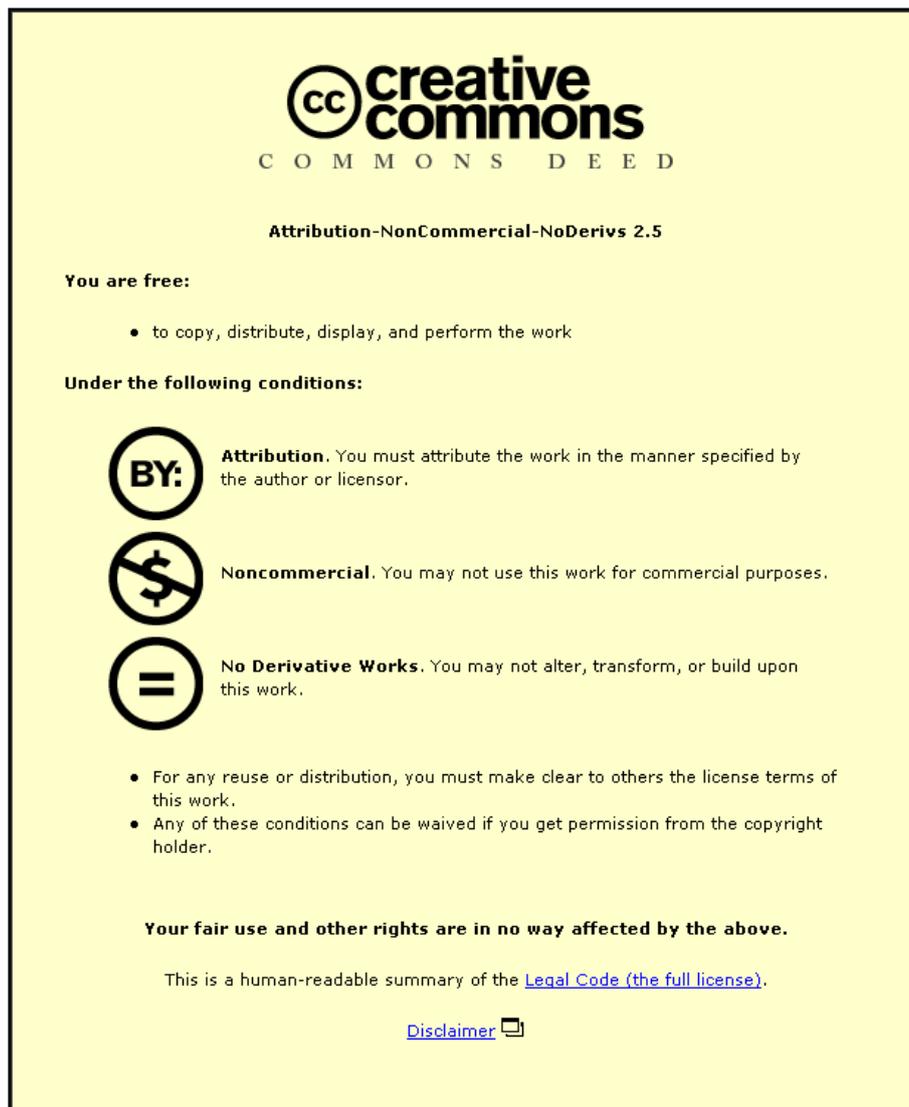


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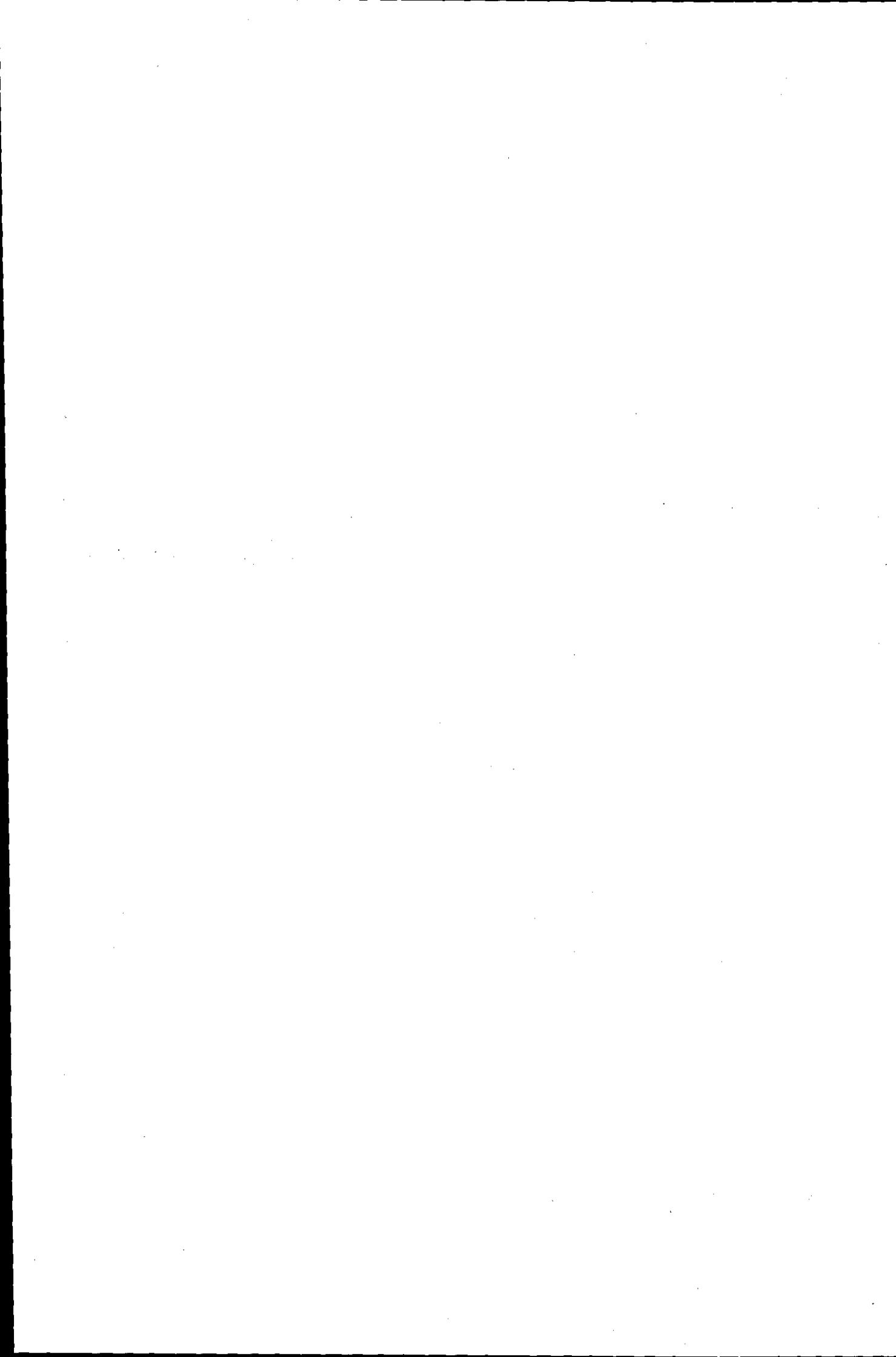
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**FACTORS INFLUENCING THE MUCOSAL IMMUNE
RESPONSE TO EXERCISE**

by

Judith Allgrove

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of
Loughborough University

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General Abstract

Despite the abundance of research conducted into the effects of exercise on mucosal immunity the results remain controversial. Much of the inconsistencies arise from the exercise protocols, the participants studied and their nutritional status, as well as methodological and analytical differences. The purpose of this thesis was to examine the influence of some of these factors, and to investigate potential means of enhancing the mucosal immune response to exercise. In study 1 (Chapter 3) it was shown that a fed or fasted state 2 h prior to exercise had no effect on the s-IgA concentration or secretion rate during prolonged exercise. However, when participants were fed during exercise (Chapter 4), the secretion rate of salivary antimicrobial proteins lysozyme and α -amylase increased, but s-IgA remained unchanged. These changes were likely due to the activation of mechanical and gustatory receptors leading to a reflex stimulation of protein secretion via the autonomic nerves, rather than changes in stress hormones, since cortisol did not change significantly during exercise. Study 3 (Chapter 5) extended these findings where it was demonstrated that chewing flavoured gum during exercise enhanced lysozyme and α -amylase secretion but resulted in a small reduction in s-IgA secretion rate. Salivary antimicrobial proteins are affected by the exercise intensity since both s-IgA and lysozyme secretion rate increased post-exercise following an incremental test to exhaustion, but not after exercise at 50% $\dot{V}O_{2max}$. Moreover, lysozyme secretion rate was also elevated following exercise at 75% $\dot{V}O_{2max}$, whereas s-IgA remained unchanged. These effects are thought to be mediated by increased sympathetic nervous system activity reflected by the concomitant increases in α -amylase and chromogranin A, rather than the hypothalamic-pituitary-adrenal axis. Resting mucosal immunity exhibits significant gender differences. In study 1 (Chapter 3) s-IgA concentration, secretion rate and osmolality were found to be lower in females than in males at rest. In addition, saliva flow rate was found to be lower in females compared with males in

study 5 (Chapter 7). However, these differences did not appear to influence the salivary responses to acute exercise or exercise training. Chronic exercise training in elite male and female swimmers resulted in lower levels of s-IgA secretion rate following periods of intense training prior to competition compared with post-competition (Chapter 7), but these levels were not directly associated with reported episodes of respiratory illness.

Key words: saliva flow rate, s-IgA, exercise, lysozyme, α -amylase, nutrition, gender, URTI

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Publications

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Conference contributions

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List of Abbreviations

ANOVA	Analysis of variance
CHO	Carbohydrate
CgA	Chromogranin A
CO ₂	Carbon dioxide
ELISA	Enzyme linked immunosorbant assay
g	Grams
h	Hours
HPA	Hypothalamic-pituitary-adrenal
HR	Heart rate
IgA	Immunoglobulin A
J	Joules
kg	Kilogram
km	Kilometre
L	Litre
m	Mili
M	Moles per litre
min	Minutes
MJ	Mega joules
n	Nano
O ₂	Oxygen
pIgR	Polymeric Ig receptor
RPE	Rating of perceived exertion
S	Seconds
SC	Secretory component

SEM	Standard error of the mean
s-IgA	Salivary immunoglobulin A
SNS	Sympathetic nervous system
URTI	Upper respiratory tract infection
$\dot{V}CO_2$	Rate of carbon dioxide production
\dot{V}_E	Rate of ventilation
$\dot{V}O_2$	Rate of oxygen uptake
$\dot{V}O_{2max}$	Maximal oxygen uptake
°C	Degrees celcius
μ	Micro

Chapter 1 - Literature Review

1.1. Exercise and infection risk

Upper respiratory tract infections (URTI) such as coughs, colds and sore throats, form the most common presentation to general medical practice (Graham, 1990). Such illness can have a substantial negative impact on the overall health and productivity of the general population, and can be even more serious for the elite athlete engaged in heavy training and competition. Any interruption of a tightly planned training schedule due to URTI could have serious adverse effects on performance, which may even mean the difference between success and failure (Pyne *et al.*, 2001).

The relationship between exercise and URTI has been modelled into the form of a “J”-shaped curve. According to this hypothesis, regular moderate exercise enhances immune responses, reducing susceptibility to URTI. In contrast, excessive prolonged exercise suppresses immunity initiating a brief period of vulnerability when the risk of URTI is increased (Nieman, 1994). Indeed, experimental studies conducted in animals have supported the concept that exhaustive exercise after inoculation with a pathogen leads to a more frequent appearance of infection and a higher fatality rate compared with sedentary animals, whereas more moderate training decreases the incidence (Davis *et al.*, 1997).

There are an increasing number of human research studies that have examined the relationship between exercise and infection risk. The evidence is primarily epidemiological in nature where the diagnosis of URTI is based upon responses to a questionnaire (defined from symptoms of sore throat, cough, runny nose, congested sinuses and headache), rather than clinical assessment (Barrett *et al.*, 2002). In general, the evidence suggests that moderate

habitual exercise is associated with decreased URTI incidence. For example, in one randomised, controlled study it was demonstrated that moderate exercise training in previously sedentary women, was associated with a significant reduction in URTI incidence. Forty-five min of exercise five times a week at 60% of the heart rate reserve reduced the number of URTI symptom days (Nieman *et al.*, 1990b). Furthermore, Matthews *et al.* (2002) found that habitual physical activity was inversely related to the incidence of colds over a 12 month period.

In contrast, there is a growing body of evidence to suggest that heavy exercise and/or training increases the prevalence of URTI. The first study to quantify this in an athletic population was published by Peters and Bateman (1983) in which 140 runners were surveyed for symptoms of URTI before and after a 56 km ultramarathon. These were compared to age-matched controls, living in the same household and sharing the same environmental conditions. During the two weeks following the race, 33% of the athletes exhibited symptoms of URTI compared with only 15% of the control subjects. These findings are consistent with a subsequent epidemiological study of over 2000 randomly selected runners competing in the 1987 Los Angeles Marathon (Nieman *et al.*, 1990a). When compared to their matched runners - who did not compete - it was found that the competitors were six times more likely to suffer from symptoms of URTI. Moreover, those runners who had the highest training load prior to the event (i.e. more than 96 km per week) were twice as likely to suffer from URTI as those with a relatively light training load (i.e. 32 km per week).

However, a more recent study failed to confirm this in a group of marathon runners, where no association was found between training volume over a 6-month period prior to a race and the post-race incidence of self-reported URTI episodes. Furthermore, there was no difference in URTI incidence during the 3 weeks post-race compared with before (Ekblom *et al.*, 2006). It

was noted though that those with infections in the 3 weeks prior to the race had a higher reported incidence of URTI episodes than the illness free competitors after the race, suggesting a possible reactivation of viral illness.

