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Exercise and immune function: the effects of acute exercise, training, nutrition and environmental stress

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EXERCISE AND IMMUNE FUNCTION: THE EFFECTS OF ACUTE EXERCISE, TRAINING, NUTRITION AND ENVIRONMENTAL STRESS

By

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1.1 Summary

The main objectives of this thesis were to investigate the effects upon leukocyte trafficking and bacterially-stimulated neutrophil degranulation of a chronic intensive training period in a military population (Chapter 4), a 48 h period of fluid, energy or combined fluid and energy restriction at rest and after exercise in healthy males (Chapter 5) and a 2 h exercise bout with and without additional heat stress in trained male cyclists (Chapter 6). Furthermore, a water-immersion technique was used to delineate the thermal effects and associated hormone and cytokine involvement in neutrophil trafficking and bacterially-stimulated neutrophil degranulation responses after prolonged exercise in healthy males (Chapter 7).

Twenty weeks of intensive physical training with periods of sleep deprivation, energy restriction, exposure to environmental extremes and psychological stressors had limited effect on leukocyte trafficking and bacterial-stimulated neutrophil degranulation. During training 48 h periods of recovery and rest were adequate to prevent cumulative decreases in circulating cell counts and neutrophil function. Interestingly, 48 h of either fluid, energy or combined fluid and energy restriction equalling ~90 % of daily energy requirements caused decreases in circulating leukocyte, lymphocyte, $CD3^+$ and $CD4^+$ counts and no alterations in circulating neutrophil counts or bacterial-stimulated neutrophil degranulation. In this incidence 6 h recovery and re-feeding was adequate to normalise circulating leukocyte and lymphocyte counts.

During 48 h period of energy and fluid restriction it may be a combination of small changes in several immune parameters such as circulating leukocyte and lymphocyte counts that compromise resistance to minor illnesses such as URTI rather than a specific alteration in one immune parameter.

Core temperature does not appear to mediate neutrophil responses to prolonged exercise. Bacterial-stimulated neutrophil degranulation decreased after prolonged cycling and deep water running regardless of heat stress and thermal clamping. This decrease appears not to be associated to activation status (CD11b), band cell counts, cortisol, Granulocyte Colony Stimulating Factor (G-CSF) and Interleukin (IL)-6).

It would appear that when athletes and military personnel are not clinically immune deficient, it may still be possible that the combined effects of small changes in several immune parameters may compromise resistance to minor illnesses such as URTI. Despite some limited findings in this thesis it should be recommended to athletes and military personnel to avoid overtraining, providing adequate rest and recovery during the training cycle and after competitions, limiting exposure to sources of infection, ensuring adequate nutrition, and limit the exposure to environmental extremes such as heat will all help to maintain leukocyte cell counts and neutrophil function.

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1.4 Publications

For all research chapters I was involved in all aspects of study and protocol design, data collection, data analysis and preparation of material for publication. I must however, acknowledge to input, advice and contribution from the other named authors for each publication. Below is a list of published manuscripts and abstracts arising from work presented in this thesis.

Full papers

Whitham, M., Laing, S.J., Dorrington, M., Walters, R., Dunklin, S., Bland, D., Bilzon, J.L., and Walsh, N.P. (2006). The influence of an arduous military training programme on immune function and upper respiratory tract infection incidence. *Military Medicine.* 171, **8,** 703-9.

Laing, S.J., Blackwell, J., Gwynne, D., Walters, R., and Walsh, N.P. (2005). Neutrophil degranulation responses to 2 hours of exercise in a 30 degrees C environment. *Aviation Space and Environmental Medicine.* **11**, 1068-73.

Laing, S.J., Jackson, A.R., Walters, R., Lloyd-Jones, E., Whitham, M., Maassen, N., Walsh, N.P. Human blood neutrophil responses to prolonged exercise with and without a thermal clamp. *Journal of Applied Physiology.* 104, **1,** 20-6.

Laing, S.J., Oliver, S.J., Wilson, S., Bilzon, J.L.J., Walsh, N.P. Neutrophil degranulation and T-Lymphocyte responses during 48 hours of restricted fluid and/or energy intake. In Press.

Abstracts

Laing, S.J., Jackson, A.R., Walters, R., Whitham, M., and Walsh, N.P. (2006). The effects of prolonged exercise with and without a thermal clamp on neutrophil degranulation. *Medicine and Science in Sports and Exercise*. **38,** S307.

Laing, S.J., Wilson, S., Oliver, S.J., Jackson, A.R., Walters, R., Lloyd-Jones, E., Walters, R., Whitham, M., Bilzon, J.L.J., Walsh, N.P. (2005). The effects of a 48 h period of fluid, calorie or fluid and calorie restriction and a 30 min time trial on circulating lymphocyte and T-lymphocyte $CD3^+$, $CD4^+$ and $CD8^+$ counts, *Journal of Sport Sciences.* **23,** 1172-1173.

Laing, S.J., Walsh, N.P., Bilzon, J.L.J., Dorrington, M., Bland, D., and Dunklin, S. (2004). Upper respiratory tract infection and saliva immunoglobulin-A during an arduous military training programme. *Medicine and Science in Sports and Exercise,* **36,** S5.

Walsh, N.P., Laing, S.J., Bilzon, J.L.J., Walters, R., Dorrington, M., Dunklin, S., and Bland, D. (2004). Upper respiratory tract infection and neutrophil degranulation during an arduous military training programme. *Medicine and Science in Sports and Exercise*, **36**. S5.

Laing, S.J., Walsh, N.P., Walters, R., Blackwell, J., Dowdle, S., and McClay, M. (2003). The effects of prolonged exercise in a hot environment on lipopolysaccharide (LPS)-stimulated monocyte TNF-α release in trained male cyclists. *Journal of Physiology,* **PC88.**

1.6 List of Tables

1.7 List of Figures

1.8 List of Abbreviations

°C	Degrees Celsius
ANOVA	Analysis of Variance
BASES	British Association of Sports and Exercise Sciences
BML	Body Mass Loss
CAMs	Cell Adhesion Molecules
CD	Cluster Designators
cm	Centimetre
$\dot{V}CO_2$	Carbon Dioxide output
CV	Coefficient of Variation
DEXA	Dual Energy X-ray Absorptiometry
$\dot{V}E$	Expired minute ventilation
ELISA	Enzyme-Linked Immunosorbent Assay
FFA	Free Fatty Acid
G-CSF	Granulocyte Colony Stimulating Factor
GH	Growth Hormone
Hb	Haemoglobin
Hct	Haematocrit
HPLC	High-pressure liquid chromatography
HR	Heart Rate
HR_{max}	Maximum Heart Rate
HRR	Heart Rate Reserve
HSD	Honestly Significant Difference
IgA	Immunoglobulin-A
IL-	Interleukin
ITCC	Infantry Training Centre Catterick
IU	International Unit
K_3EDTA	Tripotassium Ethylenediaminetetraacetic Acid
MJ	Mega Joules
NBM	Nude Body Mass
NKCA	Natural Killer Cell Cytotoxic Activity
$\dot{V}O_2$	Rate of Oxygen Uptake
$\dot{V}O_{2\,max}$	Maximum Oxygen Uptake

$\dot{V}O_{2\ peak}$	Peak Maximum Oxygen Uptake
O_2	Oxygen
PPO	Peak Power Output
RH	Relative Humidity
RMR	Resting Metabolic Rate
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
Rpm	Revolutions Per Minute
SEM	Standard Error of the Mean
s-IgA	Salivary Immunoglobulin-A
SPSS	Statistical Package for Social Sciences
SSHES	School of Sport, Health and Exercise Sciences
SST II^{tm}	Serum Separation Tube II
T_{re}	Rectal Temperature
TT	Time Trial
URTI	Upper Respiratory Tract Infection
W	Watts

CHAPTER 2

REVIEW OF LITERATURE

2.1 Infection incidence in athletes

Upper respiratory tract infections (URTI; common cold, cough, sore throat and middle ear infection) are thought to be the most common illness experienced by athletes (Berglund and Hemmingsson 1990; Mackinnon and Jenkins 1993b) and military personnel during training (Lee *et al.* 1992; Martinez-Lopez *et al.* 1993). Unlike the general population, work days lost to athletes and military personnel equate to a reduction in quality days spent training (Weidner 1994) and potentially detrimental effects on performance (Maughan and Evans 1982). Work days lost due to URTI in the British Field Army range from 5-20 per 1000 rising to 35-45 per 1000 in training establishments (Health of the Army, 2001). Medical diagnosis by an experienced practitioner is the most accurate way of determining the presence of an URTI. Within the exercise literature, symptoms of URTI are generally reported via means of self-report questionnaires. This anecdotal method allows for large epidemiological studies to be conducted with thousands of participants surveyed. Despite the inherent inaccuracies with self-report questionnaires there is evidence to suggest that athletes can accurately identify URTI's (Mackinnon *et al.* 1993a). However, the literature on URTI incidence in athletes has been identified as artificially biased because highly-trained athletes are believed to have an increased body-awareness (Gleeson and Bishop 1999) and are more likely to seek medical care because even minor illnesses may inhibit their ability to train (Douglas and Hanson 1978).

2.1.1 Upper Respiratory Tract Infection incidence and exercise

Depending on the modality, intensity and duration, exercise is generally recognised to be therapeutically beneficial to cardiovascular, respiratory and metabolic adaptation (McArdle *et al.*, 1981). Evidence suggests that taking sedentary individuals and repeatedly performing moderate exercise for 45 min, 5 $d \cdot wk^{-1}$ over 15 weeks at 60 %

heart rate reserve (HRR) can reduce the number of days spent suffering from symptoms of URTI (Nieman *et al.* 1990b). In addition, taking previously sedentary elderly women and completing 30-40 min brisk walking at 60 % HRR, 5 $d \cdot wk^{-1}$ for 12 weeks compared with callisthenic (muscular strength and flexibility work, light intensity keeping HR close to resting levels) showed that walkers experienced fewer episodes of URTI compared with those partaking callisthenic training (one episode compared to eight, for walkers and callisthenics respectively (Nieman *et al.* 1993). However, more intense heavy exercise appears to negate the beneficial effects on the immune system observed with moderate exercise. During two weeks following marathon events (Nieman *et al.* 1990a) and 56 km ultra-endurance marathons (Peters and Bateman 1983) athletes are believed to be 2 to 6 times more susceptible to URTI. In these cases the incidence of illnesses is likely to be increased as race time decreases (Peters and Bateman 1983), and pre-race training distance increases (Nieman *et al.* 1990a). These studies both used self-reported symptoms of URTI and were not determined by physician assessments. Heavy training and increased incidence of URTI as diagnosed by a physician have also been reported in professional footballers compared with untrained controls (Bury *et al.* 1998). Footballers observed over a 1 year period reported 22 episodes (group total) of URTI compared with 9 episodes (group total) in the control group. Of the URTI's diagnosed in this study 77 % occurred during the winter months; albeit, this is the heavy competitive period for professional footballers.

Other factors associated with increased susceptibility to URTI include increased exposure to pollutants (competing in large cities marathons such as Cape Town and Los Angeles), increased exposure to pathogens (sharing drinks bottles), increased time in close proximity to others (training camps, shared accommodation) poor dietary practices and elevated psychological stress (Shephard and Shek 1999). Military training

programmes are widely acknowledged to be rigorous with prolonged periods of heavy physical activity, negative energy balance, sleep deprivation, shifts in circadian rhythms, exposure to extremes of hot and cold environments and severe psychological stressors. Therefore it is not surprising that during an 11-month period of training at the U.S Naval Academy, midshipmen experienced frequent episodes of URTI which were often reported as having an adverse effect on physical performance (Gray *et al.* 2001). However there is conflicting evidence to suggest that training and heavy exercise are not associated with increased incidence of URTI. During a course of basic infantry training lasting 18.5 weeks the incidence of URTI remained unaltered (Brenner *et al.* 2000). In addition URTI has not always been reported to increase after endurance events, no significant increases in reported URTI were observed following the Stockholm marathon (Ekblom *et al.* 2006). In this study 17 % of runners reported URTI in the 3 weeks preceding the race and 19 % in the 3 weeks following the race, contradicting the earlier findings of Nieman *et al.* (1990a) and Peters and Bateman, (1983). Interestingly, 33 % of runners who had suffered from URTI prior to the race reported similar symptoms in the following 3 weeks, whilst younger runners (30-39 years) appeared to be more prone to URTI before and after the race (Ekblom *et al* 2006). Several studies have failed to correlate specific changes in immune system host defence to infection, illness (Gleeson 2000) or physical performance (Pyne *et al.* 2001). At present there is a lack of data directly linking impaired immune function (*in vivo*) and increased susceptibility to URTI's following heavy strenuous exercise.

Within the literature the mechanisms behind moderate exercise and reduced susceptibility to URTI remain inconclusive (Nieman 1997; Konig *et al.* 2000; Matthews *et al.* 2002). Increased 'surveillance' of host defence to respond to invading pathogens has been proposed (Nieman 2000b). Increased natural killer cell cytotoxic activity

(NKCA) has been observed in previously sedentary obese women after 6 weeks training of moderate (60 % HRR) exercise for 45 min, 5 times per week (Nieman *et al.* 1990b). The 57 % increase in NKCA evident after 6 weeks of training was not evident after the completion of 15 weeks training. The authors attributed the increase in NKCA after 6 weeks to seasonal variations. In addition, immunoglobulin-A (IgA) concentration, the principal antibody in saliva secretions, has been reported to increase up to 57 % after 12 weeks exercise training (3 x 30 $min \cdot wk^{-1}$ at 70 % HRR) in sedentary men and women (Klentrou *et al.* 2002). Similar associations of negative relationships between saliva IgA concentration (s-IgA) and incidence of URTI have been reported and verified by physicians in swimmers and non-exercising controls (Gleeson *et al.* 1999). Unfortunately, a direct link between heavy intense exercise and increased susceptibility to URTI has been difficult to establish. Designing a study that requires participants to perform heavy strenuous exercise during viral infections has potentially severe implications to health and thus ethically such a study would not be viable. One study investigated the whole body's ability to respond to pathogens following severe exercise without participants actually exercising with a viral load. Antigens that induce an antibody response were injected into the skin of the forearms of triathletes following a half-ironman and showed an impaired whole body response to the infectious challenge in exercised triathletes. This was suggested to be linked to an increased risk of developing a subsequent infection (Bruunsgaard *et al.*, 1997).

Based on the body of literature ahead of 1994 (Peters and Bateman 1983; Nieman *et al.* 1990a; Nieman *et al.* 1993), Nieman proposed the J-shaped model of the relationship between risk of upper respiratory tract infection and exercise intensity (Nieman, 1994). According to the hypothesised model moderate exercise would protect an individual from infections while strenuous exercise would increase the number of infectious

episodes within a given time. The increased susceptibility to URTI after exercise is attributed to the notable decline in host defence; termed the 'open window'. This open window is the period of opportunity for bacteria and viruses to gain entry into the body (Nieman 2000b). More recently the S-shaped model has been proposed to incorporate elite athletes and immune function (Figure 2.1, Ekblom *et al* 2006). Elite athletes must sustain large training volumes with severe physiological and psychological stress whilst withstanding a significant increase in the susceptibility to infection.

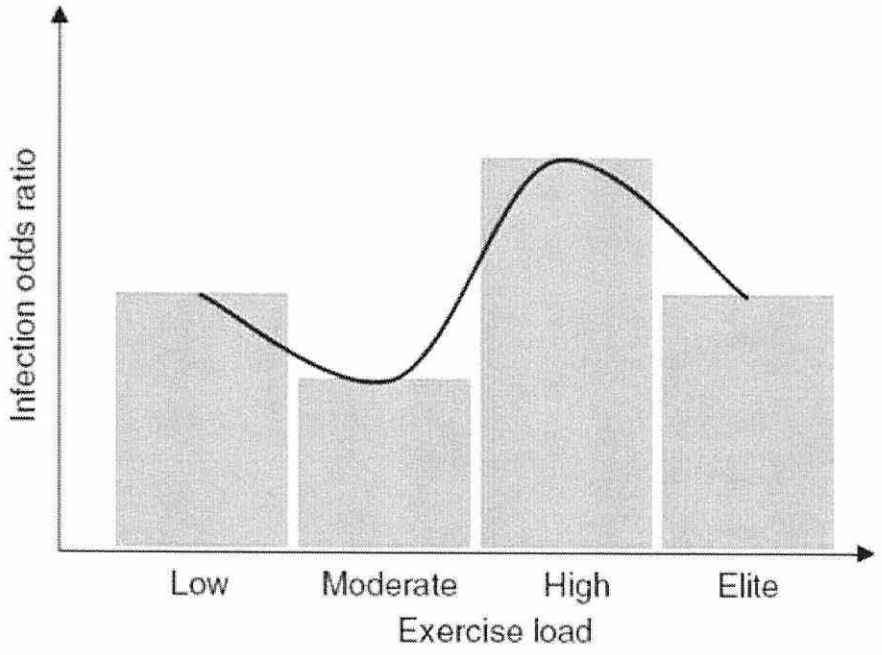

Figure 2.1 Proposed S-Shaped relationship between training load and infection rate taken from Malm (2006).

Elite athletes often train 10 to 12 times per week for the majority of the year and an elite distance runner can cover from 120 to over 200 km·wk^{-1}. For this reason it would appear logical that there is a negative correlation between the amount of training performed by an elite athlete and the number of days spent suffering from URTI. The odds ratio for chances of having a URTI were determined in a group of runners and identified that the odds increased with average running distance, but only up to 2222 km·year^{-1} (43 km·wk^{-1}) (Heath *et al.* 1991). Above 43 km·wk^{-1} the odds ratio remained

constant, thus providing some support to the suggestion that there is limited additional risk of infection with extremely high training volumes often performed by elite athletes (S-shaped curve).

2.2 Circulating neutrophils and host defence

Polymorphonuclear leukocytes or neutrophils are the most abundant of the non-specific host defence cells and constitute 50-60 % (2.0-7.5 x $10^{9} \cdot L^{-1}$) of the circulating blood leukocyte pool. Neutrophils target microbial, bacterially, fungi and viral pathogens that penetrate the body's physical barriers (Smith 1997). Neutrophils exist in dormant (resting) or activated states and also various intermediate stages depending on priming and pre-activation (Smith *et al.* 1996). At rest, approximately 90% of the neutrophil population is located in the bone marrow as newly differentiated cells; the remaining 10% are dispersed in the blood and are adhered to the vascular endothelium (Yang and Hill 1991; Pyne 1994). The neutrophil response includes a cascade of sequential steps to chemotactic factors generated by infectious agents and contact of these agents with phagocytes and other components of the immune system (Smith 1997). When necessary, neutrophils penetrate the endothelial layer (diapedesis) and move through connective tissue to sites of infection (chemotaxis and migration). Increased neutrophil efficiency means they usually bind to and engulf the pathogen (phagocytosis). This allows neutrophils to digest the infectious agent by releasing granular lytic enzymes (e.g. elastase) from azurophilic (primary) and specific (secondary) granules into the phagocytic vesicle (degranulation) and to have a 50 to 100-fold increase in O_2 consumption which results in the generation of cytotoxic reactive oxygen species (ROS, oxidative or respiratory burst) (Smith and Pyne 1997; Mackinnon 1999). These processes appear to be triggered simultaneously upon the initiation of phagocytosis.

Microbial damage caused by ROS can be enhanced by neutrophil hydrolytic enzymes such as elastase. The enzyme elastase is a serine protease that begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the bacterial cell.

2.3 Circulating lymphocytes and host defence

Lymphocytes compose about 20-25% (1.5-3.5 $x10^{9} \cdot L^{-1}$) of all circulating leukocytes. Lymphocytes are derived from the bone marrow lymphoid progenitor and are the major immune cells of adaptive immunity (B and T cells) and some aspects of innate immunity (NK cells). Approximately 60-75% of peripheral blood lymphocytes are T cells, 5-15% B cells and 10-20% NK cells (Mackinnon 1999). Lymphocyte maturation occurs in either the thymus (T cells) or the bone marrow (B cells). On entry to the circulation naïve T and B cells become distributed to secondary lymphoid organs such as the spleen, lymph nodes and mucosa-associated lymphoid tissue (Bain, 2006). The cluster designators (CD) that distinguish all T cells are $CD3^{+}$ and all B cells $CD19^{+}$ and $CD20^{+}$.

T-lymphocytes can be further divided into subsets each with distinct functions. These include helper T (T_h or $CD4^{+}$ cells), cytotoxic T (T_c or $CD8^{+}$), memory T (either $CD4^{+}$ or $CD8^{+}$), regulatory T (T_{reg}) and NK cells. T-helper cells divide upon activation and secrete cytokines (interferon-γ (IFN- γ), IL-2, IL-4, IL-5, IL-6, IL10 and IL-13) that play a role in defence against pathogens or stimulate activation and proliferation of other immune cells. Cytotoxic T cells destroy tumor and virally infected cells and with the interaction with T_h cells can be transformed into T_{reg} cells. There are two major classes of $CD4^{+}$ T_{reg} cells, the naturally occurring T_{reg} cells and the adaptive T_{reg} cells. Towards the end of an immune reaction T_{reg} cells shut down the T cell mediated response. Memory T cells can either be $CD4^{+}$ or $CD8^{+}$ cells and are present after an

immune reaction or infection. Memory T cells provide memory of past infections and can be generated in large quantities upon subsequent re-infection of an antigen. Natural killer cells are cytotoxic $CD8^+$ cells that lack the T cell receptor. The functions of NK cells include cytokine production and release of cytolytic molecules similar to both T_h and T_c cells. Further detail on acquired immune functions and T lymphocytes cells can be found in the extensive reviews by Bishop (chapter in Gleeson 2006) and Kruger and Mooren, 2007.

2.4 Exercise and leukocyte trafficking

It is well documented that exercise causes an increase in circulating leukocyte cell counts with the size and magnitude dependent on the intensity and duration of the exercise bout (McCarthy and Dale 1988). Increased fitness levels attenuate leukocyte responses to the same absolute exercise workload. For brief exercise lasting less than 1 h, intensity appears to be the most influential factor when considering the magnitude of leukocytosis (Gimenez *et al.* 1986). Upon cessation of exercise lasting up to 30 min in duration the circulating leukocyte counts fall quickly approaching normal values within 10 to 30 min. For exercise lasting longer than 1 h, duration appears to be the most important factor causing elevated circulating leukocyte cell counts (McCarthy and Dale 1988; Tvede *et al.* 1993). After high intermittent exercise spread over 1.5 h and prolonged exercise of 2.5 to 3.5 h circulating leukocyte counts can continue to rise upon cessation and return to normal more slowly than shorter duration exercise (Galun *et al.* 1987). The increase in circulating leukocyte number observed immediately after short duration exercise lasting less than 1 h is predominantly due to increases in circulating neutrophils, and to a lesser extent, lymphocytes. Interestingly, the leukocytosis observed upon cessation of prolonged exercise lasting longer than 1 h is almost exclusively due to

developing neutrophils. Circulating lymphocyte counts rise progressively with increasing work rate although the magnitude of lymphocytosis appears to be related to intensity rather than exercise duration (Shek *et al.* 1995). The general consensus is that circulating leukocyte, neutrophil and lymphocyte counts increase following exercise however to varying degrees depending on the intensity and duration of exercise. The main effects of acute and prolonged exercise and training on circulating neutrophil and lymphocyte counts, bacterially-stimulated neutrophil degranulation and T-lymphocyte subset counts will be discussed in further detail in sections 2.4.1-2.5.1.

2.4.1 Circulating neutrophil cell counts with short-term and prolonged exercise

Marked increases in circulating neutrophil cell counts are observed during and after virtually all forms of exercise and have been extensively reviewed (McCarthy and Dale 1988). During and immediately after brief (<30 min) intense exercise the increase in circulating neutrophil count appears to be transient. For example, circulating neutrophil cells accounted for 60 % of the total leukocytosis observed immediately after very brief (30 second) maximal intensity cycling, returning to normal 1 h post-exercise (Nieman *et al.* 1992). Similarly, in various modes of exercise (cycling, squash, swimming, jogging) lasting 30 min at approximately 70 % (range 48-84 %) of maximal oxygen consumption, an immediate post-exercise increase in circulating neutrophil count which returned to within baseline levels ~30 min later was observed (McCarthy *et al.* 1991; McCarthy *et al.* 1992).

Exercise performed over more prolonged periods is reported to cause circulating neutrophil counts to rise during the exercise bout, remain elevated upon cessation of exercise and remain elevated several hours later (Peake 2002). In healthy untrained

men, 1.5 h cycling at 70 % VO_2 max caused a 2.5 fold increase in neutrophil cell counts post-exercise with further elevations (>3 fold) 1 h post-exercise (Suzuki *et al.* 1996b). In recreational cyclists performing 2 h cycling at 65 % VO_2 max, evoked a 4.5 fold increases in neutrophil cell counts post-exercise (Bishop *et al.* 2004). Similar findings have been found in trained triahletes, 2.5 h cycling and 2.5 h running both at 75 % VO_2 max resulted in 4.4 and 3.6 (cycling and running respectively) fold increases in circulating neutrophil count post-exercise, remaining elevated above baseline values at 6 h post-exercise (Nieman 1998). Similar responses in magnitude were reported in trained cyclists after 2 h cycling at 75 % VO_2 max (Bishop *et al.* 2003). The magnitude and persistence of the exercise neutrophilia appears to be related to intensity, duration and mode (Hansen *et al.* 1991; Peake 2002). Significantly greater neutrophil counts were reported 1.5 and 12 h after 1 h running downhill at 70 % VO_2 max compared to similar intensity running on a level treadmill (Pizza *et al.* 1995).

2.4.2 Circulating neutrophil counts and exercise training

The effects of training status *per se* (trained versus untrained) of individuals on circulating neutrophil cell responses to exercise appears to be contradictory. In a study comparing endurance-trained long distance runners and triathletes with untrained controls, no significant differences were observed between groups both at rest and following a graded exercise test to volitional exhaustion for circulating neutrophil cell counts (Hack *et al.* 1992). Interestingly, from the same laboratory, untrained controls were compared to well-trained male distance runners using a graded exercise test to exhaustion whilst undertaking moderate or intense training periods. Circulating neutrophil counts at rest and after completion of the graded exercise test did not differ significantly between moderate training runners and untrained controls (Hack *et al.* 1994). However, athletes undertaking an intense training programme showed significant

reductions in circulating neutrophil counts and phagocytic activity at rest and immediately upon completion of a graded exercise test when compared with runners partaking in a moderate training programme and untrained controls (Hack *et al* 1994). Total exercise duration was different between groups (total exercise duration; control 14 ± 2 min, moderate training 21.4 ± 1 min and intense training 20.8 ± 1 min) which may have accounted for the lack of significant findings between moderate training runners when compared to untrained controls (Hack *et al* 1994). Similarly, trained cyclists were reported to have lower resting neutrophil cell counts compared with untrained controls (Blannin *et al.* 1996). In addition, during submaximal cycling (45 min duration) increases in circulating neutrophil cell counts occurred in trained cyclists and untrained healthy controls, although the magnitude of neutrophilia was smaller in trained cyclists (Blannin *et al.* 1996). In field based training studies in military populations 4 weeks and 18.5 weeks basic training caused no significant impact on neutrophil cell populations (Brenner *et al.* 2000; Anomasiri *et al.* 2002), while a 19 day U.S. Army training course designed to assess soldiers physical, emotional and mental stamina resulted in only a modest 8% increase in neutrophil cell count upon completion (Woods *et al.,* 2005). The different response of circulating neutrophil counts in trained athletes and after training may be because investigators have used different training regimes or different durations and intensities of exercise during training.

2.4.3 Neutrophil function and exercise

Chediak-Higashi Syndrome (CHS) although rare and passed down through families, is associated with increased infections in the lungs, skin and mucosal membranes (Curnutte *et al.* 1988). This disease does not only affect neutrophils however, the primary defect in within neutrophil cells is in specific granules present in the cytoplasm. As with CHS, patients with neutropenia are more susceptible to bacterial infections.

Neutropenia is more common than CHS and can be defined as acute or chronic depending on the duration of the illness. The causes of neutropenia are either a problem with cell production in the bone marrow or destruction of neutrophil cells elsewhere in the body (Weingarten and Bokoch 1990). It has been suggested that an impaired or depleted neutrophil function could be an important factor in the increased susceptibility of athletes to infection (Pyne 1994). It is important to note that increases in circulating cell counts does not automatically lead to increased host defence, the function of each cell must be taken into consideration (Blannin *et al.* 1996). Neutrophil degranulation appears to be induced by varying modes of exercise. Elevated plasma elastase was evident after 30 min cycling at 70 % VO_2 max (Blannin *et al.* 1996), after ~ 40 min cycling to fatigue at 80 % VO_2 max and up to 3 h cycling at 55 % VO_2 max (Robson *et al.* 1999). After correction for neutrophil cell counts reductions in neutrophil degranulation were observed (Robson *et al.* 1999). An increase in plasma myeloperoxidase (MPO; produced during neutrophil respiratory burst) has also been shown following 1 h running at 85 % VO_2 max (Peake *et al.* 2004) and following marathons (Suzuki *et al.* 2003a). Increases in plasma concentrations of elastase and MPO accompanied by increases in circulating neutrophil cell count do not necessarily reflect increased neutrophil function (in this example degranulation). Indeed, elevated plasma elastase concentration following prolonged cycling corrected on a per cell basis for the exercise-induced increase in circulating neutrophil cell count showed that the neutrophil cells were not stimulated after exercise (Suzuki *et al.* 1999).

Perhaps of more relevance is the *in-vitro* ability of neutrophils to respond to a challenge. One such method involves bacterially-stimulated elastase release per neutrophil. Sub-maximal exercise is commonly reported to decrease bacterially-stimulated elastase release per neutrophil (Robson *et al.* 1999; Walsh *et al.* 2000; Bishop *et al.* 2000;

Bishop *et al.* 2003). Exercise duration appears to be an important factor in suppressed neutrophil degranulation as bacterially-stimulated elastase release per neutrophil was significantly lower following prolonged exercise (~3 h) at 55 % VO_2 max compared to shorter duration (~40 min) at a higher intensity 80 % VO_2 max (Robson *et al.* 1999). In addition, the suppressed neutrophil degranulation was still evident 24 h post-exercise after prolonged exercise compared to recovered neutrophil degranulation after shorter duration exercise (Robson *et al.* 1999). Independent research conducted using similar exercise protocols of 2 h cycling at 60 % VO_2 max reported decreased neutrophil degranulation of 40 and 33 % post-exercise (compared to pre-exercise) respectively (Walsh *et al.* 2000; Bishop *et al.* 2000). It has been reported that neutrophil bacterial killing capacity is lower in trained cyclists before and after maximal intensity exercise (Lewicki *et al.* 1987; Blannin *et al.* 1996) and that endurance training (5 weeks training 30-60 min at 70 % VO_2 max) attenuates the neutrophil degranulation response of resting and 2.5 h post-exercise samples (Blannin *et al.* 1996).

2.4.4 Circulating lymphocytes and exercise

Circulating lymphocytes elicit a biphasic change in number following acute exercise (Nieman *et al.,* 1995; Nielsen *et al.,* 1996). Typically an increase in the number of lymphocytes (lymphocytosis) is observed during and immediately after exercise. Lymphocyte cell counts then typically fall (lymphopenia) below pre-exercising values following cessation of brief maximal exercise and before steadily returning to resting values by 6 h post exercise (Nieman *et al.* 1995; Nielsen *et al.* 1996). This pattern of cell trafficking is also evident in T-lymphocyte $CD3^+$ cells for intensive exercise of both short and prolonged duration. These changes to lymphocyte and T-lymphocyte $CD3^+$

cells are proportional to exercise intensity and to a lesser extent duration (Gray *et al.* 1993).

2.4.5 Circulating lymphocytes and short-duration exercise (<60 min)

Acute moderate exercise may lead to no change in circulating lymphocyte number, despite public health recommendations which emphasis moderate intensity physical activity. Indeed, 30 min walking at 50 % $VO_{2\ max}$ in sedentary males cause no significant change in circulating lymphocyte counts (Markovitch *et al.,* 2008). Large increases in circulating lymphocyte cell counts have been seen following brief maximal exercise. A two to three-fold increase in circulating lymphocyte count was observed after 6 min maximal rowing ergometry in trained rowers (Nielsen *et al.* 1996) and repetitive or heavy resistance training to exhaustion (Nieman *et al.,* 1995; Miles *et al.,* 2003). The lymphocytosis following short-duration exercise appears to be transient, in that lymphocyte counts tend to decline below baseline levels 1-3 hours post-exercise. Following 6 min maximal rowing ergometry in trained rowers, circulating lymphocytes counts fell 40 % below resting values. Similarly following repetitive resistance training to exhaustion, a 40-50 % decrease in circulating lymphocytes counts was observed.

2.4.6 Circulating lymphocytes and prolonged exercise (> 60 min)

Increased (58 %) in circulating T-lymphocyte $CD3^+$ cell counts was observed 30 min into a 2 h treadmill run at 65 % VO_2 max, falling to 42 % below resting values at 2 h post-exercise (Shek *et al* 1995). As $CD4^+$ cells account for 70 % of the T cell population it might be expected that the absolute changes in $CD4^+$ cell number are larger than those observed for $CD8^+$ cells. However, $CD8^+$ cell counts appear to increase to a larger extent during and immediately after exercise; suggesting a recruitment of $CD8^+$ cells into the circulation greater than $CD4^+$ cells (Pedersen *et al.* 1994; Natale *et al.* 2003).

