The effects of caffeine ingestion on immunoendocrine responses to prolonged and intensive exercise in humans

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THE EFFECTS OF CAFFEINE INGESTION ON IMMUNOENDOCRINE
RESPONSES TO PROLONGED AND INTENSIVE EXERCISE IN HUMANS

By

Gary J. Walker

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Doctor of

Philosophy of Loughborough University

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Abstract

Athletes commonly consume caffeine as an ergogenic aid. The effect of caffeine ingestion on the immune response in humans, and in particular following exercise, has received little scientific attention. Caffeine may affect immune responses via adenosine receptor antagonism or adrenaline-mediated mechanisms. Following the relatively recent removal of caffeine from the WADA list of prohibited substances, its use is expected to increase further, therefore any influence of caffeine on immune function is of particular relevance. The aim of this thesis was to investigate the influence of caffeine ingestion on immunoendocrine responses following prolonged and intensive cycling in humans.

Initially, caffeine (6 mg.kg\(^{-1}\) body mass) did not affect neutrophil functional responses when participants rested for 3.5 h following ingestion (Chapter 4). Caffeine ingestion attenuated the post-exercise decline in neutrophil oxidative burst response when stimulated by f-MLP (Chapter 6) but not PMA (Chapter 5). An in vitro study suggested that this most likely occurs as a result of adenosine-receptor antagonism by caffeine (Chapter 8). Although caffeine ingestion improved pre-loaded TT performance by 4%, there was no attenuating effect of caffeine on post-exercise f-MLP-stimulated neutrophil oxidative burst responses (Chapter 7) nor was there any benefit of co-ingesting caffeine with CHO on oxidative responses, compared with either supplement alone (Chapter 9). Caffeine supplementation did not affect total circulating leukocyte or neutrophil count at rest or following exercise, but increased circulating lymphocyte count both at rest and during exercise following ingestion. Caffeine ingestion was consistently associated with an increased plasma adrenaline concentration but not noradrenaline concentration at rest and following exercise. Caffeine generally had no effect on plasma cortisol concentration, though it was consistently associated with an increased plasma IL-6 concentration following exercise of various duration and intensity.

In conclusion, the attenuating/positive effects of caffeine ingestion on post-exercise neutrophil oxidative burst responses appear to be stimulant-dependent. None of the experimental studies, however, indicated that caffeine ingestion was more detrimental than placebo on the f-MLP-stimulated neutrophil oxidative burst response to exercise.
Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Nicolette Bishop, for her assistance throughout my PhD research. Her open door policy, honest approach and expertise was extremely helpful and I am truly grateful for her input to this thesis. In particular, Lettie always managed to make time for me, even when on maternity leave. I wish Lettie and her family every happiness in the future.

I also wish to thank all of the participants in the studies described in this thesis. Without their help, these demanding studies would not have been possible.

I am grateful to the other members of our research group, Dr. Glen Davison and Judith Allgrove for their support and encouragement throughout the past 3 years.

I would also like to thank the BSc and MSc students who assisted with the collection of physiological data during the preliminary and main experimental trials. I thank Eleanor Rogers (Chapter 5), Natalie Dixon, Phillipa Caudwell and Catrin Sheppard (Chapter 6), Anneliese Dziubak, Laura Lim, Ciaran Prendergast and Laurence Houghton (Chapter 7), and Anwen Rees, Laura Blunt, Hannah Griffiths, James Sylvester, Mark Williams and Olli Finlay (Chapter 9). In addition I would like to thank Karen Turner at Queen’s Medical Centre for the catecholamine analysis.

Finally, I thank my parents, Carol and Stewart, for all their love, help and support throughout my many years of education. I hope I can in some way repay you for everything you have done for me.
Publications

The findings of some of the material presented in this thesis have been published as follows:

*Chapters 5 & 6:*

*Chapter 6:*


*Chapter 7:*

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<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<tr>
<td>ADA</td>
<td>adenosine deaminase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3', 5'-cyclic monophosphate (cyclic AMP)</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD4+</td>
<td>Helper T cells</td>
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<td>CD8+</td>
<td>Cytotoxic T cells</td>
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<td>CD69</td>
<td>early T cell activation antigen</td>
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<td>CGD</td>
<td>chronic granulomatous disease</td>
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<td>carbohydrate</td>
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<td>chemiluminescence</td>
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<td>Chloride</td>
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<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
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<td>FFA</td>
<td>free fatty acids</td>
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<td>f-MLP</td>
<td>formyl-methionyl-leucyl-phenylalanine</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<td>h</td>
<td>hour</td>
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<td>HBSS</td>
<td>hank’s balanced salt solution</td>
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<td>HOCl</td>
<td>hydrochlorous acid</td>
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<td>H₂O₂</td>
<td>hydrogen peroxide</td>
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<td>IAUC</td>
<td>incremental area under curve</td>
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<td>IC₅₀</td>
<td>the half maximal inhibitory concentration</td>
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<td>interleukin</td>
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mg
mL
min
mmol
MPO
N
Na
NADPH
NK
nmol
O₂⁻•
OH⁻•
OZ
PAF
PBS
PHA
PMA
PKA
PL-D
PWM
RER
rh
RLU
RNS
ROS
s
SEM
TNF
TT
URTI
Vₑ
Vₒ₂ max
W
WADA
Wmax
°C
µmol
major histocompatibility complex
metre
milligram
millilitre
minute
millimole
myeloperoxidase
nitrogen
sodium
nicotinamide adenine dinucleotide phosphate
natural killer
nanomole
superoxide anion
hydroxyl radical
opsonised zymosan
platelet activating factor
phosphate buffered saline
phytohaemagglutinin
phorbol-12-myristate-13-acetate
protein kinase A
phospholipase D
pokeweeds mitogen
respiratory exchange ratio
recombinant human
relative light unit
reactive nitrogen species
reactive oxygen species
second
standard error of the mean
tumour necrosis factor
time-trial
upper respiratory tract infection
expired gas volume
maximum oxygen uptake
watts
world anti-doping agency
maximal workload
degrees celsius
micromole
Chapter 1

Introduction
Introduction

Anecdotal, survey and epidemiological data suggest that athletes who perform prolonged and intensive exercise are at an increased risk for upper respiratory tract infection (URTI) (Nieman, 2000). This may be detrimental to an athletic performer, causing them to miss training and competition days through illness or compromise the intensity of their training schedules. A direct link between immune function in vivo and actual infection has not been established, primarily due to an ethical concern about inoculating participants with a virus before or after heavy-exertion exercise (Bishop, 2006). However, exercise is a useful model to determine immune responses as it is a reproducible and quantifiable stressor that can be manipulated to evoke defined hormonal, endocrine and nervous responses, depending upon its intensity and duration (Smith, 1997). Exercise of moderate duration and intensity is associated with few disturbances to immune function (Nieman, 1997); however, following prolonged and intensive exercise, many components of the immune system exhibit change. During this period of altered immunity, opportunistic viruses and bacteria may gain a foothold, increasing the susceptibility for infection. This has subsequently been termed the 'open window for infection' (Nieman and Pedersen, 1999; Nieman, 2000).

The impairment of immune function following exercise appears to be predominantly caused by elevated concentrations of stress hormones (Smith, 1997; Gleeson and Bishop, 1999; Gleeson et al., 2004a). Not surprisingly, therefore, nutritional interventions have attempted to reduce stress hormone responses in an attempt to reduce the magnitude of this immunosuppression and attempt to 'close' this window. The consumption of carbohydrate (CHO) before and during exercise is associated with an attenuated rise of plasma adrenaline, cortisol, adrenocorticotropic hormone
(ACTH), growth hormone and a number of cytokines (Nehlsen-Canarella et al., 1997; Nieman et al., 1998; Davison and Gleeson, 2005; Nieman et al., 2005). CHO ingestion has also been reported to attenuate the decline of formyl-methionyl-leucyl-phenylalanine (f-MLP)-stimulated neutrophil oxidative burst response (Scharhag et al., 2002), bacterially-stimulated neutrophil degranulation response (Bishop et al., 2000; Davison and Gleeson, 2005), and cell-adjusted phytohaemagglutinin (PHA)-induced lymphocyte proliferation (Henson et al., 1998) following prolonged duration exercise. Nutritional anti-oxidants have also been extensively researched with regards to immune function and oxidative stress. Peters et al. (1993) reported that daily supplementation with large Vitamin C doses reduced incidence of URTI following an ultramarathon race. This has been associated with an attenuation of the post-race plasma cortisol and adrenaline concentrations following Vitamin C supplementation (Peters et al., 2001). Supplementation with Vitamins C and E significantly reduced cortisol and interleukin-6 (IL-6) responses following 3-h dynamic knee-extensor exercise (Fischer et al., 2004), but a 2-week supplementation with Vitamin C alone did not affect IL-6 or neutrophil functional responses following 2.5-h cycling (Davison and Gleeson, 2006). Furthermore, Vitamin C supplementation for 1-week did not affect any immune changes following a 10-h ultramarathon event (Nieman et al., 2002). Glutamine supplementation did not alter post-exercise leukocytosis or immune function following prolonged exercise (Walsh et al., 2000). Equivocal evidence has been reported with regards to the effects of a number of minerals, such as iron and zinc supplementation, upon immune responses following exercise and the incidence of URTI (Gleeson et al., 2004a).
Caffeine is contained in various foods, beverages and medications. It is the most widely consumed behaviourally active substance in the world (Fredholm et al., 1999), yet it is not a typical nutrient nor is it essential for health (Graham, 2001a). Athletes commonly consume caffeine as an ergogenic aid (Tarnopolsky, 1994) and its use is highly prevalent in the sport of amateur and professional cycling. Following the relatively recent removal of caffeine from the World Anti-Doping Agency (WADA) list of prohibited substances, its use is expected to increase considerably amongst athletes during periods of training and competition. The effect of caffeine ingestion on the immune response in humans, and in particular following exercise, has not been extensively researched (Section 2.10). Caffeine ingestion, however, is consistently associated with an increased secretion of adrenaline (Graham and Spriet, 1991, 1995) and is also reported to increase plasma cortisol concentration (Laurent et al., 2000). Furthermore, the main action of caffeine at physiological concentrations is that of adenosine-receptor antagonism (Ongini & Fredholm, 1996). Adenosine receptors are found in most tissues, including immune cells, therefore this thesis investigated the influence of caffeine ingestion on immunoendocrine responses following prolonged and intensive cycle ergometry exercise.

Firstly, the effect of caffeine ingestion upon hormonal and immune responses was determined in participants at rest (Chapter 4). The effect of caffeine ingestion on post-exercise neutrophil oxidative burst responses to phorbol-12-myristate-13-acetate (PMA) and f-MLP stimulation was investigated in Chapters 5 and 6, respectively. As caffeine is consumed as an ergogenic aid, its effect on neutrophil oxidative burst, hormonal and cytokine responses was investigated following a pre-loaded time-trial performance test (Chapter 7). The potential mechanisms by which caffeine may affect
neutrophil oxidative burst responses were investigated \textit{in vitro} (Chapter 8). Caffeine is commonly consumed with CHO in commercially available soft drinks and energy drinks. The effect of caffeine co-ingested with CHO on hormonal, cytokine and neutrophil responses following endurance exercise was determined in Chapter 9.
Chapter 2

Review of Literature
2.1. Are athletes more susceptible to infection?

A common perception exists among elite performers and their coaches that athletes performing chronic exercise (i.e. prolonged and intensive exercise sessions) are at increased risk of URTI (e.g. common cold, cough, sore throat and middle ear infection) (Nieman et al., 1997; Nieman, 2000). Such respiratory infections could lead to an increased number of training or competition days missed through illness, or may reduce the intensity that an athlete is able to train or compete, therefore potentially having a substantial negative impact for the elite performer. This anecdotal evidence is supported by survey-based epidemiological data (Peters and Bateman, 1983; Nieman et al., 1990a). These studies reported that the risk of URTI is between 2 and 6 times greater in the 2-week period following a prolonged endurance event (marathon/ultramarathon) and is correlated with race finishing time (Peters and Bateman, 1983) and pre-race training distance (Nieman et al., 1990a). The diagnosis of URTI in these studies was based upon responses to questionnaires, rather than clinical assessment and determination. This self-reported evidence may therefore be subject to response bias due to an increased body-awareness in highly trained athletes (Gleeson and Bishop, 1999). Furthermore, other practices may also contribute to an increased incidence of infection amongst athletes such as improper nutrition, lack of sleep, psychological stress, air pollution, travel and sharing of drinking bottles, which may confound this epidemiological evidence (Gleeson and Bishop, 1999).

Although epidemiological evidence has supported the notion that strenuous exercise leads to increased infection risk, a direct link between immune function in vivo and actual infection has not been established. One reason for this is a serious ethical concern about inoculating participants with a virus prior to heavy-exertion exercise.
A study conducted by Bruunsgaard et al. (1997) investigated whether in vivo impairment of immune response could be demonstrated following intense exercise of prolonged duration. In this study, a mixture of antigens was injected into the forearm skin of triathletes after they had completed a half-ironman race (3 km swim, 130 km cycle, 21 km run) and hypersensitivity response was measured. This is a complex immunological reaction involving several different cell types and mediators, resulting in swelling at the site of antigen administration which was measured at 48 h following injection. The response of the triathletes after exercise was significantly lower than both non-exercising triathletes and moderately trained controls, suggesting that cell-mediated immunity is depressed in the first days following prolonged high-intensity exercise. In trained swimmers, salivary immunoglobulin A (sIgA) concentration was inversely related to training volume over the course of a 7-month season (Gleeson et al., 1999b). Furthermore, the pre-season sIgA levels and the rate of decrease in pre-training sIgA levels were associated with an increase in the number of episodes of URTI as assessed by a clinician (Gleeson et al., 1999b).

This general perception that athletes are at increased risk of URTI has contrasted with the belief of recreational exercisers or 'keep-fit fanatics', that physical exercise provides protection or resistance to URTI (Mackinnon, 2000). For example, in a survey of 170 experienced non-elite marathon runners, 90% reported that they 'rarely got sick' (Nieman, 2000). Moreover, a 15-week moderate exercise training programme (5 sessions of 45 min walking per week) was associated with significantly fewer days suffering from URTI symptoms in a group of mildly obese women when compared with a sedentary control group (Nieman et al., 1990b). In this study, natural
killer (NK) cell activity was significantly elevated in the exercising group during the initial 6-week period; however there was no difference at the end of the 15-week study. A J-shaped hypothesis has therefore been proposed, suggesting that intense exercise increases the risk, whereas moderate exercise may decrease the risk of contracting URTI (Nieman, 1994). After an acute bout of prolonged and intensive endurance exercise, many components of the immune system exhibit change. This period, which may last between 3 and 72 hours depending upon the immune parameter measured and the exercise protocol utilised, has been termed the 'open window' for infection and during this time, opportunistic viruses and bacteria may gain a foothold, increasing the susceptibility of infection (Nieman and Pedersen, 1999; Pedersen et al., 1999; Nieman, 2000). The major components of the immune system that exhibit change following exercise are described in Section 2.2.

2.2. Exercise and immune responses

Exercise exerts significant effects upon the immune system. In general, acute bouts of exercise that are of moderate duration (< 60 min) and intensity (< 60% \( \dot{VO}_2 \) max) are associated with fewer perturbations to the immune system and may actually enhance immune responses (Nieman, 1997). The following changes have generally been reported to occur following prolonged (> 90 min) and intensive (\( \geq 70\% \dot{VO}_2 \) max) endurance exercise:-

- Circulating neutrophil concentration increases at post-exercise and recovery (McCarthy and Dale, 1988). Circulating lymphocyte count increases during exercise and falls below pre-exercise values following exercise (McCarthy and
Collectively, this leads to an increase of the neutrophil/lymphocyte ratio.

- Increase in circulating neutrophil phagocytosis (Blannin et al., 1996) and decreased nasal neutrophil phagocytosis (Muns, 1994).

- Increase in neutrophil degranulation response (Suzuki et al., 2003; Peake et al., 2004) but a decreased degranulation response to bacterial stimulation on a per-cell basis (Robson et al., 1999; Walsh et al., 2000).

- Decrease in bacterially-stimulated neutrophil oxidative burst response on a per-cell basis (Robson et al., 1999; Suzuki et al., 2003; Davison and Gleeson, 2005). Occasionally an increase (Smith et al., 1990) or no change (Walsh et al., 2000) in the response has been observed.

- Decrease in NK cell cytotoxic activity (Shek et al., 1995; Nieman, 1997).

- Decrease in the ratio of helper T cells (CD4\(^+\)) to suppressor T cells (CD8\(^+\)). A decrease of the CD4\(^+\):CD8\(^+\) ratio to below 1.5 is associated with an increased susceptibility to infection (Shephard et al., 1991).

- Decrease in mitogen-induced T cell proliferative responses (Nieman et al., 1995).

- Either a decrease (Tvede et al., 1989) or no change (Mackinnon et al., 1989) in mitogen-stimulated B cell Ig synthesis in vitro.

- Decreased cell-mediated delayed hypersensitivity response (Bruunsgaard et al., 1997).

- Decreased toll like receptor expression on monocytes and decreased functional responses (Lancaster et al., 2005).

- Decreased major histocompatibility complex (MHC II) expression in macrophages (Woods et al., 1997).
• Increase in a number of pro-inflammatory and anti-inflammatory cytokines such as tumour necrosis factor (TNF-α) and IL-1β, IL-6, IL-10, IL-1ra (Ostrowski et al., 1998).

• Decrease in nasal IgA concentration (Nieman, 1997) and changes in salivary IgA concentration (Gleeson et al., 2004b).

As detailed above, one aspect of the immune system that exhibits change following endurance exercise is that of neutrophil response, and it is these responses upon which this thesis will focus.

2.3. Neutrophils and host defence

Neutrophils constitute 50-60% of circulating leukocytes and act as the first-line of defence against agents that penetrate the body’s physical barriers (Smith, 1997). Their targets include bacteria, fungi and viruses, yet they require signalling mediators within the immune system to direct their attack since they have the inability to differentiate between host and foreign agents. Without this, healthy host tissues may be damaged resulting in inflammation (Pyne, 1994). The neutrophil functional cascade begins when previously mobile neutrophils adhere to the vascular endothelium. Upon signalling from various chemoattractants, neutrophils penetrate the endothelial layer (diapedesis), move through connective tissues and are localised to the sites of infection (chemotaxis and migration). At the site of infection neutrophils bind to and ingest the foreign material by a process called phagocytosis, leading to the activation of the oxidative burst and secretory function of neutrophils (Smith and Pyne, 1997; Mackinnon, 1999). Activation of the secretory function of neutrophils results in the release of cytoplasmic granules (eg. Myeloperoxidase
(MPO), elastase, lysozyme and lactoferrin) into the phagolysosomal vacuole and often occurs simultaneously with the oxidative burst. The neutrophil oxidative burst consists of a complex series of events that can be divided into 3 distinct phases: stimulation and recognition, signal transduction and activation phase (Pyne, 1994). A detailed explanation of these phases is beyond the scope of this review; for further detail the reader is directed to Peake (2002).

Neutrophils are stimulated via receptor-mediated and receptor-independent mechanisms. Receptor-mediated stimuli include the complement fragment C5a, various N-formylated methionyl peptides, bacterial endotoxins, bioactive lipids such as platelet activating factor (PAF) and leukotriene B_4 (Pyne, 1994). Receptor-independent stimuli include long-chain unsaturated fatty acids and phorbol esters, that directly activate protein kinase C (Chanock et al., 1994). Receptor-dependent stimuli usually activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase for < 5 min, while receptor-independent stimuli activate the enzyme for a much longer period (Chanock et al., 1994). When neutrophils become activated, a 50 to 100-fold increased consumption of molecular oxygen (O_2) results in the formation of superoxide ions (O_2•-) via the action of a plasma-membrane NADPH oxidase (Chanock et al., 1994; Hasegawa et al., 1997; Smith, 1997; Babior, 1999). The NADPH core enzyme consists of 5 components: 3 of these exist in the cytosol (p40^{PHOX}, p47^{PHOX}, p67^{PHOX}) and 2 are located within the membranes of secretory vesicles and are collectively known as cytochrome b_558 (p22^{PHOX}, gp91^{PHOX}). Following stimulation, the cytosolic p47^{PHOX} component is heavily phosphorylated and the component migrates to the membrane where it associates with cytochrome b_558 to assemble the active oxidase (Babior, 1999). It catalyzes the production of O_2•-
by the one-electron reduction of $\text{O}_2$. The $\text{O}_2^-$ may then initiate a cascade of reactions, involving hydrogen peroxide ($\text{H}_2\text{O}_2$), hydroxyl radicals ($\cdot\text{OH}$) and chloride ions ($\text{Cl}^-$) resulting in the production of reactive oxygen species (ROS), or through a different mechanism, reactive nitrogen species (RNS) (Smith, 1997; Babior, 1999; Peake, 2002). These act to eliminate micro-organisms and damaged tissue by damaging the cell membrane and/or rendering the cells inactive. This process is illustrated in Figure 2.1.

The importance of neutrophils in host defence cannot be underestimated and is illustrated by deficiencies in number and/or function (Smith, 1997). For example, the neutrophil oxidative burst is essential for killing a number of micro-organisms, as shown by the increased susceptibility to infections of individuals with chronic granulomatous disease (CGD). CGD is a genetic disease in which the NADPH oxidase is inactive, thus neutrophils do not undergo a burst when bacteria is ingested and do not produce oxidising radicals. Infections can occur anywhere within the body and may take many forms (Smith and Curnutte, 1991). It is therefore clear that neutrophils play an important role in the resistance against infection, thus their function during and following exercise is of importance. Neutrophils undergo several functional alterations during exercise, some of which are discussed below (for a comprehensive review refer to Smith and Pyne, 1997).
2.4. Exercise-induced neutrophilia

The concept of exercise-induced neutrophilia is not a new phenomenon, with a large body of published literature demonstrating a marked increase in circulating neutrophil count during and after virtually all forms of exercise (reviewed by McCarthy and Dale, 1988). The magnitude of this increase, however, is related to the intensity, duration and mode of exercise performed (Hansen et al., 1991; Gabriel and Kindermann, 1997; Robson et al., 1999; Pyne et al., 2000; Peake, 2002, amongst others).
Data from brief exercise suggests that only a transient disturbance in circulating neutrophil count occurs as a result of short duration exercise. For example, Nieman et al. (1992) showed that numbers of circulating neutrophils increased by approximately 40% following a 30 s maximal-effort cycle sprint, yet neutrophil counts returned to pre-exercise values after 1 h recovery. However, in studies where multiple post-exercise blood samples were taken, Hansen et al. (1991) and Gabriel et al. (1992) reported that circulating neutrophil counts exhibit a biphasic response consisting of a modest increase immediately following brief and intensive exercise, returning to baseline levels within 30 min but exhibiting a delayed and prolonged neutrophilia at 2 and 4 h following exercise cessation. This was demonstrated following a maximal 60 s cycling sprint (Gabriel et al., 1992) and in the recovery following short (1.7 km), medium (4.8 km) and long (10 km) duration running distances (Hansen et al., 1991). Immediately following exercise, neutrophil count had increased by approximately 20%, yet the delayed neutrophilia occurring at 2 h was 68%, 102% and 170% above pre-exercise values following the short, medium and long runs, respectively. The magnitude of the delayed response varied amongst subjects but was associated with the duration of the exercise performed.

In relation to endurance exercise, many researchers have reported an immediate increase in circulating neutrophils during exercise that remains elevated for several hours after the cessation of exercise (review by Peake, 2002). For example, in endurance trained cyclists, 2 h cycling at 75% VO₂ max resulted in a 4-fold increase in circulating neutrophils immediately post-exercise with a further rise at 1 h post-exercise (Bishop et al., 2003). A 4.5-fold increase was reported at post-exercise when recreationally active cyclists performed the same duration of exercise at 65%
\( \dot{V}O_{2}\max \) (Bishop et al., 2004). In healthy untrained males, a 2.5-fold increase in circulating neutrophil count was found following 1.5 h cycling at 70% \( \dot{V}O_{2}\max \), rising to greater than 3-fold at 1 h post-exercise (Suzuki et al., 1996a). Responses of a similar magnitude were reported by Walsh et al. (2000) and Scharhag et al. (2002, 2006) using cycling protocols of 2 h at 60% \( \dot{V}O_{2}\max \) and 4 h at 70% individual anaerobic threshold, respectively. In addition to an increase in circulating neutrophil count during and after exercise, an increase in the number of neutrophils in the upper respiratory tract has also been demonstrated. A study conducted by Muns (1994) used a nasal lavage technique to assess neutrophil count following a 20 km road race. Immediately following the race, a 3-fold increase in respiratory tract neutrophils was found that remained elevated by 1.6-fold above pre-race values following a 24 h recovery period.

The neutrophilia that occurs with exercise appears to be directly related to the duration rather than the intensity of the exercise performed. Circulating neutrophil count was increased by 2-fold at post-exercise when participants cycled for 1.5 h at 60% \( \dot{V}O_{2}\max \) (Li and Gleeson, 2005). However, a 4-fold increase was reported when participants cycled for 2.5 h at the same relative exercise intensity (Davison and Gleeson, 2006). Furthermore, the delayed neutrophilia is also related to exercise duration rather than intensity. Robson et al. (1999) compared the effects of cycling at 80% \( \dot{V}O_{2}\max \) (resulting in fatigue within 1 h) with cycling at 55% \( \dot{V}O_{2}\max \) (up to 3 h) and reported a significantly higher neutrophil count at 0 h, 1 h, 2.5 h and 5 h post-exercise on the prolonged exercise trial. In slight contrast to the above results, the studies of Suzuki et al. (2003) and Hessel et al. (2000) observed the effects of marathon distance running on neutrophil cell counts. Both research groups found
increases of the same magnitude (approximately 4.5-fold) even though there was a
marked difference in reported finishing times between the subjects in the 2 races
(mean finishing time: 2.6 h vs. 3.5 h).

The mode of exercise performed has also been documented as a reason for
discrepancies between research findings. In a study conducted by Pizza et al. (1995)
blood neutrophil counts were higher at 1.5 h and 12 h post-exercise following a 60
min downhill treadmill run at 70% $\text{VO}_2\text{max}$ compared with level running,
suggesting that when eccentric exercise is performed, a larger mobilisation of
neutrophils is elicited. In contrast, however, Nieman et al. (1998) reported little
difference in circulating count between exercise modes following 2.5 h of running or
cycling at 75% $\text{VO}_2\text{max}$. Pyne et al. (2000) assessed the effects of intensity and type
on neutrophil count with a protocol employing 3 separate 40-min interval treadmill
bouts (8 x 5 min). Circulating neutrophil counts were higher in the uphill treadmill
run compared to both the downhill or near-level run; however the intensity of the
uphill run was 90% $\text{VO}_2\text{max}$ compared to 52% $\text{VO}_2\text{max}$ for the other 2 conditions.

The neutrophil response to repeated exercise on the same day (Ronsen et al., 2001;
Boyum et al., 2002) and on repeated days (Suzuki et al., 1996a) has been studied. The
second of two exercise bouts separated by a 3 h recovery produced a significantly
stronger neutrophilia than a single exercise bout (Ronsen et al., 2001) or two bouts
separated by a 6 h recovery (Boyum et al., 2002). The exercise protocol of Suzuki and
coworkers (1996a) consisted of 7 consecutive days of cycling for 90 min at 70% $\text{VO}_2\text{max}$
with blood samples at 1, 4 and 7 days. A single bout of endurance exercise
induced a marked neutrophilia but the magnitude of the response to exercise on days 4
and 7 was attenuated. This may have reflected an adaptive response to repeated physiological stress, which may have confounded the results obtained at the end of the study.

2.5. Mechanisms underlying neutrophil distribution during and after exercise

The leukocytosis of exercise has been extensively studied (McCarthy and Dale, 1988). Following a prolonged bout of exercise, the leukocytosis of exercise shows a biphasic response consisting of an immediate increase in lymphocytes and neutrophils, followed by a lymphocytopenia (decrease in lymphocyte number) and a neutrophilia towards or following the cessation of exercise. The mechanisms which underly neutrophil mobilisation during and following prolonged exercise have been well documented (Pyne, 1994). Changes in the number of circulating neutrophils during exercise were originally attributed to the mechanical effect of the increase in cardiac output with exercise (Foster et al., 1986). The shear forces associated with an increased pulmonary blood flow may mobilise neutrophils from marginated pools within the vascular endothelium and lungs, and possibly the liver and spleen into the systemic circulation (Muir et al., 1984; McCarthy and Dale, 1988).

Although this appears the major mechanism for the increases in circulating neutrophil number during exercise, at high work rates the cardiac output begins to plateau while neutrophilia continues to increase (Blannin, 2006). Increased plasma catecholamine concentrations therefore make a significant contribution (Boxer et al., 1980, McCarthy and Dale, 1988, Tvede et al., 1994). The effects of catecholamines are mediated via adrenoreceptors, which are separated into 4 categories ($a_1$, $a_2$, $\beta_1$, $\beta_2$) based on their sensitivity to particular agonists (Benschop et al., 1996). Neutrophil
count has been reported to increase and neutrophil adherence to vascular endothelium is reported to decrease following adrenaline infusion (Boxer et al., 1980). When a β-
antagonist was infused prior to adrenaline infusion, there were no significant changes, suggesting that the response is mediated via β-adrenoreceptors. It was proposed that β-receptors on endothelial cells are stimulated by adrenaline to activate adenylate cyclase, which increases cAMP concentration and subsequently reduces neutrophil adherence (Boxer et al., 1980). Furthermore, adrenaline dose-dependently inhibited neutrophil adherence to endothelial cell monolayers (Bazzoni et al., 1991). In contrast, however, Benschop et al. (1996) reported that α-adrenoreceptors are involved in the effects of catecholamines on granulocyte trafficking. During exercise, there is a decrease in the expression of L-selectin (CD62L) on neutrophils (Van Eeden et al, 1999). L-selectin plays an important role in endothelial cell interaction, therefore an altered expression of cell-surface adhesion molecules may effect neutrophil mobilisation to and from the circulation.

The delayed neutrophilia that occurs following exercise has been attributed to the effects of cortisol (Gabriel et al., 1992; McCarthy et al., 1992; Pyne, 1994; Brenner et al., 1998). Cortisol is a steroid hormone, synthesised and secreted by the adrenal cortex, with its secretion controlled by the anterior pituitary ACTH (Brenner et al., 1998). Both psychological and physiological stressors are major stimuli for cortisol release. Cortisol concentrations increase in response to acute exercise with a critical level of approximately 60% VO₂ max (Davies and Few, 1973), but the effects of cortisol only appear after a lag-period that is in some cases maybe more than 1 hour (Viru and Viru, 2004). It is reported that cortisol typically stimulates the migration of neutrophils from the bone marrow into the circulation (Hetherington and Quie, 1985;
McCarthy et al., 1987). In rabbits, however, glucocorticoid infusion caused a significant granulocytosis primarily by the process of demargination with only a minor contribution from bone marrow release (Nakagawa et al., 1998). Plasma cortisol levels were found to directly correlate with granulocyte number (Moorthy and Zimmermann, 1978) and the neutrophil/lymphocyte ratio (Nieman, 1997) following endurance running. Furthermore, plasma cortisol was significantly higher at post-exercise following low-intensity, long duration exercise than high-intensity short duration exercise (Robson et al., 1999). Subsequently, circulating neutrophil counts were significantly higher throughout a 5 h recovery period following the prolonged duration exercise, yet others have reported no relationship between cortisol and post-exercise neutrophilia (Shinkai et al., 1996) or delayed neutrophilia (Hansen et al., 1991). Growth hormone and IL-6 have also been reported to mediate the delayed-onset neutrophilia (Suzuki et al., 1996b; Peake, 2002).

2.6. Exercise and neutrophil degranulation

An analysis of the number of circulating cells does not provide the whole story, because the function of those cells is an equally important consideration (Rowbottom and Green, 2000). An important microbicidal process of neutrophils is degranulation, which corresponds to the release of contents of azurophilic (primary) and specific (secondary) granules into the phagocytic vesicle to form a phagolysosome (Smith, 1997). A large body of evidence suggests that neutrophil degranulation increases after exercise of varied intensities and durations. For example, an increase in plasma MPO concentration has been reported following exercise to volitional exhaustion in both humans and rats (Suzuki et al., 1996b; Morozov et al., 2003), running for 1 h at 85% \( \dot{V}O_2 \text{max} \) (Peake et al., 2004) and marathon running (Suzuki et al., 2003). This was
reflected in a decreased neutrophil intracellular MPO concentration following exercise (Peake et al., 2004). Similarly, an increased plasma elastase concentration is reported following medium and prolonged-duration cycle ergometry exercise (Blannin et al., 1997b; Robson et al., 1999; Walsh et al., 2000; Bishop et al., 2001a, 2004). The authors concluded that exercise activates neutrophils; however Suzuki et al. (1999) reported that there was no difference in plasma elastase concentration following marathon running when adjusted on a per-cell basis, indicating that an increased plasma elastase concentration is not a useful parameter for neutrophil activation *in vivo* when accompanied by a similar neutrophilia.

It may be more clinically relevant to examine the effects of exercise on the changes in bacterially-stimulated elastase release per neutrophil, since the site of infection may only allow a limited number of cells to surround the infected tissue, regardless of the number of cells in the circulation (Bishop et al., 2001c). Bacterially-stimulated elastase release is widely reported to decrease on a per-cell basis following submaximal intensity exercise of prolonged duration (Robson et al., 1999; Walsh et al., 2000; Bishop et al., 2000; Bishop et al., 2003; Li and Gleeson, 2005). For example, research conducted by Bishop et al. (2000) and Walsh et al. (2000) using the same protocol (2 h cycling at 60 % $\text{VO}_2$ max) demonstrated post-exercise decreases in bacterially-stimulated elastase release on a per-cell basis of 40 and 33% respectively, compared with pre-exercise values. A protocol of the same duration at a higher relative exercise intensity (75% $\text{VO}_2$ max) revealed a 47% fall in bacterially-stimulated elastase release at post-exercise that remained at 45% of the resting value following a 1 h recovery (Bishop et al., 2003). The findings of Robson et al. (1999) confirmed that the duration rather than the intensity of exercise induces a larger
suppression of \textit{in vitro} neutrophil elastase release to bacterial stimulation. Neutrophil degranulation response was significantly lower at post and 2.5 h post-exercise after approximately 3 h cycling at 55\% $\tilde{V}O_2$ max compared to 80\% $\tilde{V}O_2$ max (resulting in fatigue within 1 h). At 24 h post-exercise, a significant reduction was still evident following the prolonged-duration trial, whereas neutrophil degranulation had returned to pre-exercise values on the high-intensity trial. Furthermore a 5-week endurance training period (30-60 min at 70\% $\tilde{V}O_2$ max) resulted in an attenuated degranulation response of resting blood samples to bacterial stimulation (Blannin et al., 1997a).

It has been speculated that falls in bacterially-stimulated neutrophil degranulation responses following exercise may be due to an inhibitory effect of plasma cortisol concentration, or may reflect a higher proportion of immature cells from the bone marrow into circulation that contain less cytotoxic granules and are therefore less responsive to stimulation (Robson et al., 1999). A further reason maybe that following an initial activation, the cells are less responsive to subsequent stimulation (Prasad et al., 1991). When CHO (6\% w/v) was fed prior to and during submaximal exercise of fixed duration, the rise in plasma cortisol was attenuated and there was no fall in bacterially-stimulated neutrophil degranulation response (Bishop et al., 2000); however CHO (5\% w/v) did not affect this response when exercise was performed to fatigue (Bishop et al., 2003). Furthermore, a number of studies have reported a reduced cortisol concentration following a number of dietary modifications both before and during exercise, but have reported no differences in bacterially-stimulated neutrophil elastase release, thus not supporting a role for cortisol in mediating the response (Bishop et al., 2001c; Lancaster et al., 2003; Bishop et al., 2004). It has also been reported that elastase availability is not a limiting factor for post-exercise
bacterially-stimulated elastase response on a per-cell basis (Bishop et al., 2003). Following exercise, the percentage of elastase release in response to bacterial stimulation fell to only 7-10% of the total elastase content, refuting the hypothesis that immature cells contain fewer cytotoxic granules that are available for release following bacterial stimulation (Bishop et al., 2003).

2.7. Exercise and neutrophil oxidative burst activity

As mentioned previously, the other major microbicidal process of neutrophils is the oxidative burst. This process is also referred to as the 'respiratory burst,' as it results in the production of superoxide anions ($O_2^-$), via the activation of NADPH oxidase. $O_2^-$ is rapidly converted to $H_2O_2$ and a series of reactions result in the highly toxic end-products HOCI, •OH, and $tO_2$ (Pyne, 1994; Smith, 1997). Although this is the most studied neutrophil function (Smith, 1997), researchers have reported conflicting findings from their studies. These equivocal results may have arisen due to differences in the intensity and duration of exercise protocols employed, the age, sex and training status of participants, the stimulant used to initiate the oxidative burst and assay techniques used to measure neutrophil function (Pyne, 1994; Peake, 2002).