It is now widely accepted that following an acute bout of prolonged, intense exercise, many components of the immune system exhibit change (reviewed by Gleeson, 2007). This 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). This period may last between 3 and 72 h depending on the immune parameter measured and the exercise protocol employed. Since many competitive athletes train at least daily, and often twice per day, it is possible that a chronic depression of immune function may result from the cumulative long-lasting acute effects of each successive bout (Mackinnon, 2000)

1.2. Role of the immune system

The function of the immune system is to protect against, recognise, attack and destroy elements that are foreign to the body. The immune system can be broadly separated into two divisions that work together synergistically: innate (natural or non-specific) and acquired (adaptive or specific). The innate immune system, the 'first-line of defence' comprises 3 mechanisms with a common goal to restrict the entry of infectious agents into the body: (1) physical/structural barriers (skin, epithelial linings, mucosal secretions); (2) chemical barriers (pH of bodily fluids and soluble factors such as lysozymes and complement proteins); and (3) phagocytic cells (immune cells, e.g. neutrophils and monocytes/macrophages) (Gleeson *et al.*, 2004b). The cells involved in innate immunity can recognise and act against foreign cells without prior exposure. However, innate immunity does not improve with repeated exposure.

Should the innate immune system fail to resist the micro-organism, the acquired immune system is activated. The acquired immune system consists of B-lymphocytes (produced in the bone marrow) and T-lymphocytes (mature in the thymus). Monocytes or macrophages ingest and process the invading protein, which is displayed or presented to the T-cells allowing them to initiate a response specific to that antigen. The T-helper cells produce cytokines that stimulate the proliferation of T-cells, and B-cells into plasma cells. The plasma cells then produce antibodies that are able to recognise the antigen and generate memory cells to enable the immune system to initiate a faster and more effective level of protection with subsequent exposure to the same agent. The T-cells also stimulate the innate cells: the macrophages, neutrophils and natural killer (NK) cells. The plasma cells produce the immunoglobulins; immunoglobulin A (IgA) is the principal antibody at mucosal sites (Gleeson, 2006).

1.3. Salivary secretion

Saliva is a clear, slightly acidic mucoserous exocrine secretion consisting of inorganic and organic compounds and usually more than 99% water. The average daily flow of whole saliva varies greatly between 0.5 L and 1.5 L which represents 20% of the total plasma volume (Chicharro *et al.*, 1998). Thus, salivary flow is a highly individualised measurement. The secretion of saliva into the mouth originates from three pairs of major salivary glands: the submandibular (65%), parotid (20%) and the sublingual (7-8%) and numerous minor salivary glands (less than 10%) found in the lower lip, tongue, palate, cheeks, and pharynx. In humans, the parotid glands produce mainly serous saliva since the secretion lacks mucin, the minor glands produce mainly mucous saliva and the sublingual and submandibular glands produce mixed saliva (Humphrey & Williamson, 2001). The types of cells found in the salivary glands are acinar cells, various duct system cells and myoepithelial cells. Acinar cells, from which saliva is first secreted, determine the type of secretion produced from the

different glands. They are connected by intercalated ducts and the secreted saliva is drained to the oral cavity through striated and excretory ducts. During this passage, the concentrations of several electrolytes change due to active ion transport (Aps and Martens, 2005). Myoepithelial cells, which are long cell processes wrapped around acinar cells, contract on stimulation to constrict the acinar cells. This causes saliva to be squeezed out (Garrett, 1987).

The secretion of saliva is under neural control by three principal mechanisms or stimuli including mechanical (the act of chewing), gustatory (with acid the most stimulating trigger and sweet the least stimulating), and olfactory (although a surprisingly poor stimulus) (Humphrey & Williamson, 2001). Stimulating high flow rates can drastically change percentage contributions from each gland, with the parotid contributing more than 50% of total salivary secretions compared with only 20% in the unstimulated state (Edgar, 1990).

The value of saliva as a biological fluid for the detection of diagnostic and prognostic biomarkers has become increasingly well established. Sample collection is non-invasive, painless, and able to provide accurate and reliable assessments of immune status and the unbound, biologically active, form of certain hormones and drugs. Thus, it is considered a valuable tool for investigating the impact of exercise on stress and immune function.

1.4. Salivary composition and mucosal immunity

Saliva secretions protect the oral mucosa via a mechanical washing effect and play an important role in immunity as the first line of defence against potential pathogens invading the oral and nasal cavities. Saliva contains several antimicrobial proteins e.g. immunoglobulins, lysozyme and α -amylase, which play a critical role in defence against

infection and disease by interfering with microbial entry and multiplication (Bosch *et al.*, 2002).

1.4.1. Immunoglobulin A

Immunoglobulin A is the principal antibody representing the acquired immune system, which is secreted at mucosal surfaces (Gleeson & Pyne, 2000). IgA is a dimer whose monomers are held together by a polypeptide structure known as the J-chain containing the secretory component (SC), the cleaved part of polymeric Ig receptor (pIgR). There are two subclasses of IgA, IgA1 and IgA2, the former accounting for 60-80% of IgA in the salivary glands. IgA is produced by plasma cells (differentiated B-cells) residing in the submucosa and is secreted via transport across the epithelial cells by the pIgR, which is inserted into the basal membrane (Mackinnon, 1999). IgA binds to this receptor and undergoes endocytosis and vesicular transport across the cell. At the apical surface secretory IgA is cleaved from the receptor and remains attached to the SC. The covalent binding of the secretory component acts to prevent the IgA from proteolysis (Gleeson, 2006).

Immunoglobulin A seems to function as a multilayered mucosal defence: it prevents antigens and microbes from adhering to and penetrating the epithelium (immune exclusion), interrupts replication of intracellular pathogens during transcytosis through epithelial cells (intracellular neutralisation), and binds antigens on the lamina propria facilitating their excretion through the epithelium back into the lumen (immune excretion) (Lamm, 1998). It is therefore important to host defence against certain viruses that are not carried by the blood especially those causing URTI. Indeed, low levels of salivary IgA (s-IgA) are associated with a higher incidence of URTI (Hanson *et al.*, 1983) and high levels of s-IgA are associated with a lower incidence of URTI (Rossen *et al.*, 1970).

1.4.2. Lysozyme

Lysozyme represents the main enzyme of the non-specific innate mucosal immune defence. It has antimicrobial properties and functions to aid the destruction of bacterial cell walls by cleaving the polysaccharide component (Bosch *et al.*, 2002). Lysozyme also acts to stimulate neutrophils and macrophages, and works with immunoglobulins to have further antimicrobial effects (Jolles and Jolles, 1984; West *et al.*, 2006). It is produced mainly by the submandibular and sublingual glands and it is secreted into saliva by mucous membranes and mononuclear cells entering the oral cavity through gingival crevices (Noble, 2000). Lysozyme has been shown to be lower in subjects prone to acute bronchitis infection caused by *Haemophilus influenzae* (Clancy *et al.*, 1995). Furthermore, patients with chronic bronchitis who were less prone to acute exacerbations had a greater capacity to aggregate bacteria and were shown to have higher levels of lysozyme (Taylor *et al.*, 1995).

1.4.3. Alpha-amylase

Alpha-amylase is an enzyme that breaks down starch into maltose and is also important to host defence by inhibiting the adherence and growth of certain bacteria (Scannapieco *et al.*, 1994). Alpha-amylase is synthesised locally by the acinar cells mainly from the parotid gland, accounting for 40 to 50% of the total salivary-gland produced protein (Noble, 2000). To date, the immunological significance of α -amylase in relation to URTI remains unclear.

1.5. Regulation of salivary secretion

The regulation of salivary antimicrobial proteins is via a short-term (minutes) mobilisation and/or a long-term (hours - days) modification of protein synthesis (Goodrich and McGee,

1998). This may be influenced by the two branches of the stress response: sympathetic nervous system (SNS) activity and the hypothalamic-pituitary-adrenal (HPA) axis.

1.5.1. Sympathetic nervous system activity

The salivary glands are innervated by both parasympathetic and sympathetic nerves. Generally, it is considered that sympathetic stimulation (via noradrenaline) leads to higher salivary protein concentrations (e.g. α -amylase), whereas increased rates of fluid output occur in response to parasympathetic stimulation (Chicharro *et al.*, 1998). However, parasympathetic stimulation can also affect salivary protein secretion, and protein secretion of some glands, such as the sublingual and some of the minor glands, may even be entirely under parasympathetic control. Thus, rather than acting antagonistically, it could be argued that the two branches of the autonomic nervous system may exert relatively independent effects in which the activity of one branch may synergistically augment the other (Bosch *et al.*, 2002).

Studies carried out in rodents suggest that protein secretion is upregulated into saliva following autonomic nerve stimulation. Indeed, the secretion of s-IgA was increased rapidly (within minutes) by both parasympathetic and sympathetic stimulation (Carpenter *et al.*, 2000), and adrenaline was shown to increase the entry of human IgA into saliva by rat salivary cells via increased mobilisation of the pIgR (Carpenter *et al.*, 2004). Parasympathetic and sympathetic stimulation in rats has also been shown to increase the secretion of other stored salivary proteins (e.g. peroxidase and α -amylase) into saliva, which occurs to a much greater magnitude than s-IgA (Carpenter *et al.*, 2000).

Activation of the sympathetic nervous system leads to the release of catecholamines (adrenaline and noradrenaline) into the circulation. However, catecholamines measured in saliva are regarded as a poor index of sympathetic nervous activity (Kennedy *et al.*, 2001), since they do not accurately reflect changes in circulating catecholamines (Schwab *et al.*, 1992). As a result, α -amylase has been identified as a potential surrogate marker of SNS activity in humans under a variety of stressful conditions. Studies have shown that acute psychological stressors increase salivary α -amylase activity (Bosch *et al.*, 2002; Nater *et al.*, 2006). Moreover, these increases were positively correlated with serum noradrenaline (Chatterton *et al.*, 1996). Thus, the increase in sympathetic tone that occurs during psychological stress would be similar to that produced during exercise and suggests that exercise could similarly affect α -amylase secretion. Indeed, a bout of exercise has been shown to increase α -amylase activity (Chatterton *et al.*, 1996). A five-fold increase in α -amylase activity was found after a 60-min intermittent cycle exercise bout, of twenty 1-min periods at 100 % of maximum oxygen uptake ($\dot{V}O_{2max}$) separated by 2 min recovery at 30% $\dot{V}O_{2max}$ (Walsh *et al.*, 1999). However, the use of α -amylase as a non-invasive measure of SNS activity requires further examination since its secretion is also increased by parasympathetic stimulation alone or in interaction with sympathetic stimulation (Asking and Proctor, 1989).

Another potential marker of sympathetic nervous system activity is Chromogranin A (CgA). Chromogranin A is an acidic secretory protein found in a wide variety of hormone and neurotransmitter storage vesicles and is co-stored and co-released with catecholamines from the adrenal medulla and neuronal vesicles during exocytosis (Banks and Helle, 1965). *In vitro* experiments have also demonstrated that CgA exhibits antifungal and antibacterial properties (Strub *et al.*, 1996; Lugardon *et al.*, 2000). Salivary CgA concentration has been shown to

increase rapidly under psychosomatic stress (Nakane *et al.*, 1998), although the response of CgA to a bout of exercise is yet to be determined.

1.5.2. Hypothalamic-pituitary-adrenal axis

Whereas autonomic stimulation may rapidly affect the secretion of salivary proteins, slower effects have been reported for pituitary-adrenal hormones. Exercise or stress activates the hypothalamic-pituitary-adrenal axis, the end product of which is cortisol. Cortisol is well known for its modulatory effects on immune function (Fleshner, 2000). The potent synthetic glucocorticoid dexamethasone has been shown to cause a decline in the mobilisation of s-IgA 24 h after a single injection (Wira *et al.*, 1990), and in the longer term this hormone may inhibit IgA synthesis by B cells in the submucosa (Saxon *et al.*, 1978). Cortisol has also been implicated in modifying other secretory proteins such as lysozyme production and its secretion into saliva (Perera *et al.*, 1997). Salivary cortisol has been shown to correlate highly with circulating levels of cortisol (Chicharro *et al.*, 1998) making it a reliable marker of HPA axis activity.

Exercise stimulates SNS activity and the HPA axis to secrete stress hormones, thus increasing the body's ability to meet the physical and metabolic demands of exercise. Prolonged intense exercise is associated with substantially increased secretions of catecholamines and glucocorticoids which have a strong influence on the immune system. This presents a potential pathway linking exercise, stress and infectious disease.

1.6. Methods of saliva collection

An important consideration when interpreting exercise studies on saliva flow rate and composition is the methods that were used to collect samples. There appears to be little

consistency across studies; some researchers have used cotton salivettes (Walsh *et al.*, 2002, Bishop *et al.*, 2006), while others have used the passive drool (Li and Gleeson 2004; Sari-Sarraf *et al.*, 2007a) or even a suction tube to draw saliva from the floor of the mouth (Michishige *et al.*, 2006). This is noteworthy since it was reported that cotton salivettes reduced the concentration of s-IgA compared with passive collection of saliva (Strazdins *et al.*, 2005). In addition, there may be a potential stimulatory effect on saliva production from introducing something foreign into the mouth, which may preferentially stimulate some glands more than others; although evidence to support this is lacking (Navazesh and Christensen, 1982). A previous study examined the reliability of the collection methods of spitting, draining, suction and swabs and it was found that the spitting and draining methods gave the most reproducible results (Navazesh, & Christensen, 1982).