A $CD4^+/CD8^+$ ratio greater than 1.2 is important for host defence (Mackinnon 1999) and a decreased $CD4^+/CD8^+$ ratio below 1.0 are indicators of decreased host defence and increased susceptibility to infection (Chailleux *et al.,* 1985). Host defence against bacterial infection depends on the interaction between macrophages and T lymphocytes. A lower $CD4^+/CD8^+$ ratio occurs as a result of a higher number of $CD8^+$ cells. The $CD8^+$ cell may represent functionally suppressive lymphocytes which down-regulate the proliferation of $CD4^+$ cells (Ainslie *et al.,* 1992). It has been reported that when $CD8^+$ cells are comprised (>40% of the total T lymphocyte number) the time required for improvement in disease increased (Yu *et al.,* 1995). Single bouts of endurance exercise and intensified training periods have been shown to suppress the $CD4^+/CD8^+$ ratio (Lewicki *et al.* 1988; Kajiura *et al.* 1995). Increases in circulating T-lymphocyte $CD3^+$, $CD4^+$ and $CD8^+$ counts were observed in moderately fit males after 2 h cycling at ~60-65 % maximum oxygen consumption, returning to within pre-exercising values by 3 h post-exercise (Gannon *et al.* 2001; Natale *et al.* 2003). While single bouts of prolonged exercise can cause temporary suppression in the $CD4^+/CD8^+$ ratio (Nieman and Pedersen 1999), heavy training appears to produce more persistent changes in the cell distribution of the immune system (Smith *et al.* 1990; Baj *et al.* 1994; Kajiura *et al.* 1995; Blannin *et al.* 1996; Gleeson *et al.* 1999).

2.4.7 Circulating lymphocytes and exercise training

The effects of 12-weeks light training on healthy sedentary males was assessed using a 60 min cycle test at 60 % of aerobic power. Training caused an increase in aerobic power (8% light training and 21% moderate training) and reduction in body mass and % body fat in the moderate training group. Moderate and light training caused significant decreases in circulating $CD3^+$ and $CD4^+$ cell counts with no change in the $CD4^+/CD8^+$

ratio, although moderate training had the additional stress of a negative energy balance (Shore *et al.* 1999). Lowered $CD3^+$ and $CD4^+$ absolute cell counts and no change in absolute NK cell counts were observed following a 6-month intensive training and racing season (cycling ~500 $km \cdot wk^{-1}$) in young cyclists (Baj *et al.* 1994). The effects of military training on immune responses is equivocal with large variability in training and exercise duration and intensity and often performed in multi-stressor environments (physical/ psychological/ environmental) (Karpinski *et al.* 2001). Following 4 weeks basic training (comprising moderate intermittent physical exercise) male air-force recruits were reported to have increases in circulating $CD4^+$ cell counts and decreases in neutrophil/lymphocyte ratio (Makras *et al.* 2005). In another military study in which the first 8 weeks of intense stress adaptation training were monitored circulating T-lymphocyte $CD3^+$, $CD4^+$ and $CD4^+/CD8^+$ ratio increased (Karpinski *et al.* 2001). In contrast, infantry men in an 8 week recruitment training programme were reported to have no alterations in circulating levels of $CD3^+$, $CD4^+$, and $CD8^+$ (Anomasiri *et al.* 2002). Due to the number of uncontrolled variables during these training studies it is difficult to establish clear conclusions on the effects of training on lymphocyte subset counts. What appears to be evident is that if the training intensity and duration is hard and long enough impairment of immune cell counts will be identifiable. The addition of adequate rest periods into training programmes or intermittent style training appears to cause no suppression or even increase immune cell counts.

2.5 Mechanisms of altered circulating immune cell counts with exercise

The release of leukocytes into the circulation during exercise, and then their removal from the circulation may involve mechanical factors such as increased cardiac output and the interactions between circulating blood flow and the vascular endothelium

(Bierman *et al.*, 1952). At present the location from which the marginated cells are recruited into the circulation is controversial. Evidence suggests that despite the spleen being a major reservoir for lymphocytes and neutrophils, surgical removal of the spleen has relatively little effect on changes in circulating leukocyte number in response to exercise (Shephard 2003). It appears that neutrophils, rather than lymphocytes, are recruited into the circulation from the microvasculature of the lungs (Shephard 2003). The lungs are reported to be a major source of marginated leukocytes, it remains unclear whether this is a result of increased ventilatory movements, increased cardiac output, or both (Fairbarn *et al.* 1993). Hyperventilation at rest has been associated with increases in circulating leukocytes (Staubli *et al.* 1985). Fairbarn *et al.* (1993) had participants perform exercise on a cycle ergometer whilst measuring ventilation rate. Participants then matched their ventilatory rates whilst seated and not performing exercising. Matched ventilation from non-exercising participants indicated that recruitment of leukocyte cells from marginated pools in the lungs could not be attributed to ventilatory movements and that it was most likely related to cardiac output. Increased cardiac output increases axial blood flow through the vascular system increasing the chance of demargination from vessel walls. Support for this mechanism comes from the administration of β-adrenergic receptor antagonists (propranolol) during brief (20 min) exercise. Propranolol decreased heart rate at all work loads but had no effect on the increase in cardiac output as work loads increased. Plasma catecholamine concentrations were similar at rest and during exercise in control and propranolol trials, removing any possible contribution of these hormones in the observed leukocytosis. Therefore it was concluded that exercise induced mobilisation of leukocytes demarginated from the lungs in response to increased cardiac output and pulmonary blood flow (Foster *et al.* 1986).

It has long been known that the stress hormones cortisol and adrenaline are involved in the influence of leukocyte distribution between circulation and body compartments such as the spleen, liver and bone marrow. Increases in plasma cortisol concentration are usually found at workloads above 60 % VO_2 max, whereas workloads below 50 % VO_2 max plasma cortisol decreases (McCarthy and Dale 1988). This workload threshold may be lower if the exercise is prolonged. Robson *et al.* (1999) observed higher plasma cortisol and circulating neutrophil cell counts following 3 h cycling at 55 % VO_2 max compared with ~40 min cycling 80 % VO_2 max. Cortisol also appears most important during recovery from exercise; evidently cortisol may not reach its peak until the cessation of exercise (Galbo 1983). For example, a negative association was observed between post exercise cortisol concentration and post-exercise circulating neutrophil and lymphocyte counts (Gabriel *et al.* 1992). The mechanisms by which cortisol influences the number of circulating lymphocyte count is less clear. In addition to the widely documented neutrophilia following exercise lasting between 40 min and 3 h (Hansen *et al* 1991; Robson *et al.* 1999; Bishop *et al.* 2000), infusion and exercise induced elevations in plasma cortisol can cause a decrease in circulating lymphocyte counts (Tonnesen *et al.* 1987). Cortisol is thought to either inhibit lymphocyte entry into the blood or promote lymphocyte movement into tissues (Cupps and Fauci 1982; Tonnesen *et al.* 1987).

The surface expression of adhesion molecules is an important contributing factor to leukocyte homing and trafficking. Alterations in the surface expression of adhesion molecules could reflect shedding of molecules, selective apoptosis, mechanical deformation or active biochemical processes involving catecholamines, cytokines or other hormones (Shephard, 2003; Kruger and Mooren, 2007). Exercise stress can induce redistribution or migration of lymphocytes between secondary lymphoid tissue such as

the lymph nodes, Peyer's patches, spleen and the peripheral circulation (Kruger and Mooren, 2007). Recently, researchers have investigated the redistribution and homing of T lymphocytes by administering adrenaline to male SWISS-mice to mimic the effects of exercise (Kruger *et al.,* 2008). Researchers reported a release of T cells from the spleen and liver as well as an increase in circulating blood T cells. The exercise induced decrease of lymphocytes cells from the spleen was related in an intensity-dependent manner most likely indicating exercise-induced mobilisation from the spleen (Kruger *et al.,* 2008). Exercise and exposure to catecholamines increases the density of β_2-adrenergic receptors on lymphocytes with the greatest expression found on NK cells (Shephard 2003). When adrenaline binds to β_2-adrenergenic receptors it triggers an increase in intracellular cyclic AMP (cAMP). This blocks the production of pro-inflammatory cytokines, enhances the secretion of IL-10 and lead to alterations in cell function and CAMs (Kittner *et al.,* 2002; Shephard, 2003). Alterations in lymphocyte CAMs may decrease their expression for ligands expressed by vascular endothelial cells, therefore causing lymphocytes to become detached from endothelial tissue and enter the peripheral circulation. Shephard (2003) provided support for this mechanism by inducing lymphocytosis through infusion of adrenaline and attenuating the exercise-induced rise in lymphocyte counts by infusion of β_2-adrenergic antagonists (β-blockers). *In vivo* infusion of adrenaline has also been shown to reduce neutrophil adherence (Boxer *et al.* 1980). However, the extent to which alterations in cell adherence affects circulating cell counts has been questioned (Foster *et al.* 1986).

2.5.1 Mechanisms of altered neutrophil cell functions with exercise

Moderate exercise intensities are often associated with enhanced immune cell function(s), lower cortisol secretion and increased cortisol clearance (McCarthy and Dale 1988). On the other hand, suppressed inflammatory responses associated with

prolonged exercise are widely acknowledged to be related to increases in plasma cortisol and catecholamines (Shephard 1998). Elevated plasma cortisol within the physiological range has been implicated in decreased neutrophil chemotaxis, oxidative burst and neutrophil degranulation after prolonged exercise (Forslid and Hed 1982; Salak *et al.* 1993). In addition, raised plasma cortisol after prolonged exercise has also been associated with the delayed release of band (immature) neutrophils, and it has been suggested that these immature cells maybe less responsive to stimulation (Suzuki *et al.* 1996a). Reduced bacterially-stimulated neutrophil degranulation may occur following an acute exercise stimulus, because neutrophils may enter a refractory state due to desensitisation following activation (Henson *et al.* 1981). It has been suggested that the decrease in bacterially-stimulated neutrophil degranulation after prolonged high intensity exercise may be associated with a reduction in CD11b expression indicating a decrease in neutrophil activation (Bishop *et al.* 2003). In addition, raised plasma cortisol concentration has been associated with suppressed neutrophil activation (Blalock 1989). Therefore a role for raised circulating plasma cortisol is likely in suppressed neutrophil functional responses (neutrophil activation, chemotaxis, oxidative burst and neutrophil degranulation) observed after prolonged exercise. More recently studies have suggested a strong link between growth hormone (GH) and the delayed increase in neutrophil count post-exercise (Suzuki *et al.* 1999; Suzuki *et al.* 2002). Yamada *et al.* (2002) have shown a stronger correlation between post-exercise GH and neutrophil count at 1 h post-exercise (r .59) than post-exercise plasma cortisol and neutrophil count at 1 h post-exercise (r .23).

2.6 Effects of energy and fluid intake on leukocyte trafficking and neutrophil cell function with and without exercise

Dietary practices such as energy restriction and fasting have the potential to weaken several aspects of immune function, leaving individuals more susceptible to URTI's (Woodward 1998; Fraker *et al.* 2000). Periods of forced and voluntary fluid and/ or energy restriction, often lasting for a number of days are common place in athletes making weight or with eating disorders (Brownell *et al.* 1987; Baum 2006) and occupational settings, for example, military personnel during survival training (Carins and Booth 2002).

Neutrophil counts were reported to be significantly reduced following 7 days of exercise (3 $h \cdot d^{-1}$ at 75 % VO_{2max}) with a 25 % energy deficit compared with exercising and receiving 110 % of daily energy expenditure (Galassetti *et al.* 2006). Following a 36-h fast, neutrophil chemotaxis and oxidative burst activity was reduced: although this was reversed after 4 h of refeeding (Walrand *et al.* 2001). In addition, neutrophil chemotaxis and microbial killing was depressed in obese patients on a 3-month calorie restriction programme (McMurray *et al.* 1990). Impaired phagocytic activity has been shown after 7 days of dietary restriction in rats (Ikeda *et al.* 2001) and in humans undergoing severe energy restriction for weight reduction prior to competitions (Kowatari *et al.* 2001; Suzuki *et al.* 2003b). The aforementioned studies do not distinguish between fluid restriction and energy restriction effects on immune function. Some authors make no reference to fluid intake (Walrand *et al.* 2001; Galassetti *et al.* 2006). Others report their subjects to be hypohydrated following energy restriction and have performed their investigations in a multi-stress environment (e.g. tropical climate, sleep deprivation, psychological stress) (Booth *et al.* 2003). Therefore, it is difficult to

assess whether the results are based on energy restriction alone or a combination of fluid restriction, energy restriction or other uncontrolled variables.

Little is known about the independent and combined effects of energy and fluid restriction on immune function at rest and after exercise. Cellular and humoral immunity have been shown to be depressed in soldiers training in a tropical environment and surviving for 12 days on ration packs (~1800 $kcal \cdot d^{-1}$) (Booth 2003). Energy restriction lasting between 36 h and 7 days has been shown to decrease circulating lymphocyte counts (Woodward 1998; Fraker *et al.* 2000) and decrease T-lymphocyte $CD3^+$ and $CD4^+$ cell counts (Savendahl and Underwood 1997). It appears that altered immune responses during energy restriction are transient and can be reversed within hours upon refeeding. For example, the percentage of T-lymphocyte $CD3^+$ and $CD4^+$, which had lowered during 36 h of fasting, was corrected with only 4 h of refeeding (~10 Kcal per kilo of body mass) (Walrand *et al.* 2001). Prolonged periods of under-nutrition have been associated with increased URTI incidence (Shippee *et al.* 2004) as has the sub-clinical disorder 'anorexia athletica' (Beals and Manore 1994). It is somewhat surprising then that anorexia nervosa patients do not report raised infection incidence and in some cases fewer infections than healthy controls (Bowers and Eckert 1978). It has been suggested that starvation suppresses and refeeding activates certain infections, however there is little evidence to support such a theory (Murray and Murray 1977).

2.6.1 Mechanisms behind energy and fluid intake effects on leukocyte trafficking and neutrophil cell functions with and without exercise

It is not yet clear whether a deficit in an individual macronutrient (e.g. protein) (Norris *et al.*, 1990) or micronutrient (e.g. zinc) (Hosea *et al.* 2004) or in energy intake *per se* is responsible for altered immune cell counts and function. Energy restriction and fasting may 'directly affect' immune cell metabolism, protein synthesis, cell replication and antioxidant defences through reduced substrate availability (Chandra 1997). Alternatively, decreases in substrate availability might have an 'indirect effect' on altered immune function through stimulatory effects on sympatho-adrenal activity resulting in increased secretions of stress hormones, for example, cortisol and adrenaline are known to modulate immune function (Gleeson *et al.* 2004). A role for raised circulating cortisol has been proposed to account for decreased circulating T-lymphocyte populations during starvation (Mustafa *et al.* 1997) and low lymphoid organ cell numbers in rats during energy restriction (Fraker *et al.* 2000). Indeed, decreased circulating $CD8^+$ and pre-T cells in the thymus of zinc deficient mice has been attributed to greater apoptosis due to elevated circulating cortisol (King *et al.* 2002). Lymphocyte IL-2 (Savendahl and Underwood 1997), the presence of newly produced lymphocytes (Kramer *et al.* 1997) and hemoconcentration due to fasting have been speculated to have differential effects on lymphocyte responses during energy restriction, fasting and refeeding. Lymphocyte IL-2 release in response to bacterially-stimulation was lowered following a 7-day fast in humans; this reduction accompanied a lowered circulating lymphocyte and $CD4^+$ cell counts (Savendahl and Underwood 1997). IL-2 is known to enhance a number of immune functions including NK and $CD8^+$ cell cytotoxicity. It is evident that the mechanism(s) responsible for decreased

circulating neutrophil, lymphocytes and T-lymphocyte subsets and the time course of recovery upon refeeding remains unclear.

Restricting fluid intake during exercise augments the plasma cortisol response to exercise (McGregor *et al.* 1999), and exercise performed in a hypohydrated state is associated with a greater stress hormone responses compared with exercising when euhydrated (Francesconi and Hubbard 1985). More recently, elevated plasma cortisol has been observed during dehydration in ruminants (Parker *et al.* 2003). As such, a role for hypohydration in the observed decrease in immune function during food and fluid restriction is likely and warrants investigation.

2.7 Environmental effects on circulating leukocyte and neutrophil counts at rest and after exercise

Athletes, military personnel and fire fighters regularly undertake vigorous activity in hot conditions, often when wearing protective clothing that limits evaporative heat loss, resulting in core temperatures often in excess of 40 °C (Roberts, 1989). Increases in body temperature that accompany fever increase the hypothalamic temperature set point. During passive heat exposure, or vigorous physical activity the hypothalamic temperature set point remains the same but problems with heat dissipation cause the body temperature to rise (Shephard 1998). Studies in which passive heating has been used in an attempt to isolate immunological effects of hyperthermia have demonstrated a pattern of leukocyte subset redistribution similar to that observed with exercise (Kappel *et al.* 1991a; Kappel *et al.* 1994; Kappel *et al.* 1995). Increases in circulating neutrophil cell count were observed in healthy subjects during and after 2 h immersion in a water bath at 39.5 °C until rectal temperature (T_{re}) reached 39.5 °C (Kappel *et al.*

1991a). During this study it was observed that the magnitude of neutrophilia was proportional to the rise in T_{re} from 37 to 39 °C. Support for these findings comes from passive heat exposure in air and water which evoked a substantial leukocytosis similar to that observed with exercise (Brenner *et al.* 1994). In this study Brenner and colleagues noted a T_{re} threshold of approximately 38 °C for such responses to occur. Immersion in a water bath at 39.5 °C for 2 h is substantial enough to increase T_{re} temperature similar to rigorous exercise in thermoneutral conditions (T_{re}: 39.5 °C). Although the observed changes in circulating immune cell counts were smaller in magnitude than exercise suggesting that hyperthermia alone can not account for all of the increase in circulating neutrophils during exercise (Kappel *et al.* 1991a; Kappel *et al.* 1994; Kappel *et al.* 1995).

Prolonged exercise in the heat elicits a more pronounced increase in core body temperature, increased cardiovascular drift, leads to larger increments of circulating stress hormones, causes a shift in substrate metabolism towards a greater reliance on carbohydrates and can have detrimental effects on performance (Brenner *et al.* 1997; Galloway and Maughan 1997; Maughan 1997; Febbraio 2001; Mitchell *et al.* 2002). Exercise with the addition of thermal stress also appears to amplify the immune response (Brenner *et al.* 1998). Running at 75 % VO_2 max for 1 h in hot air (28 °C) compared with thermoneutral (18 °C) conditions, (final T_{re} in hot 39.8 and thermoneutral 38.7 °C respectively) evoked a greater neutrophilia 3 h into recovery (Niess *et al.* 2003). Severs *et al.* (1996) had subjects cycle at 50 % VO_2 max for two 30 min bouts with 45 min rest between in air temperatures of 40 °C compared with 18 °C. The authors observed a larger rise in circulating neutrophil cell counts when exercise was performed under a greater thermal stress. More recently, 75 min of cycling at 55 % VO_2 peak in recreationally active males evoked a larger increase in circulating

leukocytes and neutrophil cell counts at post and 2 h post-exercise after exercise was performed in hot (38 °C) compared to thermoneutral conditions (22 °C) where final T_{re} reached approximately 38.7 and 37.7 °C, respectively (Mitchell *et al.* 2002).

Exercising in cold water (thermal clamp) allows the independent contributions of T_{re} and the physical mechanics of exercise to be separated. Cold water aids heat dissipation during exercise resulting in maintenance of resting T_{re}. Cross *et al.* (1996) performed such a study with 4 trials using a well-grounded cycle ergometer modified to include a positive-pressure water seal for use in a large water bath. Each water immersion trial was 80 min in duration. Two water immersion trials consisting of 40 min cycling at 65 % aerobic power (remaining immersed for a further 40 min) and two consisted of being seated immersed in water assuming a similar posture to the exercising trials. Cold exercise (thermal clamp) and cold seated trials were performed in 23 °C water while hot exercise and hot seated trials were performed in 39 °C water. Exercise performed with a rise in T_{tr} (hot exercise) evoked an increase in circulating neutrophil cell counts, whilst exercise performed with a thermal clamp elicited a slower rise over the immersion duration. The authors denote this rise to a slight increase in rectal temperature (T_{re}). Both hot and cold seated trials produced no change in cell counts, which is in contrast with previous literature, however the hot seated trial did not produce the T_{re} threshold temperature of ~38 °C proposed by Brenner *et al.* (1994). A similar investigation was conducted by Rhind *et al.* (1999) whereby subjects cycled for 40 min at 65 % VO_2 max whilst submerged to chest high water of 39 °C or 18 °C. Again exercise in cold water clamped the rise in T_{re} and decreased the leukocytosis observed with exercise. This study indicated that as much as half of the exercise-induced leukocytosis was attributed to the rise in T_{re}.

2.7.1 Environmental effects on circulating lymphocyte and T-lymphocyte subset counts

Lymphocyte cell counts (predominantly NK cells) increased following 2 h passive immersion in 39.5 °C water which evoked a rise in T_{re} to 39.5 °C (Kappel *et al.* 1991a; Kappel *et al.* 1994; Kappel *et al.* 1995). However, 4 h of passive heating in a thermal chamber at 40 °C only evoked a modest increase in T_{re} of 0.7 °C and did not alter NK cell counts (Severs *et al.* 1996). Total circulating lymphocyte, $CD4^+$ and $CD8^+$ cell counts were slightly elevated during two 30 min cycle bouts (separated by 45 min) in hot conditions (40 °C) in a climatic chamber. Circulating $CD4^+$ and $CD8^+$ cell counts peaked towards the end of exercise and fell post-exercise (Severs *et al* 1996). Short duration (30 min) cycling at 50 % VO_2 max, performed in thermoneutral (23 °C) and hot (40 °C) conditions, not only cause increases in T_{re} of 0.9 and 1.6 °C after thermoneutral and hot conditions, respectively but also increased circulating lymphocyte cell counts in both condition (Severs *et al* 1996). Generally, in hot conditions with large elevations in T_{re}, large increases in circulating lymphocyte, $CD3^+$, $CD4^+$ and $CD8^+$ cell counts have been observed. Cycling for 75 min at 55 % VO_2 peak evoked a larger increase in circulating lymphocyte, $CD3^+$, $CD4^+$, $CD8^+$ and NK cells counts at post-immersion after exercise was performed in hot air (38 °C) compared with thermoneutral conditions (22 °C) (Mitchell *et al.* 2002). Interestingly these observations were made despite the moderate exercise intensity and modest increases in T_{re}. Rhind *et al.,* (1999) had subjects exercise at 65 % peak oxygen consumption for 40 min in 39 °C or 18 °C water. Thermal clamping substantially reduced the exercise-induced increments in T_{re}, circulating lymphocyte, $CD3^+$, $CD4^+$, $CD8^+$ and NK cells counts at post-immersion. In a similar study Cross *et al.,* (1996) observed elevated lymphocyte counts in hot exercise conditions which were substantially higher than cold exercise from 5 min into the exercise bout.

2.7.2 Mechanisms of environmental stress and thermal clamping with and without exercise on circulating neutrophil, lymphocyte and T-lymphocyte subset counts

Authors believe that there could be a direct effect of raised temperature (heat dissipation, cardiovascular drift, dehydration) and/or an indirect effect of altered circulating stress hormones and cytokines that may alter immune function during heat stress and exercise with additional heat stress. Indeed, exercise performed with a thermal stress causes larger increases in circulating neutrophil cell counts which has been attributed to post-exercise elevations in circulating stress hormones (Niess *et al.* 2003). Specifically, running at 75% $\dot{V}O_{2max}$ for 1 h in hot air (28 °C: final T_{re} 39.8 °C) compared with thermoneutral (18°C: final T_{re} 38.7 °C) conditions led to larger increases in circulating noradrenaline, cortisol, GH and neutrophil cell counts and lower unstimulated MPO release per neutrophil (Niess *et al.* 2003). A suppressive effect of the addition of heat stress with exercise might account for lower unstimulated MPO release per neutrophil in this study. Indeed, elevations in circulating leukocyte and neutrophil counts similar to exercising levels have been observed after infusion of adrenaline (Kappel *et al.* 1991b), cortisol (Tonnesen *et al.* 1987) and GH (Kappel *et al.* 1993). In patients with solid tumors, whole body hyperthermia (41.0-41.8 °C for 60 min) increased GH, ACTH and cortisol as well as TNF-α, IL-6 and IL-12R. In addition, during the latter phases of heat exposure, increased plasma concentrations of IL-8, GH and cortisol correlated with an influx of neutrophils into the peripheral circulation (Atanackovic *et al.*, 2006).

More recent studies have also indicated a likely role for G-CSF in the mobilisation of neutrophils into the circulation following sustained hyperthermia in rats (Ellis *et al.* 2005), and brief incremental exercise to exhaustion in humans (Yamada *et al.* 2002). In

addition, IL-6 concentration 1 h after exercise correlated more strongly than cortisol with circulating neutrophil counts 2 h after exercise: also indicating a possible role for IL-6 in the delayed neutrophilia of exercise (Yamada *et al.* 2002). Unfortunately discrepancies between these studies could be due to the use of short duration exercise lasting ~10 min (Yamada *et al.* 2002) compared to 40 min (Cross *et al.* 1996; Rhind *et al.* 1999) or were conducted in animals (Ellis *et al.* 2005). Exercising in cold water (thermal clamp), albeit lasting only 40 min, substantially blunted the rise in circulating catecholamines, cortisol and GH, and was associated with smaller increases in circulating leukocytes compared with thermoneutral conditions (Cross *et al.* 1996; Rhind *et al.* 1999). Moderate (55% $\dot{V}O_{2\ peak}$) exercise performed in a hot environment was associated with moderate elevations in cortisol concentrations (~61 % and ~22 % in the euhydrate and dehydrated hot respectively). These cortisol responses could also be partly attributed to the administration of a carbohydrate/electrolyte beverage and paste during the trial (Mitchell *et al.* 2002). Interestingly, subjects completed the exercise bouts in hot and thermoneutral conditions once with adequate fluid intake and once with no fluid intake (dehydrated), and showed that dehydration had little effect on leukocyte counts after exercise. These results suggest a much greater impact of heat stress than hydration status on leukocyte trafficking after exercise.

2.8 Thesis Objectives

With this information in mind the objectives of the following experiments in this thesis were to:

I Clarify the acute and chronic effects of an intensive 20-week multi-stressor training period on URTI incidence, circulating leukocyte and lymphocyte subset counts, the $CD4^+/CD8^+$ ratio and bacterially-stimulated neutrophil degranulation.

II Determine the effects of a 48 h period of fluid, energy or combined fluid and energy restriction on circulating leukocyte and lymphocyte subset counts and bacterially-stimulated neutrophil degranulation at rest and after exercise.

III Compare the effects of prolonged exercise in thermoneutral and warm conditions on circulating leukocyte and lymphocyte subset counts, the $CD4^+/CD8^+$ ratio and bacterially-stimulated neutrophil degranulation.

IV Use a water-immersion technique to delineate the thermal effects and associated hormone and cytokine involvement in neutrophil trafficking and bacterially-stimulated neutrophil degranulation responses after prolonged exercise.

Chapter Three

General Methods

3.1 Ethical Approval

Ethical approval was obtained for all experimental chapters from Bangor University School of Sport, Health and Exercise Sciences Ethics Committee (Chapter 4, 5 and 6) and from the North West Wales Local Research Ethics Committee (Chapter 7). All participants received verbal and written instructions detailing their involvement during the experimental periods and the objectives of each research study (Appendix A). Participants gave written informed consent and were informed of their right to withdraw at any point, without question or being asked to provide a reason. To determine eligibility for inclusion into a research study, participants completed a medical and health questionnaire, a pre-experimental questionnaire (Appendix B) and reported no incidences of URTI in the 6-weeks prior to and during each study. During all experimental periods participants were required to complete a daily self-report health log for the identification of any URTI, cold and/or flu symptoms (Appendix C, Nieman *et al.*, 1998).

3.2 Determination of maximal oxygen uptake ($\dot{V}O_{2\ max}$)

Prior to the main experimental trials (Chapter 5, 6 and 7) and training (Chapter 4) an incremental exercise test to volitional exhaustion for the determination of maximal oxygen uptake ($\dot{V}O_{2\ max}$) was completed by each participant. For all laboratory based $\dot{V}O_{2\ max}$ test protocols the BASES criteria for the attainment of maximal oxygen uptake were adopted; criteria for attaining $\dot{V}O_{2\ max}$ included reaching volitional exhaustion, a heart rate (HR) within ten beats·min^{-1} of age predicted HR_{max} and a respiratory exchange ratio $\geq$1.15 (Bird and Davison 1997).

3.2.1 Multi stage fitness test

In chapter 4 maximal oxygen uptake ($\dot{V}O_{2\,max}$) was estimated using the 20 m multi stage fitness test (MSFT; (Leger and Lambert 1982). Following a 5 min warm up of light jogging each participant lined up at the end of a 20 m shuttle run course. The test began at a running speed of 8.5 $km \cdot h^{-1}$ and increases by 0.5 $km \cdot h^{-1}$ each level. The completion of each shuttle was indicated by a single bleep. Each participant was instructed to place one foot on or beyond the 20 m marker at the end of each shuttle. The participants were also instructed to keep running for as long as possible until they could no longer keep up with the speed set by the test. Participants that failed to reach the end of the shuttle before the beep were allowed 2 further shuttles to attempt to regain the required pace before being withdrawn from the test. The participants $\dot{V}O_{2\,max}$ was then determined from the MSFT table using the level and shuttle achieved (Appendix D). The test was performed at week 0 and 10 for Parachute Regiment Recruits (PARA) and week 0 and 20 for CONTROL. Unfortunately, due to PARA Company (P. Company) assessments at week 20 PARA were not permitted to perform the MSFT. The Ministry of Defence only informed us of these restrictions during week 19, hence a MSFT was not conducted in CONTROL at week 10.

3.2.2 Treadmill ergometry

In chapter 5 and 7 the incremental exercise tests to volitional exhaustion were conducted on a treadmill (Powerjog, Sports engineering Ltd, Birmingham, UK). Participants completed a short warm-up period, after which initial increments in speed were implemented every three min. Thereafter the gradient was increased by 2.5 % grade every three min. The exact incremental exercise test protocols conducted in chapter 5 and 7 are found in Table 3.1. Rate of oxygen uptake ($\dot{V}O_2$), rate of carbon dioxide output ($\dot{V}CO_2$) and expired minute ventilation ($\dot{V}E$) were measured continuously using an on-line gas analysis system (Cortex

Metalyser 3B; Leipzig, Germany). The on-line gas analysis system was calibrated according to the manufacturer's instructions. Briefly, the Cortex Metalyser 3B system was switched on and warmed up for 3 min prior to calibration. The three parameters calibrated prior to every test were pressure, gas (two point calibration with ambient air and reference gas) and volume (using a 3 litre syringe at pre-determined speeds set by the calibration software). Immediately before commencing data collection on the on-line gas analysis system a final ambient air calibration was performed. HR was measured continuously using wireless chest strap radio telemetry (Polar, Electro Kempele, Finland) and Rating of Perceived Exertion (RPE) using Borg 6-20 scale (Borg 1998) were recorded in the final 1 min of each incremental stage.

Table 3.1 Incremental exercise test protocols for treadmill ergometry (Bird and Davison, 1997).

	Chapter 5	Chapter 7
Warm up	3 min at 6 $km.h^{-1}$ and 0 % grade	5 min at 9 $km.h^{-1}$ and 0 % grade
Stage one	3 min at 9.6 $km.h^{-1}$ and 0 % grade	3 min at 10 $km.h^{-1}$ and 1 % grade
Stage two	3 min at 11.3 $km.h^{-1}$ and 0 % grade	3 min at 12 $km.h^{-1}$ and 1 % grade
Stage three	3 min at 11.3 $km.h^{-1}$ and 2.5 % grade	3 min at 14 $km.h^{-1}$ and 1 % grade
Stage four	3 min at 11.3 $km.h^{-1}$ and 5 % grade	3 min at 14 $km.h^{-1}$ and 3.5 % grade
Stage five*	3 min at 11.3 $km.h^{-1}$ and 7.5 % grade	3 min at 14 $km.h^{-1}$ and 6 % grade

* If further stages were required increments of 2.5 % gradient were made every 3 min

3.2.3 Cycle ergometry

In chapter 6 the incremental exercise test was conducted on a cycle ergometer (Monark 814e, Monark exercise, Sweden). Following a 5 min warm up at 70 W participants began cycling at 130 W, with increments of 35 W every 3 min. Expired air samples were

measured continuously using an online gas analysis system (Cortex Metalyser 3B, Leipzig, Germany). $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E$, HR and RPE were measured and recorded similar to the treadmill ergometry test.

3.3 Blood sampling and handling

Participants were seated for ten min prior to the collection of all resting blood samples, whilst post-exercise blood samples were collected in a seated position immediately upon cessation of exercise. Blood samples were collected, without venostasis by venepuncture from an antecubital vein, into four separate vacutainer tubes (Becton Dickinson, Oxford, UK). Two 4 ml vacutainers containing tripotassium ethylenediaminetetraacetic acid (K_3EDTA; 1.6 mg EDTA·mL^{-1} blood), one 6 ml vacutainer containing lithium heparin (1.5 IU heparin·mL^{-1} blood) and one 5 ml vacutainer containing serum separation gel (SST II^{tm}). Blood taken from one K_3EDTA tube and one lithium heparin tube were centrifuged at 3000 rpm for 10 min and the plasma aliquotted into snap-seal microcentrifuge tubes (1.5mL capacity: Sarstedt, Germany) and stored at -80°C. Blood collected in the second K_3EDTA tube was stored at room temperature prior to haematological analysis. Blood in the plain SST II^{tm} tube was left to clot at room temperature for 1 hour and then centrifuged (1500 g for 10 min at 5°C). Serum was removed from the tube and aliquotted into snap-seal microcentrifuge tubes (1.5mL capacity: Sarstedt, Germany) and stored at -80°C.

3.3.1 Haematological analysis

All automated haematological analysis was conducted at Ysbyty Gwynedd Haematology Department within 6 h of collection. Haematological analyses including total and differential leukocyte counts, Hb and Hct (automated Hct analysis for Chapter 4, 6 and 7)

were performed using an automated cell counter (Gen-S, Beckman Coulter, Fullerton, USA). Manual Hct analysis was performed at the School of Sport, Health, and Exercise Sciences (Chapter 5). Three plain glass capillary tubes (80 iu/ml) were filled with heparinised whole blood, plugged at one end using Cristaseal and placed onto the microhaematocrit rotor. The glass capillary tubes were spun for two min at 13000 g, removed, and read using microhaematocrit reader (Hawksley & sons Ltd, W. Sussex, England). The measured length of the column of packed erythrocytes was compared to that of the total column of blood in the tube. The average of all three Hct readings was then used in conjunction with the Hb values from the coulter counter to estimate plasma volume changes in accordance with Dill and Costill (1974).

