise on the *in vivo* immune response after exercise of varying intensity and duration. (A−D) Summed increase in skinfold thickening response to DPCP challenge for each exercise group (30MI, 30HI and 120MI) and rested CON. Data are Mean ± SD.

5.4.2 Perceived Stress Scale

Participants reported low-to-moderate PSS scores (16.5 ± 5.3). Average PSS score for young adults has been reported as 21 ± 7 and high PSS score in posttraumatic stress disorder patients as 34 ± 8 (Hu *et al.*, 2014). After accounting for the influence of exercise in step 1 of the regression model (Table 5.1), PSS score was a significant, moderate predictor (in step 2), accounting for an additional 13% of the variance in DPCP response ($P < 0.05$); together,

exercise and PSS score accounted for 33% of the variance in the dermal thickening response to DPCP (Table 5.1). This association between the perception of psychological stress in the last month (i.e. the degree to which life situations are considered stressful) and *in vivo* immunity after exercise challenge indicates that participants reporting lower life stress were more likely to have a lower DPCP response following an exercise challenge than participants reporting moderate life stress.

5.4.3 Circulating stress hormones

When comparing LOW and MOD, a significant anxiety level x time interaction was observed for circulating epinephrine concentration ($F_{(2, 88)} = 5.9$; $P < 0.01$); whereby, epinephrine was lower in LOW than MOD at pre-exercise (LOW 0.25 ± 0.17 *vs.* MOD 0.58 ± 0.46 nmol·L^{-1}; $P < 0.01$), but not different at post or 1 h post-exercise. Similarly, an independent *t*-test showed that circulating cortisol concentration was also lower pre-exercise in LOW than MOD (LOW 545 ± 190 *vs.* MOD 699 ± 289 nmol·L^{-1}; $P < 0.05$); albeit, there was no significant interaction. Nevertheless, the lower circulating epinephrine and cortisol concentration in LOW than MOD before exercise represent large ($d = 0.94$) and medium ($d = 0.63$) effects, respectively. Circulating norepinephrine was not different between LOW and MOD. No significant relationships were observed between circulating stress hormones and the summed increase in dermal thickening response to DPCP challenge.

5.5 DISCUSSION

The aim of this work was to investigate the influence of anxiety and perceived psychological stress on the *in vivo* immune response after exercise. In line with the hypothesis, the level of anxiety and perceived psychological stress reported by the individual prior to exercise play an important role in determining the strength of the subsequent *in vivo* immune response after exercise (Table 5.1 and Figure 5.2). Moreover, the findings indicate a similar, moderate strength relationship for the level of anxiety prior to exercise (STAI-S; r = 0.39) and the level of physiological stress during exercise (TRIMP; r = -0.37) with the *in vivo* immune response after exercise challenge. The ubiquitous influence of anxiety on the immune response after exercise is further evidenced by a lower *in vivo* immune response to DPCP in individuals reporting low compared with moderate anxiety, regardless of the intensity and duration of the exercise challenge (30MI, 30HI and 120MI, Figure 5.2A−C). These findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

The findings of the present study demonstrate an important interaction between the a priori level of anxiety and perceived psychological stress and the subsequent immune response after an exercise challenge. We previously showed no significant influence of 30MI or 30HI on *in vivo* immunity (Diment *et al.*, 2015), but these new insights show a lower *in vivo* immune response in individuals reporting low compared with moderate anxiety in 30MI and 30HI (Figure 5.2A−B). Moreover, although we have previously shown a suppressive effect of 120MI compared with rested control on *in vivo* immunity (Diment *et al.*, 2015), particularly striking is the 50% lower *in vivo* immune response in individuals reporting low compared with moderate anxiety on 120MI (Figure 5.2C). Given that DPCP is benign, determining the clinical significance of these findings, 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 in this regard is supportive as cutaneous immune measures are impaired in individuals with acute infectious illness (Haider *et al.*, 1973; Bennett *et al.*, 1998), type 1 diabetes and psoriasis (Bangsgaard *et al.*, 2011) and predict mortality in critically ill HIV-infected patients (Dolan *et al.*, 1995). That we show lower pre-exercise circulating cortisol and epinephrine in the low compared with moderate anxiety group raises the possibility that stress hormones may modulate the immune response to subsequent exercise; indeed, stress hormones are considered to play important roles in preparing the immune system for challenge (Dhabhar and McEwen, 1999; Dhabhar, 2014). For example, administration of physiological doses of corticosterone and epinephrine increased T-cell drainage away from the site of DTH challenge to lymph nodes, which in-turn enhanced the DTH response in rats (Dhabhar and McEwen, 1999). In addition, adrenalectomy has been shown to eliminate stress-induced immune-enhancement in rats, likely by reducing the glucocorticoid and epinephrine response (Dhabhar and McEwen, 1999). Nevertheless, no associations were observed between circulating stress hormones and the CHS response to DPCP, and post-exercise circulating cortisol and epinephrine were not different between individuals reporting low and moderate anxiety in the present study; as such, further research is required into the underlying mechanisms.

Regarding the timing of the psychological measurements, the findings were unlikely due to an acute anticipatory effect prior to exercise as our participants underwent thorough familiarisation to all procedures, including running 50% of their allocated exercise duration; indeed, the success of familiarisation is shown as similar STAI-S scores prior to exercise and rested CON (Figure 5.1A). In addition, our findings for the relationship between STAI-S score

and the *in vivo* immune response after exercise are further supported by the relationship between PSS score and the *in vivo* immune response after exercise: PSS assesses the perception of stress, and measures the degree to which life situations spanning the last month are considered stressful (whereas STAI-S provides an acute measure of anxiety) (Cohen and Williamson, 1988). As such, the PSS findings provide added confidence regarding the observed association between psychological stress and the *in vivo* immune response after exercise challenge. It remains to be shown whether individuals are predisposed to respond to stressful situations, such as competitive sport or military scenarios, in a predictable manner with regards to neuro-endocrine-immune responses. In support of this notion, there is some evidence that personality traits predict endocrine-stress-reactivity (Edelstein *et al.*, 2010; Bibbey *et al.*, 2013); nevertheless, further research is required to investigate this novel concept in exercise immunology, and to establish whether the findings of the present study extend to other immune measures e.g. vaccination responses (Burns, 2012; Whittaker, 2018) and mucosal immunity (Hanstock *et al.*, 2016). Further research is also required to disentangle the influence of psychological and physiological strain during prolonged exercise (e.g. during endurance and ultra-endurance events) on *in vivo* immunity. Psychological stress measurements were made before exercise in the present study and it is reasonable to assume that psychological stress during more prolonged exercise (e.g. 120MI) might also play a role in the observed decrease in the *in vivo* immune response (Figure 5.2C).

Research investigators have long since acknowledged a role for psychological stress in the decrease in immunity associated with heavy exercise and training but there is little empirical research to support this hypothesis (Perna *et al.*, 1997; Clow and Hucklebridge, 2001). Since Clow and Hucklebridge's Exercise Immunology Review article highlighting this working hypothesis in 2001 (Clow and Hucklebridge) there have been > 3,000 peer-reviewed

publications in exercise immunology (using the search terms ‘exercise’ and ‘immune’, Web of Science^{TM}) yet < 5% of these publications include the search terms ‘psychological stress’ or ‘anxiety’. Closer inspection of this small subset of exercise immunology publications reveals that the large majority mention a putative role for psychological stress or anxiety in exercise-immune modulation; however, only a small handful of original investigations either attempt to manipulate psychological stress or include objective measures of psychological stress (Huang *et al.*, 2010; Moreira *et al.*, 2011; Rehm *et al.*, 2013; Rehm *et al.*, 2016). The present study answers the recent calls to physiologists (Wehrwein and Carter, 2016) and exercise immunologists (Walsh and Oliver, 2016) to incorporate objective psychological measurements in their human studies.

The findings herein support the recommendation that exercise immunologists should include aspects of mental health (e.g. psychological stress and others), in a broader conceptual framework of exercise-immune interactions alongside other factors thought to decrease immunity in athletes and military personnel (e.g. prolonged training sessions, poor nutrition etc.). This will inform and direct research questions and experimental designs with the aim of improving our understanding of the complicated exercise-immune interactions and with the potential to provide effective countermeasures to immune impairment in those concerned. To this end, the exercise immunologist’s toolkit will be enhanced by joining forces with experts in the ever expanding field of psycho-neuro-immunology to begin to disentangle the psychosocial and physiological underpinning of decreased immunity and increased infection risk in high level athletes, military personnel and others in physically demanding occupations. Our finding that pre-exercise anxiety and perceived psychological stress accounted for additional variance in post-exercise *in vivo* immunity after accounting for exercise (using TRIMP) emphasises the importance of incorporating psychological measurements in studies

investigating the immune response to exercise. As do the similar strength correlations for pre-exercise anxiety (STAI-S; r = 0.39) and physiological stress during exercise (TRIMP; r = -0.37) with *in vivo* immunity after exercise. These findings indicate a beneficial effect of moderate (*vs*. low) anxiety and perceived psychological stress on *in vivo* immunity after exercise (Figure 5.3A and D); as such, the findings accord with the immune-enhancement theory of moderate stress (Edwards *et al.*, 2006; Edwards *et al.*, 2007; Dhabhar). Further research is required to investigate exercise-immune responses in athletes, military personnel and others in physically demanding occupations (e.g. firefighters and mountain rescue workers) experiencing higher levels of psychological stress than those reported in this study e.g. as might occur in relation to important competition, major life events etc. The immuno-suppressive effects of chronic high stress in rats (3 weeks of restraint and shaking stress) (Dhabhar and McEwen, 1997) and humans (examination period) (Smith *et al.*, 2004a) are widely acknowledged (Dhabhar, 2014). As such, research is required to test the hypothesis that chronic high levels of psychological stress exacerbate the decrease in *in vivo* immunity after exercise. Irrespective, the present findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise, and for coaches and support staff to monitor anxiety and psychological stress alongside more traditional physiological measures of training stress. Accordingly, recent evidence highlights that aspects of mental health such as psychological stress and depression are important risk factors for illness in Olympic athletes (Drew *et al.*, 2017). In time, studies may demonstrate the utility of interventions to alter psychological stress in order to optimise immunity and host defence in athletes, military personnel and those in physically demanding occupations. There is good reason for optimism as an 8-week mindfulness meditation programme increased the antibody response to influenza vaccine in employees working in a highly stressful environment (*vs*. waiting-list controls) (Davidson *et al.*, 2003). Also, although somewhat limited

methodologically, preliminary work in competitive athletes showed that a 3-week stress management intervention reduced the number of days out due to illness and injury (Perna *et al.*, 2003).

In summary, these findings show that anxiety and perceived psychological stress levels prior to exercise play an important role in determining the strength of the *in vivo* immune response after exercise. Moreover, these findings indicate a similar, moderate strength relationship for the level of state-anxiety prior to exercise and the level of physiological stress during exercise with the *in vivo* immune response after exercise. Future research is required to investigate exercise-immune responses in athletes and others in physically demanding occupations experiencing higher levels of psychological stress than those reported in this study e.g. related to important competition and major life events. Nevertheless, these findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

CHAPTER SIX

The level of anxiety prior to an acute psychological stressor predicts *in vivo* immunity after the stressor

CHAPTER SIX

The level of anxiety prior to an acute psychological stressor predicts *in vivo* immunity after the stressor

6.1 SUMMARY

It is widely accepted that psychological stress modulates immune function, and highly-anxious individuals are more likely to perceive and experience psychological stress resulting in a greater chronic stress burden. However, limited evidence exists examining the role of anxiety on *in vivo* immunity after an acute psychological stressor. Therefore, the aim of this study was to investigate whether the a priori level of anxiety reported by the individual prior to an acute psychological stressor was an important predictor of *in vivo* immunity after the stressor. Thirty-one healthy participants reported state-anxiety in the 10 min before undertaking a novel zip-line stressor to evoke acute psychological stress. Participants were sensitised to DPCP immediately upon cessation of stressor, and cutaneous recall to DPCP was measured as the dermal thickening and erythema responses to a low-dose series challenge 4-weeks after sensitisation. Using curvilinear regression, state-anxiety was found to be a significant predictor of the *in vivo* immune response to DPCP (STAI-S; $\Delta R^2 = 0.15$; $P < 0.05$) indicating that individuals reporting low and high levels of anxiety prior to an acute psychological stressor were more likely to have lower DPCP responses compared to those reporting moderate levels of anxiety. Additional analyses further highlighted this finding, whereby, the DPCP response was lower in participants reporting low-anxiety (LOW STAI-S; mean 24, -72%, $P < 0.01$) and high-anxiety (HIGH STAI-S; mean 55, -50%, $P < 0.05$) *vs*. moderate-anxiety (MOD STAI-S; mean 36). In conclusion, these findings highlight that the level of anxiety prior to an acute psychological stressor is an important predictor of *in vivo* immunity. Moreover, these data indicate an inverted-U association between the a priori level of anxiety and the *in vivo* immune

response after an acute psychological stressor. The present findings emphasise the importance of assessing anxiety when examining the influence of stressors on immune function and raise the possibility for interventions to be investigated that manipulate the levels of anxiety to optimise immunity.

6.2 INTRODUCTION

There are a multitude of occupations and scenarios that are considered stressful and expose individuals to situations that cause increases in anxiety e.g. military, emergency services, air traffic controllers, athletes in competition etc (Crump, 1979; Newbury-Birch and Kamali, 2001). Highly-anxious individuals are more likely to perceive and experience psychological stress resulting in a greater chronic stress burden (Dhabhar, 2014). There is a widely-known and marked influence of psychological stress on immune function and infection resistance (Cohen *et al.*, 1991; Cohen *et al.*, 2007a; Dhabhar, 2014), and it is generally agreed that impaired immune responses are at least partly responsible for the increased incidence of infection (Segerstrom and Miller, 2004). However, the influence of psychological stress on immune function can be adaptive or suppressive (Dhabhar and McEwen, 1997; Edwards *et al.*, 2007; Dhabhar, 2014), whereby, chronic psychological stress is widely accepted to decrease immunity, but acute psychological stress can enhance both *in vitro* measures of immunity and more clinically relevant *in vivo* measures of immunity instigated at the skin including vaccine responses and DTH (Altemus *et al.*, 2003; Altemus *et al.*, 2006; Edwards *et al.*, 2006; Breen *et al.*, 2016). Indeed, the prominent influence of low-to-moderate levels of anxiety on the *in vivo* immune response after exercise stress has recently been highlighted, whereby, individuals reporting low levels of anxiety prior to exercise had lower immune responses after exercise compared to individuals reporting moderate levels of anxiety (**Chapter 5**). However, it remains unknown whether anxiety is an important predictor of *in vivo* immunity after an acute psychological stressor inducing a wide range (low-moderate-high) of anxiety levels.

Acute psychological stressors have been shown to increase levels of state-anxiety (Hare *et al.*, 2013; Nagy *et al.*, 2015) and modulate immunity, principally through activation of the HPA axis and SAM axis, and subsequent immunomodulatory hormones (Schedlowski *et al.*, 1993;

Benschop *et al.*, 1996; Breen *et al.*, 2016). However, there are differential effects of stress hormones such as cortisol and catecholamines on immune function (Dhabhar, 2014). In addition to the widely reported immunosuppressive effect of stress hormones, these immunomodulatory hormones have also been suggested to play important roles in preparing the immune system for challenge (Dhabhar and McEwen, 1999; Dhabhar, 2014). Yet the biological responses to stressful situations are heavily dependent on the individuals' appraisal of the situation and cognitive and emotional responses (Frankenhauser, 1986; Tomaka *et al.*, 1997; Segerstrom and Miller, 2004). However, few studies focus on the individuals' perception of the upcoming stressor (Feldman *et al.*, 2004). Given that there are individual differences in how a stressor is perceived, and that different individuals report varying levels of anxiety prior to undertaking the same stressor, it is likely that the level of anxiety prior to an acute psychological stressor is involved in modulating the subsequent *in vivo* immune response.

With this information in mind, the aim of the current study was to investigate whether the a priori level of anxiety reported by the individual was an important predictor of *in vivo* immunity after an acute psychological stressor. *In vivo* immunity was assessed by DPCP sensitisation immediately after the stressor and recall responses measured 28 d later. In line with the hypothesis, this study provides evidence that individuals reporting low and high levels of anxiety prior to an acute psychological stressor are more likely to have lower *in vivo* immune responses compared to those reporting moderate levels of anxiety.

6.3 METHODS

6.3.1 Participants

Thirty-one healthy adult volunteers participated in the study (16 males and 15 females; age 22 ± 3 yr; height 174 ± 10 cm; body mass 71.2 ± 14.0 kg). Participants had no previous experience of the stressor, had not undertaken another similar stressor in the past year (e.g. zip line or parachute jump), and did not regularly partake in activities involving heights (e.g. rock climbing). Participants abstained from alcohol and strenuous exercise for 24 h before and 48 h after cutaneous patch applications. No participants self-reported URI symptoms during the 4 weeks prior to the study.