1.7. Methods of expressing s-IgA

The methods of expressing s-IgA can also vary greatly making direct comparisons between studies difficult. S-IgA concentrations have often been expressed not only in absolute concentrations (Gleeson *et al.*, 1999; Tharp and Barnes, 1990), but also as a ratio to total salivary protein, (Blannin *et al.*, 1998, Mackinnon *et al.*, 1987; Tomasi *et al.*, 1982), albumin (Pyne *et al.*, 2000) or osmolality (Blannin *et al.*, 1998, Laing *et al.*, 2005). It has been suggested that expressing s-IgA relative to total protein is misleading since total protein secretion rate can increase during exercise (Blannin *et al.*, 1998; Walsh *et al.*, 1999; Sari-Sarraf *et al.*, 2007a). However, expressing s-IgA relative to osmolality may be a valid measure when significant variations in saliva flow rate occur (Blannin *et al.*, 1998). This is because a reduction in saliva flow rate may have a concentrating effect on s-IgA resulting in an artificial increase in the s-IgA concentration for a given volume. It is now thought that the secretion rate of s-IgA (saliva flow rate multiplied by the s-IgA concentration) is the most

appropriate measure (Mackinnon and Hooper, 1994; Walsh *et al.*, 1999, Blannin *et al.*, 1998; Li and Gleeson 2004), since s-IgA secretion rate represents the total amount of s-IgA delivered to the mucosal surface per unit time and takes into account the saliva flow rate and IgA concentration, both of which are important for host defence (Mackinnon *et al.*, 1993a). Indeed, the study by Li and Gleeson (2004) highlights these inconsistencies since it was shown that cycling at 60% $\dot{V}O_{2max}$ for 2 h significantly increased the concentration of s-IgA but did not affect its secretion rate. These findings highlight the problems associated with the different methods of expressing s-IgA which are likely to confound the interpretation of results.

1.8. Variability in s-IgA

One of the major problems surrounding s-IgA research is the considerable variability that exists within and between individuals (Burrows *et al.*, 2002, Francis *et al.*, 2005). Francis *et al.* (2005) compared this in three populations: elite swimmers, active adults and a sedentary group and found that the within-individual and between-individual variability differed substantially in the three populations. For example, the within-subject variability was much greater in elite swimmers (47%) than the other active (23%) and sedentary (28%) groups. However, the elite swimmers were a more homogenous group, with lower between-subject variability (20%) compared with the active (54%) and sedentary (46%) group. The sources of variation are likely to be multifactorial in nature, including diurnal variation, nutritional status and psychological stress, but one of the major sources is the saliva flow rate (Gleeson, 2000), since this can be greatly affected by the hydration status of the individual and indeed exercise. Such a variation is important since it will impact on the design of studies i.e. the number of subjects needed and the ability to detect changes in mucosal immune status in response to exercise (Gleeson *et al.*, 2004).

1.9. Exercise and saliva flow rate

Several investigators have reported a marked decrease in saliva flow rate in response to short duration (less than 8 min), high intensity exercise (Engels *et al.*, 2003; Fahlman *et al.*, 2001), although others have reported no change in saliva flow rate to brief bouts of exercise at sub-maximal or maximal intensities (Dawes, 1981; Pilardeau *et al.*, 1990). Several factors associated with high-intensity exercise such as removal of parasympathetic vasodilatory influences (rather than sympathetic mediated vasoconstriction), (Proctor and Carpenter, 2007), or evaporation of saliva through increased ventilation (Pilardeau *et al.*, 1992), have been proposed to explain this lower secretion in saliva flow rate. Prolonged exercise (> 90 min) also results in a reduction in flow rate (Bishop *et al.*, 2000, Li and Gleeson 2005; Sari-Sarraf *et al.*, 2007a; Walsh *et al.* 1999), which may be further attributed to dehydration occurring through sweat loss during exercise (Ford *et al.*, 1997; Walsh *et al.*, 2004). This is supported by evidence where the decrease in saliva flow rate was prevented by providing fluids to offset losses (Walsh *et al.*, 2004). Changes in saliva flow rate following exercise are significant since they may not only affect the concentration of s-IgA, but more importantly, could lead to an increased risk of URTI. This is based on the finding that individuals suffering from xerostomia (dry mouth syndrome) have a substantially increased incidence of oral infections and more pathogenic bacteria in the buccal cavity (Fox *et al.*, 1985).

1.10. Acute exercise and s-IgA responses

Although there has been an abundance of research conducted on the s-IgA response to acute exercise in recent years, the results remain inconsistent (Table 1.1). Some have shown significant decreases post-exercise (Tomasi *et al.*, 1982; Mackinnon & Jenkins, 1993; Nieman *et al.*, 2002; Steerenberg *et al.*, 1997; Walsh *et al.*, 2002), some have shown increases (Blannin *et al.*, 1998, Reid *et al.*, 2001, Williams *et al.*, 2001), and others have

shown no change (Mackinnon and Hooper, 1994; McDowell *et al.*, 1991; Sari-Sarraf *et al.*, 2007b; Walsh *et al.*, 1999). The reasons for the inconsistencies are likely to range from the dissimilarity of exercise protocols, participant groups, collection methods (whether the sample was stimulated or unstimulated), how s-IgA was expressed i.e. as a concentration, or secretion rate and the mode of exercise performed.

Tomasi and colleagues (1982) were the first to report evidence of altered mucosal immunity following intense exercise. In a cohort of elite cross-country skiers, a 20% reduction in s-IgA concentration was observed after 2-3 h of competition. Similar findings were reported in elite male swimmers following a training session (Tharp and Barnes, 1990), and Gleeson and colleagues have consistently reported reductions (albeit small ~10%) in s-IgA concentration following 2 h of intense swimming in Australian national standard swimmers (Gleeson *et al.*, 1995; Gleeson *et al.*, 1999).

Several subsequent laboratory studies also reported decreases in s-IgA levels post-exercise. Mackinnon *et al.* (1989) found a 60% decrease in s-IgA concentration and a 65% decrease in the secretion rate immediately after 2 h high intensity cycling, which remained low for 1 h and then returned to baseline levels after 24 h. Moreover, McDowell and colleagues (1992) reported a 25% decrease in s-IgA concentration following an incremental treadmill run to exhaustion, which recovered at 1 h post-exercise. In these studies it was not stated whether fluid ingestion during exercise was restricted or *ad libitum* which could have had an impact on the saliva flow rate and resulting s-IgA secretion.

In contrast, there is now growing evidence to suggest that exercise can result in significant increases in s-IgA concentration. Studies in well-trained cyclists showed a 17% increase in s-IgA concentration following 2 h cycling at 70% $\dot{V}O_{2max}$ (Walsh *et al.*, 2002) and a 15%

increase following 2 h cycling at 65% $\dot{V}O_{2max}$ (Laing *et al.*, 2005). However, when s-IgA was expressed as secretion rate, significant reductions (20-35%) were observed. In these studies the increase in s-IgA concentration post-exercise was likely due to the observed reduction in saliva flow rate (Walsh *et al.*, 2002), which highlights the importance of accounting for changes in saliva flow rate when examining the s-IgA response to exercise.

Studies examining the s-IgA responses to more moderate soccer-specific exercise (Sari-Sarraf *et al.*, 2007a; Sari-Sarraf *et al.*, 2007b) and moderate cycling (Reid *et al.*, 2001; Li and Gleeson, 2004) have consistently shown increases in the s-IgA concentration post-exercise. However, when this was expressed as a secretion rate, some reported increases (Sari-Sarraf, *et al.*, 2007a), while others reported no change (Sari-Sarraf, *et al.*, 2007b; Li and Gleeson, 2004).

Table 1.1. Effect of an acute bout of exercise on salivary IgA

Study	Participants	Exercise	Method of expressing s-IgA	S-IgA response
Blannin <i>et al.</i> , (1998)	Young males mixed fitness	Exhaustive cycling at 55% $\dot{V}O_{2max}$ and 80% $\dot{V}O_{2max}$	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva protein • Relative to saliva osmolality 	<ul style="list-style-type: none"> • 200% increase • 60% increase • No change • 70% increase
Gleeson <i>et al.</i> , (1995)	Elite swimmers	Training sessions	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 10% decrease
Laing <i>et al.</i> , (2005)	Trained male cyclists	2 h cycle at 60% $\dot{V}O_{2max}$	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva osmolality 	<ul style="list-style-type: none"> • 15% increase • 35% decrease • 25% decrease
Li and Gleeson (2004)	Active men	2 h cycle at 60% $\dot{V}O_{2max}$	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate 	<ul style="list-style-type: none"> • 25% increase • No change
Mackinnon and Hooper (1994)	Recreational joggers Competitive distance runners	Treadmill exercise at 55% and 75% VO_{2peak} for 40 min (Recreational joggers) or 90 min (Competitive runners)	<ul style="list-style-type: none"> • Secretion rate 	<ul style="list-style-type: none"> • No change in either group
Mackinnon and Jenkins (1992)	Active men	5 supramaximal 60 s bouts of cycling	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva protein 	<ul style="list-style-type: none"> • 15% increase • 52% decrease • 21% decrease
Mackinnon <i>et al.</i> , (1989)	Competitive male cyclists	2 h cycling at 90% of ventilatory threshold	<ul style="list-style-type: none"> • Absolute concentration • Relative to saliva protein 	<ul style="list-style-type: none"> • 60% decrease • 65% decrease

Table 1.1. (continued)

Study	Participants	Exercise	Method of expressing s-IgA	S-IgA response
McDowell <i>et al.</i> , (1991)	Young active males	15-45 min at 50-80% $\dot{V}O_{2max}$ treadmill test	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • No change
McDowell <i>et al.</i> , (1992)	Trained males	Incremental treadmill test to exhaustion	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 25% decrease
Nehsen-Cannarella <i>et al.</i> , (2000)	Elite female rowers	2 h moderate intensity training session	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate 	<ul style="list-style-type: none"> • 50% decrease • 20% decrease
Nieman <i>et al.</i> , (2002)	Trained runners	Marathon race	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva protein 	<ul style="list-style-type: none"> • 21% decrease • 25% decrease • 31% decrease
Reid <i>et al.</i> , (2001)	Physically active participants	Incremental cycle test to fatigue	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate 	<ul style="list-style-type: none"> • 30% increase • No change
Sari-Sarraf <i>et al.</i> , (2007a)	Moderately trained men	Single and repeated bouts of 45 min intermittent exercise	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva osmolality 	<ul style="list-style-type: none"> • 56% increase • 15% increase • 10% increase
Sari-Sarraf <i>et al.</i> , (2007b)	Moderately trained men	Soccer-specific intermittent exercise	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva osmolality 	<ul style="list-style-type: none"> • 56% increase • No change • No change

Table 1.1. (continued)

Study	Participants	Exercise	Method of expressing s-IgA	S-IgA response
Steerenberg <i>et al.</i> , (1997)	Competitive triathletes	Race	<ul style="list-style-type: none"> • Absolute concentration • Relative to saliva protein 	<ul style="list-style-type: none"> • No change • 40% decrease
Tharp (1991)	Junior male basketball players	Training and matches	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 10% increase
Tharp & Barnes (1990)	Highly trained male swimmers	2 h training session	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 10% decrease
Tomasi <i>et al.</i> , (1982)	Elite male and female cross country skiers	2-3 h race	<ul style="list-style-type: none"> • Absolute concentration • Relative to saliva protein 	<ul style="list-style-type: none"> • 20% decrease • 40% decrease
Walsh <i>et al.</i> , (1999)	Trained male games players	Intense intermittent exercise ~ 60 min	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate 	<ul style="list-style-type: none"> • No change • No change
Walsh <i>et al.</i> , (2002)	Trained male cyclists	2 h cycle at 70% $\dot{V}O_{2max}$	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate 	<ul style="list-style-type: none"> • 17% increase • 19.5% decrease

1.10.1. Effect of exercise intensity on s-IgA

Previous research conducted in athletes suffering from recurrent infections has shown that exercise of equal duration performed at 75% $\dot{V}O_{2max}$ and 100% $\dot{V}O_{2max}$ were associated with a trend for a lower s-IgA concentration post-exercise, while levels increased slightly after exercise at 50% $\dot{V}O_{2max}$ (Williams *et al.*, 2001). It was concluded that there exists a positive association between the degree of immune suppression post-exercise and the exercise intensity level. However, McDowell and colleagues (1991) examined the effect of treadmill exercise at intensities ranging from 50-80% $\dot{V}O_{2max}$ for durations of 15-45 min in a group of college men where no significant changes in s-IgA concentration were observed after exercise irrespective of the exercise intensity.

To add further confusion, Blannin *et al.* (1998) showed that exercise to exhaustion at a higher intensity (80% $\dot{V}O_{2max}$) showed a 105% increase in the s-IgA secretion rate compared with 15% increase during moderate intensity (55% $\dot{V}O_{2max}$) exercise. However, these findings may have been influenced by the exercise duration, which was different between the two exercise intensities: ~30 versus ~160 min for the 80% $\dot{V}O_{2max}$ trial and 55% $\dot{V}O_{2max}$ trial, respectively.

1.11. Chronic exercise and s-IgA

Exercise-induced changes in s-IgA may be cumulative over time since long-term exercise training has also been suggested to influence s-IgA levels, although the research is still limited to date (Table 1.2). Results are easier to interpret since studies are more uniform and the resting measures should not be affected by changes in the saliva flow rate or increases in salivary protein secretion associated with acute exercise. Longitudinal studies monitoring elite Australian swimmers over a 7-month training period showed decreased pre- and post-

exercise s-IgA concentrations (Gleeson *et al.*, 1995; Gleeson *et al.*, 1999), and the resting s-IgA concentration fell by approximately 4.1% for every month of training (Gleeson *et al.*, 1999). Additionally, in competitive swimmers, a decrease in both pre- and post-exercise s-IgA concentration was observed over a 3-month training period (Tharp and Barnes, 1990). Conversely, increases in pre-exercise s-IgA concentration were observed in another group of elite swimmers over 12 weeks of training (Gleeson *et al.*, 2000). In this study, the swimmers had just returned from a 6-week rest period and the authors commented that there may not have been sufficient time to note any long-term negative change in immune parameters. More recently it was shown that both s-IgA concentration and secretion rate were reduced in a cohort of American Footballers during two periods of intense training and competition over a 12-month period (Fahlman and Engels, 2005). However, given that saliva flow rate exhibits a seasonal variation (decreasing during the warmer summer months; Kavanagh *et al.*, 1998; Whitham *et al.*, 2006), this may affect the s-IgA concentration during long-term training studies.