3.3.2 Bacterially-Stimulated Neutrophil Degranulation

A 1.0 mL aliquot of fresh heparinised whole blood was immediately added to a snap-seal microcentrifuge tube containing 50 μL of bacterial stimulant solution (Stimulant, Sigma, Poole, UK) as described previously (Robson *et al.* 1999). The blood and bacterial stimulant were gently mixed on a rotamixer (Hook and Tucker, Croydon, UK) and incubated for 1 h at 37 °C, with a gentle mix after 30 min. After incubation, the tube was centrifuged for 2 min at 5000 g and the supernatant immediately removed and stored at -80 °C. Plasma elastase concentration in stimulated and unstimulated samples was assessed with bacterial stimulant using sandwich-type enzyme-linked immunosorbent assay (ELISA) kits specific for elastase (Merck, Lutterworth, UK; Chapter 4, Biovendor, Heidelberg, Germany; Chapter 5 and 7; Oncogene, San Diego, USA; Chapter 6). The intra-assay CV for each ELISA kit can be found in Table 3.2. The elastase released from bacterially-stimulated neutrophils was determined according to Blannin *et al.* (1996).

3.3.3 Flow Cytometry

All flow cytometry was performed at Ysbyty Gwynedd and was conducted by either the chief or lead biomedical scientists. Standard flow cytometric techniques were used to determine T-lymphocytes ($CD3^+$), helper/inducer T-lymphocytes ($CD4^+$) and cytotoxic/suppressor T-lymphocytes ($CD8^+$) using the tetraCHROME^{TM} murine monoclonal antibody reagent (Beckman Coulter Ltd, UK), neutrophil expression of CD11b and lymphocyte NK cells.

For $CD3^+$, $CD4^+$ and $CD8^+$ a 100 µl volume of whole blood from the K_3EDTA tube was immediately added to a tube containing 10 µl reagent (FITC (anti CD45), PC5 (anti CD3), RD1 (anti CD4) and ECD (anti CD8)). The tube was incubated in the dark at room temperature for 15 min and then lysed using a T-Q prep (Beckman Coulter, Ltd, UK). The stained cell suspension was enumerated on an Epics XL flow cytometer (Beckman Coulter Ltd, UK) with 10, 000 events recorded. The lymphocyte population was determined by gating on CD45 versus side scatter. Counts for individual subsets ($CD3^+$, $CD4^+$, and $CD8^+$) were obtained by multiplying the corresponding percentages of cells derived from the Epics XL flow cytometer (by quadrant analysis of dot plots) by the total lymphocyte counts on the Gen S Coulter counter.

For CD11b a 100 µl volume of whole blood from the heparin tube was added to a tube containing 10µL of anti-human CD11b (PE; Beckman Coulter Ltd, UK). The neutrophil population was determined by gating on forward scatter versus side scatter. Non-specific staining was assessed using a PE negative control tube containing 10µL of negative mouse

immunoglobulin (IgG1: Beckman Coulter Ltd, UK). The stained cell suspensions was enumerated on an Epics XL flow cytometer (Beckman Coulter Ltd, UK) with 10, 000 events recorded. Neutrophil expression of CD11b is presented as both percentage and mean fluorescence intensity (MFI).

NK cell counts were carried out with monoclonal antibodies against $CD3^-/CD16^+$ and $CD3^-/CD56^+$ in single platform tests (Beckman Coulter Ltd, UK). 100 µl volume of whole blood from the K_3EDTA tube was immediately added to one tube containing 10 µl reagent (FITC (anti CD45), PC5 (anti CD3), ECD (anti CD16)) and one tube containing 10 µl reagent (FITC (anti CD45), PC5 (anti CD3), RD1 (anti CD56)). The tubes were incubated in the dark at room temperature for 15 min and then lysed using a T-Q prep (Beckman Coulter, Ltd, UK). The stained cell suspensions were enumerated on an Epics XL flow cytometer (Beckman Coulter Ltd, UK) with 10, 000 events recorded. The lymphocyte population was determined by gating on CD45 versus side scatter. NK cell counts were obtained by multiplying the corresponding percentages of cells derived from the Epics XL flow cytometer ($CD3^-/CD16^+$ and $CD3^-/CD56^+$) by the total lymphocyte counts on the Gen S Coulter counter.

Instrument calibration used flow-check fluorospheres for daily verification of the flow cytometer's optical alignment and fluidics system and flow-set fluorospheres for weekly standardisation of the light scatter and fluorescence intensity (Beckman Coulter, Ltd, UK). Digitalized data were acquired using auto gating and analysed on a NEC Multisync FE700+ Computer system by using Coulter System II^{tm} version 3.0 Software packages. Analysis of 10000 events from the Epics XL flow cytometer (Beckman Coulter Ltd, UK) was used as

standard throughout all analysis and was in accordance with other authors (Van Eeden *et al.*, 1999; Simpson *et al.*, 2007). Some authors have reported results with as low as 8000 events (Hong *et al.*, 2005).

3.3.4 Blood films for determination of neutrophil lobularity

Blood films were prepared using freshly drawn K_3EDTA blood and stained using the Romanowsky stain method. The blood films were fixed in methanol for 10 min, then placed in May Grunwald (BDH, Lutterworth, UK) for 5 min (which had been diluted 1:1 in Sorensons buffer (BDH, Lutterworth, UK). The slides where then placed in Giemsa (BDH, Lutterworth, UK) diluted 1:10 with Sorensons buffer for 15 min then washed in Sorensons buffer for 5 min and air dried. The number of band (non-segmented) neutrophils was determined on a count of 100 neutrophils by an experienced hematologist using the Cooke Arneth technique on a light microscope (Light Labortux 12, Leitz, Germany). The absolute number of each cell type was calculated from the total neutrophil count from the Gen-S Coulter counter and the band to total neutrophil ratio was calculated by dividing the absolute band count by the absolute neutrophil count.

3.3.5 Stress hormones, catecholamines and blood borne metabolites

Concentrations of cortisol (serum; Chapter 4, heparinised plasma; Chapter 5, 6 and 7) were determined using ELISA kits (DRG Diagnostics, Marburg, Germany). Adrenaline and noradrenaline were determined on K_3EDTA plasma using ELISA kits (Labor Diagnostica Nord, Nordhorn, Germany; Chapter 6) and HPLC (Chapter 7) method as described previously (this method did not include the addition of an antioxidant or preservative to the sample; Chudalla *et al.* 2006). The HPLC analysis was conducted by Sports and Exercise

Physiology, Medical School Hannover (Hannover Germany). Heparinised plasma was used to determine concentrations of glucose using spectrophotometric kits (GOD-PAP method, Randox, County Antrim, UK; Chapter 5; hexokinase method, Sigma, Pool, UK; Chapter 6), and G-CSF (Chapter 7) and GH (Chapter 6 and 7) using ELISA kits (DRG Diagnostics, Marburg, Germany). K_3EDTA plasma was used to determine concentrations of FFA also using ELISA kits (Randox, County Antrim, UK; Chapter 5). Finally, serum was used to determine concentrations of IL-6 (R & D Systems, Oxford, UK; Chapter 7) and prolactin (R & D Systems, Oxford, UK; Chapter 6) using ELISA methods. For each assay, all subject samples were analysed on the same day and with standards on each plate. The intra-assay co-efficient of variation (CV) for each assay can be found in Table 3.2.

Table 3.2 Intra-assay coefficient of variation (%) determined from blood samples from each research chapter for the determination of elastase, cortisol, adrenaline, noradrenaline, glucose, G-CSF, GH, FFA, IL-6 and prolactin concentrations.

	Units	Chapter 4	Chapter 5	Chapter 6	Chapter 7
Elastase	$\mu g \cdot L^{-1}$	6.3	3.0	6.3	3.0
Cortisol	$nmol \cdot L^{-1}$	3.1	3.1	4.0	2.3
adrenaline	$nmol \cdot L^{-1}$	...	...	6.9	7.8
Noradrenaline	$nmol \cdot L^{-1}$	...	...	9.8	11.0
Glucose	$mmol \cdot L^{-1}$	...	6.0	4.0	...
G-CSF	$ng \cdot L^{-1}$	...	...	4.0	2.8
GH	$pmol \cdot L^{-1}$	...	...	3.6	2.4
FFA	$mmol \cdot L^{-1}$	...	7.0	...	...
IL-6	$pg \cdot mL^{-1}$	...	...	...	3.3
Prolactin	$pmol \cdot L^{-1}$	...	...	4.3	...

3.4. Statistical Analysis

Data in text, tables and figures are presented as mean values ± SEM in Chapter 4-7 unless otherwise stated. Data examined using repeated measures ANOVA design followed assumptions of homogeneity and sphericity and, where appropriate, adjustments in the degrees of freedom were made using the Greenhouse-Geisser correction method. Significant differences were analysed using post hoc Tukey's HSD test and Bonferroni adjusted *t*-tests where appropriate. The statistical package for social scientists (SPSS version 10.1) was used for statistical analysis with statistical significance accepted at $P < 0.05$.

CHAPTER 4

The Influence of an Arduous Military Training Programme on Immune Function and Upper Respiratory Tract Infection Incidence

4.1 Abstract

The aim was to determine the effects of a 20-week military training programme on upper respiratory tract infection (URTI) incidence, circulating leukocyte and lymphocyte subset counts ($CD3^+$, $CD4^+$ and $CD8^+$) and bacterially-stimulated neutrophil degranulation responses. Fourteen Parachute Regiment recruits (PARA) and 12 recreationally active controls (CONTROL) who were not undertaking training were recruited. At 2-weekly intervals during training, and before and after Easter leave (weeks 11 and 12), venous blood samples were collected at between 0500-0700 h following an overnight fast in PARA and CONTROL. On a daily basis incidence and severity of URTI were recorded. There were no significant differences between groups for the average number or duration of URTI during the 20-weeks (total number of URTI incidence: PARA; 57 and CONTROL; 56). A significant increase in URTI incidence, typical for the time of year (early-mid February), occurred at weeks 2 and 3 for PARA and week 4 for CONTROL compared with week 0 ($P < 0.05$). Circulating leukocyte count decreased in PARA at weeks 2, 16 ($P < 0.05$) and 19 compared with week 0 ($P < 0.01$). The decrease in leukocyte count at week 2 in PARA coincides with peak incidence of URTI. Lymphocyte and $CD4^+$ counts decreased periodically throughout PARA training (weeks 2, 4, and 19, $P < 0.01$) compared with week 0. Decreased lymphocyte and $CD4^+$ count in PARA coincides with the peak incidence of URTI (weeks 2) and with the heaviest training load after P. Company (week 19). There were significant increases in neutrophil degranulation at weeks 4, 11, 12 and 16 for PARA compared with week 0 ($P < 0.01$). Significant decreases in neutrophil degranulation occurred in CONTROL at weeks 2, 4 and 16 compared with week 0 ($P < 0.01$). In conclusion these data show a limited effect of a 20 week Parachute Regiment training

programme on URTI incidence. Additionally, a 20 week Parachute Regiment training programme does not appear to depress neutrophil degranulation.

4.2 Introduction

Epidemiological studies have concluded that increased risk URTI during heavy training or following strenuous long duration events may be prevalent amongst athletes (Peters and Bateman 1983; Nieman *et al.* 1990a; Nieman 1994; Shephard 1997). While a single bout of prolonged exercise can cause temporary suppression in neutrophil degranulation, lymphocyte proliferation, natural killer (NK) cell number and activity and the ratio of $CD4^+/CD8^+$ cells (Robson *et al.* 1999; Nieman and Pedersen 1999), heavy training appears to produce more persistent changes in the cell distribution of the immune system (Smith *et al.* 1990; Baj *et al.* 1994; Kajiura *et al.* 1995; Blannin *et al.* 1996; Gleeson *et al.* 1999).

An impaired or depression of neutrophil function could possibly be an important contributing factor to the increased susceptibility of athletes to infection (Pyne 1994). Neutrophil oxidative burst and ROS production and neutrophil phagocytic activity has been shown to be lower at rest (Hack *et al* 1992; Pyne *et al.* 1995; Blannin *et al.* 1996) and after exercise (Smith *et al.* 1990; Hack *et al* 1992; Pyne *et al.* 1995) in trained runners, cyclists and swimmers compared with untrained individuals and athletes with a lower training load. Furthermore, lowered neutrophil oxidative burst activity has been shown after a 17-week period of high intensity training in horses (Raidal *et al.* 2000), a 12-week intensive training period in elite swimmers (Pyne *et al.* 1995) and 6-months of heavy training in young cyclists (Baj *et al.* 1994). Most authors have reported unchanged or slightly elevated lymphocyte proliferative responses during intense exercise training programmes (Baj *et al.*

1994; Mitchell *et al.* 1996). Single bouts of endurance exercise and intensified training periods have shown suppressed NK cell number and activity (Pedersen *et al.* 1989; Gleeson *et al.* 1995) and $CD4^+/CD8^+$ ratio (Lewicki *et al.* 1988; Kajiura *et al.* 1995). Light training over 12-weeks caused significant decreases in $CD3^+$ and $CD4^+$ cell counts with no change in the $CD4^+/CD8^+$ ratio (Shore *et al.* 1999). During a 6-month training period in young cyclists, lowered $CD3^+$ and $CD4^+$ absolute cell counts, no change in NK cell absolute counts but increased lymphocyte proliferation were observed (Baj *et al.* 1994).

There are few studies investigating the effects of military training on susceptibility to infection. Military training programmes are widely acknowledged to be rigorous with prolonged periods of heavy physical activity, negative energy balance, sleep deprivation, shifts in circadian rhythms, exposure to extremes of hot and cold environments, and severe psychological stressors. During an 11-month period of training at the U.S Naval Academy midshipmen experienced frequent episodes of URTI which were often reported as having an adverse effect on physical performance (Gray *et al.* 2001). However, during the course of basic infantry training lasting 18.5 weeks incidence of URTI remained unaltered (Brenner *et al.* 2000).

The effects of military training on immune responses are equivocal with large variability in duration (5-7 d, Boyum *et al.,* 1996; 4 weeks, Makras *et al.,* 2005; 8 weeks, Karpinski *et al.,* 2001 and Anomasiri *et al.,* 2002), intensity and physical/psychological stresses (calorie deficiency, sleep deprivation and continuous physical exercise, Boyum *et al.,* 1996; running, walking, gymnastics, loaded and un-loaded marches, Makras *et al.,* 2005; marches, crawling, drill and educational sessions, Karpinski *et al.,* 2001). As a result some

authors have reported increases in neutrophil chemotaxis (Boyum *et al.* 1996), numbers of $CD4^+$ T-lymphocytes (Makras *et al.* 2005) and numbers of $CD3^+$, $CD4^+$, $CD4^+/CD8^+$ ratio (Karpinski *et al.* 2001). Whilst others have reported decreases in NK cell counts (Fry *et al.* 1992), neutrophil cell count and neutrophil/lymphocyte ratio (Makras *et al.* 2005) and cellular immune responses, assessed using a tuberculin skin test (Anomasiri *et al.* 2002). In contrast, no alterations in circulating levels of $CD3^+$, $CD4^+$, and $CD8^+$ have also been observed (Anomasiri *et al.* 2002). To date military training studies have been investigated over a variety of periods ranging from <10 days to 8 weeks. The only chronic training study lasting 18.5 weeks had low physical demands causing no change in aerobic fitness throughout training (Brenner *et al.* 2000). With this information in mind, the purpose of this study was to investigate the acute and chronic effects of an intensive 20-week period of military training for Parachute Regiment recruits on URTI incidence, leukocyte counts, neutrophil degranulation and $CD4^+/CD8^+$ ratio. We hypothesised that an intensive 20-week period of military training for Parachute Regiment recruits would decrease circulating leukocyte counts, lymphocyte subset counts and neutrophil degranulation and that these decreases would coincide with reported incidence of URTI.

4.3 Methods

4.3.1 Participants

Fourteen Parachute Regiment recruits (PARA: mean ± SEM; age 22 ± 1 yr; height 179 ± 2 cm; body mass 76.8 ± 2.9 kg; body fat 17.6 ± 0.7 %; $\dot{V}O_{2\ max}$ 51.9 ± 0.1 $ml \cdot kg^{-1} \cdot min^{-1}$) and 12 recreationally active controls who were not undertaking the PARA training programme (CONTROL: mean ± SEM; age 22 ± 1 yr; height 178 ± 1 cm; body mass 78.2 ± 2.8 kg; body fat 17.0 ± 1.1 %; $\dot{V}O_{2\ max}$ 51.9 ± 1.2 $ml \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in this study. Ten of the CONTROL group were students and staff from within SSHES and 2 were inactive military recruits based at the Infantry Training Centre Catterick (ITCC). There were no reported symptoms of infection and none of the participants were taking any medication or nutritional supplements in the six weeks prior to the study. The absence of URTI at the start of the study was determined by means of a self-report questionnaire (Appendix C, Nieman 1998). The study commenced January 20th, 2003 and lasted for 21 weeks of the Combined Infantryman's Course for Parachute Regiment Recruits, which took place at the ITCC.

4.3.2 Combined Infantryman's Course for PARA Recruits

Parachute Regiment training is widely regarded as one of the most physically demanding courses in the British Army. First time pass-out rates average 40 %, suggesting a mismatch between the capability of the recruits and the requirements imposed by the course (Wilkinson *et al.* 2004). During the 20 weeks training (week 0-19) PARA undergo rifle lessons (stripping, assembling and cleaning), parade drill, physical training (steady state runs, agility, circuits and loaded march's), map reading and over-night exercises. After completing the first 6 weeks of training PARA were granted weekend leave for each week

thereafter until the end of the course. PARA were also given a 2 week period of Easter leave between weeks 11 and 12. Upon satisfactory completion of 20 weeks training, PARA underwent 5 days of 'P. Company' assessments which take place over arduous terrain, often in ambient temperatures >25 °C, including a series of tests: 10- and 20- mile march carrying a 35-lb bergan and weapon, assault-course, steeplechase, 1.8- mile log race in teams of six to nine, combat/self defence competition and 5 mile stretcher race in teams of twelve to sixteen. It is noteworthy that the results presented in this chapter are for 14 PARA recruits who successfully completed the training course. During PARA training there appeared to be no overall progression in both the levels of physiological stress (physical activity counts) and resultant cardiovascular strain (Wilkinson *et al* 2004).

4.3.3 Preliminary Measurements

At week 0, prior to commencing PARA training, measures of anthropometry (height, body mass, body density; (Durnin and Womersley 1974) and % body fat; (Siri, 1956) were taken in both groups, these measures were repeated at week 20 (completion of the study). Maximal oxygen uptake ($\dot{V}O_2$ max) was estimated, at week 0 and 10 for PARA and week 0 and 20 for CONTROL using the 20 m Multi Stage Fitness Test as described in Chapter 3 (Leger and Lambert 1982). Unfortunately, due to P. Company assessments at week 20 PARA were not allowed to perform the multi-stage fitness test. It was only upon completion of the study that PARA MSFT results for week 10 were made available to investigators from the physical training instructors at ITCC. By this time it was too late to test the CONTROL group mid-way through the study.

4.3.4 Experimental Procedures

On entry and at 2-weekly intervals throughout the first 20-weeks (weeks 0-19) of the training programme), both PARA and CONTROL provided a venous blood sample (19 ml) collected from the antecubital vein by a qualified Phlebotomist. Additional blood samples were collected before (week 11) and after (week 12) the Easter leave period, immediately after P. Company (week 19, Thursday) and 4 days after a recovery period during which no training took place (week 20, Monday). All blood samples were collected between 05:00 and 07:00 hours as detailed in Chapter 3. Participants were given a weekly health and sickness log (Nieman 1998) and asked to record daily incidence of health problems (e.g. 1 = no health problems today; 2 = cold symptoms today; 3 = flu symptoms today etc.) and the severity of these health problems (e.g. A = mild; B = Moderate; C = severe). A single occurrence of URTI was classified by self-reported ratings of 2a, 2b or 2c for 2 or more consecutive days (Appendix C).

4.3.5 Analytical Methods

Haematological parameters including Hb, Hct, plasma volume change, total and differential leukocyte counts, $CD3^+$, $CD4^+$, $CD8^+$ and NK ($CD16^+/CD56^+$) cell counts as described in Chapter 3. Bacterially-stimulated neutrophil degranulation was determined as described in Chapter 3. Plasma elastase and serum cortisol concentrations were measured as described in Chapter 3.

4.3.6 Statistical Analysis

The data were examined using a 2 (groups) x 11 (blood samples) repeated measures ANOVA. Assumptions and analysis described in Chapter 3. Mann-Whitney U and Friedman non parametric tests were used to determine the effects of training on URTI incidence.

4.4 Results

4.4.1 Anthropometry, fitness and plasma volume change

There was no significant changes in body mass for PARA and CONTROL across the 20-weeks. However, PARA training was associated with a 2.8 % decrease in body fat by week 20 (interaction: $F_{(1,17)} = 4.3$, $P < 0.01$) and an increase in predicted $\dot{V}O_2$ max by week 10 (interaction: $F_{(1,17)} = 4.9$, $P < 0.01$; Table 4.1). There was no change in body composition and fitness for CONTROL by week 20 (Table 4.1). There was no significant change in plasma volume for PARA and CONTROL across the 20- weeks compared with week 0. Plasma volume change in PARA fluctuated between -2.8 and 2.4 % between weeks 2 to10 and -2.9 and 3.2 % between weeks 11 to 18 compared with week 0, although not significant.

TABLE 4.1. The effects of 20-weeks PARA training on basic anthropometry and predicted $\dot{V}O_2$ max.

	Body Mass (kg) Week 0	Body Mass (kg) Week 20	Body Fat (%) Week 0	Body Fat (%) Week 20	$\dot{V}O_{2\,max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$) Week 0	$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$) Week 20
PARA	76.8 ± 2.9	77.8 ± 2.5	17.6 ± 0.7	14.8 ± 0.7**	51.9 ± 0.1	53.9 ± 0.1†**
CONTROL	78.2 ± 2.8	80.0 ± 2.9	17.0 ± 1.1	16.9 ± 0.9	51.9 ± 1.2	52.5 ± 1.2

Values are mean ± SEM. Significantly different than week 0; ** $P < 0.01$. Measurement taken at week 10; †

4.4.2 Weekly incidence of URTI

Non-parametric analysis revealed significant differences in weekly incidence of URTI during the military training programme. There was an increase in self reported incidence of URTI at weeks 2 and 3 for PARA and week 4 for CONTROL compared with week 0 ($P < 0.05$, Figure 4.1). At week 2, URTI incidences were greater in PARA compared with

CONTROL ($P < 0.05$; Figure 4.1). Both PARA and CONTROL reported a similar total number of URTI incidences (total number of URTI incidence: PARA; 57 and CONTROL; 56). There were no reported changes in URTI incidence during or after P. Company (weeks 18 and 19 respectively).

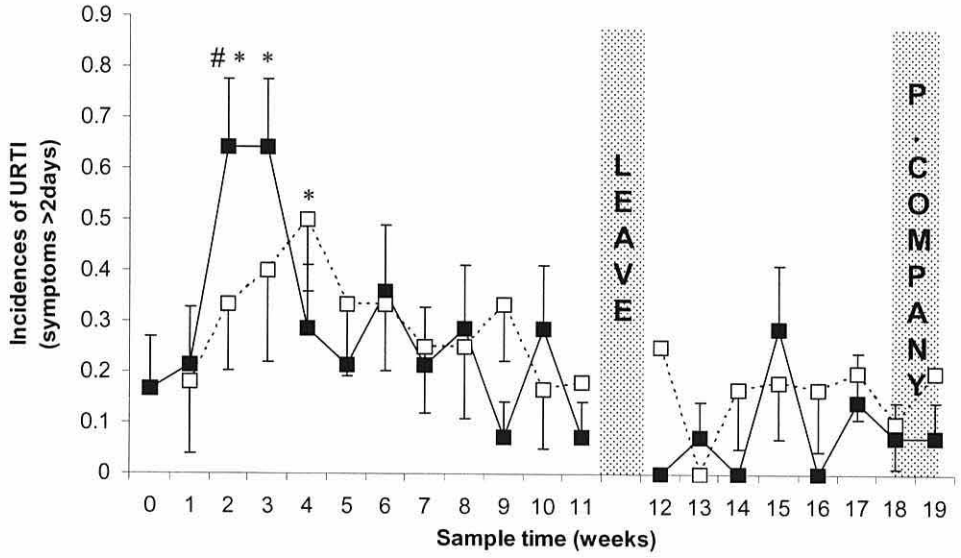

FIGURE 4.1. The effects of 20-weeks PARA training on weekly incidence of URTI. Values are mean ± SEM. (■) PARA, (□) CONTROL. Significantly higher than week 0; * $P < 0.05$, significantly higher than CONTROL; # $P < 0.05$, Leave; 2 week break in training for Easter leave

4.4.3 Leukocyte counts, lymphocyte counts and T-lymphocyte subsets

There was a significant group x time interaction observed for circulating leukocyte counts (interaction: $F_{(5,115)} = 2.6$, $P < 0.05$; Figure 4.2A), circulating lymphocyte counts (interaction: $F_{(10,210)} = 2.1$, $P < 0.05$; Figure 4.2B), T-lymphocyte $CD3^+$ (interaction: $F_{(6,123)} = 2.6$, $P < 0.05$; Figure 4.3A) and T-lymphocyte $CD4^+$ (interaction: $F_{(10,210)} = 2.2$, $P < 0.05$; Figure 4.3B). Leukocyte count decreased in PARA at weeks 2, 16 ($P < 0.05$) and 19 (post P. Company: $P < 0.01$) and increased on return from Easter leave (week 12: $P < 0.05$). The decrease in leukocyte count at week 2 in PARA coincides with peak incidence of URTI (Figure 4.1). Lymphocyte counts decreased at weeks 2, 4, and 19 during PARA training (P

< 0.01; Figure 4.2B) compared with week 0. Decreased lymphocyte counts in PARA coincides with the peak incidence of URTI (weeks 2: Figure 4.1) and with the heaviest training load after P. Company (week 19). Higher $CD4^+$ cell counts were observed at weeks 8, 10 ($P < 0.05$) and 12 ($P < 0.01$) in PARA compared with CONTROL. In addition, $CD3^+$ cell counts were significantly higher in PARA compared with CONTROL at week 12 ($P < 0.01$; Figure 4.3A). Throughout the 20-week study T-lymphocyte $CD8^+$, $CD4^+/CD8^+$ ratio and NK cell counts remained constant for both PARA and CONTROL (Figure 4.3C, D and 4.2C, $CD8^+$, $CD4^+/CD8^+$ ratio and NK cell counts, respectively).

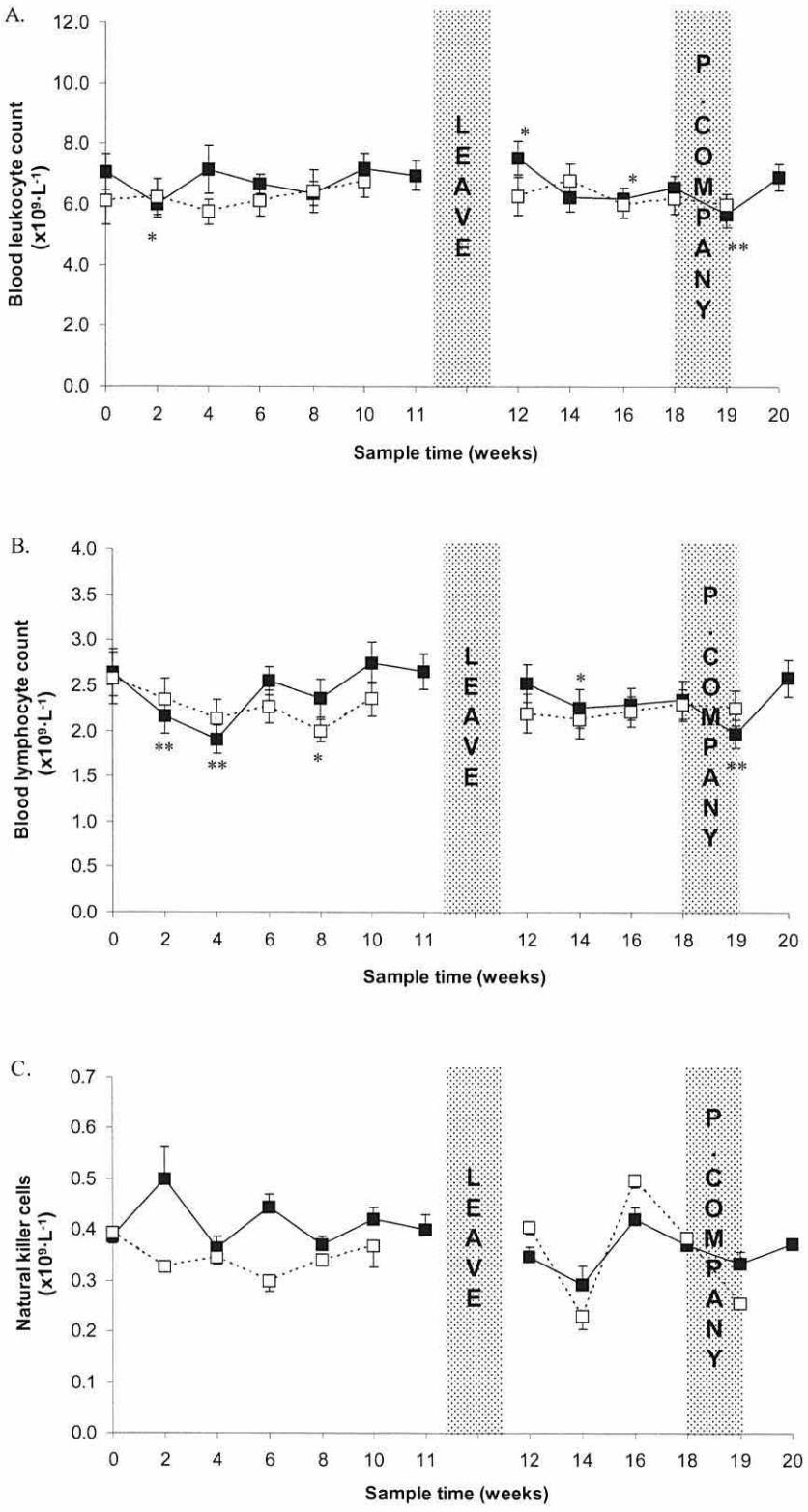

FIGURE 4.2. The effects of 20-weeks PARA training on resting (A) leukocyte counts, (B) lymphocyte counts and (C) NK cell counts. Values are mean ± SEM. (■) PARA, (□) CONTROL. Significantly different than week 0; * $P < 0.05$, ** $P < 0.01$, Leave; 2 week break in training for Easter leave, P. Company; period of physical assessments.

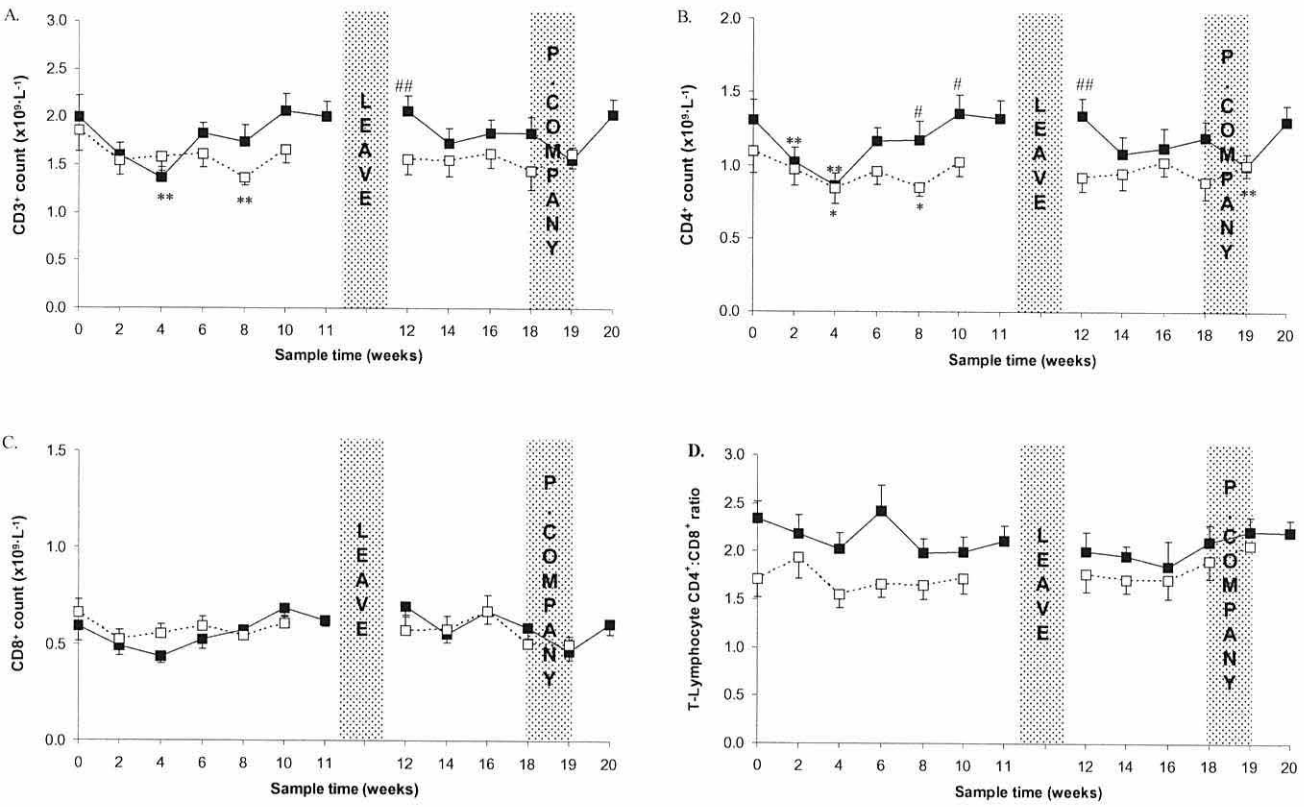

FIGURE 4.3. The effects of 20-weeks PARA training on resting (A) T-lymphocyte $CD3^+$ cell counts, (B) T-lymphocyte $CD4^+$ cell counts, (C) T-lymphocyte $CD8^+$ cell counts and (D) T-lymphocyte $CD4^+/CD8^+$ ratio. Values are mean ± SEM. (■) PARA, (□) CONTROL. Significantly different than week 0; * $P < 0.05$, ** $P < 0.01$, significantly higher than CONTROL; # $P < 0.05$, ## $P < 0.01$, Leave; 2 week break in training for Easter leave, P. Company; period of physical assessments.