6.3.2 Experimental procedures

The stressor consisted of a single ride on a ~1.6 km Zip line (Zip World Velocity, Bethesda, Gwynedd, UK) reaching speeds of ~160 $km \cdot h^{-1}$, and a duration of approximately 2 min from release to dismount. All testing took place between 0900 h and 1100 h to minimise diurnal effects and participants were transported to the top of the Zip line on the morning of the stress trial. In the 10 min prior to undertaking the stressor, state-anxiety was assessed as described in **Chapter 3, section 3.4**, and heart rate (HR) (FT7, Polar Electro, Kempele, Finland) was recorded every 2 min while participants remained seated. No physical effort was required to complete the task as trained instructors attached participants' safety harness to the line in a suspended recumbent position allowing limited movement. Following cessation of the stressor, participants were immediately sensitised to DPCP as described in **Chapter 3, section 3.4**. Participants remained seated for 20 min following stressor cessation during which time state-anxiety was assessed, and heart rate was recorded at 5 min intervals.

6.3.3 Elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP twenty-eight days after the initial sensitisation, as described in **Chapter 3, section 3.5**.

6.3.4 Assessment of CHS responses

Dermal thickness and skin erythema were determined as described in **Chapter 3, section 3.6**.

6.3.5 Statistical analysis

To examine the influence of state-anxiety on the *in vivo* immune response following an acute psychological stressor, a hierarchical regression was performed. After accounting for sex, the linear effect of STAI-S was entered into the model, followed by the curvilinear STAI-S (STAI-S x STAI-S). The STAI-S and mean HR responses were compared pre- and post- stressor using paired t-tests. Pearson correlation coefficients were used to examine the relationship between STAI-S and HR responses prior to the stressor. To illustrate the influence of anxiety on *in vivo* immunity after an acute psychological stressor, additional analyses were performed by categorising the population into low (LOW: ≤ 29; mean 24; n = 6), moderate (MOD: ≥ 30; mean 36; n = 14) and high (HIGH: ≥ 44; mean 55; n = 11) anxiety based on STAI-S scores. These STAI-S cut-offs were determined based on previous literature (**Chapter 5**; Spielberger, 1983). A one-way ANOVA was performed to compare the summed DPCP responses and heart rate between LOW, MOD and HIGH, respectively. A two-way, mixed-model ANOVA was used to analyse DPCP responses across the full dose-series challenge (anxiety level x dose). To determine the influence of anxiety on the threshold DPCP dose that elicits a response, logarithmic transformation was performed on the DPCP data (LOW and HIGH *vs*. MOD). This enabled the calculation of the *x*-intercept when $y = 0$, using linear regression on the linear

portion of the dose-response curve. A threshold dose for a response to DPCP was then calculated by back transformation (antilog). A sample size of 33 participants was estimated using data from **Chapter 5** examining the influence of anxiety prior to exercise stress on *in vivo* immunity. The sample size was deemed sufficient for regression analysis with two predictors using Cohen's F^2 effect size of 0.33, with the alpha set at 0.05, and power set at 0.8 (G*Power software, version 3.1.2).

6.4 RESULTS

6.4.1 Psychological and physiological responses to an acute psychological stressor

To examine the individuals' psychological and physiological response to the zip-line stressor, state-anxiety and heart rate were examined pre- and post-stressor. As expected, anxiety (Pre 41 ± 14; Post 29 ± 8 AU; $t_{(30)} = 6.23$; $P < 0.01$) and HR (Pre 78 ± 11; Post 73 ± 9 b·min^{-1}; $t_{(28)} = 4.16$; $P < 0.01$) were significantly higher pre-stressor compared with post-stressor, indicating that the stress protocol successfully induced anticipatory psychological and physiological responses. However, no significant relationship was observed between pre-stressor anxiety and heart rate ($r = 0.22$; $P > 0.05$).

6.4.2 Effect of anxiety on in vivo immunity after an acute psychological stressor

Using a curvilinear regression model, the association between the a priori levels of anxiety and the subsequent *in vivo* immune response to DPCP was examined. The linear effect of STAI-S was not a significant predictor of *in vivo* immunity ($\Delta R^2 = 0.01$; $R^2 = 0.01$; $\Delta F = 0.32$; $P > 0.05$). However, when the curvilinear association between STAI-S and *in vivo* immunity was examined, STAI-S was found to be a significant predictor, accounting for 16% of the variance in the dermal thickening response to DPCP ($\Delta R^2 = 0.16$; $R^2 = 0.17$; $\Delta F = 5.03$; $P < 0.05$). These findings indicate that individuals reporting low and high levels of anxiety prior to an acute psychological stressor were more likely to have lower DPCP responses compared to those reporting moderate levels of anxiety. The prominent influence of anxiety prior to an acute psychological stressor on *in vivo* immunity is illustrated in the comparisons between individuals reporting low (LOW), moderate (MOD) and high (HIGH) levels of state-anxiety (Figure 6.1A), whereby, the summed dermal thickening responses were 72% and 50% smaller, and summed erythema responses were 53% and 38% smaller in LOW and HIGH *vs.* MOD, respectively (Figure 6.1B-C). The dermal thickness responses to the full dose-series also

revealed a significant trial effect with smaller responses in LOW and HIGH *vs.* MOD (Figure 6.2). Furthermore, the threshold dose for a positive response to DPCP was calculated using the linear part of the dose–response curve, as 2.58, 0.62 and 1.80 $\mu g \cdot cm^{-2}$ for LOW, MOD and HIGH, respectively. This suggests that LOW and HIGH required a 4.2 and 2.8 times greater DPCP dose to elicit a positive response compared with MOD, respectively. Prior to the stressor, no differences in HR were observed between LOW, MOD and HIGH, respectively (LOW: 75 ± 8; MOD: 79 ± 10; HIGH: 80 ± 13 $b \cdot min^{-1}$; $P > 0.05$).

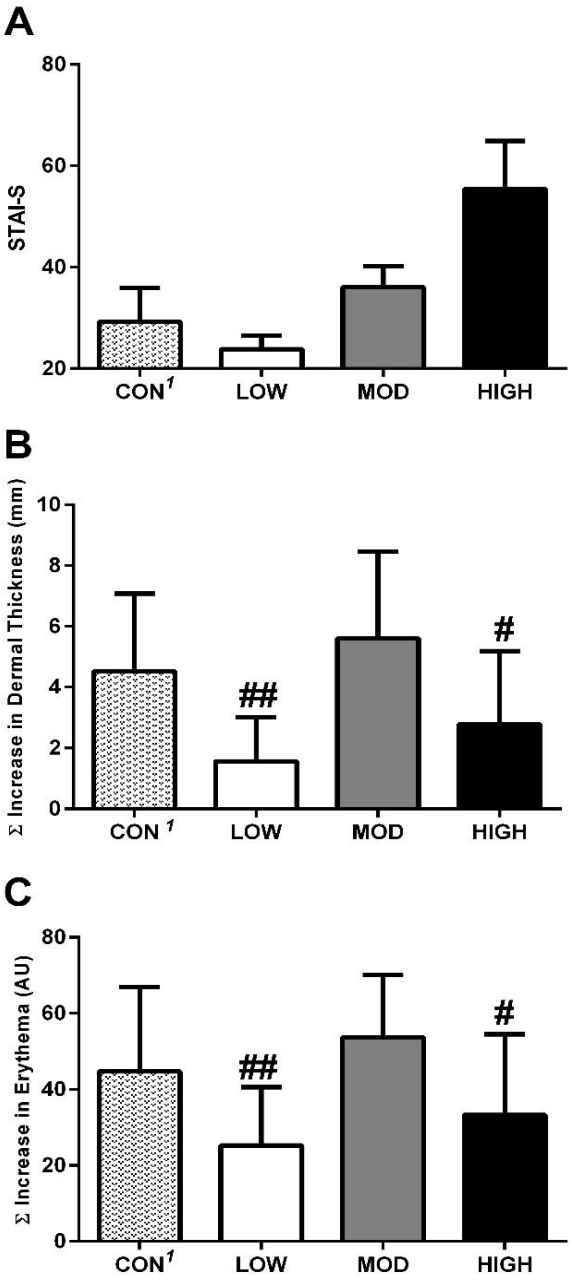

Figure 6.1 Effect of state-anxiety prior to an acute psychological stressor on the subsequent *in vivo* immune response. Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. A. Level of state-anxiety (STAI-S). B. Summed increase in dermal thickening response to DPCP challenge. C. Summed increase in erythema response to DPCP challenge. Data are shown as Mean ± SD. # $P < 0.05$, ## $P < 0.01$ significant difference *vs.* MOD. [1]Shown for comparison from Diment *et al.* (2015).

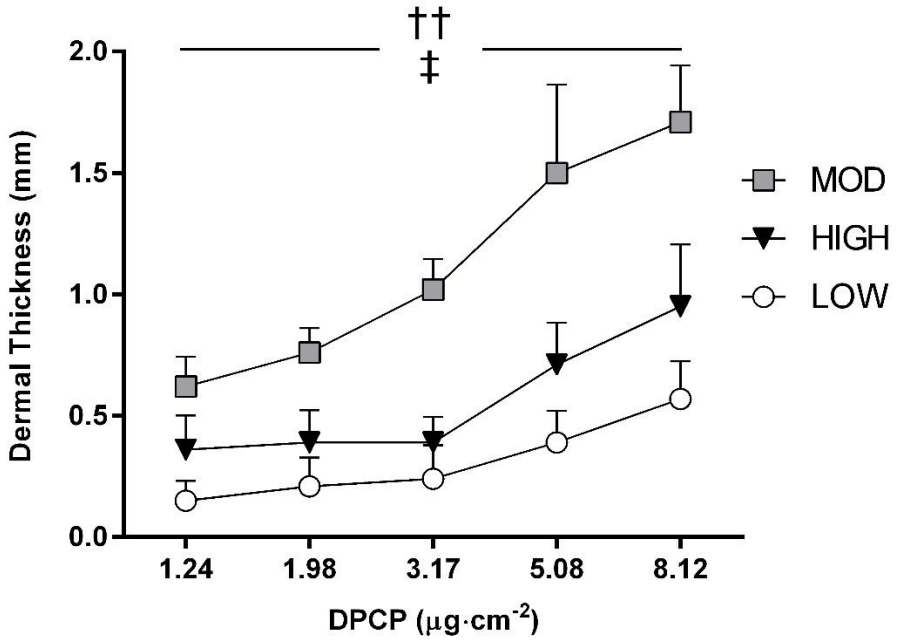

Figure 6.2 Effect of state-anxiety prior to an acute psychological stressor on the subsequent *in vivo* immune response. Contact hypersensitivity (CHS) assessed as elicitation challenge 28 d after DPCP induction. Dermal thickening response to the full dose-series challenge with DPCP is shown. Data are shown as Mean ± SEM for clarity. †† $P < 0.01$ LOW *vs.* MOD; ‡ $P < 0.05$ HIGH *vs.* MOD.

6.5 DISCUSSION

The aim of this study was to investigate whether the level of anxiety prior to an acute psychological stressor was an important predictor of *in vivo* immunity. In line with the hypothesis, the main finding was that individuals reporting low and high levels of anxiety prior to an acute psychological stressor had lower *in vivo* immune responses after the stressor. These findings indicate an inverted-U association between the a priori level of anxiety and the *in vivo* immune response after an acute psychological stressor. As such, this study emphasises the importance of assessing anxiety when examining the influence of stressors on immune function and raises the possibility for interventions to be investigated that manipulate anxiety prior to stressors to optimise immunity.

There is a well-known influence of psychological stress on immune function and infection incidence (Cohen *et al.*, 1991; Dhabhar, 2014). The novel zip-line stressor successfully induced acute psychological stress, as evidenced by higher levels of state-anxiety and heart rate immediately prior to the stressor compared with after the stressor. Furthermore, state-anxiety (STAI-S Pre 41) and heart rate responses (78 bpm) were similar to those reported during laboratory based acute psychological stressors such as memory recall and public speaking tasks (Feldman *et al.*, 2004; Nagy *et al.*, 2015). However, only a snapshot of psychological status was assessed in the current study, and it is unknown whether these findings are reflective of longer duration stress rather than just acute anticipation. For instance, we have recently shown that the levels of anxiety and perceived psychological stress reported before an exercise stressor predict the subsequent *in vivo* immune response (**Chapter 5**); perceived psychological stress assesses the perception of stress, and measures the degree to which life situations spanning the last month are considered stressful (Cohen and Williamson, 1988). There is further supporting evidence in this regard, indicating that chronic psychological stress increases cortisol

concentrations prior to an acute stressor, and that chronic stress alters the neuroendocrine and immune response to acute psychological stressors (Pike *et al.*, 1997; Viena *et al.*, 2012). It is also possible that longer-term psychological factors or personality traits could play an important confounding role in determine the response to stress, with future studies required to account for traits when examining the immune response to acute stressors. Furthermore, given that long term psychological factors such as early life experiences have been identified as risk factors for negative health outcomes (Avitsur *et al.*, 2015), future studies are required to investigate whether long term life stress predicts immune health and infection incidence in response to acute stressors. Moreover, previous trauma has been associated with diminished recovery to acute stress situations, indicating that those who experienced the highest levels of anxiety prior to the stressor may be at increased risk of experiencing prolonged stress reactions (Regehr *et al.*, 2007). Given that prolonged exercise stress is widely considered to cause an 'open window' of decreased immune function which increases the risk of illness (Walsh *et al.*, 2011b; Peake *et al.*, 2017), future studies are required to investigate the influence of recovery from psychological stress on immunity.

The findings in the present study emphasise that anxiety is an important modulator of the *in vivo* immune response after an acute psychological stressor. However, we observed no correlation between anxiety and HR responses prior to the stressor, indicating no direct relationship between psychological and cardiovascular responses. These findings are in agreement with previous literature that observed no relationship between anxiety and physiological variables during a flight in participants with flight phobia (Ekeberg *et al.*, 1990). The relative independence of autonomic measures, and their low correlation with perceptual responses have long been recognised (Marks and Huson, 1973; Ekeberg *et al.*, 1990). However, the way in which psychological and physiological responses to acute psychological stress are

linked is unclear. Given that only HR was assessed as a physiological measure in the current study, further research is required to investigate associations between psychological measures and other physiological responses (e.g. blood pressure, saliva cortisol, urine catecholamines etc) to acute stressors on *in vivo* immunity. For instance, stress hormones are widely accepted to module immune function and have been shown to have differential effects on *in vivo* immunity, whereby, administration of physiological doses of corticosterone and epinephrine have been shown to increase T-cell drainage away from the site of DTH challenge to lymph nodes, which in-turn enhanced the DTH response in rats (Dhabhar and McEwen, 1999). However, adrenalectomy has been shown to eliminate stress-induced immune-enhancement in rats, likely by reducing the glucocorticoid and epinephrine response (Dhabhar and McEwen, 1999). Given that blood sampling can influence psychological and hormonal stress responses (Hoogerwerf *et al.*, 2018), we decided against assessing blood stress hormones in the current study, but future studies using 'cannulation' or non-invasive sampling methods are required to delve into the potential mechanisms. Although saliva samples were collected, financial constraints unfortunately precluded the assessment of salivary stress hormone responses in the present study.

Here we show that individuals reporting low and high levels of anxiety prior to an acute psychological stressor were more likely to have lower *in vivo* immune responses after the stressor, indicating a beneficial effect of moderate anxiety on *in vivo* immunity. These findings accord with the immune enhancement theory of stress (Edwards *et al.*, 2007), and support an inverted-U association between anxiety and *in vivo* immunity. This is in accordance with the reactivity hypothesis which proposes that high or low stress reactivity could exacerbate day-to-day fluctuations in immune function, increase susceptibility to opportunistic infections (Cacioppo *et al.*, 1998) and indicate poor states of long-term health (Lovallo, 2011). As such,

future studies are required to investigate whether the findings in the present study extend to infection incidence and other clinically relevant immune measures such as vaccine responses. Nevertheless, we are optimistic that 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, as supported by the findings in **Chapter 4**. The available evidence in this regard is supportive as cutaneous immune measures are impaired in individuals with acute infectious illness (Haider *et al.*, 1973; Bennett *et al.*, 1998), diabetes and psoriasis (Bangsgaard *et al.*, 2011) and predict mortality in critically ill HIV-infected patients (Dolan *et al.*, 1995). However, given that DPCP is benign, studies need to determine the clinical relevance of the cutaneous recall response to DPCP with specific regard to infection.