Several studies, employing relatively long-duration exercise protocols (1 h to 2.5 h) have reported an increased ROS production at post-exercise and in the recovery period following exercise (Smith et al., 1990; Singh et al., 1994; Smith et al., 1996; Suzuki et al., 1996a; Nieman et al., 1998). The capacity of neutrophils to generate $H_2O_2$ in response to PMA and opsonised zymosan (OZ) was significantly enhanced following 1 h of exercise at 60% $\dot{V}O_2$ max and at a heart rate of 140 b.min$^{-1}$ (Smith et al., 1990; 1996). The authors reported that exercise 'primed' neutrophils for
subsequent activation. Nieman et al. (1998) showed a slight increase of 7-18% in granulocyte and monocyte oxidative burst response to *S. Aureus* bacteria following running or cycling protocols for 2.5 h at 75% \( \dot{V}O_2 \) max. These findings supported the earlier research of Singh et al. (1994) and Suzuki et al. (1996a). Singh and colleagues (1994) reported a significantly increased production of \( O_2^- \) in response to \( OZ \) stimulation following a run to exhaustion (approximately 2 h) at 70-75% \( \dot{V}O_2 \) max, that remained non-significantly elevated after a 24 h recovery period. Suzuki et al. (1996a) observed a 28% increased chemiluminescence (CL) response following a 90 min cycle at 70% \( \dot{V}O_2 \) max in untrained participants. However, both of these studies used purified neutrophils, thereby removing other influencing mediators that may be present in whole blood, therefore these findings may not accurately reflect the *in vivo* situation. In contrast however, it is claimed that the problem with whole blood use is that radical scavengers within the plasma could impede ROS metabolism of neutrophils (Suzuki et al., 1996a).

Following a marathon race, Hessel et al. (2000) noted a 1.5-fold increase in neutrophil luminol-dependent CL response to \( OZ \) stimulation. Using the same stimuli and exercise protocol, however, Suzuki et al. (2003) reported that luminol-dependent CL responses at post-race were reduced by 34% on a per-cell basis. In agreement with this, a number of studies have found that exercise decreases oxidative burst responses. For example, Davison and Gleeson (2005) reported that the PMA-stimulated neutrophil oxidative burst response was reduced by approximately 30% following 2.5 h cycling at 60% \( \dot{V}O_2 \) max. Stimulation with PMA also resulted in a 40% decreased \( H_2O_2 \) production following 1 h cycling at 60% \( \dot{V}O_2 \) max (Macha et al., 1990) and 4 h
cycling at 70% individual anaerobic threshold (IAT) reduced post-exercise f-MLP-stimulated neutrophil oxidative burst responses (Scharhag et al., 2002, 2006). In line with the equivocal nature of research in this area, a number of studies have reported no change in neutrophil oxidative burst responses following prolonged duration exercise of submaximal-intensity. Both Suzuki et al. (1994) and Walsh et al. (2000) observed no change in the neutrophil oxidative burst response following exercise at 60% \( \dot{V}O_2 \text{max} \) for 1 and 2 h respectively.

It has been proposed that neutrophil oxidative burst responses are dependent upon exercise intensity (Smith et al., 1996; Quindry et al., 2003). Quindry and co-workers (2003) measured \( O_2^- \bullet \) production before and after 4 exercise protocols: a \( \dot{V}O_2 \text{max} \) test, 10% above the lactate threshold (LT) for 45 min, 10% below LT for 45 min and 10% below LT until calorific expenditure equalled that expended when exercising at 10% above LT. Elevations in \( O_2^- \bullet \) were reported at 2 h post-exercise following the \( \dot{V}O_2 \text{max} \) test and the 10% above LT trial only, therefore it was concluded that exercise intensity is more important than total energy expenditure in the post-exercise neutrophil response. These findings are in contrast to those reported by Robson et al. (1999) who reported that approximately 3 h cycling at 55% \( \dot{V}O_2 \text{max} \) tended to cause greater falls in PMA-stimulated neutrophil oxidative burst response at 0 h, 1 h and 2.5 h post-exercise than cycling at 80% \( \dot{V}O_2 \text{max} \) to exhaustion.

Along with endurance exercise of prolonged duration, research has also been conducted using brief, high-intensity and moderate-intensity exercise protocols.
Following high-intensity exercise to exhaustion, equivocal findings have been reported. Hack et al. (1994) reported that PMA-stimulated neutrophil O$_2$• production was reduced by 37% following a graded treadmill run to exhaustion. Using a similar exercise protocol, Morozov et al. (2003) showed no change in neutrophil CL response, whereas a 3-fold increase in OZ-stimulated neutrophil O$_2$• production was found following an incremental cycle ergometry test to exhaustion (Miyazaki et al., 2001). In a study conducted by Pyne et al. (2000), neutrophil CL response was measured following 40 min of uphill interval treadmill running at 90% V$\text{O}_2$ max, compared to downhill and near-level treadmill running at 52% V$\text{O}_2$ max. Neutrophil CL response was significantly lower than baseline response at immediately post and 1 h post-exercise after the intense running, but CL was significantly enhanced at 6 h post-exercise following the 2 moderate-intensity exercise protocols. This evidence supports the notion that intense exercise may be immunosuppressive yet moderate exercise may have immunoenhancing effects.

Pyne et al. (2000) reported a decline in the neutrophil oxidative burst response during 40 min of moderate-intensity cycling that reached statistical significance after 30 min (53% reduction from pre-exercise values). This suggests that exercise impairs neutrophil oxidative burst activity on a per-cell basis for those cells in circulation. The authors subsequently termed this the 'post-exercise refractory period' in which neutrophils are less able to respond to microbial challenge (Pyne et al., 2000), in agreement with the findings of Prasad et al. (1991). In support of this, both PMA and f-MLP-stimulated neutrophil oxidative burst responses were significantly reduced following treadmill running to exhaustion at 80% V$\text{O}_2$ max (Mooren et al., 2001). It would also appear that when ultra-long duration endurance exercise is performed, a
longer period is required for neutrophil function to recover (Gabriel et al., 1994). In this study, cyclists rode to exhaustion (approximately 20 min) prior to and 8 or 9 days after completing an ultra-endurance event (762 ± 74 min). PMA-induced neutrophil oxidative burst response was not affected prior to the endurance event, but a 34% decrease was found when the protocol was repeated following the ultra-endurance event.

Many athletes train a number of times per day and as such, the effects of repeated bouts of exercise performed on the same day have been investigated. Boyum et al. (2002) compared the effects of 1 bout of 65 min exercise at 75% \( \dot{V}O_2 \) max with 2 bouts performed on the same day, separated by a 3 or 6 h recovery. PMA-induced CL per 200,000 granulocytes increased in all exercise trials, yet the total oxidative potential of the blood was significantly higher when the exercise bouts were separated by a 3 h recovery. Two bouts of 90 min cycling at 60% \( \dot{V}O_2 \) max separated by a 3 h recovery produced a larger total PMA-stimulated neutrophil oxidative burst response, but a greater decline when expressed on a per-cell basis than a single exercise bout (Li and Gleeson, 2005). In contrast, Pyne et al. (1996) reported that the magnitude of the initial decrease of the neutrophil oxidative burst response after 1 bout of treadmill running (40 min) was not exceeded by 2 bouts separated by a 1 h recovery period. The neutrophil oxidative burst response to exercise has been shown to differ between trained and untrained participants. Miyazaki et al. (2001) reported that OZ-stimulated neutrophil \( O_2^- \) release was significantly lower after a cycle ergometry test to exhaustion following a 12-week training programme, than it was prior to the training period. Furthermore, the capacity of the PMA-stimulated oxidative burst response of elite swimmers at rest was approximately half that of age and sex-matched untrained
control subjects (Pyne et al., 1995). The neutrophil oxidative burst response of elite swimmers significantly decreased during a monitored 12-week training period, reaching a nadir at a time corresponding to the peak endurance and quality training phases, but this was not associated with an increased incidence of infection.

2.8. Mechanisms of exercise-induced alterations in neutrophil response

2.8.1. Adrenaline

Neutrophils express adrenoreceptors of $\alpha_1$, $\alpha_2$, $\beta_1$ and $\beta_2$ class. Ignarro and co-workers (1974) demonstrated that cAMP is increased within the neutrophil cell following incubation with the $\beta$-agonist adrenaline. Stimulation by adrenergic agonists is reported to activate adenyl cyclase, inducing the synthesis of cAMP and this has been reported to decrease phagocytosis, chemotaxis, enzyme release and cellular adhesion of neutrophils (Ignarro et al., 1974; Dulis and Wilson, 1980; Chilcoat et al., 2002). An increase in cAMP concentration has specifically been shown to inhibit f-MLP-stimulated neutrophil oxidative burst responses but no effect was found when neutrophils were stimulated with PMA (Yu et al., 1995; Chilcoat et al., 2002; O'Dowd et al., 2004). Moreover, adrenaline decreased f-MLP-stimulated neutrophil CL measurements (Weiss et al., 1996) and $O_2$-• generation (Wenisch et al., 1996; Tintinger et al., 2001) in a dose-dependent manner. The magnitude of the inhibitory effect was most pronounced at clinically high-doses; however, in the presence of low concentrations of adenosine, physiological concentrations of adrenaline have been shown to strongly inhibit f-MLP-stimulated neutrophil oxidative burst responses (Bazzoni et al., 1991). In a more recent study, O’ Dowd et al. (2004) reported that adrenaline can inhibit f-MLP-stimulated neutrophil $O_2$-• release at physiological concentrations starting as low as 1 nmol.L$^{-1}$, with a significant decrease from control
at 5 nmol.L\(^{-1}\) adrenaline. Furthermore, a strong negative correlation \((r = -0.75, P<0.01)\) between plasma adrenaline and PMA-stimulated neutrophil \(O_2^-\) release has been reported following graded exercise to exhaustion (Hack et al., 1994).

The inhibition of f-MLP-stimulated neutrophil oxidative burst responses by adrenaline has been specifically attributed to the \(\beta_2\) adrenoreceptor subtype (Weiss et al., 1996). The recorded increase in cAMP following \(\beta_2\) adrenoreceptor stimulation may inhibit the f-MLP-stimulated neutrophil oxidative burst response through activation of Protein Kinase A (PKA), which subsequently suppresses cPLA\(_2\)-dependent arachidonic acid production. Arachidonic acid is a further pathway by which NADPH oxidase may be activated; however an adrenaline concentration of 5 nmol.L\(^{-1}\) was required for this effect to be observed (O'Dowd et al., 2004). It has also been speculated that adrenaline may deviate glucose from the NADPH producing pentose phosphate pathway, reducing substrate availability for \(O_2^-\) generation by the NADPH oxidase (Garcia et al., 1999).

2.8.2. Adenosine

Adenosine is known as a potent anti-inflammatory agent (Cronstein, 1994). Since neutrophils are the first cells to respond to bacterial invasion or injury and play a major part in acute inflammation, adenosine is an important regulator of neutrophil function (Gessi et al., 2000). Adenosine mediates its effects via activation of at least 4 cell surface receptors; \(A_1\), \(A_{2A}\), \(A_{2B}\) and \(A_3\) (Fredholm et al., 2000) and human neutrophils are known to express all 4 adenosine receptor subtypes, that are coupled to G proteins (Gessi et al., 2002).
Early *in vitro* work conducted by Cronstein and colleagues (1983) reported that adenosine inhibited f-MLP-stimulated neutrophil $O_2^\cdot$ generation but had minimal effect following PMA-stimulation. Maximal inhibition occurred with an adenosine concentration of 100 µmol.L$^{-1}$ and the removal of adenosine by adenosine deaminase (ADA) led to a marked enhancement of neutrophil $O_2^\cdot$ generation in response to f-MLP. At physiological concentrations, adenosine has also been shown to specifically inhibit $O_2^\cdot$ generation by f-MLP-stimulated neutrophils (Cronstein et al., 1985; Swain et al., 2003). f-MLP-stimulated neutrophil $O_2^\cdot$ generation was reduced to 60% of control following incubation with 0.1 µmol.L$^{-1}$ of adenosine with a further reduction to 39% following incubation with 1 µmol.L$^{-1}$ adenosine (Cronstein et al., 1985). Furthermore, a significant inverse relationship between the individual *in vivo* plasma adenosine concentration and *ex vivo* f-MLP-stimulated neutrophil $O_2^\cdot$ generation has been reported (Chouker et al., 2005). Inhibition of neutrophil $O_2^\cdot$ generation by adenosine is predominantly mediated via the $A_{2A}$ receptor subtype (Gessi et al., 2000). In contrast to this, acting via the $A_1$ receptor, adenosine is reported to promote positive neutrophil functions; chemotaxis (Cronstein et al., 1990), phagocytosis (Salmon and Cronstein, 1992) and endothelial adherence (Cronstein et al., 1992), however, the effects of adenosine acting at $A_{2A}$ receptors dominate the effects of $A_1$ receptors (Hasko and Cronstein, 2004).

A number of intracellular mechanisms have been proposed to account for the inhibitory effect of adenosine on f-MLP-stimulated neutrophil oxidative burst responses. $A_{2A}$ receptors are coupled to activation of adenyl cyclase through Gs binding proteins, and their occupancy by adenosine causes an increased intracellular cAMP concentration in neutrophils, which acts as a second messenger to alter cellular
function (Iannone et al., 1989; Thibault et al., 2002). Furthermore, Gessi et al. (2000)
reported a significant correlation between cAMP accumulation and inhibition of O$_2$–
generation by adenosine receptor agonists (Spearman rank correlation coefficient =
1.00). However, a number of studies have not found evidence to support cAMP as the
second messenger for adenosine inhibition of neutrophil oxidative burst responses
(Cronstein et al., 1988; Cronstein, 1994). The same authors also reported that A$_{2A}$
receptor occupancy does not affect calcium (Ca$^{2+}$) mobilisation from intracellular
stores (Cronstein et al., 1988). A$_{2A}$ receptor occupancy has also been reported to
inhibit the neutrophil oxidative burst response by suppressing phospholipase-D (PL-
D) activity (Thibault et al., 2000). Adenosine suppresses the signalling cascade in f-
MLP-stimulated neutrophils since PL-D catalyzes the hydrolysis of
phosphatidylincholine to yield phosphatic acid, which is further metabolised. These PL-
D second messengers regulate the oxidative burst and other neutrophil functions.
Suppression of f-MLP-induced PL-D activity has been correlated with an increased
cAMP concentration, that may activate PKA to mediate this suppression (Thibault et
al., 2000; Thibault et al., 2002). A final mechanism by which adenosine can inhibit
neutrophil oxidative burst responses is via an increased trafficking of
Flavocytochrome b, a key component of the NADPH oxidase (Swain et al., 2003).
The treatment of neutrophils with adenosine increased the trafficking of
Flavocytochrome b between cellular compartments within the neutrophil cell and
resulted in an increased movement from the plasma membrane to a higher density
membrane, coinciding with an accelerated shut-down of ROS production by f-MLP-
stimulated neutrophils.
While there is clear evidence for an inhibitory effect of adenosine on f-MLP-stimulated neutrophil oxidative burst responses, equivocal evidence has been presented with regards to the effect of adenosine on neutrophil degranulation responses. Adenosine has been reported to inhibit f-MLP-stimulated neutrophil lactoferrin release (Richter, 1992) and to dose-dependently inhibit neutrophil elastase release from neutrophils stimulated using LPS or TNF-α (Bouma et al., 1997). This was attributed to A₂ and A₃ receptor-mediated mechanisms, yet the concentrations of adenosine required to inhibit neutrophil degranulation were considerably higher than those required to inhibit neutrophil oxidative burst responses. At lower micromolar concentrations however, adenosine inhibited f-MLP-stimulated neutrophil lysozome release (Schmeichel and Thomas, 1987). In contrast however, a number of studies have reported no significant effects of adenosine on neutrophil degranulation responses (Marone et al., 1980; Cronstein et al., 1983; McGarrity et al., 1989; Swain et al., 2003). Reasons for the difference between studies may be related to the concentrations of adenosine used, incubation time prior to measurement, stimulation methods employed and the use of whole blood or isolated neutrophils.

2.8.3. Neutrophil responsiveness

As detailed in Section 2.4, endurance exercise increases the circulating number of leukocytes, of which neutrophils exhibit the greatest change in cell count. Suzuki et al. (1996b) characterised individual cell maturity and activation state using a histochemical nitro blue tetrazolium test. They reported that a neutrophil subpopulation with a less responsive $\mathrm{O}_2•^{-}$ production to in vitro stimulation was mobilised following exercise. They concluded that the reported decrease in neutrophil oxidative burst response on a per-cell basis is due to the increase of less responsive
neutrophils into circulation. Following a marathon race, a 10-fold increase in the
mobilisation of neutrophils from the bone marrow has been reported (Suzuki et al.,
2003). Immature neutrophils or those released from the bone marrow prematurely
have a lower capacity to produce ROS following stimulation (Berkow and Dodson,
1986), therefore the post-exercise neutrophilia may contribute to reduced neutrophil
functional capacity. A further speculated mechanism for a reduced neutrophil
functional response following exercise is provided by Prasad et al. (1991). In dogs,
pre-stimulated neutrophils had a reduced capacity to release ROS after subsequent
stimulation. Therefore exercise-induced activation of ROS release may reduce the
capability of neutrophils to respond to a subsequent bacterial challenge.

2.8.4. Other factors

A number of other potential factors have been proposed to mediate the exercise-
induced neutrophil oxidative burst response. The cytokines IL-6, IL-8, IL-10,
granulocyte-colony stimulating factor (G-CSF) and stress hormones, such as cortisol
and growth hormone that increase following prolonged and intensive exercise have
also been implicated as modulators of neutrophil function (Suzuki et al., 2003). Both
IL-6 and growth hormone are immunopotentiating (Smith, 1997) and IL-6 has been
positively correlated with neutrophil CL responses at post and 1 h post-exercise
(Suzuki et al., 1999). IL-6 may enhance G protein activity, contributing to the
assembly of NADPH oxidase formation (Peake, 2002). Furthermore, the activity of
several components of the signal transduction pathway are enhanced by IL-8 and G-
CSF, but IL-10 may attenuate neutrophil oxidative burst responses (Peake, 2002). The
effects of cortisol on neutrophil oxidative burst responses are equivocal. Robson et al.
(1999) attributed the lower tendency for PMA-stimulated oxidative burst response
following prolonged duration cycling to the effects of cortisol but Nieman et al. (1997) showed no relationship after prolonged and intensive running. Mooren and co-workers (2001) determined the alterations in intracellular Ca\(^{2+}\) signalling following exercise to exhaustion. Intracellular Ca\(^{2+}\) concentration increased following f-MLP-stimulation, reflecting an activated or primed neutrophil state, but oxidative burst responses were attenuated at post-exercise. This suggests an uncoupling of intracellular Ca\(^{2+}\) signals from other aspects of the neutrophil oxidative burst pathway following exhaustive exercise.

2.9. Caffeine

Athletes commonly consume caffeine as an ergogenic aid (Tarnopolsky, 1994). The effect of caffeine ingestion on the neutrophil oxidative burst response in humans, and in particular following exercise, has not been extensively researched (Section 2.10). Caffeine ingestion, however, is consistently associated with an increased secretion of adrenaline (Graham and Spriet, 1991, 1995) and is also reported to increase plasma cortisol concentration (Laurent et al., 2000). Furthermore, the main action of caffeine at physiological concentrations is that of adenosine-receptor antagonism (Ongini & Fredholm, 1996). Adenosine receptors are found in most tissues, including immune cells, therefore there are a number of potential mechanisms by which caffeine may affect neutrophil functional responses to exercise. Caffeine is a chemical substance that has been identified in about sixty plants and is obtained as a white, odourless, crystalline, bitter-tasting powder (Debry, 1994). It is a trimethyl purine base, trimethylxanthine (1, 3, 7-trimethylxanthine) with a molecular weight of 194.2. The chemical structure of caffeine is shown in Figure 2.2.
Figure 2.2. Chemical structure of caffeine (from Debry, 1994).

Caffeine is a legal and socially acceptable drug (Graham, 2001a). It is the most widely consumed behaviourally active substance in the world (Fredholm et al., 1999), yet it is not a typical nutrient nor is it essential for health (Graham, 2001a). Caffeine is contained in various foods, beverages and medications. Coffee undoubtedly serves as the primary source of caffeine in the adult, but caffeine is also contained in tea and cocoa, many soft drinks, chocolate and several drug preparations, including many over-the-counter products (Mandel, 2002). Energy drinks represent a relatively new category of beverages that contain caffeine in amounts that exceed those found in soft drinks, approaching the low end of the concentration range found in coffee (Magkos and Kavouras, 2005). Table 2.1 shows the content of common caffeine-containing products. Caffeine consumption from all sources is reported to be 70-76 mg.day\(^{-1}\) worldwide; however, this is much higher in the Western and developed world (Fredholm et al, 1999). It is estimated that caffeine consumption in the UK and US is approximately 200 mg.day\(^{-1}\) (3 mg.kg\(^{-1}\) body mass), whereas in parts of Scandinavia it may be as high as 400 mg.day\(^{-1}\) (6 mg.kg\(^{-1}\) body mass). There is considerable
variation in habitual caffeine intake, since half of the population do not consume coffee and some individuals consume substantial amounts (Mandel, 2002).

The liver demethylates this trimethylxanthine to three dimethylxanthines: paraxanthine, theophylline and theobromine, which are then further catabolised (Graham, 2001a). Caffeine absorption from the gastrointestinal tract is rapid and reaches 99% in humans approximately 45 to 60 min following ingestion, with a half-life in humans of between 2.5 and 4.5 h (Debry, 1994). It has been reported that gender, exercise and thermal stress have no effect on caffeine pharmacokinetics in men and women (Mclean and Graham, 2002).

<table>
<thead>
<tr>
<th>Source (serving size)</th>
<th>Caffeine content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee (150 ml)</td>
<td>40 – 180*</td>
</tr>
<tr>
<td>Tea (150 ml)</td>
<td>25 – 50*</td>
</tr>
<tr>
<td>Cola (330 ml)</td>
<td>50</td>
</tr>
<tr>
<td>Energy drinks (250 ml)</td>
<td>30 - 85</td>
</tr>
<tr>
<td>Chocolate bars (50 g)</td>
<td>40</td>
</tr>
<tr>
<td>Over the counter medication (1 tablet)</td>
<td>30 - 200</td>
</tr>
</tbody>
</table>

* Large variation in caffeine content due to source and preparation of product.

Anecdotal evidence has shown that caffeine occupies a prominent position in the athletic world. A 1993 survey of Canadian teenagers showed that 27% of athletes had used caffeine in the previous year (Graham, 2001a), and approximately 70% of US young athletes claimed to have used caffeine for social reasons (Wagner, 1991), yet it appears to be more prevalent in the sport of amateur and professional cycling.
(Delbeke and Debackere, 1984). A 1997 survey conducted during the US Professional Championship road race found that roughly 90% of the teams consumed caffeine, usually in the form of Coca-Cola at some point during the race (Martin, 1997). Cyclists that completed the survey were largely from North America, Europe and Australia.

Prior to 2004, the International Olympic Committee’s acceptable maximum level of caffeine was 12 µg.ml\(^{-1}\) of urine, however this was identified as a poor reflection of either dose or plasma caffeine concentration due to large individual variability (Graham, 2001a). With effect from January 2004, the WADA removed caffeine from its list of prohibited substances and caffeine is currently placed on a monitoring programme for 2006. The removal of caffeine from the WADA prohibited list may lead to an increased use by athletes during training and competition.

2.9.1. Caffeine consumption and exercise performance

Self-reported anecdotal evidence has shown that caffeine is consumed, particularly by endurance athletes as it has been demonstrated to provide an ergogenic benefit (Tarnopolsky, 1994). Initial research was conducted by Costill and colleagues (1978) who examined the effects of adding 330 mg of caffeine to decaffeinated coffee, that was given to trained cyclists 60 min prior to completing an exhaustion ride at 80% \(\dot{VO}_2\)\(_{max}\). Time to exhaustion was increased from 76 ± 5 min to 90 ± 7 min following the ingestion of caffeine. Subsequent work was conducted by Ivy et al. (1979) who reported an increased work output (7.4%) with caffeine ingestion (250 mg) before and during 2 h of isokinetic cycling exercise, than when an equivalent
dose of a glucose polymer drink or placebo was administered. Following on from this early research, many studies have also reported an ergogenic benefit of caffeine ingestion, which due to its rapid absorption from the gastrointestinal tract, is generally given 60 min prior to the start of exercise (Graham and Spriet., 1991; Spriet et al., 1992; Pasman et al., 1995; Cohen et al., 1996; Jackman et al., 1996; Van Soeren and Graham, 1998; Bruce et al., 2000 amongst many others). A caffeine dose of 6 mg.kg\(^{-1}\) body mass increased cycling endurance capacity at 80% \(\text{VO}_2\)\(_{\text{max}}\) by 22% (Greer et al., 2000) and doses of 9 mg.kg\(^{-1}\) body mass increased running and cycling time to exhaustion at 85% \(\text{VO}_2\)\(_{\text{max}}\) by 21 and 20 min respectively (Graham and Spriet, 1991). The mode of caffeine intake is important as it appears that coffee alone does not produce the ergogenic benefits of pure caffeine (Graham et al., 1998). The authors concluded that other compounds present in the coffee act to antagonise the responses occurring when caffeine is consumed alone, even in the presence of elevated plasma caffeine concentrations. A summary of studies investigating the effects of caffeine ingestion on endurance capacity at \(\geq 80\%\) \(\text{VO}_2\)\(_{\text{max}}\) is provided in Table 2.2.
Table 2.2. A summary of investigations employing various doses and timings of caffeine ingestion on endurance capacity (i.e. time to exhaustion at a fixed % of \( \dot{V}O_2 \) max).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Participants</th>
<th>Caffeine dose/timing</th>
<th>Protocol</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Costill et al.</td>
<td>9 cyclists (7 M: 2 F)</td>
<td>330 mg caffeine added to decaf coffee, given 60 min prior to exercise</td>
<td>Cycle to exhaustion at 80% ( \dot{V}O_2 ) max</td>
<td>CAF: 90 ± 7 min*, PLA: 76 ± 5 min</td>
</tr>
<tr>
<td>Graham and Spriet</td>
<td>7 trained athletes</td>
<td>9 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>Run and cycle to exhaustion at 85% ( \dot{V}O_2 ) max</td>
<td>Run: CAF: 71 ± 11 min*, PLA: 49 ± 7 min</td>
</tr>
<tr>
<td>Graham and Spriet</td>
<td>8 trained runners</td>
<td>0, 3, 6 or 9 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>Run to exhaustion at 85% ( \dot{V}O_2 ) max</td>
<td>CAFE: 59 ± 10 min*, PLA: 39 ± 7 min</td>
</tr>
<tr>
<td>Graham et al.</td>
<td>9 trained runners</td>
<td>4.45 mg.kg(^{-1}) given 60 min prior to exercise in various coffee products</td>
<td>Run to exhaustion at 85% ( \dot{V}O_2 ) max</td>
<td>Caffeine resulted in 7.5 – 10 min increase time to exhaustion in the absence of coffee, but had no difference in coffee products</td>
</tr>
<tr>
<td>Greer et al.</td>
<td>8 active volunteers</td>
<td>6 mg.kg(^{-1}) given 90 min prior to exercise</td>
<td>Cycle to exhaustion at 80% ( \dot{V}O_2 ) max</td>
<td>CAF: 41 ± 5 min*, PLA: 33 ± 3 min</td>
</tr>
<tr>
<td>Jackman et al.</td>
<td>14 varsity athletes</td>
<td>6 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>Cycle to exhaustion at 100% ( \dot{V}O_2 ) max</td>
<td>CAF: 4.9 ± 0.6 min*, PLA: 4.1 ± 0.4 min</td>
</tr>
<tr>
<td>Pasman et al.</td>
<td>9 trained cyclists</td>
<td>0, 5, 9 or 13 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>Cycle to exhaustion at 80% ( \dot{V}O_2 ) max</td>
<td>0: 47 ± 13 min, 5: 58 ± 11 min*, 9: 59 ± 12 min*, 13: 58 ± 12 min*</td>
</tr>
<tr>
<td>Spriet et al.</td>
<td>8 recreational cyclists</td>
<td>9 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>Cycle to exhaustion at 80% ( \dot{V}O_2 ) max</td>
<td>CAF: 96 ± 9 min*, PLA: 76 ± 5 min</td>
</tr>
<tr>
<td>Van Soeren and</td>
<td>6 recreational cyclists</td>
<td>6 mg.kg(^{-1}) given 60 min prior to exercise following 0, 2 or 4 days caffeine withdrawal</td>
<td>Cycle to exhaustion at 80 - 85% ( \dot{V}O_2 ) max</td>
<td>0 days: CAF: 75 ± 9 min*, PLA: 59 ± 4 min</td>
</tr>
<tr>
<td>Graham (1998)</td>
<td></td>
<td></td>
<td></td>
<td>2 days: CAF: 81 ± 7 min*, PLA: 60 ± 8 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 days: CAF: 82 ± 7 min*, PLA: 64 ± 6 min</td>
</tr>
</tbody>
</table>

Significantly improved endurance capacity (*P*<0.05).
The dose of caffeine administered has also been extensively studied. Graham and Spriet (1995) gave a 3, 6 or 9 mg.kg\(^{-1}\) body mass dose of caffeine 60 min before a run to exhaustion at 85\% \(\dot{VO}_2\) max. The low and moderate doses increased time to exhaustion by approximately 22\%, yet there was no significant increase with the high caffeine dose. Similarly, caffeine ingestion significantly increased cycling time to exhaustion at 80\% \(\dot{VO}_2\) max but there were no differences between 5, 9 and 13 mg.kg\(^{-1}\) body mass doses of caffeine (Pasman et al., 1995). Collectively, these studies demonstrate that there is not a linear relationship between intake and ergogenic benefit. A study by Conway et al. (2003) compared the effect of a single caffeine dose (6 mg.kg\(^{-1}\) body mass) versus a divided caffeine dose containing the same amount of caffeine (3 mg.kg\(^{-1}\) body mass given at 60 min prior to exercise and again at the midpoint of a 90 min cycle at 68\% \(\dot{VO}_2\) max). Following the fixed duration ride, a time-trial showed that performance did not significantly differ between doses, yet there was a clear trend of an ergogenic benefit of caffeine versus a placebo condition. A similar study reported that when a large bolus of caffeine (5 mg.kg\(^{-1}\) body mass) is given, the need to consume additional smaller doses of caffeine in order to maintain blood caffeine concentration is not required (Bell and McLellan, 2003). The effect of adding caffeine to a carbohydrate (CHO) electrolyte solution has also been shown to provide ergogenic benefit. Participants completed a cycling time-trial (TT) faster when 225 and 320 mg.L\(^{-1}\) caffeine were added to a 7\% CHO-electrolyte solution than when CHO alone was consumed (Kovaecs et al., 1998). An 11\% CHO solution containing 1.5 mg.kg\(^{-1}\) body mass of caffeine (Coca-Cola) was also associated with a performance enhancement (3.3\%) in a cycling TT following 2 h of submaximal intensity cycling. The improved performance was similar to that associated with larger caffeine doses (Cox et al., 2002).
Although it is clear that caffeine improves exercise time to exhaustion, there is equivocal evidence for the effect of caffeine on performance outcomes when there is a fixed end-point. Cohen et al. (1996) reported no significant differences between 0, 5 and 9 mg.kg\(^{-1}\) body mass of caffeine on 21 km race time performed in a hot environment (25°C-28°C), yet a 4% body mass loss may have confounded the ergogenic benefits of caffeine. In the laboratory setting, no effect of caffeine was found on 10 km treadmill time when participants could control their treadmill running velocity (Bell et al., 2002). Similarly, caffeine did not affect the time to completion or average power output throughout a 100 km cycling TT (Hunter et al., 2002). The authors argued that when a trial is close-ended with a fixed end-point, caffeine does not affect an athlete’s pacing strategy. However, this is in contrast to a number of TT and work output studies (Kovacs et al., 1998; Cox et al., 2002; Conway et al., 2003). Recently, a field-based study reported that a single dose of caffeine (3 mg.kg\(^{-1}\) body mass) ingested 1 h before exercise significantly reduced 8 km race time by 23.8 s, resulting in a 1.2% improvement in running performance (Bridge and Jones, 2006). A summary of studies investigating the effects of caffeine ingestion on performance outcomes in laboratory and field-based settings is shown in Table 2.3.

The effect of caffeine on brief and intense exercise has also produced equivocal findings. A 6 mg.kg\(^{-1}\) body mass dose of caffeine provided a significant increase in cycling time to exhaustion at 100% \(\dot{V}O_{2}\) max (Jackman et al., 1996), yet in a mock TT following a 10 min cycle at 90% \(\dot{V}O_{2}\) max, an absolute caffeine dosage of 250 mg did not affect mean power output. The participants used in the study however, were normally active and were non-cyclists, thus they may have been unaccustomed to cycling at heavy workloads (Collomp et al., 2002). In highly trained athletes, 2000
m row time was significantly improved by 1.3% with a 3% increased power output following the ingestion of 6 or 9 mg.kg$^{-1}$ body mass of caffeine. The lower dose resulted in a slightly faster rowing performance time (Bruce et al., 2000).
Table 2.3. A summary of investigations employing various doses and timings of caffeine ingestion on performance measures in laboratory and field-based settings.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Participants</th>
<th>Caffeine dose/timing</th>
<th>Protocol</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bell et al. (2002)</td>
<td>12 recreational runners</td>
<td>4 mg.kg(^{-1}) given 90 min prior to exercise</td>
<td>10 km run time, speed of treadmill self-controlled</td>
<td>No difference between CAF and PLA on run time</td>
</tr>
<tr>
<td></td>
<td>(10 M: 2 F)</td>
<td>3 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>8 km race in a field setting</td>
<td>CAF resulted in mean performance improvement of 23.8 s* (1.2% improvement)</td>
</tr>
<tr>
<td>Bridge and Jones</td>
<td>8 trained runners</td>
<td>6 or 9 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>2000 m row time on ergometer</td>
<td>6 mg.kg(^{-1}): 1.3% performance improvement*, 9 mg.kg(^{-1}): 1.0% performance improvement. Mean power was 2.7% higher in CAF trials</td>
</tr>
<tr>
<td>Jones (2006)</td>
<td></td>
<td></td>
<td></td>
<td>No difference between CAF and PLA trials. All times within 1.1% of each other</td>
</tr>
<tr>
<td>Bruce et al. (2000)</td>
<td>8 trained rowers</td>
<td></td>
<td></td>
<td>PLA: 28.3 ± 3.1 min</td>
</tr>
<tr>
<td>Cohen et al.</td>
<td>7 competitive runners</td>
<td>5 or 9 mg.kg(^{-1}) given 60 min prior to exercise</td>
<td>21 km race time in a field setting</td>
<td>Single dose: 24.2 min*, Divided dose: 23.4 min*</td>
</tr>
<tr>
<td>(1996)</td>
<td>(5 M: 2 F)</td>
<td>6 mg.kg(^{-1}) given 60 min prior to exercise, 3 mg.kg(^{-1}) given 60 min prior to exercise with 3 mg.kg(^{-1}) at 45 min of exercise</td>
<td>90 min cycle at 68% (\dot{V}O_2) max followed by TT (80% Wmax for 30 min)</td>
<td></td>
</tr>
<tr>
<td>Conway et al.</td>
<td>9 trained cyclists</td>
<td></td>
<td></td>
<td>Precaf: 3.4% improvement in performance*</td>
</tr>
<tr>
<td>(2003)</td>
<td></td>
<td></td>
<td></td>
<td>Durcaf: 3.1% improvement in performance*</td>
</tr>
<tr>
<td>Cox et al. (2002)</td>
<td>12 trained cyclists</td>
<td>6 mg.kg(^{-1}) given 60 min prior to exercise, 6 x 1 mg.kg(^{-1}) given at 20 min intervals</td>
<td>120 min cycle at 70% (\dot{V}O_2) max followed by TT (7kJ/kg)</td>
<td>No difference in 100 km TT time to completion, high intensity period time to completion or average power output between CAF and PLA trials</td>
</tr>
<tr>
<td>Hunter et al.</td>
<td>8 trained cyclists</td>
<td>6 mg.kg(^{-1}) given 60 min prior to exercise, 0.33 mg.kg.min(^{-1}) caffeine and 7% CHO given every 15 min of exercise</td>
<td>100 km cycling TT, interspersed with high intensity activity on cycle ergometer</td>
<td>PLA: 62.5 ± 1.3 min; 150 mg: 60.4 ± 1.0 min 225 mg: 58.9 ± 1.0 min*; 320 mg: 58.9 ± 1.2 min*. 225 mg and 320 mg significantly lower time than PLA.</td>
</tr>
<tr>
<td>Kovacs et al.</td>
<td>15 trained cyclists and</td>
<td>PLA or 150 mg, 225 mg or 320 mg caffeine contained in 7% CHO drink consumed throughout trial</td>
<td>Cycling TT requiring energy expenditure equivalent to 75% Wmax for 60 min</td>
<td></td>
</tr>
<tr>
<td>(1998)</td>
<td>triathletes</td>
<td></td>
<td></td>
<td>Significantly improved performance (*P&lt;0.05).</td>
</tr>
</tbody>
</table>

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2.9.2. Potential mechanisms for ergogenic benefit

It is well understood that caffeine provides ergogenic benefit in the majority of exercise protocols investigated. However, the precise mechanism by which this occurs remains less clear and a number of potential mechanisms have been proposed to explain the ergogenic benefit of caffeine ingestion. In the late 1970’s and early 1980’s, a number of researchers (Costill et al., 1978; Ivy et al., 1979; Essig et al., 1980) proposed a theory that caffeine ingestion resulted in an increased mobilisation of free fatty acids (FFA), leading to a sparing of muscle glycogen stores. These studies reported an increased rate of FFA oxidation and a decreased respiratory exchange ratio (RER) during exercise performed following caffeine ingestion. Spriet et al. (1992) reported that muscle glycogenolysis was reduced by 55% following caffeine ingestion but only during the first 15 min of exercise. It was hypothesised that the spared glycogen was available late in exercise, coinciding with an increased endurance capacity. More recent studies have reported no glycogen sparing during exercise following caffeine ingestion (Jackman et al., 1996; Greer et al., 2000; Laurent et al., 2000). It is difficult to explain why early studies consistently reported a glycogen sparing effect whereas latter studies have consistently not found such an effect, given that the exercise protocols employed were similar. Furthermore, caffeine has been shown to provide ergogenic benefit in activities lasting only a few minutes in which glycogen depletion could not be a limiting factor (Jackman et al., 1996; Bruce et al., 2000).