4.4.4 Neutrophil Responses

There was a significant trial x time interaction observed for circulating neutrophil count (interaction: $F_{(10,210)} = 2.4$, $P < 0.05$, Table 4.2), unstimulated plasma elastase concentration (interaction: $F_{(5,97)} = 3.6$, $P < 0.05$, Table 4.2), and bacterially stimulated elastase release per cell (interaction: $F_{(10,210)} = 9.3$, $P < 0.01$, Table 4.2). There was a significant neutrophilia at weeks 10 and 14 for CONTROL compared with week 0 ($P < 0.05$, Table 4.2). However, neutrophil counts were greater at week 0 ($P < 0.05$) and week 4 ($P < 0.01$) in PARA compared with CONTROL (Table 4.2). Progressive increases in unstimulated elastase concentration reached significance at week 6 in PARA compared with week 0 ($P < 0.01$; Table 4.2), however, decreased below baseline levels from week 14 to 19 (weeks 14, 18 and 19 $P < 0.05$; and week 16 $P < 0.01$; Table 4.2). Bacterially stimulated elastase release per cell increased periodically throughout PARA training (weeks 4, 11, 12 and 16; $P < 0.01$; Table 4.2). At weeks 4, 12 and 16 the bacterially stimulated release elastase per cell was greater in PARA compared with CONTROL ($P < 0.01$; Table 4.2). Decreased bacterially-stimulated elastase release per cell was only observed in CONTROL (week 2, 4 and 16; $P < 0.01$; Table 4.2). Interestingly, the decreased bacterially stimulated elastase release per cell only coincides with peak incidence in URTI at week 4.

TABLE 4.2. The effects of 20-weeks PARA training on resting neutrophil count, plasma elastase concentration and bacterially-stimulated elastase release per neutrophil cell.

	Week No.						
	0	2	4	6	8	10	11
Neutrophil count ($x\ 10^9 \cdot L^{-1}$)							
PARA	3.6 ± 0.3$^{\#}$	3.0 ± 0.3	4.1 ± 0.5$^{\#\#}$	3.3 ± 0.2	3.2 ± 0.2	3.6 ± 0.3	3.4 ± 0.3
CONTROL	2.7 ± 0.5	3.0 ± 0.4	2.8 ± 0.2	3.1 ± 0.3	3.5 ± 0.7	3.4 ± 0.3**	...
Unstimulated elastase ($\mu g \cdot L^{-1}$)							
PARA	118 ± 26	131 ± 29	158 ± 48	167 ± 25**	93 ± 12	138 ± 37	95 ± 15
CONTROL	93 ± 28	99 ±14	240 ± 59	70 ± 12	75 ± 21	106 ± 25	...
Bacterially-stimulated elastase ($fg \cdot cell^{-1}$)							
PARA	121 ± 10	122 ± 20	193 ± 17$^{\#\#**}$	104 ± 12	138 ± 18	139 ± 19	184 ± 17**
CONTROL	126 ± 16	91 ± 17**	83 ± 11**	109 ± 14	148 ± 11	113 ± 16	...

	Week No.					
	12	14	16	18	19	20
Neutrophil count ($x\ 10^9 \cdot L^{-1}$)						
PARA	4.2 ± 0.4	3.2 ± 0.2	3.1 ± 0.2	3.4 ± 0.2	2.9 ± 0.3	3.5 ± 0.2
CONTROL	3.3 ± 0.4	3.8 ± 0.5**	3.1 ± 0.4	3.0 ± 0.4	2.8 ± 0.2	...
Unstimulated elastase ($\mu g \cdot L^{-1}$)						
PARA	82 ± 18	82 ± 10*	60 ± 8**	75 ± 12*	74 ± 10*	63 ± 10*
CONTROL	80 ± 16	80 ± 11	112 ± 57	79 ± 32	75 ± 11	
Bacterially-stimulated elastase ($fg \cdot cell^{-1}$)						
PARA	236 ± 25$^{\#\#**}$	146 ± 15	189 ± 22$^{\#\#**}$	130 ± 14	155 ± 11	97 ± 15
CONTROL	134 ± 17	134 ± 14	69 ± 14**	110 ± 21	158 ± 17	...

LEAVE

Values are mean ± SEM. Significantly different than week 0; * $P < 0.05$, ** $P < 0.01$, significantly higher than CONTROL; # $P < 0.05$, ## $P < 0$.

4.4.5 Serum Cortisol

There was a significant increase in serum cortisol concentration at week 4 for PARA compared with week 0 and CONTROL (interaction: $F_{(10,210)} = 4.3$, $P < 0.01$). Elevated serum cortisol concentration at week 4 in PARA ($P < 0.01$) coincided with increased neutrophil count and neutrophil degranulation (Figure 4.2A and 4.2C). Decreased serum cortisol concentrations were observed after a period of rest in PARA (week 12, return from 2-week Easter leave and week 20 after 4 days rest following P. Company; $P < 0.05$) and week 16 in CONTROL ($P < 0.01$; Figure 4.4).

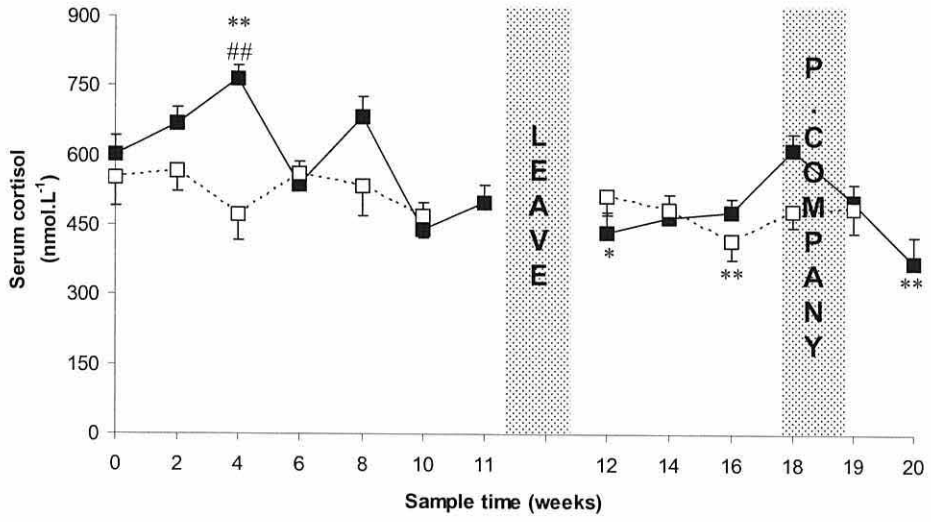

FIGURE 4.4. The effects of 20-weeks PARA training on serum cortisol concentration. Values are mean ± SEM. (■) PARA, (□) CONTROL. Significantly different than week 0; * $P < 0.05$, ** $P < 0.01$, significantly higher than CONTROL; ## $P < 0.01$, Leave; 2 week break in training for Easter leave, P. Company; period of physical assessments.

4.5 Discussion

The purpose of this study was to investigate the short-term and chronic effects of an intensive 20-week period of military training for Parachute Regiment recruits on URTI incidence and immune function. The results of the present investigation suggest a limited effect of PARA training on URTI incidence, circulating leukocyte counts, $CD4^+/CD8^+$ ratio, and neutrophil degranulation. Self-reported incidence of URTI increased at weeks 2 and 3 with no significant alterations in URTI after this point for PARA (Figure 4.1). These findings are in partial support of previous studies reporting no alterations in URTI after 18.5 weeks of basic training (Brenner *et al.* 2000), and in contrast with reports that very intense military training is associated with increased susceptibility to infectious illness (Linenger *et al.* 1993; Martinez-Lopez *et al.* 1993). Compared with basic training, PARA training is more physically demanding with PARA becoming leaner and increased their aerobic fitness (Table 4.1; Brenner *et al.* 2000). Increased reports of URTI within PARA on entry to training (Figure 4.1) might be attributed to living conditions, where recruits stay in compact shared accommodation (Edwards *et al.* 2003). Furthermore, seasonality could account for the findings in the present study as self reported incidence of URTI also increased during the winter at week 4 in CONTROL in addition to weeks 2 and 3 in PARA (Heikkinen and Jarvinen 2003).

During the initial weeks of training a significant decrease in leukocyte (week 2), lymphocyte (weeks 2 and 4), $CD3^+$ (week 4) and $CD4^+$ (weeks 2 and 4) cell counts was observed (Figures 4.3A, 4.3B, 4.4A and 4.4B, respectively). The decrease in these cell counts coincides with peak incidence of URTI in PARA (weeks 2-3) and significant elevations in serum cortisol concentrations (week 4). Any haemodilution effects upon leukocyte, lymphocyte , $CD3^+$ and $CD4^+$ cell counts is unlikely as there were no

significant plasma volume changes observed during the 20-week period. Haemodilution effects are typically observed when alteration in $\dot{V}O_2$ max accompanies endurance training (Shephard and Åstrand, 2000; Kargotich *et al.,* 1997); an observation made in the present study by week 10 in PARA predicted $\dot{V}O_2$ max scores but without the associated blood parameter changes. The decreased leukocyte, lymphocyte, $CD3^+$ and $CD4^+$ cell counts in the present study is consistent with previous training studies lasting 12-weeks (Shore *et al.* 1999) and 6 months (Baj *et al.* 1994). Precise mechanisms for changes in $CD3^+$ and $CD4^+$ cell counts in response to exercise training remain unclear. PARA training induced some changes in cortisol concentration at similar weeks to altered $CD3^+$ and $CD4^+$ cell counts. Interestingly, both the $CD4^+/CD8^+$ ratio and NK cell counts remained constant throughout the 20-week study. Evidence for alterations in these immune parameters during and after training remains equivocal. Previous training studies have reported unaltered $CD4^+/CD8^+$ ratio and NK cell counts after 12-weeks light training (Shore *et al.* 1999) and 6 months intense training in cyclists (Baj *et al.* 1994). Lowered NK cell counts were seen after several weeks of intensified training in competitive swimmers (Gedge *et al.* 1997; Gleeson 2000), after 3 weeks intense military training (Gomez-Merino *et al.* 2005), after sleep deprivation (Irwin *et al.* 1996) and prolonged psychological stress (Perna *et al.* 1997). An acute bout effect from exercise performed the previous day on leukocyte and lymphocyte counts during weeks 2 and 4 is unlikely, because PARA recruits undertook only light activities over the weekends (weeks 0-6) before sample collection on Monday morning. The reduced leukocyte, lymphocyte and $CD4^+$ cell counts (Figures 4.3A, 4.3B and 4.4B, respectively) observed the morning after P.Company (week 19) more than likely reflect acute exercise effects (Gabriel and Kindermann 1997). All cell counts returned to within baseline levels following 4 days of rest, supporting an effect of acute exercise rather than a chronic adaptation caused by training (Gabriel and Kindermann 1997).

Throughout the 20-week training programme no significant decreases in circulating neutrophil counts and bacterially-stimulated neutrophil degranulation were observed, indicating little effect of PARA training on neutrophil responses (Table 4.2). Instead, increased bacterially-stimulated neutrophil degranulation occurred periodically throughout training (weeks 4, 11, 12 and 16; Table 2). The increased neutrophil degranulation at week 4 in PARA coincides with peak serum cortisol concentrations and is approximately at the time of peak URTI incidence (weeks 2 and 3). Increased cortisol concentrations have been implicated in altered neutrophil responses (Robson *et al.* 1999), and increased neutrophil activation and function are known to occur with rhinovirus-induced colds (Douglas *et al.*, 1966; Winther *et al.* 1984; Levandowski *et al.* 1988). However, increased neutrophil degranulation at weeks 11, 12 and 16 can not be attributed to increased serum cortisol or URTI as these remained unaltered at these times (Figure 4.6 and 4.1, respectively). During weeks 0-6 PARA remained at ITCC and were permitted only light activities during the weekends prior to sample collection. Following week 6 PARA were granted weekend leave making it difficult to control PARA activity (i.e. alcohol intake, amount of sleep, energy intake). In part this could account for the variable results in neutrophil degranulation throughout this study.

Overall, the design of the present field study had several strengths. The inclusion of a non-training control group assured that where increases in URTI occurred at similar times in CONTROL and PARA, these were not incorrectly attributed to PARA training. Previous military training studies (Brenner *et al.* 2000; Gray *et al.* 2001; Anomasiri *et al.* 2002; Gomez-Merino *et al* 2005) where controls were omitted cannot identify any seasonal effects or results that may occur naturally without training. All blood samples were collected at the same time of day avoiding any diurnal effects and collected and

handled by the same researchers to avoid any experimenter variability. Several reason may account for why we did not observe the immunosuppression previously documented (Anomasiri *et al.* 2002; Makras *et al.* 2005). Although blood samples were collected on a Monday morning following an overnight fast, PARA had undergone 2 days without heavy enforced training. This time may have been adequate for any suppressed immune parameters to recover to within baseline (week 0) values. A delimitation of this study could be the geographical location of the control group; only 2 of the 12 were actually at ITCC and had similar living conditions. Finally, the validity of self-report health logs should be highlighted. Previous research which used physicians to determine the presence of URTI still used self-referral (Gray *et al.* 2001) and highlighted the potential for underestimated URTI symptoms with self-reports. Our recommendations would be to have physicians collect mouth swabs at weekly intervals to study the virology during training or at least verify symptoms of URTI lasting >2 days (as used by Bury *et al.,* 1998).

4.6 CONCLUSION

The results from the present study show a limited effect of 20-weeks Parachute Regiment training on self-report incidence of URTI, neutrophil degranulation and T-lymphocyte $CD4^{+}/CD8^{+}$ ratio. We did observe increased URTI incidences upon commencing PARA training however; this is likely to be related to the compact living conditions and seasonality. We also observed transient decreases in leukocyte counts and lymphocyte subsets although this was the morning after a week of heavy exercise and full recovery was achieved after 4 days.

Chapter 5

Neutrophil degranulation and lymphocyte subset response to at rest and after exercise following a 48 h period of fluid and/or energy restriction

5.1 Abstract

The aim was to investigate the effects of a 48 h period of fluid, energy or combined fluid and energy restriction on circulating leukocyte and lymphocyte subset counts ($CD3^+$, $CD4^+$ and $CD8^+$) and bacterially-stimulated neutrophil degranulation at rest and after exercise. Thirteen healthy males (mean ± SEM: age 21 ± 1 y) participated in four randomised 48-h trials. During control (CON) participants received their estimated energy (2903 ± 17 $kcal.d^{-1}$) and fluid (3912 ± 140 $ml.d^{-1}$) requirements. Estimated energy and fluid requirements were determined beforehand by indirect calorimetry and body mass changes during a 24 h period when participants lived in the laboratory environment. During fluid restriction (FR) participants received their energy requirements and 193 ± 19 $ml.d^{-1}$ water to drink and during energy restriction (ER) participants received their fluid requirements and 290 ± 6 $kcal.d^{-1}$. Fluid and energy restriction (F+ER) was a combination of FR and ER. After 48 h, participants performed a 30 min self-paced treadmill time trial (TT) followed by rehydration (0-2 h) and refeeding (2-6 h). Circulating leukocyte and lymphocyte counts remained unchanged on CON and FR. Circulating leukocyte, lymphocyte and $CD3^+$ and $CD4^+$ counts decreased ~20% on ER and ~30% on F+ER by 48 h ($P<0.01$) returning to within 0 h values by 6 h post-TT. Circulating neutrophil count and degranulation were unaltered by dietary restriction at rest and after TT. In conclusion, a 48 h period of ER and F+ER decreased circulating leukocyte, lymphocyte and $CD3^+$ and $CD4^+$ counts but not neutrophil count or degranulation. Circulating leukocyte and lymphocyte counts normalised upon refeeding. Finally, a 48 h period of dietary restriction did not alter circulating leukocyte, lymphocyte and neutrophil responses to 30 min maximal exercise.

5.2 Introduction

Dietary restriction has the potential to weaken several aspects of immune function, potentially leaving individuals more susceptible to infection (Fraker *et al.* 2000) Periods of forced or voluntary fluid and energy restriction, often lasting for a number of days, are common place in athletes making weight or with eating disorders (Brownell *et al.* 1987; Baum 2006) and in occupational settings e.g. military personnel during survival training (Carins and Booth 2002). Energy restriction may decrease immune cell metabolism, protein synthesis, cell replication and antioxidant defences directly through reduced substrate availability (Chandra 1997). Alternatively, a decrease in substrate availability might weaken immune function indirectly through raised sympatho-adrenal activity resulting in increased secretions of stress hormones (e.g. cortisol and catecholamines) known to have immuno-suppressive effects (Gleeson *et al.* 2004).

Little is known about the independent and combined effects of fluid and energy restriction on immune function at rest and after exercise. Cellular and humoral immunity were depressed in soldiers training in a tropical environment and surviving for 12 days on limited rations (1800 $kcal \cdot d^{-1}$) (Booth 2003). A 36-h fast decreased circulating lymphocyte counts (Walrand *et al.* 2001) and a 7-day fast decreased circulating T-lymphocyte ($CD3^+$) and helper T-lymphocyte ($CD4^+$) counts (Savendahl and Underwood 1997). The mechanism(s) responsible for decreased circulating lymphocytes and lymphocyte subsets during fasting remains unclear although a role for elevated circulating cortisol has been proposed (Mustafa *et al.* 1997). Circulating neutrophil counts were lower after 7 days of exercise (3 $h \cdot d^{-1}$ at 75 % $\dot{V}O_{2max}$) with a 25 % energy deficit compared with when participants received 110 % of their estimated daily energy requirements (Galassetti *et al.* 2006). Neutrophil chemotaxis and oxidative burst activity were decreased after a 36-hour fast but this was reversed after only 4 h of

refeeding (Walrand *et al.* 2001). Impaired phagocytic activity has also been shown in athletes restricting food and fluid intake to make weight prior to competition (Kowatari *et al.* 2001; Suzuki *et al.* 2003). Unfortunately, these studies do not distinguish between energy restriction and fluid restriction effects on immune function. Studies have either provided limited information about fluid intake, reported their participants to be hypohydrated following dietary restriction and/or were performed in a multi-stressor setting (e.g. tropical climate, psychological stress). Therefore, it is difficult to assess whether the observed immune responses reflect energy restriction effects or a combination of fluid and energy restriction and/or effects of other uncontrolled variables. Indeed, elevated plasma stress hormones have been observed during dehydration in ruminants (Parker *et al.* 2003) and during exercise with restricted fluid intake in humans (McGregor *et al.* 1999). As such, a role for hypohydration in the observed decrease in immune function during dietary restriction warrants enquiry.

Therefore, the purpose of the present study was to investigate the effects of a 48-h period of fluid, energy or combined fluid and energy restriction on circulating leukocyte and lymphocyte subset counts ($CD3^+$, $CD4^+$ and $CD8^+$) and bacterially-stimulated neutrophil degranulation at rest and after exercise. We hypothesised that fluid or energy restriction would decrease circulating leukocyte counts, lymphocyte subset counts and neutrophil degranulation at rest and after exercise and that the effects of combined fluid and energy restriction would be additive.

5.3 Methods

5.3.1 Participants

Thirteen recreationally active healthy males (mean ± SEM: age 21 ± 1 yr; height 179 ± 1 cm; body mass 74.7 ± 1.3 kg; body fat 16.8 ± 1.5 %; $\dot{V}O_{2max}$ 50.9 ± 1.2 $ml \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in the study. All participants gave written informed consent before commencing the study, which received local Ethics Committee approval. There were no reported symptoms of infection and participants did not take any medication or nutritional supplements in the six weeks prior to or during the study.

5.3.2 Preliminary measurements

Prior to the main experimental trials, each participant completed a continuous incremental exercise test on a treadmill for the determination of maximal oxygen uptake ($\dot{V}O_{2max}$) as described in **Chapter 3**. From the VO_2 – work rate relationship, the work rate equivalent to 50 % VO_{2max} was estimated and used for submaximal exercise during the experimental trials. On a separate day, 7-10 d prior to beginning the experimental trials, participants returned to the laboratory for individual energy expenditure estimation and familiarisation. Participants arrived euhydrated at 08:00 h after an overnight fast, having consumed water equal to 40 $ml \cdot kg^{-1}$ of body mass the previous day. On arrival and after voiding, anthropometric measurements of height and nude body mass (NBM) were collected. Following these measures, body composition was estimated using whole body dual energy x-ray absorptiometry (DEXA; Hologic, QDR1500, software version 5.72, Bedford, USA) and resting metabolic rate (RMR) was estimated using a portable breath by breath system (Metamax 3B, Biophysik, Leipzig, Germany). After breakfast, participants performed a 1.5 h treadmill walk at 50 % VO_{2max} during which energy expenditure was estimated (Cortex Metalyser 3B, Biophysik, Leipzig, Germany). For short periods during the day participants wore the

portable breath by breath system (Metamax 3B, Biophysik, Leipzig, Germany) to estimate the energy expenditure incurred during habitual living in the laboratory environment (participants were familiarised to wearing the breath by breath mask prior to data collection and the first 5 min of each period of use was excluded from analysis). This additional energy expenditure data was used, along with the RMR data, to estimate the energy intake required for the experimental trials. Additionally, during this 24 h period, fluid requirements were estimated by assessing changes in body mass at hourly intervals. Physical activity was standardised throughout the familiarisation and all experimental trials by recording 24 h step counts with pedometers (Digi-walker SW-200, Yamax, Tokyo, Japan).

5.3.3 Experimental Trials

Separated by 7-10 d, participants were required to complete four experimental trials in a randomised order (Table 1). The four dietary interventions included a control trial (CON), a fluid restriction trial (FR), an energy restriction trial (ER) and a combined fluid and energy restriction trial (F+ER). On the day prior to the experimental trial, to control nutritional and hydration status, participants were provided with their estimated energy requirements (12154 ± 71) $kJ \cdot d^{-1}$ of which 49, 36, 15 % were carbohydrate, fat and protein respectively and water equal to 40 $ml \cdot kg^{-1}$ of body mass (Figure 1). Participants were also instructed to refrain from exercise. Participants arrived at the laboratory at 22:00 h the evening prior to each trial. From the evening prior to the completion of each trial participants slept for 8 h per night in a temperate laboratory (19.7 ± 0.3 °C; 58.8 ± 1.9 % RH). The intervention began at 08:30 h the following morning after participants had voided and a NBM was obtained. Following NBM and 10-15 min seated, a baseline (0 h) blood sample was obtained. Further blood samples were collected after 24 h (08:30 h, day 2) and 48 h (08:30 h, day 3). Participants

performed a 1.5 h treadmill walk at a set workload equivalent to 50 % VO_{2max} after breakfast on day one and two. During the 1.5 h walks, water was consumed equal to fluid losses on CON and ER whereas no fluids were provided on FR and F+ER. Following lunch and evening meals, participants also completed a 20 min walk at similar intensities to the 20 min walk following breakfast. After providing a 48 h sample participants performed a self-paced 30 min treadmill time trial (TT), the data from which are presented elsewhere (Oliver *et al.* 2007). Participants were instructed to 'run as far as possible in 30 min' and to control the speed of the treadmill (gradient set at 1 %) as and when they felt appropriate. No fluids were consumed during the treadmill TT. Further blood samples were obtained immediately post-TT, 2 h post-TT and 6 h post-TT. During the first 2 h of recovery, fluid was provided as a citrus flavoured electrolyte only solution (50 $mmol \cdot l^{-1}$ sodium, Science in Sport, Blackburn, UK). The rehydration solution was divided evenly across the 2 h and consumed equal to 100 % body mass loss (BML) or up to 29 $ml \cdot kg^{-1}$ of body mass; which reflects the approximate maximal gastric emptying rate for this solution (Mitchell *et al.* 1994). During 2-3 and 4-5 h of recovery, participants consumed a total of 1950 ± 37 kcal (49, 36, 15 % were carbohydrate, fat and protein, respectively) divided equally into two meals. Water was available *ad-libitum* during these two meals.

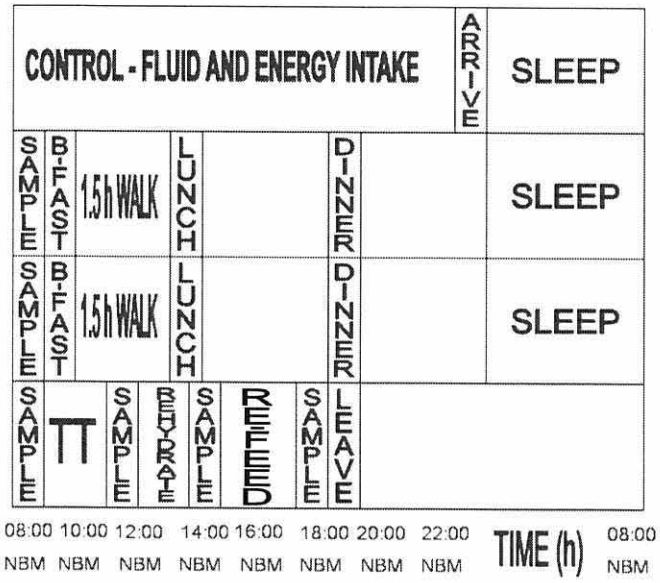

Figure 5.1 Schematic of trial events. B-fast, breakfast; NBM, nude body mass.

Table 5.1 Nutrient intake for a 24 h period.

	CON	FR	ER	F+ER
Fluid				
Fluid consumed (mL)	3912 ± 140	960 ± 15	3893 ± 136	962 ± 16
Water to drink (mL)	3145 ± 134	193 ± 19	3816 ± 135	885 ± 15
Water in food (mL)	767 ± 11	767 ± 11	77 ± 1	77 ± 1
Energy				
Energy consumed (MJ)	12.2 ± 1	12.2 ± 1	1.2 ± 0	1.2 ± 0
Carbohydrates (g)	387 ± 8	387 ± 8	39 ± 1	39 ± 1
Fat (g)	119 ± 3	119 ± 2	12 ± 0	12 ± 0
Protein (g)	104 ± 1	104 ± 1	10 ± 0	10 ± 0

Values are mean ± SEM (n=13). Abbreviations: control (CON); fluid restriction (FR); energy restriction (ER); fluid and energy restriction (F+ER). Macronutrient composition was the same across all trials and equal to 50, 36, 14 % where carbohydrate, fat and protein respectively.

5.3.4 Analytical Methods

Haematological parameters including Hb, Hct, plasma volume change, total and differential leukocyte counts, $CD3^+$, $CD4^+$ and $CD8^+$ cell counts as described in Chapter 3. Bacterially-stimulated neutrophil degranulation was determined as described in Chapter 3. Plasma elastase, cortisol, glucose and FFA concentrations were measured as described in Chapter 3.

5.3.5 Statistical Analysis

One-way analysis of variance (ANOVA) was performed on pre-experimental body mass and trial physical activity. Two-way fully repeated measured ANOVA was performed on BML, and blood parameters.

5.4 Results

5.4.1 Body mass loss and physical activity

At baseline (0 h) body mass was not significantly different between trials (CON, 73.4 ± 2.0; FR, 73.9 ± 2.0; ER, 73.9 ± 2.1 and F+ER, 73.7 ± 2.1 kg, $P > 0.05$). After 48 h, BML on FR 3.2 ± 0.1, ER 3.4 ± 0.1 and F+ER 3.6 ± 0.1 % was significantly greater than CON 0.6 ± 0.1 %. BML was also greater on F+ER than FR at 48 h ($P < 0.01$). Following the TT, BML was; 1.7 ± 0.1 on CON, 4.1 ± 0.1 on FR, 4.3 ± 0.1 on ER and 4.4 ± 0.1 % on F+ER ($P < 0.01$). The 6 h recovery protocol re-established 0 h body mass on CON and FR (-1.0 ± 0.1 and -1.0 ± 0.2 % respectively) although significant BML was still evident on ER and F+ER (0.9 ± 0.2 and 0.5 ± 0.2 % respectively, $P < 0.01$). The mean experimental trial physical activity ranged from 18,459 to 19,230 steps·d^{-1} and was not significantly different between trials ($P > 0.05$).

5.4.2 Plasma volume change, free fatty acids, glucose and cortisol

Plasma volume did not change significantly during the 48 h period (CON: -1.4 ± 1.6; FR: -0.9 ± 1.4; ER: -5.2 ± 0.9 and F+ER: -5.1 ± 1.2 %). Plasma volume was increased on all trials following the 2 h rehydration protocol (CON: 6.4 ± 1.3; FR: 7.6 ± 1.2; ER: 9.0 ± 1.8 and F+ER: 10.5 ± 1.5 %, $P < 0.05$). Plasma FFA concentration increased on energy restriction trials by 24 h (ER and F+ER: $P < 0.05$: Table 5.2) and was significantly greater than CON and FR from this point onwards ($P < 0.05$). Plasma glucose concentration was lower on ER and F+ER at 48 h compared with CON and FR ($P < 0.05$, Table 5.2). Following the TT, plasma glucose concentration was significantly greater on CON and FR compared with 0 h, ER and F+ER ($P < 0.05$). Plasma cortisol concentration did not alter significantly throughout the 48 h period but was greater following the TT and the 2 h rehydration period on ER and F+ER compared with CON and FR ($P < 0.05$: Table 5.2). Diurnal variation most likely accounts for the decrease in

plasma cortisol concentration at 6 h post-TT (17:00 h) compared with 0 h (08:30 h) on all trials.

Table 5.2 The effects of a 48 h period of fluid (FR), energy (ER) or combined fluid and energy (F+ER) restriction and a 30 min treadmill TT on plasma FFA, glucose and cortisol concentrations.

		CON	FR	ER	F+ER
FFA	0 h	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
($mmol \cdot l^{-1}$)	24 h††	0.6 ± 0.0	0.5 ± 0.0	1.6 ± 0.2^{ab*}	1.7 ± 0.1^{ab*}
	48 h††	0.6 ± 0.1	0.6 ± 0.1	1.8 ± 0.2^{ab*}	2.0 ± 0.2^{ab*}
	Post-TT††	1.1 ± 0.1*	1.1 ± 0.1*	2.2 ± 0.1^{ab*}	2.4 ± 0.2^{ab*}
Glucose	0 h	5.1 ± 0.2	5.0 ± 0.2	5.3 ± 0.1	5.1 ± 0.2
($mmol \cdot l^{-1}$)	24 h	5.3 ± 0.2	5.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.2
	48 h	5.4 ± 0.2	5.5 ± 0.2	4.2 ± 0.2^{ab}	4.1 ± 0.2^{ab}
	Post-TT†	8.2 ± 0.6*	7.9 ± 0.5*	4.5 ± 0.3^{ab}	4.4 ± 0.2^{ab}
	2 h Post	5.8 ± 0.2	5.7 ± 0.2	5.5 ± 0.3	5.3 ± 0.2
	6 h Post	6.6 ± 0.2*	6.4 ± 0.2*	7.2 ± 0.3*	7.2 ± 0.4*
Cortisol	0 h	494 ± 24	455 ± 16	479 ± 17	502 ± 16
($nmol \cdot l^{-1}$)	24 h	528 ± 24	502 ± 23	554 ± 25	544 ± 17
	48 h	516 ± 22	500 ± 24	559 ± 32	564 ± 21
	Post-TT	459 ± 29	448 ± 27	571 ± 41^{ab*}	556 ± 38^{ab}
	2 h Post††	263 ± 17*	284 ± 17*	377 ± 27^{ab*}	379 ± 34^{ab*}
	6 h Post††	246 ± 14*	186 ± 14*	247 ± 18*	243 ± 26*

Values are mean ± SEM, (n = 13). a *vs.* CON, b *vs.* FR.* *vs.* 0 h ; $P < 0.05$. Main effect of time; significantly different from 0 h; † $P < 0.05$, †† $P < 0.01$.

5.4.3 Circulating leukocyte counts, lymphocyte counts and lymphocyte subsets

There was a significant trial x time interaction for circulating leukocyte counts (interaction: $F_{(6,12)} = 3.4$, $P < 0.05$), lymphocyte count (interaction: $F_{(6,72)} = 3.9$, $P < 0.01$), $CD3^+$ cell count (interaction: $F_{(6,60)} = 3.9$, $P < 0.01$) and $CD4^+$ cell counts (interaction: $F_{(6,60)} = 4.0$, $P < 0.01$). Circulating leukocyte counts decreased by 48 h on energy restriction trials compared with 0 h (ER and F+ER: $P<0.01$: Table 5.3). In addition, circulating leukocyte count was significantly lower on ER compared with FR at 48 h ($P < 0.01$: Table 5.3). Following the TT, circulating leukocyte counts increased and remained elevated at 2 and 6 h post ($P < 0.01$: Table 5.3). Circulating lymphocyte counts decreased by 24 h on ER and 48 h on F+ER compared with 0 h ($P < 0.01$: Figure 5.2A). On ER and F+ER circulating lymphocytes were lower than CON at 48 h and after the TT, and this was apparent by 24 h on ER ($P < 0.01$: Figure 5.2A). $CD3^+$ and $CD4^+$ counts demonstrated a decrease on ER and F+ER at 24 h ($P < 0.05$) and 48 h ($P < 0.01$) and did not change significantly from 0 h on CON and FR (Figure 5.2B and 5.3A). Circulating lymphocyte, $CD3^+$ and $CD4^+$ counts returned to within baseline (0 h) by 6 h post-TT except for $CD3^+$ on FR, which remained elevated ($P < 0.01$). There was no significant trial x time interaction for $CD8^+$ cell counts (Figure 5.3B). However, $CD8^+$ cell counts were significantly lower than baseline (0 h) at 48 h and 2 h post TT ($P < 0.05$, Figure 5.3B). The $CD4^+$:$CD8^+$ ratio tended to decrease during the 48 h on ER (0 h: 1.95 ± 0.17 and 48 h: 1.61 ± 0.12) and F+ER (0 h: 1.99 ± 0.24 and 48 h: 1.44 ± 0.11) trials, but did not reach significance ($P > 0.05$, Figure 5.3C).