The importance of incorporating psychological measures within physiology and exercise immunology has recently been highlighted (**Chapter 5**; Wehrwein and Carter, 2016). The findings of the current study reiterate this recommendation by demonstrating the prominent influence of anxiety on the *in vivo* immune response following an acute psychological stressor. These findings provide reason to speculate that athletes, military personnel and others in physically demanding occupations (e.g. firefighters and mountain rescue workers) experiencing high levels of anxiety and psychological stress will have altered immune responses to exercise, yet evidence supporting this notion is lacking. Further research is also required to investigate whether the findings of the current study are replicated with acute laboratory stressors designed to characterise the transient stresses of daily life (Marsland *et al.*, 2002). Furthermore, as individuals are likely to experience multiple stressors throughout the day, future studies should investigate the influence of repeated acute stressors on anxiety and immune function. Another interesting avenue for future research is to examine associations between stress perception and subsequent immune responses, particularly given that the type

of psychological stressor that individuals are exposed to evoke differential effects on immunity (Bosch *et al.*, 2001). Research has shown enhanced immune responses in mice that experienced an escapable foot-shock stress compared to mice that were forced to undergo the foot-shock stress (Steel *et al.*, 2015). As such, it is possible that a stressor perceived as 'positive' or 'exciting' may evoke differential immune responses to stressors that are perceived as 'negative' or 'aversive'. Irrespective of these exciting research opportunities, there is an increasing awareness and prevalence of mental health in those in psychologically demanding occupations such as soldiers (Hoge *et al.*, 2004; Hoge *et al.*, 2006) and emergency services personnel (Bennett *et al.*, 2004) which have a negative impact on work performance, absence and economic impact for the employer. Additionally, the impact of mental health on other aspects of health are profound i.e. respiratory disease, cancer cardiovascular disease (Prince *et al.*, 2007; Kleinman *et al.*, 2016). Our findings raise the possibility that anxiety manipulation interventions can be implemented in stressful occupations i.e. military, emergency services and athletes to optimise immune health. For instance, stress reduction interventions such as meditation have been shown to increase vaccination responses (Davidson *et al.*, 2003) and improve wound healing (Meesters *et al.*, 2018), and stressors such as acute video gaming or virtual reality have been proposed to increase levels of acute anxiety from low to moderate (Dhabhar, 2014; Siervo *et al.*, 2018). Moreover, given the overlap between symptoms of anxiety and depression (Dobson, 1985), individuals reporting high levels of anxiety to acute stressors may be more likely to experience depressive symptoms. Given that depression has been identified as a risk factor for common cold incidence (Kim *et al.*, 2011) and cardiovascular disease (Grippo and Johnson, 2002), and assessing the physiological responses to acute stressors have been associated with future disease mortality (Carroll *et al.*, 2012), this leads us to speculate that the assessment of psychological measures prior to acute stressors may predict infection incidence and even future disease and mortality outcomes.

In conclusion, these findings show that individuals reporting low and high levels of anxiety prior to an acute psychological stressor are more likely to have lower *in vivo* immune responses after the stressor, supporting an inverted-U association between anxiety and *in vivo* immunity. These findings highlight the importance of assessing anxiety when examining the influence of stressors on immune function and raise the possibility for interventions to be investigated that manipulate anxiety to optimise immunity.

CHAPTER SEVEN

Impaired *in vivo* immunity during pathogen confirmed upper respiratory tract infection and upper respiratory common cold symptoms

CHAPTER SEVEN

Impaired *in vivo* immunity during pathogen confirmed upper respiratory tract infection and upper respiratory common cold symptoms

7.1 SUMMARY

The aim of the present study was to assess the clinical relevance of DPCP with regard to the common cold by examining *in vivo* immunity using DPCP during a common cold. Twenty-six participants were recruited during the common cold season, with participants receiving a sensitising dose of DPCP either during a common cold or after 4-week daily monitoring for common cold symptoms. Four weeks after sensitisation, the strength of immune reactivity was quantified by measuring the cutaneous responses to a low dose-series challenge with DPCP. Common cold pathogens were determined using quantitative real-time PCR at the time of sensitisation. Sixteen participants reported URS during the 4-week monitoring period, with pathogen confirmed URTI identified in 8 (50%) of these participants. Dermal thickening responses to DPCP were 42% lower in URS compared with HEALTHY (URS: 2.26 ± 2.48 mm, HEALTHY: 3.87 ± 2.47 mm, $P < 0.05$), and 54% lower in URTI compared with HEALTHY ($P = 0.06$). In summary, impaired *in vivo* immunity during a common cold supports the notion that DPCP is a clinically relevant immune marker. Future research is required to investigate the predictive utility of DPCP with regard to the common cold.

7.2 INTRODUCTION

Respiratory infections and other infections such as those that affect the gastrointestinal system are major problems for athletes that may limit availability to train and take part in major competition (Walsh, 2019). As such, identifying risk factors for respiratory infection in athletes has been a major focus of exercise immunology over the last 30 years. However, it is often not practical for studies investigating the influence of stressors on immunity to follow up with infection incidence, primarily due to the relatively low incidence of illness and the subsequent need for large samples that make controlled laboratory studies unfeasible. As such, utilising clinically relevant immune measures are of crucial importance within stress and exercise immunology studies. The clinical relevance of *in vitro* measures of immunity are questionable because the function of cells *in vitro* loses the tissue-specific context within which immune responses operate and may behave differently to those that are activated *in vivo* (Akbar *et al.*, 2013). *In vivo* measures of immunity instigated at the skin, including DTH and CHS, provide an integrated, whole-body immune response that are considered more clinically meaningful than *in vitro* measures to assess immune competence (Albers *et al.*, 2013). There is substantial supporting evidence that skin *in vivo* immune measures are clinically relevant whereby, DTH reactions to common recall antigens in the Mérieux CMI Multitest and Mantoux test are impaired in immune deficient populations (Haider *et al.*, 1973; Lloyd *et al.*, 1992; Blatt *et al.*, 1993; Gordin *et al.*, 1994; Dolan *et al.*, 1995; Zaman *et al.*, 1997). Furthermore, DTH responses are decreased during various viral illnesses (Reed *et al.*, 1972; Kauffman *et al.*, 1974b; Kauffman *et al.*, 1976), relate to the time of progression to AIDS (Blatt *et al.*, 1993; Gordin *et al.*, 1994) and even relate to survival time in HIV-infected patients (Dolan *et al.*, 1995). However, the use of common recall antigens does not permit the assessment of new immune memory, and findings may be confounded by the lack of control over immunological memory given that the sensitising dose and time elapsed since sensitisation influence immunological

memory (Friedmann, 2007). Experimental CHS responses to novel antigens such as DPCP provide less invasive measures of CMI than DTH tests, and allow rigorous control of both the dose and timing of sensitisation. Moreover, there is strong supporting evidence that DPCP relates to clinical outcomes in immune deficient populations, whereby, i*n vivo* immune induction to DPCP successfully predicted allograft rejection in liver transplant patients (Bathgate *et al.*, 2001). Those with lower (or non-detectable) immune responses were less likely to reject the transplant, indicating that those with an immune system unable to respond to a challenge did not reject the transplant whereas those able to mount an immune response to DPCP were more likely to reject the liver transplant (Bathgate *et al.*, 2001). However, the clinical relevance of the cutaneous recall response to DPCP with regards to the common cold in otherwise healthy populations needs to be established. As such, the aim of this study was to assess the clinical significance of DPCP with specific relevance to the common cold by examining the *in vivo* immune response in participants reporting upper respiratory symptoms (URS) and pathogen-identified URTI at the time of sensitisation compared with healthy controls (HEALTHY).

7.3 METHODS

7.3.1 Participants

Twenty-six recreationally active volunteers (12 males and 14 females; age 22 ± 4 yr; height 172 ± 10 cm; body mass 68.2 ± 11.5 kg) were recruited across two common cold seasons in the UK (2014/15 and 2015/16). Participants did not have any underlying health conditions and were not regularly taking medication known to influence immune indices. Participants were required to abstain from alcohol and strenuous exercise for 24 h before and 48 h after visiting the laboratory for DPCP sensitisation and elicitation.

7.3.2 Experimental procedures

Participants completed the Jackson common cold questionnaire on a daily basis for a 4-week period (Hanstock *et al.*, 2016; Jackson *et al.*, 1958). The questionnaire included one dichotomous global question and eight symptom items (headache, sneezing, chills, sore throat, nasal discharge, nasal obstruction, malaise, and cough) scored on a four-point Likert scale (0, not at all; 1, mild; 2, moderate; 3, severe). If participants reported upper respiratory symptoms (URS) by answering ‘‘yes’’ to the dichotomous question ‘‘Do you think you are suffering from a common cold today?’’ or reporting a symptom score ≥ 6 for at least two consecutive days, they were asked to attend the laboratory as previously described (Hanstock *et al.*, 2016). In the laboratory, URS completed IPAQ, PSS and BRUMS questionnaires (**Chapter 3, sections 3.3 and 3.4**), had nasopharyngeal and throat swabs collected for screening of a battery of viral and bacterial pathogens, and were sensitised to DPCP, as described (**Chapter 3, section 3.5**). Those participants who did not report symptoms according to the predefined criteria for 4 weeks were defined as HEALTHY. At the end of the 4-week period, HEALTHY participants reported to the laboratory to complete the same IPAQ, PSS and BRUMS questionnaires as URS, had nasopharyngeal and throat swabs collected, and were sensitised to DPCP.

7.3.3 Elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP. Twenty-eight days after the initial sensitisation to DPCP, participants received a challenge with a low-concentration dose-series of DPCP on individual patches, as previously described (**Chapter 3, section 3.6**).

7.3.4 Assessment of CHS responses

Dermal thickness, skinfold thickness and skin erythema were determined as described in **Chapter 3, section 3.7**.

7.3.5 Nasopharyngeal and throat swabs.

Nasopharyngeal swab collection was performed by inserting a flexible, cotton-tip swab into one nostril until reaching resistance at the posterior nasopharynx and rotating it gently (Spence *et al.*, 2007). Throat swab collection was performed by swabbing the back of the throat at the tonsillar fossa (Spence *et al.*, 2007). The nasopharyngeal and throat samples were collected with different foam swabs, which were subsequently placed in the same viral transport medium and stored at -80 °C. Samples were shipped on dry ice to the microbiology laboratory at Queens University Belfast, and were analysed using quantitative real-time polymerase chain reaction (qPCR) analysis to screen for a battery of common upper respiratory pathogens: influenza types A and B; respiratory syncytial virus types A and B; metapneumovirus; adenovirus; coronavirus; parainfluenza virus types 1, 2, 3, and 4; human rhinovirus, bocavirus, *Pneumocystis jirovecii, Chlamydophila pneumoniae, Mycoplasma pneumoniae, and Bordetella pertussis*.

7.3.6 Statistical analysis

Independent *t*-tests were used to compare physical and psychological characteristics between HEALTHY and URS, and to compare summed DPCP responses between HEALTHY and URS, and HEALTHY and URTI. Independent *t*-tests were also used to compare common cold duration and peak symptom severity between URTI and those with unconfirmed-upper respiratory illness (U-URI). Pearson correlation coefficients were used to determine the relationships between summed DPCP responses and URS symptom severity and duration. To determine the influence of URS on the threshold DPCP dose that elicits a response, logarithmic transformation was performed on the DPCP data (URS *vs*. HEALTHY). This enabled the calculation of the *x*-intercept when $y = 0$, using linear regression on the linear portion of the dose-response curve. A threshold dose for a response to DPCP was then calculated by back transformation (antilog). Sample size was estimated using an effect size of 0.81 (G*Power software, version 3.1.9.2), which was calculated based on previous data, whereby, 87% of healthy control participants responded to the CMI multitest, with only 54% responding to this test during acute infectious illness (Hickie *et al.*, 1993; Bennett *et al.*, 1998).

7.4 RESULTS

7.4.1 Laboratory identification of respiratory pathogens

Of the 26 participants sensitised to DPCP, 16 reported upper respiratory symptoms (URS), and 9 were symptom and pathogen-free (HEALTHY). One male participant reported the onset of URS whilst the sensitisation patch was applied, and was subsequently removed from data analysis. This participant was confirmed positive for human Rhinovirus. Of the 16 participants reporting URS, URTI was confirmed in 8 (50%) by qPCR analysis; specifically, 7 participants were confirmed positive for human Rhinovirus and 1 participant was confirmed positive for adenovirus. Interestingly, the common cold episode at the time of DPCP sensitisation was significantly longer and more severe in URTI compared to U-URI (Figure 7.1).

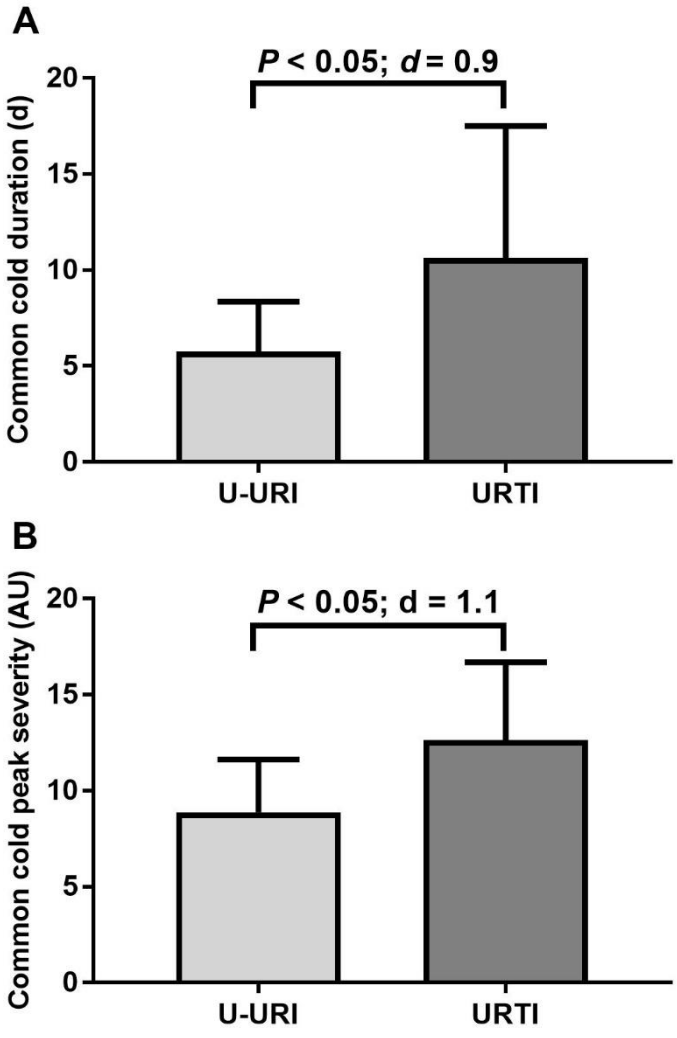

Figure 7.1 Comparison of common cold duration and severity between individuals with an unconfirmed upper respiratory illness (U-URI) and individuals with a pathogen confirmed upper respiratory tract infection (URTI). A, common cold duration. B, common cold peak severity. Data are shown as Mean ± SD.

7.4.2 Comparison of physical and psychological characteristics

There were no significant differences between HEALTHY and URS for physical characteristics (age: HEALTHY 23 ± 4, URS 21 ± 3 years; height: HEALTHY 172 ± 8, URS 172 ± 11 cm; body mass: HEALTHY 72 ± 15, URS 66 ± 9 kg). The level of physical activity was significantly greater in URS *vs.* HEALTHY in the week before sensitisation (Table 7.1). The level of perceived psychological stress and mood was comparable between HEALTHY and URS, except for fatigue, which was significantly greater in URS *vs.* HEALTHY (Table 7.1).

Table 7.1 Comparison of physical activity, perceived psychological stress and mood between participants who had not experienced upper respiratory symptoms in the previous 4-weeks (HEALTHY) and those reporting upper respiratory symptoms (URS) at the time of diphenylcyclopropenone (DPCP) sensitisation.

	HEALTHY	**URS**	**Sig**
IPAQ (MET·h·wk^{-1})	28 ± 23	51 ± 31	< 0.05
PSS	14.2 ± 11.6	13.9 ± 4.6	NS
BRUMS			NS
Anger	1.3 ± 2.5	1.1 ± 2.0	NS
Confusion	2.6 ± 2.6	1.9 ± 1.7	NS

Depression	1.1 ± 2.5	2.1 ± 2.5	NS
Fatigue	5.4 ± 3.0	8.0 ± 3.3	< 0.05
Tension	2.1 ± 2.0	1.5 ± 2.4	NS
Vigour	5.6 ± 4.1	5.5 ± 3.5	NS

IPAQ = International Physical Activity Questionnaire; PSS = Perceived Stress Scale; BRUMS = Brunel Mood Scale

7.4.3 The common cold and CHS responses to DPCP

The summed dermal thickening response to the five challenge doses of DPCP was 42% lower in URS than HEALTHY ($t_{(23)}$ = 1.77, $P < 0.05$; d = 0.7; Figure 7.2A). Similarly, summed erythema responses were 38% lower in URS than HEALTHY ($t_{(23)}$ = 2.04, $P < 0.05$; Figure 7.2B). To further investigate the differences between URS and HEALTHY, the threshold dose for a positive dermal thickening response to DPCP was calculated using the linear part of the dose-response curves, and compared with HEALTHY, URS required a 2.34 times greater DPCP dose (0.81 $\mu g.cm^{-2}$) to elicit a positive response. In the subgroup with confirmed URTI, skinfold thickness was 54% lower in URTI than HEALTHY; a finding that approached statistical significance (HEALTHY: 4.14 ± 3.7 mm, URTI: 1.92 ± 1.13 mm; $t_{(15)}$ = 1.63, P = 0.06; d = 0.8). No significant relationships were observed between summed DPCP responses and common cold duration or symptom severity.