Caffeine ingestion has consistently been reported to increase plasma adrenaline concentration but not plasma noradrenaline concentration (Graham and Spriet, 1991; Graham et al., 1991; Spriet et al., 1992; Graham and Spriet, 1995; Jackman et al.,
1996; Graham et al., 2000 amongst numerous others). Collectively this would suggest that caffeine acts on the sympathetic nervous control of the adrenal medulla rather than increasing the activity of the sympathetic nervous system in general (Graham and Spriet, 1991). It was initially thought that an increased plasma adrenaline concentration, occurring with caffeine ingestion would stimulate FFA mobilisation; however, a number of studies have found an increased adrenaline response without a similar increase in FFA (Tarnopolsky et al., 1989; Graham and Spriet, 1991; Graham et al., 2000; Laurent et al., 2000). Alternatively, in spinal cord injury patients, in which the entire sympathetic and motor outflow is separated from the brain, the ingestion of 6 mg.kg⁻¹ body mass of caffeine did not increase plasma adrenaline concentration, yet plasma FFA and glycerol concentration increased rapidly and remained elevated throughout the experimental protocol (Van Soeren et al., 1996). Following on from these findings, Mohr et al. (1998) administered the same caffeine dose to tetraplegic patients 1 h prior to the electrical stimulation of their paralysed limbs to exhaustion. Caffeine ingestion significantly increased time to exhaustion (6%) and was associated with a significant increase of serum FFA concentration without a significant increase in adrenaline concentration. Furthermore, Graham and Spriet (1995) reported a 22% increase in endurance capacity following the ingestion of 3 mg.kg⁻¹ body mass of caffeine without a significant increase in adrenaline concentration, leading to the conclusion that a rise in adrenaline is not essential for the ergogenic benefit of caffeine.

Numerous other studies have failed to support the classical metabolite theory proposed in the 1970's. A lowering of the RER with caffeine ingestion has frequently been opposed (Graham and Spriet, 1991, 1995; Graham et al., 2000). Furthermore, in
a well controlled study using techniques of direct Fick measures and muscle biopsies
during a 1-h cycle at 70% \( \text{VO}_2 \text{max} \), Graham et al. (2000) reported an increased
arterial FFA concentration following the ingestion of 6 mg kg\(^{-1}\) body mass of caffeine,
yet the net uptake by the exercising leg was not enhanced. Net leg uptake of FFA was
calculated by multiplying the blood flow by the arterial-venous difference in
concentration.

Greer et al. (2000) speculated that there are three possible mechanisms through which
caffeine and other methylxanthines produce their effects at the cellular metabolic level
including an increased Ca\(^{2+}\) release, inhibition of cAMP phosphodiesterase and
antagonism of adenosine receptors. Caffeine induces the mobilisation of intracellular
Ca\(^{2+}\) from the sarcoplasmic reticulum and may improve neuromuscular function
(Debry, 1994). A concentration of 100 µmol L\(^{-1}\) caffeine is reported to increase the
sensitivity of Ca\(^{2+}\) release channels to endogenous activators, however
pharmacological caffeine concentrations (mmol L\(^{-1}\)) are required to directly activate
Ca\(^{2+}\) release channels (Magkos and Kavouras, 2005). At concentrations that have
provided ergogenic benefit, however, caffeine (9 mg kg\(^{-1}\) body mass) is reported to
attenuate the exercise-induced increase in plasma K\(^+\) by stimulating \( \text{Na}^+ / \text{K}^+ \) pump
activity in contracting and inactive skeletal muscles (Lindinger et al., 1993). These
changes may help maintain the membrane potential in contracting muscle and produce
increases in skeletal muscle force production that may contribute to the ergogenic
effect of caffeine during exercise (Spriet and Howlett, 2000). Caffeine can also inhibit
phosphodiesterase activity leading to an accumulation of intracellular cAMP, yet
pharmacological doses of methylxanthines are required to elicit these effects that are
not achievable \textit{in vivo} (Fredholm et al., 1999). It is now agreed that the most relevant
mechanism of action of caffeine at physiological doses (µmol.L⁻¹) is that of adenosine receptor antagonism. Methylxanthines are non-selective adenosine receptor antagonists at A₁ and A₂ receptors (Biaggioni et al., 1991). Adenosine receptors are found in most tissues of the body, including the brain, heart, adipocytes, skeletal muscle, smooth muscle and immune cells (Gessi et al., 2000; Graham et al., 2001a). The effects of caffeine on immune responses and potential mechanisms for action are described in Section 2.10. The increases in FFA concentration that occur with caffeine ingestion have been attributed to a stimulation of lipolysis as caffeine directly antagonises the A₁ receptors on adipocytes. Strong correlations between caffeine and FFA (r=0.93) and caffeine and glycerol (r=0.94) have been reported (Van Soeren et al., 1996). A strong correlation between paraxanthine (major metabolite of caffeine) and the appearance of FFA (r = 0.93) has also been observed (Hetzler et al., 1990). Paraxanthine is a more potent adenosine receptor antagonist than caffeine, hence may increase lipolysis further. These reports are in support of the spinal cord injury studies previously discussed (Van Soeren et al., 1996; Mohr et al., 1998) supporting the theory that methylxanthines act directly on the tissues, independent of the central nervous system.

Anecdotal evidence has suggested that caffeine can ‘stimulate the mind’ (Graham, 2001b) and is reported to improve alertness during prolonged wakefulness (Kamimori et al., 2000). In scientific terms, it is known that caffeine can cross the blood/brain barrier due to its lipophilic properties (Oldendorf, 1971). The CNS contains areas of adenosine-receptor populations and adenosine is known to inhibit the release of a number of brain excitatory transmitters, particularly dopamine (Okada et al., 1997). Decreases in dopamine and an increase in serotonin have been linked to the
development of central fatigue during exercise (Davis and Bailey, 1997). In support of this, the intracerebroventricular administration of a small dose of caffeine (200 µg) significantly increased run time to exhaustion by 60% compared to a placebo condition in rats. Following the administration of an A₁/A₂ adenosine receptor agonist, run time compared to placebo was reduced by 68%. When caffeine was administered 5 min prior to the A₁/A₂ agonist drug, it blocked the reduction in time to fatigue and was comparable with placebo (Davis et al., 2003). The same dose of caffeine (~0.6 mg.kg⁻¹ body mass) given peripherally was ineffective. The authors suggested that the blockade of adenosine receptors by caffeine within the CNS may explain the fatigue-delaying effects observed. As caffeine is likely to alter the release, binding or activity of neurotransmitters in the brain, it may alter the perception of work intensity. It has been reported that work output is increased at a given RPE following caffeine ingestion (Ivy et al., 1979; Cole et al., 1996) or more typically, a reduced RPE is reported for a given work intensity (Costill et al., 1978; Doherty et al., 2002, 2004). Caffeine ingestion may also provide ergogenic benefit by lowering the threshold for exercise-induced β-endorphin release (Laurent et al., 2000). An increase in β-endorphin is reported to promote euphoria and decrease pain perception and hypoalgesia has been reported during exercise following caffeine ingestion (O'Connor et al., 2004). Caffeine also affects changes in cognitive function after exercise. This may be important in many sports where reaction time, thought processes and tactical decisions are vital to success. Furthermore caffeine ingestion is also associated with increased feelings of happiness and pleasure during exercise (Backhouse et al., 2004).
2.9.3. Habitual caffeine consumption

There are surprisingly few studies that have determined the effect of caffeine ingestion on physiological and performance responses in habituated and naïve users. Equivocal findings have been reported. In animal models, habitual caffeine consumption is associated with an upregulation of adenosine receptors in the vascular and neural tissue of the brain (Fredholm et al., 1999). In habitual caffeine users (200 mg.day⁻¹), Tarnopolsky et al. (1989) reported no neuromuscular or metabolic effects (with the exception of increased FFA concentration) that would be beneficial for endurance exercise following the ingestion of 6 mg.kg⁻¹ body mass caffeine. This was attributed to caffeine tolerance. Similarly, the ingestion of a high caffeine dose (500 mg.day⁻¹) for a 6-week habituation period, significantly reduced the adrenaline concentration in response to an acute caffeine dose compared to that of naïve users (Bangsbo et al., 1992). Given the lack of a clear relationship between adrenaline concentration and endurance capacity (Graham and Spriet, 1995), the significance of this finding is unknown. Bell and McLellan (2002) determined the effects of 5 mg.kg⁻¹ body mass of caffeine on cycling time to exhaustion at 80% VO₂ max in caffeine users versus non-users. The magnitude of the effect differed between users and non-users, with a 19% improved performance for users but a 28% increase for non-users.

The majority of studies that have determined the influence of caffeine on physiological and performance parameters have employed a 48 – 72 h caffeine abstention, prior to dose administration. Following a 48 h withdrawal, caffeine ingestion did not effect substrate utilization during 60 min running (Hetzler et al., 1994). Caffeine ingestion also increased endurance capacity in both users and non-users following a similar abstention period (Graham and Spriet, 1991; Spriet et al.,
Furthermore, adrenaline responses were similar at exhaustion with caffeine ingestion regardless of 0, 2 or 4 days withdrawal in users with a high caffeine intake (760 mg.day⁻¹). Caffeine provided a clear ergogenic benefit on time to exhaustion at 80 – 85% \( \text{VO}_2 \text{max} \) but there was no effect of the number of days of withdrawal on the magnitude of the ergogenic impact (Van Soeren and Graham, 1998). Similarly, Tarnopolsky and Cupido (2000) showed that 6 mg.kg⁻¹ body mass caffeine potentiated dorsiflexor muscle force development during electrical stimulation with no difference between habituated and naïve users.

2.9.4. Negative effects of caffeine

Caffeine has a number of unwanted side effects that may limit its use in high doses by sensitive individuals: these effects include insomnia, headache and gastrointestinal distress (Maughan, 2002). The most widely held notion is that as caffeine is a mild diuretic it should not be consumed by athletes, since exercise stimulates sweating that may lead to dehydration. The belief is that caffeine exaggerates dehydration and impairs exercise performance. This viewpoint, however, is formed in the absence of any real supporting scientific evidence (Grandjean et al., 2000). Following a 6 day period of caffeine ingestion (3 mg.kg⁻¹ body mass), the ingestion of 0, 3 or 6 mg.kg⁻¹ body mass caffeine for an additional 5 days did not affect any indices of hydration (Armstrong et al., 2005). Furthermore, during an exercise heat tolerance test, acute caffeine ingestion did not alter fluid-electrolyte or thermoregulatory responses in habituated caffeine consuming subjects (Roti et al., 2006). There is therefore no evidence to suggest that moderate caffeine ingestion results in a fluid imbalance that is detrimental to health or performance (Armstrong, 2002).
2.10. Caffeine and immune function

A number of studies have demonstrated that caffeine can modulate various aspects of both innate and adaptive immune function (Horrigan et al., 2004, 2005, 2006). Very few studies, however, have been conducted using human subjects and fewer still using physiological caffeine concentrations that are relevant to normal consumption.

2.10.1. Innate Immunity

In vivo administration of 6 mg.kg.day\(^{-1}\) dose of caffeine significantly inhibited NK cell cytotoxicity to pokeweed mitogen (PWM) in rats but there was no effect of either 2 or 18 mg.kg.day\(^{-1}\) caffeine doses. In vitro, caffeine had no effect on NK activity (Kantamala et al., 1990). Also in rats, caffeine ingestion has been shown to increase \(O_{2}^{-}\) production of macrophages lavaged from the lung; however, this response was only seen with pharmological caffeine doses in the mmol.L\(^{-1}\) range (Jafari and Rabani, 2000). Ramanaviciene et al. (2004) reported that serum lysozyme activity was 1.4, 1.6 and 1.8 times higher than control when caffeine doses of 2 mg.kg\(^{-1}\), 20 mg.kg\(^{-1}\), and 40 mg.kg\(^{-1}\) were administered to mice. Circulating neutrophil counts were also significantly elevated by approximately 2.5-fold following the ingestion of 20-40 mg.kg\(^{-1}\) caffeine.

In blood obtained from healthy humans, 100 µmol.L\(^{-1}\) caffeine enhanced the CL response of mixed leukocytes (neutrophils and PBMC) primed by LPS but the same caffeine dose had no effect on neutrophil \(O_{2}^{-}\) production (Sullivan et al., 1995). Caffeine also enhanced MPO release from neutrophils and as the luminol-enhanced CL response is dependent upon both \(O_{2}^{-}\) production and MPO release, this accounted for the increased CL response following caffeine incubation. This response
only occurred in the presence of adenosine (30 nmol.L\(^{-1}\)), yet there was no effect at higher adenosine concentrations (µmol.L\(^{-1}\) range). The authors concluded that adenosine is necessary for the actions of caffeine to occur. The potential mechanisms by which caffeine may affect neutrophil function following exercise are detailed in Section 2.10.3. A further in vitro study reported that caffeine suppressed TNF-α production in LPS-stimulated whole-blood cultures (Horrigan et al., 2004). The response was reduced in each and every human subject to 47-69% of control but caffeine had no effect on IL-1β, IL-12 or IL-10 responses. The authors reported that as TNF-α production is an essential part of a healthy immune response, the impairment of TNF-α production by caffeine may have implications for the immune response to an infectious challenge. In contrast to these potentially negative effects of caffeine ingestion, Bishop et al. (2006) reported that caffeine ingestion (6 mg.kg\(^{-1}\)) 1 h prior to prolonged and intensive submaximal exercise is associated with elevations in salivary-IgA concentration, salivary-IgA secretion rate and α-amylase during exercise. These findings tentatively suggest that caffeine can enhance measures of mucosal immune function, since negative associations between resting salivary-IgA concentration and risk for URTI have been reported in elite swimmers during a training season (Gleeson et al., 1999a).

2.10.2. Adaptive Immunity
Kantamala et al. (1990) reported differential effects of caffeine on lymphocyte proliferation in rats. In vivo 6 mg.kg\(^{-1}\) caffeine significantly reduced B lymphocyte responses following PWM stimulation, yet 18 mg.kg\(^{-1}\) caffeine significantly increased T lymphocyte proliferative response to PHA-P. Caffeine dose-dependently inhibited both T and B lymphocyte responses in vitro. Caffeine also significantly inhibited
human T lymphocyte proliferative responses to tetanus toxoid, staphylococcal endotoxin B and PHA in a dose-dependent manner (Rosenthal et al., 1992). The authors argued that T lymphocyte function is inhibited via a cAMP dependent pathway as caffeine can inhibit specific cAMP-phosphodiesterases. Similarly, Horrigan et al. (2005) reported that caffeine reduced T lymphocyte proliferation in humans following Con A stimulation. The only published study that has investigated the in vivo effects of caffeine on adaptive immunity in humans, reported an immunostimulatory role for caffeine in T lymphocytes (Bishop et al., 2005). A 6 mg.kg\(^{-1}\) body mass dose of caffeine increased the natural state of lymphocyte activation, assessed by an increased percentage of CD4\(^{+}\) and CD8\(^{+}\) cells expressing the early activation marker CD69, both before and after a bout of prolonged intensive submaximal exercise. CD69 is expressed rapidly following T lymphocyte receptor expression and is associated with T lymphocyte proliferation and cytotoxic activity (Mueller et al., 2002); however, as the study did not expose the cells to mitogenic or antigenic challenge, it is unclear if this would lead to an altered T lymphocyte responsiveness.

In summary, caffeine has been demonstrated to have immunomodulatory properties, that are potentially both positive and negative for immune response. Few studies have been conducted using human subjects and of those that have used ‘physiological’ concentrations, it is unlikely that even these can be achieved with normal caffeine consumption. For example, Varani et al. (2005) reported a mean peak plasma concentration of 63 µmol.L\(^{-1}\) following the ingestion of 600 mg.day\(^{-1}\) caffeine for 7 days. Furthermore at 1 h following the ingestion of a 9 mg.kg\(^{-1}\) body mass caffeine
dose, plasma caffeine only increased to approximately 60 µmol.L\(^{-1}\) (Graham and Spriet, 1995).

### 2.10.3. Potential effects of caffeine on neutrophil function following exercise

Plasma adenosine concentration is known to increase during periods of metabolic stress such as ischaemia, hypoxia and exercise (Hasko and Cronstein, 2004). As detailed in Section 2.8.2, adenosine has been reported to stimulate chemotaxis and phagocytosis \textit{in vitro} via the $A_1$ adenosine receptor expressed by neutrophils, but inhibit neutrophil oxidative burst responses via the $A_{2A}$ receptor. Caffeine is a non-selective $A_1$ and $A_{2A}$ adenosine receptor antagonist, therefore it can inhibit the occupancy and/or action of adenosine receptors on neutrophil responses. Furthermore, caffeine ingestion is consistently associated with an increased secretion of adrenaline (Section 2.9.2). Stimulation of neutrophil $\beta_2$-adrenoreceptors by adrenaline is frequently reported to increase cAMP synthesis, leading to decreased neutrophil functional responses. Caffeine also increases the release of Ca\(^{2+}\) from the endoplasmic reticulum of cells (Biaggini et al., 1991) and is a phosphodiesterase inhibitor (Sullivan et al., 1995). Phosphodiesterase breaks down cAMP to the inactive 5'-AMP, therefore inhibition by caffeine and its metabolites can stimulate measurable concentrations of cAMP within neutrophils, potentially decreasing neutrophil function. However, caffeine concentrations of between 1 and 100 mmol.L\(^{-1}\) are required to cause these events, which are not achievable at physiological doses \textit{in vivo} (Biaggioni et al., 1991). A summary of the potential mechanisms by which caffeine may affect neutrophil function is outlined in Figure 2.3.
Caffeine induced increases in catecholamines, particularly adrenaline

Adrenaline stimulates β-2 adrenoreceptors on neutrophils

Caffeine inhibition of phosphodiesterase

Adenosine (via A2A receptors)

Caffeine induced mobilisation of Ca^{2+}

Caffeine antagonises A1 and A2A receptors

**Figure 2.3.** A summary of the proposed mechanisms by which caffeine can affect neutrophil function.
Chapter 3

General Methods
3.1. Ethical Approval

All study protocols presented in this thesis received prior approval by Loughborough University Ethical Advisory Committee. Each participant was fully informed about the rationale for the particular study, the experimental procedures to be undertaken and possible risks and discomforts that may have been experienced during the investigation. This was explained both verbally and in writing. Following an opportunity to ask any questions, participants provided written informed consent and were made aware that they could withdraw from the study at any time without providing reason.

Participants completed an initial health screening questionnaire (Appendix A) and a physical activity questionnaire (Appendix B) to ascertain if they were suitable candidates for the study. If participants reported taking any medication or experiencing symptoms of infection in the 4 weeks prior to participating in the study, they were unable to take part. A habitual caffeine consumption questionnaire (Appendix C) was also administered at the beginning of each study to classify participants as low, moderate or high habitual caffeine users, according to Daly (1993). On the day of each experimental trial, participants were required to complete a health questionnaire (Appendix D) to confirm that they were not experiencing any symptoms of URTI.

3.2. Preliminary procedures

Preliminary experimental measurements were conducted within a 2-week period prior to the beginning of the main trials. Maximal oxygen uptake ($\dot{V}O_2$ max) was estimated using a continuous incremental exercise test performed on an
electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands). Following an explanation of the protocol, participants began cycling at a work rate of 95 W, with increments of 35 W every 3 min and continued until volitional fatigue. Verbal encouragement was provided to each participant to ensure a maximum effort. Maximal work rate (Wmax) was determined according to the following formula: Wmax = Wout + (t/180) * 35, where Wout is the last completed stage and t is the time in seconds in the final stage (Jeukendrup et al., 1996). Samples of expired gas were collected into Douglas bags during the third min of each work rate increment and the final min of the exercise test. Heart rates were recorded continuously using short-range telemetry (Polar Beat, Polar Electro, Oy, Finland). The percentages of O₂ and CO₂ in the expired gas samples were measured using a paramagnetic O₂ analyser (Servomex 1420B, Crowborough, UK) and an infrared CO₂ analyser (Servomex 1415B) respectively. These were used alongside a dry gas meter (Harvard Apparatus, Edenbridge, UK) to determine 

\[ \dot{V}_{E}, \dot{V}_{O_2}, \text{and } \dot{V}_{CO_2}. \]

From the \( \dot{V}_{O_2} (l.min^{-1}) \) – Work rate (W) relationship, the work rate equivalent to 70% \( \dot{V}_{O_2} \text{max} \) (Chapters 5, 6, 7) or 65% \( \dot{V}_{O_2} \text{max} \) (Chapter 9) for each participant was interpolated.

Participants returned to the laboratory on a second occasion to undertake a familiarisation period that consisted of cycling for 60 min at a work rate corresponding to 65% or 70% \( \dot{V}_{O_2} \text{max} \). Heart rates were monitored continuously and 1-min expired gas samples were taken at 15 min intervals to ensure that the participants were exercising at the correct intensity. Work rate was adjusted if intensity was more than 5% higher or lower than the required intensity.
3.3. Standardisation of pre-trial conditions

Each participant was provided with a list of caffeine containing products (Appendix E) and instructed to abstain from these during the 60 h prior to each main experimental trial. They were also instructed to avoid strenuous physical activity and to record their dietary intake in the 24 h prior to the first experimental trial. This diet was then replicated as closely as possible in the 24 h prior to each additional trial in an attempt to standardise their nutritional status.

For each trial, participants arrived at the laboratory following an overnight fast of between 10 and 12 h. All participants were instructed to ingest ~300 ml of water upon waking on the morning of each trial in an attempt to standardise their hydration status prior to the exercise bouts.

3.4. Main trial procedures

In the experimental trials of Chapters 4-7, the method of caffeine administration was identical. Participants received 6 mg.kg\(^{-1}\) body mass of caffeine (BDH Laboratory Supplies, Poole, UK) or placebo (dextrose powder; BDH Laboratory Supplies, Poole, UK) dissolved in 200 ml no-added sugar pink grapefruit juice drink. Grapefruit juice was used to disguise the taste of the treatments. In Chapter 9 the same quantity of caffeine or placebo (6 mg.kg\(^{-1}\) body mass) was taken in the form of a cellulose capsule (Blackfirs Health, Cheshire, UK) with 2 ml.kg\(^{-1}\) body mass of water.

The mode of exercise in all exercise chapters was cycling performed using a electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands). The ergometer saddle and handlebar height was specifically adjusted to
each participant’s physical characteristics to optimise cycling performance and comfort. These parameters were recorded at the initial visit to the laboratory and set prior to each additional visit to speed up the transition time from rest to exercise.

Heart rate was recorded continuously during the exercise bouts via short range telemetry (Polar Beat, Polar Electro, Oy, Finland). Subjective ratings of perceived exertion (RPE) were measured every 15 min throughout each exercise protocol, according to Borg (1982). In addition, 1-min expired gas samples were collected throughout exercise to determine $\dot{V}_O_2$ and $\dot{V}_C_O_2$, to ensure that the participants were exercising at the correct intensity and to estimate carbohydrate and fat oxidation and contribution towards energy expenditure, as described by Peronnet and Massicote (1991).

3.5. Blood sampling and analysis

This section details blood analyses methods that have been used on more than one occasion throughout this thesis. All blood samples were collected by venepuncture from an antecubital forearm vein with the participant in the seated position. For each sample, approximately 23 ml of blood was collected into four evacuated monovette tubes (Sarstedt, Leicester, UK), two containing K$_3$EDTA (1.6 mg.ml$^{-1}$), one containing lithium heparin (1.5 IU.ml$^{-1}$) and one containing no additive to obtain serum.

3.5.1. Haematological analysis

Blood collected into one of the K$_3$EDTA monovettes (2.7 ml) was used to determine the total and differential leukocyte count, haematocrit and haemoglobin content using
a haematology analyser (AC-TM 5diff analyser, Beckman Coulter, UK). The intra-assay coefficient of variation was 1.3%, 0.9% and 1.1% for leukocyte counts, haemoglobin content and haematocrit respectively. Plasma volume changes were estimated from haemoglobin and haematocrit values according to Dill and Costill (1974) and cell counts were corrected for these plasma volume changes relative to the initial blood sample.

3.5.2. Measures of neutrophil function

**PMA-stimulated oxidative burst response**

*In vitro* neutrophil oxidative burst response following PMA-stimulation was measured using the ABEL® (Analysis By Emitted Light) cell activation test kit on a microplate according to manufacturer’s instructions (Knight Scientific Limited, Plymouth, UK). In duplicate analysis, 10 µl K$_3$EDTA blood was diluted 1:100 with blood dilution buffer (Hank’s Balanced Salt Solution; HBSS containing 20 mM Hepes, pH 7.4 but without magnesium or calcium). A 20 µl sample of this solution was added to the microplate, followed by the addition of 90 µl reconstitution and assay buffer (HBSS containing 20 mM Hepes, pH 7.4), 20 µl Adjuvant-K and 50 µl Pholasin (10 µg.ml$^{-1}$). For stimulated oxidative burst response, 20 µl of PMA stimulant (5 µg.ml$^{-1}$ working solution) was added to the microplate. Unstimulated oxidative burst response was determined by adding 20 µl reconstitution and assay buffer to the appropriate well of the microplate, which was placed in a luminometer (Anthos Lucy 1 Microplate Luminometer, Anthos, Labtec Instrument, Austria) and shaken for 60 s at 37°C. Pholasin-enhanced CL was measured every min for 30 min and the incremental area under the curve (IAUC) for stimulated and unstimulated oxidative burst responses were determined. Unstimulated IAUC was subtracted from stimulated IAUC to give
total oxidative burst response, which was divided by the neutrophil count per well to give oxidative burst response per neutrophil. Neutrophil count per well was determined from the cell counter results which were adjusted for the dilution of whole blood at the beginning of the assay. The intra-assay coefficient of variation was 6.4% for the PMA-stimulated oxidative burst response.

**f-MLP-stimulated neutrophil oxidative burst response**

In vitro neutrophil oxidative burst response following f-MLP-stimulation was measured using the ABEL® (Analysis By Emitted Light) cell activation test kit on a microplate according to manufacturer's instructions (Knight Scientific Limited, Plymouth, UK). The process of whole blood dilution and microplate preparation was identical to that of PMA-stimulated oxidative burst response to the point of cell stimulation. Briefly, upon the addition of 20 µl f-MLP stimulant (10 µmol.L⁻¹ working solution), the microplate was placed in a luminometer (Anthos Lucy I Microplate Luminometer, Anthos, Labtec Instrument, Austria) and incubated for 30 s at 37 °C. Following the 30 s incubation, CL was recorded every 1 s for 100 s and peak CL (total oxidative burst response) was determined. This value was then divided by the neutrophil count per well to calculate the oxidative burst response per neutrophil. The intra-assay coefficient of variation was 9.0% for the f-MLP-stimulated oxidative burst response.

**In vitro neutrophil degranulation assay**

Neutrophil plasma elastase release was determined as according to Robson et al. (1999). Briefly, 1 ml of heparinised whole blood was added to an eppendorf tube containing 50 µl of stimulant (10 mg.ml⁻¹), containing a mixture of both gram positive
and gram negative bacterial extracts (Sigma, Poole, UK). The contents were mixed by
inversion and incubated at 37°C for 1 h, with a gentle inversion of the eppendorf tubes
at 30 min. Following the incubation period, the eppendorf tubes were centrifuged at
5000 g for 2 min and the supernatant was immediately stored at -80°C for later
analysis. The elastase concentration in plasma before and after bacterial stimulation
was determined using an Enzyme-Linked Immunosorbent Assay (ELISA) kit
(BioVendor Laboratory Medicine Inc, Brno, Czech Republic). Unstimulated
neutrophil plasma elastase concentration was determined and subtracted from the
stimulated elastase concentration to give a total neutrophil release. This was adjusted
for neutrophil count to calculate elastase release on a per-cell basis in response to the
bacterial stimulant. The intra-assay coefficient of variation was 3% for the
degranulation assay.

3.5.3. Stress hormones and IL-6
The remaining heparinised blood (approximately 6.5 ml) and blood collected into the
second K3EDTA monovette (7.5 ml) were spun at 1500 g for 10 min in a refrigerated
centrifuge (4 °C) to obtain plasma. Of the heparinised plasma obtained, 2 ml was
immediately added to chilled tubes containing 200 µl of a glutathione (100 mM) and
EGTA (100 mM) anti-oxidant preservative (pH 6.5). Tubes were mixed and frozen at
-80°C before later determination of plasma adrenaline and noradrenaline
concentration by high-performance liquid chromatography with electrochemical
detection, as previously described (Forster and MacDonald, 1999). The remaining
heparinised plasma was stored at -80°C for later analysis of plasma cortisol, which
was determined using a commercially available ELISA method (DRG Diagnostics,
DRG instruments, Germany). The K3EDTA plasma was also stored at -80°C until
later analysis for IL-6 cytokine concentration using a commercially available ELISA procedure (Diaclone, Besancon Cedex, France). The intra-assay coefficient of variation was 6.6%, 1.9%, 1.6% and 2.9% for adrenaline, noradrenaline, cortisol and IL-6 respectively.

3.5.4. Blood-borne metabolites

Serum was obtained from whole blood which was left to clot on ice for 1 h, then spun at 1500 g for 10 min at 4 °C. Serum was transferred to eppendorf tubes and frozen at -80 °C. Serum caffeine concentration was determined using a commercially available kit (Emit Caffeine Assay, Dade-Behring, Milton Keynes, UK) on an automatic photometric analyser (COBAS Miras Plus, Roche Diagnostic Systems, Switzerland). Aliquots of K3EDTA plasma were used for determination of glucose (GOD-PAP method, Randox Laboratories, Co. Antrim, N. Ireland, UK) and free fatty acids (FFA) (Wako Chemicals, Neuss, Germany) on an automatic photometric analyser. Aliquots of heparinised plasma were also deproteinised by adding 100 µl plasma to 1 ml perchloric acid (0.3 M). Lactate concentration was determined in the deproteinised samples using a spectrophotometric method as described by Fink and Costill (1995). The intra-assay coefficient of variation was 2.9% and 3.3% for caffeine and lactate respectively and was less than 1% for both glucose and FFA.

3.6. Statistical analysis

All statistical analysis was conducted using SPSS 12.0 software for Windows (SPSS Inc., Chicago IL, USA). Statistical significance was accepted at the P<0.05 level and all data presented in the text, tables and figures throughout this thesis are expressed as mean values and the standard errors of the mean (± SEM). The data was checked for
normality and examined using two-factor (trial x time-point) analysis of variance (ANOVA) with repeated measures design. If the data was not normally distributed, analysis was performed on logarithmic transformed data. Assumptions of sphericity were checked and appropriate adjustments in the degrees of freedom of ANOVA, as according to Atkinson (2001) were made. Any significant differences were assessed using Student’s paired t-tests with Holm-Bonferroni adjustments for multiple comparisons. Single comparisons between trials were assessed using Student’s paired t-tests with Bonferroni adjustment.
Chapter 4

The effect of caffeine ingestion on resting hormonal and neutrophil functional responses.
4.1. Abstract

The aim of the study was to determine the effect of a single 6 mg.kg\(^{-1}\) body mass dose of caffeine on both PMA and f-MLP-stimulated neutrophil oxidative burst responses and bacterially-stimulated neutrophil degranulation response when participants rested for 3.5 h following caffeine ingestion. In a single-blind, counter-balanced design, 6 male participants (mean ± SEM: age 25 ± 2 years; body mass 75.6 ± 3.8 kg; height 1.79 ± 0.05 m) ingested 6 mg.kg\(^{-1}\) body mass of caffeine (CAF) or placebo (PLA) and rested in the laboratory for a 3.5 h period. During this time water was consumed *ad libitum*. Venous blood samples were obtained upon arrival (0 h), 60 min after CAF or PLA ingestion (1 h), 2.5 h after CAF or PLA ingestion (2.5 h) and a further sample 1 h later (3.5 h). The sampling points were chosen to coincide with the time-points that were used in the exercise trials of Chapters 5 and 6. CAF ingestion did not affect the neutrophil oxidative burst response following PMA (Time, \(P=0.55\); Trial, \(P=0.99\)) or f-MLP-stimulation (Time, \(P=0.46\); Trial, \(P=0.63\)) nor did it effect bacterially-stimulated neutrophil elastase release (Time, \(P=0.28\); Trial, \(P=0.84\)). Plasma adrenaline concentration was significantly higher on CAF than PLA at 1 h and 2.5 h (both \(P<0.05\)), but there were no significant differences for plasma noradrenaline concentration. There were no significant differences between CAF and PLA trials for either total leukocyte, neutrophil or lymphocyte count. Plasma FFA concentration was significantly elevated from rest at 2.5 h and 3.5 h on CAF trial only (\(P<0.05\)) but there were no differences between CAF and PLA for plasma glucose or cortisol concentrations. The findings of the study suggest that caffeine ingestion does not affect the PMA or f-MLP-stimulated neutrophil oxidative burst response or bacterially-stimulated neutrophil elastase release when participants are at rest.
4.2. Introduction

Caffeine is a legal, socially acceptable drug and is the most widely consumed behaviour influencing substance in the world (Graham, 2001a). Caffeine is contained in various foods, beverages and medications, yet caffeine is not a typical nutrient and is not essential for health. It is estimated that approximately 200 mg caffeine per person per day is consumed in the UK and US, whereas this intake may be doubled in certain Scandinavian countries (Fredholm et al., 1999).

There are relatively few studies that have investigated the effects of caffeine on specific indices of immune function in healthy volunteers using ‘normal’ caffeine doses. *In vitro*, caffeine has been demonstrated to have little effect on the production of the anti-inflammatory cytokine IL-10 or the pro-inflammatory cytokines IL-1β or IL-12, but it suppressed TNF-α production in LPS-stimulated human whole blood (Horrigan et al., 2004). Caffeine has also been reported to inhibit the proliferative responses of human lymphocytes to PHA, SEB and tetanus toxoid (Rosenthal et al., 1992). In contrast however, an immunostimulatory role for caffeine in T cells has been reported (Bishop et al., 2005). In this study, a 6 mg.kg⁻¹ body mass dose of caffeine increased the natural state of lymphocyte activation, assessed by an increased number of CD4⁺ and CD8⁺ cells expressing the early activation marker CD69, both before and after a bout of submaximal intensity exercise. Furthermore, the same caffeine dose was associated with increased salivary-IgA concentration and secretion rate during exercise, suggesting that mucosal immunity may be enhanced by caffeine (Bishop et al., 2006).
It is possible that caffeine may affect neutrophil function via a number of speculated mechanisms. Adenosine is reported to inhibit f-MLP-stimulated neutrophil oxidative burst responses (Cronstein et al., 1983, 1985; Swain et al., 2003) and may also inhibit neutrophil degranulation responses (Bouma et al., 1997). As caffeine is an antagonist of adenosine receptors, caffeine may modulate the effects of endogenous adenosine on neutrophil function. It has been reported that 100 µmol.L⁻¹ caffeine had no effect on neutrophil luminol-enhanced CL response when adenosine was not present. In the presence of adenosine however, the response was significantly increased (Sullivan et al., 1995). The same authors also reported no effects of caffeine on neutrophil O₂⁻• production, therefore an increased CL response was probably due to increased MPO release as luminol-enhanced CL reflects both O₂⁻• production and the release of MPO from neutrophilic granules. Caffeine ingestion also increases the secretion of adrenaline (Graham and Spriet, 1991, 1995; Jackman et al., 1996; Bishop et al., 2005) that is known to stimulate the β-2 adrenoreceptors expressed by neutrophils. Adrenaline has previously been reported to inhibit both PMA (Garcia et al., 1999) and f-MLP (Wenisch et al., 1996) stimulated neutrophil oxidative burst responses and may also inhibit neutrophil degranulation responses following stimulation (Tintinger et al., 2000).

The purpose of the present study, therefore, was to determine the effects of a single 6 mg.kg⁻¹ body mass dose of caffeine on both PMA and f-MLP-stimulated neutrophil oxidative burst responses and bacterially-stimulated neutrophil degranulation responses when participants rested for 3.5 h following ingestion. It was hypothesised that caffeine ingestion would have little effect on neutrophil functional responses when participants were at rest.
4.3. Methods

Participants

Six healthy males (mean ± SEM: age 25 ± 2 years; body mass 75.6 ± 3.8 kg; height 1.79 ± 0.05 m) volunteered to participate in the study. Participants were informed of the rationale and experimental procedures involved in the study, before providing written consent to participate. The study protocol had earlier received approval by Loughborough University Ethical Advisory Committee. Average daily caffeine intake was 242 ± 72 mg.day\(^{-1}\). One participant was characterised as a light user (< 50 mg.day\(^{-1}\)), 2 as moderate users (50-250 mg.day\(^{-1}\)) and 3 as having a high caffeine intake (> 250 mg.day\(^{-1}\)).

Experimental procedures

Participants undertook 2 trials separated by 7 days. In a single-blind, counter-balanced design, participants were randomly assigned to one of two experimental conditions: ingesting caffeine (CAF) or placebo (PLA). Participants arrived at the laboratory at 10.00 am following an overnight fast of between 10 and 12 h. Following a void, participants ingested 6 mg.kg\(^{-1}\) body mass of either CAF or PLA dissolved in 200 ml no-added sugar pink grapefruit juice drink and rested in the laboratory for a 3.5 h period. During this time, water was consumed \textit{ad libitum}. Venous blood samples were obtained upon arrival (0 h), 60 min after CAF or PLA ingestion (1 h), 2.5 h after CAF or PLA ingestion (2.5 h) and a further sample 1 h later (3.5 h). The sampling points were chosen to coincide with the time-points that were used during the exercise trials described in Chapters 5 and 6. Laboratory conditions were 23.0°C ± 0.3 °C and 45 ± 2% relative humidity.
Blood analytical methods

Blood sampling and analyses methods are detailed in Chapter 3.5.