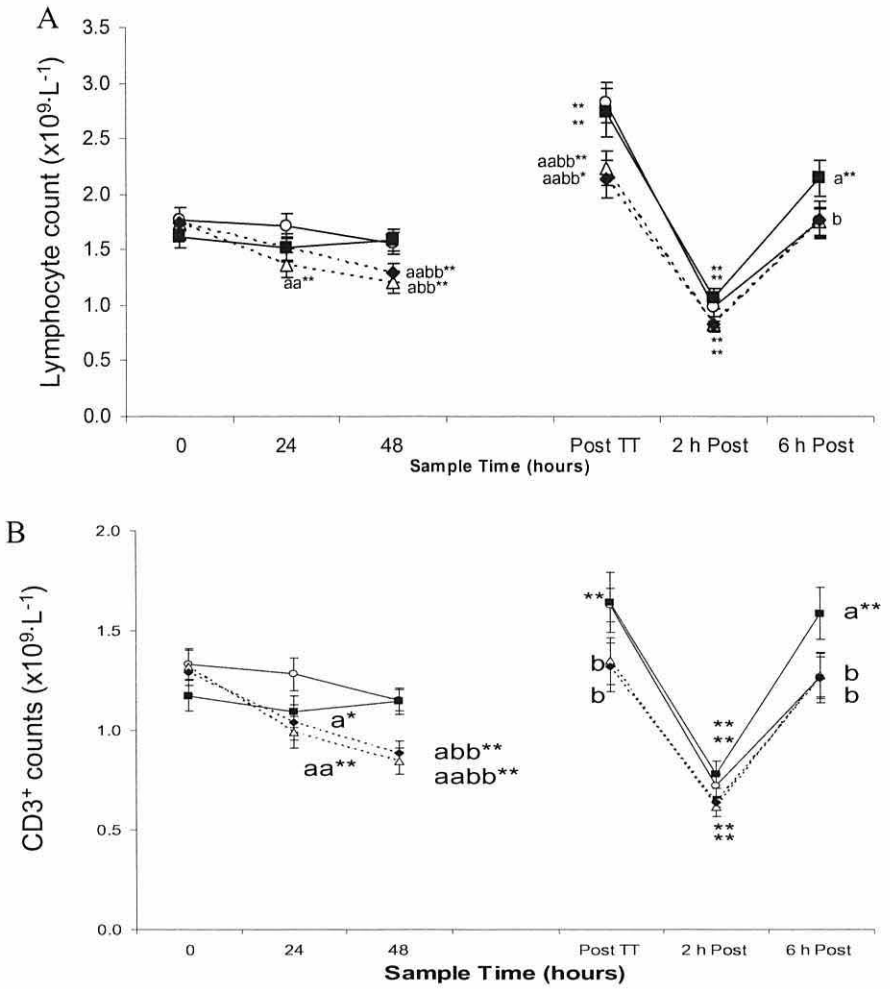

Figure 5.2 The effects of a 48 h period of fluid restriction (■), energy restriction (Δ), fluid and energy restriction (♦) compared with control (○) and a 30 min treadmill TT on circulating (A) lymphocyte cell counts and (B) $CD3^+$ cell counts. Values are mean ± SEM, (n = 13). a *vs.* control, b *vs.* fluid restriction and * *vs.* 0 h, $P < 0.05$. aa *vs.* control, bb *vs.* fluid restriction and ** *vs.* 0 h, $P < 0.01$.

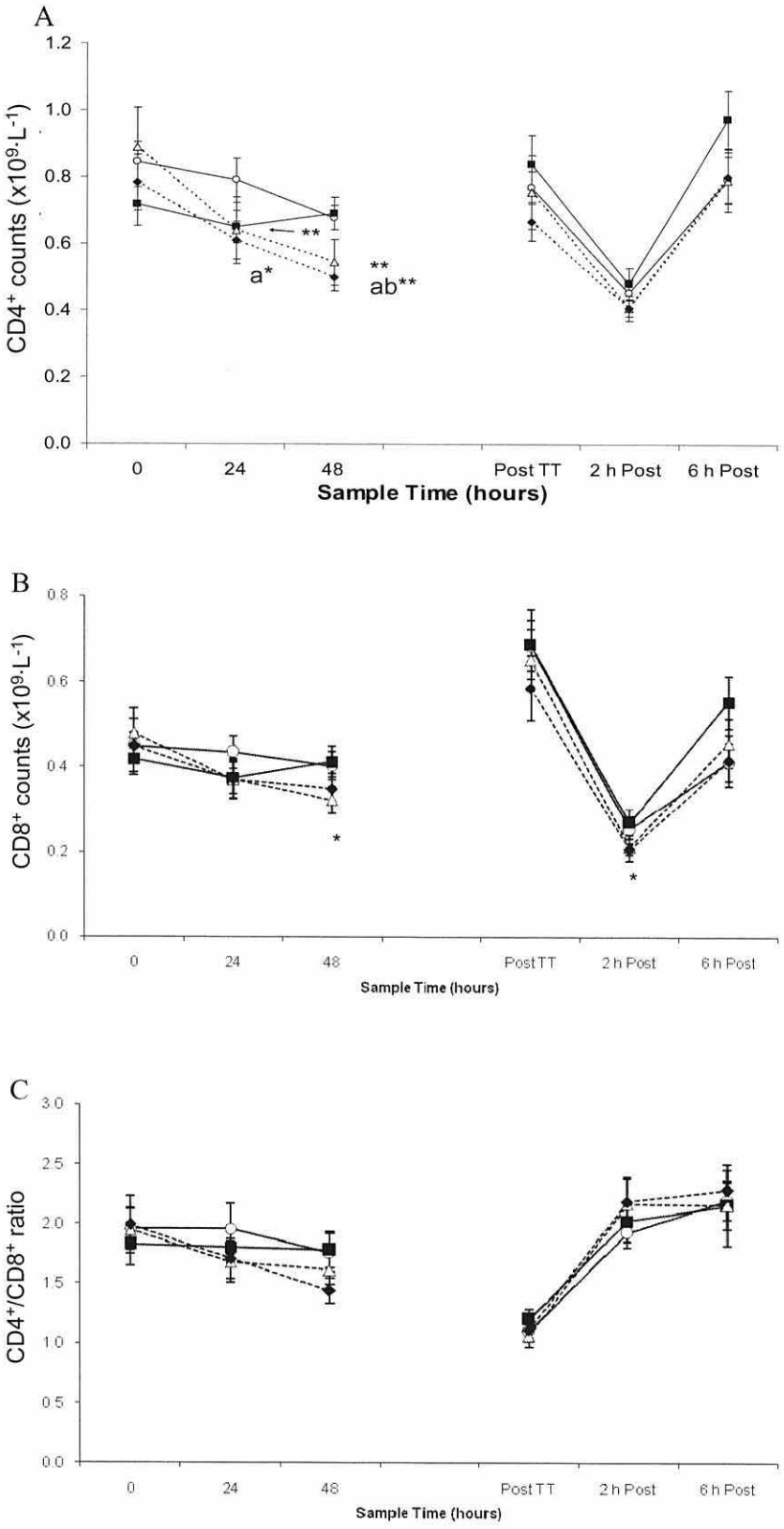

Figure 5.3 The effects of a 48 h period of fluid restriction (■), energy restriction (Δ), fluid and energy restriction (♦) compared with control (○) and a 30 min treadmill TT on circulating (A) $CD4^+$ cell counts, (B) $CD8^+$ cell counts and (C) $CD4^+$ /$CD8^+$ ratio. Values are mean ± SEM, (n = 13). a *vs.* control, b *vs.* fluid restriction and * *vs.* 0 h, $P < 0.05$. and ** *vs.* 0 h, $P < 0.01$.

5.4.4 Circulating neutrophil counts and bacterially-stimulated neutrophil degranulation

There were no significant trial x time interactions observed for circulating neutrophil count, plasma elastase concentration and bacterially-stimulated elastase release per neutrophil (Table 5.3). Circulating neutrophil counts were elevated at 2 and 6 h post-TT compared with 0 h ($P < 0.01$: Table 2). Plasma elastase concentration increased immediately post-TT compared with 0 h ($P < 0.01$, Table 5.3). There was a trend for reduced bacterially-stimulated elastase release per neutrophil 2 h post-TT compared with 0 h, however this did not reach significance (Table 5.3).

Table 5.3 The effects of a 48 h period of fluid (FR), energy (ER) or combined fluid and energy (F+ER) restriction and a 30 min treadmill TT on circulating leukocyte counts, circulating neutrophil counts, plasma elastase concentration and bacterially-stimulated elastase release per neutrophil.

		CON	FR	ER	F + ER
Leukocytes	0 h	4.8 ± 0.4	4.4 ± 0.2	4.6 ± 0.3	4.8 ± 0.3
($x\ 10^9 \cdot L^{-1}$)	24 h	4.5 ± 0.3	4.2 ± 0.2	4.0 ± 0.3	4.4 ± 0.2
	48 h†	4.2 ± 0.3	4.3 ± 0.2	3.7 ± 0.2bb**	4.0 ± 0.2**
	Post-TT†	6.5 ± 0.3	6.6 ± 0.5	6.0 ± 0.3	5.9 ± 0.3
	2 h Post††	8.0 ± 0.6	8.3 ± 0.5	8.0 ± 0.8	7.5 ± 0.5
	6 h Post††	7.1 ± 0.4	7.7 ± 0.4	7.1 ± 0.5	7.1 ± 0.5
Neutrophils	0 h	2.3 ± 0.1	2.5 ± 0.2	2.4 ± 0.2	2.6 ± 0.2
($x\ 10^9 \cdot L^{-1}$)	24 h	2.1 ± 0.1	2.3 ± 0.1	2.5 ± 0.2	2.7 ± 0.2
	48 h	2.2 ± 0.2	2.4 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
	Post-TT	3.4 ± 0.2	3.7 ± 0.3	4.0 ± 0.3	3.8 ± 0.2
	2 h Post††	6.6 ± 0.5	6.3 ± 0.4	7.2 ± 0.8	6.4 ± 0.6
	6 h Post††	4.5 ± 0.4	5.6 ± 0.2	4.5 ± 0.5	4.4 ± 0.3
Plasma elastase	0 h	48 ± 5	45 ± 5	50 ± 5	45 ± 5
Concentration	24 h	49 ± 4	56 ± 7	49 ± 4	46 ± 4
($ng \cdot ml^{-1}$)	48 h	51 ± 8	48 ± 5	47 ± 6	54 ± 6
	Post-TT††	99 ± 10	154 ± 33	101 ± 11	106 ± 13
	2 h Post	62 ± 8	69 ± 7	52 ± 4	70 ± 7
	6 h Post	84 ± 9	71 ± 9	70 ± 9	78 ± 13
Stimulated	0 h	611 ± 32	574 ± 47	635 ± 40	641 ± 55
elastase release	24 h	632 ± 26	590 ± 51	639 ± 37	653 ± 41
($fg \cdot cell^{-1}$)	48 h	652 ± 42	598 ± 37	624 ± 44	638 ± 29
	Post-TT	598 ± 48	665 ± 52	620 ± 58	568 ± 35
	2 h Post	498 ± 30	588 ± 51	513 ± 29	569 ± 29
	6 h Post	606 ± 43	655 ± 69	680 ± 54	618 ± 26

Values are mean (SEM), (n = 13). bb *vs.* FR.** *vs.* 0 h, $P < 0.01$. Main effect of time; significantly different from 0 h; † $P < 0.05$, †† $P < 0.01$.

5.5 Discussion

The aim of the present study was to investigate the effects of a 48-h period of fluid, energy or combined fluid and energy restriction on circulating leukocyte and lymphocyte subset counts ($CD3^+$, $CD4^+$ and $CD8^+$) and bacterially-stimulated neutrophil degranulation at rest and after exercise. In contrast to our hypothesis, 48 h of FR (~2.9 $l.d^{-1}$ deficit) did not alter immune responses either at rest or after the 30 min treadmill TT. In partial support of our hypothesis 48 h of ER (~2600 $kcal.d^{-1}$ deficit) and F+ER decreased circulating leukocyte, lymphocyte $CD3^+$ and $CD4^+$ counts but did not alter circulating neutrophil count or neutrophil degranulation. Circulating leukocyte and lymphocyte counts returned to within baseline values after rehydration and refeeding (by 6 h post). Finally, a 48 h period of fluid, energy or combined fluid and energy restriction did not alter circulating leukocyte, lymphocyte and neutrophil responses to the 30 min treadmill TT.

Despite the relatively modest nutrient restriction in the current study, reduced circulating leukocyte, lymphocyte and lymphocyte $CD4^+$ counts were observed. Studies using shorter duration (36 h; (Walrand *et al.* 2001) and long duration (7 days, (Savendahl and Underwood 1997); 64 days, (Kramer *et al.* 1997) restrictions showed similar findings of reduced lymphocyte, $CD3^+$ and $CD4^+$ counts. However, in addition decreases in $CD8^+$ cell counts were also reported by previous authors (Walrand *et al.* 2001). Albeit not significant in the present study, 36 h fasting caused a reduced $CD4^+/CD8^+$ ratio (Walrand *et al.* 2001). Discrepant findings in the literature can partially be attributed to the various lengths and severity of the restrictions ranging from 36 h complete nutrient fasting (Walrand *et al.* 2001) through to several months of repeated periods of energy restriction (Kramer *et al.* 1997; McFarlin *et al.* 2006). Unlike Walrand et al, (2001) and Savendahl and Underwood, (1997) the energy restriction

during the present study still allowed for 290 Kcal.d^{-1} (39 g carbohydrates, 12 g fat and 10 g protein). In previous fasting studies some authors have provided vitamin supplementation thus having a macronutrient restriction only (Savendahl and Underwood 1997), whilst most attempt to offset the effects of dehydration and haemoconcentration by providing water in set volumes (Savendahl and Underwood 1997; Walrand *et al.* 2001). The strict laboratory condition in the present study enabled the independent and combined effects of fluid and energy restriction to be identified without inclusion of confounding multi stressors such as sleep deprivation, large periods of physical exertion and severe psychological stress as often experienced in field based studies (Kramer *et al.* 1997). It would appear that re-feeding (6 hours) is enough to return lymphocytes and T-lymphocyte subsets to within normal values. Again the discrepant findings within the literature regarding re-feeding could be affected by the prior intervention, however after 4 h re-feeding (Walrand *et al.* 2001) and 9 days removal of the energy restriction (Kramer *et al.* 1997) similar recovery patterns were observed for lymphocytes and T-lymphocyte subset cell counts.

The mechanism(s) responsible for the decreased circulating lymphocyte and T-lymphocyte subset counts remain to be clarified. Several authors have highlighted the importance of IGF-1 and prolactin in the involvement lymphocyte proliferation (Reiss *et al.,* 1992; Clark *et al.,* 1993; Clevenger *et al.,* 1992). Indeed, IGF-1 potentiates IL-2 production in human peripheral T cells and prolactin has been shown to increase the expression of IL-2 receptors on T helper cells (Gala *et al.,* 1993). Both IGF-1 and prolactin have been shown to be reduced during 7-days total fasting with only mineral water (Savendahl and Underwood 1997). In the present study neither IGF-1 nor prolactin were measured due to financial constraints. In hindsight these may have

provided further evidence towards a mechanism for the decreased circulating lymphocyte and T-lymphocyte subset counts when energy restrictions are in place.

In support primary local immune responses against antigen exposure, migration of lymphocytes to extra vascular lymphoid tissues, particularly at the intestinal level upon re-feeding has been documented in patients with irritable bowel syndrome and healthy adults (Komaki *et al.,* 1997; Walrand *et al.,* 2001). Within the present study refeeding of 1950 ± 37 kcal (49, 36, 15 % were carbohydrate, fat and protein, respectively) divided equally into two meals on the two energy restriction trials returned circulating lymphocyte, $CD3^+$ and $CD4^+$ counts to within baseline levels (Figures 5.2A, 5.2B and 5.2C). The transient nature of lymphocyte trafficking with energy restriction and refeeding suggest that the mechanism behind altered cell counts is reliant of adequate energy provision. In the present study no independent effect of 48 h FR on lymphocyte, $CD3^+$, $CD4^+$, $CD8^+$ cell counts and the $CD4^+/CD8^+$ ratio were observed, again this could be attributed to the mild severity and duration of the restriction imposed.

Interestingly, neutrophil counts, plasma elastase concentration and bacterially stimulated elastase release per neutrophil remained unaltered throughout 48 h of energy restriction, fluid restriction and combined fluid and energy restriction. These findings support those showing no change in neutrophil cell counts after a 7 day fast (Savendahl and Underwood 1997) and after 20 days with a 800-1500 $Kcal \cdot d^{-1}$ deficit (Kowatari *et al.,* 2001). However, these findings do not support those showing lower circulating neutrophil counts after a 6-week very low energy diet (consuming 400 $kcal \cdot d^{-1}$) (Field *et al.* 1991) and higher circulating neutrophil counts following a 36-h fast (Walrand *et al.* 2001) Similar to lymphocytes cell counts, a number of factors are likely to contribute to the discrepant findings in the literature with respect to energy availability and neutrophil

trafficking (fasting v's restriction, duration, additional vitamin supplementation). Fasting has been hypothesized to limit glucose uptake and utilization by neutrophils (Mowat and Baum 1971; McMurray *et al* 1990). The energy restriction undertaken in the current study albeit modest was probably not severe enough to cause alterations in neutrophil cell counts or bacterially stimulated neutrophil degranulation. These findings conflict with previous reports of decreased neutrophil phagocytic activity following weight reduction, low diet and severe energy restriction (Kowatari *et al.* 2001; Suzuki *et al.* 2003b; Galassetti *et al.* 2006), although the current study did investigate different neutrophil functional parameters (elastase release per neutrophil cell following bacterially challange). Typically, the stress hormone cortisol has been associated with increased neutrophil cell count and decreased neutrophil function, it is not surprising that in the current study unaltered neutrophil function during the 48 h restriction period was accompanied by unaltered plasma cortisol levels during at the same time points (Table 2 and 3). In addition, elevated plasma cortisol has been observed during dehydration in ruminants (Parker *et al.* 2003). These data suggest (BML: FR 3.2 ± 0.1 %) no independent effect of 48 h FR on plasma cortisol concentration, neutrophil cell counts and neutrophil degranulation.

There would appear to be a limited effect of prior nutritional status on leukocyte trafficking following the 30 min TT (Table 2). It remains unclear if a similar dietary restriction affects circulating leukocyte responses to more prolonged exercise (>1 h). Lymphocytes, and T-lymphocyte $CD3^+$, $CD4^+$ and $CD8^+$ show no effect of prior nutritional status on post-exercise responses, with 6 h refeeding being adequate to return cell counts to within baseline values (Figure 5.2). There was a significant decrease in circulating plasma cortisol 2 h post-exercise which accompanied a neutrophilia at 2 and 6 h post exercise. Given the proposed involvement of cortisol in post exercise

neutrophilia it would appear that the increased cell counts in the present study are induced by something other than cortisol. Recent evidence from our laboratories (discussed in further detail in chapter 7) negates the role of cortisol in post exercise neutrophillia and suggests the possibility of demargination caused by mechanical effects of raised cardiac output (Foster *et al.*, 1986).

5.6 Conclusion

In conclusion, a 48 h period of energy restriction and combined fluid and energy restriction decreased circulating leukocyte, lymphocyte and $CD3^+/CD4^+$ counts but did not alter circulating neutrophil count or degranulation. Circulating leukocyte and lymphocyte counts normalised rapidly upon refeeding. A 48 h period of fluid restriction alone did not alter immune responses either at rest or after a 30 min maximal exercise bout. Finally, nutrient restriction did not alter circulating leukocyte, lymphocyte and neutrophil responses to a 30 min maximal exercise bout.

Chapter 6

Neutrophil Degranulation and Lymphocyte Subset Responses to 2 Hours of Exercise in a 30 °C Environment

6.1 Abstract

It was hypothesized that prolonged exercise in the heat would evoke a greater stress hormone response, a greater decrease in neutophil degranulation (bacterially-stimulated release) and altered $CD3^+/CD4^+$ ratio than when the same exercise was performed in thermoneutral conditions. In counterbalanced order and separated by 7 d, 13 male cyclists cycled for 2 h at 62 ± 3 % $\dot{V}O_{2\,max}$ (mean ± SEM), with *ad-libitum* water intake, on one occasion with heat (HOT: 30.3 ± 0.1 °C and 76 ± 1 % RH) and on another occasion without (CONTROL: 20.4 ± 0.1 °C and 60 ± 1 % RH). Venous blood samples were collected pre-, post-, and 2 h post-exercise. Exercising HR, RPE, T_{re}, corrected body mass loss and plasma cortisol concentration at post- and 2 h post-exercise were greater during HOT compared to CONTROL. A marked neutrophillia was evident at post- and 2 h post-exercise with no difference between trials (Neutrophil count post-exercise: 12.7 ± 1.3 and 13.2 ± 1.2 x $10^{-9} \cdot L^{-1}$, HOT and CONTROL respectively). Bacterially-stimulated elastase release per neutrophil decreased post-exercise with no difference between trials. There was no effect of exercise or trial on neutrophil CD11b expression (activation index) or band cell percentage (neutrophil maturity index). Leukocyte and lymphocyte counts, $CD3^+$, $CD4^+$ and $CD8^+$ cells were significantly elevated immediately post-exercise compared to pre-exercise ($P < 0.05$). There were no additional effects observed for leukocyte and lymphocyte counts and T-lymphocyte subsets when exercising in the heat (Lymphocyte count post-exercise: 2.6 ± 0.3 and 2.8 ± 0.3 x $10^{-9} \cdot L^{-1}$, HOT and CONTROL respectively). In conclusion prolonged exercise results in a decrease in neutrophil degranulation and elevated leukocyte and lymphocyte counts that is unaffected by performing the exercise in hot conditions despite the increase in physical stress. Additionally, these data suggest that the decrease in neutrophil degranulation after prolonged exercise is not associated with a change in neutrophil activation or maturity as previously suggested.

6.2 Introduction

Performing prolonged strenuous exercise can lead to suppression of immune function(s) lasting between 1 and 24 h (Brenner *et al.* 1998). There is a relationship between the neuro-endocrine response to exercise and the immune response to exercise (Hoffman-Goetz and Pedersen 1994; Pedersen *et al.* 1997). Stress hormones are widely recognised to influence immune cell trafficking (McCarthy and Dale, 1988; Tonnesen *et al.,* 1987). In Addition, the expression of β-adrenergic surface receptors on immune cells are well documented as the targets for catecholamine signaling during stress (Shephard, 1998).

Reports of reduced *in vitro* neutrophil degranulation (bacterially-stimulated) and neutrophil oxidative burst have been observed following prolonged exercise (Gabriel *et al.* 1994; Robson *et al.* 1999; Bishop *et al.* 2003) and endurance training (Blannin *et al.* 1996). Elevated plasma cortisol within the physiological range has been implicated in decreased neutrophil chemotaxis, oxidative burst after prolonged exercise (Forslid and Hed 1982; Salak *et al.* 1993). Raised plasma cortisol after prolonged exercise has also been associated with delayed release of band (immature) neutrophils, and it has been suggested that these immature cells may be less responsive to stimulation (Suzuki *et al.* 1996a). After prolonged exercise the increase in expression of neutrophil activation marker CD11b promotes the release of elastase into the circulation (Smith *et al.* 1996). It has been suggested that the decrease in bacterially-stimulated neutrophil degranulation after prolonged high intensity exercise may be associated with a reduction in CD11b expression indicating a decrease in neutrophil activation (Bishop *et al.* 2003). In addition, raised plasma cortisol concentration has been associated with suppressed neutrophil activation (Blalock 1989). It remains to be seen if decreased bacterially-stimulated neutrophil degranulation after prolonged exercise coincides with a decrease

in neutrophil activation status and/or an increase in the percentage of band (immature) neutrophils.

Prolonged exercise in the heat elicits a more pronounced increase in core body temperature, increased cardiovascular drift, leads to larger increments of circulating stress hormones, causes a shift in substrate metabolism towards a greater reliance on carbohydrates and can have detrimental effects on performance (Brenner *et al* 1997; Maughan 1997; Galloway and Maughan 1997; Febbraio 2001; Mitchell *et al.* 2002). Compared with exercise in thermoneutral conditions, exercise in the heat is associated with elevated: circulating leukocyte and neutrophil cell counts and plasma cortisol concentrations (Severs *et al* 1996). Previously, exercise performed in a hot environment was associated with moderate elevations in cortisol concentrations (~61 % and ~22 % in the euhydrate and dehydrated hot respectively). These cortisol responses could be partly attributed to the modest exercise intensity of 55 % $\dot{V}O_{2\ peak}$ and administration of a carbohydrate/electrolyte beverage and paste during the trials (Mitchell *et al.* 2002). Reduced neutrophil degranulation, oxidative burst and suppressed elevations in cortisol concentrations have been observed following carbohydrate beverage ingestion during exercise (Nieman 1998; Bishop *et al.* 2000; Scharhag *et al.* 2002; Bishop *et al.* 2002; Bishop *et al.* 2003). Increases in neutrophil cell count and plasma cortisol concentrations have also been seen when exercising at 75 % $\dot{V}O_{2\ max}$ for 60 min at high ambient temperatures (28 °C, 50 % RH) when compared to room temperature (18 °C, 50 % RH) (Niess *et al.* 2003), However, this study reported no functional measures and the acute exercise was not prolonged.

$CD3^+$, $CD4^+$ and $CD8^+$ cell counts have been reported to increase immediately after prolonged exercise, and return to resting values by 3 hours post-exercise (Gannon *et al.*

2001; Natale *et al.* 2003). In addition, exercise-induced redistribution of lymphocytes is mediated by adrenaline, because $CD8^+$ T cells have more β_2-adrenergic receptors on the surface compared with $CD4^+$ cells (Pedersen and Hoffman-Goetz, 2000). $CD8^+$ cell counts have been reported to increase to a greater extent than the $CD4^+$ cell counts, resulting in a decreased $CD4^+/CD8^+$ ratio (Pedersen *et al.* 1994; Natale *et al.* 2003). A $CD4^+/CD8^+$ ratio greater than 1.2 is important for host defense (Mackinnon 1999). Additionally, the effects of prolonged exercise with the additive effects of heat stress have not been investigated. Therefore, we hypothesised that prolonged exercise in the heat would be associated with a greater disturbance in neutrophil function and T-lymphocyte subsets. We also hypothesized that the decrease in neutrophil degranulation post-exercise would be associated with an increase in the proportion of band (immature) neutrophils and activation status (CD11b expression).

6.3 Methods

6.3.1 Participants

Thirteen, healthy male trained cyclists (mean ± SEM: age 28 ± 2 yr; height 178 ± 4 cm; body mass 75.4 ± 4.9 kg; peak power output 348 ± 26 W; $\dot{V}O_{2\ max}$ 61.2 ± 2.3 ml·kg^{-1}·min^{-1}) volunteered to participate in this study. All participants were club level road or mountain bike cyclists with a minimum of 5 years competitive experience. Weekly training distance was 99 ± 12 miles with a range of 50-150 miles. The duration of the study spanned the winter training season when road cyclists undertake a reduced training volume. There were no reported symptoms of infection and all participants were taking no medication in the six weeks prior to the study.

6.3.2 Preliminary Measurements

Prior to the main experimental trials, an incremental exercise test to volitional exhaustion for the determination of maximal oxygen uptake ($\dot{V}O_{2\ max}$) and peak power output (PPO) was completed by each participant using cycle ergometry as described in Chapter 3.

6.3.3 Experimental Procedures

On two occasions separated by at least 7 d, participants reported to the laboratory at 12:00 h following a 4 h fast. Subjects drank only water from 08:00 h. Subjects cycled for 2 h on a stationary ergometer at 55% peak power output (194 ± 4 W; 62 ± 3 % $\dot{V}O_{2\ max}$) in an environmental chamber (Delta Environmental Services, Chester, UK) on one occasion at a temperature and relative humidity of 30.3 ± 0.1 °C and 76 ± 1 % (HOT) and on another occasion at a temperature and relative humidity of 20.4 ± 0.1 °C and 60 ± 1 % (CONTROL). The trials were completed in a randomised order. On arrival at the laboratory participants were asked to empty their bladder and bowels and nude body mass was obtained (Seca 705, Hamburg, Germany). Participants were then seated for 10

min prior to the resting blood sample collection as described in Chapter 3. The 2 h exercise bout was performed with a fan placed 1 m in front of the cycle ergometer with the wind speed set at 2.0 $m \cdot s^{-1}$. In both trials participants wore cycle shorts only. HR (Polar Electro, Kempele, Finland), RPE and T_{re} (YSI 4000A, Daytona, Ohio) were recorded at 10 min intervals throughout exercise. Water consumption was permitted *ad libitum*. Immediately post-exercise a venous blood sample was obtained prior to nude body mass being recorded. All participants remained fasted until a further venous blood sample was obtained 2 h post-exercise. For all blood samples, 19 ml of blood was collected, with the participants in a seated position.

6.3.4 Analytical Methods

Haematological parameters including Hb, Hct, plasma volume change, total and differential leukocyte counts, $CD3^+$, $CD4^+$, $CD8^+$ and CD11b counts as described in Chapter 3. Blood films were prepared for the analysis of neutrophil lobularity using the Cook Arneth technique and the bacterially-stimulated neutrophil degranulation was determined as described in Chapter 3. Plasma concentrations of elastase, glucose and cortisol and serum concentrations of prolactin and GH concentrations were measured as described in Chapter 3.

6.3.5 Statistical Analysis

The sample size was estimated to be N = 6 (Cohen 1988) using previous data examining the effects of exercise on neutrophil degranulation responses (Robson *et al.* 1999). The estimated effect size for the decreased neutrophil degranulation during exercise was 1.3. Alpha and power levels were set at 0.05 and 0.8 respectively, both of which are standard estimates. The data were examined using a two-factor (2 trial x 3 time measurements) repeated measures ANOVA. Paired samples T-tests were used to compare body mass losses.

6.4 Results

6.4.1 Physiological Variables and RPE

Heart rate (main effect of time: $F_{(12,108)} = 41.4$, $P < 0.01$), RPE (main effect of time: $F_{(12,108)} = 47.3$, $P < 0.01$) and T_{re} (main effect of time: $F_{(12,108)} = 60.4$, $P < 0.01$) increased throughout the 2 h exercise bout. Heart rate was significantly higher in the HOT trial between 10 min and 120 min (interaction: $F_{(12,108)} = 4.9$, $P < 0.01$, mean trial HR: 134 ± 3 and 146 ± 3 beats·min^{-1} for CONTROL and HOT trials respectively). Mean trial RPE were 12 ± 0.3 and 13 ± 0.4 for CONTROL and HOT respectively. RPE was significantly higher on the HOT trial between 40 min and 120 min (interaction: $F_{(12,108)} = 4.7$, $P < 0.01$). Pre-exercise T_{re} were similar for both trials (CONTROL: 37.0 ± 0.1 °C and HOT: 37.1 ± 0.1 °C). T_{re} was significantly higher on the HOT trial between 50 min and 120 min (interaction: $F_{(12,108)} = 4.7$, $P < 0.01$; Table 6.1).

Body mass losses were not significantly different between trials (Table 6.1). After correction for fluid intake body mass losses were greater on the HOT trial ($P < 0.01$, Table 6.1). The decrease in plasma volume post-exercise was similar on both trials. Plasma glucose concentration was similar on both trials post-exercise (Table 6.1). Plasma cortisol (main effect of time: $F_{(2,24)} = 24.8$, $P < 0.01$; Figure 6.1A), serum prolactin (main effect of time: $F_{(2,16)} = 21.2$, $P < 0.01$; Figure 6.1B) and GH concentrations (main effect of time: $F_{(2,22)} = 22.2$, $P < 0.01$; Figure 6.1C) increased throughout the 2 h exercise bout. Plasma cortisol and serum prolactin concentration were significantly higher on the HOT trial compared with CONTROL trial immediately post-exercise, with plasma cortisol remaining elevated 2 h post-exercise (plasma cortisol interaction: $F_{(2,24)} = 3.6$, $P < 0.05$; Figure 6.1A; Serum prolactin interaction: $F_{(2,16)} = 13.2$, $P < 0.01$; Figure 6.1B).

TABLE 6.1. The effects of 2 h cycling at 55 % PPO in a HOT and CONTROL environment in trained male cyclists on HR, RPE, T_{re}, body mass loss, plasma volume change during exercise and post exercise plasma glucose concentration. (N = 13).

	HOT trial	CONTROL trial
HR at 120 min exercise (beats·min^{-1})[a] ##	161 (3)**	146 (4)**
RPE at 120 min exercise ##	16 (1)**	14 (0)**
T_{re} at 120 min exercise (°C) ##	38.7 (0.1)**	38.1 (0.1)**
Body mass loss (Kg)[a]	+0.4 (0.3)	-0.2 (0.2)
Corrected body mass loss (Kg)[b] ##	+2.2 (0.2)	+1.6 (0.1)
Plasma volume change (%)[c]	-7.1 (1.1)	-6.9 (1.3)
Post exercise plasma glucose (mmol·L^{-1})	4.5 (0.2)	4.4 (0.2)

Significant difference from pre-exercise; ** $P < 0.01$, Significant differences between trials; ## $P < 0.01$, [a] mean body mass losses post exercise, [b] after correction for fluid intake and [c] immediately post-exercise compared with pre-exercise.