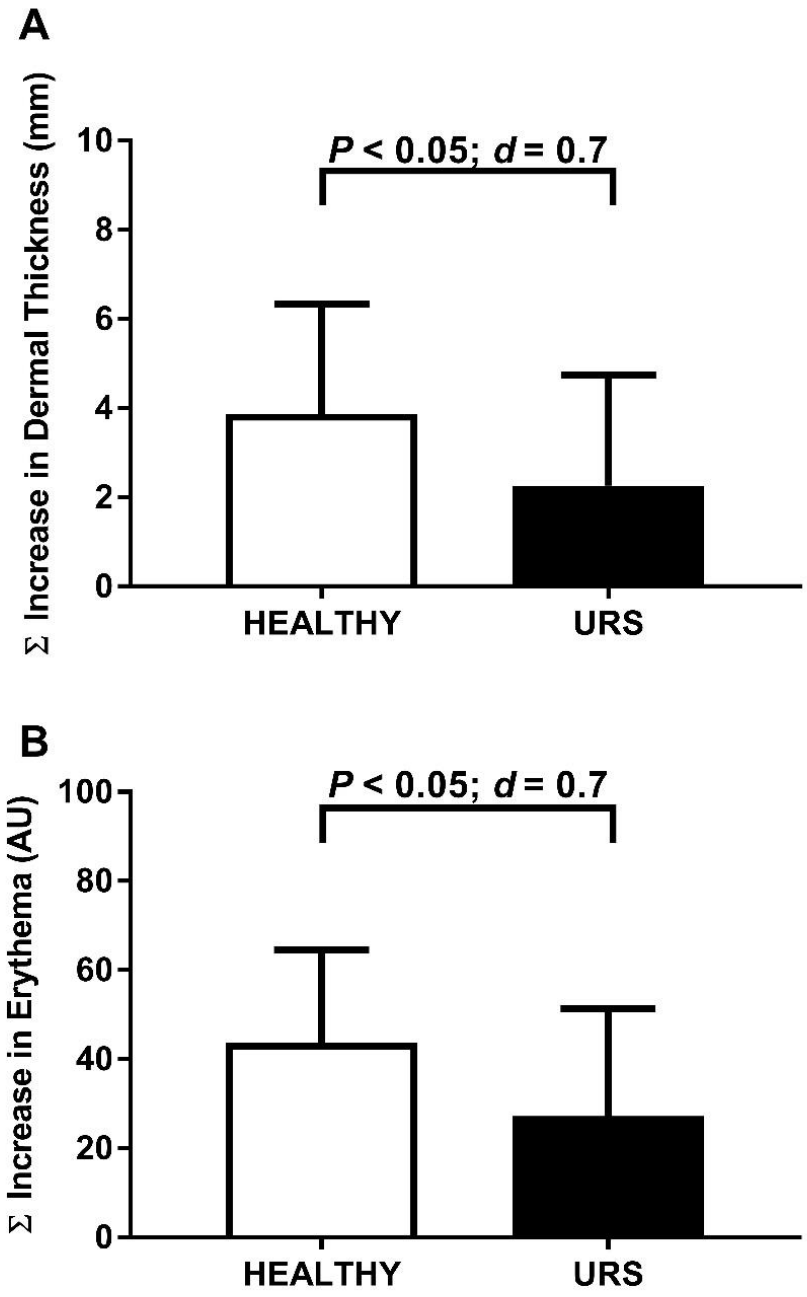

Figure 7.2 Comparison of *in vivo* immunity in individuals reporting upper respiratory symptoms (URS) and individuals not reporting symptoms (HEALTHY). Contact hypersensitivity assessed as the summed response to elicitation challenge with diphenylcyclopropenone (DPCP) 28 days after DPCP induction. A, URS *vs.* HEALTHY for summed dermal thickening response. B, URS *vs.* HEALTHY for summed erythema response. Data are shown as Mean ± SD.

7.5 DISCUSSION

The novel findings of the present study point to the clinical relevance of *in vivo* immunity using DPCP by demonstrating lower *in vivo* immunity in participants with common cold symptoms compared with healthy controls (-42%; Figure 7.2A). The current study also verified confirmed pathogens, and demonstrated lower DPCP responses in participants with pathogen confirmed URTI *vs.* healthy controls (-54%). The findings herein support the notion that DPCP is a clinically meaningful *in vivo* biomarker of immune competence. Nevertheless, future studies are required to prospectively examine the association between DPCP and common cold susceptibility.

It is widely acknowledged that intradermal DTH measures of *in vivo* immunity are impaired during viral illnesses (Reed *et al.*, 1972; Haider *et al.*, 1973; Kauffman *et al.*, 1974b; Kauffman *et al.*, 1976; Zaman *et al.*, 1997). The findings of the present study support that less invasive experimental CHS models are also clinically relevant by demonstrating impaired CHS responses to DPCP during URS, both with and without pathogen-confirmed URTI, compared with HEALTHY. The similar effect sizes between URS ($d = 0.7$) and URTI ($d = 0.8$) compared with HEALTHY provide added confidence regarding the findings observed between pathogen confirmed URTI and HEALTHY, despite not quite achieving statistical significance ($P = 0.06$). It is likely that lower DPCP responses represent compromised host defence and increased susceptibility to the common cold given the short incubation time of common cold pathogens such as HRV (Harris and Gwaltney, 1996), but we recognise that the present findings do not determine whether a lower DPCP response represents a lowered systemic immune status or whether the common cold alters the DPCP response. For example, it is possible that viral pathogens impair the migration of Langerhans' cells (LCs) which play a crucial role in CHS induction (Toebak *et al.*, 2009). Human rhinoviruses (HRV) use the cell surface receptor,

intracellular adhesion molecule 1 (ICAM-1), which is also an essential receptor on LCs, and an elegant study demonstrated inhibited LC migration into regional lymph nodes in ICAM-1 deficient mice (Xu *et al.*, 2001). Furthermore, ICAM-1 deficient APCs have an impaired capacity to induce T-cell responses (Smith *et al.*, 1996; Gaglia *et al.*, 2000). As such, it is possible that inhibition of T-cell proliferation is dependent on HRV binding to ICAM-1 on APCs, indicating that viral pathogens may interfere with lymphocyte activation indirectly through effects on APCs (Gern *et al.*, 1996; Kirchberger *et al.*, 2007).

Of those reporting URS, 50% had pathogen confirmed URTI, with 7 of 8 (87.5%) participants confirmed positive for HRV, in line with previous findings that HRV caused around 80% of common colds in adults during the seasonal autumn peak when assessed by qPCR (Arruda *et al.*, 1997; Makela *et al.*, 1998). The proportion of pathogen confirmed URTI is lower than previously reported by some (Arruda *et al.*, 1997; Makela *et al.*, 1998), but comparable or greater than others (Spence *et al.*, 2007; Cox *et al.*, 2008). Although viruses can be detected in small quantities for up to 3 weeks after infection, we cannot discount the possibility that the virus was undetectable in our participants given the short incubation time of HRV (shedding peaks on the second day and decreases rapidly thereafter); our participants were swabbed at least 2 days after the onset of common cold symptoms (Heikkinen and Jarvinen, 2003). There are over 200 serologically different viral types responsible for respiratory infections (Eccles, 2005), and newly identified viruses within the last 20 years highlight the potential for previously unknown pathogens to cause common cold like illnesses (Van den Hoogen *et al.*, 2001; Heikkinen and Jarvinen, 2003; Sloots *et al.*, 2006). As such, it is possible that those with U-URI had a viral pathogen not assessed in the current study. Our findings showed that common cold duration and peak severity were greater in URTI compared with U-URI, which raises the question about the cause of the unidentified illnesses. One possibility is that the

nature of symptoms may be allergic or inflammatory based rather than infectious (Bermon, 2007; Robson-Ansley *et al.*, 2012), particularly given that the level of physical activity was greater in URS compared with HEALTHY in the week prior to DPCP sensitisation. However, we recruited participants in the common cold season (when allergy is typically low) to minimise this risk. Another possibility is that some of the U-URI symptoms might be due in part to EBV viral shedding, which has been implicated as a cause of URI symptoms in athletes, albeit in small participant numbers (Gleeson *et al.*, 2002). EBV reactivation also provides a biomarker of cellular immunity indicating that immune function is compromised, thus providing an opportunity for infectious pathogens to take hold (Gleeson *et al.*, 2002; Spence *et al.*, 2007). Given that both *in vivo* immunity and assessments of viral latency are T-cell driven immune responses, future studies are required to disentangle the complex interactions between various aspects of immune function and URS (Mehta *et al.*, 2000). Irrespective of the cause of illness in U-URI, lower DPCP responses in URS compared with HEALTHY provides confidence that individuals reporting common cold symptoms were actually ill.

The level of perceived psychological stress was similar at the time of sensitisation between URS and HEALTHY. In addition, assessments of mood were similar between groups except for fatigue. The increased levels of fatigue in URS *vs.* HEALTHY was not surprising given that malaise is a widely accepted symptom of the common cold (Jackson *et al.*, 1958; Barrett *et al.*, 2002). Although similar levels of other mood measures (e.g. tension, confusion) were reported between URS and HEALTHY, it is possible that changes in mood occur later as the time course of mood assessment was taken during early onset of URS in the present study, however; little evidence of mood disturbances has been found when rhinovirus infection was experimentally induced (Drake *et al.*, 2000). Also, it is likely that changes in mood only occur in severe cases of upper respiratory symptoms (e.g. influenza) which result in changes in the

cytokine cascade and increased levels of inflammation. There is increasing evidence that psychological changes may be caused by the effects of cytokines on the central nervous system (Mahoney and Ball, 2002; Eccles, 2005). As such, future studies examining URS should consider assessing psychological measures in conjunction with cytokines and inflammatory biomarkers.

Experimental CHS using DPCP provides an attractive measure of *in vivo* immunity that 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 novel antigens 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 present findings lend support to the notion that *in vivo* immunity assessed by DPCP is also clinically meaningful by demonstrating lower *in vivo* immunity during a common cold. As such, exercise and stress immunologists are encouraged to utilise clinically relevant *in vivo* immune methods such as DPCP where possible. Notwithstanding, the use of experimental CHS is not without limitations. For instance, the use of novel antigens requires purposefully inducing CHS, albeit benign antigens such as DPCP overcome ethical constraints associated with using live pathogens; the doses are also low and the responses are temporary. Furthermore, the current twenty-eight day period between sensitisation and elicitation allows numerous confounders (e.g. life events) to affect the recall response (Harper Smith *et al.*, 2011). Nevertheless, the formation of immune memory occurs in less than 28 days with other antigens and future studies should determine the minimal amount of time required between sensitisation and elicitation to assess the primary immune response (Friedmann, 2007).

In conclusion, impaired *in vivo* immunity during a common cold supports the notion that DPCP is a clinically relevant immune marker. Future research is required to investigate the predictive potential of DPCP with regard to the common cold.

CHAPTER EIGHT

A preliminary investigation prospectively examining the association between upper respiratory illness and *in vivo* immunity using diphenylcyclopropenone

CHAPTER EIGHT

A preliminary investigation prospectively examining the association between upper respiratory illness and *in vivo* immunity using diphenylcyclopropenone

8.1 SUMMARY

There is a need for scientists examining immune health to utilise clinically relevant measures of immunity. With this in mind, this study aimed to prospectively examine whether *in vivo* immunity using skin sensitisation with DPCP is associated with upper respiratory symptoms in otherwise healthy individuals. In a prospective design, 53 healthy participants were monitored for URI symptoms on a daily basis for 13 weeks. Cutaneous recall to DPCP was measured as the dermal thickening response to a low-dose series DPCP challenge 14-days after sensitisation. EBV IgG antibody titre was also assessed as a measure of cellular immunity using ELISA. The CHS response to DPCP challenge was a significant predictor of peak URI severity (ΔR_2 = 0.26; R_2 = 0.37; $P < 0.05$). Furthermore, significantly lower DPCP responses were observed in those reporting the most severe URI episodes compared with those reporting the least severe URI episodes (Low URI severity 5.42 ± 3.47 mm *vs.* High URI severity 3.08 ± 2.47 mm; $P < 0.05$). Compared with high DPCP responders, low DPCP responders reported greater peak URI symptom severity (13.5 ± 2.4 *vs.* 10.6 ± 2.8 AU; $P < 0.05$), longer total URI duration (8.9 ± 6.4 d *vs.* 4.8 ± 3.4 d; $P < 0.05$) and greater total URI symptom score (159 ± 150 *vs.* 47 ± 32 AU; $P < 0.05$). Finally, the summed increase in dermal thickening responses to DPCP challenge were 40% lower in EBV seropositive individuals *vs.* EBV seronegative individuals (3.02 ± 2.75 mm *vs.* 5.06 ± 3.15 mm; $P < 0.05$). In summary, these findings provide promising preliminary support for the clinical utility of DPCP as a measure of *in vivo* immunity with regard to URI. Future studies with larger populations are required to confirm these findings.

8.2 INTRODUCTION

There are a vast array of biomarkers used to assess immune function with over 75 markers previously identified (Albers *et al.*, 2013). Many of these measures are considered *in vitro* where immune cells, typically from peripheral blood, are extracted from their normal environment and analysed in artificial cultures (Albers *et al.*, 2013). Although biologically interesting, the clinical relevance of investigations utilising *in vitro* measures of immunity are often questioned (Walsh *et al.*, 2011b). As such, there is a need for scientists examining immune health to use clinically relevant biomarkers of immunity where possible. Arguably the optimal means of evaluating global immune competency is via assessment of the immune response to *in vivo* challenge (Campbell and Turner, 2018). Experimental CHS using the novel antigen, DPCP, provides an attractive biomarker of *in vivo* cell-mediated immunity that is sensitive to the influence of numerous stressors including anxiety, psychological stress (**Chapters 5 and 6**) and prolonged heavy exercise stress (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). However, the clinical significance of differences in *in vivo* immunity to DPCP remains unknown. The available evidence in other *in vivo* immune measures are supportive, as cutaneous immune measures are impaired in individuals with acute infectious illness, diabetes, and psoriasis, and predict mortality in critically ill HIV-infected patients (Dolan *et al.*, 1995; Bennett *et al.*, 1998; Bangsgaard *et al.*, 2011). Furthermore, the findings in **Chapter 7** support the clinical significance of DPCP as a marker of *in vivo* immunity, whereby, *in vivo* immunity assessed during a common cold was over 40% lower compared to healthy controls. However, no studies have prospectively examined the association between DPCP and URI, with a recent call made to determine the clinical relevance of *in vivo* immunity using DPCP with regard to URI (Campbell and Turner, 2018).

In addition to experimental CHS responses to DPCP providing a measure of cell-mediated immunity, increases in antibody titres to latent viruses reflect a downregulation of the virus-specific cell-mediated T-cell response and the ability to control the steady-state expression of the latent virus (Glaser et al., 2005). Some evidence shows that increased shedding of latent viruses is associated with diminished DTH responses in Antarctic expeditioners (Mehta et al., 2000). Furthermore, herpesviruses serostatus have previously been suggested to relate to upper respiratory infection in athletes, whereby, common cold incidence occurred in 82% of athletes who were seropositive for Epstein Barr Virus, but no common colds were reported in athletes who were seronegative (Gleeson et al., 2002). Furthermore, given that both *in vivo* immunity and the assessment of antibody titres to latent herpesviruses are considered measures of cellular immunity mediated principally by T-cells, it seems plausible that *in vivo* immune responses to DPCP will negatively associate with antibody titres to latent herpes viruses, providing additional supporting evidence that impaired DPCP responses are reflective of lower cellular immune function.

The aim of this study was to prospectively examine whether *in vivo* immunity using DPCP relates to upper respiratory symptoms. To achieve this objective, we investigated the predictive utility of DPCP using regression analyses, prospectively examined the DPCP response in those reporting low and high URI severity, and compared common cold incidence, severity and duration between low and high DPCP responders. Finally, given that both *in vivo* immunity to DPCP and EBV viral latency are markers of cell-mediated immunity, we also examined the association between EBV viral latency and the *in vivo* immune response to DPCP.

8.3 METHODS

8.3.1 Participants

Fifty-three recreationally active males and females were recruited across two common cold seasons in the UK (2018/19 and 2019/20). Participants were 29 males and 24 females (males: age 21 ± 4 years, height 180 ± 8 cm, body mass 76.5 ± 12.9 kg; females: age 21 ± 3 years, height 165 ± 6 cm, body mass 63.6 ± 10.4 kg), did not have any underlying health conditions and were not regularly taking medication known to influence immune indices. Participants were required to abstain from alcohol and strenuous exercise for 24 h before and 48 h after visiting the laboratory for DPCP sensitisation and elicitation. All females were sensitised to DPCP in the early follicular phase of the menstrual cycle (3 – 7 d after onset of menstruation), with contraceptive pill users sensitised at least 24 h after starting the no pill or inactive phase of oral contraceptive use.