Statistical analysis

Statistical analysis methods are detailed in Chapter 3.6.
4.4. Results

**Blood metabolites and hormones**

Serum caffeine was significantly higher at 1 h, 2.5 h and 3.5 h on CAF compared with PLA (all $P<0.01$; Figure 4.1). Plasma adrenaline concentration was significantly elevated above 0 h at 1 h and 2.5 h on CAF (both $P<0.01$) but there was no change on PLA. At 1 h and 2.5 h, plasma adrenaline concentration was significantly higher on CAF than PLA (both $P<0.05$; Figure 4.2). There were no significant differences over time or between CAF and PLA trials for either plasma noradrenaline, cortisol or glucose concentrations (Table 4.1). Plasma FFA concentration was significantly higher than 0 h at 2.5 h and 3.5 h on CAF only (both $P<0.05$; Table 4.1).

![Figure 4.1. Serum caffeine concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point (↑↑ $P<0.01$). Significantly higher than rest within trial (★★ $P<0.01$).](image-url)
Figure 4.2. Plasma adrenaline concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point († P<0.05). Significantly higher than rest within trial (*** P<0.01).

Table 4.1. Plasma concentrations of noradrenaline, cortisol and blood-borne metabolites on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>1 h</th>
<th>2.5 h</th>
<th>3.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenaline (nmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.59 (0.17)</td>
<td>1.91 (0.22)</td>
<td>1.77 (0.28)</td>
<td>-</td>
</tr>
<tr>
<td>PLA</td>
<td>1.35 (0.26)</td>
<td>2.26 (0.81)</td>
<td>1.63 (0.42)</td>
<td>-</td>
</tr>
<tr>
<td>Cortisol (nmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>301 (43)</td>
<td>297 (38)</td>
<td>295 (32)</td>
<td>243 (34)</td>
</tr>
<tr>
<td>PLA</td>
<td>315 (44)</td>
<td>272 (35)</td>
<td>261 (25)</td>
<td>270 (32)</td>
</tr>
<tr>
<td>FFA (mmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.30 (0.09)</td>
<td>0.52 (0.11)</td>
<td>0.71 (0.14)*</td>
<td>0.86 (0.21)*</td>
</tr>
<tr>
<td>PLA</td>
<td>0.36 (0.08)</td>
<td>0.34 (0.07)</td>
<td>0.41 (0.08)</td>
<td>0.54 (0.10)</td>
</tr>
<tr>
<td>Glucose (mmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>5.8 (0.4)</td>
<td>5.7 (0.2)</td>
<td>5.1 (0.3)</td>
<td>4.9 (0.3)</td>
</tr>
<tr>
<td>PLA</td>
<td>5.6 (0.4)</td>
<td>5.6 (0.3)</td>
<td>5.9 (0.4)</td>
<td>5.2 (0.3)</td>
</tr>
</tbody>
</table>

Significantly higher than 0 h within trial (*P<0.05).
Total and differential leukocyte counts

There were no significant differences between CAF and PLA trials for circulating leukocyte, neutrophil or lymphocyte counts. These counts did not significantly change throughout the course of the experimental protocol. Total and differential blood leukocyte count during the 3.5 h resting period is presented in Table 4.2.

**Table 4.2. Total and differential blood leukocyte counts on CAF and PLA trials.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Leukocytes ($x 10^9 \cdot L^{-1}$)</th>
<th>Neutrophils ($x 10^9 \cdot L^{-1}$)</th>
<th>Lymphocytes ($x 10^9 \cdot L^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>CAF 5.0 (0.4)</td>
<td>CAF 2.5 (0.3)</td>
<td>CAF 1.68 (0.17)</td>
</tr>
<tr>
<td></td>
<td>PLA 4.6 (0.5)</td>
<td>PLA 2.2 (0.2)</td>
<td>PLA 1.68 (0.17)</td>
</tr>
<tr>
<td>1 h</td>
<td>CAF 5.6 (0.4)</td>
<td>PLA 2.7 (0.3)</td>
<td>PLA 2.00 (0.14)</td>
</tr>
<tr>
<td></td>
<td>CAF 5.5 (0.6)</td>
<td>CAF 3.1 (0.4)</td>
<td>PLA 1.73 (0.13)</td>
</tr>
<tr>
<td>2.5 h</td>
<td>CAF 5.6 (0.6)</td>
<td>PLA 3.1 (0.4)</td>
<td>CAF 1.73 (0.13)</td>
</tr>
<tr>
<td></td>
<td>PLA 3.4 (0.4)</td>
<td>PLA 2.6 (0.2)</td>
<td>PLA 1.73 (0.19)</td>
</tr>
<tr>
<td>3.5 h</td>
<td>CAF 5.8 (0.5)</td>
<td>PLA 2.6 (0.2)</td>
<td>CAF 1.73 (0.15)</td>
</tr>
<tr>
<td></td>
<td>PLA 2.6 (0.3)</td>
<td>PLA 2.6 (0.2)</td>
<td>PLA 1.63 (0.13)</td>
</tr>
</tbody>
</table>

Neutrophil Function

There were no significant differences between CAF and PLA trials ($P = 0.99$; Figure 4.3) for PMA-stimulated neutrophil oxidative burst responses. The response did not change over time throughout the resting protocol ($P = 0.55$). Likewise, there were no significant differences between CAF and PLA trials ($P = 0.63$), or over time ($P = 0.46$) for f-MLP-stimulated neutrophil oxidative burst responses (Figure 4.4). Neutrophil elastase release in response to bacterial stimulation was similar between CAF and PLA conditions ($P = 0.84$; Figure 4.5). Elastase release did not significantly change over the 3.5 h resting period ($P = 0.28$).
Figure 4.3. PMA-stimulated neutrophil oxidative burst response expressed as total chemiluminescence (IAUC) in relative light units on CAF and PLA trials.

Figure 4.4. f-MLP-stimulated neutrophil oxidative burst response expressed as peak chemiluminescence per neutrophil cell on CAF and PLA trials.
Figure 4.5. Neutrophil elastase release (expressed as a % of baseline response) following bacterial stimulation on CAF and PLA trials.
4.5. Discussion

The main findings of the present study suggest that caffeine ingestion (6 mg.kg$^{-1}$ body mass) has no effect on either PMA or f-MLP-stimulated neutrophil oxidative burst responses or bacterially-stimulated neutrophil degranulation responses when participants rested for 3.5 h following caffeine ingestion. Although CAF was associated with an increased adrenaline concentration above PLA, it was not associated with a decreased neutrophil function. In vitro studies have shown that PMA-stimulated rat neutrophils have a reduced capacity to produce O$_2^-$ following incubation with 5 nmol.L$^{-1}$ adrenaline (Garcia et al., 1999). A similar adrenaline concentration is known to inhibit the f-MLP-stimulated neutrophil oxidative burst response (Wenisch et al., 1996) whereas incubation of isolated human neutrophils with β-2 adrenoreceptor agonists may inhibit neutrophil degranulation responses following stimulation (Tintinger et al., 2000). In the present study, it is likely that an increase of approximately 0.3 nmol.L$^{-1}$ adrenaline following caffeine ingestion is insufficient to produce these immunosuppressive effects observed with higher adrenaline concentrations.

The major action of caffeine at physiological concentrations is as an adenosine receptor antagonist (Ongini and Fredholm, 1996). Adenosine is reported to inhibit f-MLP-stimulated oxidative burst responses when incubated with neutrophils in vitro (Cronstein et al., 1985; Swain et al., 2003). Incubation with 100 nmol.L$^{-1}$ and 1 µmol.L$^{-1}$ adenosine inhibited neutrophil ROS production by 38% and 60% respectively (Swain et al., 2003). Adenosine, acting via A$_2$ and A$_3$ receptors has also been shown to inhibit LPS stimulated human neutrophil elastase release with an IC$_{50}$ of 14 µmol.L$^{-1}$ (Bouma et al., 1997). As the participants were at rest in the present
study, their basal adenosine concentrations would be expected to be low. Mean plasma adenosine concentrations of 50 nmol.L\(^{-1}\) (Chouker et al., 2005) and 82 nmol.L\(^{-1}\) (Moser et al., 1989) have been reported in humans at rest, therefore this would suggest that in the present study, caffeine ingestion has no effect on neutrophil responses when endogenous adenosine concentration is low. It is likely that caffeine may exert greater effects in situations such as intense exercise where there is a discrepancy between the rate of ATP consumption and ATP synthesis in muscle, leading to an increased adenosine concentration in blood that may exert suppressive effects upon neutrophil function (Fredholm et al., 1999).

A study conducted by Sullivan et al. (1995) reported no effect of caffeine (100 µmol.L\(^{-1}\)) on O\(_2\)-• generation in an LPS-stimulated mixed leukocyte mixture and in pure neutrophils primed with rhTNF-α. Similarly in the present study, caffeine did not affect PMA or f-MLP stimulated neutrophil oxidative burst responses at serum concentrations of 40 to 50 µmol.L\(^{-1}\). The average caffeine dose administered in the present study was 450 mg, equating to 3 or 4 cups of very strong coffee (Fredholm, 1980). It is likely therefore, that 100 µmol.L\(^{-1}\) caffeine concentrations that previous studies have termed ‘physiological’ are not possible for the average person unless extraordinarily large caffeine doses are consumed. For example, Varani et al., (2005) reported a mean peak plasma concentration of only 63 µmol.L\(^{-1}\) following the ingestion of 600 mg caffeine per day for 7 days.

As mentioned previously, CAF significantly increased plasma adrenaline concentration, but had no effect on plasma noradrenaline concentration. An increased secretion of adrenaline following caffeine ingestion has consistently been reported in
both resting and exercise studies (Spriet et al., 1992; Graham and Spriet, 1995; Jackman et al., 1996; Graham et al., 2000; Bishop et al., 2005). In contrast to the findings for adrenaline, studies have rarely found an increase in plasma noradrenaline concentrations, which is consistent with the findings of the present study. CAF increased FFA concentration at 2.5 h and 3.5 h following ingestion but there was no difference for blood glucose concentration. Since adenosine is known to inhibit lipolysis (Fredholm, 1978), it is likely that caffeine antagonises $A_1$ receptors of adipocytes and this enhances lipolysis (Graham, 2001a). It may also be that caffeine-induced increases in adrenaline concentration augment lipolysis via $\beta$-adrenergic receptor stimulation (Kobayashi-Hattori et al., 2005); however, it has been reported that FFA mobilisation occurred without an increased adrenaline concentration in tetraplegic participants at rest (Van Soeren et al., 1996).

There was no effect of CAF on resting total leukocyte count, neutrophil or lymphocyte count for 3.5 h following ingestion. In mice studies, doses of 2 mg.kg$^{-1}$ and 40 mg.kg$^{-1}$ body mass caffeine did not influence neutrophil count, whereas 20 mg.kg$^{-1}$ body mass caffeine significantly increased neutrophil count by 2.5 fold (Ramanaviciene et al., 2004). In humans, a 6 mg.kg$^{-1}$ body mass dose of caffeine significantly increased circulating lymphocyte count above PLA at 60 min following caffeine ingestion and was attributed to an increased adrenaline concentration at that time (Bishop et al., 2005). In the present study, circulating lymphocyte count was $2.0 \times 10^9$.L$^{-1}$ on CAF and $1.71 \times 10^9$.L$^{-1}$ on PLA in the presence of a significantly higher adrenaline concentration on CAF than PLA, yet this difference was not statistically significant.
In summary, the findings of this study suggest that caffeine ingestion significantly increased plasma adrenaline concentration but had no effect on neutrophil function as measured by PMA and f-MLP-stimulated oxidative burst and bacterially-stimulated degranulation responses when participants rested for 3.5 h following caffeine ingestion.
Chapter 5

The effect of caffeine ingestion on hormonal and neutrophil functional responses following prolonged and intensive submaximal cycling.
5.1. Abstract

The purpose of the present study was to determine the effect of caffeine ingestion on PMA-stimulated neutrophil oxidative burst and bacterially-stimulated neutrophil degranulation responses following exercise. Eleven endurance trained males (mean ± SEM: age 23 ± 1 years; body mass 73.0 ± 2.6 kg; \( \dot{V}O_2 \max \) 61.0 ± 1.6 ml.kg\(^{-1}\).min\(^{-1}\); Peak Power Output 327 ± 10 W) ingested 6 mg.kg\(^{-1}\) body mass of caffeine (CAF) or placebo (PLA) 60 min before cycling for 90 min at 75% \( \dot{V}O_2 \max \). Venous blood samples were obtained at rest, 60 min after CAF or PLA ingestion (pre-exercise), immediately post-exercise and at 1 h post-exercise. CAF ingestion did not affect either PMA-stimulated oxidative burst or bacterially-stimulated elastase release of neutrophils compared with PLA. Regardless of condition, PMA-stimulated oxidative burst was decreased at post-exercise on a per-cell basis \((P<0.01)\) whereas elastase release (% of baseline response) tended to be lower at this time-point \((P = 0.076)\). CAF was associated with increased plasma adrenaline at pre \((P<0.01)\) and post-exercise \((P<0.05)\). There was no effect of CAF on circulating leukocyte or neutrophil count but lymphocyte count was significantly higher at pre-exercise on CAF \((P<0.05)\). The RPE of participants was significantly lower from mid-way \((P<0.05)\) through to the end of the exercise protocol on CAF than PLA \((P<0.01)\). These results suggest that CAF does not affect PMA-stimulated oxidative burst or bacterially-stimulated neutrophil degranulation responses following exercise. The insensitivity of the PMA-activated response of human neutrophils to cAMP elevating agents suggests that PMA may not be a suitable stimulant for investigating the influence of caffeine ingestion on neutrophil oxidative burst responses.
5.2. Introduction

The findings of Chapter 4 suggest that caffeine ingestion (6 mg.kg\(^{-1}\) body mass) does not affect either PMA or f-MLP-stimulated neutrophil oxidative burst response in humans at rest. To the author’s knowledge there are no studies that have specifically investigated the influence of caffeine ingestion on neutrophil responses following exercise. In response to prolonged and intensive submaximal exercise, PMA-stimulated neutrophil oxidative burst (Robson et al., 1999; Davison and Gleeson, 2005) and bacterially stimulated neutrophil degranulation responses typically decrease on a per-cell basis (Bishop et al., 1999, 2003; Walsh et al., 2000).

There are a number of speculated mechanisms by which caffeine may affect neutrophil function following exercise. Plasma adenosine concentration increases during exercise by the dephosphorylation of AMP (Vizi et al., 2002). Human neutrophils express all four classes of adenosine receptor subtypes on their surfaces (Gessi et al., 2002) and adenosine is well known to modulate neutrophil functional responses (Cronstein, 1994). Adenosine, acting via \(A_1\) receptors promotes neutrophil chemotaxis and phagocytosis (Cronstein et al., 1990), whereas acting through \(A_{2A}\) receptors, it has opposite effects, inhibiting phagocytosis and superoxide generation (Cronstein et al., 1983, 1990; Gessi et al., 2002). This inhibition occurred following f-MLP but not PMA-stimulation of neutrophils (Cronstein et al., 1983). Adenosine may also inhibit neutrophil degranulation responses (Sullivan et al., 1995; Bouma et al., 1997), but this has not been consistently reported (Cronstein et al., 1985; Swain et al., 2003). Caffeine and its derivatives are non-selective adenosine receptor antagonists (Ongini and Fredholm, 1996), hence their action may affect both oxidative burst and degranulation aspects of neutrophil function.
It is well known that caffeine increases plasma levels of catecholamines, particularly adrenaline (Graham and Spriet, 1991, 1995; Jackman et al., 1996; Graham et al., 2000 amongst others) which stimulates β-2 adrenoreceptors expressed by neutrophils. This stimulation activates adenyl cyclase, inducing cAMP synthesis which is reported to decrease the phagocytosis, chemotaxis and enzyme release of neutrophils (Dulis and Wilson, 1980). In rats, the rate of PMA-induced neutrophil $O_2$-$\bullet$ production was significantly reduced by 5 nmol.L$^{-1}$ adrenaline in the presence of glucose (Garcia et al., 1999), whereas the incubation of isolated human neutrophils with β-2 adrenoreceptor agonists may (Tintinger et al., 2000) or may not (Barnett et al., 1997) inhibit neutrophil degranulation response following stimulation.

Taking this information together, caffeine may affect neutrophil functional responses following prolonged exercise via adenosine receptor antagonism, or via enhanced adrenaline concentrations. The purpose of the present study therefore, was to investigate the influence of caffeine ingestion on the PMA-stimulated oxidative burst response and bacterially-stimulated degranulation response of human neutrophils following prolonged intensive submaximal exercise.
5.3. Methods

Participants

Eleven endurance trained males (mean ± SEM: age 23 ± 1 years; body mass 73.0 ± 2.6 kg; \(\bar{\text{VO}}_2\text{max} \ 61.0 \pm 1.6 \text{ml.kg}^{-1}.\text{min}^{-1}\); Peak Power Output 327 ± 10 W) volunteered to participate in the study. Participants were informed of the rationale and experimental procedures involved in the study, before providing written consent to participate. The study protocol had earlier received approval by Loughborough University Ethical Advisory Committee. Average daily caffeine intake was 160 ± 39 mg.day\(^{-1}\). Four participants were characterised as light users (< 50 mg.day\(^{-1}\)), 5 as moderate users (50-250 mg.day\(^{-1}\)) and 2 as having a high caffeine intake (> 250 mg.day\(^{-1}\)).

Experimental procedures

Preliminary measurements (\(\bar{\text{VO}}_2\text{max} \) and familiarisation) were conducted within a 2-week period prior to the start of the main trials. Preliminary measurements and pre-trial standardisation procedures are described in Chapter 3. Participants performed 2 sessions of exercise separated by 7 days. The participants acted as their own controls in a repeated-measures, single-blind, cross-over design and were randomly assigned to one of two experimental conditions: ingesting caffeine (CAF) or placebo (PLA). Participants arrived at the laboratory at 10.00 am following an overnight fast of between 10 and 12 h. Following a void, body mass was recorded before participants ingested 6 mg.kg\(^{-1}\) body mass of either CAF or PLA dissolved in 200 ml no-added sugar pink grapefruit juice drink. After resting quietly for 60 min, participants began cycling for 90 min at 75\% \(\bar{\text{VO}}_2\text{max} \). Heart rate and RPE were measured every 15 min throughout exercise and 2 ml.kg\(^{-1}\) body mass of water was administered at these
time points. At 20, 50 and 80 min of exercise, 1-min expired gas samples were collected. Following the cessation of exercise, body mass was recorded and participants consumed 3 ml.kg\(^{-1}\) body mass of water before resting for a further 60 min. Venous blood samples were obtained at rest, 60 min after CAF or PLA ingestion (pre-exercise), immediately post-exercise and at 1 h post-exercise. Laboratory conditions were 16\(^\circ\)C - 20\(^\circ\)C and 57 ± 3% relative humidity.

**Blood analytical methods**

Blood sampling and analyses methods are detailed in Chapter 3.5.

**Statistical analysis**

Statistical analysis methods are detailed in Chapter 3.6.
5.4. Results

*Physiological variables and RPE*

Exercise intensity did not significantly differ between trials; mean % VO₂ max throughout exercise was 74.9 ± 0.8% and 74.2 ± 0.6% on CAF and PLA trials, respectively. Following exercise, body mass change (corrected for fluid consumed) fell similarly on both trials (-1.8 ± 0.2 kg and -1.7 ± 0.2 kg on CAF and PLA respectively). At post-exercise, plasma volume had decreased by 9.2 ± 1.8% on CAF and 6.6 ± 2% on PLA compared with resting values, but this difference was not statistically significant (P = 0.375). Heart rate during exercise was similar between trials (157 ± 1 beats.min⁻¹ and 156 ± 1 beats.min⁻¹ on CAF and PLA respectively, mean of all recordings). RPE increased on both trials with exercise duration but was significantly lower at 45 (P<0.05), 60, 75 and 90 min on CAF than PLA (P<0.01; Figure 5.1).
Figure 5.1. Perceived ratings of exertion (RPE) during 90 min cycling at 75% VO2max on CAF and PLA conditions. Significantly lower on CAF than PLA at that time point († P<0.05, †† P<0.01). Significantly higher RPE than at 15 min within trial (*** P<0.01).

**Substrate Oxidation and Energy Expenditure**

Mean RER was similar between CAF and PLA trials during the 90 min exercise period (CAF: 0.93 ± 0.01, PLA: 0.92 ± 0.01, P = 0.552). CHO oxidation rate decreased during the exercise period (main effect of time, P<0.05) but was similar between trials throughout, with mean values of 3.47 ± 0.13 g.min\(^{-1}\) and 3.40 ± 0.15 g.min\(^{-1}\) on CAF and PLA trials respectively. Similarly there were no significant differences between fat oxidation rates between trials (CAF: 0.40 ± 0.03 g.min\(^{-1}\), PLA: 0.44 ± 0.04 g.min\(^{-1}\)) however, a main effect of time showed that fat oxidation significantly increased throughout the exercise period (P<0.05; Table 5.1). Total energy expenditure was similar for both trials with mean values of 69 ± 1 kJ.min\(^{-1}\) and
68 ± 1 kJ.min\(^{-1}\) on CAF and PLA respectively. Likewise, the energy derived from the oxidation of CHO (CAF: 55.6 ± 2.0 kJ.min\(^{-1}\), PLA: 54.5 ± 2.4 kJ.min\(^{-1}\)) and fat (CAF: 15.6 ± 1.2 kJ.min\(^{-1}\), PLA: 17.1 ± 1.6 kJ.min\(^{-1}\)) did not significantly differ between trials throughout the exercise period. The energy derived from CHO oxidation decreased and contribution from fat increased with exercise duration (main effect of time, \(P<0.05\)). There were no significant differences between trials for the percentage contribution of fat (CAF: 23 ± 2%, PLA: 25 ± 2%) and CHO (CAF: 77 ± 2%, PLA: 75 ± 2%) towards energy expenditure during the exercise period.
Table 5.1: Substrate oxidation rates and contribution towards energy expenditure during the exercise period in the CAF and PLA trials.

<table>
<thead>
<tr>
<th>Time during Exercise</th>
<th>20</th>
<th>50</th>
<th>80</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHO oxidation (g.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>3.67 (0.29)</td>
<td>3.51 (0.20)</td>
<td>3.24 (0.17)</td>
<td>3.47 (0.13)</td>
</tr>
<tr>
<td>PLA</td>
<td>3.55 (0.29)</td>
<td>3.59 (0.31)</td>
<td>3.07 (0.15)</td>
<td>3.40 (0.15)</td>
</tr>
<tr>
<td><strong>Fat oxidation (g.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.31 (0.03)</td>
<td>0.35 (0.04)</td>
<td>0.47 (0.05)</td>
<td>0.40 (0.03)</td>
</tr>
<tr>
<td>PLA</td>
<td>0.32 (0.06)</td>
<td>0.41 (0.15)</td>
<td>0.52 (0.05)</td>
<td>0.44 (0.04)</td>
</tr>
<tr>
<td><strong>Energy derived from CHO oxidation (kJ.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>58.7 (4.4)</td>
<td>56.2 (3.2)</td>
<td>51.9 (2.7)</td>
<td>55.6 (2.0)</td>
</tr>
<tr>
<td>PLA</td>
<td>56.8 (4.6)</td>
<td>57.5 (5.0)</td>
<td>49.2 (2.3)</td>
<td>54.5 (2.4)</td>
</tr>
<tr>
<td><strong>Energy derived from fat oxidation (kJ.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>12.2 (1.3)</td>
<td>13.8 (1.7)</td>
<td>14.1 (1.9)</td>
<td>15.6 (1.2)</td>
</tr>
<tr>
<td>PLA</td>
<td>12.5 (2.2)</td>
<td>15.9 (3.1)</td>
<td>20.3 (1.9)</td>
<td>17.1 (1.6)</td>
</tr>
<tr>
<td><strong>% contribution to total energy expenditure from CHO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>81 (2)</td>
<td>80 (3)</td>
<td>74 (3)</td>
<td>77 (2)</td>
</tr>
<tr>
<td>PLA</td>
<td>80 (4)</td>
<td>77 (5)</td>
<td>71 (3)</td>
<td>75 (2)</td>
</tr>
<tr>
<td><strong>% contribution to total energy expenditure from fat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>19 (2)</td>
<td>20 (3)</td>
<td>26 (3)</td>
<td>23 (2)</td>
</tr>
<tr>
<td>PLA</td>
<td>20 (3)</td>
<td>23 (4)</td>
<td>29 (3)</td>
<td>25 (2)</td>
</tr>
</tbody>
</table>

Blood metabolites and hormones

Serum caffeine was significantly higher at pre-exercise, post-exercise and 1 h post-exercise on CAF compared with PLA (all P<0.01; Figure 5.2). Plasma adrenaline concentration was significantly higher on CAF compared with PLA at pre-exercise (P<0.01) and increased significantly above resting values on both trials by post-exercise (CAF: P<0.01, PLA: P<0.05). At this point, plasma adrenaline was significantly higher on CAF than PLA (P<0.05; Figure 5.3). There were no significant differences between trials for plasma noradrenaline or cortisol concentrations, which both increased to a similar extent on CAF and PLA trials following exercise (main effect of time, P<0.01; Table 5.2).
Figure 5.2. Serum caffeine concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point (†† $P<0.01$). Significantly higher than rest within trial (** $P<0.01$).

Figure 5.3. Plasma adrenaline concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point (†† $P<0.01$, † $P<0.05$). Significantly higher than rest within trial (** $P<0.01$, * $P<0.05$).
Plasma FFA concentration increased significantly from rest to pre-exercise on CAF trial only ($P<0.01$). FFA concentration increased significantly from pre to post-exercise on both trials ($P<0.01$), however at 1 h post-exercise, plasma FFA concentration was significantly higher on CAF than PLA ($P<0.01$; Table 5.2). Plasma glucose concentration was similar between trials and no significant main effects were observed. Plasma lactate concentration increased significantly from rest to pre-exercise on CAF only ($P<0.05$) and was significantly increased at post-exercise on both trials ($P<0.01$). At this time-point, plasma lactate concentration was significantly higher on CAF than PLA ($P<0.01$; Table 5.2).

Table 5.2. Plasma concentrations of noradrenaline, cortisol and blood-borne metabolites on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Pre Exercise</th>
<th>Post Exercise</th>
<th>1 h Post</th>
</tr>
</thead>
</table>
| **Noradrenaline (nmol.L$^{-1}$)**
| CAF            | 1.81 (0.24)| 2.42 (0.19)  | 8.63 (0.92)  | -        |
| PLA            | 1.66 (0.20)| 2.08 (0.16)  | 6.23 (0.66)  | -        |
| **Cortisol (nmol.L$^{-1}$)**
| CAF            | 370 (58)   | 344 (42)     | 612 (55)     | 512 (66) |
| PLA            | 369 (50)   | 315 (51)     | 545 (65)     | 482 (43) |
| **FFA (mmol.L$^{-1}$)**
| CAF            | 0.24 (0.04)| 0.54 (0.06)$^{**\dagger}$ | 1.60 (0.11)$^{**}$ | 1.38 (0.14)$^{**\dagger}$ |
| PLA            | 0.28 (0.06)| 0.24 (0.02)  | 1.30 (0.13)$^{**}$ | 0.95 (0.12)$^{**}$ |
| **Glucose (mmol.L$^{-1}$)**
| CAF            | 5.3 (0.2)  | 5.9 (0.2)    | 5.3 (0.2)    | 4.8 (0.2) |
| PLA            | 5.4 (0.2)  | 5.3 (0.2)    | 4.9 (0.4)    | 5.0 (0.3) |
| **Lactate (mmol.L$^{-1}$)**
| CAF            | 1.5 (0.1)  | 1.9 (0.2)$^{**\dagger}$ | 4.1 (0.2)$^{**\dagger}$ | -        |
| PLA            | 1.4 (0.1)  | 1.4 (0.1)    | 3.0 (0.3)$^{**}$ | -        |

Significantly higher on CAF than PLA at that time point ($\dagger P<0.01$, $\dagger P<0.05$). Significantly higher than rest within trial ($^{**}P<0.01$). $^a$ Main effect of time, significantly higher at post-exercise than rest and pre-exercise ($P<0.01$). $^b$ Main effect of time, significantly higher at post-exercise than pre-exercise. Significantly lower at 1 h post-exercise than post-exercise (both $P<0.01$).
Total and differential leukocyte counts

All participants had resting total and differential blood leukocyte counts within the normal range for healthy adults. There were no significant differences for total number of circulating leukocytes or neutrophils between CAF and PLA trials. There was however, a significant main effect of time ($P<0.01$; Table 5.3) for total leukocytes with a leukocytosis evident at post-exercise which increased further at 1 h post-exercise. Likewise, neutrophil count was significantly higher following the cessation of exercise with a delayed neutrophilia at 1 h post-exercise compared with resting values (main effect of time, $P<0.01$; Table 5.3). Circulating lymphocyte count increased from rest to pre-exercise on CAF trial only and was significantly higher at this point on CAF compared with PLA, in which counts remained at resting levels ($P<0.05$; Table 5.3). Immediately following the exercise bout, a marked lymphocytosis was evident on both trials (both $P<0.05$ compared to resting values). There was a significant main effect of time for the neutrophil/lymphocyte ratio with higher values at 1 h post-exercise compared to rest, pre and post-exercise values ($P<0.01$), yet this occurred independent of trial.
Table 5.3. Total and differential blood leukocyte counts and the neutrophil/
lymphocyte ratio on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Pre-Exercise</th>
<th>Post-Exercise</th>
<th>1 h Post</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes (x 10⁷.L⁻¹) a</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>4.8 (0.4)</td>
<td>5.9 (1.3)</td>
<td>9.1 (0.9)</td>
<td>12.2 (1.4)</td>
</tr>
<tr>
<td>PLA</td>
<td>5.6 (0.5)</td>
<td>5.8 (0.5)</td>
<td>9.3 (0.8)</td>
<td>12.9 (1.0)</td>
</tr>
<tr>
<td><strong>Neutrophils (x 10⁹.L⁻¹) a</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>2.5 (0.3)</td>
<td>3.2 (0.3)</td>
<td>5.4 (0.6)</td>
<td>9.7 (1.2)</td>
</tr>
<tr>
<td>PLA</td>
<td>3.1 (0.5)</td>
<td>3.4 (0.5)</td>
<td>5.7 (0.7)</td>
<td>10.0 (1.0)</td>
</tr>
<tr>
<td><strong>Lymphocytes (x 10⁹.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.63 (0.17)</td>
<td>1.98 (0.16)</td>
<td>2.74 (0.28)*</td>
<td>1.52 (0.11)</td>
</tr>
<tr>
<td>PLA</td>
<td>1.75 (0.15)</td>
<td>1.69 (0.11)</td>
<td>2.51 (0.20)*</td>
<td>1.64 (0.07)</td>
</tr>
<tr>
<td><strong>Neutrophil/Lymphocyte ratio b</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.6 (0.2)</td>
<td>1.7 (0.2)</td>
<td>2.0 (0.2)</td>
<td>6.4 (0.6)</td>
</tr>
<tr>
<td>PLA</td>
<td>1.9 (0.3)</td>
<td>2.2 (0.4)</td>
<td>2.4 (0.3)</td>
<td>6.3 (0.6)</td>
</tr>
</tbody>
</table>

Significantly higher on CAF than PLA at that time point (†P<0.05). Significantly higher than resting values within trial (*P<0.05). a Main effect of time, significantly higher at post-exercise and 1 h post-exercise than rest (P<0.01). b Main effect of time, significantly higher at 1 h post-exercise than rest, pre and post-exercise (P<0.01).

**Neutrophil Function**

There were no significant differences between trials for PMA-stimulated neutrophil oxidative burst response expressed as total CL response. Total CL response was significantly higher at post-exercise and 1 h post-exercise than resting values (main effect of time, P<0.01; Figure 5.4). When total CL was adjusted for neutrophil count, a significant main effect of time showed that the response on a per-cell basis was significantly lower at pre-exercise, post-exercise and 1 h post-exercise than at rest (P<0.01; Figure 5.5). This decrease occurred independently of the trial followed.
Figure 5.4. Neutrophil oxidative burst response expressed as total CL (relative light units) following PMA stimulation. Main effect of time ($P<0.01$).

Figure 5.5. Neutrophil oxidative burst response expressed per neutrophil cell (relative light units) following PMA stimulation. Main effect of time ($P<0.01$).
In response to bacterial stimulation, neutrophil degranulation response was similar between CAF and PLA trials. Neutrophil elastase release tended to be lower at post-exercise, however this did not reach statistical significance (main effect of time, $P = 0.076$; Figure 5.6).

**Figure 5.6.** Neutrophil elastase release following bacterial stimulation.
5.5. Discussion

The main findings of the present study suggest that caffeine ingestion (6 mg.kg\(^{-1}\) body mass) has no effect on PMA-stimulated oxidative burst or bacterially-stimulated neutrophil degranulation responses following prolonged submaximal intensity cycling.

Regardless of experimental condition, the total neutrophil oxidative burst response (as indicated by IAUC CL values) increased with exercise and was approximately 2 to 3-fold higher than rest at 1 h post-exercise. Similar findings have been previously reported (Smith et al., 1996; Suzuki et al., 1996a). Using PMA as a stimulant, Smith et al. (1996) reported a 3-fold increase in neutrophil \(H_2O_2\) production following 1 h of cycling at a heart rate of 140 beats.min\(^{-1}\). Average heart rate in this study was 158 beats.min\(^{-1}\) in which similar findings were observed. In accordance with Boyum et al. (2002), the present findings suggest that the oxidative potential of the blood (calculated as the CL response multiplied by the circulating neutrophil count) is increased following exercise; however, when the response was adjusted to account for the increases in neutrophil number, a significant reduction on a per-cell basis was found at post-exercise and at 1 h post-exercise compared with resting values. These findings support previous work (Pyne et al., 1996; Robson et al., 1999; Davison and Gleeson, 2005) yet are in contrast to the findings of Walsh et al. (2000) who reported no effect of 2 h cycling at 60% \(\bar{VO}_2\) max on the PMA-stimulated oxidative burst response of isolated neutrophils. A reduced PMA-stimulated oxidative burst response per neutrophil may be explained by the post-exercise neutrophilia, as it is known that immature neutrophils have a lower activity of NADPH oxidase and a reduced capacity to generate \(O_2^-\) than fully mature neutrophils (Berkow and Dodson, 1986).
There are a number of possible reasons to explain why CAF did not affect the PMA-stimulated neutrophil oxidative burst response compared with PLA. One of the speculated mechanisms for caffeine affecting neutrophil function is via adenosine receptor antagonism as caffeine and its metabolites are non-selective A$_1$ and A$_{2A}$ adenosine receptor antagonists (Ongini and Fredholm, 1996). Adenosine acting via A$_{2A}$ adenosine receptors is widely reported to inhibit the oxidative burst response when stimulated by f-MLP (Cronstein et al., 1983, 1985; Thibault et al., 2002; Swain et al., 2003), whereas minimal inhibition was observed following PMA stimulation (Cronstein et al., 1983). Furthermore, it is reported that cAMP elevating agents such as adrenaline, do not affect the PMA stimulated oxidative burst response in humans (Yu et al., 1995; Tintinger et al., 2001). In rat studies however, PMA stimulated oxidative burst response was significantly reduced following incubation with 5 nmol.L$^{-1}$ adrenaline (Garcia et al., 1999). The findings of the present study are in accordance with the previous research involving human subjects as plasma adrenaline was approximately 2-fold higher at post-exercise on CAF compared with PLA trial, yet there was no difference in oxidative burst response. It should be noted however, that plasma adrenaline concentration in the CAF trial only reached 1.8 nmol.L$^{-1}$.

Neutrophils are activated by a number of different pathways depending on the stimulant used to initiate the oxidative burst response. PMA is known to directly activate protein kinase C in blood neutrophils (Lopez et al., 1995; Hazan et al, 1997) producing a prolonged and maximum response. Alternatively, f-MLP acts via a G protein-coupled receptor (Lopez et al., 1995) invoking a shorter and more subtle response than PMA. It has been reported that an increase in cAMP by physiological cAMP agonists (ie. adenosine or β-adrenergic stimulants) exerts an effect on the
receptor-mediated oxidative burst of neutrophils but has no effect on neutrophils stimulated with PMA (Bengis-Garber et al., 1996). The insensitivity of the PMA-activated response of human neutrophils to cAMP elevating agents (Tintinger et al., 2001) may suggest that PMA is not a suitable stimulant for investigating the influence of caffeine ingestion on neutrophil oxidative burst responses.

The effect of adenosine and adrenaline on neutrophil degranulation responses in vitro, have produced equivocal findings. It is reported that adenosine acts via A2 and A3 receptors to dose-dependently inhibit degranulation responses (Bouma et al., 1997), whereas others have reported no effects (Cronstein et al., 1985; Swain et al., 2003). In addition, incubation of neutrophils with adrenaline has also been found to inhibit (Tintinger et al., 2001) or have no effect (Barnett et al., 1997) upon neutrophil elastase release. In the present study, there was no effect of CAF on neutrophil elastase release following bacterial stimulation even in the presence of an increased adrenaline concentration. This suggests that at physiological doses, adrenaline has no effect on bacterially stimulated neutrophil degranulation response. Neutrophil elastase release at post-exercise was reduced to 65% and 67% of baseline response on CAF and PLA respectively but this did not reach statistical significance ($P = 0.076$). A decreased elastase release per neutrophil cell has been frequently reported following prolonged intensive submaximal cycling (Bishop et al., 1999; Walsh et al., 2000; Li and Gleeson, 2004). It is likely that this non-significant difference is due to large inter-individual variation of neutrophil elastase release in the present study.