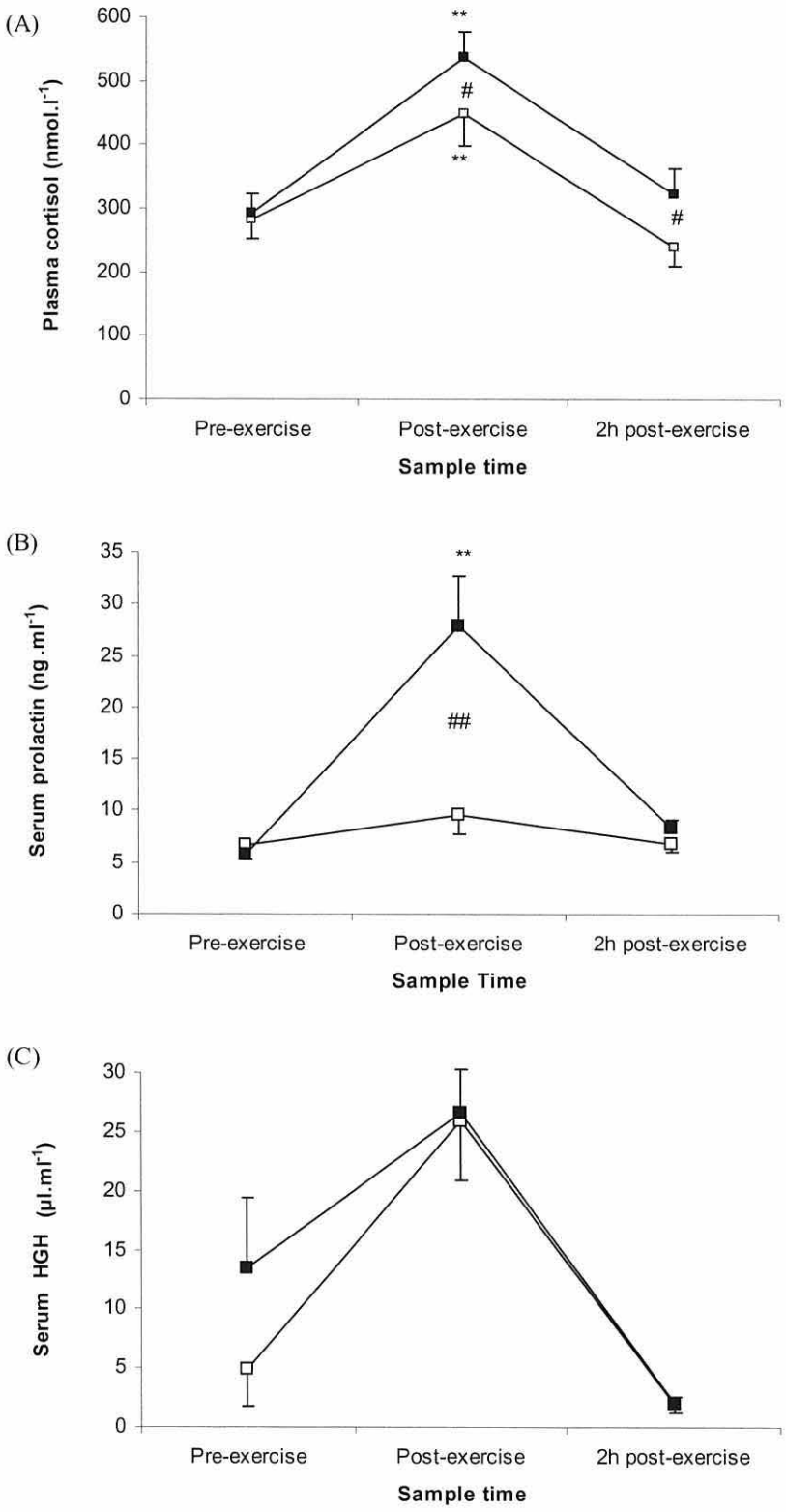

FIGURE 6.1. (A) Plasma cortisol (N = 13), (B) serum prolactin (N = 9) and (C) serum GH (N = 13) responses to 2 h exercise at 55 % PPO in a HOT (■) and CONTROL (□) environment in trained male cyclists. Significant difference from pre-exercise; ** $P <$ 0.01, Significant differences between trials; # $P < 0.05$, ## $P < 0.01$.

6.4.2 Neutrophil responses

There were no trial x time interaction effects observed for neutrophil counts, plasma elastase concentration, total bacterially-stimulated elastase release and bacterially-stimulated elastase release per cell; nevertheless, there was a significant main effect of time where neutrophil counts increased immediately post-exercise and remained elevated 2 h post-exercise (main effect of time; $F_{(2,20)} = 10.1$, $P < 0.01$, Table 6.2). Plasma elastase concentration increased above pre-exercise values immediately post-exercise (main effect of time; $F_{(2,20)} = 10.1$, $P < 0.01$, Table 6.2). Total bacterially-stimulated elastase release increased significantly immediately post-exercise and remained elevated 2 h post-exercise (main effect of time, $F_{(1.3,13.3)} = 29.0$, $P < 0.01$, Table 6.2). Adjusting these values to take into account changes in circulating neutrophils revealed a significant decrease in bacterially-stimulated plasma elastase release per cell immediately post-exercise returning to pre-exercise values 2 h post-exercise (main effect of time; $F_{(2,16)} = 10.7$, $P < 0.01$, Figure 6.2). Band (immature) neutrophil counts were significantly elevated immediately post-exercise and 2 h post-exercise (main effect of time, $F_{(2,24)} = 22.5$, $P < 0.01$, Table 6.2). There was no significant interaction or main effect of time for the percentage of band neutrophils.

TABLE 6.2. The effects of 2 h cycling at 55 % PPO in a HOT and CONTROL environment in trained male cyclists on neutrophil count, plasma elastase, total bacterially-stimulated elastase release, band counts (as a percentage of total neutrophil cell counts) and neutrophil expression of CD11b. (N = 13).

	Pre-exercise	Post-exercise	2 h post-exercise
Neutrophils (x $10^9 \cdot L^{-1}$) ‡‡ ††			
HOT	3.3 (0.3)	12.7 (1.3)	11.1 (1.0)
CONTROL	3.9 (0.5)	13.2 (1.2)	10.9 (0.8)
Plasma elastase ($\mu g \cdot L^{-1}$) ‡			
HOT	47 (8)	89 (14)	67 (9)
CONTROL	47 (8)	96 (14)	73 (12)
Total bacterially-stimulated elastase ($\mu g \cdot L^{-1}$) ‡‡††			
HOT	620 (69)	1735 (287)	1881 (228)
CONTROL	793 (114)	1903 (303)	1885 (276)
Band neutrophils (x $10^9 \cdot L^{-1}$) ‡‡ ††			
HOT	0.2 (0.0)	0.4 (0.1)	0.5 (0.2)
CONTROL	0.2 (0.0)	0.7 (0.1)	0.5 (0.1)
Band neutrophils (%)			
HOT	4.3 (0.9)	4.0 (0.8)	4.9 (1.2)
CONTROL	3.5 (0.7)	4.7 (0.7)	4.5 (1.0)
Neutrophil expression of CD11b (%)			
HOT	96.5 (1.1)	96.2 (2.8)	98.0 (1.0)
CONTROL	95.0 (3.5)	98.2 (1.2)	96.4 (2.0)
CD 11b MFI			
HOT	7.8 (1.4)	8.3 (1.4)	8.0 (1.3)
CONTROL	5.9 (0.9)	6.8 (1.1)	6.3 (0.9)

MFI = mean florescence intensity. Significant main effect of time; post-exercise significantly greater than pre-exercise; ‡ $P < 0.05$, post-exercise significantly greater than pre-exercise; ‡‡ $P < 0.01$, 2 h post-exercise significantly greater than pre-exercise; †† $P < 0.01$.

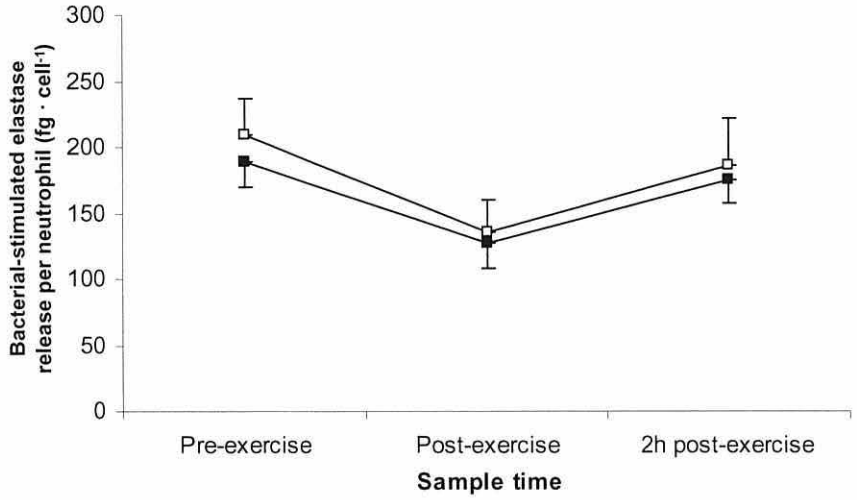

FIGURE 6.2 Bacterially-stimulated elastase release per neutrophil to 2 h cycling at 55 % PPO in a HOT (■) and CONTROL (□) environment in trained male cyclists. (N = 13).

6.4.3 Leukocyte counts, lymphocyte counts and T-lymphocyte subsets

There were no trial x time interaction effects observed for leukocyte and lymphocyte counts and T-lymphocyte subsets. Leukocyte and lymphocyte count, $CD3^+$, $CD4^+$ and $CD8^+$ cells were significantly elevated immediately post-exercise compared to pre-exercise (main effect of time, leukocyte count, lymphocyte count, $CD3^+$ and $CD4^+$ $P < 0.01$; $CD8^+$ $P < 0.05$). Leukocyte count and $CD4^+$:$CD8^+$ ratio was significantly elevated above pre-exercise levels at 2 h post-exercise ($P < 0.01$).

TABLE 6.3. The effects of 2 h cycling at 55 % PPO in a HOT and CONTROL environment in trained male cyclists on leukocyte counts, lymphocyte counts and subsets, ($N = 13$).

	Pre-exercise	Post-exercise	2 h post-exercise
Leukocytes (x $10^9 \cdot L^{-1}$) ‡‡ ††			
HOT	5.8 (0.3)	16.5 (1.3)	13.3 (1.0)
CONTROL	6.4 (0.5)	16.2 (1.2)	12.4 (0.7)
Lymphocytes (x $10^9 \cdot L^{-1}$) ‡‡			
HOT	1.9 (0.1)	2.6 (0.3)	1.4 (0.1)
CONTROL	1.9 (0.1)	2.8 (0.3)	1.7 (0.2)
CD3+ T-cells (x $10^9 \cdot L^{-1}$) ‡‡			
HOT	1.4 (0.2)	1.9 (0.4)	1.1 (0.1)
CONTROL	1.3 (0.1)	2.0 (0.3)	1.2 (0.1)
CD4+ $T_{helper/inducer}$ cells (x $10^9 \cdot L^{-1}$) ‡‡			
HOT	0.7 (0.1)	1.0 (0.1)	0.7 (0.1)
CONTROL	0.8 (0.1)	1.0 (0.1)	0.8 (0.1)
CD8+ $T_{cytotoxic/suppressor}$ cells (x $10^9 \cdot L^{-1}$) ‡			
HOT	0.6 (0.1)	1.0 (0.2)	0.4 (0.0)
CONTROL	0.6 (0.1)	0.8 (0.1)	0.5 (0.0)
CD4+/CD8+ ratio †			
HOT	1.3 (0.1)	1.2 (0.2)	1.7 (0.1)
CONTROL	1.6 (0.2)	1.5 (0.3)	1.8 (0.2)

Significant difference from pre-exercise; ** $P < 0.01$, Significant differences between trials; ## $P < 0.01$. Significant main effect of time; post-exercise significantly greater than pre-exercise; ‡ $P < 0.05$, post-exercise significantly greater than pre-exercise; ‡‡ $P < 0.01$, 2 h post-exercise significantly greater than pre-exercise; † $P < 0.05$, 2 h post-exercise significantly greater than pre-exercise; †† $P < 0.01$.

6.5 Discussion

The aim of the present study was to determine the effects of prolonged exercise in the heat on neutrophil degranulation and the $CD4^+/CD8^+$ ratio. It was hypothesised that prolonged exercise in the heat would evoke a greater stress hormone response and potentially a greater decrease in bacterially-stimulated neutrophil degranulation, $CD3^+$, $CD4^+$, and $CD8^+$ cell counts compared with prolonged exercise in a thermoneutral environment. We also hypothesised that the decrease in bacterially-stimulated neutrophil degranulation post exercise would be associated with an increase in the proportion of band (immature) neutrophils and a decrease in neutrophil activation status (CD11b expression). These results show that prolonged cycling in the heat causes a greater increase in T_{re}, HR, RPE and stress hormones namely cortisol compared with a thermoneutral environment. Prolonged cycling in HOT and thermoneutral CONTROL caused an increase in leukocytes (neutrophil and lymphocyte cell counts), lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$) and band neutrophil count, a decrease in bacterially-stimulated neutrophil degranulation and no change in the percentage of band neutrophils and CD11b expression. There was no effect of the additional heat stress other than on monocyte count at post- and 2 h post-exercise; therefore, these data do not support the initial hypotheses.

The results show an increase in leukocyte and neutrophil cell counts after prolonged exercise regardless of the addition of heat stress. These findings are similar to those of McFarlin and Mitchell, (2003) however, contrast with studies showing greater circulating leukocyte counts, (Rhind *et al.* 1999; Mitchell *et al.* 2002) and neutrophil responses (Cross *et al.* 1996; Niess *et al.* 2003) after exercising in the heat compared to cooler conditions. The final T_{re} reported by McFarlin and Mitchell (2003) was similar to our study (38.7 °C in the heat) whereas Rhind *et al.*(1999) and Niess *et al.*(2003)

reported 39.3 °C and 39.8 °C respectively; this might explain the lack of an additional effect of exercise in the heat on the leukocyte and neutrophil responses in the present study. Rhind *et al.* (1999) suggested that hyperthermia mediates the exercise induced immune cell redistribution through sympathoadrenal activation with resultant alterations in circulating adrenaline, noradrenaline and cortisol. The plasma cortisol and GH responses to exercise in the heat were more pronounced in another study (Niess *et al.* 2003) which was associated with higher final T_{re} after exercise in the heat. In contrast to Niess *et al.* (2003) we show a modest (19 %) albeit significant, increase in plasma cortisol concentration when exercise was performed in the heat versus control at post-exercise (Figure 6.1A). Substantial biological effects of cortisol have been demonstrated when the plasma cortisol concentration exceeds the capacity of the corticosteroid binding globulins (550 $nmol.l^{-1}$) (McCarthy and Dale 1988). In the present study the delayed effects of plasma cortisol on neutrophil count may have been missed with the recovery sample 2 h post-exercise and may have occurred after this time. Previous studies have suggested a strong link between GH and the delayed increase in neutrophil count post-exercise (Suzuki *et al.* 1999; Suzuki *et al* 2002). Yamada *et al.* (2002) have shown a stronger correlation between post-exercise GH and neutrophil count at 1 h post-exercise (r .59) than post-exercise plasma cortisol and neutrophil count at 1 h post-exercise (r .23). Kappel *et al.* (1998) suggest that GH is partly responsible for the neutrophillia. Given that we observed similar GH responses in the heat and in thermoneutral conditions (Figure 4.1C) and a modest response of elevated plasma cortisol concentration to prolonged exercise in the heat it is not surprising that we find indifferent neutrophil counts with the addition of heat stress.

In direct response to prolonged exercise, $CD3^+$, $CD4^+$ and $CD8^+$ counts all increased; however, there was no additional affect of exposure to heat stress on T-lymphocyte

subsets. Similar to previous authors the post-exercise lymphocytopenia was exacerbated after 2 h recovery (Gannon *et al.* 2001; Natale *et al.* 2003; Niess *et al.* 2003). McFarlin and Mitchell (2003) reported lymphocyte counts to be elevated by 24 % post-exercise in the heat compared with cooler conditions. In contrast to Pedersen *et al.* (1994) and Natale *et al.* (2003), the $CD4^+/CD8^+$ ratio in this study remained unaltered immediately following prolonged exercise. The $CD4^+/CD8^+$ ratio resulted in a delayed increase 2 h post-exercise, which is in accordance with previous literature (Gannon *et al.* 2001; Natale *et al.* 2003). Within the literature, adrenaline and to a lesser extent noradrenaline concentration have been associated with the post-exercise increase in lympocytes (Pedersen *et al.* 1997). Elevated cortisol concentration is also thought to contribute to the post-exercise lymphopenia via selective retention of recirculating lymphocytes within the spleen and lymph nodes (Nielsen *et al.* 1997; Miller *et al.* 1998; Rhind *et al.* 1999). With the increase in plasma cortisol concentration potentially being responsible for the lymphopenia after prolonged exercise we would have expected greater lymphocyte response to prolonged exercise in the heat (similar to the plasma cortisol concentration). However, we found no additional effects of exercise in the heat upon this response.

In accordance with other studies (Blannin *et al.* 1996; Robson *et al.* 1999; Bishop *et al.* 2002; Bishop *et al.* 2003) bacterially-stimulated elastase release per cell was significantly decreased post-exercise. Given that authors have cited the increase in plasma cortisol concentration as potentially being responsible for the decreased neutrophil function after prolonged exercise, we hypothesised that prolonged exercise in the heat with associated increases in plasma cortisol responses would result in a greater reduction in bacterially-stimulated neutrophil degranulation. However, we found no additional effects of exercise in the heat upon this response.

Bishop *et al.* (2001a, 2004) reported a less likely role of cortisol causing a decreased neutrophil degranulation response when performing exercising with carbohydrate beverage ingestion or fluid ingestion alone. Alternatively, the increased cortisol response observed in the present study after prolonged exercise in the heat might not have been great enough to elicit a significant biological effect. Plasma cortisol concentration was only 19 % higher when exercising in the heat compared with thermoneutral conditions. It should be noted that subjects in this study consumed *ad-libitum* water, potentially masking the effects of exercise in the heat on the cortisol response, similar to Bishop *et al.* (2004). Subjects consumed significantly more water on the HOT trial resulting in similar plasma volume change (-7.1 and -6.9 % change post-exercise in hot and control respectively). It has been that fluid intake during prolonged exercise in heat can blunt the plasma cortisol response (Mitchell *et al.* 2002). It has been reported that delayed elevations in neutrophil cell counts are a result of plasma cortisol acting upon neutrophils from bone marrow (Pyne 1994). In this respect the recovery sample 2 h post-exercise may have been premature and missed the delayed effects of plasma cortisol.

These findings do not support the hypothesis that elevated plasma stress hormone concentration indirectly decreases neutrophil degranulation by up-regulating bone marrow release of band (immature) neutrophils that may not be functionally mature (Suzuki *et al.* 1996a). Although the direct effect of cortisol on neutrophil degranulation appears less likely, it has been suggested that elevated plasma cortisol concentration may indirectly reduce superoxide radical production of neutrophils by increasing the bone marrow release of band neutrophils that might not be functionally mature (Suzuki *et al.* 1996a). To investigate this possibility we have reported the percent and absolute band (immature) neutrophil cell counts in this study. Band or nonsegmented neutrophil

counts were significantly elevated immediately post and 2 h post-exercise as a direct consequence of increased neutrophil counts. More importantly, though, there was no increase in percentage band (immature) neutrophil cells at 2 h post-exercise, particularly given the increase in plasma cortisol and serum GH at post-exercise which might be expected to mobilise bone marrow reserves of neutrophils (Pyne 1994; Suzuki *et al.* 1996a; Suzuki *et al.* 1999). This finding contrasts with a regularly reported finding of others showing an increase in circulating band (immature) neutrophil cells after acute exercise (Suzuki *et al.* 1999; Suzuki *et al.* 2000; Suzuki *et al* 2002).

This data shows that a decrease in bacterially-stimulated neutrophil degranulation does not appear to be associated with an increase in the proportion of band neutrophils. Researchers have reported that 60 % of corticosteroid-induced neutrophillia occurs by demargination from intravascular pools of cells at the blood vessel walls, with minor contributions from the bone marrow (Nakagawa *et al.* 1998). This could explain why no increase in band (immature) neutrophils within the present study was observed. Mature neutrophils have a greater number of cytotoxic granules and thus greater total elastase content compared with immature neutrophils (Roitt and Delves 2001). A recent study showing no effects of 2 h of cycling at 75 % $\dot{V}O_{2\ max}$ on neutrophil total elastase content tends to suggest that prolonged intense exercise does not cause an increase in the proportion of immature cells within the circulation (Bishop *et al.* 2003). It should be acknowledged that the delayed effect of raised plasma cortisol concentration and serum GH concentration on % band (immature) neutrophils cells may have been missed by not taking further recovery samples following the 2 h post-exercise sample. It has been reported to take up to 6 h for significant increases in band (immature) neutrophil cells to be detected after infusion of dexamethasone compared with saline (Nakagawa *et al.* 1998).

Neutrophil expression of CD11b is known to increase upon activation (Smith *et al.* 1996). Previously it has been speculated that the decrease in bacterially-stimulated degranulation response might be associated with altered neutrophil activation status (Bishop *et al.* 2003). The effects of exercise on neutrophil CD11b expression are somewhat equivocal. Neutrophil CD11b expression was unaffected by an ultra-distance run (Gabriel *et al.* 1995), increased after an incremental cycle test to exhaustion (van Eeden *et al.* 1999) and decreased after 1 h of running at both 60 and 80 % $\dot{V}O_{2\ max}$ (Peake *et al.* 2004). Our data show that prolonged exercise evokes a reduction in bacterially-stimulated neutrophil degranulation that is unrelated to altered neutrophil activation status as we observed no change in the percentage or the mean fluorescence intensity of neutrophil expression of CD11b. In partial agreement with our findings, a recent study (Peake *et al.* 2004) has shown that changes in CD11b expression after 1 h running did not influence neutrophil degranulation indicated by changes in plasma intracellular myeloperoxidase concentration.

Although a direct or indirect effect of cortisol on neutrophil degranulation cannot be completely discounted, the results of this study and others (Bishop *et al.* 2002; Bishop *et al.* 2003; Bishop *et al.*, 2004) suggest that the decrease in bacterially-stimulated neutrophil degranulation after prolonged exercise may be due to another mechanism. Bishop *et al,* (2003) suggested future attention should be directed at the influence of intracellular calcium signalling, whilst others have suggested further investigation into complement and fragments of C3a and C5a associated with prolonged-exercise (Dufaux and Order, 1989; Dufaux *et al.*, 1991). The prolonged exercise bout in this study was performed in the heat in which only modest core temperature rises were achieved (38.7 °C). It remains to be seen if similar neutrophil responses are observed when exercising with larger increases in core temperature.

6.6 Conclusion

These data suggest that prolonged exercise results in an increase in lymphocyte cell counts and T-lymphocyte subsets, and these are unaffected when performing exercise in hot conditions. These data also suggest that performing prolonged exercise results in a decrease in neutrophil degranulation that is unaffected by performing the exercise in the heat. In addition, the decrease in neutrophil degranulation after prolonged exercise was not associated with a change in neutrophil band cell percentage or neutrophil activation status.

Chapter 7

Human blood neutrophil responses to prolonged exercise with and without a thermal clamp

7.1 Abstract

The purpose of this study was to investigate the effects of prolonged exercise with and without a thermal clamp on neutrophil trafficking, bacterially-stimulated neutrophil degranulation, stress hormone and cytokine responses. Thirteen healthy male volunteers (mean ± SEM: age; 21 ± 1 yr, height; 180 ± 2 cm, mass; 74.9 ± 2 kg, $\dot{V}O_{2\ max}$ 58 ± 1.2 $ml \cdot kg^{-1} \cdot min^{-1}$) completed four randomly assigned, 2 h water immersion trials separated by 7 d. Trials were exercise-induced heating (EX-H: water temperature 36°C) exercise with a thermal clamp (EX-C: 24°C), passive heating (PA-H: 38.5°C) and control (CON: 35°C). EX-H and EX-C was comprised of 2 h deep water running at 58 maximal oxygen uptake. Blood samples were collected at pre-, post- and 1 h post-immersion. Core body temperature was unaltered on CON, clamped on EX-C (-0.02°C) and rose by 2.23°C and 2.31°C on EX-H and PA-H, respectively. Exercising with a thermal clamp did not blunt the neutrophilia post-exercise (EX-C post-exercise: 9.6 ± 1.1 and EX-H post-exercise: 9.8 ± 1.0 x 10^9 $cells.l^{-1}$). Neutrophil degranulation decreased to a similar extent immediately after EX-C and EX-H (-28%; $P<0.05$). EX-C blunted the circulating noradrenaline, cortisol, G-CSF and IL-6 response ($P < 0.01$) but not the plasma adrenaline and serum GH response. These results show a similar neutrophilia and decrease in neutrophil degranulation after prolonged exercise with and without a thermal clamp. As such, the rise in core body temperature does not appear to mediate neutrophil trafficking and degranulation responses to prolonged exercise. In addition, these results suggest a limited role for cortisol, G-CSF and IL-6 in the observed neutrophil responses to prolonged exercise.

7.2 Introduction

Although evidence supports a relationship between neuro-endocrine and immune responses to exercise (Hoffman-Goetz and Pedersen 1994) a clear role for stress hormones in the neutrophilia of prolonged exercise (lasting >1 h) has not been identified. Compared with thermoneutral conditions, greater circulating leukocyte counts, catecholamine and cortisol concentrations have been observed following short fixed-duration exercise in hot conditions and passive heat exposure (Severs *et al* 1996; Brenner *et al* 1997; Maughan 1997; Galloway and Maughan 1997; Febbraio 2001; Mitchell *et al.* 2002). Indeed, elevations in circulating leukocyte and neutrophil counts similar to exercising levels have been observed after infusion of adrenaline (Kappel *et al.* 1991b), cortisol (Tonnesen *et al.* 1987) and growth hormone (GH) (Kappel *et al.* 1993). Exercising in cold water (thermal clamp), albeit lasting only 40 min, substantially blunted the rise in circulating catecholamines, cortisol and GH, and was associated with smaller increases in circulating leukocytes compared with thermoneutral conditions (Cross *et al.* 1996; Rhind *et al.* 1999). However, recent studies have indicated a likely role for granulocyte-colony stimulating factor (G-CSF) in the mobilization of neutrophils into the circulation following sustained hyperthermia in rats (Ellis *et al.* 2005) and brief incremental exercise to exhaustion in humans (Yamada *et al.* 2002). In addition, interleukin-6 (IL-6) concentration 1 h after exercise correlated more strongly than cortisol with circulating neutrophil counts 2 h after exercise which also indicated a possible role for IL-6 in the delayed neutrophilia of exercise (Yamada *et al.* 2002). Unfortunately, these studies used short duration exercise lasting ~10 min (Yamada *et al.* 2002) or 40 min (Cross *et al.* 1996; Rhind *et al.* 1999) or were conducted in animals (Ellis *et al.* 2005). Therefore, a role for raised circulating catecholamines, stress hormones and more recently cytokines (G-CSF and IL-6) as potential mediators of the neutrophilia of prolonged exercise has not been studied in

humans. In the present study a thermal clamp study was adopted to delineate the influence of stress hormones and cytokines in the neutrophilia of prolonged exercise.

Little is known about the possible mechanism(s) responsible for suppressed *in vitro* neutrophil degranulation after prolonged exercise (Robson *et al.* 1999; Bishop *et al.* 2003). Elevated cortisol within the physiological range decreases *in vitro* neutrophil chemotaxis (Forslid and Hed 1982; Salak *et al.* 1993) and has been implicated in the decreased bacterially-stimulated neutrophil degranulation after prolonged exercise (Robson *et al.* 1999). However, a more recent study showed that blunting the circulating cortisol response to prolonged exercise with carbohydrate feeding did not significantly alter neutrophil degranulation responses during recovery, indicating a less likely role for cortisol in the decreased bacterially-stimulated neutrophil degranulation after prolonged exercise (Bishop *et al.* 2003). Controversy also surrounds the temperature dependency of neutrophil function(s). Although some authors report enhanced neutrophil bactericidal capacity and migration at higher *in vitro* temperatures (38-39 °C) (Nahas *et al.* 1971; Roberts and Sandberg 1979; Pramanik *et al.* 2004), others report inhibited neutrophil motility and unaltered oxidative burst activity as *in vitro* temperature is raised (Roberts and Sandberg 1979; Pedersen *et al.* 1994). Running at 75% $\dot{V}O_{2max}$ for 1 h in hot (28 °C: final rectal temperature (T_{re}) 39.8 °C) compared with thermoneutral (18°C: T_{re} final 38.7 °C) conditions led to larger increases in circulating noradrenaline, cortisol, GH and neutrophils and lower unstimulated myeloperoxidase (MPO) release per neutrophil (Niess *et al.* 2003). Since a larger neutrophilia after exercise in hot conditions was not paralleled by a larger increase in plasma MPO, the authors speculated that this might reflect a suppressive effect of heat stress on neutrophil activation (Niess *et al.* 2003).

With this information in mind, using a water-immersion technique to control body temperature, the purpose of this study was to investigate thermal effects and associated hormone and cytokine involvement in neutrophil trafficking and neutrophil degranulation responses after prolonged exercise. We hypothesised that a thermal clamp would blunt circulating stress hormone and cytokine responses and the associated neutrophilia. We also hypothesized that a thermal clamp would decrease neutrophil degranulation after prolonged exercise.

7.3 Methods

7.3.1 Participants

Thirteen healthy, recreationally active males (mean ± SEM: age 21 ± 1 yr; height 180 ± 2 cm; body mass 74.9 ± 2.1 kg; $\dot{V}O_{2\ max}$ 58.1 ± 1.2 $ml \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in this study. All subjects gave written informed consent before starting the study, which received local ethics committee approval. There were no reported symptoms of infection and all subjects were taking no medication or nutritional supplements in the six weeks prior to the study.

7.3.2 Preliminary Measurements

Prior to the main experimental trials, and after abstaining from exercise for 24 h, each subject completed an incremental exercise test to volitional exhaustion to determine maximal oxygen uptake ($\dot{V}O_{2\ max}$). The test was conducted on a treadmill (Powerjog, Sports engineering Ltd, Birmingham, UK) as described in Chapter 3. During the initial visit to the laboratory, subjects were also familiarized with the deep-water running technique used in the experimental trials (Reilly *et al.* 2003). Subjects ran for 30 min in a tank immersed in thermoneutral water to shoulder level, suspended by a harness and ropes for support.

7.3.3 Experimental Procedures

In a randomized order, subjects performed four 2 h water immersion trials separated by 7 d. Trials were thermoneutral exercise (TE: water temperature 36.3 ± 0.3 °C) cold exercise (CE: 23.5 ± 0.9 °C), thermoneutral seated (TS: 35.3 ± 0.2 °C) and hot seated (HS: 38.5 ± 0.2 °C). Exercise trials (TE and CE) comprised deep water running at 58.1 ± 0.7 and 58.9 ± 0.5 % $\dot{V}O_{2max}$ determined using expired gas collected in Douglas bags (Harvard Apparatus, Edenbridge, UK). Rate of oxygen uptake ($\dot{V}O_2$), rate of expired

carbon dioxide ($\dot{V}CO_2$) and expired minute ventilation ($\dot{V}E$) were determined using a combined paramagnetic oxygen and infrared carbon dioxide analyzer (Servomex 1420B, Crowborough, UK), and a dry gas meter (Harvard Apparatus, Edenbridge, UK). During the 24 h period prior to each experimental trial subjects were required to refrain from exercise, caffeine and alcohol and record their food intake in an effort to standardize their nutritional status. At 08:00 h on the morning of each experimental trial subjects consumed a standardized breakfast (816 kcals; carbohydrate 78 %; fat 14 % and protein 8 %) and drank only water until their arrival at the laboratory at 11:30 h. The experimental trials were performed at 12:00 h. On arrival at the laboratory subjects were asked to empty their bladder and bowels and nude body mass was obtained (Seca 705, Hamburg, Germany). To ensure subjects arrived euhydrated a urine osmolality measure was performed and all recorded samples were below the 900 $mOsmol \cdot kg^{-1}$ threshold for euhydration (Shirreffs and Maughan 1998). Subjects were then seated for 15 min prior to a resting blood sample which was collected from an antecubital vein by venepuncture. In all trials subjects wore swimming shorts only. Heart rate (Polar Electro, Kempele, Finland), rectal temperature (T_{re}: YSI 4000A, Daytona, USA) and thermal sensation (McGinnis 1-13 rating of thermal comfort) (Hollies 1977) were recorded at 10 min intervals throughout exercise and seated immersion trials; whilst expired gas and RPE were recorded at 10 min intervals during exercise trials only. Water consumption was permitted *ad-libitum.* Immediately post immersion a venous blood sample was obtained prior to nude body mass being recorded. All subjects remained fasted until a further venous blood sample and nude body mass was obtained 1 h post-immersion. For all blood samples, 19 ml of blood was collected, with the subject in a seated position.

7.3.4 Analytical Methods

Haematological parameters including Hb, Hct, plasma volume change, total and differential leukocyte counts, bacterially-stimulated neutrophil degranulation was determined as described in Chapter 3. Plasma concentrations of elastase, cortisol and G-CSF and serum concentrations of IL-6 and GH concentrations were measured as described in Chapter 3. Aliquots of EDTA plasma were used for the determination of adrenaline and noradrenaline using high-pressure liquid chromatography (Knauer, Berlin, Germany; cColumn, Lichtopher 60, RP Select B, 5 μm/ 250x4 mm, Merck, Germany). Catecholamine concentrations were determined by means of electrochemical detector (2143-RPE, Pharmacia LKB, Freidburg, Germany).

7.3.5 Statistical Analysis

The data were examined using a repeated measures ANOVA design. The Gpower power calculator was used to determine sample sizes. The sample size was estimated to be N = 10 using previous data examining the effects of exercise on neutrophil degranulation responses (Walsh *et al.* 2000). Alpha and power levels were set at 0.05 and 0.8 respectively, both of which are standard estimates. To allow for dropout 13 subjects were recruited.