8.3.2 Common cold monitoring

Participants were prospectively monitored for URI symptoms on a daily basis for a period of 13-weeks using the Jackson common cold questionnaire (Jackson *et al.*, 1958) (Figure 8.1). The questionnaire included eight symptom items (headache, sneezing, chills, sore throat, nasal discharge, nasal obstruction, malaise, and cough) scored on a four-point Likert scale (0, not at all; 1, mild; 2, moderate; 3, severe), with a URI bout determined if participants reported a symptom score ≥ 6 for at least two consecutive days (equivalent to three moderate or two severe symptoms). To be considered a separate URI bout, seven consecutive days not achieving the criteria for URI was required, as previously described (Spence *et al.*, 2007) .

8.3.3 *Laboratory visits*

Participants first visited the laboratory to provide a blood sample for the assessment of latent EBV serology. Two days later, participants returned to the laboratory and completed STAI-S, STAI-T, PSS, BRUMS and IPAQ questionnaires (**Chapter 3, sections 3.3 and 3.4**) before being sensitised to DPCP. Exactly 14 days after the initial sensitisation to DPCP, participants received a challenge with a low-concentration dose-series of DPCP, with elicitation patches removed after 6 h and the strength of immune reactivity assessed as cutaneous responses 48 h after application. All skin patch applications were applied between 0800 h and 1200 h to minimise diurnal effects.

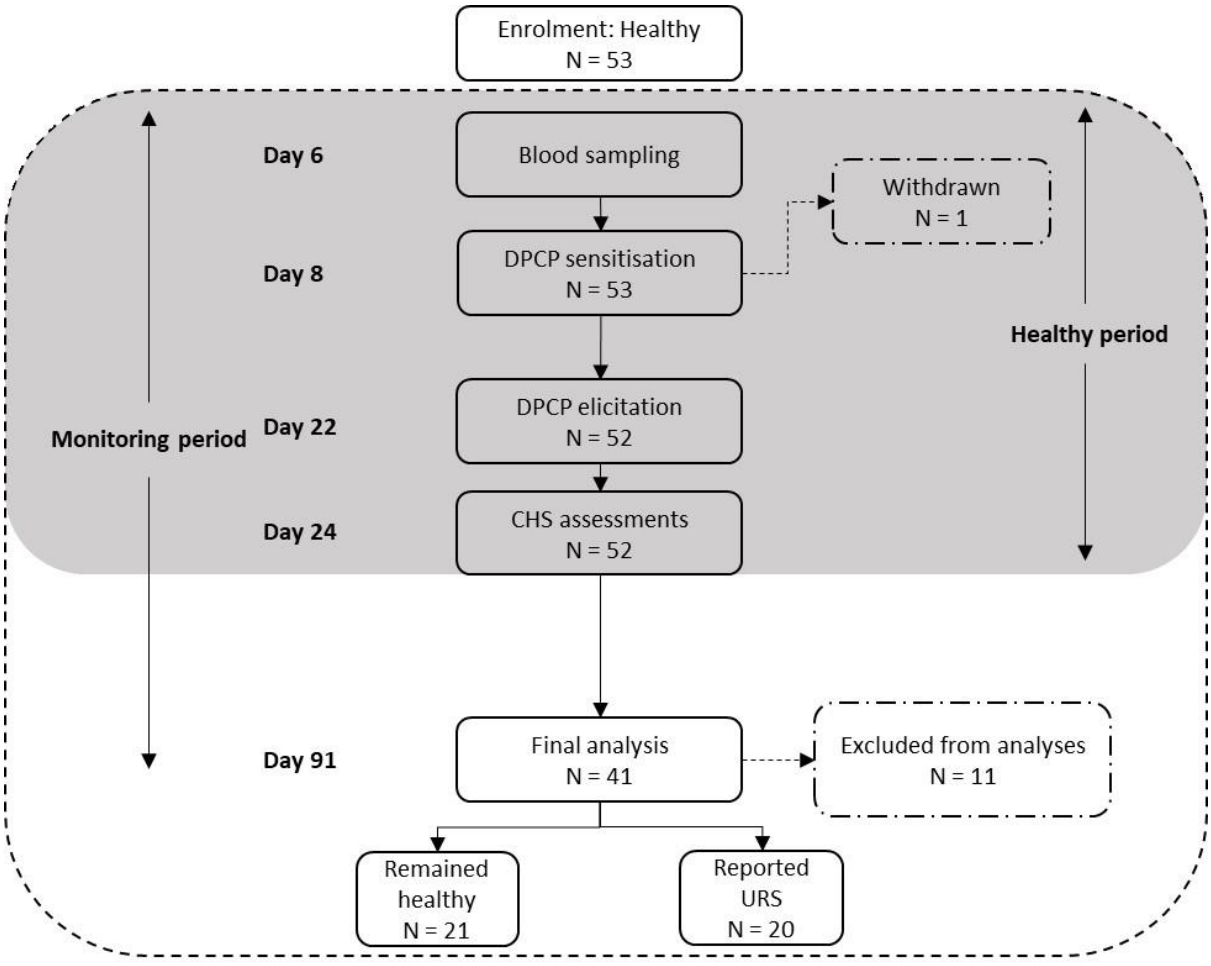

Figure 8.1 Flow chart depicting study protocol.

8.3.4 Blood collection and analysis

A resting venous blood sample was obtained by venepuncture from an antecubital vein into a 6 mL vacutainer tube (Becton–Dickinson, Oxford, UK) containing no additives following a 15 min period of seated rest. Following collection, the sample clotted for exactly 60 min at room temperature before being spun at 1500 g for 10 min in a refrigerated centrifuge. Serum was aliquoted into Eppendorf tubes, and frozen at -80 ºC for later analysis.

Samples were analysed for EBV serostatus and EBV VCA-IgG antibody titre using ELISA (Cat. No. EI 2791-9601 G; Euroimmun, Luebeck, Germany) to assess control over viral latency. Samples <16 RU/mL were considered seronegative with samples >22 RU/mL considered seropositive as recommended by the manufacturer. In EBV seropositive samples, antibody titre was determined by interpolation from a calibration curve using standards of 2, 20 and 200 RU/mL provided with the kit. Mean intra-assay coefficient of variation was 2.9% from duplicate standards and samples across all plates.

8.3.5 DPCP sensitisation

Participants were sensitised to DPCP as described in **Chapter 3, section 3.4**.

8.3.6 DPCP elicitation

The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP. Exactly fourteen days after the initial sensitisation to DPCP, participants received a challenge with a low-concentration dose-series of DPCP on individual patches in the following concentrations: 10 μL of DPCP: 0.0036%, 0.82 $\mu g \cdot cm^{-2}$; 0.0048%, 1.24 $\mu g \cdot cm^{-2}$; 0.0064%, 1.45 $\mu g \cdot cm^{-2}$; 0.0085%, 1.92 $\mu g \cdot cm^{-2}$; 0.0113%, 2.56 $\mu g \cdot cm^{-2}$; and, 10 μL of 100% acetone control for background subtraction. A pilot study within our

laboratory demonstrated no differences in cutaneous responses 14 days after sensitisation compared to the typically used 28-day induction period (**Appendix D**).

8.3.7 Assessment of CHS responses

Dermal thickness and skin erythema were determined as described in **Chapter 3, section 3.7**.

8.3.8 Statistical analysis

A sample size of 20 URI episodes was estimated using alpha of 0.05 and beta of 0.80 to calculate a Cohen's *d* effect size of 0.81 using data from **Chapter 7**, whereby, those with a confirmed URTI had 54% lower DPCP responses compared to healthy controls. Of the 52 participants with CHS data, 11 were excluded from the final analyses due to reporting the onset of URI prior to completion of CHS readings (N = 7) or not achieving the daily monitoring adherence criteria of 80% (N = 4). Independent t-tests were used to examine differences in summed CHS responses between those who reported URI and those who remained healthy during monitoring. Multiple linear regression was used to examine the predictive utility of DPCP responses on URI severity and duration, accounting for sex, BMI and EBV serostatus. To examine whether differences in URI symptom severity and duration were observed between different DPCP responders, we categorised the population (N = 41) using a median split based on the summed increase in dermal thickening response to DPCP challenge into low (LOW DPCP Mean: 1.23 mm) and high (HIGH DPCP Mean: 6.06 mm) DPCP responders. Similarly, low URI severity and high URI severity were categorised using median split. To determine the influence of URI severity on the threshold DPCP dose that elicits a response, logarithmic transformation was performed on the DPCP data (Low URI severity *vs*. High URI severity). This enabled the calculation of the *x*-intercept when $y = 0$, using linear regression on the linear portion of the dose-response curve. A threshold dose for a response to DPCP was then

calculated by back transformation (antilog). The CHS comparisons between low URI severity and high URI severity were examined using independent t-tests, as were differences in URI symptom severity and duration between LOW DPCP and HIGH DPCP. The following variables were examined for URI symptom severity and duration:

- Peak URI severity: defined as the maximum reported URI severity score.
- Mean URI severity: defined as the mean reported URI severity score.
- Total URI duration: defined as the total number of days reporting URI.
- Mean URI duration: defined as the mean reported URI bout duration.
- Total URI symptom score: defined as the total number of days reporting URI x URI symptom severity score (Gleeson *et al.*, 2011).

Comparisons of DPCP responses between individuals reporting ≥ 2 URI episodes and individuals reporting 0 or 1 URI episode during monitoring were examined using independent t-tests. Chi-squared analysis was used to examine whether the proportion of individuals reporting URI differed between EBV seropositive and seronegative individuals during monitoring, with independent t-tests used to examine differences in summed increases in DPCP responses between EBV seropositive and seronegative individuals. The relationships between the EBV IgG antibody titre and DPCP responses, and the EBV IgG antibody titre and URI symptom severity and duration variables were examined using Pearson correlation coefficients and Spearman rank correlation coefficients where necessary.

8.4 RESULTS

8.4.1 URI incidence, severity and duration

Of the 41 participants included in the final analyses, 20 (49%) reported at least one URI bout during the monitoring period. No differences in the summed increase in CHS responses were observed between those who reported URI and those who remained healthy ($P > 0.05$). However, the CHS response was a significant predictor of peak URI severity, accounting for 26% of the variance over and above control variables ($\Delta R_2 = 0.26$; $R_2 = 0.37$; $P < 0.05$). In addition, we compared those reporting the most severe URI episodes (High URI severity) with those reporting the least severe URI episodes (Low URI severity), and found significantly lower increases in dermal thickening responses to DPCP in High URI severity *vs.* Low URI severity ($P < 0.05$; Figure 8.2). To further investigate the differences between Low URI severity and High URI severity, the threshold dose for a positive dermal thickening response to DPCP was calculated using the linear part of the dose-response curves, and compared with Low URI severity (1.2 $\mu g.cm^{-2}$), High URI severity required a 2.75 times greater DPCP dose (3.3 $\mu g.cm^{-2}$) to elicit a positive response.

We next compared low and high DPCP responders to examine differences in URI incidence, severity and duration. LOW DPCP reported greater peak URI symptom severity, longer total URI duration and greater total URI symptom scores compared with HIGH DPCP (Figure 8.3A-C). No differences were observed for URI incidence, mean URI symptom severity or mean URI duration between LOW DPCP and HIGH DPCP (data not shown for brevity; $P > 0.05$). No differences in psychological and lifestyle factors at the time of sensitisation between LOW DPCP and HIGH DPCP indicate that the differences observed were not due to the influence of psychological and lifestyle factors ($P > 0.05$; Table 8.1).

As total URI symptom score incorporates URI duration (those with a longer duration are more likely to report more than one URI episode), it seems logical that those who report frequent URI bouts are more likely to have impaired *in vivo* immunity compared with those reporting URI bouts less frequently. Although supplementary data as only five participants reported recurring URI episodes during monitoring (≥ 2), interestingly, these individuals had 51% lower summed increases in dermal thickening responses compared with those reporting 0 or 1 URI (≥ 2 URI: 1.90 ± 1.43 mm; *vs.* 0 or 1 URI: 3.86 ± 3.09 mm; $t_{(39)} = 1.39$; $P = 0.08$).

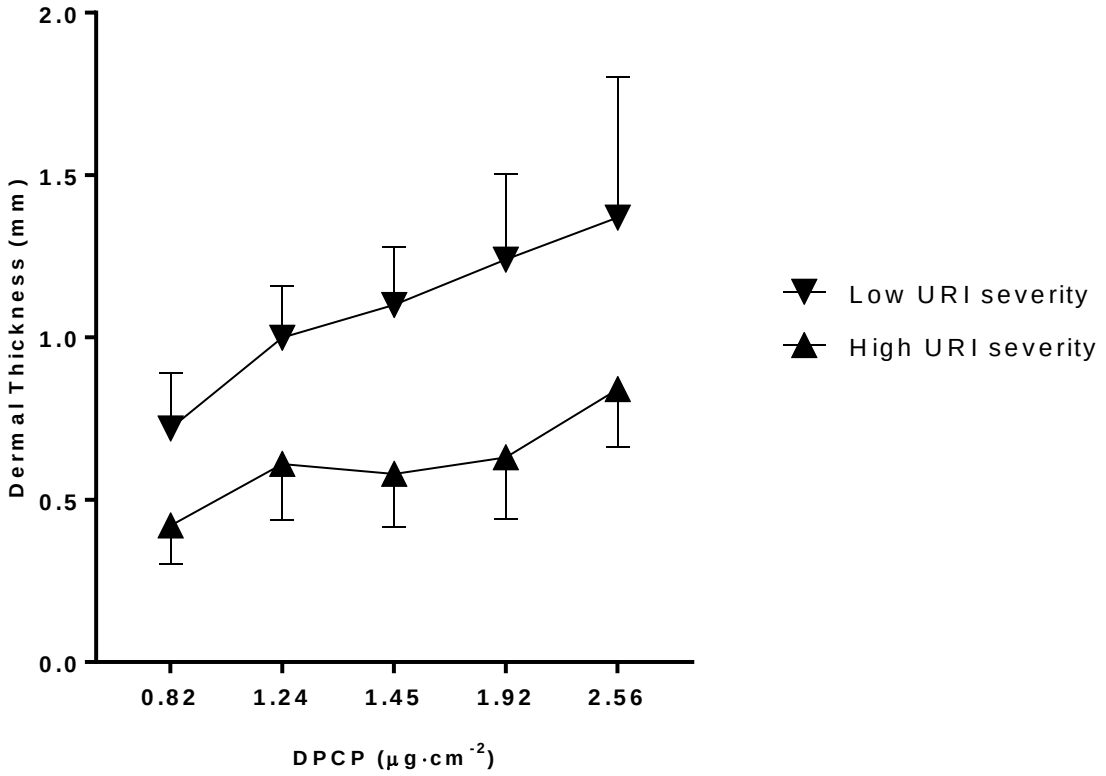

Figure 8.2 Comparison of *in vivo* immunity in those reporting low and high upper respiratory illness (URI) severity. Contact hypersensitivity (CHS) assessed as elicitation challenge 14 d after diphenylcyclopropenone (DPCP) induction. Dermal thickening response to the full dose-series challenge with DPCP is shown. URI symptom severity defined as the maximum reported

URI symptom severity score. LOW URI severity and HIGH URI severity were determined by median split. Low URI severity had significantly lower summed increases in dermal thickness compared with High URI severity. Data are Mean ± SEM for clarity.