The effects of short periods of intense training on s-IgA are less clear. A short training season in elite kayakers resulted in reductions in pre-exercise s-IgA concentrations over 3 weeks (Mackinnon *et al.*, 1993a) and 4 weeks of training in female hockey players reduced the s-IgA concentration, secretion rate and s-IgA to protein ratio (Mackinnon *et al.*, 1992). A 3-week intense military training course induced no change in s-IgA concentration though it was reduced after the 5-day combat course (Tiollier *et al.*, 2005). However, an increase in pre- and post-exercise s-IgA concentration was observed in junior basketball players measured 3 times over a 2-month training period (Tharp, 1991).

Moderate exercise training has resulted in increases in s-IgA levels in elderly men and women during 12 weeks of exercise (75% heart rate reserve for 30 min 3 times per week)

(Klentrou *et al.*, 2002). Salivary IgA concentration and s-IgA concentration-salivary albumin concentration ratio increased significantly in the exercise group in comparison to the control group.

Collectively, the evidence from these studies suggests that intense training (> 3 months) in well-trained athletes may be associated with a chronic suppression of s-IgA levels. This is thought to be an accumulative effect of reduced s-IgA levels following prolonged bouts of intense exercise. Conversely, more moderate training, particularly in more sedentary groups could result in increased s-IgA.

Table 1.2. Effect of exercise training on salivary IgA.

Study	Participants	Exercise	Method of Expressing s-IgA	S-IgA Response
Fahlman and Engels, (2005)	Competitive American footballers	12-month training season	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate 	<ul style="list-style-type: none"> • 38% decrease • 30% decrease
Gleeson et al., (1995) Gleeson et al., (1999)	Highly trained swimmers	7-month training season	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 16-17% decrease
Gleeson <i>et al.</i> , (2000)	Highly trained swimmers	12-week training cycle	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 50% increase
Klentrou <i>et al.</i> , (1999)	Sedentary elderly men	12-weeks moderate aerobic training	<ul style="list-style-type: none"> • Absolute concentration • Relative to albumin 	<ul style="list-style-type: none"> • 57% increase • 13% increase
Mackinnon <i>et al.</i> , (1992)	Elite female hockey players	4-weeks of training	<ul style="list-style-type: none"> • Absolute concentration • Secretion rate • Relative to saliva protein 	<ul style="list-style-type: none"> • ~ 30% decrease in all measures
Mackinnon <i>et al.</i> , (1993a)	Elite male kayakers	3-month intensive training	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 34% increase
Tharp (1991)	Junior basketball players	2-month season	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 15% increase
Tharp and Barnes (1990)	Highly trained swimmers	3-month training season	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 22% decrease
Tiollier <i>et al.</i> , (2005)	Military personnel	3-week intense training and 5-day combat course	<ul style="list-style-type: none"> • Absolute concentration 	<ul style="list-style-type: none"> • 40% decrease

1.12. Exercise and other salivary antimicrobial proteins

Presently, the research investigating the influence of exercise on other salivary antimicrobial proteins is scant, although there have been several published findings of their response to psychological stress. Lysozyme concentrations have been shown to decrease in final year students during acute stress immediately prior to an exam, compared with one month after the end of all examinations (Perera *et al.*, 1997). Furthermore, lysozyme concentrations have also shown a negative correlation with perceived stress scores (Perera *et al.*, 1997). In contrast, significant increases in the antimicrobial protein lactoferrin were found following an acute laboratory stressor (Bosch *et al.*, 2003). There is only one published abstract examining the effects of acute exercise on lysozyme following intense training sessions in elite swimmers (Koutedakis *et al.*, 1996). In this study, significant reductions in salivary lysozyme concentration and secretion rate were reported post-exercise. These preliminary findings highlight the need for further well-controlled studies designed to investigate the responses of salivary antimicrobial proteins to both acute and chronic exercise.

The response of α -amylase to exercise appears to be more consistent. Generally, it is reported that it increases following an acute bout of exercise (Bishop *et al.*, 2000; Chatterton *et al.*, 1996; Walsh *et al.*, 1999), and this increase is related to the exercise intensity (Bishop *et al.*, 2000; Ljungberg *et al.*, 1997; Walsh *et al.*, 1999). These transient increases in α -amylase are likely to be induced by the increases in catecholamines and SNS activity with exercise (Dawes, 1981). However, the relevance of these findings in terms of URTI risk is not fully understood.

1.13. Salivary antimicrobial proteins and infection risk in athletes

It is well known that individuals with IgA deficiency experience a higher incidence of URTI, and a significant relationship between s-IgA concentration and URTI incidence in the general population has been reported (Jemmott and McClelland, 1989). A decrease in salivary IgA levels has also been implicated as a possible causal factor for the increased susceptibility of athletes to URTI. In a much cited study by Gleeson and colleagues (1999), this was assessed in a cohort of elite swimmers during a 7-month training period. S-IgA concentration fell during this training period and an inverse correlation was found between reported infections and the pre-session s-IgA concentration. Mackinnon *et al.* (1993b) also reported that the levels of s-IgA in squash players decreased on days preceding illness, whereas the levels were higher on days that did not precede URTI. Significant associations have also been observed between URTI episodes and s-IgA secretion rate in American college football players over a 12-month competitive season (Fahlman and Engels, 2005). An increase in s-IgA in elderly subjects in response to moderate exercise was associated with a decreased incidence of URTI symptoms (Klentrou *et al.*, 2002).

However, several other investigations have been unable to replicate these findings. Following a marathon, when expressed as a ratio to total saliva protein, post-race levels were lower in those developing URTI. However, when expressed as absolute s-IgA concentration and s-IgA secretion rate no association was observed (Nieman *et al.*, 2002). Furthermore, no significant correlation between URTI and s-IgA was found in another cohort of swimmers over 12 weeks (Gleeson *et al.*, 2000), in elite female rowers over 2 months (Nehlsen-Cannarella *et al.*, 2000) or in elite tennis players studied over 12 weeks (Novas *et al.*, 2003). Given the high degree of variability in s-IgA for an individual, it would appear that s-IgA may only be a marginal predictor of short-term URTI risk (Pyne *et al.*, 2000). Nonetheless, to date, this is

the only measure of immune function that has been directly linked to URTI susceptibility in athletes and thus, warrants further investigation.

1.14. Circadian Variation of Salivary Components

Many components of the immune system exhibit rhythmic changes (Shephard and Shek, 1997) and circadian variations in s-IgA and α -amylase have been shown in a number of previous studies (Gleeson *et al.*, 2001; Hucklebridge *et al.*, 1998; Li and Gleeson, 2004). A significant decrease in s-IgA throughout the day is observed from its highest value in the early morning to its lowest value in the evening. In contrast, α -amylase secretion rate increases during the day (Li and Gleeson, 2004). Consequently, reports of a decrease in s-IgA and an increase α -amylase after prolonged exercise performed in the morning may not give a clear representation of whether the response was a result of the exercise *per se*, or a reflection of the circadian rhythm. The effects of a diurnal variation on the salivary responses were assessed following prolonged cycling (Li and Gleeson, 2004) and intensive intermittent swimming (Dimitriou *et al.*, 2002), however, no influence of diurnal variations on the responses were found.

1.15. Nutritional influences on mucosal immunity

Several researchers have investigated the effects of certain nutritional supplements on the s-IgA response to exercise but with varying success. The supplementation of ginseng (Engels *et al.*, 2003), Vitamin C (Palmer *et al.*, 2003) and glutamine (Kreiger *et al.*, 2004) appear to have little impact on s-IgA. More important perhaps is the effect of carbohydrate (CHO) and fluid intake (Bishop *et al.*, 2000; Costa *et al.*, 2005), bovine colostrum (Crooks *et al.*, 2006) and echinacea purpurea (Hall *et al.*, 2007).

1.15.1. Carbohydrate, exercise and s-IgA

Carbohydrate ingestion during prolonged exercise has been shown to attenuate the rise in stress hormones such as plasma catecholamines and cortisol and appears to limit the degree of exercise-induced immune depression (Gleeson and Bishop, 2000). In contrast, an athlete exercising in a CHO-depleted state experiences larger increases in circulating stress hormones and a greater perturbation of several immune function indices (Gleeson *et al.*, 2004b).

Bishop *et al.* (2000) demonstrated that CHO and fluid intake during prolonged sub-maximal exercise were important in maintaining the saliva flow rate and s-IgA concentration (but not secretion rate) when compared with restricted fluid intake. However, there appears to be little effect of CHO ingestion during exercise on s-IgA when compared with placebo (Bishop *et al.*, 2000; Nehlsen-Cannarella *et al.*, 2000; Nieman *et al.*, 2002). In the longer term, a high CHO diet appeared to enhance s-IgA levels post-exercise in ironman athletes (Costa *et al.*, 2005) and it was found that a 24 – 48 h period of combined fluid and energy restriction decreased the s-IgA secretion rate (Oliver *et al.*, 2007).

Since few studies have quantified the nutritional status of individuals, its effect on the immune response to exercise cannot be readily determined (Gleeson *et al.*, 2004a). Moreover, it is thought that the nutritional status of an individual may be a contributing factor to the large variation of s-IgA observed within-individuals, since fasting saliva was reported to yield higher and more variable concentrations of s-IgA concentration than non-fasting samples (Gleeson *et al.*, 1990; Gleeson *et al.*, 2004a).

1.16. Gender differences

The majority of available literature has focused on male subjects or a mixed gender population. Very little research has been conducted on females alone (Fahlman *et al.*, 2001; Nehlsen-Cannarella *et al.*, 2000). This may be important since significantly lower levels of resting s-IgA concentrations in females compared with males have been reported in elite swimmers (Gleeson *et al.*, 1999). Whether these gender differences may influence the salivary responses to acute exercise or exercise training is yet to be determined. Furthermore, whether there are gender differences in resting s-IgA levels for other sporting populations remains unknown.

1.17. Stimulated saliva flow

Stimulating whole mouth saliva secretion can increase the flow rate up to $7 \text{ mL}\cdot\text{min}^{-1}$ compared with $0.3 \text{ mL}\cdot\text{min}^{-1}$ in unstimulated saliva (Humphrey and Williamson 2001). Navazesh & Christensen (1982) compared different methods of salivary stimulation: gustatory: citric acid drops, straight onto the tongue or onto hardened ashless filter paper, and masticatory: chewing polyvinyl acetate gum base for one minute. All three methods successfully increased saliva flow rates. However, despite controlling chewing rate at 20 jaw strokes per minute, the masticatory method was found to produce the most variable results which were most likely due to differences in force and duration of force when chewing.

Stimulating saliva flow can also influence the composition of saliva produced. Proctor and Carpenter (2001) showed that chewing increased the s-IgA secreted into saliva by epithelial cell transcytosis. Salivary secretion was stimulated by chewing on a tasteless piece of polythene tube, which therefore only stimulated masticatory salivary secretion. Similar increases were also found in the secretion rates of total protein and α -amylase. Such changes

in the quantity and quality of saliva by chewing gum may have potential health benefits, not only for individuals suffering with xerostomia (dry mouth syndrome), but also for athletes as a measure of counteracting the reported post-exercise drop in s-IgA secretion rate. This was examined in one previous study where the effects of stimulated and unstimulated salivary flow on the s-IgA response to high intensity exercise were compared. Participants cycled at 85% $\dot{V}O_{2max}$ until volitional fatigue. Saliva secretion was stimulated by sucking a mint (taste stimulation only) for 1 min prior to saliva collection. S-IgA secretion rate was found to decrease with exercise but was higher in stimulated compared with unstimulated saliva (Gleeson *et al.*, 2003).

1.18. Summary

This chapter presents a review of the effect of exercise on salivary markers of stress and antimicrobial proteins. Specifically, it highlights the responses of s-IgA and lysozyme to exercise and how these changes may be related to the salivary markers of adrenal activation: cortisol and α -amylase. Exercise causes a marked increase in the activity of the SNS and the HPA-axis which can act to modify both the synthesis and translocation of antimicrobial proteins into saliva. However, whether this is in a positive or negative manner remains controversial. It is clear that the s-IgA response to acute and chronic exercise requires further attention and the response of other antimicrobial proteins to exercise are yet to be fully investigated. Therefore, the main objectives of the present research studies were to:

- 1) Investigate the effect of *pre-exercise* nutritional status (fed versus overnight fasted) on the s-IgA response to prolonged cycling
- 2) Investigate the effect of feeding versus fasting *during* prolonged exhaustive exercise on salivary antimicrobial proteins

- 3) Compare the effects of stimulated and unstimulated salivary flow on the s-IgA, lysozyme and α -amylase responses to prolonged exhaustive cycling
- 4) Examine the effect of short duration cycling exercise at different intensities on salivary antimicrobial proteins and markers of adrenal activation
- 5) Determine the effect of a 6-month period of training and competition on s-IgA and URTI incidence in elite male and female swimmers

Chapter 2 - General Methods

2.1. Ethical approval

Approval of the study protocols included in this thesis was obtained prior to their commencement by the Loughborough University Ethical Advisory Committee. The aims and procedures were explained fully to each participant verbally and in writing. All participants were given an opportunity to ask questions before providing written informed consent (Appendix A), and were made fully aware that they were free to withdraw from the study at any time without providing any reason.

Prior to beginning the study, the participants completed a health screen questionnaire (Appendix B) and a physical activity questionnaire (Appendix C) to confirm that they were suitable to take part. On each testing day, participants were required to complete a separate health questionnaire (Appendix D) to confirm that they were not experiencing any symptoms of illness.