7.4 Results

7.4.1 Rectal temperature (T_{re}), Physiological Variables and RPE

There was a significant trial x time interaction for T_{re} ($F_{(4,44)}$ = 80.0, P < 0.01). Progressive increases in T_{re} occurred during the 2-h immersion on EX-H and PA-H (P < 0.01; Figure 7.11). T_{re} increased by 2.23 ± 0.08 °C and 2.31 ± 0.07 °C on EX-H and PA-H respectively, remained unaltered on CON and was successfully clamped on EX-C (T_{re} peak at 20 min: 0.18 ± 0.08 °C, P < 0.01).

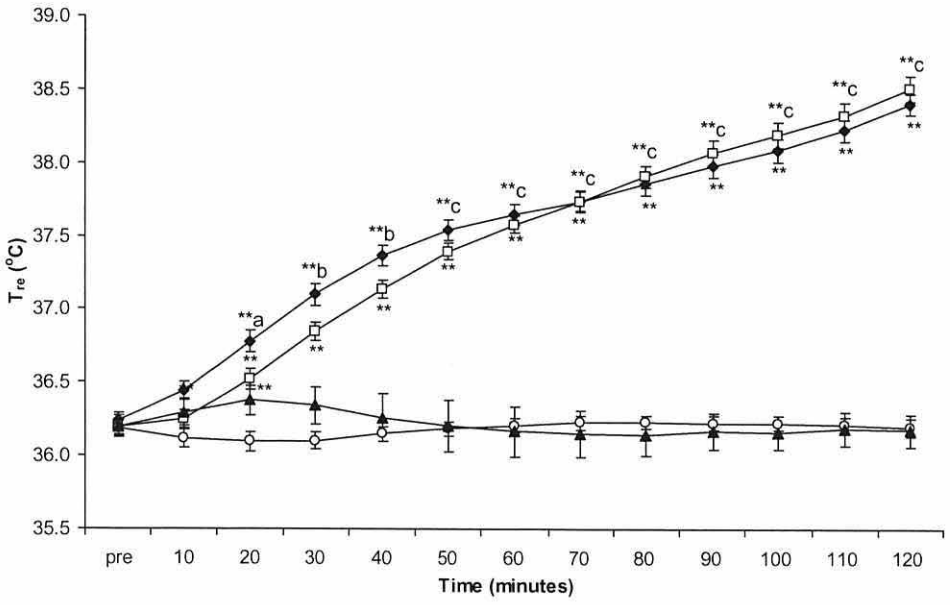

FIGURE 7.1 The effects of 2 h exercise at 58 % $\dot{V}O_{2max}$ immersed in thermoneutral water (exercised induced heating; EX-H; ♦) and cold water (EX-C; ▲) and 2 h seated in thermoneutral water (CON; ○) and hot water (PA-H; □) on rectal temperature (T_{re}). Values are mean ± SE. Significantly greater than pre-immersion ** P < 0.01, a; EX-H>PA-H+EX-C>CON (P < 0.05), b; EX-H>PA-H>EX-C+CON (P < 0.01), c; EX-H+PA-H>EX-C+CON (P < 0.01).

There was a significant trial x time interaction for HR ($F_{(36,432)}$ = 28.2; P < 0.01; Table 7.1), RPE ($F_{(12,144)}$ = 30.7; P < 0.01) $\dot{V}E$ ($F_{(3,27)}$ = 238; P < 0.01), and thermal sensation ($F_{(5,56)}$ = 13.4; P < 0.01). HR increased during 2-h immersion on EX-H, EX-C and PA-H compared with pre-immersion (P < 0.01; Table 7.1). In contrast, there were no

significant changes from pre-immersion observed for HR during CON. Exercising with a thermal clamp (EX-C) resulted in lower HR and RPE responses but similar $\dot{V}E$ compared with exercise associated with a rise in T_{re} (EX-H; $P < 0.01$; Table 1). Reports of thermal sensation were significantly higher on EX-H and PA-H between 10 and 120 min compared with pre-immersion and both EX-C and CON (10 to 20 min, $P < 0.05$; 30 to 120 min, $P < 0.01$). In addition, there were no differences in thermal sensation during CON and EX-C compared with pre immersion; however, exercising with a thermal clamp (EX-C) evoked significantly lower thermal sensation than CON for the entire duration of immersion ($P < 0.05$; Table 7.1).

Body mass losses were significantly greater on EX-H compared with CON ($P < 0.05$; Table 1). After correction for fluid intake, estimated total sweat loss was significantly greater on EX-H and PA-H compared with EX-C and CON ($P < 0.01$; Table 7.1). Plasma volume decreased on all trials compared with pre-immersion (CON, $P < 0.05$; EX-H, PA-H and EX-C, $P < 0.01$; Table 7.1). The decrease in plasma volume post-immersion was significantly greater on EX-C and PA-H compared with CON ($P < 0.01$; Table 7.1).

TABLE 7.1 The effects of 2 h exercise at 58 % $\dot{v}O_{2max}$ immersed in thermoneutral water (EX-H) and cold water (EX-C) and 2 h seated in thermoneutral water (CON) and hot water (PA-H) on HR, RPE, ($\dot{v}E$) ventilation, thermal sensation, body mass loss (BML), sweat loss (estimated) and plasma volume change (PVC).

	EX-H	EX-C	PA-H	CON	Interaction
HR (beats·min^{-1})[a]	164 ± 3**	134 ± 2**	120 ± 4**	73 ± 3	EX-H>EX-C>PA-H>CON
RPE[a]	18 ± 1**	13 ± 0**	—	—	EX-H>EX-C
$\dot{v}E$ (l·min^{-1})[b]	68.6 ± 2.5	69.2 ± 2.5	26.1 ± 2.6	13.9 ± 1.5	EX-H+EX-C>PA-H>CON
Thermal sensation[a]	11 ± 0**	6 ± 0	11 ± 0**	7 ± 0	EX-H+PA-H>CON>>EX-C
BML (Kg)[a]	1.1 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	0.4 ± 0.2	EX-H>>CON
Sweat loss (L)[b]	2.3 ± 0.3	0.7 ± 0.1	2.2 ± 0.2	0.5 ± 0.1	EX-H+PA-H>EX-C+CON
PVC (%)[a]	-6.7 ± 1.4**	-9.7 ± 1.0**	-9.0 ± 1.6**	-4.2 ± 1.0*	EX-C+PA-H >CON

Values are mean ± SE. [a] Immediately post-immersion compared with pre-immersion, [b] after correction for fluid intake. Significant difference from pre-immersion; * $P < 0.05$, ** $P < 0.01$, Interaction; >> $P < 0.05$ and > $P < 0.01$.

7.4.2 Neutrophil Responses

There was a significant trial x time interaction observed for circulating neutrophil count ($F_{(6,72)} = 13.2$; $P < 0.01$; Figure 7.2A), plasma elastase concentration ($F_{(6,72)} = 3.8$; $P < 0.05$; Figure 7.2B), and bacterially-stimulated elastase release per neutrophil ($F_{(6,66)} = 3.4$; $P < 0.05$; Figure 7.2C). The magnitude of the neutrophilia was similar when exercising with (EX-C) and without (EX-H) a thermal clamp and was greater after exercise (EX-C and EX-H) than passive heat stress (PA-H) at post and 1 h post immersion ($P < 0.01$; Figure 7.2A). Unstimulated plasma elastase concentration increased following exercise (EX-C and EX-H) compared with pre-immersion and was greater than PA-H and CON at this time (Figure 7.2B). Unstimulated plasma elastase concentration remained elevated on EX-C and EX-H trials above baseline, CON and PA-H at 1 h post-immersion ($P < 0.01$; Figure 7.2B). Bacterially-stimulated elastase release per neutrophil decreased significantly after exercise (EX-C and EX-H)

compared with pre-immersion (Figure 2C). Heat stress alone evoked a 21 % reduction in neutrophil degranulation at post-immersion although this was not significant. Bacterially-stimulated elastase release per neutrophil remained 29 % below pre-immersion values 1 h post-immersion on the EX-C trial and was significantly lower than CON at this point ($P < 0.01$; Figure 7.2C).

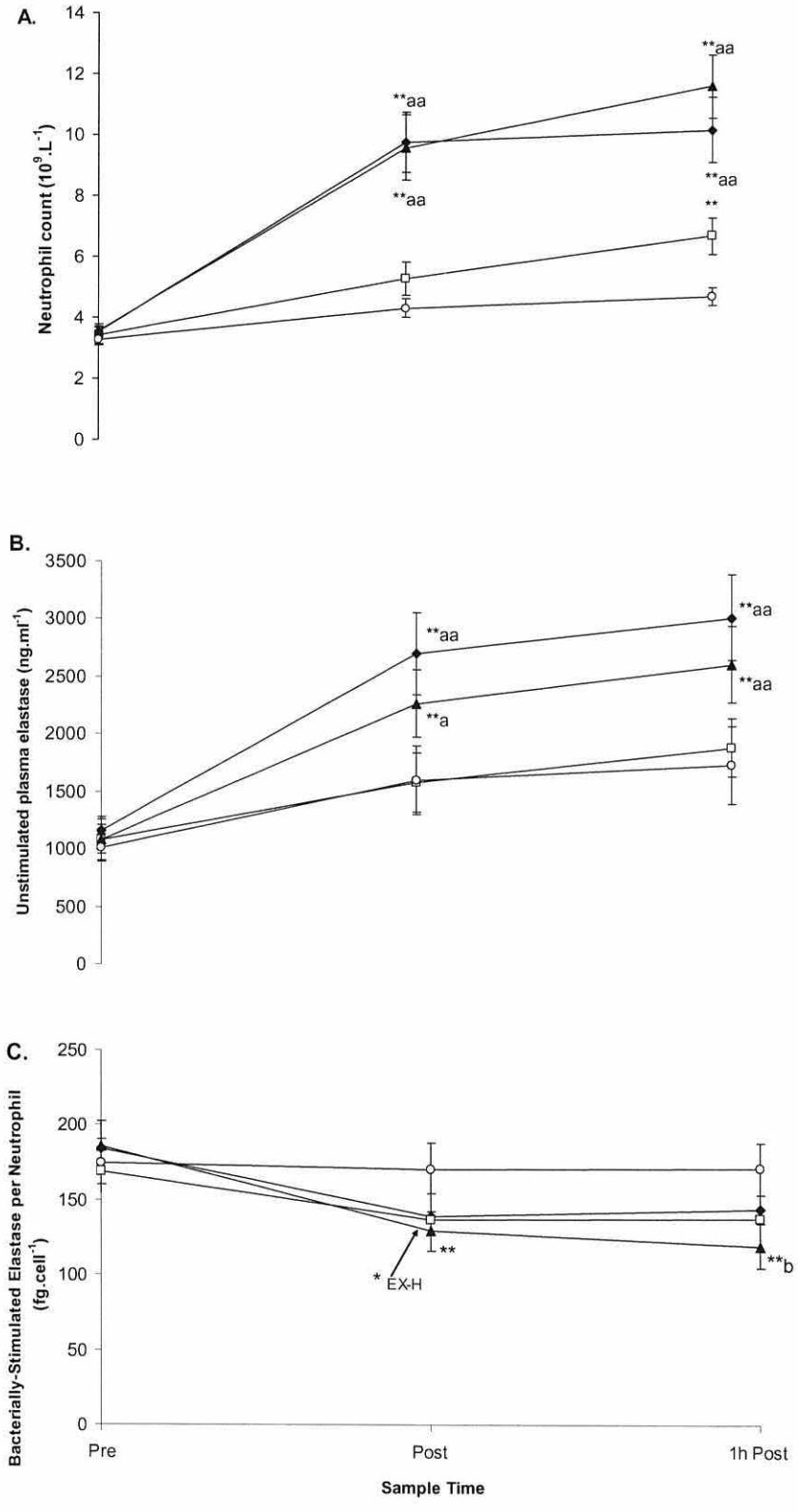

FIGURE 7.2 The effects of 2 h exercise at 58 % $\dot{V}O_{2max}$ immersed in thermoneutral water (exercised induced heating; EX-H; ♦) and cold water (EX-C; ▲) and 2 h seated in thermoneutral water (CON; ○) and hot water (PA-H; □) on (A) neutrophil count, (B) unstimulated plasma elastase (N = 11) and (C) bacterially-stimulated elastase release per neutrophil (N = 10). Values are mean ± SE. Significant difference from pre-immersion * $P < 0.05$ and ** $P < 0.01$, a; > CON+PA-H ($P < 0.05$), aa; > CON+PA-H ($P < 0.01$), and b; EX-C < CON ($P < 0.01$).

7.4.3 Circulating Hormone and Cytokine Responses

There was a significant trial x time interaction observed for plasma adrenaline ($F_{(6,48)}$ = 4.4; $P < 0.01$; Figure 7.3A), plasma noradrenaline ($F_{(6,48)} = 8.2$; $P < 0.01$; Figure 7.3B), plasma cortisol concentration ($F_{(4,42)}$ = 24.9; $P < 0.01$; Figure 4A), serum GH concentration ($F_{(2,24)} = 9.3$; $P < 0.01$; Figure 7.4B), plasma G-CSF concentration ($F_{(2,24)}$ = 8.8; $P < 0.01$; Figure 7.4C) and serum IL-6 concentration ($F_{(2,26)}$ = 7.4; $P < 0.01$; Figure 7.4D). These results indicate that exercising with a thermal clamp (EX-C) blunts the circulating noradrenaline, cortisol, G-CSF and IL-6 response ($P < 0.01$; Figure 7.3B, 4, A, C and D). In contrast, exercising with a thermal clamp (EX-C) did not significantly blunt the plasma adrenaline and serum GH response (Figure 7.3A and 7.4B). Plasma adrenaline and noradrenaline were significantly elevated post-immersion on EX-H, PA-H and EX-C ($P < 0.01$; Figure, 7.3, A and B) compared with pre-immersion. Post-immersion increases in circulating cortisol, G-CSF and IL-6 tended to occur only when T_{re} increased (EX-H and PA-H), with the exception being plasma G-CSF, which also increased at post-immersion on CON ($P < 0.01$). Circulating cortisol, G-CSF and IL-6 remained elevated 1 h post-immersion on EX-H and PA-H ($P < 0.01$; Figure 7.4, A, C and D). Serum GH increased post-immersion on EX-H, PA-H and EX-C ($P < 0.01$; Figure 7.3B) and returned to within pre-immersion levels by 1 h post-immersion.

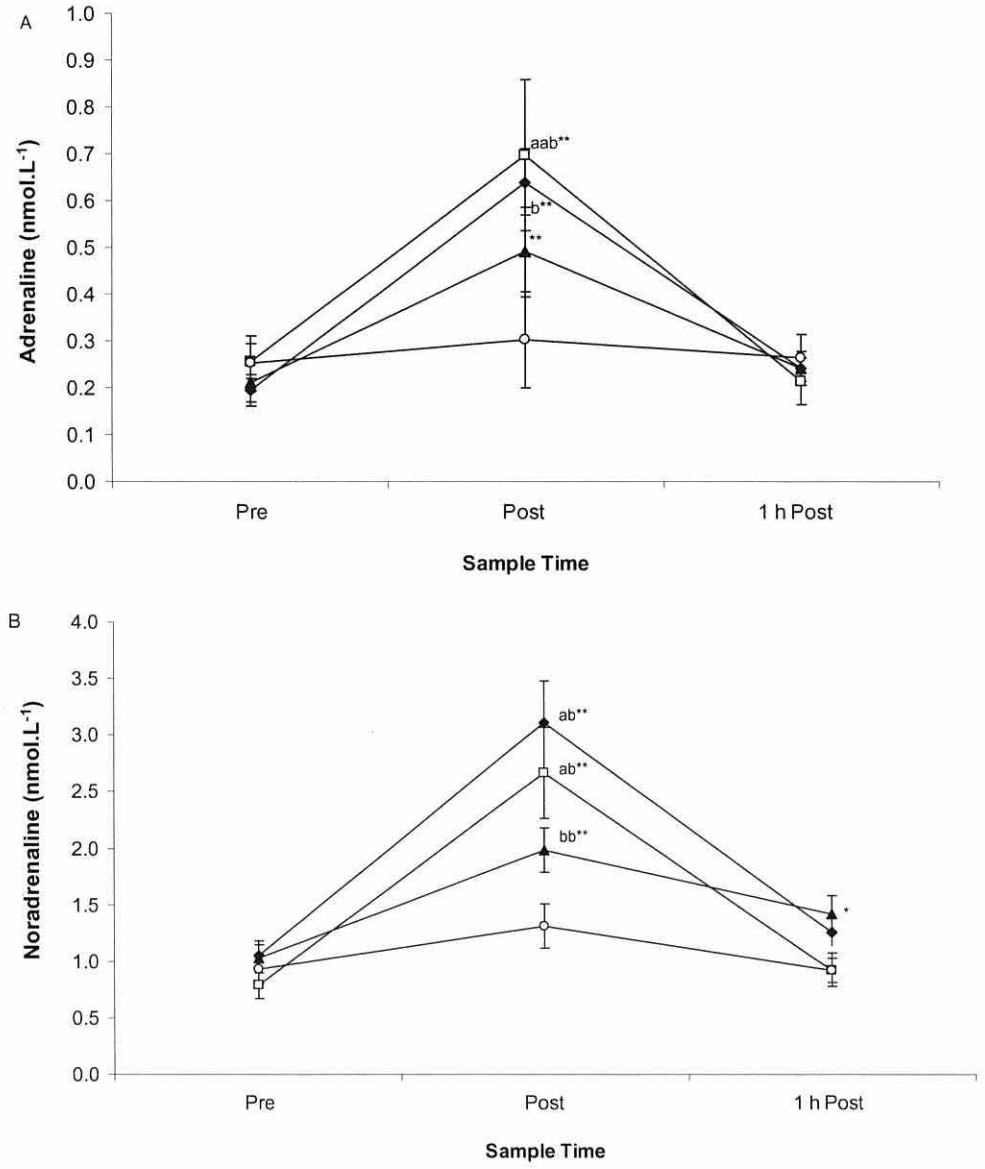

FIGURE 7.3 The effects of 2 h exercise at 58 % $\dot{v}O_{2max}$ immersed in thermoneutral water (exercised induced heating; EX-H; ♦) and cold water (EX-C; ▲) and 2 h seated in thermoneutral water (CON; ○) and hot water (PA-H; □) on (A) Plasma adrenaline and (B) Plasma noradrenaline concentration (N = 9). Values are mean ± SE. Significant greater than pre-immersion * P <0.05, ** P <0.01, a; > EX-C (P < 0.01), aa; > EX-C (P < 0.05), b; > CON (P < 0.01) and bb; > CON (P < 0.05).

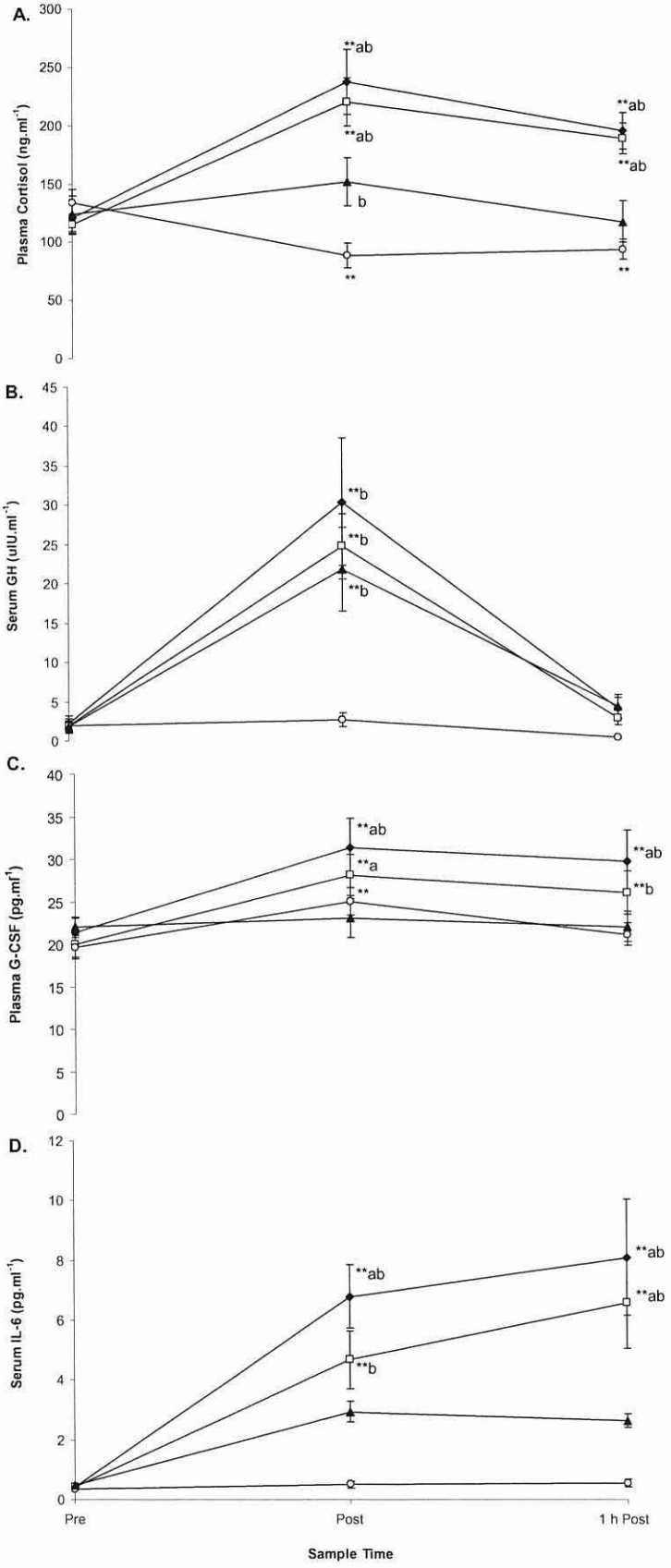

FIGURE 7.4 The effects of 2 h exercise at 58 % $\dot{v}O_{2max}$ immersed in thermoneutral water (exercised induced heating; EX-H; ♦) and cold water (EX-C; ▲) and 2 h seated in thermoneutral water (CON; ○) and hot water (PA-H; □)on (A) Plasma cortisol, (B) Serum GH (N = 12), (C) Plasma G-CSF and (D) Serum IL-6 concentration. Values are

mean ± SE. Significant difference from pre-immersion ** P <0.01, a; > EX-C (P < 0.01), b; > CON (P < 0.01).

7.5 Discussion

The purpose of this study was to investigate thermal effects and associated hormone and cytokine involvement in neutrophil responses after prolonged exercise. By using a water immersion technique, the rise core body temperature associated with prolonged exercise was successfully clamped (T_{re} -0.02 °C on EX-C and +2.2 °C on EX-H vs. pre-immersion; Figure 7.1). The addition of the PA-H trial was a particular strength of the study design because this enabled us to identify the effect of a rise in core body temperature alone on neutrophil responses (T_{re} +2.31 °C vs pre-immersion). In direct contrast to our hypothesis, these results show a similar neutrophilia and similar decrease in bacterially-stimulated neutrophil degranulation after prolonged exercise with and without a thermal clamp. As such, the rise in core body temperature does not appear to mediate the observed neutrophil responses to prolonged exercise.

In partial support of our hypothesis, EX-C blunted increases in circulating noradrenaline, cortisol, G-CSF and IL-6 but not adrenaline or GH. Given the similar neutrophilia on EX-C and EX-H, these results suggest a more limited role for cortisol, G-CSF and IL-6 in the neutrophilia of prolonged exercise than previously indicated after short-term exercise (Cross *et al.* 1996; Rhind *et al.* 1999; Yamada *et al.* 2002). Similar increases in circulating catecholamines and GH immediately after PA-H and EX-H with no significant neutrophilia on PA-H at this time indicate a less likely role for catecholamines and GH in the neutrophilia immediately after prolonged exercise. A less likely role for adrenaline is in accordance with the finding that β-blockade (propranolol) did not alter the neutrophilia of submaximal exercise (lasting ≤ 25 min) (Foster *et al.* 1986). Propranolol lowered existing HR (~ 14%) but did not alter cardiac output.

Consequently, the authors proposed a mechanical effect of raised cardiac output to account for the neutrophilia immediately after exercise via demargination from the pulmonary circulation (Foster *et al.* 1986). Since we recorded HR and not cardiac output (HR was lowered during EX-C vs. EX-H), measurement of cardiac output using the CO_2 re-breathing technique might support a pivotal role for raised cardiac output in the neutrophilia immediately after more prolonged exercise. Similar $\dot{V}E$ on EX-C and EX-H supports early work indicating a role for ventilatory patterns in neutrophil recruitment into the circulation (Bierman *et al.,* 1952). However, more recent work has discounted an involvement of raised $\dot{V}E$ in the neutrophilia of short-term exercise (Fairbarn *et al.* 1993).

Approximately one-half of all neutrophils in the blood are marginated to the vascular endothelium, and one-half are freely circulating (Athens *et al.,* 1961). Consequently, demargination of neutrophils (e.g. via the increase in cardiac output) might account for an approximate doubling of circulating neutrophil number immediately after exercise. It might then be reasonable to assume that a small component of the three-fold increase in circulating neutrophils on EX-C and EX-H at post-immersion, and possibly a larger component at 1 h post-immersion, is accounted for by increased bone marrow release and/or increased half-life of neutrophils in circulation. Typically, bone marrow neutrophil release is indicated by an increase in circulating band neutrophils (Orr *et al.* 2005). However, large variation exists in the percentage of band neutrophils reported in resting healthy males: large variation even exists in papers from the same research group e.g. 10% (Suzuki *et al.* 2003a) and 29% (Yamada *et al.* 2002). Exercise of similar duration and intensity to the present study did not alter the percentage of band neutrophils (Chapter 6). In addition, since EX-C abolished increases in circulating levels of known mediators of bone marrow neutrophil release (e.g. cortisol, G-CSF and

IL-6) (Suzuki *et al.* 1996a; Yamada *et al.* 2002), the contribution of bone marrow release to the observed neutrophilia at post-immersion and 1 h post-immersion might be considered small. A more objective and quantitative method of determining bone marrow contribution to the neutrophilia of prolonged exercise may be to determine neutrophil expression of $CD10^{-}/CD16^{low}$ using flow cytometry (Orr *et al.* 2005). Using flow cytometry before and after cardiac surgery a unique subpopulation of neutrophils expressing $CD10^{-}/CD16^{low}$ was identified indicative of active bone marrow band forms. $CD10^{-}/CD16^{low}$ neutrophils were reported to emerge at the same operative stages as active bone marrow band forms and represented over 40% of circulating neutrophils postoperatively (Orr *et al.*, 2005). The present findings indicate that the mediators of the neutrophilia immediately after and 1 h after prolonged exercise may be different from those identified for short term exercise and require further clarification.

In direct contrast to our hypothesis, EX-C evoked a similar reduction in bacterially-stimulated neutrophil degranulation as with EX-H. In addition, PA-H evoked a 21% reduction in bacterially-stimulated neutrophil degranulation although this was not statistically significant. Since EX-C blunted increases in circulating cortisol, G-CSF and IL-6, it is unlikely that these are important mediators of neutrophil degranulation as suggested previously (De Haas *et al.* 1994; Johnson *et al.* 1998; Robson *et al.* 1999). Raised circulating adrenaline and GH and decreased neutrophil degranulation immediately after EX-C and EX-H (and to a certain degree PA-H) indicate a more likely role for these hormones in the decrease in neutrophil degranulation. In cardiopulmonary bypass patients, cardiac-selective β-blockade (esmolol) prevented the increase in circulating elastase 5 h after surgery, indicating a depressive effect of adrenaline on neutrophil degranulation (Boldt *et al.* 2004). Adrenaline (albeit $\geq$ 10 nM) has been showed to down regulate *in vitro* bacterially-stimulated neutrophil elastase

release via cAMP-mediated enhancement of the clearance of cytosolic Ca^{2+} (Tintinger *et al.* 2001). Since propranolol attenuated this effect, a role for β_2-adrenoreceptors in the anti-inflammatory interaction of adrenaline and neutrophils was suggested. Further support comes from studies showing that β-agonists have favorable outcomes for recovery from acute lung injury where suppressing the harmful effects of activated neutrophils is beneficial (Ware and Matthay 2000). Research examining a role for adrenaline in the decrease in neutrophil degranulation after prolonged exercise is limited. One recent study showed that carbohydrate ingestion blunted the plasma adrenaline response (post-immersion 0.6 nM vs. 1.4 nM on placebo) but did not alter the neutrophil degranulation response to the second of two 90 min bouts of cycling at 70 $VO_{2\ max}$ (Li and Gleeson 2005). Previous research has not identified a role for GH in the decrease in neutrophil degranulation after prolonged exercise. Repeating PA-H with propranolol (β-blockade) and somatostatin (GH-blocker) may verify whether these hormones are involved in the decrease in neutrophil degranulation immediately after and also possibly 1 h after immersion. There is some evidence that neutrophils remain in a refractory state for up to two hours following activation (Henson *et al.* 1978). A role for complement C5a in secretary desensitization in human neutrophils has been shown (Henson *et al.* 1978), and circulating C5a concentration increased six-fold after a marathon race (Castell *et al.,* 1997). A potential for raised circulating C5a in the decrease in bacterially-stimulated neutrophil degranulation after prolonged exercise also warrants investigation. Exposing neutrophils collected at pre-immersion with plasma collected at post-immersion may unravel if circulating factors in the blood are responsible for the decrease in neutrophil degranulation after prolonged exercise (and passive heating).

In line with Chapter 6 these findings indicate a limited effect of additional heat stress (EX-H +2.2°C T_{re} *vs.* EX-C), at least within the range of T_{re} reported here, on the neutrophil degranulation response to prolonged exercise. A limitation of the previous study was that T_{re} was only 0.6°C greater immediately after exercise in hot compared with thermoneutral condition. We recognise that, due to ethical committee restrictions, the absolute final T_{re} (EX-H 38.4 °C) represents relatively modest heat stress compared with responses reported in individuals undertaking vigorous exercise in hot conditions (Roberts, 1989). We also recognise that more frequent blood sampling during immersion and for a longer time period during recovery may provide further insight into the potential mediators of neutrophil responses. For example, neutrophil cell counts had not returned to pre-immersion levels at 1 h post-immersion (EX-C, EX-H and PA-H). In addition, the time course of recovery for neutrophil degranulation after prolonged exercise remains a topic of debate. Neutrophil degranulation recovered to within pre-exercise levels by 2 h post-exercise after cycling for 120 min at 60% VO_{2max} (Chapter 6) but remained depressed for 24 h after cycling to fatigue (lasting on average 164 min) at 55% VO_{2max} (Robson *et al.* 1999).

7.6 Conclusion

These results show a similar neutrophilia and decrease in neutrophil degranulation after prolonged exercise with and without a rise in core body temperature. As such, the rise in core body temperature does not appear to mediate neutrophil trafficking and degranulation responses to prolonged exercise. In addition these results suggest a limited role for cortisol, G-CSF and IL-6 in the observed neutrophil trafficking and degranulation responses to prolonged exercise.

Chapter Eight

General Discussion

Background

Athletes and military personnel frequently undergo rigorous heavy training and often compete in strenuous long duration events. In addition, these physically demanding periods are often coupled with forced or voluntary fluid and energy restriction, sleep deprivation, shifts in circadian rhythms, exposure to extremes of hot and cold environments, and severe psychological stressors. It is not surprising that URTI's are thought to be the most common illness experienced by athletes (Berglund and Hemmingsson 1990; Mackinnon and Jenkins 1993b) and military personnel during training (Lee *et al* 1992; Martinez-Lopez *et al.* 1993). Increased susceptibility to infection may arise due to suppressed neutrophil functions (degranulation, oxidative burst and phagocytic activity) and lowered T-lymphocyte subset counts ($CD3^+$ and the ratio of $CD4^+$ and $CD8^+$ cell counts), lymphocyte proliferation and NKCA. The main objectives of this thesis were to investigate the effects upon leukocyte trafficking and bacterially-stimulated neutrophil degranulation of a chronic intensive training period (Chapter 4), a 48 h period of fluid, energy or combined fluid and energy restriction at rest and after exercise (Chapter 5), a 2 h exercise bout with and without additional heat stress (Chapter 6). Furthermore, a water-immersion technique was used to delineate the thermal effects and associated hormone and cytokine involvement in neutrophil trafficking and bacterially-stimulated neutrophil degranulation responses after prolonged exercise (Chapter 7).

Circulating neutrophil counts, neutrophil function and potential immune function mediators

Circulating neutrophil cell counts and bacterially-stimulated neutrophil degranulation was unaffected by performing prolonged exercise in the heat. Similar increases in circulating neutrophil cell counts and similar decreases in bacterially-stimulated

neutrophil degranulation were observed following 2 hours cycling in HOT (30.3 °C) and CONTROL (20.4 °C; Chapter 6) conditions and following 2 hours deep water running with (24 °C water) and without (36 °C water) a thermal clamp (Chapter 7). These results suggest that the rise in core body temperature does not appear to mediate the observed neutrophil responses to prolonged exercise at least within the range of T_{re} reported. These findings are in contrast with those previously reported by Cross *et al.* 1996 and Rhind *et al.* 1999. A limitation of Chapter 6 was that T_{re} was only 0.6°C greater immediately after exercise in hot compared with thermoneutral conditions. It has to be acknowledged that due to ethical committee restrictions, the absolute final T_{re} achieved in Chapter 7 represents relatively modest heat stress compared with responses reported in individuals undertaking vigorous exercise in hot conditions (Roberts, 1989). Sustained exposure to febrile-range hyperthermia (FRH) core temperatures (39.5-40 °C) has been show to induce expression of G-CSF, increase peripheral neutrophil cell counts (Ellis *et al.,* 2005), augment delivery of neutrophils to sites of inflammation (Hasday *et al.,* 2003), accelerate neutrophil apoptosis (Nagarsekar *et al.,* 2008). Based on the body of evidence presented in this thesis and in the literature it would appear that the important factor in altered neutrophil responses to heat stress is the absolute final core temperature rather than the absolute change from pre- to post-exercise/immersion. However, due to the lengthy exposures reported in FRH studies the duration of heat exposure can not be discounted as a factor and warrants further investigation.