Caffeine ingestion did not affect the leukocyte and neutrophil counts in response to exercise yet it was associated with a significant increase in circulating lymphocyte
number at pre-exercise (i.e. 1 h following caffeine ingestion). This is in accordance with Bishop et al. (2005) who reported similar findings using the same exercise protocol and method of caffeine administration as that employed in the present study. An increase in circulating lymphocyte count may be due to the elevated plasma adrenaline concentration at this time point altering endothelial adhesion via activation of β-adrenoreceptors present on lymphocytes. A greater number of β-adrenoreceptors are present on lymphocytes than neutrophils (Mackinnon, 1999) which may explain the relatively small influence that caffeine exerts on this leukocyte subset.

Participants on the CAF trial reported a significantly lower RPE from the mid-point of exercise through to completion of the exercise protocol. It has been suggested that caffeine may alter the release, binding or activity of neurotransmitters in the brain (Davis et al., 2003). This may affect the perception of work intensity (Cole et al., 1996) or reduce feelings of leg pain associated with strenuous cycle exercise (O‘Connor et al., 2004). The ability to influence pain perception and psychological state is an important factor in the physiological performance of athletes during periods of training and competition.

In summary, the findings of this study suggest that caffeine ingestion has no effect on either the PMA-stimulated neutrophil oxidative burst response or bacterially-stimulated neutrophil degranulation response following prolonged and intensive submaximal cycling exercise. This effect was observed despite a higher adrenaline concentration on the CAF trial.
Chapter 6

The effect of caffeine ingestion on f-MLP-stimulated neutrophil oxidative burst responses following prolonged and intensive submaximal cycling.
6.1. Abstract

The purpose of the present study was to determine the effect of caffeine ingestion on f-MLP-stimulated neutrophil oxidative burst responses following exercise. Eight endurance trained male cyclists (mean ± SEM: age 24 ± 1 years; body mass 72.8 ± 2.8 kg; height 1.79 ± 0.03 m, $\bar{V}O_2$ max 65.6 ± 1.9 ml.kg$^{-1}$.min$^{-1}$, Peak Power Output 344 ± 5 W) ingested 6 mg.kg$^{-1}$ body mass of caffeine (CAF) or placebo (PLA) 60 min before cycling for 90 min at 70% $\bar{V}O_2$ max. Venous blood samples were obtained at rest, 60 min after CAF or PLA ingestion (pre-exercise), immediately post-exercise and at 1 h post-exercise. f-MLP-stimulated neutrophil oxidative burst response decreased following the exercise period, but was significantly lower at post-exercise compared with rest and pre-exercise values on the PLA trial only (both $P<0.01$). When neutrophil oxidative burst was expressed as a percentage of baseline response, values were significantly higher on CAF compared with PLA (main effect of trial, $P<0.05$). CAF was associated with significantly elevated plasma IL-6 response at post-exercise ($P<0.05$), which may in part be the result of an increased adrenaline response at post-exercise on the CAF trial ($P<0.05$). There was no effect of CAF on circulating leukocyte or neutrophil count but lymphocyte count was significantly higher at pre-exercise on CAF ($P<0.05$). The RPE of participants was significantly lower during the exercise protocol on CAF than PLA (main effect of trial, $P<0.01$). Therefore, although CAF ingestion was associated with an increase in adrenaline, this was not associated with an expected decrease in neutrophil function. This suggests that in the present study, CAF ingestion influenced neutrophil oxidative burst responses via alternative mechanisms.
6.2. Introduction

The neutrophil oxidative burst response consists of a pathway of complex events and results in the formation of $O_2^\cdot$ anions via the activation of the NADPH oxidase (Peake, 2002). A cascade of reactions may follow forming ROS and other reactive species (Pyne, 1994). Neutrophils are activated by both protein kinase C-mediated and receptor-coupled mechanisms (Peake, 2002). PMA is known to directly activate protein kinase C in blood neutrophils (Lopez et al., 1995) producing a prolonged and maximum response. f-MLP on the other hand, acts via a G protein-coupled receptor (Lopez et al., 1995) invoking a quicker yet more subtle response than PMA. The stimulant used to initiate the oxidative burst has been proposed as a factor for the equivocal findings that are frequently published in the literature (Peake, 2002).

The findings of the study described in Chapter 5 demonstrated that there was no effect of caffeine ingestion on PMA-stimulated neutrophil oxidative burst responses following 90 min cycling at 70% $\dot{V}O_2$ max. It has been reported that the PMA-stimulated neutrophil response is insensitive to cAMP elevating agents such as adenosine or $\beta$-adrenergic agonists (Bengis-Garber et al., 1996; Tintinger et al., 2001), suggesting that PMA is not a suitable stimulant for investigating the influence of caffeine ingestion on neutrophil oxidative burst responses. When neutrophils are stimulated by f-MLP however, adenosine is widely reported to inhibit the oxidative burst response (Cronstein et al., 1983; 1985; 1990; Thibault et al., 2002; Swain et al., 2003), mediating these effects via the $A_{2A}$ adenosine receptor expressed on neutrophil cell surfaces (Thibault et al., 2000; Gessi et al., 2002). The methylxanthine caffeine is a non-selective adenosine receptor antagonist with affinity in the $\mu$mol.L$^{-1}$ range (Ongini and Fredholm, 1996). Furthermore, it has been reported that neutrophil
activation is inhibited following \( \beta_2 \) adrenoreceptor stimulation (Chilcoat et al., 2002) and catecholamines are reported to decrease f-MLP stimulated neutrophil oxidative burst responses in a dose-dependent manner (Wenisch et al., 1996).

Therefore, the purpose of the present study was to investigate the influence of caffeine ingestion on the f-MLP-stimulated neutrophil oxidative burst response (ie. the production of reactive species by neutrophils when stimulated \textit{in vitro} by f-MLP) following prolonged intensive submaximal exercise. It was hypothesised that if adenosine receptor antagonism was the predominant mechanism involved, caffeine ingestion would attenuate the exercise-induced fall in neutrophil oxidative burst responses. However, if an increased adrenaline response was the overriding influence, caffeine ingestion would be detrimental to the neutrophil response.
6.3. Methods

Participants

Eight endurance trained male cyclists (mean ± SEM: age 24 ± 1 years; body mass 72.8 ± 2.8 kg; height 1.79 ± 0.03 m, \( \dot{V}O_2 \) max 65.6 ± 1.9 ml.kg\(^{-1}\).min\(^{-1}\), Peak Power Output 344 ± 5 W) volunteered to participate in the study. Participants were informed of the rationale and experimental procedures involved in the study, before providing written consent to participate. The study protocol had earlier received approval by Loughborough University Ethical Advisory Committee. Average daily caffeine intake was 201 ± 38 mg.day\(^{-1}\). One participant was characterised as a light user (< 50 mg.day\(^{-1}\)), 4 as moderate users (50-250 mg.day\(^{-1}\) ) and 3 as having a high caffeine intake (> 250 mg.day\(^{-1}\)).

Experimental procedures

Preliminary measurements (\( \dot{V}O_2 \) max and familiarisation) were conducted within a 2-week period prior to the start of the main trials. Preliminary measurements and pre-trial standardisation procedures are described in Chapter 3. Participants performed 2 sessions of exercise separated by 7 days. The participants acted as their own controls in a repeated-measures, single-blind, counter-balanced, cross-over design and were randomly assigned to one of two experimental conditions: ingesting caffeine (CAF) or placebo (PLA). Participants arrived at the laboratory at 10.00 am following an overnight fast of between 10 and 12 h. Following a void, body mass was recorded before participants ingested 6 mg.kg\(^{-1}\) body mass of either CAF or PLA dissolved in 200 ml no-added sugar pink grapefruit juice drink. After resting quietly for 60 min, participants began cycling for 90 min at 70% \( \dot{V}O_2 \) max. Heart rate and RPE were measured every 15 min throughout exercise and 2 ml.kg\(^{-1}\) body mass of water was
administered at these time points. At 20, 50 and 80 min of exercise, 1-min expired gas samples were collected. Following the cessation of exercise, body mass was recorded and participants consumed 3 ml.kg⁻¹ body mass of water before resting for a further 60 min. Venous blood samples were obtained at rest, 60 min after CAF or PLA ingestion (pre-exercise), immediately post-exercise and at 1 h post-exercise. Laboratory conditions were 23.5 ± 0.6°C and 42 ± 2% relative humidity.

Blood analytical methods

Blood sampling and analyses methods are detailed in Chapter 3.5.

Statistical analysis

Statistical analysis methods are detailed in Chapter 3.6.
6.4. Results

Physiological variables and RPE

There was no difference in overall exercise intensity between trials; mean $\dot{V}O_2$ max was 72.3 ± 0.6% and 72.0 ± 0.9% on CAF and PLA trials, respectively. Following exercise, body mass change (corrected for fluid intake) fell similarly on both trials (-2.0 ± 0.2 kg on both CAF and PLA) as did plasma volume (CAF: -12.9 ± 0.9%, PLA: -12.8 ± 1.9%). Mean heart rate during exercise was similar on both trials (160 ± 2 beats.min⁻¹ and 162 ± 1 beats.min⁻¹ on CAF and PLA, respectively). Mean RPE throughout the 90-min exercise bout was significantly lower on CAF than PLA (CAF: 13.3 ± 0.3, PLA: 14.0 ± 0.2, $P<0.01$).

Substrate Oxidation and Energy Expenditure

Mean RER was similar between CAF and PLA trials during the 90 min exercise period (CAF: 0.92 ± 0.01, PLA: 0.92 ± 0.01). CHO oxidation rate decreased during the exercise period (main effect of time, $P<0.05$; Table 6.1) but was similar between trials throughout, with mean values of 3.41 ± 0.17 g.min⁻¹ and 3.43 ± 0.18 g.min⁻¹ on CAF and PLA trials, respectively. Similarly there were no significant differences for fat oxidation rates between trials (CAF: 0.46 ± 0.03 g.min⁻¹, PLA: 0.43 ± 0.04 g.min⁻¹) however, fat oxidation significantly increased throughout the exercise period (main effect of time, $P<0.05$; Table 6.1).

Mean rate of energy expenditure was similar for both trials with values of 72 ± 2 kJ.min⁻¹ and 71 ± 2 kJ.min⁻¹ on CAF and PLA respectively. Accordingly, the energy derived from the oxidation of CHO (CAF: 54.5 ± 2.7 kJ.min⁻¹, PLA: 54.8 ± 2.9...
kJ.min⁻¹) and fat (CAF: 17.3 ± 1.6 kJ.min⁻¹, PLA: 16.7 ± 1.4 kJ.min⁻¹) did not significantly differ between trials throughout the exercise period. The energy derived from the oxidation of CHO decreased and contribution from fat increased with exercise duration (main effect of time, P<0.05). There were no significant differences between trials for the percentage contribution of CHO (CAF: 74 ± 3%, PLA: 76 ± 2%) or fat (CAF: 26 ± 2%, PLA: 24 ± 2%) towards energy expenditure during the exercise period. The percentage CHO contribution decreased and fat contribution increased as a function of exercise duration (P<0.01).

Table 6.1: Substrate oxidation rates and contribution towards energy expenditure during the exercise period in the CAF and PLA trials.

<table>
<thead>
<tr>
<th>Time during Exercise</th>
<th>20</th>
<th>50</th>
<th>80</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHO oxidation (g.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>3.80 (0.31)</td>
<td>3.29 (0.28)</td>
<td>3.13 (0.25)</td>
<td>3.39 (0.20)</td>
</tr>
<tr>
<td>PLA</td>
<td>3.67 (0.35)</td>
<td>3.41 (0.21)</td>
<td>3.23 (0.30)</td>
<td>3.43 (0.18)</td>
</tr>
<tr>
<td><strong>Fat oxidation (g.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.31 (0.06)</td>
<td>0.49 (0.06)</td>
<td>0.57 (0.06)</td>
<td>0.47 (0.04)</td>
</tr>
<tr>
<td>PLA</td>
<td>0.32 (0.06)</td>
<td>0.42 (0.03)</td>
<td>0.51 (0.06)</td>
<td>0.43 (0.04)</td>
</tr>
<tr>
<td><strong>Energy derived from CHO oxidation (kJ.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>60.9 (5.0)</td>
<td>52.7 (4.4)</td>
<td>50.1 (4.0)</td>
<td>54.3 (3.2)</td>
</tr>
<tr>
<td>PLA</td>
<td>58.8 (5.7)</td>
<td>54.5 (3.4)</td>
<td>51.7 (4.9)</td>
<td>54.8 (2.9)</td>
</tr>
<tr>
<td><strong>Energy derived from fat oxidation (kJ.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>10.7 (2.7)</td>
<td>19.1 (2.2)</td>
<td>22.2 (2.2)</td>
<td>17.6 (1.7)</td>
</tr>
<tr>
<td>PLA</td>
<td>12.6 (2.2)</td>
<td>16.2 (1.3)</td>
<td>19.7 (2.5)</td>
<td>16.4 (1.4)</td>
</tr>
<tr>
<td><strong>% contribution to total energy expenditure from CHO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>82 (4)</td>
<td>73 (4)</td>
<td>69 (3)</td>
<td>74 (3)</td>
</tr>
<tr>
<td>PLA</td>
<td>81 (4)</td>
<td>77 (2)</td>
<td>72 (4)</td>
<td>76 (2)</td>
</tr>
<tr>
<td><strong>% contribution to total energy expenditure from fat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>18 (4)</td>
<td>27 (4)</td>
<td>31 (3)</td>
<td>27 (2)</td>
</tr>
<tr>
<td>PLA</td>
<td>19 (4)</td>
<td>23 (2)</td>
<td>28 (4)</td>
<td>24 (2)</td>
</tr>
</tbody>
</table>
Blood metabolites and hormones

Serum caffeine was significantly higher at pre-exercise, post-exercise and 1 h post-exercise on CAF compared with PLA (all $P<0.01$; Figure 6.1). Plasma adrenaline concentration was higher than rest at post-exercise on both trials ($P<0.05$). At this time, plasma adrenaline concentration was significantly higher on CAF than PLA ($P<0.05$; Figure 6.2). Plasma noradrenaline concentration was significantly higher than rest at post-exercise ($P<0.01$; Table 6.2) but there were no significant differences between CAF and PLA trials. A significant main effect of trial ($P<0.01$) for plasma cortisol was observed with values significantly higher on CAF compared with PLA (mean values were $489 \pm 34$ nmol.L$^{-1}$ and $391 \pm 25$ nmol.L$^{-1}$ on CAF and PLA respectively; Table 6.2). Plasma FFA was significantly higher on CAF compared with PLA (mean values were $1.13 \pm 0.15$ mmol.L$^{-1}$ and $0.76 \pm 0.11$ mmol.L$^{-1}$ for CAF and PLA respectively, main effect of trial, $P<0.05$; Table 6.2) whereas there were no significant differences for plasma glucose. Plasma lactate concentration was significantly higher at post-exercise than rest ($P<0.01$; Table 6.2) yet this occurred independently of the trial followed.
Figure 6.1. Serum caffeine concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point († † P<0.01). Significantly higher than rest within trial (‡‡ P<0.01).

Figure 6.2. Plasma adrenaline concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point († P<0.05). Significantly higher than rest within trial (* P<0.05).
Table 6.2. Plasma concentrations of noradrenaline, cortisol and blood-borne metabolites on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Pre Exercise</th>
<th>Post Exercise</th>
<th>1 h Post</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Noradrenaline (nmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.43 (0.18)</td>
<td>1.68 (0.12)</td>
<td>11.66 (1.44)</td>
<td>-</td>
</tr>
<tr>
<td>PLA</td>
<td>1.59 (0.34)</td>
<td>1.84 (0.30)</td>
<td>8.57 (1.88)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cortisol (nmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>414 (61)</td>
<td>447 (52)</td>
<td>597 (59)</td>
<td>462 (81)</td>
</tr>
<tr>
<td>PLA</td>
<td>384 (45)</td>
<td>386 (47)</td>
<td>402 (53)</td>
<td>355 (45)</td>
</tr>
<tr>
<td><strong>FFA (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.32 (0.06)</td>
<td>0.70 (0.10)</td>
<td>1.71 (0.22)</td>
<td>1.78 (0.30)</td>
</tr>
<tr>
<td>PLA</td>
<td>0.29 (0.05)</td>
<td>0.25 (0.05)</td>
<td>1.28 (0.14)</td>
<td>1.23 (0.19)</td>
</tr>
<tr>
<td><strong>Glucose (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>5.5 (0.2)</td>
<td>5.8 (0.2)</td>
<td>5.7 (0.2)</td>
<td>5.5 (0.3)</td>
</tr>
<tr>
<td>PLA</td>
<td>5.5 (0.3)</td>
<td>5.5 (0.3)</td>
<td>5.3 (0.2)</td>
<td>5.0 (0.2)</td>
</tr>
<tr>
<td><strong>Lactate (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.3 (0.1)</td>
<td>1.6 (0.2)</td>
<td>3.8 (0.6)</td>
<td>-</td>
</tr>
<tr>
<td>PLA</td>
<td>1.2 (0.1)</td>
<td>1.5 (0.3)</td>
<td>2.7 (0.2)</td>
<td>-</td>
</tr>
</tbody>
</table>

Main effect of trial, significantly higher on CAF than PLA (‡ P<0.01; † P<0.05).

Main effect of time, significantly higher than rest at post-exercise and 1 h post-exercise (P<0.01). Main effect of time, significantly higher than rest at post-exercise (P<0.01).

Total and differential leukocyte counts

All participants had resting total and differential leukocyte counts within the normal range for healthy adults. There was a significant leukocytosis and neutrophilia evident at post-exercise and 1 h post-exercise (main effect of time; P<0.01; Table 6.3) but there were no significant differences between CAF and PLA trials. At pre-exercise, circulating lymphocyte count was significantly higher on CAF compared with PLA (P<0.05; Table 6.3). Immediately post-exercise, a marked lymphocytosis was evident on both trials that reached statistical significance on CAF trial only (P<0.05) and a lymphocytopenia was observed at 1 h post-exercise. There was a significant main effect of time for the neutrophil/lymphocyte ratio with higher values at 1 h post-
exercise compared to rest, pre and post-exercise values (P<0.01), yet this occurred independently of the trial followed.

**Table 6.3.** Total and differential blood leukocyte counts and the neutrophil/lymphocyte ratio on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Pre-Exercise</th>
<th>Post-Exercise</th>
<th>1 h Post</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes (x 10^9.L^-1)</strong> a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>4.6 (0.3)</td>
<td>5.0 (0.4)</td>
<td>8.7 (0.7)</td>
<td>12.0 (1.2)</td>
</tr>
<tr>
<td>PLA</td>
<td>4.8 (0.3)</td>
<td>4.8 (0.3)</td>
<td>8.0 (0.9)</td>
<td>10.1 (1.1)</td>
</tr>
<tr>
<td><strong>Neutrophils (x 10^9.L^-1)</strong> a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>2.3 (0.2)</td>
<td>2.6 (0.3)</td>
<td>5.5 (0.5)</td>
<td>9.4 (1.1)</td>
</tr>
<tr>
<td>PLA</td>
<td>2.5 (0.2)</td>
<td>2.7 (0.3)</td>
<td>5.3 (0.9)</td>
<td>7.7 (1.0)</td>
</tr>
<tr>
<td><strong>Lymphocytes (x 10^9.L^-1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.64 (0.16)</td>
<td>1.79 (0.17)</td>
<td>2.37 (0.26)*</td>
<td>1.53 (0.17)</td>
</tr>
<tr>
<td>PLA</td>
<td>1.71 (0.17)</td>
<td>1.56 (0.14)</td>
<td>2.00 (0.25)</td>
<td>1.54 (0.17)</td>
</tr>
<tr>
<td><strong>Neutrophil/Lymphocyte ratio</strong> b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.5 (0.1)</td>
<td>1.4 (0.2)</td>
<td>2.2 (0.2)</td>
<td>6.4 (0.9)</td>
</tr>
<tr>
<td>PLA</td>
<td>1.5 (0.2)</td>
<td>1.8 (0.3)</td>
<td>2.5 (0.5)</td>
<td>5.3 (0.6)</td>
</tr>
</tbody>
</table>

Significantly higher on CAF than PLA at that time point (†P<0.05). Significantly higher than resting values within trial (*P<0.05). a Main effect of time, significantly higher at post-exercise and 1 h post-exercise than rest (P<0.01). b Main effect of time, significantly higher at 1 h post-exercise than rest, pre and post-exercise (P<0.01).

**Neutrophil Function**

The peak neutrophil CL response to f-MLP stimulation decreased following the exercise period, but was significantly lower at post-exercise compared with rest and pre-exercise values on the PLA trial only (both P<0.01; Figure 6.3). When peak neutrophil CL was expressed as percentage of baseline response, values were significantly higher on CAF compared with PLA (main effect of trial: P<0.05; Figure 6.4).
Figure 6.3. Neutrophil oxidative burst response expressed as peak CL values in relative light units (RLU) following f-MLP stimulation. Significant decrease from rest within trial (** P<0.01). Significant decrease from pre-exercise within trial († P<0.01).

Figure 6.4. f-MLP stimulated neutrophil oxidative burst (peak CL) expressed as % of baseline response. Main effect of trial (P = 0.019).
**IL-6**

There were no differences in IL-6 concentration between rest and pre-exercise (ie. 60 min following CAF or PLA ingestion). Plasma IL-6 concentration increased significantly on both trials following the exercise bout ($P<0.01$). At this time (post-exercise), plasma IL-6 concentration was significantly higher on CAF than PLA (CAF: $5.2 \pm 0.8$ ng.L$^{-1}$; PLA: $3.3 \pm 0.8$ ng.L$^{-1}$, $P<0.05$; Figure 6.5), which corresponded to a $436 \pm 81\%$ and $249 \pm 43\%$ change from pre-exercise values on the CAF and PLA trials, respectively.

![Figure 6.5. Plasma IL-6 concentration. Significantly higher on CAF than PLA at that time point ($\dagger P<0.05$). Significantly higher than rest within trial (** $P<0.01$, * $P<0.05$).](image_url)
6.5. Discussion

The major finding of the present study suggests that caffeine (6 mg.kg\(^{-1}\) body mass) attenuated the exercise-induced fall in f-MLP-stimulated neutrophil oxidative burst response when ingested 60 min prior to cycling for 90 min at 70% \(\dot{V}O_2\) max. At post-exercise, f-MLP-stimulated neutrophil oxidative burst responses (as indicated by peak CL values) were significantly decreased after the PLA trial only. A decline in f-MLP-stimulated response following exercise has been previously reported (Scharhag et al., 2002). This finding cannot be explained as a diurnal effect as a resting control study (Chapter 4) used the same treatment and time course as that employed in the current study. The results of Chapter 4 demonstrated that there were no significant changes in f-MLP-stimulated neutrophil oxidative burst response over time, or differences between the CAF and PLA trials when exercise is not performed.

A study conducted by Sullivan and colleagues (1995) reported that caffeine had no direct action on the \textit{in vitro} f-MLP stimulated neutrophil CL response; however, caffeine enhanced the response when neutrophils had been previously treated with physiological concentrations of adenosine. In the present study, when expressing the f-MLP stimulated neutrophil oxidative burst data as a \% decline from resting values, a main effect of trial was found with higher neutrophil oxidative burst responses on CAF than PLA. Although plasma adenosine concentration was not measured in the present study, it is known that adenosine is produced during ATP metabolism and is increased during times of metabolic stress such as exercise (Vizi et al., 2002; Chouker et al., 2005). Adenosine produced intracellularly or formed extracellularly, diffuses to cell membranes of surrounding cells where it binds to adenosine receptors. Acting via A\(_{2A}\) receptors expressed on neutrophils, adenosine is known to inhibit phagocytosis.
and neutrophil $O_2\cdot$ generation (Cronstein et al, 1985; 1990; Gessi et al., 2002; Thibault et al., 2002). Specifically, *in vitro* studies have shown that incubation with relatively low physiological concentrations of adenosine (100 nmol/L) for 3 to 5 min can reduce f-MLP stimulated neutrophil oxidative burst responses by approximately 40% (Cronstein et al., 1985; Swain et al., 2003). Furthermore, Chouker et al., (2005) observed a significant inverse relationship between *in vivo* plasma adenosine concentration and *ex vivo* neutrophil oxidative burst response following f-MLP stimulation. These negative effects may be caused by an increased synthesis of cAMP (Thibault et al., 2000), an inhibition of PL-D activation (Thibault et al., 2002) or a redistribution of a key component of the NADPH oxidase, Flavocytochrome b (Swain et al., 2003).

The potency of caffeine to antagonise $A_{2A}$ receptors occurs at a $K_i$ of 40-44 µmol.L$^{-1}$ (Daly, 1993). In the present study, the caffeine concentrations measured at pre, post and 1 h post-exercise were 53 µmol.L$^{-1}$, 46 µmol.L$^{-1}$ and 42 µmol.L$^{-1}$ respectively, suggesting that caffeine may possibly inhibit the occupancy and/or activation of $A_{2A}$ adenosine receptors. Since inhibition of oxidative burst is predominantly $A_{2A}$-mediated (Gessi et al., 2002) it could be argued that in the present study, caffeine attenuated the post-exercise decline in neutrophil oxidative burst response following f-MLP-stimulation by inhibiting the occupancy and/or activation of $A_{2A}$ adenosine receptors. Alternatively, it could be argued that caffeine affects the intracellular calcium signalling process at post-exercise following stimulation with f-MLP. It is reported that post-exercise decreases in neutrophil oxidative burst responses are related to a decoupling of intracellular calcium signalling with cellular function, suggesting a blockade in intracellular signalling pathways (Mooren et al., 2001).
the present study, caffeine may have interfered with this blockade leading to further effects downstream on the signalling pathway and the observed attenuation of the post-exercise neutrophil oxidative burst response.

It has been suggested that elevated concentrations of adrenaline stimulate β-2 adrenoreceptors expressed on neutrophils (Weiss et al., 1996; Tintinger et al., 2000), activating adenyl cyclase and inducing the synthesis of cAMP (Chilcoat et al., 2002; O'Dowd et al., 2004). An increase in cAMP has been shown to specifically inhibit neutrophils stimulated with f-MLP (Yu et al., 1995) via activation of PKA and suppression of PLA2-dependent arachidonic acid production (O'Dowd et al., 2004). It has frequently been reported that adrenaline can inhibit f-MLP stimulated neutrophil oxidative burst response in a dose-dependent manner (Weiss et al., 1996; Tintinger et al., 2001; O'Dowd et al., 2004). In the present study, CAF was associated with a significant increase in plasma adrenaline concentration above values on PLA after exercise but there were no differences between trials for noradrenaline concentration, which is consistent with previous findings (Tarnopolsky et al., 1989; Graham and Spriet, 1991; Jackman et al., 1996; Graham et al., 1998; Bishop et al., 2005). However, the elevated levels of adrenaline did not appear to inhibit oxidative burst response following stimulation with f-MLP. It has been reported that an adrenaline concentration of 5 nmol.L⁻¹ significantly inhibits f-MLP-stimulated neutrophil oxidative burst response in humans (O'Dowd et al., 2004). However, in the present study, post-exercise adrenaline values were only 2.8 nmol.L⁻¹ and 1.3 nmol.L⁻¹ for the CAF and PLA trials, respectively. It is likely that any effect of caffeine is multifactorial and the net result of a number of positive and negative influences. In the
present study, there may be a stronger influence on neutrophils from $A_{2A}$ adenosine-receptor antagonism than a catecholamine effect.

Caffeine ingestion did not affect the leukocyte and neutrophil counts in response to exercise yet it was associated with a significant increase in circulating lymphocyte number at pre-exercise (i.e. 1 h following caffeine ingestion). Similar findings were reported in Chapter 5 in which the same method of caffeine administration, exercise protocol and sampling time points were used. As previously discussed, an increase in circulating lymphocyte count may be due to the elevated plasma adrenaline concentration at this time point altering endothelial adhesion via activation of $\beta$-adrenoreceptors present on lymphocytes.

In accordance with frequently reported findings, plasma IL-6 concentration was significantly elevated following exercise (Nehlsen-Canarella et al., 1997; Bishop et al., 2001b; Starkie et al., 2001; Febbraio et al., 2003 amongst numerous others). The magnitude of the increase however (approximately 3-fold), was much smaller than previous reports of 20 to 30-fold increases following 60 to 150 min of submaximal intensity exercise (Nieman et al., 1998; Starkie et al., 2001; Steensberg et al., 2001). Plasma IL-6 concentration was significantly higher at post-exercise on CAF compared with PLA. As adrenaline concentration at this time was also significantly higher on CAF than PLA, it may be speculated that adrenaline lead to an increased IL-6 production. Increases in adrenaline concentration have previously been related to increased IL-6 production in both rats (Yu et al., 2001; Frost et al., 2004) and humans (Sondergaard et al., 2001; Papanicolaou et al., 1996). Adrenaline infusion at concentrations that are reached during strenuous exercise has been reported to elicit a
6-fold increase in plasma IL-6 concentration (Steenberg et al., 2001). In this study the IL-6 response was of a significantly lower magnitude than that observed following strenuous exercise and the authors' concluded that adrenaline plays a relatively minor role in stimulating IL-6 during exercise (Steenberg et al., 2001). Alternatively, adrenaline may affect IL-6 clearance from the circulation in humans. It is known that hepatosplanchnic viscera remove IL-6 from the circulation (Febbraio et al., 2003) thus adrenaline mediated vasoconstriction of hepatosplanchnic viscera may augment the level of IL-6 through a decreased rate of clearance. Furthermore, adenosine receptors are present on smooth muscle of various organs such as the liver (Graham, 2001a), therefore a vasoconstriction due to adenosine-receptor antagonism by caffeine may also contribute to reduced clearance of IL-6 from the systemic circulation.

CAF was associated with a lower RPE response compared with PLA (mean RPE was 13.3 ± 0.3 and 14.0 ± 0.2 on CAF and PLA respectively, main effect of trial). An attenuated RPE during submaximal-exercise following caffeine ingestion has been consistently reported (Cole et al., 1996; Cox et al., 2002; Doherty et al., 2004; Doherty and Smith, 2005). It has been suggested that caffeine may alter the release, binding or activity of neurotransmitters in the brain (Davis et al., 2003), release β-endorphins into plasma during physical exertion (Laurent et al., 2000) and reduce feelings of leg muscle pain during exercise (O'Connor et al., 2004). In addition, mood states and feelings of pleasure during exercise are increased following caffeine ingestion (Backhouse et al., 2004).

In summary, the main findings of this study indicate that caffeine ingestion attenuated the exercise-induced fall in f-MLP-stimulated neutrophil oxidative burst response.
(measured as peak CL values). Although caffeine ingestion was associated with an increase in plasma adrenaline concentration, it would appear that this is not the major pathway by which caffeine affected neutrophil function in this study. This suggests that other actions of caffeine may have exerted a stronger influence here.
Chapter 7

The effect of caffeine ingestion on immunoendocrine responses following time-trial cycling.
7.1. Abstract

Following high-intensity, fixed duration exercise, caffeine ingestion has been associated with an attenuation of the exercise-induced decline in f-MLP-stimulated neutrophil oxidative burst response (i.e. the capacity to generate reactive oxygen species in response to *in vitro* stimulation). However, the response following high-intensity exhaustive exercise is unknown. In this study, nine highly endurance-trained male cyclists (mean ± SEM: age 23 ± 1 years; body mass 67.4 ± 1.7 kg; $\dot{V}O_2$ max 71.2 ± 2.3 ml.kg$^{-1}$.min$^{-1}$; Peak Power Output 362 ± 13 W) ingested 6 mg.kg$^{-1}$ body mass of caffeine (CAF) or placebo (PLA) 60 min before cycling for 90 min at 70% $\dot{V}O_2$ max. This was followed by a time-trial (TT) requiring an energy expenditure equivalent to cycling for 30 min at 70% Wmax. Participants completed the TT faster in CAF than PLA, resulting in a 4% improvement in performance ($P<0.05$). CAF was associated with increased plasma adrenaline concentration at post-90 min and post-TT ($P<0.05$) and increased serum caffeine concentration ($P<0.01$, at all time points), yet there were no significant differences observed for f-MLP-stimulated neutrophil oxidative burst responses. CAF was also associated with significantly increased plasma IL-6 concentration at post-TT compared with PLA ($P<0.05$) but had no effect on IL-1ra concentration. There was no effect of CAF on circulating leukocyte or neutrophil count, but lymphocyte count was significantly higher at post-TT on PLA ($P<0.05$). These data suggest that high-intensity exhaustive exercise negates the attenuation of the exercise-induced decline in the f-MLP-stimulated neutrophil oxidative burst response previously observed when caffeine is ingested prior to exercise of fixed duration and intensity. This may be associated with the greater increase in adrenaline concentration observed in the present study.
7.2. Introduction

Athletes commonly consume caffeine as an ergogenic aid (Tarnopolsky, 1994). The use of caffeine is highly prevalent in the sport of amateur and professional cycling (Martin, 1997). Following the relatively recent removal of caffeine from WADA list of prohibited substances, its usage is expected to increase considerably amongst athletes during periods of training and competition. Laboratory based studies have reported increases in performance following the ingestion of 3-6 mg.kg\(^{-1}\) body mass of caffeine. For example, 6 mg.kg\(^{-1}\) body mass of caffeine was associated with a 3.4% increase in performance time to completion of a pre-loaded TT (Cox et al., 2002) while divided caffeine doses have also been shown to enhance pre-loaded TT performance (Kovacs et al., 1998; Conway et al., 2003). It has been reported however, that similar caffeine doses have not altered performance during a 100 km cycling TT (Hunter et al., 2002).

There is limited published literature that has looked specifically at the effects of caffeine ingestion on neutrophil oxidative burst responses following exercise. It has previously been reported (Chapter 6) that caffeine ingestion attenuated the decline of f-MLP stimulated neutrophil oxidative burst responses observed at post-exercise following submaximal intensity cycling of fixed duration. Since caffeine and its derivatives are potent adenosine receptor antagonists (Ongini & Fredholm, 1996) the attenuated decline was hypothesised to have been due to caffeine acting to antagonise A\(_{2A}\) adenosine receptors expressed on neutrophils. The occupancy of A\(_{2A}\) receptors is linked to an increase in cAMP (Thibault et al., 2002), inhibition of PL-D activation (O'Dowd et al., 2004) and a redistribution of Flavocytochrome b (Swain et al., 2003); all factors that may contribute to the inhibition of the f-MLP stimulated neutrophil
oxidative burst response which occurs following the incubation of neutrophils with physiological concentrations of adenosine (Cronstein et al., 1985; Swain et al., 2003).

Caffeine ingestion also increases plasma adrenaline concentration (Graham, 2001a). Stimulation of neutrophil β-2 adrenoreceptors by adrenaline activates adenylyl cyclase and induces cAMP synthesis which has been demonstrated to inhibit the activation of neutrophils (Chilcoat et al., 2002). It is reported that catecholamines decrease neutrophil ROS production in a dose-dependent manner at pharmacological (~65 nmol.L\(^{-1}\)) (Wenisch et al., 1996). At physiological concentrations, however, adrenaline (5 nmol.L\(^{-1}\)) inhibits f-MLP-stimulated O\(_2^{-}\) release (O'Dowd et al., 2004) and the reported decline in neutrophil oxidative burst activity following exercise is attributed to elevated concentrations of adrenaline (Hack et al., 1994).

Given this information and the reported ergogenic benefit of caffeine ingestion on TT performance, the aim of the study was to investigate the influence of 6 mg.kg\(^{-1}\) body mass of caffeine on f-MLP-stimulated neutrophil oxidative burst responses (i.e. the capacity to generate ROS in response to in vitro stimulation) following a TT after 90 min cycling at 70% \(\dot{V}O_2\) max. It is well known that the magnitude of adrenaline release is related to the intensity of exercise (Kotchen et al., 1971); therefore, the current study was designed to determine the effect of caffeine ingestion on neutrophil oxidative burst responses when adrenaline concentration increases to a greater extent than during prolonged submaximal exercise of fixed intensity. It was hypothesised that a higher adrenaline concentration following the TT may become the predominant mechanism mediating the f-MLP-stimulated neutrophil oxidative burst response, and
thus may negate the attenuating effects of caffeine ingestion previously observed following submaximal exercise of fixed duration.
7.3. Methods

Participants
Nine highly-endurance trained male cyclists (mean ± SEM: age 23 ± 1 years; height 1.77 ± 0.03 m; body mass 67.4 ± 1.7 kg; \( \dot{V}O_2 \) max 71.2 ± 2.3 ml.kg\(^{-1}\).min\(^{-1}\); Peak Power Output 362 ± 13 W) volunteered to participate in the study. Participants were informed of the rationale and experimental procedures involved in the study, before providing written consent to participate. The study protocol had earlier received approval by Loughborough University Ethical Advisory Committee. Average daily caffeine intake was 172 ± 66 mg.day\(^{-1}\). Three participants were characterised as light users (< 50 mg.day\(^{-1}\)), 4 as moderate users (50-250 mg.day\(^{-1}\)) and 2 as having a high caffeine intake (> 250 mg.day\(^{-1}\)).