2.2. Protocol for the determination of maximal oxygen uptake ($\dot{V}O_{2max}$)

Measurements of $\dot{V}O_{2max}$ were conducted within 1-2 weeks before commencing the main trials. Participants performed a continuous incremental exercise test on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, Netherlands) to volitional exhaustion to determine their maximal oxygen uptake ($\dot{V}O_{2max}$). Following a 3-min warm-up, participants began cycling at 70 W for Chapter 7 and 95 W for all other trials with increments of 35 W every 3 min. Verbal encouragement was provided to each participant to ensure maximal effort. Samples of expired gas were collected in Douglas bags (Harvard

Apparatus, Edenbridge, UK) during the third minute of each work rate increment and heart rate (HR) was measured continuously using short-range radio telemetry (Polar Beat, Polar Electro Ltd., Oy, Finland). An oxygen/carbon dioxide analyser (Servomex 1400, Crowbridge, UK) was used along with a dry gas meter (Harvard Apparatus, Edenbridge, UK) for the determination of \dot{V}_E , $\dot{V}O_2$ and $\dot{V}CO_2$. From the $\dot{V}O_2$ work rate relationship, the work rates equivalent to 65% $\dot{V}O_{2max}$ (Chapter 4), 50% and 75% $\dot{V}O_{2max}$ (Chapter 7) and 60% and 75% $\dot{V}O_{2max}$ (Chapter 6) for each participant were interpolated.

2.3. Familiarisation trials

In Chapters 4, 5 and 6 participants performed a familiarisation ride prior to their main trials. The purpose of this was to familiarise them with the exercise protocol and to check that the correct relative exercise intensity ($\% \dot{V}O_{2max}$) was being performed. Participants cycled at the specific work rate and amount of time that was to be performed for each of the main trials. Expired gas samples were collected over a 1-min period into Douglas bags after 10 min and 30 min of exercise and then every 30 min thereafter. Heart rate and RPE were also measured every 15 min. Prior to all main trials the participants were familiarised with the saliva collection procedure. This enabled them to feel comfortable with the collection method and to establish individual flow rates and an appropriate collection time to ensure that an adequate volume (~ 1.5 mL) of saliva was collected for analysis.

2.4. Analytical methods

2.4.1. Saliva sampling and analysis

All saliva collections were made with the participants seated, leaning forward and with their heads tilted down. Participants were instructed to swallow in order to empty the mouth before

an unstimulated whole saliva sample was collected over a pre-determined time period into a pre-weighed, sterile vial. Care was taken to allow saliva to dribble into the collecting tubes with eyes open making minimal orofacial movement. Samples were then stored at -80°C until analysis.

2.4.2. Determination of saliva flow rate

Saliva volume was estimated by weighing to the nearest mg and the saliva density was assumed to be 1.0 g.mL^{-1} (Cole and Eastoe, 1988). Saliva flow rate (mL.min^{-1}) was determined by dividing the volume of saliva by the collection time.

2.4.3. Determination of salivary IgA concentration

After thawing, the stored saliva samples were analysed for IgA using a sandwich-ELISA method similar to that described by Gomez *et al.* (1991). Briefly, flat-bottomed microtitration plates (Corning medium affinity plates, Sigma, Poole Dorset) were coated with the primary antibody, rabbit anti-human IgA (I-8760, Sigma, Poole, UK) at a dilution of 1 in 800 in carbonate buffer, pH 9.6. After washing with phosphate buffered saline (PBS, pH 7.2) the plates were coated with blocking protein solution (2% w/v bovine serum albumin in PBS). Sample analysis was performed in duplicate using saliva samples diluted 1 in 1000 with PBS and a range of standards (Human colostrum IgA, I-2636, Sigma) up to 500 mg.L^{-1} . A set of standards was incorporated into each micro-well plate, and all samples from a single subject were analysed on a single plate. The plates were incubated for 90 min at room temperature. Following a washing step, peroxidase-conjugated goat anti-human IgA (A-4165, Sigma) was added and the plate was incubated for a further 90 min at room temperature. Following another washing step, the substrate, ABTS (Boehringer Mannheim, Lewes, UK) was added and after 15 min the absorbance was measured at 405nm. Salivary IgA concentration was

determined by interpolation from the linear standard calibration curve. The intra-assay coefficient of variation for s-IgA concentration was 7.2%.

2.4.4. Determination of α -amylase, cortisol, chromogranin A, lysozyme and osmolality

Alpha-amylase activity was measured using a spectrophotometric method as previously described by Li and Gleeson (2004). Salivary cortisol (DX-SLV-2930, DRG Instruments, Marburg, Germany), Chromogranin A (CgA) (Cosmo Bio Co., Ltd, Japan) and lysozyme (Biomedical Technologies Inc., USA) concentration were analysed using commercially available ELISA kits. Osmolality was determined using a cryoscopic (freezing point depression) osmometer (Osmomat 030, Gonotec, GbBH, Berlin, Germany) calibrated with 300 mOsmol.kg⁻¹ NaCl solution. The intra-assay coefficient of variation for the analytical methods used in all studies combined were 2.4%, 1.3%, 7.9% and 8.2% for α -amylase, cortisol, CgA and lysozyme, respectively.

2.4.5. Determination of secretion rate

The secretion rate of s-IgA, lysozyme, α -amylase and CgA was calculated by multiplying the saliva flow rate by the concentration of the measured analyte.

2.5. Reporting of respiratory illness

In Chapter 8 symptoms of URTI were recorded weekly by means of a questionnaire (Appendix E), adapted from the Wisconsin Upper Respiratory Symptom Survey (WURSS-21; Barrett *et al.*, 2002). This referred to symptoms of URTI exhibited during the previous week. A positive incidence of URTI was recorded when two or more symptoms were exhibited on two consecutive days, at least 1 week apart from a previous episode.

2.6. General statistical methods

All statistical analysis was performed using SPSS 12.0 software for Windows (SPSS Inc., Chicago IL, USA). Data are presented as mean values and standard error of the mean (\pm SEM). Data were checked for normality, homogeneity of variance and sphericity before statistical analysis. If the data were not normally distributed, analysis was performed on logarithmic transformed data. In Chapter 8 data were analysed using a one-factor analysis of variance (ANOVA) with repeated measures design. Chapters 4, 5, 6 and 7 employed a two-factor (trial x timepoint) repeated measures ANOVA. Significant differences were assessed using Student's paired *t*-test with Holm-Bonferroni adjustments for multiple comparisons. Single comparisons between trials were assessed using Student's paired *t*-tests with Holm-Bonferroni adjustments. Statistical significance was accepted at $P < 0.05$.

Chapter 3 - Study 1: Influence of a fed or fasted state on the s-IgA response to prolonged exercise

Abstract

It has been previously suggested that the nutritional status of an individual can influence the levels of salivary immunoglobulin A (s-IgA) at rest, yielding higher and more variable concentrations in fasting saliva compared to non-fasting saliva (Gleeson *et al.*, 2004a). Prolonged, strenuous exercise has been associated with changes in the levels of this antibody (Gleeson *et al.*, 2003a); however the influence of the fed or fasted state on the s-IgA response is unknown. Thus, the present study investigated the effect of a fed or fasted state on the s-IgA response to prolonged exercise. Using a randomised cross-over design, 8 males and 8 females of mixed physical fitness, mean \pm SEM age 22 ± 1 yr, performed 2 h cycling on a stationary ergometer at 65% of their maximal oxygen uptake on one occasion following an overnight fast (FAST) and on another occasion following the consumption of a 2.2 MJ high carbohydrate meal (FED) 2 h before. Timed, unstimulated whole saliva samples were collected immediately before ingestion of the meal, immediately pre-exercise, at 5 min before cessation of exercise, immediately post-exercise and at 1 h post-exercise. The samples were analysed for s-IgA concentration, osmolality and cortisol and saliva flow rates were determined to calculate the s-IgA secretion rate. Carbohydrate oxidation was significantly higher ($P < 0.05$) and fat oxidation tended to be lower ($P = 0.06$) in FED compared with FAST. Saliva flow rate decreased during exercise from 0.50 ± 0.04 mL.min⁻¹ to 0.37 ± 0.03 mL.min⁻¹ ($P < 0.05$), s-IgA concentration increased during exercise from 163 ± 20 to 232 ± 24 mg.L⁻¹ ($P < 0.05$) but the s-IgA secretion rate remained unchanged, pre-exercise: 79 ± 11 μ g.min⁻¹, post-exercise: 74 ± 9 μ g.min⁻¹. There was a significant reduction in the s-IgA:osmolality ratio from 2.3 ± 0.2 pre-exercise to 1.5 ± 0.1 post-exercise ($P < 0.05$).

Salivary cortisol increased from 9.9 ± 1.6 pre-exercise to 16.7 ± 2.9 nmol.L⁻¹ post-exercise ($P < 0.05$). There was no effect of FED versus FAST on these salivary responses. The immediately pre-exercise values of s-IgA were 165 ± 27 and 161 ± 27 mg.L⁻¹ in the FED and FAST trials, respectively. The s-IgA concentration and secretion rate were found to be significantly lower in females than in males across all time points ($P < 0.05$); however, there was no significant difference between genders in the saliva flow rate or the s-IgA:osmolality ratio. These data demonstrate that the nutritional status does not influence resting s-IgA or the s-IgA response to prolonged exercise. Furthermore, these data suggest a significant effect of gender on resting s-IgA levels without affecting the acute response to exercise.

3.1. Introduction

Mucosal secretions protect the oral mucosa via a mechanical washing effect and play an important role in immunity as the first line of defence against potential pathogens invading the oral and nasal cavities (Gleeson and Pyne 2000). The principal antibody present in mucosal fluids is immunoglobulin A (IgA) which functions to inhibit the colonisation of pathogens, bind antigens for transport across the epithelial barrier and neutralise viruses (Lamm, 1998). A reduction in the concentration of s-IgA has been implicated as a causal factor for the reported increased incidence of URTI during heavy training in athletes (Fahlman and Engels, 2005; Gleeson *et al.*, 1999). Indeed, several studies have reported significant reductions in s-IgA levels following an acute bout of intense exercise (Gleeson *et al.*, 1999, Nieman *et al.*, 2002; Steerenberg *et al.*, 1997; Tomasi *et al.*, 1982) and it is thought that these effects are cumulative over time, since s-IgA concentration fell during a 7-month training period, and the pre-training levels were inversely correlated with symptoms of URTI (Gleeson *et al.*, 1999).

The responses of s-IgA to exercise appear to be related to perturbations in sympatho-adrenal activation and resulting increases in circulating stress hormones. Carbohydrate (CHO) ingestion during exercise has been shown to attenuate the rise in stress hormones, whereas an athlete exercising in a CHO-depleted state experiences larger increases in circulating stress hormones (Gleeson *et al.*, 2004b). Bishop *et al.* (2000) demonstrated that the ingestion of a high CHO beverage during exercise helped to maintain saliva flow rate and s-IgA concentration when compared with restricted fluid intake; however, when compared with placebo, no effect of CHO on s-IgA concentration or secretion rate has been observed (Bishop *et al.*, 2000; Nehlsen-Cannarella *et al.*, 2000; Nieman *et al.*, 2002).

The prior nutritional status of an individual may also influence s-IgA. Gleeson and colleagues (1990) reported that s-IgA concentration at rest was higher and more variable in fasting saliva compared with non-fasting saliva. Moreover, Oliver *et al.* (2007) reported that 48 h of energy and fluid restriction resulted in a fall in s-IgA secretion rate.

The effects of nutritional status (fed vs fasted) on the s-IgA response to exercise remains unknown. However, some researchers have studied fasted participants (Bishop *et al.*, 2006; Blannin *et al.*, 1998), while others have studied fed participants (Gleeson *et al.*, 1999; Gleeson *et al.*, 2000; Laing *et al.*, 2005; Walsh *et al.*, 2002). Given the apparent link between s-IgA and nutritional status at rest (Gleeson *et al.*, 1990), understanding the impact of this factor on the s-IgA responses to exercise will have potential methodological considerations for further research studies as well as implications for the elite athlete. Thus, the present study examined the effects of a fed or fasted state on the s-IgA responses to prolonged cycling. A secondary aim was to assess whether gender would influence the levels of s-IgA at rest and during exercise.

3.2. Methods

3.2.1. Participants

Sixteen fit, healthy men and women (8 males and 8 females) volunteered to take part in the study. Their physical characteristics are outlined in Table 3.1. Following a preliminary $\dot{V}O_{2\max}$ test and familiarisation ride, participants completed 2 main trials in a counterbalanced order, on separate occasions separated by at least 1 week.

Table 3.1. Participant Characteristics ($N = 16$).

Variable	Mean (\pm SEM)	
	Males ($n=8$)	Females ($n=8$)
Age (yr)	22 \pm 1	22 \pm 1
Body Mass (kg)	80.6 \pm 5.8	60.0 \pm 0.9 *
Height (cm)	182 \pm 1	165 \pm 1*
$\dot{V}O_{2\max}$ (mL.kg ⁻¹ .min ⁻¹)	51.0 \pm 3.6	39.5 \pm 0.7 *

* Significantly different to Males ($P < 0.05$).

3.2.2. Experimental procedures

Participants reported to the laboratory at 09:00 h following an overnight fast (10-12 h). A resting fingertip blood sample was obtained using an autoclick lancet, and the blood glucose concentration was analysed using an Accutrend GC (Roche, Germany) glucose analyser. This was to confirm the participants were in a fasted state. Mean values were 4.4 ± 0.3 mmol.L⁻¹ on the treatment days. Participants then consumed either: 3 cereal bars and 500 mL of a commercially available sports drink (FED; total energy content: 2210 kJ) or 500 mL of a dilute low calorie cordial drink (FAST; total energy content: 60 kJ). The nutritional content of the breakfast is outlined in Table 3.2. The participants were required to consume the

breakfast within a 15-min time period. They then rested for 1 h 45 min before performing 2 h cycling at 65% $\dot{V}O_{2\max}$ on a stationary cycle ergometer. The order of the trials was randomised. Body mass was measured pre- and post-exercise. Heart rate and RPE were obtained at 15-min intervals during and expired gas samples were collected every 30 min of exercise using a Douglas bag (Harvard Apparatus, Edenbridge, UK). Saliva samples were obtained upon arrival at the laboratory, immediately pre-exercise, 5 min before cessation of exercise, immediately post-exercise and 1 h post-exercise. Water ingestion was permitted *ad libitum* before, during and after exercise with the exception of the 10-min period prior to each saliva sample collection. During both experimental trials, the participants were instructed to remain fasted until the 1 h post-exercise sample had been collected. Fat and carbohydrate oxidation and energy expenditure were calculated by indirect calorimetry using stoichiometric equations (Frayn, 1983) and appropriate energy equivalents, with the assumption that the urinary nitrogen excretion rate was negligible. For the 24 h preceding each trial, the participants were requested to follow the same (pre-trial 1) diet and eating schedule before the subsequent trial. They were also requested to abstain from alcohol, caffeine and heavy exercise for 48 h prior to each trial. The environmental conditions of the laboratory were $25.4 \pm 0.2^\circ\text{C}$ and relative humidity of $41 \pm 2\%$.