Circulating neutrophil cell counts and bacterially-stimulated neutrophil degranulation remained unaltered following 48-h energy restriction, fluid restriction or combined fluid and energy restriction (Chapter 5), and showed small transient increases during 20-week arduous military training (Chapter 4). Increased cortisol concentrations have been implicated in altered neutrophil responses (McCarthy and Dale, 1988; Robson *et al.*

1999; Mitchell *et al.*, 2002). Where 48h-periods of energy restriction, fluid restriction or combined fluid and energy restriction (Chapter 5) were undertaken, it would appear that the stressor was not severe enough to cause alterations in either circulating neutrophil cell counts or circulating cortisol concentrations. However, increases in circulating neutrophil cell counts can not be attributed to increased circulating cortisol concentrations during arduous military training (weeks 11, 12 and 16), following 2 hours cycling in HOT (30.3 °C) and CONTROL (20.4 °C; Chapter 6) conditions and following 2 hours deep water running with (24 °C water) and without (36 °C water) a thermal clamp (Chapter 7). In fact there appears to be a more limited role for not only cortisol but also G-CSF, IL-6, catecholamines and GH in elevated circulating neutrophil counts as suggested previously (De Haas *et al* 1994; Johnson *et al* 1998; Robson *et al.* 1999).

Beyond the scope of the cytokines and cell surface markers measured in this thesis neutrophil priming may have a significant effect on altered cell trafficking observed following exercise stress and energy restriction. There is some evidence that TNF-α primed neutrophils for migration (to CCL3 via CCR5) and that TNF-α upregulated CD11b/CD18 (Mac-1) on neutrophil cell surface (Montecucco *et al.*, 2008). Wengner *et al.* (2008) have shown *in vitro* and *in vivo* that infusion of G-CSF induced neutrophil mobilization by disrupting their SDF-1α-mediated retention in the bone marrow. They also demonstrated that G-CSF acted cooperatively with the chemokine KC/MIP-2 to mobilise neutrophils (Wengner *et al.*, 2008). Interesting the association between increased circulating neutrophil cell counts and decreased neutrophil degranulation and the cytokine G-CSF could not be made when performing prolonged exercise with or without a thermal clamp (Chapter 7). There is some evidence that neutrophils remain in a refractory state for up to two hours following activation (Henson *et al.* 1978). A role

for complement C5a in secretary desensitisation in human neutrophils has been shown (Henson *et al.* 1978), and circulating C5a concentration increased six-fold after a marathon race (Castell *et al.,* 1997). A potential for raised circulating C5a in the decrease in bacterially-stimulated neutrophil degranulation after prolonged exercise also warrants investigation. Exposing neutrophils collected at pre-exercise with plasma collected at post-exercise may unravel if circulating factors in the blood are responsible for the decrease in neutrophil degranulation after prolonged exercise (and passive heating).

Obtaining a general consensus within the literature as to the time course of recovery for altered cell counts and function (i.e. neutrophil degranulation) related to exercise training, prolonged exercise and energy restriction remains a topic of debate. Results are difficult to interpret due to the differing training and exercise durations and intensities and energy restriction durations and types (i.e. complete fast v's supplementation or restriction). It would appear that the two days recovery experienced by Parachute Regiment Recruits during the 20-weeks training (chapter 4) was adequate to maintain circulating neutrophil cell counts and neutrophil cell function. However, 2 hours recovery following prolonged exercise and 6 hours recovery and refeeding following energy restriction and a 30 min self-paced TT was not adequate for neutrophil cell counts to have reduced to pre-exercising level. The duration of the exercise or energy restriction and the magnitude of altered cell counts will impact on the time course of recovery, however further investigations should take this into account with regards to frequency of blood samples during recovery periods.

Circulating lymphocyte and T-lymphocyte subset counts and potential trafficking mediators

Prolonged cycling caused an increase in circulating lymphocyte, $CD3^+$, $CD4^+$ and $CD8^+$ cell counts with no additional influence of exposure to heat stress observed (Chapter 6). However, during the initial weeks of Parachute Regiment Training (weeks 2 and 4) a significant decrease in circulating lymphocyte, $CD3^+$ (week 4) and $CD4^+$ (weeks 2 and 4) cell counts was observed. Similarly, following a 48-h period of energy restriction and a combination of fluid and energy restriction decreases in circulating lymphocyte and $CD4^+$ cell counts was noted. These alterations in circulating lymphocyte and T-lymphocyte subsets appear to be transient in nature as; post-exercise lymphopenia following 2 h prolonged cycling was exacerbated after 2 h recovery (Chapter 6); reduced circulating lymphocyte and $CD4^+$ cell counts observed the morning after P.Company (week 19) during arduous military training returned to within baseline levels following 4 days of rest (Chapter 4); 6 hours re-feeding was enough to return lymphocytes and T-lymphocyte subsets to within normal values following 48-h energy and fluid and energy restriction (Chapter 5).

It would appear that that modest rise in T_{re} (Final T_{re}: 38.7 °C) during prolonged cycling in the heat (Chapter 6) may not have been severe enough to elicit any additive effects of exercising in the heat previously observed in the literature (McFarlin and Mitchell, 2003). Certainly the adaptations to lymphocyte trafficking following P.Company during PARA training support an effect of acute exercise rather than a chronic adaptation caused by training. Additionally, the transient nature of lymphocyte trafficking with energy restriction and refeeding suggests that the mechanism, which remains to be clarified, is reliant upon adequate energy provision because no independent effect of 48

h of fluid restriction (~2.9 $l.d^{-1}$ deficit) on circulating leukocyte, lymphocyte, or lymphocyte subsets was observed.

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The initial decrease in circulating lymphocyte, $CD3^+$ (week 4) and $CD4^+$ (weeks 2 and 4) cell counts coincided with peak incidence of URTI in PARA (weeks 2-3) and significant elevations in serum cortisol concentrations (week 4). It is possible that training programmes induce alterations in cortisol secretion that promote lymphopenia through inhibited entry of lymphocytes into the blood or by promoting lymphocyte movement to tissues (Cupps and Fauci, 1982; Tonnensen *et al.,* 1987). However, during periods of energy restriction (ER and F+ER; Chapter 5) the results do not support such a putative role for cortisol. We observed a decreased circulating lymphocyte, $CD3^+$ and $CD4^+$ counts during 48 h of ER and F+ER in the absence of a change in circulating cortisol. Given that elevated cortisol concentrations are also thought to contribute to the post-exercise lymphopenia via selective retention of recirculating lymphocytes within the spleen and lymph nodes (Nielsen *et al.* 1997; Miller *et al.* 1998; Rhind *et al.* 1999). We might have expected greater lymphocyte response to prolonged exercise in the heat (similar to the plasma cortisol concentration). However, no additional effects of exercise in the heat upon this response were found and this could be due to the relatively small increase (19 %) observed in plasma cortisol concentration (Chapter 6). With reflection of the current findings the mechanism(s) behind altered lymphocyte and T-lymphocyte subset trafficking with exercise training, energy restriction and prolonged exercise remains to be identified. Based on previous literature one can only postulate as to the likely involvement of altered surface expression of adhesion molecules which could reflect shedding of molecules, selective apoptosis, mechanical deformation or active biochemical processes involving catecholamines, cytokines or other hormones (Shephard, 2003; Kruger and Mooren, 2007).

Conclusions

The major conclusions from this thesis are:

I 20-weeks of Parachute Regiment training has a limited effect on self-report incidence of URTI, circulating neutrophil and lymphocyte counts and bacterially-stimulated neutrophil degranulation.

II Increased incidence of URTI upon commencing Parachute Regiment training is likely to be related to the compact living conditions and time of year (seasonality) rather than the physical and psychological stresses of training.

III Forty-eight hours of energy restriction and combined fluid and energy restriction decreased circulating leukocyte, lymphocyte, $CD3^+$ and $CD4^+$ counts but did not alter circulating neutrophil count or degranulation. However, a 48 h period of fluid restriction alone did not alter immune responses either at rest or after a 30 min maximal exercise bout.

IV Six hours refeeding of approximately 100% BML fluids and 1950 Kcal is adequate to restore circulating leukocyte and lymphocyte counts following 48 h energy restriction and combined fluid and energy restriction.

V Forty-eight hours nutrient restriction does not alter circulating leukocyte, lymphocyte and bacterially-stimulated neutrophil degranulation responses to acute maximal intensity exercise.

VI Performing prolonged exercise in hot conditions does not exacerbate the increase circulating neutrophil, lymphocyte T-lymphocyte subset cell counts or the decrease in bacterially-stimulated neutrophil degranulation.

VII The decrease in bacterially-stimulated neutrophil degranulation after prolonged exercise was not associated with a change in neutrophil band cell percentage or neutrophil activation status (CD11b).

VIII The increase in core body temperature does not appear to mediate neutrophil trafficking and degranulation responses to prolonged exercise.

IX The results do not support a likely involvement for raised cortisol, G-CSF and IL-6 in the observed neutrophil trafficking and degranulation responses to prolonged exercise.

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APPENDIX A

The University of Wales, Bangor
School of Sport, Health and Exercise Sciences

Subject Information Sheet

Research Title: The effects of restricted food and/or fluid intake on markers of hydration status, immune function and incidence of upper respiratory tract infections

Research Co-ordinator: Dr Neil Walsh
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Additional Investigators: Sally Wilson, Sam Oliver and Stewart Laing.

Invitation to take part

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

Do I have to take part?

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

Background

An athlete's health and physical performance are negatively affected by body fluid losses (dehydration). Therefore, it is important to be able to identify body fluid losses before severe dehydration disrupts health and performance. Recent investigations by our group have found that simple measurements performed on saliva samples are able to track fluid losses and therefore determine the hydration status of athletes over short periods of time (2-3 hours). Dehydration often results from a combination of improper fluid intake and sweat losses over a number of days. It is not clear if these simple salivary measurements can track hydration status over longer periods of time (e.g. over a number of days). One of the aims of the study is to compare these new saliva markers of hydration with more traditionally used blood and urine hydration markers over a period lasting 3 days. This will enable us to conclude whether saliva markers can be used outside of the laboratory setting to determine an athlete's hydration status.

This study will also examine if a 2 day period of food and/or fluid restriction will decrease immunity and in turn increase the susceptibility to infections (e.g. colds). Military personnel often have to carry out demanding exercises, lasting for many days, on restricted rations and fluid intake. The effects of restricted rations and fluid intake on immunity and infection incidence are relatively unknown. In summary, the aims of this study are to examine the

effects of restricted food and/or fluid intake on markers of hydration status, immunity and the incidence of infection.

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

- to avoid drinking alcohol, caffeinated drinks (i.e. coffee, tea, coke and diet coke) and avoid participating in any exercise for 24 hours before arriving for each visit.

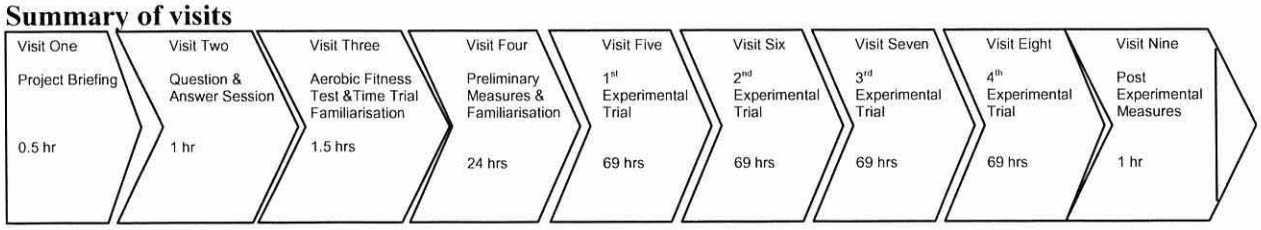
Whilst completing the experimental trials in the laboratory you will be expected;

- to spend 3.5 consecutive days in the laboratory on 4 separate occasions.
- to live and sleep in the laboratory during each 3.5 day trial (Soft furnishings, bedding, computers, TV, video, DVD will be provided and living temperature will be between 17-21°C)
- to eat and drink only what is given to you.
- to follow the study's daily timetable, including going to bed and waking from sleep when asked.
- to perform prescribed physical activity (45 min walking per day).
- to be weighed nude (behind screens to maintain privacy).
- to have 7 blood samples taken during each 3.5 day trial (~98 ml during each trial) totalling 29 blood samples in all during the 6- week study period (~400 ml blood)
- to perform a maximal aerobic test and four 30 min time trials.

You will be excluded if you are a smoker or you are on specific types of medication or supplements (i.e. Creatine)

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~300 HOURS OF YOUR TIME.

Summary of visits



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

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

Visit three: Aerobic fitness test and time trial familiarisation. (~ 1.5 hours)
On an agreed day between 09:00-10:00 hours you will be required to perform a 10-15 min treadmill test that will be used to measure your aerobic fitness (maximal oxygen uptake).

This test will require you to run at your maximal aerobic capacity for approximately one min. After a recovery period, this test will be followed by a familiarisation of the 30 min treadmill time trial that will be used in the main experimental trials. This time trial will require you to run as far as possible in 30 min on a treadmill. You will control the speed of the treadmill throughout the time trial. Completing a practice time trial will help you to pace yourself during the time trial in the main experimental trials.

Visit four: Preliminary measurements and familiarisation (~24 hours)
As you will be required to spend approximately 69 hours residing in the physiology laboratory for each of the four trials we feel it is necessary to familiarise you with the surroundings and measures we will be taking from you. This will help to reduce any anxiety you may have about any of the visits. This visit will also provide us with the time to perform body composition measurements and procedures to estimate your daily energy expenditure. You will also have blood pressure, urine, saliva and blood taken at this visit.

You will arrive at the Physiology laboratory at 08:00 hours after an over night fast (**no breakfast**). You will also have refrained from exercising or consuming alcohol and caffeine (tea, coffee, coke, and diet coke) the day before your visit. Measurements performed on your blood and urine will be used to ensure you are properly hydrated on arrival at the laboratory.

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

Your energy expenditure will be assessed using breath by breath gas analysis which involves you wearing a face mask at rest and during normal activities moving around the laboratory. Familiarisation of blood, urine and saliva techniques will also be performed at this visit. All blood samples will be collected by a qualified member of staff from a forearm vein using a small needle (~14 ml of blood at each collection). A saliva sample will be obtained by placing a cotton swab in your mouth for 2 min and urine will be collected in privacy by passing urine into a plastic container. In order to complete the investigation these samples are a necessity. Breakfast will be provided (~10:00 hours) followed by lunch (~15:30 hours) and an evening meal (~21:30 hours). After the overnight stay you will also receive breakfast and be free to leave the laboratory.

Experimental trials
You will be required to complete four experimental trials in a random order each separated by eight days. Each experimental trial will consist of a 69 hour period where you will reside in the laboratory under treatment conditions (Visit Five). **Including the briefing meeting, familiarisation visit and all four experimental trials you will be required to visit the laboratory on nine occasions for a total of ~300 hours.**

Visit five (~69 hours)
You will arrive at the laboratory at 22:00 hours. We will provide you with water before you go to bed and when you wake up in the morning to make sure you are well hydrated at the start of the trial. The next morning, after confirmation that you are well-hydrated you will begin one of the four 48 hour experimental conditions at random. The trials are as follows: control trial, fluid restriction trial, calorie restriction trial and a fluid and calorie restriction

trial. The control trial will involve you consuming a range of foods (approx. 3000 kcal per day), and sufficient water (approx. 3.0 litres per day), to cover your normal living requirements. The fluid restriction trial will involve you consuming the same foods as the control trial (approx. 3000 kcal per day) but with restricted fluid intake (approx. 0.2 litres per day). The calorie restriction trial will involve you consuming a range of foods providing 1/10 of your estimated energy requirements (approx. 400 kcal per day), and sufficient water (approx. 3.0 litres per day), to maintain hydration. The fluid and calorie restriction trial will involve you consuming a range of foods providing 1/10 of your estimated energy requirements (approx. 400 kcal per day) and restricted fluid intake (approx. 0.2 litres per day). In addition and on both days of the trial you will be asked to perform a 1.5 hour bout of moderate ('brisk') paced walking (4mph/6.4kph).

After 48 hours (day 3) you will be required to perform a 30 min treadmill time trial. You will then begin the rehydration and refeeding process. During the first 2 hours after the time trial, you will be provided with a carbohydrate drink (similar to Lucozade) that will help rehydrate any losses that may have occurred during the trial. You will be provided with two meals one at 2 and 4 hours of recovery. After 7 hours of recovery you will be free to leave the laboratory. On leaving the laboratory you will be provided with additional meals to replace any calorie deficit that has occurred during the trial for you to consume over the proceeding week. You will also be given a simple 8 day health and sickness log where for each day you are asked to record the occurrence of any health problems (e.g. cold symptoms). We will ask that you bring the completed health and sickness log to the laboratory the next time you visit.

During each trial you will have blood, urine and saliva samples taken every 24 hours and immediately after and 2 and 6 hours after the treadmill time trial (**6 blood samples in each trial**). In the fluid restriction trial, in addition to the previously mentioned measures, we will also ask you to drink a small volume of water containing a safe dose of deuterium oxide. After a 4 hour period for the deuterium oxide to mix with body fluids an additional blood sample will be collected. This is a well established and safe method to allow us to estimate the amount of water in your body at the start of this trial.

Visit Six, Seven and Eight (~69 hours each)
Six days after visit four you will be required to return to complete one of the three remaining experimental trials. Once again, you will be asked to complete a 48 hour experimental trial (see visit 5). You will be required to repeat this cycle until you have completed all four experimental trials.

Visit Nine (1 hour)
After completing all experimental trials you will be asked to return to the laboratory after 8 days for a final visit. During this visit you will have your weight assessed and also undergo a whole body x-ray scan as in the familiarisation visit.

Advantages and disadvantages of taking part

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

The disadvantages of taking part in this study, which you will probably be most concerned about, are giving blood samples, the food and fluid restriction treatments and the amount of

time you will be asked to commit to the study. Firstly, regarding the blood samples, we will only be collecting a single blood sample every 24 hours with the addition of the three samples after the 30 min treadmill time trial (6 samples in each trial). Only qualified Phlebotomists with experience at performing this procedure will collect blood samples. The blood samples will be taken with a very small butterfly needle without a tourniquet. In addition, the blood sample volume will only be approximately 14 ml at each collection. We will collect ~84 ml of blood during each 3 day trial giving a total blood volume of ~340 ml over the 4 trials spread across 6 weeks. The second of your concerns will probably be the 2 days of restricted food and fluid intake. Military recruits, of similar age and fitness to you, consume on average 330 kcal per day, with fluid restriction, for periods lasting 2-3 weeks on escape and evasion training. On top of the energy and fluid restriction the recruits cover 10-15 km per day on foot in difficult desert conditions with severe sleep deprivation. Clearly, the food and fluid restriction in this study is modest in comparison with that experienced during escape and evasion training. In case of emergency there will be an experimenter qualified in First Aid present through-out the study. The third disadvantage is that to complete all aspects of the study we will require you to visit the laboratory on eight occasions for a total of approximately 300 hours.

We understand that this study will ask a lot of you as a participant and so we are offering a financial incentive. As a participant you will be eligible for a £50 reward on completion of each of the first three experimental trials and a further £100 bonus on successful completion of the final experimental trial (**£250 total**). This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime. Any further questions will be happily answered by Dr Neil Walsh or any of the additional investigators.

The University of Wales, Bangor
School of Sport, Health and Exercise Sciences

Subject Information Sheet

Project Title: Effect of exercise in a hot environment on immune function in humans

Project Investigators:

Stewart Laing	**07753 747 557**	**pepc09@bangor.ac.uk**
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Co-ordinator: Dr Neil Walsh Tel: **01248 383480** Email: **n.walsh@bangor.ac.uk**

Invitation to take part

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

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time without giving a reason.

Background

Endurance athletes tend to suffer from more regular and persistent infections of the upper respiratory tract (e.g. colds, sore throats). Prolonged exercise and training have been shown to suppress some aspects of immune function, this may explain the increased occurrence of infections in endurance athletes. Increases in blood levels of stress hormones during and after endurance exercise have often been blamed for the suppression in immune function associated with endurance exercise. Exercising in a hot environment places greater stress on the body than when performing the same exercise in a cooler environment. Further increases in blood stress hormones would be expected when performing exercise in a hot compared to a cool environment. The purpose of this study is therefore to determine the effects of exercising in a hot environment on immune function in endurance athletes.

Visit One (approximately 45 min)

At this visit an incremental exercise test to volitional exhaustion will be performed in order to determine maximal oxygen uptake. Non-invasive measures of breathing and

heart rate will be taken. This session will also familiarize you with the tests and procedures to be used during the exercise protocol.

Visit Two (approximately 4 hours)
Prior to arriving for visit two you must not have completed any exercise for the preceding twenty-four hours. Arrive at the lab in appropriate clothing (shorts, T-shirt, water bottle + trainers), at 12:00 hours. You should arrive at the laboratory (lab 202) having fasted for 4 hours so do not eat or consume any caffeinated drinks after 08:00am. Please ensure you consume the same breakfast (before 08:00am) before visits two and three. To ensure you arrive at the laboratory properly hydrated please drink 1 pint of water along with your normal breakfast. You may drink **only water** after 08:00am. A venous blood sample (12-14 ml) will be taken before, and immediately after, and then 2 hours after a 2-hour bout of exercise in an environmental chamber at 55 % of your peak power output. This 2-hour bout of exercise will be performed in an environmental chamber, with fan cooling, on one occasion at normal room temperature (control trial ~20 °C), and on another occasion with the chamber temperature set to replicate hot conditions (experimental trial ~30°C). The order of the trials that you will perform will be selected randomly. Half of the subjects will complete the experimental trial first (at visit two) and the other half will perform the control trial first (at visit two) and vice versa. At visit three you will complete the exercise in the other environmental temperature. A disinfected rectal probe, which is a thin lead that you will place 10 cm past the anal sphincter, will be used to monitor core temperature during both trials for your safety. Heart rate and a subjective rating of perceived exertion will be recorded throughout the exercise period. You will be encouraged to drink plenty of water during visits two and three. You will be required to remain fasted until after the 2 hour post-exercise blood sample has been taken.

Visit Three (approximately 4 hours)
Between 7 and 14 days after visit two you will perform the 2-hour exercise protocol in the other temperature that you have not yet completed. All procedures and protocols (except the environmental temperature) remain the same as visit two.

Advantages and disadvantages of taking part

A useful benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, for both your fitness level and blood measures. This feedback should help you with planning and monitoring your athletic training program. The feedback you will receive regarding your fitness level is similar to that which many testing facilities provide as a fee-paying service.

The disadvantages of taking part in this study, which you will probably be most concerned about are, giving venous blood samples (7 in total), the use of a rectal probe and exercising in a hot environment. Firstly, regarding the blood samples, we will only take 1 blood sample at visit one, and 3 blood samples at visits two and three. A qualified phlebotomist with many years experience at performing this procedure will take these samples. The rectal probe is a thin lead placed only 10 cm past the anal sphincter and is used to monitor your body core temperature. We use the rectal probe as a safety measure whereby we will stop you exercising if your temperature rises more than 2°C during any trial. In my experience subjects have not complained that the rectal probe is uncomfortable during exercise. In fact most subjects forget they have the rectal probe fitted! The use of the rectal probe along with regular water consumption during exercise

in all trials should mean the risks of symptoms of heat stress (e.g. head ache, weakness, dizziness, and cramps) are negligible.

Dr Neil Walsh and any of the above contacts will happily answer any further questions.

PARTICIPANT INFORMATION SHEET & CONSENT FORM

Study title: Exercise induced immune alterations with and without a thermal clamp.

Project investigators:

Stewart Laing	07753747557	Pepc09
Anna Jackson	07736323556	Pep00a
Jade Leung	07990513952	Peuc8d
Lee Marshall	07766541385	Peua5d
Richard Wiggett	07764749803	Peuc75

Co-ordinators: Dr N Walsh (n.walsh@bangor.ac.uk)
Dr M Whitham (m.whitham@bangor.ac.uk)

Invitation to take part

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

What is the purpose of the study?

Previous research has identified aspects of the immune system as being suppressed following prolonged exercise. While there have been observed alterations in immune cell functions, the mechanisms behind these changes are largely unknown. Both the increase in body temperature and hormones released during exercise have been suggested as potential mechanisms. However, since these "stress" hormones released during exercise have been associated with changes in body temperature, it is difficult to determine which mechanism prevails. The present study aims to use a "thermal clamping" method which controls body temperature during exercise to assess the independent contribution of heat and stress hormones on selected immune functions.

Why have I been chosen?

We have chosen fit, healthy males as participants. We will use 25 participants in total. All participants should NOT be taking any medications or nutrition supplements.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You will be free to withdraw at any time without giving a reason. If you decide not to participate, it will not affect your relationship with the School of Sport, Health and Exercise Sciences (SHES), or with any of the investigators involved in the study. All the information collected will be treated confidentially.

What will happen to me if I take part?

You will be asked to visit the School of Sport, Health, and Exercise Science on five occasions separated by approximately one week.

Visit one (approximately 45 min) Maximal test & familiarization

At this visit you will be asked to exercise until you feel so tired that you do not want to go on. This will allow us to measure how much oxygen your body takes up under normal circumstances. We shall measure your breathing and heart rate by simple means. We shall also show you the tests and procedures we will be using in the other tests.

Visits Two, Three, Four, and Five (approximately 5 hours): At least 2 days after visit one you will be asked to arrive at the SHES Physiology laboratory in the appropriate clothing (with swimming costume). All exercise testing will be performed in the morning. A small resting venous blood sample (19ml) will be collected using a small needle. This will be performed by one of the qualified phlebotomists in the research team (Dr Whitham, Dr Walsh, Mr Laing (postgraduate student).
Over the next 4 weeks, you will then be asked to carry out four different trials in a randomised order. Your body temperature will be monitored throughout all of the trials. This will involve the use of a rectal thermister which is a very thin lead that is inserted 10cm beyond the anal sphincter. It is commonly used in hospitals and in all trials of this nature. It has no side effects. As well as providing us with important information about your body temperature, we will also use it as a safety measure so that we can tell when your body temperature has risen or fallen to unsafe levels and we can stop the tests prematurely. In our experience subjects do not find the rectal probe is uncomfortable. In fact most subjects forget that they have one fitted.

Cold Immersion Exercise Trial
This will involve 2 hours of "aqua jogging" at a predetermined intensity whilst immersed in 19° - 20°C water to the neck. Aqua jogging basically replicates the movements of running, but underwater using a special floatation jacket. You will also wear a safety harness as a precautionary measure.
Thermoneutral Immersion Exercise Trial
In a similar trial, you will be asked to exercise for 2 hours at the same intensity as the cold immersion exercise trial, but this time in water maintained at a temperature of 30° C.

Hot Immersion Trial
In order for us to examine the independent effects of exercise and a rise in body temperature, the hot immersion trial will involve no exercise. You will be seated immersed up to the neck in water controlled at a temperature of 39°C. It is intended that core temperature in this trial will reach the same values seen in the thermoneutral immersion exercise trial (~39°C).

Thermoneutral Immersion Trial
To control for the effects of time of day and water immersion, you will be asked to carry out a control trial where you will remain seated as in the hot immersion trial, but this time in thermoneutral (~normal body temperature) water (33-35°C). Aside from body temperature, heart rate will be monitored throughout each trial using a chest belt and watch receiver. We will measure your breathing using a system that requires wearing a simple face mask which covers your mouth and nose. In addition to the blood sample we take before you start each trial, we will take an identical sample immediately after the trial and after a further two hours.

You will be asked to refrain from eating during this two hour period after each trial.

What do I have to do? Before each trial we will ask you to

- Abstain from caffeine (coffee, tea, coke etc), alcohol and smoking, as well as from any vigorous physical activity for a period of 24h before each trial
- Refrain from eating and drinking for 4 hours before the experiments, (water is allowed)
- You will be provided with a standard breakfast to consume between 7.30-8am. This will be repeated before each of the four trials.

What are the possible disadvantages and risks of taking part?
The disadvantages of taking part in this study are the discomfort associated with the use of a temperature thermisters, blood sampling and exercising in water. Any discomfort you experience will be short lived. There are standard risks associated with exercise. Accordingly, persons trained in CPR and AED will be readily available or present at all exercise trials.

What are the possible benefits of taking part?
Taking part in this study will enable you to receive a comprehensive feedback, with full explanations of your fitness level. The feedback you will receive regarding your fitness level is similar to that which many fitness testing centres provide as a fee-paying service.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the department/hospital will have your name and address removed so that you cannot be recognised from it.

APPENDIX B

MEDICAL QUESTIONNAIRE

Name: .....

Age:.....

Are you in good health? Yes/No
If no, please explain:

How would you describe your present level of training? Tick intensity level and indicate approx. duration.

vigorous moderate low intensity

Duration (Min).

How Often:

2-3 times per week
4-5 times per week
5-6 times per week
7 times a week.

Have you suffered from a serious illness or accident? Yes/No
If yes, please give particulars:

Do you suffer, or have you ever suffered from:

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

Are you currently taking medication ? Yes/No
If yes, please give particulars:

Are you currently attending your GP for any condition or have you consulted your doctor in the last three months? Yes/No

If yes, please give particulars:

Are you currently taking any dietary supplements e.g. Vitamin C tablets?

Yes/No

If yes, please give particulars:

Have you, or are you presently taking part in any other laboratory experiment? Yes/No

PLEASE READ THE FOLLOWING CAREFULLY

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

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

DECLARATION
I agree that I have none of he above conditions and I hereby volunteer to be a participant in testing during 2004.

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 tests and possible risks involved.

Signature of Participant.....

Date: .....

Signature of Experimenter.....

Date: .....

PRE-TEST QUESTIONNAIRE

NAME:

DATE:

		YES/NO
1.	HAVE YOU HAD ANY KIND OF ILLNESS OR INFECTION IN THE LAST 2 WEEKS?	.....
2.	ARE YOU TAKING ANY FORM OF MEDICATION?	.....
3	DO YOU HAVE ANY FORM OF INJURY?	.....
4.	HAVE YOU EATEN IN THE FOUR HOURS?	.....
5.	HAVE YOU CONSUMED ANY ALCOHOL IN THE LAST 24 HOURS?	.....
6.	HAVE YOU PERFORMED EXHAUSTIVE EXERCISE WITHIN THE LAST 24 HOURS?	.....

IF THE ANSWER TO ANY OF THE ABOVE QUESTIONS IS YES, THEN YOU MUST CONSULT A MEMBER OF STAFF BEFORE UNDERGOING ANY EXERCISE TEST.

SIGNATURE OF PARTICIPANT .....

APPENDIX C

HEALTH AND SICKNESS LOG

Name:_____

Date:

Heart Rate in 20 secs:	**Heart Rate in 20 secs:**	**Heart Rate in 20 secs:**	**Heart Rate in 20 secs:**	**Heart Rate in 20 secs:**	**Heart Rate in 20 secs:**	**Heart Rate in 20 secs:**

Instructions:

A. Fill in **health code(s) (**as many as apply)

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

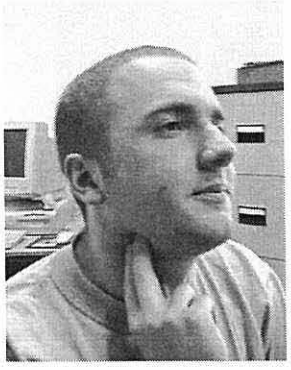
Please rate **severity** of symptoms:

A = Mild, B = Moderate, C = Severe

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

B. Fill in **Resting Heart Rate**

Each morning on waking, record your resting heart rate by taking a 20 second count from the carotid artery on the right hand side of your neck (in line with your Adams apple).



APPENDIX D

Level	Shuttle	Predicted VO2 max
4	2	26.8
4	4	27.6
4	6	28.3
4	9	29.5
5	2	30.2
5	4	31
5	6	31.8
5	9	32.9
6	2	33.6
6	4	34.3
6	6	35
6	8	35.7
6	10	36.4
7	2	37.1
7	4	37.8
7	6	38.5
7	8	39.2
7	10	39.9
8	2	40.5
8	4	41.1
8	6	41.8
8	8	42.3
8	11	43.3
9	2	43.9
9	4	44.5
9	6	45.2
9	8	45.8
9	11	46.8
10	2	47.4
10	4	48
10	6	48.7
10	8	49.3
10	11	50.2
11	2	50.8
11	4	51.4
11	6	51.9
11	8	52.5
11	10	53.4
11	12	53.8
12	2	54.3
12	4	54.8
12	6	55.4
12	8	56
12	10	56.5
12	12	57.1
13	2	57.6
13	4	58.2
13	6	58.7
13	8	59.3
13	10	59.8
13	13	60.6
14	2	61.1
14	4	61.7
14	6	62.2
14	8	62.7
14	10	63.2
14	13	64
15	2	64.6
15	4	65.1
15	6	65.5
15	8	66.2
15	10	66.7
15	13	67.5
16	2	68
16	4	68.5
16	6	69
16	8	69.5
16	10	69.9
16	12	70.5
16	14	70.9
17	2	71.4
17	4	71.9
17	6	72.4
17	8	72.9
17	10	73.4
17	12	73.9
17	14	74.4
18	2	74.8
18	4	75.3
18	6	75.8
18	8	76.2
18	10	76.7
18	12	77.2
18	15	77.9
19	2	78.3
19	4	78.6
19	6	79.2
19	8	79.7
19	10	80.2
19	12	80.6
19	15	81.3
20	2	81.8
20	4	82.2
20	6	82.6
20	8	83
20	10	83.5
20	12	83.9
20	14	84.3
20	16	84.8
21	2	85.2
21	4	85.6
21	6	86.1
21	8	86.6
21	10	86.9
21	12	87.4
21	14	87.8
21	16	88.2

Multi Stage Fitness Test