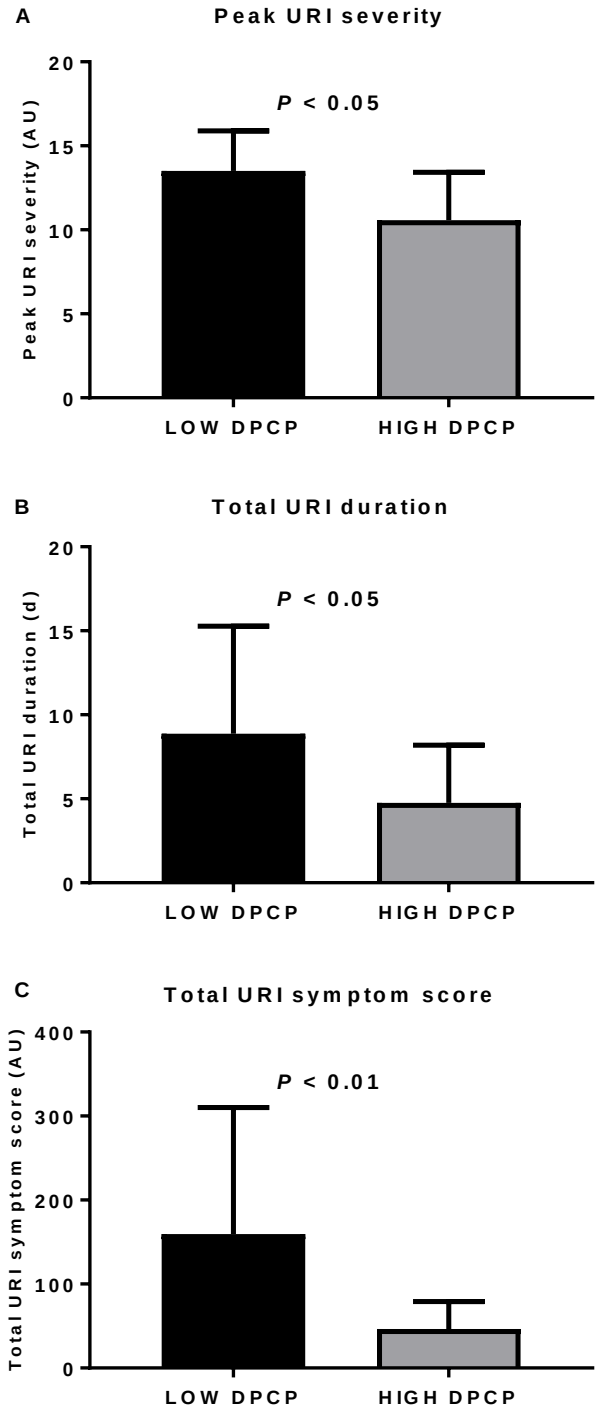

Figure 8.3 Comparison of upper respiratory illness (URI) peak symptom severity (A), total URI duration (B) and total URI symptom score (C) between low and high *in vivo* immune responders, using skin sensitisation to diphenylcyclopropenone (DPCP). Contact hypersensitivity (CHS) assessed as the summed increase in dermal thickening response to elicitation challenge with DPCP 14 d after DPCP induction. Peak URI symptom severity defined as the maximum reported URI symptom severity score. Total URI duration defined as the total number of days reporting URI. Total URI symptom score defined as the total number of days reporting URI x URI symptom severity score. An URI bout was defined as an URI score ≥ 6 on at least two consecutive days. LOW DPCP and HIGH DPCP were determined by median split based on the summed increase in dermal thickening response to DPCP challenge. Data presented are mean ± SD.

Table 8.1 Comparison of sleep, physical activity and psychological factors at the time of sensitisation between low and high *in vivo* immune responders using skin sensitisation to diphenylcyclopropenone (DPCP), assessed as the summed increase in dermal thickening response to elicitation challenge with DPCP 14 d after DPCP induction. No significant differences were observed between LOW DPCP and HIGH DPCP for sleep, physical activity and psychological factors.

	LOW DPCP	**HIGH DPCP**
Global PSQI	4.6 ± 2.1	4.3 ± 3.1
IPAQ ($MET \cdot h \cdot wk^{-1}$)	48 ± 43	43 ± 45
STAI-S	27 ± 7	28 ± 8
STAI-T	35 ± 11	36 ± 8
PSS	13.5 ± 4.8	13.8 ± 6.9

BRUMS		
Anger	2.4 ± 1.8	3.2 ± 2.7
Confusion	2.3 ± 1.6	2.7 ± 3.4
Depression	1.4 ± 1.5	2.5 ± 3.1
Fatigue	7.9 ± 4.1	6.7 ± 3.4
Tension	4.0 ± 3.0	4.3 ± 2.6
Vigour	9.9 ± 2.4	8.9 ± 3.7

BRUMS = Brunel Mood Scale; IPAQ = International Physical Activity Questionnaire; PSS = Perceived Stress Scale; PSQI = Pittsburgh Sleep Quality Index; STAI-S = state aspect of the State-Trait Anxiety Inventory; STAI-T = trait aspect of the State-Trait Anxiety Inventory.

8.4.2 Epstein-Barr Virus serostatus and IgG antibody titre

Thirty-eight participants were included in the analyses as we were unable to collect venous blood from three participants. The EBV serostatus of one participant was considered borderline with this participant subsequently excluded from analyses. Of 37 participants, 26 (70%) were seropositive with 11 seronegative (30%). There were no differences in the proportion of seropositive (50%) and seronegative (45%) individuals reporting URI during monitoring ($P > 0.05$). The summed increase in dermal thickening responses to DPCP challenge were 40% lower in EBV seropositive individuals *vs.* EBV seronegative individuals ($t_{(35)} = 1.97$, $P < 0.05$; Figure 8.4). However, in seropositive individuals, no associations were observed between the EBV IgG antibody titre and summed increases in dermal thickening responses to DPCP. A non-significant positive ($r = 0.37$; $P = 0.10$) relationship was observed between the EBV IgG antibody titre and peak URI symptom severity, with no associations observed between the EBV IgG antibody titre and other URI severity and duration variables. The activation of latent herpesviruses in seropositive individuals is widely recognised as a marker of cell-mediated

immunity by assessing immune system control over viral latency, whereby, increases in antibody titres reflect a downregulation of cell-mediated immunity (Fagundes *et al.*, 2014; Morey *et al.*, 2015).

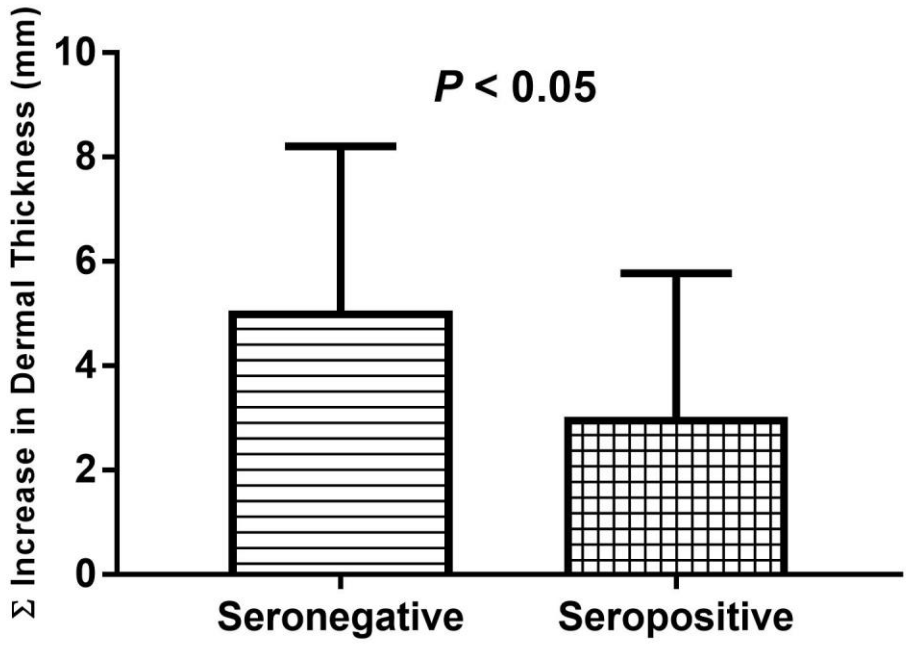

Figure 8.4 Comparison of *in vivo* immunity in Epstein-Barr virus seronegative and seropositive individuals. Contact hypersensitivity (CHS) assessed as the summed increase in dermal thickening response to elicitation challenge with diphenylcyclopropenone (DPCP) 14 d after DPCP induction. Data presented are mean ± SD.

8.5 DISCUSSION

The aim of this study was to prospectively examine whether *in vivo* immunity using DPCP relates to upper respiratory symptoms in otherwise healthy individuals. To our knowledge, this preliminary investigation provides the first evidence to prospectively show an association between *in vivo* immunity to DPCP and URI outcomes, thus providing initial supporting evidence that DPCP is a clinically relevant measure of *in vivo* immunity with regard to URI. To this end, we found that the CHS response to DPCP was a significant predictor of peak URI severity, and we showed lower CHS responses to DPCP in those who reported more severe URI episodes compared with those reporting less severe URI episodes. Moreover, low DPCP responders reported more severe URI episodes, longer total URI duration and greater total URI symptom scores compared with high DPCP responders. Finally, we observed 40% lower DPCP responses in EBV seropositive individuals compared with EBV seronegative individuals. Taken together, these findings provide preliminary support for DPCP as an *in vivo* marker of immune competence with regard to URI, and potentially implicate a role of EBV serostatus on *in vivo* immunity. Future studies with larger populations are required to confirm these findings.

The findings of the present study support the utility of DPCP as a clinically relevant biomarker of *in vivo* immunity. These findings showed that the CHS response to DPCP was a predictor of URI severity, with differences in CHS responses between those reporting low URI severity compared with those reporting high URI severity. Furthermore, we observed that peak URI severity, total URI duration and total URI symptom score was greater in low DPCP responders *vs.* high DPCP responders, with these findings unlikely due to differences in psychological and lifestyle factors as no differences in these factors were observed between low and high DPCP responders at the time of sensitisation. These findings partly accord with other studies utilising *in vivo* immune measures, whereby impaired DTH responses were observed in individuals with

acute infectious illness (Bennett *et al.*, 1998), and children who produced anergic responses to the CMI Multitest were at 20% greater risk of developing URI in malnourished children than immunocompetent children (Zaman *et al.*, 1997). However, the findings in the present study found that the DPCP response was not associated with URI incidence. A new concept within exercise immunology has recently been proposed focusing on 'immune tolerance', which is defined as the ability to endure microbes (Ayres and Schneider, 2012; Walsh, 2019). Given that the findings in the present study support an association between DPCP responses and URI symptom severity and duration, but not URI incidence, align with the 'immune tolerance' concept. For instance, URI incidence is influenced by many other lifestyle factors such as infection exposure and hygiene (Drew *et al.*, 2017). The severity and duration of URI episodes provide better reflections of the clinical relevance of immune markers because these variables indicate how well the immune system can regulate responses to infection. It is possible that cytokines play an important role in regulating responses to infection given the widely postulated 'cytokine storm' associated with a wide variety of infectious pathogens (Tisoncik *et al.*, 2012). However, further research is required into the underlying mechanisms.

Although only five participants reported recurrent URI episodes (≥ 2), CHS responses were 51% lower in these individuals compared with those who reported 0 or 1 URI during monitoring. One possible explanation is that those reporting more frequent URI may have chronic low-grade inflammation that subsequently impacts immune health, with evidence indicating impaired inflammatory regulation in illness-prone individuals (Cox *et al.*, 2007). Furthermore, recent evidence suggests that increases in pro-inflammatory gene expression are associated with down-regulation of antiviral type 1 interferon genes in response to psychosocial stress (Cole, 2019). As such, it is possible that upregulated pro-inflammatory responses and down-regulated cellular immune processes are prominent in those experiencing recurring URI

episodes, and may even be mediated by psychosocial factors not assessed in the current study such as depression and early life trauma (Cole, 2019). Of course, these findings need to be interpreted cautiously with future research in larger populations required.

In the current study, 70% of participants were considered positive for previous EBV infection, with this proportion similar to previous studies (Gleeson *et al.*, 2002; Pottgiesser *et al.*, 2006; He *et al.*, 2013). However, no association was observed between EBV IgG antibody titre and the CHS response to DPCP. Although EBV reactivation provides a biomarker of cellular immunity indicating that immune function is compromised, the antibody titre of EBV is already elevated due to prior infection, of which 70-90% are seropositive (Gleeson *et al.*, 2002; He *et al.*, 2013). As such, this limits the ability to discern differences in viral reactivation to EBV, potentially explaining the lack of association between EBV antibody and the CHS response to DPCP. As such, future studies assessing viral DNA shedding should be considered in future studies, although detecting shedding requires frequent sampling (Gleeson *et al.*, 2002). Nevertheless, 40% lower CHS responses were observed in EBV seropositive individuals compared with EBV seronegative individuals. These findings support previous investigations identifying the salient role of herpesvirus serostatus on immune outcomes and URI (Gleeson *et al.*, 2002; Pottgiesser *et al.*, 2006; Turner *et al.*, 2014; Reed *et al.*, 2017; Merani *et al.*, 2018). Latent herpesviruses can cause a general suppression of immune function via the production of a viral homologue of human IL-10, a potent inhibitory cytokine (Chang and Barry, 2010). Furthermore, CMV seropositivity impairs the Th1 response to influenza, and given that CMI is primarily a Th1 cell mediated immune response, further research is required to elucidate the role of herpesvirus serostatus on *in vivo* immunity (Merani *et al.*, 2018). Future research is also required to examine the role of other latent viruses on *in vivo* immunity (e.g. CMV) because only EBV serostatus was assessed in the current study.

Given that most viral infections enter the host via the respiratory tract, and that DPCP is benign and challenges immunity at the skin, one may question the relevance of differences in immunity with regard to URI. The findings in this study provide preliminary evidence regarding the clinical significance of DPCP as a measure of *in vivo* immunity with regard to URI. However, this study is not without limitation. For instance, it is acknowledged that a relatively small sample size was used in the current study, and larger populations are required to confirm these findings. In addition, causality cannot be determined from these findings due to the associative nature of the study, although we did utilise a prospective design with concerted efforts made to rigorously ensure that participants were healthy before sensitisation and until completion of CHS assessments: URI symptoms were assessed daily and individuals who reported a URI episode prior to completion of CHS assessments were excluded from analyses. We aimed to maximise the number of URI episodes and reduce the risk of allergies being mistaken for respiratory symptoms by utilising the Autumn-Winter common cold seasons, but future studies examining DPCP responses across the whole year will increase the generalisability of the findings. Given the geographic proximity of the participants to each other, we can assume that that most participants will have been exposed to infectious pathogens during the monitoring period. Indeed, over half of the participants reported respiratory symptoms during monitoring in the current study, but even when others have experimentally inoculated individuals with common cold pathogens, only around 75% experienced respiratory symptoms (Cohen *et al.*, 1991). As such, confirming the pathogenic nature of self-reported respiratory symptoms will add strength to future studies.

In summary, these findings provide promising initial support for the clinical utility of DPCP as a measure of *in vivo* immunity with regard to URI. These findings also potentially implicate a

role of EBV serostatus on *in vivo* immunity. Future studies with larger populations are required to confirm these findings.

CHAPTER NINE

General discussion

CHAPTER NINE

General discussion

9.1 Summary of main findings

The overall objectives of this thesis were to examine the role of anxiety and psychological stress on the *in vivo* immune response, investigate the role of these factors in exercise-immune modulation, and investigate the efficacy of experimental CHS using DPCP as a clinically relevant, systemic measure of *in vivo* immunity. **Chapter 4** demonstrated no influence of prolonged exercise stress on local cutaneous processes to a local irritant (croton oil), supporting the notion that lower CHS responses to DPCP induction likely represents a systemic suppression of *in vivo* immunity. **Chapter 5** provided the first empirical evidence that the level of anxiety and psychological stress prior to exercise plays a role in moderating the subsequent *in vivo* immune response after exercise. For instance, we showed that the DPCP response after exercise was 62% lower in those reporting low *vs*. moderate state-anxiety before exercise. Similar strength relationships were also observed between physiological stress (heart rate training impulse) and the summed dermal response to DPCP (r = -0.37), and state-anxiety and the summed dermal response to DPCP (r = 0.39). **Chapter 6** utilised an acute psychological stressor to induce a wide range of anxiety levels, and demonstrated that the DPCP response was lower in those reporting low and high anxiety, supporting an inverted-U association between the a priori level of anxiety and the *in vivo* immune response after an acute psychological stressor. The findings from **Chapters 7 and 8** provide promising preliminary support for the utility of experimental CHS using DPCP as a clinically relevant *in vivo* biomarker of immune competence in relation to the common cold by showing impaired *in vivo* immunity during pathogen confirmed upper respiratory tract infection and upper respiratory common cold symptoms (**Chapter 7**), and by prospectively showing an association between

DPCP responses and URI (**Chapter 8**). Taken together, the findings in this thesis highlight the prominent role of psychological factors on *in vivo* immunity and their role on exercise-immune modulation, and provide initial support the use of DPCP as a systemic, clinically relevant measure of *in vivo* immunity. As such, it is recommended that exercise scientists account for anxiety and psychological stress when examining the immune response to exercise, and that exercise immunologists and psychoneuroimmunologists utilise *in vivo* measures of immunity such as experimental CHS using DPCP where possible.