Table 3.2. Participant Characteristics ($N = 16$).

Variable	Sports Drink (500 mL bottle)	Cereal bar (37 g)
Energy (kJ)	590	540
Protein (g)	-	4
Carbohydrate (g)	32	67
Fat (g)	-	8
Sodium (mg)	250	250

3.2.3. Saliva collection and analysis

All saliva collections and analyses were performed as described in Chapter 2.

3.2.4. Statistical analysis

For comparison of the effects of nutritional status (FED vs FAST) and gender (males vs females) the data were examined using a 2 (trials) x 5 (times of measurement) repeated measures ANOVA for saliva flow rate, salivary IgA concentration, salivary IgA secretion rate, salivary osmolality and salivary cortisol concentration. Significant differences were assessed using Student's paired *t*-test with Holm-Bonferroni adjustments for multiple comparisons. Physiological variables, RPE and blood glucose were examined using Student's paired *t*-tests. Gender comparisons at pre-exercise in the fasted trial were made using Student's unpaired *t*-tests. Statistical significance was accepted at $P < 0.05$.

3.3. Results

3.3.1. Physiological variables and RPE

Results for exercise intensity ($\% \dot{V}O_{2max}$), HR, RPE and body mass changes are presented in Table 3.3. There were no significant differences between trials in any of the physiological variables.

Table 3.3. Mean (\pm SEM) physiological and RPE values obtained during each trial ($N = 16$).

	FED	FAST
% $\dot{V}O_{2max}$	65.8 (0.7)	65.1 (0.9)
HR (beats.min ⁻¹)	154 (3)	151 (3)
RPE	12 (1)	12 (1)
Body Mass Change (kg)	-0.2 (0.2)	-0.2 (0.2)
Fluid Intake (mL)	1203 (120)	1270 (155)
Fluid Loss (L)	1.41 (0.16)	1.49 (0.18)

Carbohydrate oxidation was significantly higher ($P = 0.019$) and fat oxidation tended to be lower ($P = 0.060$) in FED compared with FAST. Mean rate of energy expenditure was similar in both trials: FED: 44.9 ± 3.7 kJ.min⁻¹, FAST: 43.3 ± 3.4 kJ.min⁻¹.

Table 3.4. Mean (\pm SEM) substrate oxidation rates and contribution to energy expenditure obtained during each trial. ($N = 16$).

	FED	FAST
Carbohydrate oxidation (g.min ⁻¹)	2.26 (0.25) *	1.92 (0.19)
Fat oxidation (g.min ⁻¹)	0.22 (0.05)	0.32 (0.03)
Energy derived from CHO (kJ.min ⁻¹)	36.2 (4.0) *	30.8 (3.1)
Energy derived from Fat (kJ.min ⁻¹)	8.8 (1.8)	12.5 (1.2)
% contribution from CHO	79 (5)	70 (3)
% contribution from Fat	21 (5)	30 (3)

* Significantly different from FAST ($P < 0.05$).

3.3.2. Salivary variables

Gender differences

There were no effects of gender on the salivary responses to exercise. However, salivary IgA concentration, IgA secretion rate and osmolality were found to be significantly lower in females compared with males ($P < 0.05$; Table 3.5). There were no significant differences between genders in saliva flow rate and s-IgA:Osmolality ratio.

Table 3.5. Effect of gender on mean salivary variables at baseline. Means (\pm SEM); Males $N = 8$, Females $N = 8$.

	Males	Females
Saliva flow rate (mL.min ⁻¹)	0.39 (0.45)	0.36 (0.71)
S-IgA concentration (mg.L ⁻¹)	224 (38)	116 (20) **
S-IgA secretion rate (ug.min ⁻¹)	85 (13)	38 (9) *
Osmolality (mOsmol.kg ⁻¹)	94 (11)	47 (5) **
S-IgA:Osmolality	2.4 (0.3)	2.5 (0.4)

* Significantly different compared with Males ($P < 0.05$; ** $P < 0.01$).

Saliva Flow rate

Saliva flow rate showed an initial increase from baseline to pre-exercise. Saliva flow rate then decreased significantly during exercise and returned to baseline at 1 h post-exercise (main effect of time: $F_{3, 46} = 13.928$, $P < 0.001$). There was no effect of FED or FAST on this response (Figure 3.1).

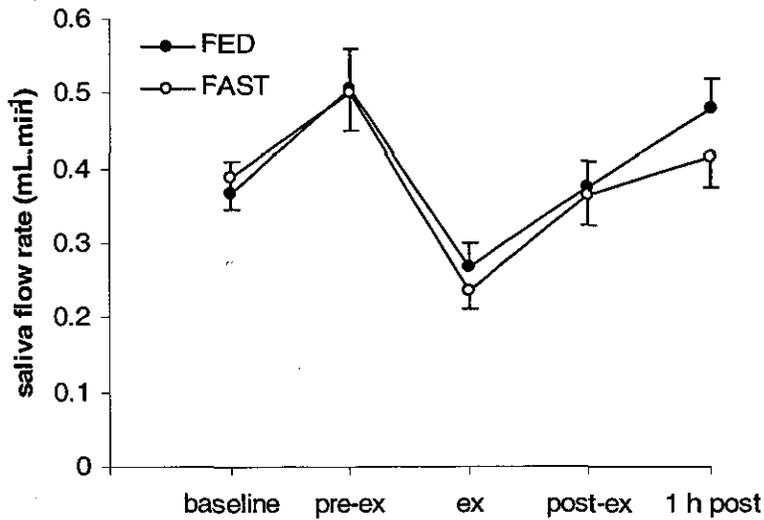


Figure 3.1. The effect of a fed (FED) or fasted (FAST) state on the saliva flow rate response to exercise. Values are mean \pm SEM ($N = 16$).

Salivary IgA concentration

Salivary IgA concentration increased with exercise (main effect of time: $F_{3, 39} = 8.226$, $P < 0.001$), but there was no effect of FED or FAST on this response (Figure 3.2).

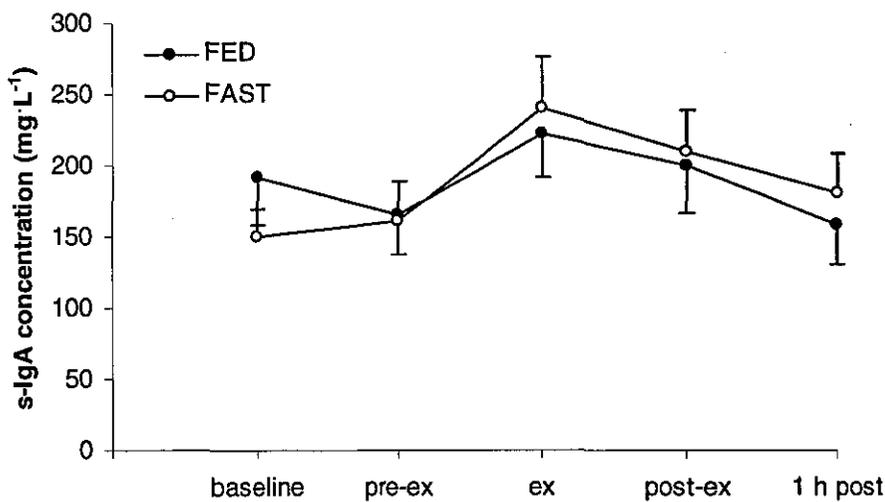


Figure 3.2. The effect of a fed (FED) or fasted (FAST) state on the s-IgA concentration response to exercise. Values are mean \pm SEM ($N = 16$).

Salivary IgA secretion rate

The s-IgA secretion rate did not change significantly throughout the exercise protocol (Figure 3.3).

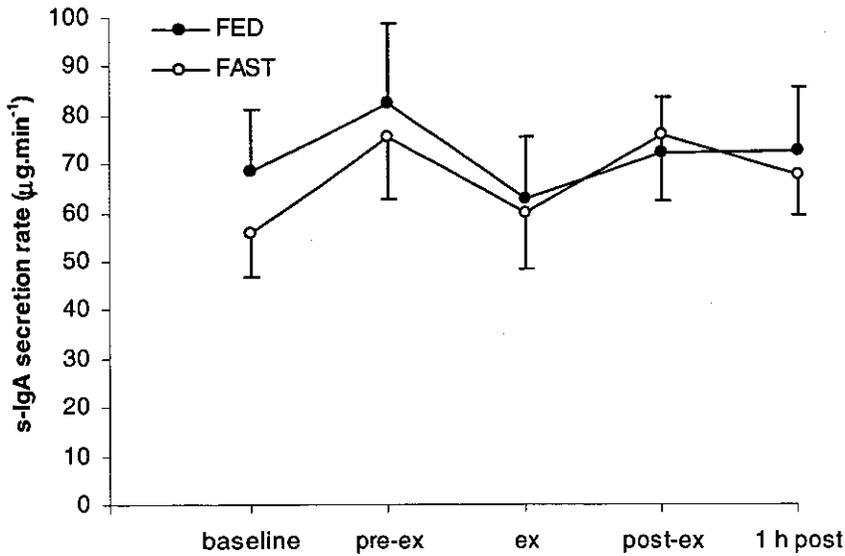


Figure 3.3. The effect of a fed (FED) or fasted (FAST) state on the s-IgA secretion response to exercise. Values are mean \pm SEM ($N = 16$).

Salivary osmolality

Salivary osmolality increased with exercise (main effect of time: $F_{1,21} = 19.587$, $P < 0.001$).

Salivary IgA:Osmolality ratio decreased post-exercise (main effect of time: $F_{3,44} = 13.830$, $P < 0.001$). There was no effect of FED or FAST on these responses (Table 3.6).

Table 3.6. Mean (\pm SEM) osmolality values and s-IgA:Osmolality ratio obtained during each trial ($N = 16$).

	Baseline	Pre-ex	Ex	Post-ex	1 h post-ex
Osmolality (mOsmol.kg ⁻¹)					
FED	77 (12)	71 (8)	144 (21)	148 (23)	69 (9)
FAST	64 (6)	66 (8)	152 (25)	152 (24)	73 (11)
s-IgA- to-osmolality ratio					
FED	2.6 (0.3)	2.2 (0.2)	1.7 (0.2)	1.5 (0.2)	2.4 (0.4)
FAST	2.4 (0.3)	2.4 (0.3)	1.7 (0.2)	1.5 (0.2)	2.7 (0.3)

Values are means \pm SEM (FED, FAST, $N = 16$).

Salivary cortisol

Salivary cortisol increased post-exercise compared with pre-exercise (main effect of time: $F_{1,6} = 6.856$, $P = 0.040$). There was no effect of FED or FAST on this response (Table 3.7).

Table 3.7. Mean (\pm SEM) cortisol (nmol.L⁻¹) values obtained during each trial ($N = 16$).

	Pre-ex	Post-ex
FED	10.44 (2.16)	16.65 (3.74)
FAST	9.35 (1.44)	16.60 (3.82)

3.4. Discussion

The primary aim of this study was to examine the influence of a fed or fasted state on the s-IgA response to prolonged cycling. This was based on previous reports from an Australian laboratory that fasting saliva samples yield higher and more variable s-IgA concentrations than non-fasting samples (Gleeson *et al.*, 1990; Gleeson *et al.*, 2004a). A secondary aim was to examine the effects of gender on the s-IgA responses, since this had not previously been investigated under controlled laboratory conditions.

It has been previously suggested that fasting saliva samples yield higher and more variable s-IgA concentrations than non-fasting samples at rest (Gleeson *et al.*, 1990; Gleeson *et al.*, 2004a). The present findings do not support this idea since none of the salivary parameters were affected by the pre-exercise nutritional status. Furthermore, the spread of the data – indicated by the SEM - for this group of individuals under the two conditions were very similar.

It is possible that the differences in s-IgA between fed and fasted states that have been previously reported (Gleeson *et al.*, 1990) may be related to the amount of fluid ingested, since this can affect the saliva flow rate and resulting s-IgA concentration (Bishop *et al.*, 2000). Gleeson and colleagues (1990) did not report the saliva flow rate or whether fluid ingestion in the fasted state was controlled thus, the increase in s-IgA concentration observed may have been due to lack of fluid intake and dehydration. In the present study the quantity of fluid ingested before exercise was the same between conditions (500 mL); however, had this not been equal, it is possible that higher and more variable samples would result.

In addition, it is possible that the timing of the meal is important in determining the s-IgA response. The present study provided a high CHO meal 2 h before commencing the exercise as this is what is typically recommended for individuals engaging in physical activity (Williams and Serratos, 2006). However, since the timing of meals was not reported in the previously cited study, a direct comparison cannot be made. Thus, future studies may attempt to address this. Nevertheless, from a methodological point of view, what these results suggest is that exercise performed in either a fed or fasted state (of approximately 10 h) would have little influence on the response pattern or variability of s-IgA.