Experimental procedures
Preliminary measurements (\( \dot{V}O_2 \) max and familiarisation) were conducted within a 2-week period prior to the start of the main trials. Preliminary measurements and pre-trial standardisation procedures are described in Chapter 3. This preliminary period also allowed the participants to familiarise themselves with the TT protocol that was to be used in the main experimental trials as a performance measure. The TT was performed on an electromagnetically braked ergometer interfaced with a computer and required the participants to complete a predetermined quantity of work (in joules) equal to cycling for 30 min at 70% \( W_{\text{max}} \), as described by Jeukendrup et al. (1996). Participants performed 2 sessions of exercise separated by 7 days. The participants acted as their own controls in a repeated-measures, single-blind, cross-over design and were randomly assigned to one of two experimental conditions: ingesting caffeine (CAF) or placebo (PLA). Participants arrived at the laboratory at 10.00 am following
an overnight fast of between 10 and 12 h. Following a void, body mass was recorded and a resting blood sample obtained, before participants ingested 6 mg.kg\(^{-1}\) body mass of either CAF or PLA dissolved in 200 ml no-added sugar pink grapefruit juice drink. After resting quietly for 60 min, participants began cycling for 90 min at 70% \(\dot{V}O_2\) max. Heart rate and RPE were measured every 15 min throughout exercise and 2 ml.kg\(^{-1}\) body mass of water was administered at these time points. At 20, 50 and 80 min of exercise, 1-min expired gas samples were collected. A further blood sample was collected immediately post-90 min exercise.

Immediately following the post-90 min exercise blood sample, participants completed a TT that required them to complete a predetermined amount of work corresponding to an energy expenditure equivalent to cycling for 30 min at 70% Wmax. Participants were given an instruction to complete the amount of work as quickly as they were able to do so. During the TT, the only on-screen information available to the participants was the total amount of work (joules) that they had completed, the percentage of the work they had completed and a general profile of their power output to provide an indication of their progress. Feedback such as total exercise time, pedal cadence, power output and heart rate was not available to the participants. No motivational factors (i.e. music, encouragement etc.) were permitted during the TT. Heart rate was recorded at 5-min intervals and water was available \textit{ad libitum} during the TT. Upon completion of the TT, a blood sample (post TT) was obtained and body mass (shorts only) was recorded. Following this, participants consumed 3 ml.kg\(^{-1}\) body mass of water and rested for a further 60 min before a final blood sample was taken (1 h post TT). Laboratory conditions were 17.3 ± 0.3°C and 46 ± 2% relative humidity.
Blood analytical methods

Blood sampling and analyses methods are detailed in Chapter 3.5. In addition to these procedures, IL-1ra was determined from K$_3$EDTA plasma using a commercially available ELISA method (R & D Systems Inc., Abingdon, Oxfordshire, UK).

Statistical analysis

In addition to the statistical analysis methods detailed in Chapter 3.6, associations between plasma adrenaline concentration and f-MLP-stimulated neutrophil oxidative burst responses were determined using Pearson’s Product Moment correlation and linear regression analysis.
7.4. Results

Physiological variables and RPE

Following the exercise protocol, body mass change (corrected for fluid intake) fell similarly on both trials (-2.1 ± 0.2 kg and -2.0 ± 0.2 kg on CAF and PLA, respectively) as did plasma volume (CAF: -10.4 ± 1.8%, PLA: -11.7 ± 2%). There was no difference in overall exercise intensity during the 90 min steady state exercise between trials; mean % \( \dot{V}O_2 \) max was 73.7 ± 0.6% and 72.6 ± 0.5% on CAF and PLA trials, respectively. Mean heart rates during the 90 min exercise were similar between the two experimental conditions (158 ± 2 beats.min\(^{-1}\) and 160 ± 1 beats.min\(^{-1}\) on CAF and PLA, respectively) as were mean perceived ratings of exertion (RPE; CAF: 11.9 ± 0.1, PLA: 12.3 ± 0.2). Significant main effects of time were found for both heart rate and RPE (\(P<0.01\)) with values increasing as a function of exercise duration.

Substrate Oxidation and Energy Expenditure

There was no significant difference between the two experimental conditions for RER during the first 90 min of exercise (0.93 ± 0.01 on CAF and PLA, mean of all recordings). CHO oxidation rates were similar between trials throughout the exercise bout with mean values of 3.58 ± 0.16 g.min\(^{-1}\) and 3.55 ± 0.13 g.min\(^{-1}\) on CAF and PLA trials respectively (Table 7.1). Similarly there were no significant differences for fat oxidation rates between trials (CAF: 0.39 ± 0.06 g.min\(^{-1}\), PLA: 0.38 ± 0.04 g.min\(^{-1}\) however, fat oxidation significantly increased throughout the 90 min exercise period (\(P<0.05\); Table 7.1). Mean rates of energy expenditure was similar on both trials with values of 73 ± 2 kJ.min\(^{-1}\) and 71 ± 1 kJ.min\(^{-1}\) on CAF and PLA respectively. Likewise, the energy derived from the oxidation of CHO (CAF: 57.3 ± 2.7 kJ.min\(^{-1}\), PLA: 56.5 ± 2.1 kJ.min\(^{-1}\)) and fat (CAF: 15.4 ± 2.2 kJ.min\(^{-1}\), PLA: 14.9
± 1.4 kJ.min⁻¹) did not significantly differ between trials throughout the 90 min exercise period, but the energy derived from the oxidation of fat increased throughout this period (P<0.05; Table 7.1). There were no significant differences between trials for the % contribution to total energy expenditure from CHO (CAF: 78 ± 3%, PLA: 79 ± 2%) or fat (CAF: 22 ± 3%, PLA: 21 ± 2%) during the 90 min steady state exercise bout. However, the contribution from fat increased and CHO contribution decreased as the exercise duration increased (both P<0.05; Table 7.1).

Table 7.1: Substrate oxidation rates and contribution towards energy expenditure during the steady state exercise period in the CAF and PLA trials.

<table>
<thead>
<tr>
<th>Time during Exercise</th>
<th>20</th>
<th>50</th>
<th>80</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO oxidation (g.min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>3.80 (0.27)</td>
<td>3.65 (0.36)</td>
<td>3.30 (0.25)</td>
<td>3.58 (0.16)</td>
</tr>
<tr>
<td>PLA</td>
<td>3.69 (0.20)</td>
<td>3.55 (0.28)</td>
<td>3.35 (0.21)</td>
<td>3.55 (0.13)</td>
</tr>
<tr>
<td>Fat oxidation (g.min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.29 (0.06)</td>
<td>0.36 (0.10)</td>
<td>0.54 (0.11)</td>
<td>0.39 (0.06)</td>
</tr>
<tr>
<td>PLA</td>
<td>0.30 (0.07)</td>
<td>0.40 (0.08)</td>
<td>0.43 (0.04)</td>
<td>0.38 (0.04)</td>
</tr>
<tr>
<td>Energy derived from CHO oxidation (kJ.min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>60.8 (4.3)</td>
<td>58.3 (5.8)</td>
<td>52.9 (4.0)</td>
<td>57.3 (2.7)</td>
</tr>
<tr>
<td>PLA</td>
<td>59.1 (3.2)</td>
<td>56.7 (4.5)</td>
<td>53.6 (3.4)</td>
<td>56.5 (2.1)</td>
</tr>
<tr>
<td>Energy derived from fat oxidation (kJ.min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>11.2 (2.5)</td>
<td>13.9 (3.8)</td>
<td>21.1 (4.3)</td>
<td>15.4 (2.2)</td>
</tr>
<tr>
<td>PLA</td>
<td>11.9 (2.4)</td>
<td>15.7 (1.7)</td>
<td>17.0 (1.4)</td>
<td>14.9 (1.4)</td>
</tr>
<tr>
<td>% contribution to total energy expenditure from CHO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>84 (4)</td>
<td>80 (4)</td>
<td>72 (3)</td>
<td>78 (3)</td>
</tr>
<tr>
<td>PLA</td>
<td>83 (3)</td>
<td>78 (4)</td>
<td>76 (3)</td>
<td>79 (2)</td>
</tr>
<tr>
<td>% contribution to total energy expenditure from fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>16 (4)</td>
<td>20 (6)</td>
<td>28 (5)</td>
<td>22 (3)</td>
</tr>
<tr>
<td>PLA</td>
<td>17 (3)</td>
<td>22 (4)</td>
<td>25 (3)</td>
<td>21 (2)</td>
</tr>
</tbody>
</table>
Time-Trial Performance

Participants completed the TT faster in the CAF (27.1 ± 0.6 min) compared with PLA trial (28.2 ± 0.7 min, \(P<0.05\)). This corresponded to a 4% improvement in TT performance. Mean power output during the TT was 282 ± 14 W and 271 ± 15 W in the CAF and PLA trials, respectively. Mean heart rate during the TT tended to be higher on CAF compared with PLA but this did not reach statistical significance (177 ± 2 beats.min\(^{-1}\) and 175 ± 1 beats.min\(^{-1}\) on CAF and PLA, respectively, \(P = 0.064\)).

Blood metabolites and hormones

Serum caffeine was significantly higher at post-90 min, post-TT and at 1 h post-TT on CAF compared with PLA (all \(P<0.01\); Figure 7.1). Plasma adrenaline was significantly increased from rest at post-90 min and immediately post-TT on both trials \(P<0.01\). However, at these time points, plasma adrenaline concentration was significantly higher on CAF compared with PLA (both \(P<0.05\); Figure 7.2). Plasma cortisol concentration was higher than resting values at post-TT on both CAF and PLA trials \(P<0.01\); Table 7.2). At 1 h post-TT, plasma cortisol concentration tended to be higher on CAF than PLA \((P = 0.054\). There were no significant differences between CAF and PLA trials for plasma free fatty acid (FFA) concentration. At 1 h post-TT, plasma glucose concentration was significantly lower than resting values on PLA trial only \((P<0.01\); Table 7.2) and at immediately post-TT, plasma lactate concentration tended to be higher on CAF than PLA \((P = 0.051\); Table 7.2).
Figure 7.1. Serum caffeine concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point (†† P<0.01). Significantly higher than rest within trial (*** P<0.01).

Figure 7.2. Plasma adrenaline concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point († P<0.05). Significantly higher than rest within trial (*** P<0.01).
Table 7.2. Plasma concentrations of cortisol and blood-borne metabolites on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Post-90 min</th>
<th>Post-TT</th>
<th>1 h Post-TT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortisol (nmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>388 (30)</td>
<td>478 (27)</td>
<td>590 (23)**</td>
<td>597 (38)**</td>
</tr>
<tr>
<td>PLA</td>
<td>421 (44)</td>
<td>469 (29)</td>
<td>598 (25)**</td>
<td>497 (22)</td>
</tr>
<tr>
<td><strong>FFA (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.23 (0.04)</td>
<td>1.19 (0.15)</td>
<td>1.08 (0.13)</td>
<td>1.60 (0.19)</td>
</tr>
<tr>
<td>PLA</td>
<td>0.21 (0.03)</td>
<td>0.93 (0.11)</td>
<td>1.07 (0.12)</td>
<td>1.38 (0.18)</td>
</tr>
<tr>
<td><strong>Glucose (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>4.7 (0.1)</td>
<td>4.9 (0.1)</td>
<td>6.1 (0.6)</td>
<td>4.7 (0.3)</td>
</tr>
<tr>
<td>PLA</td>
<td>4.8 (0.1)</td>
<td>5.0 (0.1)</td>
<td>5.1 (0.5)</td>
<td>4.1 (0.1)**</td>
</tr>
<tr>
<td><strong>Lactate (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>0.9 (0.1)</td>
<td>2.6 (0.3)**</td>
<td>9.3 (1.1)**</td>
<td>-</td>
</tr>
<tr>
<td>PLA</td>
<td>1.3 (0.2)</td>
<td>2.1 (0.2)**</td>
<td>6.6 (1.0)**</td>
<td>-</td>
</tr>
</tbody>
</table>

Significantly different from rest within trial (**P<0.01). † Main effect of time, significantly higher than rest at all time points (P<0.01).

Total and differential leukocyte counts

All participants had resting total and differential blood leukocyte counts within the normal range for healthy adults. There was a significant leukocytosis and neutrophilia evident at post-90 min, post-TT and 1 h post-TT (main effect of time; P<0.01; Table 7.3) but there were no significant differences between CAF and PLA trials. A significant lymphocytosis occurred on both trials at post-90 min (P<0.01); however, at post-TT, values were significantly higher on PLA than CAF (P<0.05; Table 7.3). There was a significant main effect of time (P<0.01; Table 7.3) for the neutrophil/lymphocyte ratio with higher values calculated at post-TT than at rest and post-90 min. A further increase was evident at 1 h post-TT which occurred independent of trial.
Table 7.3. Total and differential blood leukocyte counts and the neutrophil/lymphocyte ratio on CAF and PLA trials.

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Post-90 min</th>
<th>Post-TT</th>
<th>1 h Post-TT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes (x 10^9 L^-1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>5.5 (0.3)</td>
<td>8.1 (0.5)</td>
<td>13.2 (1.6)</td>
<td>14.8 (1.4)</td>
</tr>
<tr>
<td>PLA</td>
<td>5.2 (0.1)</td>
<td>8.3 (0.7)</td>
<td>15.6 (1.4)</td>
<td>15.6 (1.5)</td>
</tr>
<tr>
<td><strong>Neutrophils (x 10^9 L^-1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>2.9 (0.3)</td>
<td>5.9 (0.3)</td>
<td>8.1 (1.3)</td>
<td>12.1 (1.3)</td>
</tr>
<tr>
<td>PLA</td>
<td>2.6 (0.1)</td>
<td>4.9 (0.5)</td>
<td>10.4 (1.2)</td>
<td>12.5 (1.3)</td>
</tr>
<tr>
<td><strong>Lymphocytes (x 10^9 L^-1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.90 (0.15)</td>
<td>2.16 (0.18)**</td>
<td>2.98 (0.26)**</td>
<td>1.46 (0.13)</td>
</tr>
<tr>
<td>PLA</td>
<td>1.86 (0.10)</td>
<td>2.30 (0.23)**</td>
<td>3.61 (0.27)**†</td>
<td>1.55 (0.15)</td>
</tr>
<tr>
<td><strong>Neutrophil/Lymphocyte ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF</td>
<td>1.5 (0.1)</td>
<td>2.4 (0.2)</td>
<td>3.0 (0.4)</td>
<td>8.4 (0.6)</td>
</tr>
<tr>
<td>PLA</td>
<td>1.4 (0.1)</td>
<td>2.3 (0.3)</td>
<td>2.9 (0.3)</td>
<td>8.2 (0.6)</td>
</tr>
</tbody>
</table>

Significantly higher on PLA than CAF at that time point († P<0.05). Significantly higher than rest within trial (**P<0.01). Main effect of time, significantly higher than rest at all time points (‡P<0.01). Main effect of time, significantly higher than rest at Post-TT and 1 h Post-TT (* P<0.01).

**Neutrophil Function**

f-MLP-stimulated neutrophil oxidative burst response (as indicated by peak CL) was significantly lower at post-90 min, post-TT and 1 h post-TT compared with resting values (P<0.01; Figure 7.3) but there were no differences between CAF and PLA conditions. Neutrophil oxidative burst response (expressed as a % of resting response) was significantly lower than rest at post-TT and 1 h post-TT (P<0.01; Figure 7.4), yet occurred independent of trial. A significant negative correlation was found between the increase in plasma adrenaline concentration and neutrophil oxidative burst response at post-TT in the CAF trial only (r = -0.836, P<0.01).
Figure 7.3. Neutrophil oxidative burst response expressed as peak CL values in relative light units (RLU) following f-MLP-stimulation. Main effect of time, significantly lower than rest at Post-90, Post-TT and 1 h Post-TT (P<0.01).

Figure 7.4. f-MLP-stimulated neutrophil oxidative burst (peak CL) expressed as % of baseline response. Main effect of time, significantly lower than rest at Post-TT and 1 h Post-TT (P<0.01).
Cytokine Response

Plasma IL-6 concentration was significantly higher than resting values at post-90 min, post-TT and 1 h post-TT on both CAF and PLA trials ($P<0.01$). At post-TT, plasma IL-6 concentration was significantly higher on CAF than PLA ($P<0.05$; Figure 7.5). Plasma IL-6 on CAF increased 22-fold above resting values to $15.2 \pm 2.3 \text{ ng.L}^{-1}$ compared with a 17-fold increase in PLA to values of $11.1 \pm 1.7 \text{ ng.L}^{-1}$.

![Figure 7.5. Plasma IL-6 concentration on CAF and PLA trials. Significantly higher on CAF than PLA at that time point († $P<0.05$), significantly higher than rest within trial (** $P<0.01$).](image)

Plasma IL-1ra concentration was significantly higher than resting values at post-90 min, post-TT and 1 h post-TT on both CAF and PLA trials ($P<0.05$). Plasma IL-1ra values peaked at 1 h post-TT on both trials (CAF: $1226 \pm 398 \text{ ng.L}^{-1}$, PLA: $755 \pm 244 \text{ ng.L}^{-1}$; Figure 7.6); this represented increases of 12- and 7-fold on CAF and PLA, respectively, but there were no significant differences between trials.
Figure 7.6. Plasma IL-1ra concentration on CAF and PLA trials. Main effect of time, significantly higher than rest at post-90 min, post-TT and 1 h post-TT ($P<0.05$).
7.5. Discussion

The results of the present study demonstrate that ingesting 6 mg.kg\(^{-1}\) body mass of caffeine 60 min prior to the onset of exercise significantly improves pre-loaded TT performance following prolonged submaximal intensity cycling. CAF ingestion was associated with increased adrenaline concentration at post-90 min and post-TT, yet there were no significant differences observed for f-MLP-stimulated neutrophil oxidative burst responses following exercise. CAF ingestion was also associated with significantly increased IL-6 concentration at post-TT compared with PLA but had no effect on IL-1ra concentration.

The time taken to complete a pre-loaded TT performance test was significantly faster on CAF than PLA, corresponding to a 4% improvement in performance. The ergogenic effect of caffeine found here is in accordance with the findings of a number of recent studies (Kovacs et al., 1998; Cox et al., 2002; Conway et al., 2003). It is generally agreed that caffeine produces its effects at physiological concentrations via adenosine receptor antagonism. The antagonism of adenosine receptor subpopulations within the central nervous system may explain caffeine's fatigue-delaying effects (Davis et al., 2003), by decreasing perceptions of work intensity. In addition, a reported increase in the release of β-endorphin associated with caffeine ingestion (Laurent et al., 2000) may reduce feelings of pain associated with intense exercise (O'Connor et al., 2004). These effects may be reflected here in the 11 W higher average power output on CAF during the TT performance test.

There were no significant main effects of caffeine ingestion on f-MLP-stimulated neutrophil oxidative burst responses, which fell significantly from resting values
following the exercise protocol. A decline in the f-MLP-stimulated response following exercise has been previously reported (Scharhag et al., 2002) and may be attributed to increases in plasma adenosine and/or adrenaline concentration following exercise. Plasma adenosine concentration is reported to increase during exercise (Vizi et al., 2002; Chouker et al., 2005) by the dephosphorylation of AMP. As plasma adenosine is increased during times of metabolic stress, it is likely to be higher following the TT performance test than following submaximal exercise due to the higher overall exercise intensity (mean power output was 232 W during steady state exercise compared with 276 W during TT); however, plasma adenosine concentration was not measured in the present study.

Caffeine and its metabolites are non-selective neutrophil A₁ and A₂A adenosine receptor antagonists (Ongini & Fredholm, 1996). As the potency for caffeine to antagonise A₂A receptors occurs at a Kᵢ of 40-44 µmol.L⁻¹ (Daly, 1993), the serum caffeine concentrations of 37 µmol.L⁻¹ and 34 µmol.L⁻¹ obtained at post-TT and 1 h post-TT in the present study may not inhibit the occupancy and/or activation of neutrophil A₂A adenosine receptors and therefore not attenuate the exercise-induced response as previously observed (Chapter 6). At immediately post-90 min, however, caffeine concentration was 42 µmol.L⁻¹ and f-MLP stimulated oxidative burst values at this time had only fallen by 9% below resting values on CAF. This is compared with a 26% fall on PLA at the same time. However, this difference was not statistically significant.

Neutrophil oxidative burst responses are also influenced by elevations in plasma catecholamine concentration, particularly adrenaline (Peake, 2002). It is known that
Caffeine ingestion increases plasma adrenaline concentration (Graham, 2001a) and this is supported by significantly higher values in the present study on CAF than PLA at both post-90 min and post-TT. Stimulation of neutrophil $\beta_2$ adrenoreceptors by adrenaline activates adenyl cyclase and induces cAMP synthesis, which has been demonstrated to inhibit the activation of neutrophils (Chilcoat et al., 2002). At low physiological levels adrenaline has little effect on neutrophil oxidative burst responses (Wenisch et al., 1996); however, adrenaline at a concentration of 5 nmol.L$^{-1}$ has been reported to inhibit f-MLP-stimulated $O_2^\cdot$ release (O'Dowd et al., 2004). In the present study, adrenaline values at post-TT were 5.2 nmol.L$^{-1}$ and 3.7 nmol.L$^{-1}$ on CAF and PLA trials, respectively, suggesting a possible inhibitory effect on neutrophil function in the CAF trial. Moreover, a significant negative correlation was found between the increase in plasma adrenaline concentration and f-MLP-stimulated neutrophil oxidative burst response at post-TT in the CAF trial. Since the effect of caffeine ingestion on neutrophil function following exercise is likely to be multifactorial and the net result of both stimulatory and inhibitory factors, these findings may suggest that during high-intensity exhaustive exercise the inhibitory effects of adrenaline on neutrophil function occur alongside the potentially positive effects of $A_{2A}$ adenosine receptor antagonism by caffeine. Therefore, such negative effects may be speculated to effectively cancel out the effects of caffeine that have previously been observed following submaximal exercise of a fixed intensity and duration (Chapter 6).

Increases in plasma adrenaline concentration have also been linked to an increased IL-6 release into circulation in both rats (Yu et al., 2001; Frost et al., 2004) and humans (Papanicolaou et al., 1996). The increase in cAMP production from $\beta_2$
adrenoreceptor stimulation has been shown to enhance IL-6 production (Dendorfer et al., 1994). IL-6, however, is largely produced in contracting skeletal muscle and is released into circulation, where it is reported to produce a number of anti-inflammatory effects, one of these being to stimulate the appearance of IL-1ra in the circulation (Steensberg et al., 2003). IL-1ra is an anti-inflammatory cytokine, with a function to bind to the IL-1 receptor and inhibit the functions of IL-1 (Dinarello, 1998).

Plasma IL-6 concentration was significantly elevated at post-90 min, with an approximate 20-fold increase at post-TT. The magnitude of IL-6 elevation reported here is in accordance with previously reported findings (Nieman et al., 1998; Starkie et al., 2001). Plasma IL-6 concentration was significantly higher at immediately post-TT on CAF than PLA. It was previously speculated that a significantly higher adrenaline concentration on CAF may have lead to an increased IL-6 production (Papanicolaou et al., 1996, Sondergard et al., 2000) or may have increased IL-6 concentration via reduced clearance from the circulation by the hepatosplanchnic tissues due to adrenaline mediated vasoconstriction. It has been previously reported that following adrenaline infusion at physiological concentrations, the IL-6 response is of a lower magnitude than that observed during exercise, leading to the conclusion that adrenaline plays a minor role in stimulating IL-6 during exercise (Steensberg et al., 2001). It is also reported, however, that adrenaline infusion peaking with a plasma adrenaline concentration of 2.1 nmol.L$^{-1}$ caused a 26-fold increase in IL-6 mRNA expression in adipose tissue with only a small increase in systemic IL-6 (Keller et al., 2004). It could be argued therefore, that the higher adrenaline concentration in the CAF trial (5.2 nmol.L$^{-1}$ vs. 3.7 nmol.L$^{-1}$) may also increase IL-6 in adipose tissue to a
greater extent than the PLA trial without a further increase of systemic IL-6. There were no significant effects of caffeine ingestion on plasma IL-1ra concentration following exercise. In accordance with previous published literature (Nieman et al., 1998; Bishop et al., 2001b; Steensberg et al., 2001, Ronsen et al., 2002), plasma IL-1ra concentration exhibited a delayed response, peaking at 1 h post-TT. Plasma IL-1ra concentration increased by 12-fold on CAF compared with a 7-fold increase on PLA. However, it is likely that no significant differences between trials were observed due to a large inter-individual variation of IL-1ra response in the present study.

Caffeine ingestion did not affect the total or differential blood leukocyte counts in response to exercise, however there was a significant increase in lymphocyte number at post-TT on the PLA trial only. At the corresponding time-point, plasma adrenaline concentration was significantly higher on CAF than PLA. This is in contrast to our previous findings and would appear an unusual finding as it is known that adrenaline can alter endothelial adhesion via activation of β-adrenoreceptors present on lymphocytes, recruiting them into the circulation (Mackinnon, 1999). One possible reason for this is a different power output distribution between the two conditions in the latter stages of the TT. However, upon closer examination of the power output profile, which was recorded every 20 s throughout the TT, one can discount this as power output was similar between CAF and PLA trials during both the final 10 and 5 min of the performance test. The reason for the increased lymphocytosis at post-TT on PLA is therefore unclear.

In summary, the ingestion of 6 mg.kg⁻¹ body mass of caffeine 60 min prior to the onset of exercise significantly improved TT performance following 90 min cycling.
Caffeine ingestion was associated with increased adrenaline concentration at post-90 min and post-TT, yet there were no significant differences observed for f-MLP-stimulated neutrophil oxidative burst responses following exercise. This is in contrast to our previous observation of an attenuation of the exercise-induced fall in peak f-MLP-stimulated oxidative burst response with caffeine ingestion following submaximal intensity exercise of a fixed duration. Therefore, the findings of the present study may suggest that during high-intensity exhaustive exercise, adrenaline exerts a greater negative influence on neutrophil function. From a performance perspective, however, caffeine provided an ergogenic benefit for a TT without exacerbating the exercise-induced decline in neutrophil function.
Chapter 8

The effect of physiological concentrations of caffeine, adenosine and adrenaline on the oxidative burst response of isolated human neutrophils in vitro.
8.1. Abstract

The effects of caffeine ingestion on f-MLP-stimulated neutrophil oxidative burst responses, reported in the previous experimental chapters were speculated to be the net result of adenosine receptor antagonism and adrenaline-mediated inhibition. The purpose of the present study was to determine the relative contribution of physiological concentrations of adenosine, caffeine and adrenaline on the f-MLP-stimulated neutrophil oxidative burst response in an attempt to mimic the conditions of the exercise trials. A resting blood sample was obtained by venepuncture from 6 healthy males (age: 27 ± 1 years) following an overnight fast and 60 h abstention from caffeine containing products. Neutrophils were separated from whole blood, washed and re-suspended in reconstitution and assay buffer at a final concentration of 1 x 10^6 neutrophils/ml. The incubation of isolated human neutrophils with 100 nmol.L\(^{-1}\) adenosine for 2 min at 37°C decreased oxidative burst response by 40% compared with control (P = 0.001). Following the addition of 50 µmol.L\(^{-1}\) caffeine, the response was 9% lower than control but this difference was not statistically significant (P = 0.58). The response of neutrophils incubated with adenosine, caffeine and adrenaline was 37% and 40% lower than control for 2 nmol.L\(^{-1}\) (P = 0.002) and 5 nmol.L\(^{-1}\) (P = 0.002) adrenaline respectively. There was no significant difference in the f-MLP-stimulated neutrophil oxidative burst response to the different concentrations of adrenaline added to the suspension (P = 0.78). This suggests that caffeine attenuates the decline in the f-MLP-stimulated oxidative burst response in the presence of adenosine alone, but not when adrenaline is also present. These findings do not easily explain the apparent attenuating effect of caffeine on post-exercise neutrophil oxidative burst responses (i.e. when both adenosine and adrenaline are
present), however, it may be that in this \textit{in vitro} environment, adenosine may potentiate the potency of adrenaline to inhibit the oxidative burst response.
8.2. Introduction

It is well known that adenosine is produced during adenine nucleotide breakdown and that its rate of production is increased during metabolic stress (Klinger et al., 2002; Hasko and Cronstein, 2004). An interest in the immunomodulatory effects of adenosine arose following the discovery that patients with abnormally high plasma adenosine levels due to defective ADA expression developed severe combined immuno-deficiency (SCID), characterised by a marked susceptibility to infection (Giblett et al., 1972). A number of in vitro studies have shown that adenosine at physiological concentrations can inhibit f-MLP-stimulated neutrophil \( \text{O}_2^- \) production (Cronstein et al., 1985; Swain et al., 2003). Indeed, a significant inverse relationship was observed between in vivo plasma adenosine concentration and ex vivo neutrophil \( \text{O}_2^- \) production in humans (Chouker et al., 2005). This inhibition by adenosine may (Varani et al., 1998) or may not (Cronstein et al., 1988) be due to an increase in the ‘secondary messenger’ cAMP.

Inhibition of neutrophil oxidative burst is predominantly \( \text{A}_{2A} \) adenosine receptor mediated (Gessi et al., 2002). The methylxanthine caffeine is a non-selective adenosine receptor antagonist with affinity in the \( \mu \text{mol.L}^{-1} \) range (Ongini and Fredholm, 1996). In the presence of adenosine (100 nmol.L\(^{-1} \)), caffeine increased f-MLP-stimulated luminol-enhanced CL but had no effect on \( \text{O}_2^- \) production (Sullivan et al., 1995). The findings of Chapter 4 demonstrated that caffeine ingestion alone does not affect the f-MLP or PMA-stimulated neutrophil oxidative burst response in humans at rest (i.e. where adenosine concentrations would be very low).
Neutrophils are also known to respond to adrenergic agonists *in vitro*, with increased cAMP synthesis and decreased immunological responses (Dulis and Wilson, 1980). Caffeine ingestion increases adrenaline release and an increase in cAMP concentration has been shown to reduce f-MLP-induced $O_2^•$ generation (Yu et al., 1995). Moreover, adrenaline can decrease neutrophil luminol-enhanced CL measurements (Weiss et al., 1996) and $O_2^•$ generation (Wenisch et al., 1996) in a dose-dependent manner. Although this effect of adrenaline is most pronounced at clinically-high doses, in the presence of low concentrations of adenosine, physiological concentrations of adrenaline strongly inhibited neutrophil function (Bazzoni et al., 1991).

Little information is known about the combined effects of adenosine, caffeine and adrenaline on the neutrophil oxidative burst response. It has been previously stated in this thesis that the net effect of caffeine on f-MLP stimulated neutrophil oxidative burst responses may be the result of a number of stimulatory (via adenosine receptor antagonism) and suppressive (via elevated adrenaline concentration) influences. The findings of Chapter 6 demonstrated that caffeine ingestion attenuated the exercise-induced decline in f-MLP stimulated neutrophil oxidative burst response following 90 min cycling at 70% $VO_2$ max, even in the presence of an elevated adrenaline concentration (CAF: 2.7 nmol.L$^{-1}$, PLA: 1.5 nmol.L$^{-1}$). When adrenaline concentration was increased further however, following maximal effort TT cycling (CAF: 5.2 nmol.L$^{-1}$, PLA: 3.7 nmol.L$^{-1}$), no attenuating effects of caffeine ingestion were observed (Chapter 7).
The aim of this study therefore was to investigate the potential mechanisms that may account for the effects observed in the previous exercise studies, by first determining the effect of physiological concentrations of adenosine on f-MLP-stimulated oxidative burst responses of isolated human neutrophils and secondly to compare the response following the addition of caffeine at a concentration detected in serum after caffeine ingestion (6 mg.kg\(^{-1}\) body mass). Finally, the study aimed to determine the effects of adrenaline (at 2 different concentrations previously observed following submaximal and high-intensity exercise) on f-MLP-stimulated neutrophil oxidative burst responses in the presence of both adenosine and caffeine.
8.3. Methods

*Chemicals and stimuli*

Both adenosine and adrenaline (Sigma-Aldrich Co, Gillingham, UK) were dissolved in phosphate buffered saline (PBS) at stock concentrations of 0.8 mmol.L\(^{-1}\) and 40 mmol.L\(^{-1}\) respectively. Caffeine (BDH Laboratory Supplies, Poole, UK) was dissolved in PBS at a stock concentration of 4 mmol.L\(^{-1}\). All stock solutions were made immediately before use and were protected from light. Further dilutions in PBS to working concentrations were made immediately prior to use. Reconstitution and assay buffer was used as a medium to re-suspend the neutrophils in following separation from whole blood. This consisted of Hank's Balanced Salt Solution (HBSS) with 20 mM Hepes at pH 7.4 (Knight Scientific Limited, Plymouth, UK). f-MLP was reconstituted according to manufacturer's instructions at a working concentration of 5 μg.ml\(^{-1}\) (Knight Scientific Limited, Plymouth, UK).

*Cell Isolation Procedure*

Six healthy males (age 27 ± 1 years) volunteered to participate in the study. Participants arrived at the laboratory at 08:00 am following an overnight fast and 60 h abstention from caffeine containing products. Following a 10 min seated rest period, a blood sample (7.5 ml) was collected into a K\(_3\)EDTA monovette from an antecubital forearm vein. Neutrophils were isolated from whole blood by adding, into a centrifuge tube and in the following order, 2 ml of Histopaque 1.119 separating medium (Sigma-Aldrich Co, Gillingham, UK), 2 ml of Histopaque 1.077 separating medium (Sigma-Aldrich Co, Gillingham, UK) and 3 ml of whole blood. The tubes were then centrifuged at 400 g for 30 min at 24°C. After centrifugation, the halo of neutrophils was carefully removed using a Pasteur pipette and transferred to separately labelled
tubes containing 5 ml of PBS. The cells were re-suspended and then centrifuged at
200 g for 10 min at 24°C. The supernatant was removed and the cells were re-
suspended in a further 5 ml of PBS and centrifuged again at 200 g for 10 min at 24°C.
Following this, the supernatant was discarded and the cells were re-suspended in 1 ml
of reconstitution and assay buffer. Neutrophils were counted in a Neubauer
haemocytometer under a light microscope and the suspension was adjusted using
reconstitution and assay buffer to a final concentration of 1 x 10^6 neutrophils/ml.

Chemiluminescence assay

The neutrophils were exposed to five separate experimental conditions that were
performed in duplicate. In the control condition (Con), 250 µl of neutrophil
suspension was added to an eppendorf containing 150 µl reconstitution and assay
buffer. The solution was mixed by vortex for 5 s then incubated for 2 min at 37°C in a
dry block heater. Immediately following the incubation, 20 µl of suspension was
removed and added to a well of a 96 well microplate containing 90 µl reconstitution
and assay buffer, 20 µl Adjuvant K and 50 µl Pholasin (as previously described in
Chapter 3). Upon the addition of 20 µl f-MLP, pholasin-enhanced CL was recorded
every 1 s for 100 s and peak CL (oxidative burst response) was determined.

To determine the effect of physiological concentrations of adenosine (Aden) on the
oxidative burst response, 50 µl adenosine (100 nmoL^-1 final concentration) was
added to 250 µl neutrophil suspension and 100 µl reconstitution and assay buffer,
prior to incubation and f-MLP stimulation described above. The third condition (Aden
+ Caf) was designed to evaluate the effect of physiological concentrations of caffeine
on the adenosine-mediated oxidative burst response and consisted of 250 µl

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neutrophil suspension, 50 µl reconstitution and assay buffer, 50 µl caffeine (50 µmol.L⁻¹ final concentration) and 50 µl adenosine (100 nmol.L⁻¹). In an attempt to replicate the conditions of the exercise studies described in Chapters 5, 6 and 7, the final two conditions consisted of the addition of 50 µl adrenaline at either 2 nmol.L⁻¹ (final concentration) or 5 nmol.L⁻¹ (final concentration) to 250 µl neutrophil suspension and 50 µl caffeine (50 µmol.L⁻¹) followed by the addition of 50 µl adenosine (100 nmol.L⁻¹). The results of an initial pilot study are shown in Appendix F.

Statistical Analysis

Differences between conditions were assessed using Student’s paired t-tests with Bonferroni adjustment for multiple comparisons. Data in Figure 8.1 is presented as % of control condition (Mean ± SEM).
8.4. Results

The incubation of human neutrophils with physiological concentrations of adenosine (100 nmol.L\(^{-1}\)) resulted in a significant inhibition (40%) of the CL response compared to the control condition \((P = 0.001)\). Following the addition of 50 µmol.L\(^{-1}\) caffeine \((\text{Aden} + \text{Caf})\), the response was 9% lower than control but was not statistically different from control. Although the response on \(\text{Aden} + \text{Caf}\) was higher than \(\text{Aden}\) alone, this difference was not statistically significant \((P = 0.12)\). The CL response of neutrophils incubated with adenosine (100 nmol.L\(^{-1}\)), caffeine (50 µmol.L\(^{-1}\)) and adrenaline at 2 nmol.L\(^{-1}\) and at 5 nmol.L\(^{-1}\) were both significantly lower than control \((\text{Adren} 2 \text{nM}: 37\% (P = 0.002); \text{Adren} 5 \text{nM}: 40\% (P = 0.001))\). There was no significant difference between the concentration of adrenaline added to the suspension and neutrophil response \((P = 0.78)\).

![Figure 8.1. Peak CL values of isolated neutrophils (stimulated with f-MLP) expressed as % of control condition (Con) in the five experimental conditions. Significantly lower than Con (**P<0.01).](image-url)
8.5. Discussion

The major findings of the present study were that physiological concentrations of adenosine significantly inhibited the f-MLP-stimulated oxidative burst response of isolated human neutrophils \textit{in vitro}. In the presence of caffeine however, adenosine did not significantly reduce the oxidative burst response. The addition of adrenaline at both 2 nmol.L\(^{-1}\) and at 5 nmol.L\(^{-1}\) significantly reduced the neutrophil oxidative burst response in the presence of caffeine and adenosine, but there was no significant difference between the adrenaline concentrations administered.