Chapter 4 aimed to determine whether the previously observed decrease in DPCP responses following prolonged exercise reflect local processes at the skin or point towards a measure of systemic immune status (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). One way to achieve this objective was to examine the role of prolonged exercise on cutaneous responses to a topically applied irritant such as CO. Unlike DPCP which stimulates an antigen-specific, T-cell mediated immune response, CO is an irritant that stimulates a localised non–T-cell mediated inflammatory response mediated principally via innate immune mechanisms (Nosbaum *et al.*, 2009). **Chapter 4** demonstrated no influence of prolonged exercise on local processes at the skin using CO, indicating that the previously observed impaired DPCP responses following prolonged exercise extend beyond localised effects at the skin and reflect systemic changes in *in vivo* immunity. Given the overlapping processes during the early stages of cutaneous responses with both irritants (CO) and sensitisers (DPCP), these findings indicate that the exercise induced immune suppression to DPCP does not occur during the initial stages of CHS at the skin, and likely occurs later in the process of CHS induction (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). Although further research is required to delve into the underlying mechanisms by which prolonged exercise stress impairs *in vivo* immunity to DPCP, altered LC function resulting in impaired LC migration is one possible explanation (Cumberbatch *et al.*,

2006). Epidermal LC play pivotal roles in the induction of cutaneous immune responses, whereby, upon initial encounter with antigen in the skin, cytokine upregulation initiates the migration and maturation of LC to draining lymph nodes where they localise within the paracortex (Toebak *et al.*, 2009). In the future, animal models or the use of skin biopsies to assess changes in LC counts at the skin may shed more light on the mechanisms by which prolonged exercise reduces the induction of immune memory using experimental CHS to DPCP and the role of LC migration in this process.

Chapter 5 used the CHS responses to exercise from a previous study to present novel insights regarding the influence of anxiety and perceived psychological stress on *in vivo* immunity after exercise (Diment *et al.*, 2015). These findings provided the first empirical evidence to demonstrate an important interaction between the a priori level of anxiety and perceived psychological stress, and the subsequent *in vivo* immune response after an exercise challenge. Moreover, similar strength relationships were observed between anxiety prior to exercise and the *in vivo* immune response after exercise challenge, and the level of physiological stress after exercise with the *in vivo* immune response after exercise challenge. The salient influence of anxiety on the immune response after exercise was further highlighted by a lower *in vivo* immune response to DPCP in individuals reporting low compared with moderate anxiety, regardless of the intensity and duration of the exercise challenge. Particularly noteworthy was that the *in vivo* immune response was 50% lower in individuals reporting low compared with moderate anxiety following 2 h moderate intensity exercise, even though we have previously shown a suppressive effect of 120MI compared with rested control on *in vivo* immunity (Diment *et al.*, 2015). The levels of anxiety and perceived psychological stress prior to exercise were low-to-moderate, and the influence of high anxiety and psychological stress on the *in vivo* immune response to exercise challenge requires investigation. The findings in **Chapter 6**

provide promising support that those experiencing high levels of anxiety will have impaired *in vivo* immune responses to an exercise challenge, but we acknowledge that **Chapter 6** investigated a direct effect of acute psychological stress on *in vivo* immunity whereas **Chapter 5** examined the moderating role of psychological factors on the *in vivo* immune response after exercise. Despite being widely postulated some 25 years ago, a scarcity of empirical evidence exists examining the interactions between psychological factors and exercise on immune modulation (Laperriere *et al.*, 1994; Perna *et al.*, 1997; Clow and Hucklebridge, 2001). Thus, future research should investigate whether the moderating role of psychological factors on the immune response to exercise extends to other clinically relevant immune measures such as vaccination responses (Burns, 2012; Whittaker, 2018) and mucosal immunity (Hanstock *et al.*, 2016; Hanstock *et al.*, 2018).

The findings in **Chapter 5** also showed lower pre-exercise circulating cortisol and epinephrine in the low compared with moderate anxiety group, supporting the notion that stress hormones are involved in modulating the *in vivo* immune response to subsequent exercise. This is hardly surprising as stress hormones are widely considered to play important roles in preparing the immune system for challenge (Dhabhar and McEwen, 1999; Dhabhar, 2014), with one study demonstrating that administration of physiological doses of corticosterone and epinephrine increased T-cell drainage away from the site of DTH challenge to lymph nodes, which in-turn enhanced the DTH response in rats (Dhabhar and McEwen, 1999). Also supporting this assertion, pharmacological blockade of corticosteroids or catecholamines exacerbated the exercise-associated immune suppression in mice (Kohut *et al.*, 2005b). Nevertheless, post-exercise circulating cortisol and epinephrine were not different between individuals reporting low and moderate anxiety, and further research is required to delve into the underlying mechanisms.

In **Chapter 6**, a zip-line stressor was utilised to induce a wide-range of anxiety levels to investigate whether the a priori level of anxiety was an important predictor of *in vivo* immunity after an acute psychological stressor. The stressor was novel to all participants, over 150 m high at the top and reached speeds of 160 $km \cdot h^{-1}$ whilst participants were immobilised face down in a prone position. The novel zip-line stressor successfully induced acute psychological stress, as evidenced by higher levels of state-anxiety and heart rate immediately prior to the stressor compared with after the stressor. Mean anxiety levels were comparable with laboratory-based stressors such as public speaking and memory recall tasks (Feldman *et al.*, 2004; Nagy *et al.*, 2015), but it was somewhat surprising that anxiety responses from this stressor were not greater than laboratory-based stressors, although interestingly, responses were comparable to novice skydivers prior to a parachute jump (Hare *et al.*, 2013). Nevertheless, we chose this stressor to provide a wide range of anxiety levels prior to the stressor which was successfully achieved. The findings in **Chapter 6** mirrored the findings in **Chapter 5** whereby individuals reporting low levels of anxiety prior to the stressor challenge had lower *in vivo* immunity after the stressor, but interestingly, those reporting high levels of anxiety prior to the stressor challenge had lower *in vivo* immune response to DPCP compared with those reporting moderate anxiety. As such, these findings support an inverted-U association between the a priori level of anxiety and the *in vivo* immune response after an acute psychological stressor. This is in agreement with the reactivity hypothesis whereby both diminished and exaggerated responses to an acute stress have been associated with negative health outcomes (Carroll *et al.*, 2003; Carroll *et al.*, 2011; Lovallo, 2011; Phillips *et al.*, 2011; Carroll *et al.*, 2012). Although anxiety levels were assessed shortly before stressor onset (within 5 minutes), the duration of a stressor is an important factor in determining the immune outcome (Dhabhar, 2014). It is possible that individuals had elevated anxiety levels upon waking, and we acknowledge that only a snapshot of anxiety was assessed meaning we were unable to quantify changes in anxiety

prior to stressor onset. As such, assessing anxiety more frequently on the morning of the stressor may have provided additional insight. Stress hormones may also have provided further insight into the potential mechanisms given the important bi-directional role of stress hormones in immune modulation (Dhabhar, 2014). We decided against assessing blood stress hormones as blood sampling likely influences psychological and hormonal stress responses, potentially confounding the influence of the novel zip line stressor on anxiety levels (Hoogerwerf *et al.*, 2018). However, studies should identify the influence of blood sampling on immune function as the psychological and physiological stress response induced by blood sampling may account for some of the equivocal findings in the exercise immunology literature examining the immune response to exercise.

Chapter 7 aimed to determine the clinical significance of experimental CHS using DPCP with regard to the common cold by examining the *in vivo* immune response in participants reporting URS and pathogen-confirmed URTI compared with healthy controls. Cutaneous responses were 42% lower in those reporting URS compared with healthy controls. These findings were not due to psychological stress as similar PSS levels were reported between URS and healthy participants at the time of sensitisation. A particular strength of this study was that throat and nasal swabs were collected and analysed for viral pathogens using qPCR at the time of sensitisation, and we observed 54% lower DPCP responses in participants with pathogen-confirmed URTI compared with controls. These findings support the clinical relevance of experimental CHS using DPCP with regard to common cold, and accord with previous literature whereby intradermal measures of DTH are impaired during viral illnesses (Reed *et al.*, 1972; Kauffman *et al.*, 1974a; Kauffman *et al.*, 1976; Zaman *et al.*, 1997). However, only 50% had pathogen-confirmed URTI, which is lower than expected given that we recruited participants during the autumn and winter when common cold pathogens are most prevalent

(Monto, 2002). We cannot discount that viral pathogens were undetected in some participants due to the short incubation time of HRV, the predominant pathogen for common colds. Shedding peaks on the second day and decreases rapidly thereafter; our participants were swabbed at least swabbed at least 2 days after common cold onset. We also cannot discount that those with U-URI had a viral pathogen not assessed in the current study. Nevertheless, it could be argued that upper respiratory symptoms that impact training and performance are more important for athletes and military personnel, irrespective of infectious aetiology. However, we did observe longer and more severe common cold bouts in those who were infectious. Although it is likely that lower DPCP responses represent compromised host defence and increased susceptibility to the common cold given the short incubation time of common cold pathogens such as HRV (Harris and Gwaltney, 1996), it is recognised that the present findings do not determine directional causality (e.g. whether a lower DPCP response represents a lowered systemic immune status or whether the common cold alters the DPCP response). As such, we cannot discount that viral pathogens interfere with lymphocyte activation indirectly through effects on APCs (Kirchberger *et al.*, 2007).

The aim of **Chapter 8** was to build upon the promising findings of **Chapter 7** and prospectively examine whether *in vivo* immunity using DPCP relates to upper respiratory symptoms in otherwise healthy individuals. The findings provide promising initial support for the use of DPCP as a clinically relevant measure of *in vivo* immunity with regard to URI as the CHS response to DPCP was a significant predictor of peak URI severity, and we showed lower CHS responses to DPCP in those who reported more severe URI episodes compared with those reporting less severe URI episodes. Moreover, low DPCP responders reported more severe URI episodes, longer total URI duration and greater total URI symptom scores compared with high DPCP responders. Examining URI symptom severity and duration provide better

reflections of the clinical relevance of immune markers because these variables indicate how well the immune system can regulate responses to infection whereas URI incidence is confounded by lifestyle factors (e.g. hygiene).

Interestingly, in **Chapter 8**, the CHS responses to DPCP were 40% lower in EBV seropositive individuals compared with EBV seronegative individuals. The salient role of herpesvirus serostatus on immune outcomes and URI is widely recognised, with CMV seropositivity impairing the Th1 response to influenza vaccination (Merani *et al.*, 2018). Given that CMI is primarily a Th1 cell mediated immune response, it is possible that EBV seropositivity downregulates the DPCP response to cellular immunity or induces a shift in the Th1/Th2 balance, thus impairing IFN-γ production important for antiviral defence. However, further research is required to elucidate the role of herpesvirus serostatus on *in vivo* immunity.

9.2 Perspectives and future directions

Experimental CHS with DPCP provides an attractive measure of *in vivo* immunity that is practical, safe, and can be administered without the need for expensive equipment, invasive injections, or blood sampling. Utilising the 'gold standard' measure of dermal thickness using a high-frequency ultrasound scanner also provides strong scientific rigour by allowing images to be analysed by a blinded investigator at a later timepoint. Moreover, the utility of experimental CHS with DPCP allows researchers to rigorously control the timing of initial exposure in relation to stressors, the dose of exposure and the time elapsed since sensitisation providing scientists with a valuable and robust immunological tool for investigations within the fields of stress and exercise immunology. Furthermore, this model overcomes many of the limitations of commonly used *in vitro* measures, and the ethical implications of using live pathogens that have been used previously in nasal inoculation studies (Cohen *et al.*, 1991;

Prather *et al.*, 2015; Janicki-Deverts *et al.*, 2016). The findings within this thesis highlight that this measure relates to URI, thereby providing scientists with confidence about the clinical meaningfulness of differences observed. Nevertheless, we recognise that the methodology is currently limited to Caucasian skin tones, although the key measure of CHS responses is dermal thickening which can be assessed in those with different skin tones; future studies are required to determine ethnic differences in CHS responses. In addition, assessing *in vivo* immunity using this model requires purposefully inducing CHS, and strong CHS responses can result in bullae developing, potentially impacting the efficacy of erythema measures by changing the colour of the skin at the reaction site from a red scale to a yellow. Refinement of the protocol to find a balance between using doses sufficient to elicit a response and minimising large bullae responses would be advantageous, and may also increase the sensitivity of the test by providing a better signal-to-noise ratio. The lower recall doses used in **Chapter 8** highlight that these doses can be used successfully under rested conditions, but studies need to determine whether these doses are optimal under stress conditions where immunity may be impaired and lower responses observed or a greater threshold dose required. Moreover, refining the protocol with lower recall doses may feasibly allow participants to be taken to a 'plateau' to investigate secondary immune responses and utilise repeated measures study designs to examine changes in the immune status of athletes and military personnel during training. An interesting anecdotal observation in **Chapter 8** where lower recall doses were used was that delayed responses occurred in some individuals, whereby, low responses were observed at 48 h, with these responses becoming more pronounced approximately 5 – 8 d after recall patches were applied. As such, it is possible that using lower recall doses results in altered kinetics of the CHS response that may also provide information on the ability of the immune system to respond to a challenge. Further refinement of the methodology will potentially allow a single

recall dose in the middle of the dose-response curve to be used in the future, making this test field-worthy in large population settings (e.g. military).

We have demonstrated the inhibitory effect of prolonged exercise (2 h) on cutaneous responses to DPCP, but future research is required to delve into the mechanisms by which prolonged exercise influences the DPCP response (Harper Smith *et al.*, 2011; Diment *et al.*, 2015). The findings in **Chapter 4** indicate that exercise induced immune suppression to DPCP is not inhibited during the initial stages of CHS at the skin, and likely occurs later in the CHS induction process. One approach to examine the mechanisms of prolonged exercise on DPCP responses include taking skin punch biopsies at a DPCP site and a control site to provide an assessment of LC migration from the epidermis (Cumberbatch *et al.*, 2003; Cumberbatch *et al.*, 2006). Recent advances in technology such as intravital multi-photon microscopy indicate that the use of cellular imaging may also be used in the future to identify cellular interactions in the lymph nodes in humans, but this method is currently limited to animal models (Stein and Gonzalez, 2017). Animal research permitting the assessment of more invasive measures may also provide further insight into the suppressive effects of prolonged exercise on events further downstream in CHS induction (e.g. in the lymph nodes). Moreover, *in vitro* systems that allow stimulation and analysis of the cellular elements involved in the CHS response with DPCP are important to further our understanding of the effects of stress on CHS induction. Thus, the development of a functional *in vitro* test to stimulate hapten specific T-cells to DPCP may aid in identifying the mechanisms associated with stress-related changes to DPCP. Preliminary work indicates that it is possible to detect lymphocyte responses to DPCP *in vitro*, but further research is required to refine and optimise this method (Friedmann *et al.*, 2017). In addition to identifying the mechanisms by which prolonged exercise inhibits CHS induction with DPCP, the duration of the inhibitory effect of prolonged exercise on CHS induction remains unknown,

and could be investigated by manipulating the timing of sensitisation after prolonged exercise. We sensitised participants to DPCP 20 minutes after exercise cessation in previous studies (Harper Smith *et al.*, 2011; Diment *et al.*, 2015) to allow cutaneous blood flow to return to baseline, but future research should determine the influence of this timing of sensitisation as lymphocytosis may have returned to normal or even below baseline 20 min after exercise cessation (Shek *et al.*, 1995). Further studies are also required to assess URI in the weeks following stress-induced immune modulation, but it is acknowledged that assessing URI with sufficiently large sample sizes in rigorously controlled laboratory studies are invariably unfeasible, and thus there is a need to utilise clinically relevant immune measures to examine the influence of stressors on immunity.

The findings in **Chapter 8** revealed differences in CHS responses between those reporting the least severe and most severe URI episodes, and more severe URI bouts and longer total URI duration were observed in those with low DPCP responses compared to those with high DPCP responses, but no differences in URI incidence were reported. Although simple practical recommendations such as frequent handwashing are effective strategies to help avert URI incidence (Jefferson *et al.*, 2011; Savolainen-Kopra *et al.*, 2012), preventing exposure to infections are difficult in team environments (e.g. athletic teams and military personnel on a barracks) and during common cold seasonal peaks. A contemporary view is that immune resistance is not impaired in athletes under heavy training, but that immune tolerance may be more susceptible to the influence of stressors (Walsh 2019). The findings in **Chapter 8** support this assertion as those with lower *in vivo* immunity reported more severe common colds compared to those with greater *in vivo* immunity. As such, future research should investigate the efficacy of preventative strategies on immune tolerance and the ability to reduce URI symptom severity and duration. It is perhaps more likely that interventions (e.g. psychological,

nutritional) employed to optimise immune health or attenuate immune suppression are more likely to be effective in reducing URI symptom severity and duration than incidence, potentially through tolerogenic effects (Walsh 2019). Furthermore, preliminary evidence indicates that illness-prone athletes are likely to have greater immune disturbances and genetic predisposition to pro-inflammatory cytokine responses (Gleeson *et al.*, 2017), raising the question that either genetics or early life experiences widely suggested to increase inflammation in adulthood, may influence susceptibility to infectious disease or the ability of the immune system to respond to stressors in later life (Avitsur *et al.*, 2015; Elwenspoek *et al.*, 2017; Murphy *et al.*, 2017). It has been suggested that early life adversity is an important factor for sporting success in 'super-elite' athletes (Collins and MacNamara, 2012; Hardy *et al.*, 2017), forming the foundation for the high levels of mental toughness and resilience required to rise to the top, but the impact of early life adversity remains a concept relatively unexplored within exercise immunology.