The present results show a significant reduction (~ 50%) in saliva flow rate with exercise returning to baseline levels at 1 h post-exercise. A decrease in saliva flow rate with exercise is a consistent finding reported in many studies (Steerenberg *et al.*, 1997; Mackinnon and Jenkins, 1993; Walsh *et al.*, 2002; Blannin *et al.*, 1998). Salivary secretion is predominantly under the control of the autonomic nervous system (Chicharro *et al.*, 1998) and is dependent on both parasympathetic cholinergic nerves and sympathetic adrenergic nerves. It has been previously suggested that the reduction in saliva flow rate during exercise is a result of an increase in SNS activity inducing vasoconstriction in the arterioles supplying the salivary glands (Chicharro *et al.*, 1998). However, since only sympathetic secretomotor, rather than vasoactive nerve fibres are activated under reflex conditions (Proctor and Carpenter, 2007), this would suggest that the exercise-induced reduction in saliva flow rate is related to a removal of parasympathetic vasodilatory influences. Indeed, the saliva flow rate was lowest at the time point during exercise when parasympathetic activity would be largely inhibited, and this started to recover upon cessation of exercise when parasympathetic innervation would return. Although parasympathetic nervous system activity was not directly examined in the present study, it is likely that the exercise intensity was sufficient to affect this activity

and based on this assumption; it is not surprising that significantly lower saliva flow rates were observed during exercise.

Dehydration can also impact negatively on saliva flow rate (Bishop *et al.*, 2002; Walsh *et al.* 2004), which usually occurs following body mass losses of ~3% (Walsh *et al.* 2004). In the present investigation, fluid intake was permitted *ad libitum*, which resulted in small body mass losses (~0.5%) despite this, a decrease in saliva flow rate and an increase in osmolality was still observed. These findings contrast Bishop *et al.* (2000) and Walsh *et al.* (2004), where fluid intake (to match pre-determined sweat losses in the latter study) prevented the decrease in flow rate and increase in osmolality. This suggests a greater effect of neural activation on saliva electrolyte secretion and flow rate during exercise rather than dehydration *per se*. These findings may question the reliability of salivary osmolality as an indicator of hydration status post-exercise.

Such a reduction in saliva flow rate can impair the mechanical washing effect of micro-organisms from the oral cavity and may also limit the availability of salivary antimicrobial proteins such as s-IgA. This is supported by findings showing that people suffering from xerostomia (dry mouth syndrome) have a substantially increased incidence of oral infections and more pathogenic bacteria in the buccal cavity (Fox *et al.*, 1985). It is therefore possible that a reduction in saliva flow rate resulting from exercise may lead to an increased risk of developing URTI.

It is noteworthy that an initial increase in the saliva flow rate from baseline to the pre-exercise time point (2 h after) was observed. This could be due to a diurnal variation since saliva flow rate increases throughout the day (Dawes, 1974). On the other hand, it may relate to an anticipatory increase in SNS activity and inhibition of parasympathetic innervation,

thereby reducing the saliva flow rate (Blannin *et al.*, 1999), which should be considered if a true baseline measure is to be obtained.

Salivary IgA concentration increased significantly with exercise and this is in accordance with many studies involving prolonged cycling (Blannin *et al.*, 1998; Walsh *et al.*, 2002; Li and Gleeson, 2004). However, it is likely that these increases are a result of the reduction of saliva flow rate and dehydration, commonly reported following sub-maximal cycling, rather than genuine alterations in the mucosal immune response (Walsh *et al.*, 2002).

Significant reductions in s-IgA secretion rate post-exercise have been previously reported following prolonged cycling (Laing *et al.*, 2005; Mackinnon *et al.*, 1993; Walsh *et al.*, 2002), though others have reported no change (Li and Gleeson, 2004). Although s-IgA secretion rate did not change significantly with exercise in the present study, a 25% reduction was observed during exercise compared with pre-exercise. However, whether this trend has any physiological significance in terms of URTI risk is yet to be determined.

A further method of expressing s-IgA is as a ratio to osmolality. This is worthwhile since unlike the total protein, osmolality (electrolyte) secretion rate does not change with exercise (Blannin *et al.*, 1998). Correcting s-IgA to osmolality ratio is recommended where significant changes in flow rate are observed (Blannin *et al.*, 1998). Few researchers have expressed s-IgA as a ratio to osmolality; of those who have, some have reported decreases (Laing *et al.*, 2005), while others reported no change (Walsh *et al.*, 2002; Sari-Sarraf *et al.*, 2007b). A clear reduction in the s-IgA:Osmolality was observed post-exercise in the present study. This lends some support to a transient depression of mucosal immunity post-exercise, which returns to baseline levels following 1 h of recovery. However, since significant reductions in the other

measures of s-IgA were not demonstrated, this highlights the continual discrepancies of which measure to use when interpreting the effects of exercise on mucosal immune function.

Carbohydrate (CHO) has been shown to attenuate the cortisol response during exercise and it has been shown that both CHO and fluid intake during prolonged sub-maximal exercise are important in maintaining the saliva flow rate and s-IgA concentration compared with restricted fluid intake (Bishop *et al.*, 2000). The present study sought to examine the effect of a high CHO meal ingested 2 h before exercise on the salivary responses compared with a control low-energy beverage. Significant increases in salivary cortisol were observed post-exercise; however, no significant effects of treatment on salivary cortisol were observed. Since alterations in mucosal immune function during exercise have been previously attributed to the elevation of stress hormones including cortisol, it is perhaps not surprising that no differences in the s-IgA responses were observed between the two conditions.

Lower values in resting s-IgA concentration in females compared with males have been previously demonstrated in elite swimmers (Gleeson *et al.*, 1999); as well as the general population (Evans *et al.*, 2000). However, no previous study has reported whether this has an effect on the s-IgA response to acute exercise. Striking differences between genders were found in many of the salivary parameters measured. No differences in saliva flow rate were found between genders; however, significantly higher values of s-IgA concentration were observed in the males compared with the females. This is in accordance with the above findings (Evans *et al.*, 2000; Gleeson *et al.*, 1999); however, these researchers did not measure the s-IgA secretion rate or salivary osmolality. Our results extend these findings since higher values in these measures were also observed in the male group. Although there was no effect of gender on the acute response to exercise, differences in resting s-IgA levels between groups would have practical importance if exercise scientists are to use this

parameter as a predictor of infection risk. If laboratories are to establish clinically significant ranges, such differences would need to be taken into consideration.

The physiological significance of the observed gender differences are yet to be elucidated. It is known that sex hormones play an important role in the immune system at rest (Timmons *et al.*, 2005). However, Burrows *et al.*, (2002) found no differences in s-IgA concentration, s-IgA secretion rate or saliva flow rate in a group of highly trained female endurance athletes over three consecutive menstrual cycles. Moreover, there was no relationship between s-IgA concentration and progesterone. It is possible that the differences relate to the absolute training status of the participants (weight-specific $\dot{V}O_{2max}$ was 20% lower in females), as it has been previously shown that s-IgA concentration is higher in elite athletes compared with both active and non-active individuals (Francis *et al.*, 2005). Finally, the diet of the participants may have differed between genders and thus, it is not known whether the differences were related to deficiencies in certain nutrients such as protein (Chandra, 1997; Gleeson *et al.*, 2004a). Given that females appear to be more resistant to viral infections than males in the general population (Beery, 2003), further research is required to investigate s-IgA in different athletic populations to determine how it might be important in infection risk.

In summary, the results from the present study show that prior nutritional status does not affect resting s-IgA levels, or their acute response to exercise. Additionally, these results demonstrate significant gender differences in resting s-IgA levels with no effect on the acute s-IgA response to exercise.

Chapter 4 - Study 2: Effect of feeding versus fasting during prolonged exhaustive exercise on s-IgA, lysozyme and α -amylase

Abstract

The purpose of the present study was to investigate the effect of feeding versus fasting during prolonged exhaustive exercise on salivary antimicrobial proteins. Using a randomised cross-over design and following a 10 – 12 h overnight fast, 24 fit healthy men (mean \pm SEM: age 23 ± 1 yr; height 179 ± 2 cm; body mass 73.8 ± 1.6 kg; $\dot{V}O_{2\max}$ 56.6 ± 1.0 mL.kg⁻¹.min⁻¹) cycled for 2.5 h at 60% $\dot{V}O_{2\max}$ (with regular water ingestion) and then cycled to exhaustion at 75% $\dot{V}O_{2\max}$. Immediately before exercise, and at 55 min and 115 min during the 60% $\dot{V}O_{2\max}$ cycle participants received a placebo beverage of 300 mL of artificial sweetened and flavoured water (FAST) or a commercially available high CHO cereal bar intended for nutritional support of athletes (FED). Timed, unstimulated saliva samples were collected immediately before exercise, mid-exercise, at the end of the 2.5 h moderate exercise bout and immediately after the exhaustive exercise bout. The samples were analysed for s-IgA concentration, salivary lysozyme, α -amylase and cortisol and the saliva flow rates were determined to calculate the secretion rates. Time to exhaustion was 44% longer in FED compared with FAST ($P < 0.05$). Saliva flow rate did not change significantly during the exercise protocol. Exercise was associated with increases in the secretion rates of s-IgA, lysozyme and α -amylase ($P < 0.01$). Feeding during exercise caused a higher secretion rate of lysozyme (43% higher) and α -amylase (15% higher) compared with fasting ($P < 0.05$), but the s-IgA levels but were not different between the two treatments. Salivary cortisol increased post-exhaustion in the FAST trial only ($P < 0.05$). Increases in lysozyme and α -amylase suggest that ingesting a high CHO cereal bar during prolonged exhaustive exercise may acutely enhance oral immune protection.

4.1. Introduction

Nutritional strategies are commonly employed by athletes not just to improve performance, but also to modify the exercise-induced immune response. Carbohydrate (CHO) ingestion before and during intense prolonged exercise has emerged to date as the most effective measure to attenuate the stress response which appears to limit the degree of immunodepression (Nieman, 2007). Previous research has examined the influence of CHO ingestion compared with placebo on the s-IgA response to prolonged exercise, but such studies have shown little effect (Bishop *et al.*, 2000; Nehlsen-Cannarella *et al.*, 2000; Nieman *et al.*, 2002). In Chapter 4 it was shown that the feeding of a high CHO meal 2 h before exercise did not affect s-IgA. Moreover, Nieman *et al.* (2002) showed s-IgA concentration and secretion rate were unaffected by the consumption of a CHO beverage during exercise. However, in this case CHO was administered in the form of a beverage rather than as a meal. This could be significant in terms of mucosal immunity since eating elicits a large increase in salivary flow over and above resting levels (Hector and Linden, 1999), and mastication (chewing) has been shown to temporarily enhance the secretion of s-IgA into saliva (Proctor and Carpenter, 2001).

Salivary IgA is only one of the many protective proteins secreted in the fluids covering the mucosa. Other secretory proteins important in mucosal defence include lysozyme and amylase. They are part of the innate immune system, produced locally in the mucous membranes and have antimicrobial and antiviral properties (Tenovuo, 1998). Previous research has shown that salivary lysozyme levels may be affected by psychological stress, where an inverse correlation between the perceived stress response (by means of a questionnaire) to an undergraduate examination and lysozyme levels was reported (Perera *et al.*, 1997). The relationship between exercise and lysozyme is unclear at present although one

study reported decreased lysozyme concentration and secretion rate post-exercise in elite swimmers (Koutedakis *et al.*, 1996). Generally it is accepted that α -amylase increases with intense exercise (Bishop *et al.*, 2000; Li and Gleeson, 2004; Walsh *et al.*, 1999), and that this effect is related to the exercise intensity (Ljungberg *et al.*, 1997). However, the influence of feeding versus fasting during exercise on these secretory proteins is presently unknown.

Thus, the aims of the present study were to investigate the effect of ingestion of a commercially available high CHO cereal bar compared with a control low energy, artificially sweetened beverage during prolonged exhaustive exercise on salivary antimicrobial proteins and exercise capacity.

4.2. Methods

4.2.1. Participants

Twenty four trained male volunteers (mean \pm SEM: age 23 ± 1 yr; height 179 ± 2 cm; body mass 73.8 ± 1.6 kg; $\dot{V}O_{2\max}$ 56.6 ± 1.0 mL.kg⁻¹.min⁻¹; HRmax 190 ± 2 beats.min⁻¹) volunteered to participate in the study. Participants who regularly engaged in a high level of exercise (2 h per day at least three times per week), with cycling as one of their main sports, were recruited to participate. Following preliminary measurements, participants completed 2 main trials, in a counterbalanced order, which were separated by at least 1 week.

4.2.2. Experimental procedures

Participants were asked to abstain from alcohol and chocolate intake for the 24 h prior to each visit to the lab and to refrain from strenuous exercise for two days prior to each visit. Participants completed a food diary for the 48 h period prior to the familiarisation trial and

were required to follow the same diet during the 48 h prior to each of the 2 main trials. The participants arrived at the laboratory at 8:30 h following an overnight fast (10-12 h). They were asked to sit quietly for 5 min, before giving a saliva sample. Immediately after the saliva collection a blood sample was obtained from an ear lobe, using an autoclick lancet, and the blood glucose concentration was determined using an Accutrend GC (Roche, Germany) blood glucose analyser. The participants were then asked to empty their bladders before body mass was measured wearing their shorts only. Participants then consumed either FED: a commercially available coconut flavoured cereal bar (PowerbarTM, Nestle, Switzerland) consisting of carbohydrate (44.9 g), protein (5.4 g), fat (3.2 g), fibre (4 g), sodium (0.6 g), vitamins and minerals or FAST: a placebo beverage of 300 mL of artificial sweetened and flavoured water intended to not provide energy or nutritional ingredients. The participants were required to consume the food and drink within a 5-min time period. The order of the trials was randomised. They then performed 2.5 h cycling at 60% $\dot{V}O_{2max}$ on a stationary cycle ergometer. Experimental supplementation of either the 300 mL of the beverage or cereal bar was administered at 55 and 115 min. In addition, 200 mL of water was consumed at the beginning of exercise and every 20 min during the exercise bout. Heart rate and RPE were measured at 20 min intervals and expired gas was collected at 30 min, 90 min and 114 min of exercise using a Douglas bag (Harvard Apparatus, Edenbridge, UK). Ear lobe blood samples for the analysis of blood glucose were obtained at 65 and 125 min during the exercise and saliva samples were collected at 70 min and 130 min. It was ensured that fluid was not consumed during the 10 min before each saliva collection. Fat and carbohydrate oxidation and energy expenditure were calculated by indirect calorimetry using stoichiometric equations (Frayn, 1983) and appropriate energy equivalents, with the assumption that the urinary nitrogen excretion rate was negligible. Following completion of the 2.5 h cycling, the participants were given a 5-min rest before performing the ride to exhaustion trial at 75% $\dot{V}O_{2max}$. Participants were instructed to maintain a pedal cadence of

more than 50 rpm while cycling, to remain seated at all times and to attempt to cycle for as long as possible. However, no form of verbal encouragement or information on time elapsed were given. Heart rate was recorded every 5 min during the exhaustion ride.