Resting plasma adenosine concentration in humans at rest is approximately 40 – 50 nmol.L\(^{-1}\) (Vizi et al., 2002; Chouker et al., 2005), however a large inter-individual variation of between 20 – 200 nmol.L\(^{-1}\) (mean 82 nmol.L\(^{-1}\)) has been reported (Moser et al., 1989). Adenosine concentration is known to increase with exercise or metabolic stress. It is reported that plasma adenosine concentration increased from 40 to 60 nmol.L\(^{-1}\) following 6 min exercise at 90% heart rate maximum (Vizi et al., 2002) and was 80 nmol.L\(^{-1}\) following a hike at low altitude (Chouker et al., 2005). In the present study, isolated neutrophils were incubated with 100 nmol.L\(^{-1}\) adenosine to attempt to reflect the increase following high-intensity exercise. Adenosine significantly reduced the f-MLP-stimulated oxidative burst response of isolated human neutrophils by 40% compared to the control condition. The inhibition reported here is of the same magnitude as that reported when neutrophils were incubated with 100 nmol.L\(^{-1}\) adenosine for 3 min (38% inhibition) and 5 min (40% inhibition) prior to f-MLP-stimulation (Cronstein et al., 1985; Swain et al., 2003). Furthermore, Chouker et al., (2005) observed a significant inverse relationship between \textit{in vivo} plasma adenosine
concentration and ex vivo neutrophil $O_2\cdot^-$ generation, reporting an IC$_{50}$ of 80 nmol.L$^{-1}$.

Adenosine produced intracellularly or formed extracellularly, diffuses to cell membranes of surrounding cells where it binds to adenosine receptors (Hasko and Cronstein, 2004). Inhibition of the f-MLP-stimulated neutrophil oxidative burst response, as observed here, is reported to occur via $A_{2A}$ adenosine receptors expressed on neutrophils (Gessi et al., 2002). Adenosine has been reported to inhibit neutrophil oxidative burst response by suppressing PL-D activity (Thibault et al., 2000). PL-D catalyzes the hydrolysis of phosphatidylycholine to yield phosphatic acid, which is further metabolised. These PL-D second messengers may regulate the oxidative burst and other neutrophil functions. Suppression of f-MLP-induced PL-D activity has been correlated with an increased cAMP concentration, that may activate protein kinase A (PKA) to mediate this suppression (Thibault et al., 2002). In addition to this, adenosine is reported to increase the trafficking of flavocytochrome b (Swain et al., 2003). This key component of NADPH oxidase may be translocated from plasma membrane to a higher density membrane by adenosine, coinciding with an accelerated shut down of the oxidative burst response by neutrophils (Swain et al., 2003).

In the present study, the suppressive effects of adenosine on the f-MLP stimulated neutrophil oxidative burst response were attenuated following the addition of caffeine (50 µmol.L$^{-1}$). Although the f-MLP stimulated neutrophil oxidative burst response was not significantly higher with caffeine and adenosine than adenosine alone ($P=0.12$) the response was only 9% lower than in the control condition, compared to a 40% decrease following incubation with adenosine alone. The concentration of caffeine used in the present study corresponded to that previously measured in serum.
following 90 min cycling at 70% \( \overline{V}O_2 \) max after the ingestion of 6 mg.kg\(^{-1}\) body mass caffeine. Serum caffeine concentration at post-exercise in Chapter 5, 6 and 7 was 55 \( \mu \)mol.L\(^{-1}\), 46 \( \mu \)mol.L\(^{-1}\) and 42 \( \mu \)mol.L\(^{-1}\) respectively. The potency of caffeine to antagonise A\(_{2A}\) adenosine receptors occurs at a \( K_i \) of 40-44 \( \mu \)mol.L\(^{-1}\) (Daly, 1993) suggesting that in the present study, caffeine may have inhibited the occupancy and/or activation of A\(_{2A}\) adenosine receptors thus attenuating the suppressive effects that adenosine exerts.

The addition of adrenaline at both 2 nmol.L\(^{-1}\) and at 5 nmol.L\(^{-1}\) significantly reduced the magnitude of the f-MLP stimulated neutrophil oxidative burst response in the presence of caffeine and adenosine. The concentration of adrenaline used in the present study reflected the measured concentration in plasma following 90 min cycling at 70% \( \overline{V}O_2 \) max (Chapter 5 & 6) and high-intensity TT cycling (Chapter 7). Adrenaline is known to stimulate \( \beta-2 \) adrenoreceptors expressed by neutrophils, activating adenyl cyclase and inducing cAMP synthesis (Tintinger et al., 2000). An increase in cAMP has been shown to specifically inhibit neutrophils stimulated with f-MLP (Yu et al., 1995). It has been suggested that adrenaline causes an inhibition of f-MLP mediated oxidative burst via production of cAMP and activation of PKA which subsequently suppresses PLA\(_2\)-dependent arachidonic acid production (O’Dowd et al., 2004). Arachidonic acid is another pathway by which NADPH oxidase may be activated; however, a concentration of 5 nmol.L\(^{-1}\) adrenaline was required for this effect to be observed (O’Dowd et al., 2004). Nevertheless, in the present study, there was no significant difference between 2 nmol.L\(^{-1}\) and 5 nmol.L\(^{-1}\) adrenaline on the f-MLP stimulated neutrophil oxidative burst response in the presence of adenosine and caffeine. It has frequently been reported that adrenaline alone can dose-dependently
inhibit neutrophil oxidative burst responses (Weiss et al., 1996; Tintinger et al., 2001; O’Dowd et al., 2004). A study by Bazzoni et al., (1991) reported that adrenaline inhibited the f-MLP-stimulated neutrophil oxidative burst response with an IC$_{50}$ of 69 nmol.L$^{-1}$. In the presence of low concentrations of adenosine however, a strong inhibitory effect of adrenaline was exhibited even at physiological concentrations in the subnanomolar range. Given that human neutrophils in suspension spontaneously generate adenosine (Iannone et al., 1989), it may be that in this in vitro environment, adenosine markedly potentiates the potency of adrenaline to inhibit the oxidative burst response.

In summary, this study supported previous findings that adenosine markedly reduces the oxidative burst response of isolated human neutrophils. In the presence of caffeine however, the response was not significantly lower than control, in accordance with the known antagonistic actions of caffeine on adenosine. The addition of adrenaline in the presence of caffeine and adenosine significantly reduced the oxidative burst response below control but there was no concentration–dependent effect. Unfortunately this study was not able to clarify the contributing mechanisms by which caffeine attenuates the post-exercise decrease in f-MLP-stimulated neutrophil oxidative burst responses in the presence of elevated adrenaline concentrations (Chapter 6). This reinforces the point that in vitro experimentation can not always truly reflect the in vivo situation.
Chapter 9

The effect of caffeine co-ingested with carbohydrate on immunoendocrine responses following prolonged submaximal cycling.
9.1. Abstract

The purpose of the present study was to determine the effect of caffeine consumed with and without CHO on f-MLP-stimulated neutrophil oxidative burst responses following exercise. Twelve recreationally active male cyclists (mean ± SEM: age 22 ± 1 years; body mass 69.9 ± 1.9 kg; \( \dot{V}O_2 \) max 59.8 ± 2.0 ml.kg\(^{-1}\).min\(^{-1}\)) participated in 4 exercise trials consisting of cycling for 2 h at 65% \( \dot{V}O_2 \) max. Sixty min prior to exercise, participants ingested 6 mg.kg\(^{-1}\) body mass of caffeine (CAF) or placebo (PLA), then during exercise they consumed a 6% CHO or placebo (PLA) drink, providing 4 experimental conditions: CAF/CHO, PLA/CHO, CAF/PLA and PLA/PLA. The f-MLP-stimulated neutrophil oxidative burst response was significantly higher at post-exercise on CAF/CHO and PLA/CHO (both \( P<0.05 \)) than PLA/PLA when expressed as % of baseline value. Oxidative burst response on CAF/PLA tended to be higher than PLA/PLA at this point (\( P=0.056 \)). There were no significant differences between CAF/CHO, PLA/CHO and CAF/PLA at post-exercise; however, only PLA/CHO showed no significant post-exercise decline. The co-ingestion of CAF/CHO significantly attenuated the adrenaline (\( P<0.05 \)) and IL-6 (\( P<0.05 \)) responses that occurred following the ingestion of CAF alone (CAF/PLA) and significantly attenuated the transient alterations in circulating total leukocyte (\( P<0.05 \)) and neutrophil (\( P<0.01 \)) count. Plasma cortisol was significantly lower on PLA/CHO than both CAF/PLA and PLA/PLA at post-exercise (\( P<0.05 \)). Perceived exertion during the exercise was significantly lower on the CAF/CHO condition than the other 3 trials (\( P<0.05 \)). These results suggest that the co-ingestion of CAF/CHO provides no additional effects on the f-MLP-stimulated neutrophil oxidative burst response following exercise compared with that observed in response to either supplement alone. It may be however, that the effects of CHO on neutrophil oxidative...
burst responses are of greater magnitude than the previously reported effects of CAF, therefore effectively masking any influence of CAF on the response.
9.2. Introduction

The findings reported in Chapter 6 of this thesis showed that caffeine ingestion attenuated the decline of the f-MLP-stimulated neutrophil oxidative burst response following submaximal intensity cycling of fixed duration. As caffeine and its derivatives are potent adenosine receptor antagonists (Ongini & Fredholm, 1996) the attenuated decline was hypothesised to have been due to caffeine acting to inhibit the occupancy and/or activation of A2A adenosine receptors expressed on neutrophils. This is plausible since inhibition of the neutrophil oxidative burst response is predominantly A2A mediated (Gessi et al., 2002) and adenosine has been shown to specifically inhibit O2•− generation by f-MLP-stimulated neutrophils in vitro (Cronstein et al., 1985; Swain et al., 2003). Caffeine, however, also increases plasma levels of catecholamines (Graham, 2001a). The decline in the neutrophil oxidative burst response following exercise is attributed to elevated concentrations of adrenaline (Hack et al., 1994) and adrenaline acting via neutrophil β-2 adrenoreceptors has been reported to inhibit neutrophil ROS production (Chilcoat et al., 2002; O'Dowd et al., 2004).

The ingestion of CHO before and during exercise is associated with a higher plasma glucose concentration and an attenuation of the stress hormone response. Both adrenaline (Nieman et al., 2005) and cortisol (Nehlsen-Cannarella et al., 1997) concentrations are significantly lower following prolonged submaximal exercise when CHO is consumed. CHO ingestion is also associated with fewer perturbations to immune cell counts, particularly an attenuation of the neutrophilia seen following prolonged exercise (Bishop et al., 2003; Nieman et al., 2005). The effect of CHO ingestion on neutrophil oxidative burst responses has produced equivocal findings. It
is reported that the post-exercise decline of the f-MLP-stimulated neutrophil oxidative burst response is attenuated by CHO beverage ingestion (Scharhag et al., 2002) whereas no effect has been observed on the PMA-stimulated neutrophil response following moderate exercise (Smith et al., 1996).

CHO ingestion prior to and during exercise (Nehlsen-Cannarella et al., 1997; Starkie et al., 2001; Nieman et al., 2003) is also shown to attenuate the increases in plasma IL-6 concentration but not muscle mRNA expression (Starkie et al., 2001; Nieman et al., 2005) following prolonged exercise. Low muscle glycogen levels are associated with elevated IL-6 responses (Bishop et al., 2001b; Steensberg et al., 2001) and it is suggested that IL-6 may be involved in the regulation of glucose homeostasis by affecting hepatic glucose production to prevent hypoglycaemia (Gleeson, 2000; Febbraio and Pedersen, 2002).

Many commercially available drinks contain both caffeine and CHO. Given that the co-ingestion of CHO and caffeine increases TT performance (Cox et al., 2002) and that its use is commonly reported by professional cyclists during competition (Martin, 1997), the aim of the study was to investigate the influence of caffeine co-ingested with CHO upon the f-MLP-stimulated neutrophil oxidative burst and plasma IL-6 response following 2 h prolonged cycling at 65% $\dot{V}O_2$ max.
9.3. Methods

Twelve recreational male cyclists (mean ± SEM: age 22 ± 1 years; height 1.78 ± 0.03 m; body mass 69.9 ± 1.9 kg; \(\dot{V}{\text{O}}_2\) max 59.8 ± 2.0 ml.kg\(^{-1}\).min\(^{-1}\); Peak Power Output 336 ± 14 W) volunteered to participate in the study. Participants were informed of the rationale and experimental procedures involved in the study, before providing written consent to participate. The study protocol had earlier received approval by the local University Ethical Advisory Committee. Average daily caffeine intake was 140 ± 37 mg.day\(^{-1}\). Four participants were characterised as light users (< 50 mg.day\(^{-1}\)), 6 as moderate users (50-250 mg.day\(^{-1}\)) and 2 as having a high caffeine intake (> 250 mg.day\(^{-1}\)).

Preliminary experimental measurements were conducted within a 2-week period prior to the beginning of the main trials. Preliminary measurements and pre-trial standardisation procedures are detailed in Chapter 3. Participants performed 4 main experimental trials separated by a minimum of 5 days. The participants acted as their own controls in a double-blind, cross-over design and were randomly assigned to one of four experimental conditions; caffeine/carbohydrate (CAF/CHO), placebo/carbohydrate (PLA/CHO), caffeine/placebo (CAF/PLA) or placebo/placebo (PLA/PLA). The order of treatments was randomised using a basic Latin squares design so that no participant completed the trials in the same order as another. Participants arrived at the laboratory at 08:00 am following an overnight fast of between 10 and 12 h. Following a void, body mass was recorded and participants ingested 6 mg.kg\(^{-1}\) body mass of either caffeine or placebo (dextrose powder), taken in the form of a cellulose capsule (Blackfirs Health, Cheshire, UK) with 2 ml.kg\(^{-1}\) body mass of water before resting quietly for 60 min. Caffeine was consumed in two
of the trials and placebo in the remaining two trials. At the end of the 60 min period, participants consumed 5 ml.kg\textsuperscript{-1} of a 6 % CHO solution (in the CAF/CHO and PLA/CHO trials) or a similar tasting artificially sweetened solution (in the CAF/PLA and PLA/PLA trials). The composition of the CHO drink consisted of 150 ml of low calorie lemon cordial, 850 ml water and dextrose monohydrate (6.6% w/v) per litre of solution. The PLA drink contained 20 calorie-free sweeteners as a substitute for dextrose monohydrate. Participants were instructed to consume this drink as quickly as possible, after which they immediately began cycling for 120 min at 65% VO\textsubscript{2} max. Heart rate and RPE were measured every 15 min throughout exercise and 2 ml.kg\textsuperscript{-1} body mass of CHO or PLA was administered at these time points according to the experimental condition. At 15, 45 and 75 and 105 min of exercise, 1-min expired gas samples were collected. Following the cessation of exercise, body mass was recorded and participants consumed 5 ml.kg\textsuperscript{-1} body mass of CHO or PLA before resting for a further 60 min. Venous blood samples were obtained at rest, post-exercise and at 1 h post-exercise. Laboratory conditions were 22.8 ± 0.4°C and 37 ± 2% relative humidity.

**Blood analytical methods**

Blood sampling and analyses methods are detailed in Chapter 3.5.

**Statistical analysis**

Statistical analysis methods are detailed in Chapter 3.6.
9.4. Results

**Physiological variables and RPE**

There were no significant differences in overall exercise intensity between trials; mean % \( \dot{V}O_2 \) max was 67.1 ± 0.7%, 65.7 ± 0.5%, 67.3 ± 0.5% and 66.3 ± 0.5% on CAF/CHO, PLA/CHO, CAF/PLA and PLA/PLA trials, respectively. Following exercise, body mass change (corrected for fluid intake) fell similarly on all trials (CAF/CHO: -1.9 ± 0.1 kg, PLA/CHO: -1.8 ± 0.1 kg, CAF/PLA: -1.8 ± 0.2 kg, PLA/PLA: -1.9 ± 0.1 kg) as did plasma volume (CAF/CHO: -8.0 ± 2.4%, PLA/CHO: -8.0 ± 1.3%, CAF/PLA: -8.5 ± 1.4%, PLA/PLA: -9.5 ± 1.4%). Heart rate during exercise was similar between the four experimental conditions (150 ± 2 beats.min\(^{-1}\), 152 ± 2 beats.min\(^{-1}\), 150 ± 3 beats.min\(^{-1}\) and 151 ± 2 beats.min\(^{-1}\) on CAF/CHO, PLA/CHO, CAF/PLA and PLA/PLA trials respectively, mean of all recordings) and heart rate values increased with exercise duration (main effect of time; \( P<0.01 \)). Mean RPE was significantly lower on CAF/CHO trial (11.2 ± 0.3, \( P<0.05 \)) than PLA/CHO (11.8 ± 0.2), CAF/PLA (11.5 ± 0.3) and PLA/PLA (11.6 ± 0.4). A significant main effect of time (\( P<0.01 \)) was found for RPE with values increasing as a function of exercise duration.
Substrate Oxidation and Energy Expenditure

Mean RER was significantly lower during the 120 min exercise period on CAF/PLA compared with PLA/CHO ($P<0.05$; Table 9.1). No other differences between trials were observed. There were no significant differences for CHO oxidation rate between trials, however CHO oxidation decreased with exercise duration ($P<0.01$; Table 9.1). Mean fat oxidation rate was significantly higher on CAF/PLA than PLA/CHO ($P<0.05$; Table 9.1). Fat oxidation increased with exercise duration on CAF/CHO, CAF/PLA and PLA/PLA, yet there were no differences during the exercise on the PLA/CHO trial (Table 9.1).
Table 9.1: RER and substrate oxidation rates during the 120 min exercise period.

<table>
<thead>
<tr>
<th>Time</th>
<th>15</th>
<th>45</th>
<th>75</th>
<th>105</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF/CHO</td>
<td>0.95 (0.01)</td>
<td>0.94 (0.01)</td>
<td>0.93 (0.01)</td>
<td>0.92 (0.02)</td>
<td>0.93 (0.01)</td>
</tr>
<tr>
<td>PLA/CHO</td>
<td>0.93 (0.01)</td>
<td>0.94 (0.01)</td>
<td>0.94 (0.01)</td>
<td>0.92 (0.01)</td>
<td>0.93 (0.01)</td>
</tr>
<tr>
<td>CAF/PLA</td>
<td>0.94 (0.01)</td>
<td>0.92 (0.01)</td>
<td>0.91 (0.01)</td>
<td>0.87 (0.01)</td>
<td>0.91 (0.01) a</td>
</tr>
<tr>
<td>PLA/PLA</td>
<td>0.94 (0.01)</td>
<td>0.93 (0.01)</td>
<td>0.92 (0.01)</td>
<td>0.90 (0.01)</td>
<td>0.92 (0.01)</td>
</tr>
</tbody>
</table>

| **CHO oxidation (g.min⁻¹)** |      |      |      |      |      |
| CAF/CHO | 3.03 (0.14) | 2.98 (0.17) | 2.91 (0.19) | 2.80 (0.18) | 2.93 (0.08) |
| PLA/CHO | 2.77 (0.22) | 2.97 (0.17) | 2.93 (0.18) | 2.71 (0.18) | 2.84 (0.09) |
| CAF/PLA | 2.92 (0.20) | 2.80 (0.16) | 2.96 (0.25) | 2.27 (0.18) | 2.74 (0.10) |
| PLA/PLA | 3.07 (0.18) | 2.75 (0.16) | 2.68 (0.15) | 2.58 (0.13) | 2.77 (0.08) |

| **Fat oxidation (g.min⁻¹)** |      |      |      |      |      |
| CAF/CHO | 0.23 (0.05) | 0.29 (0.05) | 0.32 (0.06) | 0.41 (0.07) | 0.31 (0.03) |
| PLA/CHO | 0.30 (0.06) | 0.27 (0.04) | 0.29 (0.03) | 0.35 (0.04) | 0.30 (0.02) |
| CAF/PLA | 0.25 (0.06) | 0.36 (0.06) | 0.36 (0.06) | 0.59 (0.05) | 0.39 (0.03) b |
| PLA/PLA | 0.22 (0.06) | 0.35 (0.05) | 0.39 (0.05) | 0.45 (0.04) | 0.35 (0.03) |

Significantly lower than PLA/CHO (a P<0.01). Significantly higher than PLA/CHO (b P<0.05). Significantly higher than at 15 min within trial († P<0.05, †† P<0.01).

There were no significant differences for mean rates of energy expenditure between trials (CAF/CHO: 59 ± 1 kJ.min⁻¹, PLA/CHO: 58 ± 1 kJ.min⁻¹, CAF/PLA: 59 ± 1 kJ.min⁻¹, PLA/PLA: 58 ± 1 kJ.min⁻¹). The energy derived from the oxidation of CHO during the 120 min exercise period decreased with exercise duration (P<0.01; Table 9.2). The energy derived from the oxidation of fat was significantly higher on CAF/PLA than PLA/CHO (mean of all calculations, P<0.05; Table 9.2). A significant time x trial interaction (P<0.05) showed that the energy contributed by fat significantly increased with exercise duration on CAF/PLA and PLA/PLA, yet there
were no differences on PLA/CHO trial (Table 9.2). There were no significant differences between trials for the mean percentage contribution of CHO or fat towards total energy expenditure during the exercise period, however a time x trial interaction ($P<0.05$) showed that % CHO contribution at 105 min was significantly higher on PLA/CHO than CAF/PLA. Accordingly, at this time point, % fat contribution was significantly higher on CAF/PLA than PLA/CHO (Table 9.2).
Table 9.2: CHO and fat contribution towards energy expenditure.

<table>
<thead>
<tr>
<th>Time</th>
<th>15</th>
<th>45</th>
<th>75</th>
<th>105</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy derived from CHO oxidation (kJ.min⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF/CHO</td>
<td>48.5 (2.3)</td>
<td>47.8 (2.8)</td>
<td>46.5 (3.1)</td>
<td>44.9 (2.8)</td>
<td>46.9 (1.3)</td>
</tr>
<tr>
<td>PLA/CHO</td>
<td>44.4 (3.6)</td>
<td>47.5 (2.7)</td>
<td>46.9 (2.9)</td>
<td>43.3 (2.3)</td>
<td>45.7 (1.4)</td>
</tr>
<tr>
<td>CAF/PLA</td>
<td>46.8 (3.1)</td>
<td>44.8 (2.6)</td>
<td>47.4 (4.03)</td>
<td>36.4 (2.9)</td>
<td>43.8 (1.7)</td>
</tr>
<tr>
<td>PLA/PLA</td>
<td>49.1 (2.9)</td>
<td>44.0 (2.5)</td>
<td>42.9 (2.5)</td>
<td>41.3 (2.0)</td>
<td>44.3 (1.3)</td>
</tr>
</tbody>
</table>

| **Energy derived from fat oxidation (kJ.min⁻¹)** |       |       |       |       |       |
| CAF/CHO | 9.0 (2.0) | 11.2 (2.1) | 12.5 (2.5) | 16.0 (3.0) † | 12.2 (1.2) |
| PLA/CHO | 11.7 (2.2) | 10.4 (1.6) | 11.3 (1.3) | 13.6 (1.7) | 11.8 (0.8) |
| CAF/PLA | 9.9 (2.2) | 14.0 (2.2) † | 14.0 (2.8) † | 22.8 (1.8) ‡ | 15.2 (1.3)a |
| PLA/PLA | 8.4 (2.3) | 13.6 (1.8) † | 15.3 (2.1) ‡ | 17.5 (1.6) ‡ | 13.7 (1.1) |

| **% contribution to total energy expenditure from CHO** |       |       |       |       |       |
| CAF/CHO | 85 (3) | 81 (3) | 79 (4) | 74 (4) | 80 (2) |
| PLA/CHO | 78 (4) | 82 (3) | 80 (3) | 76 (3) | 79 (2) |
| CAF/PLA | 82 (4) | 76 (4) | 76 (5) | 61 (3) ‡b | 74 (2) |
| PLA/PLA | 85 (4) | 76 (3) † | 74 (4) ‡ | 70 (3) ‡ | 76 (2) |

| **% contribution to total energy expenditure from fat** |       |       |       |       |       |
| CAF/CHO | 15 (3) | 19 (3) | 21 (4) | 26 (4) | 20 (2) |
| PLA/CHO | 22 (4) | 18 (3) | 20 (3) | 24 (3) | 21 (2) |
| CAF/PLA | 18 (4) | 24 (4) | 24 (5) | 39 (3) ‡a | 26 (2) |
| PLA/PLA | 15 (4) | 24 (3) ‡ | 26 (3) ‡ | 30 (3) ‡ | 24 (2) |

Significantly higher than PLA/CHO (a \( P<0.05 \)). Significantly lower than PLA/CHO (b \( P<0.05 \)). Significantly different from 15 min within trial († \( P<0.05 \), ‡ \( P<0.01 \)).
**Blood metabolites and hormones**

Serum caffeine was significantly higher at post-exercise and 1 h post-exercise on both CAF/CHO and CAF/PLA than PLA/CHO and PLA/PLA ($P<0.01$; Figure 9.1).

![Figure 9.1. Serum caffeine concentration. Significantly higher than PLA/CHO and PLA/PLA at that time point (a $P<0.01$). Significantly higher serum caffeine above resting within trial (** $P<0.01$).](image)

Plasma adrenaline concentration significantly increased on all conditions following the exercise protocol ($P<0.01$; Figure 9.2). At post-exercise, plasma adrenaline concentration was significantly higher on CAF/PLA than PLA/CHO ($P<0.01$), CAF/CHO and PLA/PLA (both $P<0.05$). At this time, adrenaline concentration on CAF/CHO and PLA/PLA was significantly higher than on PLA/CHO ($P<0.05$).
Figure 9.2. Plasma adrenaline concentration at rest and post-exercise. Significantly higher than PLA/CHO at that time point (a $P<0.01$). Significantly higher than CAF/CHO and PLA/PLA at that time point (b $P<0.05$). Significantly higher than PLA/CHO at that time point (c $P<0.05$). Significantly higher than rest within trial (** $P<0.01$).

Plasma cortisol concentration was significantly higher at post-exercise on both CAF/PLA and PLA/PLA than PLA/CHO but not CAF/CHO ($P<0.05$; Figure 9.3).

Plasma cortisol concentration at 1 h post-exercise had significantly decreased from post-exercise on all four conditions. At this time, cortisol concentration was significantly lower than resting values on CAF/CHO and PLA/CHO only ($P<0.01$).
Figure 9.3. Plasma cortisol concentration. Significantly higher than PLA/CHO at that time point (a $P<0.05$). Significantly lower than rest within trial (** $P<0.01$).

Plasma glucose concentration was significantly higher than rest at post-exercise on CAF/CHO and PLA/CHO trials (both $P<0.01$; Table 9.3). At this time, glucose concentration was significantly higher than CAF/PLA and PLA/PLA ($P<0.01$). There were no differences between trials at 1 h post-exercise yet plasma glucose concentration had decreased below rest on PLA/CHO ($P<0.05$) and PLA/PLA ($P<0.01$). Plasma FFA concentration was significantly higher than rest at post-exercise on all experimental trials ($P<0.01$; Table 9.3). At this point, plasma FFA concentration was significantly higher on CAF/PLA than both CAF/CHO and PLA/CHO ($P<0.01$) and FFA concentration on PLA/PLA was significantly higher than PLA/CHO ($P<0.01$). Plasma FFA concentration remained elevated at 1 h post-exercise on CAF/PLA and PLA/PLA compared to CAF/CHO and PLA/CHO ($P<0.01$), yet it was significantly higher on CAF/PLA than PLA/PLA ($P<0.01$). Plasma lactate concentration was also significantly elevated above rest following the
exercise period \( (P<0.01; \text{Table 9.3}) \). At post-exercise, plasma lactate concentration was significantly higher on CAF/PLA than both PLA/CHO and PLA/PLA \( (P<0.05) \) and lactate concentration on CAF/CHO was significantly higher than PLA/CHO \( (P<0.05) \).

<table>
<thead>
<tr>
<th>Table 9.3. Plasma concentrations of blood-borne metabolites.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose ( \text{mmol.L}^{-1} )</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>CAF/CHO</td>
</tr>
<tr>
<td>PLA/CHO</td>
</tr>
<tr>
<td>CAF/PLA</td>
</tr>
<tr>
<td>PLA/PLA</td>
</tr>
<tr>
<td><strong>FFA ( \text{mmol.L}^{-1} )</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>CAF/CHO</td>
</tr>
<tr>
<td>PLA/CHO</td>
</tr>
<tr>
<td>CAF/PLA</td>
</tr>
<tr>
<td>PLA/PLA</td>
</tr>
<tr>
<td><strong>Lactate ( \text{mmol.L}^{-1} )</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>CAF/CHO</td>
</tr>
<tr>
<td>PLA/CHO</td>
</tr>
<tr>
<td>CAF/PLA</td>
</tr>
<tr>
<td>PLA/PLA</td>
</tr>
</tbody>
</table>

Significantly higher than CAF/PLA and PLA/PLA \( (a \text{P}<0.01) \), Significantly higher than PLA/PLA \( (b \text{P}<0.01) \) and CAF/PLA \( (b \text{P}<0.05) \), Significantly higher than CAF/CHO and PLA/CHO \( (c \text{P}<0.01) \), Significantly higher than PLA/CHO \( (d \text{P}<0.01) \), Significantly higher than CAF/CHO, PLA/CHO and PLA/PLA \( (e \text{P}<0.01) \), Significantly higher than CAF/CHO and PLA/CHO \( (f \text{P}<0.01) \), Significantly higher than PLA/CHO \( (g \text{P}<0.05) \), Significantly higher than PLA/CHO and PLA/PLA \( (h \text{P}<0.05) \), Significantly different from rest within trial **P<0.01, *P<0.05**.
Total and differential leukocyte counts

There was a marked leukocytosis evident at post-exercise with a further increase at 1 h post-exercise on all experimental conditions ($P<0.01$; Figure 9.4). At post-exercise, circulating leukocyte count was significantly lower on PLA/CHO ($P<0.01$) and CAF/CHO ($P<0.05$) than both CAF/PLA and PLA/PLA. At 1 h post-exercise, leukocyte count remained significantly lower on PLA/CHO and CAF/CHO than CAF/PLA and PLA/PLA (both $P<0.01$).

![Figure 9.4. Total circulating leukocyte count. Significantly lower than CAF/PLA and PLA/PLA at that time point (a $P<0.01$), Significantly lower than CAF/PLA and PLA/PLA at that time point (b $P<0.05$). Significantly higher than rest within trial (** $P<0.01$).](image)

Circulating neutrophil count was significantly higher than resting counts at post-exercise and 1 h post-exercise, irrespective of the trial followed ($P<0.01$; Figure 9.5). At post-exercise, circulating neutrophil count was significantly lower on PLA/CHO than CAF/PLA and PLA/PLA ($P<0.01$). At this point, neutrophil count was...
significantly lower on CAF/CHO than PLA/PLA ($P<0.05$). Neutrophil count remained significantly lower on PLA/CHO and CAF/CHO than CAF/PLA and PLA/PLA (both $P<0.01$) at 1 h post-exercise.

Figure 9.5. Circulating neutrophil count. Significantly lower than CAF/PLA and PLA/PLA at that time point (a $P<0.01$), Significantly lower than PLA/PLA at that time point (b $P<0.05$), Significantly higher than rest within trial (** $P<0.01$).

Circulating lymphocyte count at post-exercise was significantly lower on PLA/CHO ($P<0.01$) and CAF/CHO ($P<0.05$) than PLA/PLA. A significant lymphopenia was observed at 1 h post-exercise on all trials and at this time, circulating lymphocyte count was significantly higher on PLA/CHO than CAF/PLA ($P<0.05$; Figure 9.6).
Figure 9.6. Circulating lymphocyte count. Significantly lower than PLA/PLA at that time point (a $P<0.01$), Significantly lower than PLA/PLA at that time point (b $P<0.05$), Significantly higher than CAF/PLA at that time point (c $P<0.05$), Significantly lower than rest within trial (** $P<0.01$, * $P<0.05$).

Neutrophil oxidative burst

There were no between trial differences for the f-MLP-stimulated oxidative burst response when values were expressed as peak CL response per neutrophil; however, at post-exercise and 1 h post-exercise the f-MLP-stimulated oxidative burst response was lower than resting values on CAF/CHO ($P<0.05$; Figure 9.7 A) and PLA/PLA ($P<0.01$; Figure 9.7 A). No statistically significant decreases were observed for PLA/CHO and CAF/PLA. When peak CL response was expressed as a % of baseline, f-MLP-stimulated oxidative burst response was significantly higher at post-exercise on CAF/CHO ($P<0.05$; Figure 9.7 B) and PLA/CHO ($P<0.05$; Figure 9.7 B) than PLA/PLA. CAF/PLA also tended to be higher than PLA/PLA at this time but did not quite reach statistical significance ($P = 0.056$). There were no significant differences
between trials at 1 h post-exercise, yet the response on CAF/CHO ($P<0.05$), CAF/PLA ($P<0.05$) and PLA/PLA ($P<0.01$) was significantly lower than baseline. No significant decreases from baseline response were observed for PLA/CHO.

**Figure 9.7 A.** Peak CL values expressed as relative light units (RLU) for neutrophils (stimulated with f-MLP). Significantly lower than rest within trial (**$P<0.01$, *$P<0.05$). **Figure 9.7 B.** Peak CL of neutrophils (stimulated with f-MLP) expressed as % of baseline. Significantly higher than PLA/PLA at that time point (a $P<0.05$). Significantly lower than rest within trial (**$P<0.01$, *$P<0.05$).
Plasma IL-6 concentration was significantly elevated above resting values at post-exercise, with values remaining significantly higher than rest at 1 h post-exercise ($P<0.01$; Figure 9.8). Plasma IL-6 concentration was significantly higher at post-exercise on CAF/PLA than both CAF/CHO and PLA/CHO ($P<0.05$). There were no significant differences between trials at 1 h post-exercise.

**Figure 9.8.** Plasma IL-6 concentration. Significantly higher than CAF/CHO and PLA/CHO (a $P<0.05$). Significantly higher than rest within trial (** $P<0.01$).
9.5. Discussion

In accordance with previous reports, the findings of the present study indicate that cycling for 2 h at 65% $\dot{V}O_2_{\text{max}}$ resulted in a significant decrease in f-MLP-stimulated neutrophil oxidative burst responses (Scharhag et al., 2002). The ingestion of a 6% CHO drink (PLA/CHO trial) before and during exercise significantly attenuated this exercise-induced decrease. The reported decline in neutrophil oxidative burst response is thought to be largely mediated by elevated concentrations of adrenaline (Hack et al., 1994). Adrenaline is known to stimulate the $\beta_2$ adrenoreceptors expressed by neutrophils, activating adenyl cyclase and inducing the synthesis of cAMP that can act to inhibit various aspects of neutrophil function (Dulis and Wilson, 1980). It is reported that catecholamines decrease ROS production in a dose-dependant manner following in vitro incubation (Wenisch et al., 1996). In the present study, the plasma adrenaline concentration at post-exercise was 0.53 nmol.L$^{-1}$ on PLA/CHO and 1.05 nmol.L$^{-1}$ on PLA/PLA. At this time, the f-MLP-stimulated neutrophil oxidative burst response was significantly higher on PLA/CHO. Furthermore, for this condition, values had only fallen 15% below baseline values at this time, compared with a fall of 50% on PLA/PLA.

Following the ingestion of 6 mg.kg$^{-1}$ caffeine only (CAF/PLA trial), the f-MLP-stimulated neutrophil oxidative burst response at post-exercise tended to be higher than when placebo (PLA/PLA) was ingested throughout the trial (70% vs. 50% of pre-exercise response, $P = 0.056$). This finding is in accordance with the results observed in Chapter 6, in which caffeine attenuated the post-exercise decline in the f-MLP-stimulated neutrophil oxidative burst response following 90 min cycling at 70% $\dot{V}O_2_{\text{max}}$. It is known that caffeine at physiological concentrations, exerts its
effects via adenosine receptor antagonism (Graham, 2001a). Plasma adenosine is reported to increase following exercise (Vizi et al., 2002) and oxidative burst inhibition is mediated via A2A adenosine receptors expressed on neutrophils (Gessi et al., 2002). Adenosine is inversely correlated with the ability of granulocytes to produce O2− and is reported to dose-dependently inhibit O2− generation following f-MLP stimulation (Chouker et al., 2005). The adenosine-mediated mechanism for this response may involve an increase in cAMP (Thibault et al., 2002), inhibition of PL-D activation (O’Dowd et al., 2004) and/or a redistribution of Flavocytochrome b (Swain et al., 2003), factors that may result in the exercise-induced fall in neutrophil oxidative burst response. The potency of caffeine to antagonise A2A receptors occurs at a Ki of 40-44 µmol.L⁻¹ (Daly, 1993). In the present study, mean serum caffeine concentration at post-exercise on CAF/PLA was 45 µmol.L⁻¹. Therefore, caffeine may attenuate the exercise-induced decline in the f-MLP-stimulated oxidative burst response by inhibiting the occupancy and/or activation of A2A adenosine receptors. In accordance with previous findings, caffeine ingestion alone (CAF/PLA) also increased plasma adrenaline concentration at post-exercise (Graham, 2001a). However, although plasma adrenaline was 3-fold higher in this trial than on PLA/CHO, there were no significant differences in the f-MLP-stimulated neutrophil oxidative burst response between CAF/PLA and PLA/CHO.