In **Chapter 5**, individuals reporting moderate state-anxiety before exercise had greater *in vivo* immune responses after subsequent exercise of various intensities and durations than those reporting low state anxiety before exercise. Thus, examining the potential of acute stress to enhance *in vivo* immunity with DPCP by manipulating the dose of sensitisation is also an area of interest, as observed when half-dose vaccinations have been administered following acute moderate intensity exercise (Edwards *et al.*, 2012). Further research is also required to investigate exercise-immune responses in athletes, military personnel and others in physically demanding occupations (e.g. firefighters and mountain rescue workers) experiencing higher levels of psychological stress than those reported in **Chapter 5**. The immuno-suppressive effects of chronic high stress in rats (3 weeks of restraint and shaking stress) and humans (examination period) are widely acknowledged (Dhabhar and McEwen, 1997; Smith *et al.*,

2004a; Dhabhar, 2014). Also, the findings in **Chapter 6** whereby we demonstrated lower *in vivo* immunity in those reporting low and high levels of anxiety prior to an acute psychological stressor provide reason to believe that those experiencing high levels of anxiety and/or psychological stress will have lower *in vivo* immune responses following prolonged exercise, but research is required to test this hypothesis. Further research is also required to disentangle the influence of psychological and physiological strain during prolonged exercise (e.g. during endurance and ultra-endurance events) on *in vivo* immunity. Evidence suggests that superimposing a psychological stressor during exercise exacerbates lymphocyte redistribution in firefighters (Huang *et al.*, 2010), but more research is required to elucidate the role of psychological strain during exercise on immune modulation during prolonged exercise bouts. Also, studies investigating a wider framework of psychological factors on immune health are lacking. Some evidence indicates that mental fatigue, mood state, psychological stress and pain tolerance influence exercise performance and running economy (Williams *et al.*, 1991; Crews, 1992; Marcora *et al.*, 2009; Otter *et al.*, 2016), but studies investigating the role of these psychological factors on immune health in athletes are topics ripe for investigation. Moreover, the role of personality traits on *in vivo* immunity response has previously been reported (Phillips *et al.*, 2005), and it is acknowledged personality traits could have partly accounted for the findings in **Chapter 5 and 6**. As such, future studies examining the *in vivo* immune response to exercise and other stressors should consider including trait-like psychological measures.

Identifying the role of stressors on immune function is important because it allows preventative strategies to be employed to minimise URI risk and limit disruption to training and performance for athletes and military personnel. However, it is acknowledged that the studies within this thesis were conducted in recreationally active individuals, and in time, studies are required to

corroborate these findings in elite athletes and military personnel. Nevertheless, numerous stressors have been identified with compromising immune health (e.g. heavy exercise, sleep, life stress, environmental stress, nutritional deficits, long haul travel) leaving athletes and military personnel at increased risk of URI, with preventative strategies proposed to reduce the risk of succumbing to URI (Walsh *et al.*, 2011a; Walsh *et al.*, 2011b; Walsh, 2018), albeit with varying degrees of success. For example, nutritional interventions are commonly employed by athletes and military personnel as countermeasures to blunt immune impairment, yet many studies have failed to show consistent and clinically meaningful beneficial effects of nutritional interventions on athlete immune health, particularly when individuals have sufficient dietary intake (i.e. not energy restricted) and in studies assessing clinically relevant *in vivo* immunity (Bermon *et al.*, 2017; Walsh, 2018). Psychological interventions have received far less attention than other preventative strategies, but there is reason for optimism in this regard with evidence indicating psychological strategies can improve immune competence and reduce URI incidence (Davidson *et al.*, 2003; Perna *et al.*, 2003). In **Chapters 5 and 6**, we demonstrated that the level of anxiety prior to challenge (exercise or acute psychological stress) influenced the subsequent *in vivo* immune response after the stressor. These findings raise the possibility for psychological interventions to alter psychological stress in order to optimise immunity and host defence in athletes, military personnel and those in physically demanding occupations. Stress reduction interventions such as meditation or even virtual reality interventions have been proposed to manipulate anxiety and psychological stress in athletes and military personnel (Rumbold *et al.*, 2012; Pallavicini *et al.*, 2016). In time, interventions manipulating anxiety and psychological stress to optimise immune health may be able to attenuate or even eliminate exercise induced immune impairment if the role of psychological factors on exercise immune modulation outweigh the influence of prolonged exercise and heavy training. In the future, novel techniques may be developed to protect against psychological stress and optimise

immune function, with one approach proposed to inoculate individual against repeated stress much like how individuals are inoculated against viral pathogens through vaccines (Dantzer *et al.*, 2018). It is possible that stress confers an "immunological memory" through alterations within the adaptive immune cell compartment thus inoculating against future stress exposure (Dantzer *et al.*, 2018). Vaccination with a weak myelin-derived peptide prevented the emergence of depression like behaviours following chronic mild stress in rats (Lewitus *et al.*, 2009), but future studies need to examine the efficacy of stress inoculation in humans. In addition, future T-cell therapy strategies may be designed towards adapting T cell responses to promote protection against stress. Preliminary work suggests that transplantation of lymph node cell suspensions from previously stressed mice produced antidepressant effects and reduced inflammatory cytokine levels in lymphocyte deficient mice (Brachman *et al.*, 2015). Pharmacological interventions using psychobiotics provide interesting future avenues to manipulate and protect against psychological stress: psychobiotics are defined as bacteria which when ingested in adequate amounts have a positive mental health benefit and promote psychological resilience (Dinan *et al.*, 2013). While much of the work on psychobiotics is at a pre-clinical level, there are human interventions supporting the view that psychobiotics provide protection against stress by increasing resilience (Allen *et al.*, 2016). In time, psychobiotics may be used by athletes and military personnel to protect against stress and optimise host defence much like nutritional countermeasures are currently used. Irrespective of the exciting future research endeavours presented here, the findings within this thesis support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise, and for coaches and support staff to monitor anxiety and psychological stress alongside more traditional physiological measures of training stress. Additionally, exercise immunologists and psychoneuroimmunologists are encouraged

to utilise clinically relevant *in vivo* measures of immunity such as experimental CHS using DPCP where possible.

9.3 Conclusions

The major conclusions from this thesis are:

1. No influence of prolonged exercise stress on local cutaneous processes to a local irritant supports the notion that the decrease in CHS responses to DPCP induction represents a systemic suppression of *in vivo* immunity (**Chapter 4**).

2. Low-to-moderate levels of state-anxiety and perceived psychological stress before exercise play an important role in determining the strength of the *in vivo* immune response after exercise, irrespective of the exercise bout performed (**Chapter 5**)

3. A similar, moderate strength relationship was observed for the level of state-anxiety prior to exercise and the level of physiological stress during exercise with the *in vivo* immune response after exercise (**Chapter 5**).

4. Lower DPCP responses in those reporting low and high levels of anxiety prior to an acute psychological stressor support an inverted-U association between the a priori level of anxiety and the *in vivo* immune response after an acute psychological stressor (**Chapter 6**).

5. Lower DPCP responses during a common cold supports the use of *in vivo* immunity to DPCP as a clinically relevant *in vivo* marker of immune competence (**Chapter 7**).

6. The CHS response to DPCP was a significant predictor of peak URI severity, and lower CHS responses were observed in those who reported more severe URI episodes compared with those reporting less severe URI episodes (**Chapter 8**).

7. Low DPCP responders reported more severe URI episodes, longer total URI duration and greater total URI symptom scores compared with high DPCP responders (**Chapter 8**).

8. Lower *in vivo* immune responses to DPCP were observed in Epstein-Barr Virus seropositive individuals, suggesting an important role of herpesvirus serostatus on *in vivo* immunity **(Chapter 8).**

9. These findings support the recommendation that exercise scientists should account for anxiety and psychological stress when examining the immune response to exercise.

10. These findings support the recommendation that exercise immunologists and psychoneuroimmunologists should utilise *in vivo* measures of immunity such as experimental CHS where possible.

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APPENDIX A

Example Informed Consent

Bangor University
SCHOOL OF SPORT, HEALTH AND EXERCISE SCIENCES

1	Title of project	
2	Name and e-mail address(es) of all researcher(s)	Jason Edwards: j.edwards@bangor.ac.uk Prof. Neil Walsh: n.walsh@bangor.ac.uk

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) Patients: ☐

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.

(ii) Students: ☐

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 or with other staff members of the School.

(iii) General members of the public: ☐

I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason.

3 I understand that I may register any complaint I might have about this experiment with Dr. Jamie MacDonald, Head of 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. ☐

4 I confirm that I have read, understand and do not knowingly present with any of the exclusion criteria listed on the participant Information sheet. ☐

Name of Participant …….

Signature ……. Date ……..

Name of Person taking consent…….

Signature ……. Date ……..

APPENDIX B

Example Medical Questionnaire

Bangor University
SCHOOL OF SPORT, HEALTH AND EXERCISE SCIENCES

Name of Participant ……..……..

Age ……

Are you in good health? ☐ YES ☐ NO

If no, please explain

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

Vigorous		Moderate		Low intensity	

Duration (minutes)…….

How often?

< Once per month		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					

Are you currently taking medication? ☐ YES ☐ NO

If yes, please give particulars:

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

If yes, please give particulars:

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

☐ YES ☐ NO

Have you ever taken part in a study involving skin patch application (DPCP)?

☐ YES ☐ NO

Do you currently have eczema or dermatitis on your arms or back?

☐ YES ☐ NO

If yes, please give particulars:

Have you ever been diagnosed with, or tested for glandular fever, chronic fatigue (ME), or overtraining syndrome? ☐ YES ☐ NO

If yes, please give particulars:

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 ……

APPENDIX C

Biological variability of local cutaneous responses to the irritant croton oil

C.1 Purpose

Cutaneous application of croton oil (CO) triggers a localised, non-specific, T-cell-independent inflammatory response that is initiated by the release of inflammatory mediators (e.g. cytokines) from damaged cells which then activate leukocytes in a non-specific manner (Berg *et al.*, 1995; Nosbaum *et al.*, 2009) Before examining the influence of stressors on local cutaneous responses to CO, determining the biological variability of this measure is an important consideration. As such, the aim of this study was to determine the intra- and inter-participant variability of local cutaneous responses to CO challenge.

C.2 Methods

In ten healthy recreationally active males, three experimental trials were conducted under rested conditions, each separated by 14 days. During each trial, a dose-series comprising of five 8 mm Finn Chamber patches of croton oil diluted in ethanol (0% control, 0.3%, 0.55%, 1%, 3%) were topically applied to the volar aspect of the upper arm for 24 h in a randomised order. After 24 h, the skin patches were removed, with cutaneous responses measured 2 h after removal of patches.

Statistical analysis

All data were checked for normality and sphericity, with none of the data violating these assumptions. Comparisons between trials for each dependent variable were analysed using repeated measures ANOVA. Intra- and inter- individual variability was assessed by biological variation analysis. To this end, average within-subject coefficient of variation (CV_W) was

calculated from three repeat measures, and between subject inter-individual coefficient of variation (CV_G) was also calculated. Coefficient of variation was calculated as (SD/mean) × 100.

C.3 Results

No significant differences were observed in population means between trials for erythema (P = 0.25) or dermal thickening responses (P = 0.90) (Table C.1). The measures of biological variability are reported in table C.2.

Table C.1. Population means for summed increases in erythema and dermal thickening responses across three repeat trials, each separated by fourteen days. Data are Mean ± SD.

Measure	**Trial 1**	**Trial 2**	**Trial 3**
Erythema (AU)	30 ± 10	35 ± 10	37 ± 7
Dermal thickness (mm)	1.1 ± 0.5	1.2 ± 0.3	1.2 ± 0.7

Table 2. Within-subject (CV_W) and between-subject (CV_G) variability for erythema and dermal thickening responses across three repeat trials, each separated by fourteen days.

Measure	**CV_W (%)**	**CV_G (%)**
Erythema	25	28
Dermal thickness	33	36

C.4 Conclusions

The aim of this investigation was to establish the biological variability of local cutaneous responses to the irritant CO. Small mean differences in population means were observed between trials. Furthermore, CV_W values of 25 - 33% are comparable with other measures of

immunity (Bagger 2003). Moreover, the CV_W and CV_G values were lower for erythema than dermal thickening responses highlighting that erythema is the key measure to assess local cutaneous responses to CO. These findings highlight the utility of CO as a repeatable measure to examine local cutaneous responses.

APPENDIX D

A pilot study to determine whether reducing the diphenylcyclopropenone induction period influences the subsequent *in vivo* immune response.

D.1. Purpose

In experimental contact sensitisation to DPCP, the immune response is initiated by application of a known sensitising dose of DPCP, never previously encountered by the individual. After 28 days, a period of time necessary to allow the establishment of immune memory (typically 7-14 days), the strength of antigen-specific in vivo immune reactivity is assessed by measuring the skin reaction (oedema and erythema) evoked by secondary exposure to DPCP. However, a practical limitation of the current approach is the long period of time between sensitisation (primary exposure) and elicitation (secondary exposure). Evidence indicates that the formation of immune memory can occur in as little as 8 days with other sensitisers such as dinitrochlorobenzene (DNCB) (Friedmann, 2007). Furthermore, using a 12 day period between sensitisation and elicitation, DPCP responses were able to successfully predict acute hepatic allograft rejection (Bathgate et al., 2001). As such, we are optimistic that secondary in vivo immune responses to DPCP can be measured after only 14 days without the need to wait until 28 days, as has become routine in many studies. With this information in mind, the aim of this investigation is to determine whether reducing the period of time between primary and secondary exposure to DPCP (from 28 days to 14 days) influences the subsequent in vivo immune response.

D.2. Methods

In ten healthy recreationally active males (14 d group: n = 6; 28 d group: n = 4), participants were sensitised to the novel antigen DPCP as described in **Chapter 3.4**. Either 14 or 28 days

after the initial sensitisation to DPCP, the magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to DPCP. Patches were applied to the volar 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 $\mu g \cdot cm^{-2}$; 0.0122%, 3.17 $\mu g \cdot cm^{-2}$. Elicitation patches were removed after 6 h, and the strength of immune reactivity was assessed as the dermal thickening and erythema responses 48 h after application (**Chapter 3.6**).

D.3. Results

Similar cutaneous responses to DPCP between 14 d and 28 d induction support that 14 days is sufficient to induce immunological memory with the current sensitising dose of 0.125% (Figure D.1.).

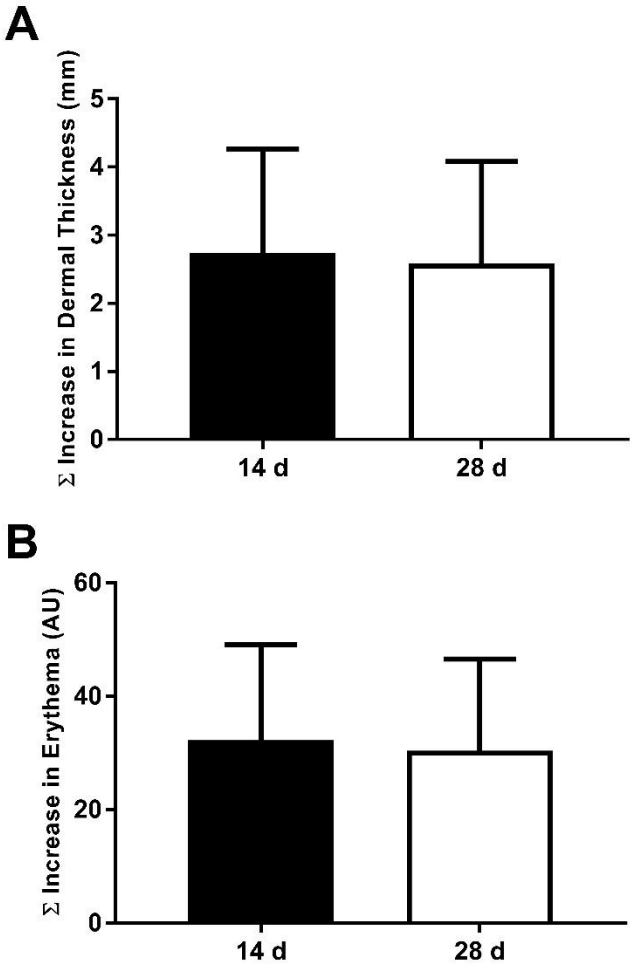

Figure D.1. Contact hypersensitivity (CHS) assessed as elicitation challenge either 14 days (14 d) or 28 days (28 d) after diphenylcyclopropenone induction. (A) Summed increase in dermal thickening response. (B) Summed increase in erythema response. Data are Mean ± SD.

D.4. Conclusions

The aim of the investigation was to determine whether reducing the period of time between primary and secondary exposure to DPCP (from 28 days to 14 days) influences the subsequent *in vivo* immune response. Similar DPCP responses between 14- and 28-day groups indicate that a period of 14 days is sufficient to induce immunological memory with the current sensitising dose of 0.125%.