It is likely that the effects of caffeine on neutrophil oxidative burst response are multifactorial and the net-result of a number of stimulatory and inhibitory influences. In light of this, the study was designed to determine the effect of caffeine co-ingested with CHO (CAF/CHO). It was hypothesised that the co-ingestion of CAF/CHO may provide additional benefit over each supplement alone, by combining A2A receptor
antagonism with an attenuation of the stress hormone response to exercise. Although the adrenaline response on CAF/CHO (0.99 nmol.L\(^{-1}\)) was significantly lower than on CAF/PLA (1.64 nmol.L\(^{-1}\)), it was significantly higher than PLA/CHO (0.53 nmol.L\(^{-1}\)) at post-exercise and was comparable to the response on PLA/PLA (1.05 nmol.L\(^{-1}\)). At this time, the f-MLP-stimulated neutrophil oxidative burst response was significantly higher on CAF/CHO than PLA/PLA and had fallen by 26% below baseline values on CAF/CHO, compared with a 50% fall below baseline on PLA/PLA. Mean serum caffeine concentration at post-exercise on CAF/CHO was 47 µmol.L\(^{-1}\), yet there was no significant difference in the f-MLP-stimulated neutrophil oxidative burst response between trials for CAF/CHO compared to either PLA/CHO or CAF/PLA. When post-exercise neutrophil oxidative burst responses were compared to resting values within trial, only PLA/CHO trial did not exhibit a significant decrease from rest. Taken together, these findings may suggest that the effects of CHO are of greater magnitude than the effects of caffeine on neutrophil responses, but this is not to say that caffeine has no effect on f-MLP-stimulated neutrophil oxidative burst responses in the presence of CHO. When observing the % oxidative burst response at post-exercise, it could be argued that caffeine inhibits the actions of CHO; however, it is more likely that any effects of caffeine are very small in magnitude and are masked by the more pronounced effects of CHO when the two are co-ingested.

CHO supplementation before and during prolonged and strenuous exercise is known to maintain plasma glucose concentrations, leading to an attenuation of the cortisol response following exercise (Nehlsen-Canarella et al., 1997; Nieman et al., 2005). In the present study, plasma glucose concentration was significantly higher on CAF/CHO and PLA/CHO than both CAF/PLA and PLA/PLA at post-exercise.
Accordingly, plasma cortisol concentration was significantly higher on CAF/PLA and PLA/PLA than PLA/CHO. This is most likely to occur via the activation of hypothalamic-pituitary-adrenal axis, resulting in an increased release of ACTH and cortisol (Nehlsen-Canarella et al., 1997). It has also been suggested that caffeine ingestion is associated with an elevated cortisol response following exercise. Following the same exercise protocol employed here, Laurent et al. (2000) reported that cortisol was significantly higher following caffeine ingestion, however, the present data do not support this finding as plasma cortisol concentration was similar between CAF/PLA and PLA/PLA conditions. Following cortisol release, neutrophils are recruited from the bone marrow into circulation (Pyne, 1994). This is likely to explain the larger leukocytosis and neutrophilia on CAF/PLA and PLA/PLA conditions and the attenuation of these responses following CHO ingestion. The previously reported reduction in f-MLP-stimulated neutrophil oxidative burst response following exercise has been attributed to this increased influx of less mature cells into circulation, since these have a reduced capacity to produce superoxide (Scharhag et al., 2002). It has also been suggested that as the oxidative burst is dependent on a source of glucose to provide NADPH, the oxidative burst response may be affected by falling plasma glucose concentration. It would appear however, that decreases in plasma glucose concentration exert no significant effect on oxidative burst responses in healthy participants (Lin et al., 1995).

In accordance with previous findings, plasma IL-6 concentration significantly increased following the exercise protocol (Nehlsen-Canarella et al., 1997; Bishop et al., 2001b; Starkie et al., 2001). It should be noted, however, that the magnitude of the IL-6 response observed here was significantly lower than a study employing a
protocol of the same intensity and duration (Febbraio et al., 2003). Plasma IL-6 concentration was significantly higher at post-exercise on CAF/PLA than both CAF/CHO and PLA/CHO. Since adrenaline concentration at post-exercise was significantly higher on CAF/PLA, it may be speculated that elevations in adrenaline lead to increased IL-6 production and therefore elevated plasma concentrations of IL-6 because increases in adrenaline have been previously related to increased IL-6 production in both rats (Yu et al., 2001) and humans (Sondergaard et al., 2000; Papanicolaou et al., 1996). However, it is reported that following adrenaline infusion at physiological concentrations, the IL-6 response is of a lower magnitude than that observed during exercise (that elicits a similar adrenaline response) leading the authors to conclude that adrenaline plays only a minor role in stimulating IL-6 during exercise (Steensberg et al., 2001). Nevertheless, adrenaline infusion did still induce a six-fold increase in the plasma concentration of IL-6 (Steensberg et al., 2001). Furthermore, adrenaline mediated vasoconstriction of hepatosplanchnic viscera may augment plasma levels of IL-6 through a decrease in cytokine clearance. In addition, adenosine receptors are present on smooth muscle of various organs such as the liver, thus a vasococonstriction by adenosine receptor-antagonism may also contribute to reduced clearance of IL-6 from the systemic circulation.

Although the co-ingestion of caffeine and CHO significantly reduced the IL-6 response to exercise compared with caffeine ingestion alone, there were no differences between CAF/CHO (3.5 pg.ml\(^{-1}\)) and PLA/CHO (3.5 pg.ml\(^{-1}\)) when compared with PLA/PLA (3.6 pg.ml\(^{-1}\)). CHO ingestion before and during exercise is reported to attenuate the increases in plasma IL-6 concentration following prolonged exercise (Nehlsen-Canarella et al., 1997; Starkie et al., 2001; Nieman et al., 2005) and
it is suggested that IL-6 may be involved in the regulation of glucose homeostasis by affecting hepatic glucose production to prevent hypoglycaemia (Gleeson, 2000; Febbraio and Pedersen, 2002). In the present study, there were no significant differences between CAF/CHO, PLA/CHO or PLA/PLA for carbohydrate oxidation rate. Furthermore, although blood glucose at post-exercise on PLA/PLA (~4.8 mmol.L⁻¹) was significantly lower than CAF/CHO and PLA/CHO, it was not significantly lower than rest and could not be considered as hypoglycaemic. It is well known that low muscle glycogen levels are associated with elevated IL-6 responses (Steensberg et al., 2001) and that carbohydrate ingestion does not attenuate the rate of muscle glycogenolysis during cycling (Coyle et al., 1986; Jeukendrup et al., 1999; Febbraio et al., 2003). This coupled with the relatively small magnitude of the response may explain why CHO did not affect the IL-6 response following exercise compared to placebo.

In summary, there was no additional benefit of co-ingesting caffeine and CHO compared with ingesting either supplement alone when the f-MLP-stimulated oxidative burst was expressed per neutrophil cell. When post-exercise f-MLP-stimulated neutrophil oxidative burst responses were expressed as a % of baseline response, only CHO ingestion alone (PLA/CHO) was associated with a maintenance of neutrophil function; ingestion of caffeine alone (CAF/PLA), co-ingestion of caffeine with CHO (CAF/CHO), or ingestion of placebo alone (PLA/PLA) all resulted in significant post-exercise declines in the f-MLP-stimulated neutrophil oxidative burst response. Caffeine and CHO (CAF/CHO) significantly attenuated the adrenaline and IL-6 responses that occurred following the ingestion of caffeine alone (CAF/PLA) and significantly attenuated the transient alterations in circulating total
leukocyte and neutrophil count. This may suggest that the effects of CHO on neutrophil oxidative burst responses are of greater magnitude than the previously reported effects of caffeine. There was however no detrimental effect of consuming caffeine (6 mg.kg\(^{-1}\) body mass) on leukocyte trafficking and f-MLP-stimulated neutrophil oxidative burst responses to exercise.
Chapter 10

General Discussion
10.1. Background

Athletes commonly consume caffeine as an ergogenic aid (Tarnopolsky, 1994). The effect of caffeine ingestion on the immune response in humans, and in particular following exercise, has received little scientific investigation. Caffeine is no longer a WADA prohibited substance, therefore its use is expected to increase considerably amongst athletes during periods of training and competition. The aim of the work described in this thesis was to investigate the influence of caffeine ingestion on immunoendocrine responses following prolonged and intensive cycle ergometry exercise.

Initially, the effect of caffeine ingestion upon hormonal and immune responses was determined when participants were at rest. Caffeine ingestion was found to have little effect on immune responses at rest despite an elevated plasma adrenaline concentration (Chapter 4). Furthermore, following 90 min cycling at 70% \( \dot{V}O_2 \) max, caffeine ingestion was also associated with elevated plasma adrenaline concentration compared with placebo, yet had little effect on neutrophil oxidative burst responses to PMA (Chapter 5). However, when neutrophils were stimulated with f-MLP, caffeine attenuated the post-exercise decline in the neutrophil oxidative burst response (Chapter 6). As caffeine is consumed as an ergogenic aid, its effect on immunoendocrine responses was investigated following a pre-loaded TT performance test (Chapter 7). In contrast to the response observed following submaximal intensity exercise of a fixed duration, there were no significant differences observed for f-MLP-stimulated neutrophil oxidative burst responses following exercise. At post-TT, plasma adrenaline and IL-6 concentrations were both significantly elevated following caffeine ingestion (Chapter 7). An \textit{in vitro} study confirmed that caffeine antagonises
the suppressive effects of adenosine on f-MLP-stimulated neutrophil oxidative burst responses, but was not able to clarify the contributing mechanisms by which caffeine attenuated the post-exercise decrease in f-MLP-stimulated neutrophil oxidative burst responses in the presence of elevated adrenaline concentrations (Chapter 8). Caffeine is commonly consumed with CHO in commercially available soft drinks and energy drinks. The co-ingestion of caffeine with CHO significantly attenuated the adrenaline and IL-6 responses that occurred following the ingestion of caffeine alone and significantly attenuated the transient alterations in circulating total leukocyte and neutrophil count, but there was no benefit of co-ingesting caffeine with CHO on the f-MLP-stimulated neutrophil oxidative burst response compared with either supplement alone (Chapter 9).

10.2. Neutrophil function

There was no effect of caffeine ingestion (6 mg.kg⁻¹ body mass) on neutrophil functional responses, assessed by the oxidative burst response to PMA and f-MLP-stimulation and by the degranulation response following bacterial-stimulation when participants were at rest (Chapter 4). Caffeine ingestion also did not affect bacterially-stimulated neutrophil elastase release or PMA-stimulated oxidative burst responses following 90 min cycling at 70% VO₂ max (Chapter 5). The effects of cAMP elevating agonists (such as adenosine and adrenaline) on neutrophil degranulation responses have produced equivocal findings. At μmol.L⁻¹ concentrations, both adenosine (Bouma et al., 1997) and adrenaline (Tintinger et al., 2000) are reported to decrease neutrophil degranulation responses following bacterial-stimulation, however, it is likely that in Chapter 4 and 5, the relatively small increases in adrenaline and adenosine concentration (at nmol.L⁻¹ concentrations) are not of sufficient magnitude
to affect the response. PMA directly activates PKC in blood neutrophils in a receptor-independent mechanism, producing a prolonged and maximum response (Lopez et al., 1995; Hazan et al., 1997). The PMA-stimulated neutrophil oxidative burst response has previously been reported to be insensitive to cAMP elevating agents, therefore, in hindsight PMA may not be a suitable stimulant for investigating the influence of caffeine ingestion on neutrophil oxidative burst responses (Yu et al., 1995; Chilcoat et al., 2002). In rats, however, 5 nmol.L⁻¹ adrenaline significantly reduced the PMA-stimulated neutrophil oxidative burst response (Garcia et al., 1999). The plasma adrenaline concentration at post-exercise in Chapter 5 was 1.8 nmol.L⁻¹ following caffeine ingestion in which no effect on PMA-stimulated neutrophil oxidative burst was observed.

Caffeine ingestion attenuated the exercise-induced decline in f-MLP-stimulated neutrophil oxidative burst responses when ingested 1 h prior to cycling for 90 min at 70% VO₂ max (Chapter 6). However, following a pre-loaded TT, no attenuating effect of caffeine on the f-MLP-stimulated neutrophil oxidative burst response was observed (Chapter 7). It is suggested that caffeine antagonises A₂A adenosine receptors with a Ki of 40 - 44 μmol.L⁻¹ (Daly, 1993). In Chapters 6 and 9, post-exercise serum caffeine concentration was 46 μmol.L⁻¹ and 45 μmol.L⁻¹, respectively and f-MLP-stimulated oxidative burst responses were 62% of the baseline condition compared to 42% on PLA (Chapter 6) and 70% compared with 50% in Chapter 9. Similarly at post-exercise in Chapter 7, serum caffeine concentration was 42 μmol.L⁻¹ and f-MLP-stimulated oxidative burst response was only 9% lower than resting values compared with a 26% decrease on PLA trial. At post-TT, however, serum caffeine concentration was 37 μmol.L⁻¹ and f-MLP stimulated neutrophil oxidative burst
response was similar between the 2 experimental conditions. The findings of Chapters 5, 6, 7 and 8 suggest that the effect of caffeine on the f-MLP-stimulated neutrophil oxidative burst response is multi-factorial, but is predominantly the net result of stimulatory (via A2A adenosine receptor antagonism) and inhibitory (via adrenaline-mediated inhibition) factors.

In Chapter 6, caffeine ingestion attenuated the exercise-induced decline in the f-MLP-stimulated neutrophil oxidative burst response in the presence of a significantly elevated plasma adrenaline concentration (2.8 nmol.L\(^{-1}\) and 1.3 nmol.L\(^{-1}\) on CAF and PLA, respectively). In this situation, it is likely that caffeine inhibits the occupancy/activation of A2A receptors and dominates the response. Adenosine is an indicator of metabolic distress (Klinger et al., 2002). It is well known that adenosine is produced during exercise by the increased dephosphorylation of ATP. Although adenosine is rapidly cleared by erythrocytes (Heptinstall et al., 2005), plasma adenosine concentration is reported to increase significantly following exercise (Vizi et al., 2002; Chouker et al., 2005). In vitro, adenosine (100 nmol.L\(^{-1}\)) reduced the f-MLP-stimulated oxidative burst of isolated neutrophils by 40% from a control condition (Chapter 8). This is in support of previous findings (Cronstein et al., 1985; Swain et al., 2003; Chouker et al., 2005) and was attributed to the A2A adenosine receptor subtype expressed on neutrophils (Gessi et al., 2000). Following the addition of caffeine (50 μmol.L\(^{-1}\)) to the neutrophil suspension before incubation with adenosine, the response was only 9% lower than control, which did not represent a statistically significant decrease (Chapter 8). As caffeine did not affect the f-MLP-stimulated neutrophil oxidative burst response at rest when adenosine concentration is expected to be low (Chapter 4), yet attenuated the response at post-exercise (Chapter
6) and following incubation with 100 nmol.L\textsuperscript{-1} adenosine (Chapter 8), this supports the notion that adenosine is necessary for the actions of caffeine to occur (Sullivan et al., 1995). A limitation of this work, however, is that adenosine concentration was not measured in the series of studies presented in this thesis.

Incubation of human neutrophils with 5 nmol.L\textsuperscript{-1} adrenaline is reported to significantly inhibit the f-MLP-stimulated neutrophil oxidative burst response (O'Dowd et al., 2004). Following a maximal effort TT performed after 90 min prolonged cycling (Chapter 7), plasma adrenaline concentration was 5.2 nmol.L\textsuperscript{-1} and 3.7 nmol.L\textsuperscript{-1} on CAF and PLA conditions, respectively. At this time-point, there was no attenuating effect of caffeine on the f-MLP-stimulated neutrophil oxidative burst response as observed in Chapter 6. It may be speculated that there is a threshold, above which adrenaline exerts a greater negative influence on neutrophil oxidative burst responses. An attempt was made to determine the relative influence of physiological concentrations of adrenaline, observed at post-exercise in Chapters 5 and 6 (~2 nmol.L\textsuperscript{-1}) and at post-TT in Chapter 7 (5 nmol.L\textsuperscript{-1}) on the f-MLP-stimulated oxidative burst of isolated neutrophils in the presence of physiological concentrations of both caffeine and adenosine. The findings of Chapter 8 do not support a threshold theory, since the f-MLP-stimulated oxidative burst response of isolated neutrophils was similar following incubation with both 2 nmol.L\textsuperscript{-1} and 5 nmol.L\textsuperscript{-1} adrenaline. This study was therefore not able to clarify the contributing mechanisms by which caffeine attenuated the post-exercise decline in the f-MLP-stimulated neutrophil oxidative burst response in the presence of an elevated adrenaline concentration, as reported in Chapter 6.
10.3. Circulating leukocyte counts

There were no significant effects of caffeine ingestion on total blood leukocyte count or circulating neutrophil count at rest (Chapter 4) or following a number of different exercise protocols (Chapters 5-9). Adrenaline has been reported to dose-dependently inhibit neutrophil adherence to endothelial cell monolayers (Bazzoni et al., 1991). Furthermore, neutrophil count has been reported to increase and neutrophil adherence to vascular endothelium is reported to decrease following adrenaline infusion (Boxer et al., 1980). As caffeine ingestion was consistently associated with a significantly elevated adrenaline concentration (Chapters 4-9), it would appear that this is not a major mechanism for neutrophil mobilisation following exercise. Caffeine ingestion did not significantly affect plasma noradrenaline concentration. This may therefore support the findings of Benschop et al. (1996) that α-adrenoreceptors are predominantly involved in the effects of catecholamines on granulocyte trafficking.

Caffeine ingestion was however, consistently associated with an increase in circulating lymphocyte count at pre-exercise (i.e. 1 h following caffeine ingestion). This was observed following caffeine ingestion in Chapters 5, 6 and 7. The circulating lymphocyte count was elevated following caffeine ingestion in Chapter 4, but this did not reach statistical significance. These findings are in accordance with Bishop et al. (2005) who reported similar findings using the same dose and timing of caffeine administration as that employed in the studies of the present thesis. An increased circulating lymphocyte count is most likely due to the elevated plasma adrenaline concentrations at the corresponding time-points altering endothelial adhesion via activation of β-adrenoreceptors present on lymphocytes. A greater density of β-adrenoreceptors are present on lymphocytes than neutrophils (Mackinnon, 1999). In
contrast, however, there was a significantly elevated circulating lymphocyte count at post-TT on PLA compared with the CAF trial. As discussed in Chapter 7, the reasons for this are unclear.

10.4. Stress hormones and cytokines

Caffeine ingestion consistently increased plasma adrenaline concentration at pre-exercise (i.e. 1 h following caffeine ingestion) by approximately 2.5 to 3-fold (Chapters 4, 5 and 6). Plasma adrenaline concentration was also significantly higher following caffeine ingestion at post-exercise following 90 min cycling at 70% \( \dot{\text{VO}}_{2}\text{max} \) (Chapter 5, 6 and 7), a maximal effort TT (Chapter 7) and 2 h cycling at 65% \( \dot{\text{VO}}_{2}\text{max} \) (Chapter 9). This is in accordance with the considerable research demonstrating an increased plasma adrenaline concentration at both rest and following exercise with caffeine ingestion (Graham and Spriet, 1991; Graham et al., 1991, 2000; Spriet et al., 1992; Graham and Spriet, 1995; Jackman et al., 1996; Bishop et al., 2005 amongst numerous others). In line with the majority of this research evidence, caffeine ingestion did not affect plasma noradrenaline concentration at either rest (Chapter 4) or following prolonged and intensive submaximal exercise (Chapter 5 and 6). Collectively this suggests that caffeine acts on the sympathetic nervous control of the adrenal medulla rather than increasing the activity of the sympathetic nervous system in general (Graham and Spriet, 1991). However it is reported that circulating noradrenaline results from the release or ‘washout’ of tissues, therefore tissue exchange measurements must be directly measured to determine whether or not the sympathetic nervous system is enhanced (Graham, 2001a).
Caffeine ingestion did not significantly increase plasma cortisol concentration at rest (Chapter 4), following prolonged and intensive submaximal cycling (Chapter 5, 7 and 9) or following a maximal intensity effort TT (Chapter 7). In Chapter 6, there was a significant main effect of trial for plasma cortisol concentration, with significantly higher values on CAF than PLA. This is the only study of the present thesis that demonstrated such an effect and is in support of the work of Bishop et al. (2005) who also reported a main effect of trial following the same caffeine dose and exercise protocol. Furthermore, Laurent et al. (2000) reported a significantly higher plasma cortisol concentration following 2 h cycling at 65% V\text{O}_2\text{max}. The reason for a discrepancy between Chapter 6 and the other studies presented in this thesis is unclear, however the mean plasma cortisol concentration was 30 nmol.L\textsuperscript{-1} higher on CAF than PLA when participants were in a rested state, prior to caffeine administration. From the studies of the present thesis, it is concluded that caffeine ingestion does not significantly increase plasma cortisol concentration compared to PLA condition.

Caffeine ingestion significantly increased plasma IL-6 concentration following 90 min cycling at 70% V\text{O}_2\text{max} (Chapter 6) and following a pre-loaded cycling TT (Chapter 7). Plasma IL-6 concentration also tended to be higher on CAF/PLA than PLA/PLA (P = 0.062) following 2 h cycle at 65% V\text{O}_2\text{max} (Chapter 9). Plasma IL-6 concentration increased by approximately 4-fold at post-exercise on CAF compared with a 2.5-fold increase on PLA (Chapter 6) and by 22-fold and 17-fold at post-TT on CAF and PLA, respectively in Chapter 7. As plasma adrenaline concentration was significantly higher on CAF than PLA at both of these time-points, it may be speculated that adrenaline lead to an increased IL-6 production. Increases in plasma
adrenaline concentration have previously been linked to an increased IL-6 release into circulation in both rats (Yu et al., 2001; Frost et al., 2004) and humans (Papanicolaou et al., 1996). Furthermore, an increase in cAMP production from β-2 adrenoreceptor stimulation has been shown to enhance IL-6 production (Dendorfer et al., 1994). The ingestion of caffeine did not increase plasma IL-6 concentration at pre-exercise (1 h following ingestion). This suggests that the magnitude of the adrenaline response is not sufficient to affect IL-6 production when participants are at rest. During exercise however, adrenaline may affect IL-6 clearance from the circulation in humans. It is known that hepatosplanchnic viscera remove IL-6 from the circulation (Febbraio et al., 2003) thus adrenaline-mediated vasoconstriction of hepatosplanchnic viscera may augment the level of IL-6 through a decreased clearance. Furthermore, a vasoconstriction due to adenosine-receptor antagonism by caffeine may also contribute to reduced clearance of IL-6 from the systemic circulation.

IL-6 has previously been reported to mediate the delayed-onset neutrophilia following exercise (Suzuki et al., 1996b; Peake, 2002). An increased plasma IL-6 concentration at post-exercise (Chapter 6) or post-TT (Chapter 7) following caffeine ingestion was not associated with a significant neutrophilia or leukocytosis after a 1 h recovery period. Furthermore, IL-6 is also reported to be immunopotentiating (Smith, 1997) and has been associated with enhanced neutrophil CL responses and G protein activity (Suzuki et al., 1999; Peake, 2002). It may be speculated therefore, that the attenuated decline of the f-MLP-stimulated neutrophil oxidative burst response at post-exercise in Chapter 6 following caffeine ingestion was associated with an increased plasma IL-6 concentration at that time-point, effectively priming the neutrophils for an enhanced oxidative burst response. In contrast however, plasma IL-6 concentration was
significantly higher on CAF at post-TT (Chapter 7) and post-exercise (Chapter 9) without an enhancement of the f-MLP-stimulated neutrophil oxidative burst response. IL-6 is reported to produce a number of anti-inflammatory effects, one of these being to stimulate the appearance of IL-1ra into circulation (Steensberg et al., 2003). There were no significant effects of caffeine ingestion on plasma IL-1ra concentration following exercise (Chapter 7). Plasma IL-1ra concentration increased by 12-fold on CAF compared with a 7-fold increase on PLA at 1 h post-TT, however a large inter-individual variation of the IL-1ra response may have prevented statistical significance from being achieved.

10.5. Blood-borne metabolites

Serum caffeine concentration was < 0.1 μmol.L⁻¹ in all studies of the present thesis when participants initially arrived at the laboratory. This indicates a full compliance and adherence to the caffeine abstinence procedure and suggests that a 60 h caffeine withdrawal was a sufficient time-period for clearance of previously ingested caffeine. Serum caffeine concentration reached a peak at 1 h following ingestion, with decreases at post-exercise and 1 h post-exercise. This is in support of the notion that caffeine absorption from the gastro-intestinal tract is rapid, with a half life of between 2.5 and 4.5 h (Debry, 1994). Furthermore, the rate of caffeine clearance did not appear to be affected by exercise since similar reductions were observed at corresponding time-points at rest (Chapter 4) and following exercise (Chapter 6).

Caffeine ingestion significantly increased plasma FFA concentration at rest (Chapter 4), at pre-exercise and 1 h post-exercise (Chapter 5) and with a main effect of trial in Chapter 6. In contrast, there was no effect observed in the studies presented in
Chapters 7 and 9. Caffeine ingestion has previously been reported to stimulate lipolysis *in vitro* and *in vivo* (Hetzler et al., 1990). It is likely that caffeine ingestion increases FFA concentration via the antagonism of A1 receptors of adipocytes that stimulates lipolysis, or via increased sympathetic activity, resulting in β-adrenergic lipolytic stimulation (Graham, 2001a; Acheson et al., 2004). An increased FFA concentration, however, did not affect the net uptake by the exercising leg during exercise following caffeine ingestion (Graham et al., 2000). There were no significant effects of caffeine ingestion on plasma glucose concentration at rest (Chapter 4) or following exercise (Chapter 5, 6, 7, 9). This finding has frequently been reported (Tarnopolsky et al., 1989; Graham et al., 1991; Cole et al., 1996; Mohr et al., 1996; Graham et al., 1998; Van Soeren et al., 1998); however, a few studies have reported an increased plasma glucose concentration during and following exercise with caffeine ingestion (Spriet et al., 1992; Graham and Spriet, 1995; Graham et al., 2000).

Plasma lactate concentration was significantly higher at pre and post-90 min exercise (Chapter 5), post 2 h exercise (Chapter 9) and tended to be higher at post-TT (Chapter 7) following caffeine ingestion. In contrast, no effect of caffeine on plasma lactate concentration was observed at post-90 min exercise in Chapters 6 and 7. An increased blood lactate concentration has frequently been reported with caffeine ingestion and appears paradoxical, considering that glycogen sparing is proposed as a mechanism for caffeine’s ergogenic effects (Graham, 2001a). An increase in blood lactate concentration may be the result of increased muscle glycogenolysis, without an increase in CHO oxidation (Raguso et al., 1996). However, Graham et al. (2000) observed an increased blood lactate concentration, without a difference in the release or clearance of lactate by the exercising muscle. The authors concluded that the
clearance of lactate by the liver or resting muscle must be reduced or the release from other tissues is increased to account for this increase in blood lactate concentration.

10.6. RER and indirect calorimetry

Although plasma FFA concentration was significantly elevated on CAF than PLA (Chapters 5 and 6), there were no significant differences for RER between CAF and PLA trials in either of these experimental chapters. Furthermore, mean RER ranged from 0.91 to 0.93 on both CAF and PLA during all submaximal exercise protocols (Chapter 5, 6, 7 and 9). The early proposed mechanism for caffeine as an ergogenic aid was that it increased FFA oxidation and spared muscle glycogen for use later during exercise (Essig et al., 1980; Spriet et al., 1992). A number of studies have reported a decreased RER value during exercise following caffeine ingestion (Costill et al., 1978; Ivy et al., 1979; Essig et al., 1980; Bangsbo et al., 1992; Nishijima et al., 2002; Bishop et al., 2005). The findings of Chapter 5, 6, 7 and 9 of the present thesis showed that caffeine ingestion did not affect CHO or fat oxidation rates during exercise, as determined by indirect calorimetry. These findings are in accordance with a number of studies that failed to show a decreased RER when caffeine is ingested prior to exercise (Tarnopolsky et al., 1989; Graham and Spriet, 1991, 1995; Graham et al., 1991; Van Soeren et al., 1996; Mohr et al., 1998; Graham et al., 2000; Greer et al., 2000 amongst others). Although many of these studies have failed to show a decreased RER during exercise following caffeine ingestion, many authors repeatedly propose glycogen sparing as the mechanism for caffeine's ergogenic effects (Graham, 2001b). To assess this, Graham et al. (2000) obtained muscle biopsies and quantified the carbohydrate metabolism of the exercising leg in humans. They observed no difference for glucose uptake and muscle glycogen utilisation following caffeine
ingestion. Similarly Greer et al. (2000) and Laurent et al. (2000) reported no effect of caffeine ingestion on glycogen sparing supporting the theory that other actions of caffeine are responsible for its ergogenic effects.

10.7. RPE and TT performance

RPE was significantly lower following caffeine ingestion during 90 min cycling at 70% \( \text{VO}_2 \text{max} \) (Chapters 5 and 6) but there were no significant differences between CAF and PLA during 2 h cycling at 65% \( \text{VO}_2 \text{max} \) (Chapter 9). Furthermore, mean power output during a pre-loaded TT was improved by 11 W following caffeine ingestion, corresponding to a 4% improvement in TT performance (Chapter 7). A dampening of the perceptual response for a given work rate following caffeine ingestion has been previously reported (Costill et al., 1978; Doherty et al., 2002; 2004). It is likely that caffeine crosses the blood-brain barrier and antagonises adenosine-receptor subpopulations within the brain (Davis et al., 2003). An increased adenosine concentration is known to inhibit the release of brain neurotransmitters such as dopamine and a decreased dopamine concentration is linked to the development of central fatigue during exercise (Davis and Bailey, 1997). Furthermore, caffeine ingestion is associated with an increased \( \beta \)-endorphin concentration that is reported to increase feelings of euphoria and decrease perceptions of pain (Laurent et al., 2000). Leg hypoalgesia (decreased sensitivity to a painful stimulus) has been previously reported during cycling exercise following caffeine ingestion (O’Connor et al., 2004). Positive self-rated mood states such as happiness and alertness during exercise are also associated with caffeine ingestion (Backhouse et al., 2004). The capability of caffeine to influence pain perception and
psychological state may be an important factor in the exercise performance of athletes.

10.8. Conclusions

The major conclusions from this thesis are:

1) Caffeine ingestion (6 mg.kg$^{-1}$ body mass) did not affect PMA or f-MLP-stimulated neutrophil oxidative burst or bacterially-stimulated degranulation responses when participants were at rest.

2) Caffeine ingestion attenuated the post-exercise decline in neutrophil oxidative burst response when stimulated by f-MLP but not PMA. An in vitro study confirmed that this is most likely the result of adenosine-receptor antagonism by caffeine. Following a TT however, there was no attenuating effect of caffeine on f-MLP-stimulated neutrophil oxidative burst responses. There was also no benefit of co-ingesting caffeine with CHO on the oxidative burst response compared with either supplement alone.

3) None of the experimental studies indicated that caffeine ingestion was more detrimental than placebo on the f-MLP-stimulated neutrophil oxidative burst response.

4) Caffeine ingestion had no effect on total circulating leukocyte or neutrophil count at rest or following exercise, but generally increased circulating lymphocyte count following ingestion.
5) Caffeine ingestion was consistently associated with an increased plasma adrenaline concentration but not noradrenaline concentration at rest and following exercise. Caffeine ingestion had no effect on plasma cortisol concentration but was associated with increased plasma IL-6 concentration following exercise.

6) Caffeine ingestion increased plasma FFA concentration but had no effect on plasma glucose concentration.

7) Despite a higher plasma FFA concentration, there was no effect of caffeine ingestion on RER or fat oxidation rate as determined via indirect calorimetry during submaximal exercise.

8) Caffeine ingestion was occasionally associated with a reduced RPE during submaximal exercise and an improved pre-loaded TT performance compared to placebo.
References


References


References


References


HEALTH SCREEN FOR STUDY VOLUNTEERS

Name or Number

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. At present, do you have any health problem for which you are:
   (a) on medication, prescribed or otherwise.............................. Yes [ ] No [ ]
   (b) attending your general practitioner................................. Yes [ ] No [ ]
   (c) on a hospital waiting list.................................................... Yes [ ] No [ ]

2. In the past two years, have you had any illness which require you to:
   (a) consult your GP .............................................................. Yes [ ] No [ ]
   (b) attend a hospital outpatient department............................ Yes [ ] No [ ]
   (c) be admitted to hospital..................................................... Yes [ ] No [ ]

3. Have you ever had any of the following:
   (a) Convulsions/epilepsy.......................................................... Yes [ ] No [ ]
   (b) Asthma.............................................................................. Yes [ ] No [ ]
   (c) Eczema............................................................................. Yes [ ] No [ ]
   (d) Diabetes ........................................................................... Yes [ ] No [ ]
   (e) A blood disorder.............................................................. Yes [ ] No [ ]
   (f) Head injury ...................................................................... Yes [ ] No [ ]
   (g) Digestive problems........................................................... Yes [ ] No [ ]
   (h) Heart problems ............................................................... Yes [ ] No [ ]
   (i) Problems with bones or joints ......................................... Yes [ ] No [ ]
   (j) Disturbance of balance/co-ordination................................. Yes [ ] No [ ]
   (k) Numbness in hands or feet ................................................. Yes [ ] No [ ]
   (l) Disturbance of vision........................................................ Yes [ ] No [ ]
   (m) Ear / hearing problems.................................................... Yes [ ] No [ ]
   (n) Thyroid problems ............................................................ Yes [ ] No [ ]
   (o) Kidney or liver problems.................................................. Yes [ ] No [ ]
   (p) Allergy to nuts .................................................................. Yes [ ] No [ ]

4. Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ........ Yes [ ] No [ ]

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.) ........................................................................................................

5. Have you had a cold or feverish illness in the past month? Yes [ ] No [ ]

6. Are you accustomed to vigorous exercise (1-3 hours per week)? Yes [ ] No [ ]
Physical Activity Questionnaire

The following questions are designed to give us an indication of your current level of physical activity.

Name:_________________________ Date:_____/_____/______

Are you currently ENDURANCE TRAINING? YES [ ] NO [ ]

If Yes, how many days each week do you usually train? ____________

How many minutes does each session last? ____________

What is your weekly mileage? ____________

Are you involved in any of the following training programmes?

Weight training [ ] Interval training [ ] Skills training [ ]

If Yes, how many days each week do you usually train?

__________________________

How many minutes does each session last?

__________________________
CAFFEINE CONSUMPTION QUESTIONNAIRE

Please complete the questionnaire concerning your caffeine usage. List the number of times you consume the following substances during a typical day.

<table>
<thead>
<tr>
<th></th>
<th>Morning</th>
<th>Afternoon</th>
<th>Evening</th>
<th>Night</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COFFEE (Regular 5 oz. Mugs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brewed</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Percolated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Espresso (2 oz. Serving)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Instant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decaffeinated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TEA (Regular 5 oz mugs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot Chocolate (Regular 5 oz mugs)</td>
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<tr>
<td>Chocolate (Regular Bar)</td>
<td></td>
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<td></td>
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<tr>
<td>Chocolate Biscuits / Cakes / Cereals</td>
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<td></td>
<td></td>
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<tr>
<td>Chocolate / Coffee Dairy Products</td>
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<tr>
<td><strong>SOFT DRINKS (330ml Can Size)</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Coke</td>
<td></td>
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<tr>
<td>Dr Pepper</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Energy Drinks (Please state which ones)</td>
<td></td>
<td></td>
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<tr>
<td><strong>OVER THE COUNTER DRUGS</strong></td>
<td></td>
<td></td>
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<tr>
<td>Pain Killers e.g. Anadin (Please state which ones)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro Plus</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Do you take caffeine prior to exercise? _______________
If Yes, How much? __________________________________
   What do you take? __________________________________
   Why? ____________________________________________
Appendix D

Health Questionnaire

Please complete the following brief questions to confirm your fitness to participate:

At present do you have any health problems for which you are:

1) On medication, prescribed or otherwise  YES [ ] NO [ ]

2) Attending your general practitioner  YES [ ] NO [ ]

Have you any symptoms of ill health, such as those associated with a cold or other common infection?  YES [ ] NO [ ]

If you have answered yes to any of the above questions please give more details below:

_________________________________________________________________
_________________________________________________________________
_________________________________________________________________

Do you want to take part in today’s experiments?  YES [ ] NO [ ]

Signature:_________________________  Date:__________________
NAME:

As you are aware, the main of this study is to investigate the effects of caffeine ingestion on the immune response to prolonged cycling. It is therefore vital that you \textbf{DO NOT} exercise for 24 hours, or consume \textbf{ANY} caffeine for 60 hours prior to each main test.

Please do ensure that you \textbf{DO NOT EAT ANYTHING} AFTER MIDNIGHT on the night prior to each main trial, and only consume water during this period.

Caffeine is found in many foods, and below is a list of some products that you should avoid. \textbf{We will be able to detect if any caffeine has been ingested during the 60 hours prior to each test, and if found, the test will be invalid.}

\textbf{DRINKS}

- Tea
- Coffee
- Coke / Pepsi / Dr Pepper
- Hot Chocolate
- Energy Drinks e.g. Red Bull, Red Card, Solstis
- NB Alcoholic energy drinks also contain caffeine!

\textbf{CHOCOLATE AND COFFEE PRODUCTS}

- Biscuits / cakes
- Chocolate / coffee bars
- Chocolate / coffee ice cream
- Chocolate / coffee yoghurts
- Chocolate / coffee mousse
- Chocolate / coffee spreads

\textbf{PHARMACEUTICAL PRODUCTS}

- Pain Killers e.g. Anadin
- Cough / cold remedies e.g. Lemsip, Day Nurse
- Hang Over Remedies e.g. Alka Seltza XS!
- Pro Plus

\textbf{NB} These are rough guidelines only. If you are unsure of the contents of any products please err on the side of caution!

After each exercise bout, you will have a period of 1 hour in which you will be able to shower, etc, prior to the final sample.

\textbf{MANY THANKS FOR YOUR TIME AND COOPERATION.}
Appendix F

Preliminary results for in vitro protocol (Chapter 8)

Chemiluminescence (RLU) vs. Time (s)

- Control
- Aden
- Aden + Caff
- Aden/Adren 5nM/Caff
- Aden/Adren 2nM/Caff
- Adren 5 nM
- Adren 2 nM