Immediately after completion of the exercise to exhaustion, a final saliva sample was obtained and an earlobe blood sample was collected. Finally, body mass was measured again in shorts only. Mean laboratory temperature and humidity during the trials were 23 ± 0.5 °C and 32 ± 4 %, respectively.

4.2.3. Saliva analysis

All saliva collections and analyses were performed as described in Chapter 2.

4.2.4. Statistical analysis

A two-way ANOVA (2 conditions x 4 sample times) with repeated measures design was used to examine the salivary data. Significant differences were assessed using Student's paired *t*-tests with Holm-Bonferroni adjustments for multiple comparisons. Physiological variables, blood glucose and RPE were examined using Students paired *t*-tests. Statistical significance was accepted at $P < 0.05$.

4.3. Results

4.3.1. Physiological variables and RPE

Values for physiological variables and RPE are presented in Table 4.1. The mean time to exhaustion was significantly longer in FED (1294 ± 141 s) compared with FAST (897 ± 122 s; $P < 0.05$). There were no significant differences between trials for % $\dot{V}O_{2max}$, HR, RPE and fluid loss, although body mass loss was greater in FED compared with FAST ($P < 0.05$). Blood glucose was significantly higher on FED trial compared with FAST ($P = 0.005$).

Table 4.1. Effect of a fed (FED) or fasted (FAST) state on physiological variables and RPE during steady state exercise. Blood glucose measurements were made at 65 min and 125 min during exercise. Values are mean \pm SEM ($N = 24$).

	FED	FAST
% $\dot{V}O_{2max}$	58.8 (0.53)	59.0 (0.48)
HR (beats.min ⁻¹)	143 (3)	141 (2)
RPE	12 (1)	12 (1)
Body mass loss (kg)	0.90 (0.92)	0.53 (0.10) *
Food intake (kg)	0.18 (0.00)	0 (0.00)
Fluid intake (L)	1.47 (0.08)	1.97 (0.08) *
Fluid loss (L)	2.55 (0.10)	2.50 (0.11)
Glucose (mM)	4.3 (0.1)	3.9 (0.1)

* Significantly different to FED ($P < 0.001$).

Carbohydrate oxidation was significantly higher ($P < 0.001$) and fat oxidation was significantly lower ($P < 0.001$) in FED compared with FAST during the steady state exercise (Table 4.2). Mean rate of energy expenditure was similar in both trials: FED: 51.8 ± 1.0 kJ.min⁻¹, FAST: 50.9 ± 0.9 kJ.min⁻¹ (Table 4.2).

Table 4.2. Mean (\pm SEM) substrate oxidation rates and contribution to energy expenditure obtained during each trial. ($N = 24$).

	FED	FAST
Carbohydrate oxidation ($\text{g}\cdot\text{min}^{-1}$)	2.47 (0.08) *	2.08 (0.07)
Fat oxidation ($\text{g}\cdot\text{min}^{-1}$)	0.31 (0.03) *	0.45 (0.02)
Energy derived from CHO ($\text{kJ}\cdot\text{min}^{-1}$)	39.52 (1.26) *	33.26 (1.10)
Energy derived from Fat ($\text{kJ}\cdot\text{min}^{-1}$)	12.23 (1.18) *	17.69 (0.94)
% contribution from CHO	76 (2) *	65 (2)
% contribution from Fat	24 (2) *	35 (2)

* Significantly different from FAST ($P < 0.001$).

4.3.2. Salivary variables

Saliva flow rate

Saliva flow rate was similar between trials and did not change significantly during the exercise protocol (Figure 4.1). A trend for a higher saliva flow rate in FED compared with FAST approached significance ($P = 0.061$).

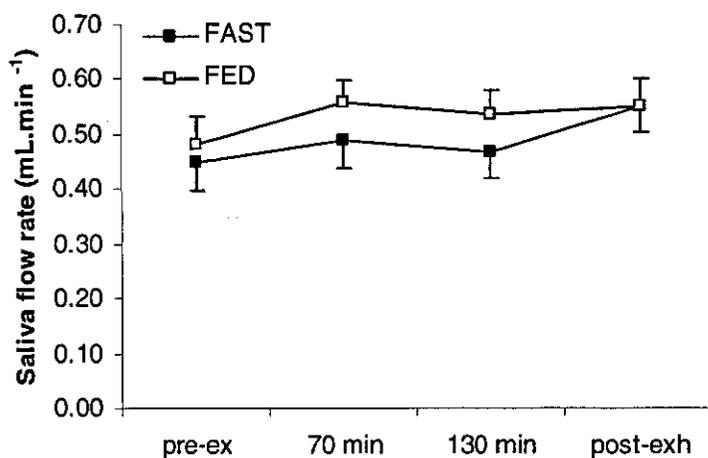


Figure 4.1. Effect of a fed (FED) or fasted (FAST) state on saliva flow rate. Values are mean \pm SEM ($N = 24$).

S-IgA concentration

Salivary IgA concentration increased with exercise between 70 min and post-exh (main effect of time: $F_{2,47} = 3.732$, $P = 0.031$). Although not significant (main effect of treatment: $P = 0.091$), higher levels of mean s-IgA concentration in FAST compared with FED were observed across all time points (Figure 4.2).

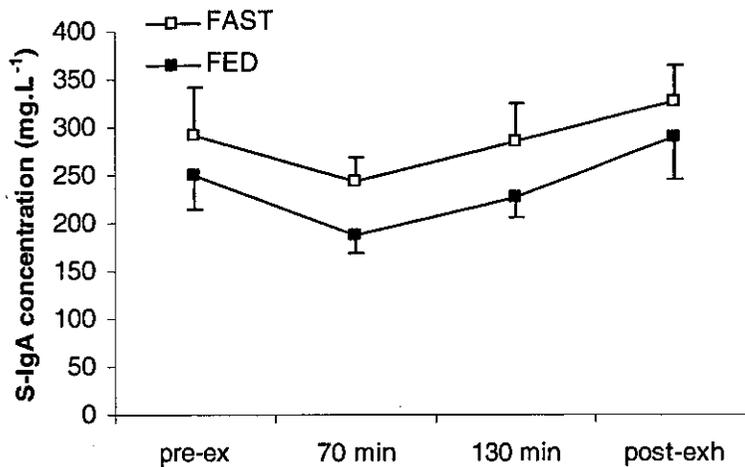


Figure 4.2. Effect of a fed (FED) or fasted (FAST) state on s-IgA concentration. Values are mean \pm SEM ($N = 24$).

S-IgA secretion rate

S-IgA secretion rate increased with exercise duration (main effect of time: $F_{2,50} = 7.413$, $P = 0.001$), but there were no differences between treatments (Figure 4.3).

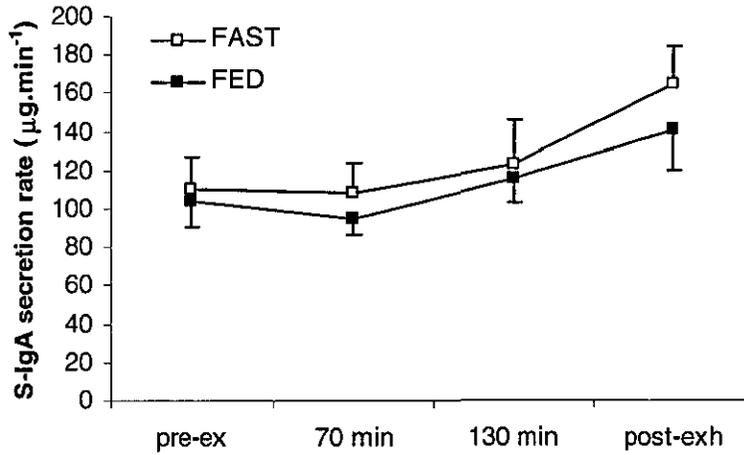


Figure 4.3. Effect of a fed (FED) or fasted (FAST) state on s-IgA secretion rate. Values are mean \pm SEM ($N = 24$).

Lysozyme concentration

Salivary lysozyme concentration increased with exercise duration (main effect of time: $F_{2,27} = 28.164$, $P < 0.001$), and was significantly higher in FED compared with FAST across all time points (main effect of treatment: $F_{1,11} = 9.107$, $P = 0.012$; Figure 4.4).

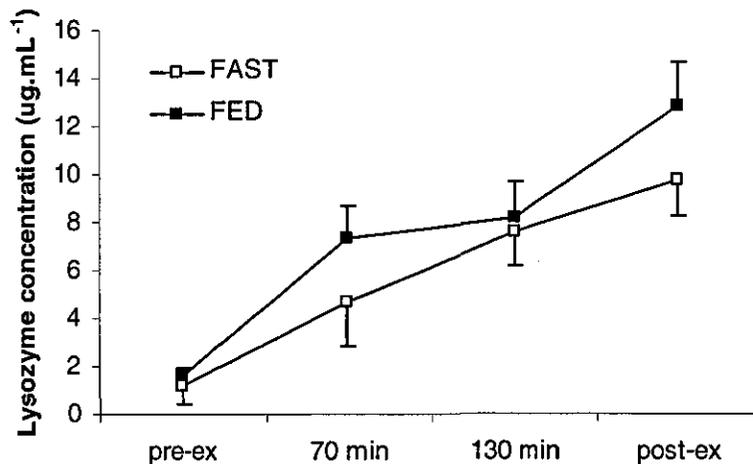


Figure 4.4. Effect of a fed (FED) or fasted (FAST) state on lysozyme concentration. Values are mean \pm SEM ($N = 24$).

Lysozyme secretion rate

Lysozyme secretion rate increased with exercise duration (main effect of time: $F_{2, 20} = 18.378$, $P < 0.001$) and was significantly higher in FED compared with FAST across all time points (main effect of treatment: $F_{1, 11} = 7.496$, $P = 0.019$; Figure 4.5)

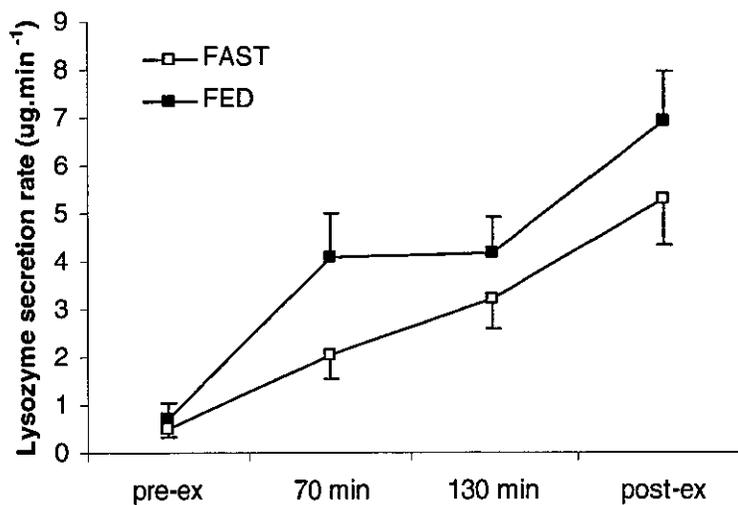


Figure 4.5. Effect of a fed (FED) or fasted (FAST) state on lysozyme secretion rate. Values are mean \pm SEM ($N = 24$).

Salivary α -amylase activity

Salivary α -amylase activity increased with exercise duration (main effect of time: $F_{2, 42} = 96.388$, $P < 0.001$). There was also a time \times trial interaction in α -amylase ($F_{2, 56} = 10.254$, $P < 0.001$), where salivary α -amylase was significantly higher in FED compared with FAST at the 70 min time point; Figure 4.6).

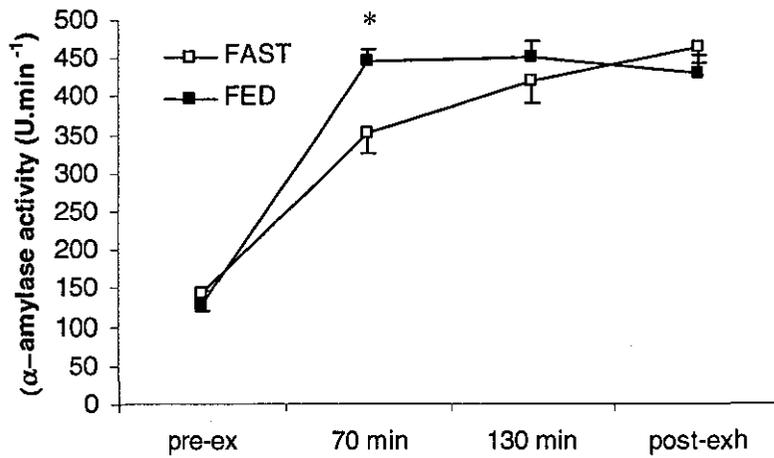


Figure 4.6. Effect of a fed (FED) or fasted (FAST) state on α -amylase activity. Values are mean \pm SEM ($N = 24$). * Significantly higher than FAST at that time point ($P < 0.01$).

Salivary α -amylase secretion rate

Salivary α -amylase secretion rate increased with exercise duration (main effect of time: $F_{2, 50} = 44.486$, $P < 0.001$). There was also a trial \times time interaction in amylase secretion rate ($F_{2, 53} = 16.414$, $P < 0.001$), where it was higher in FED compared with FAST at the 70 min and 130 min time points (Figure 4.7).

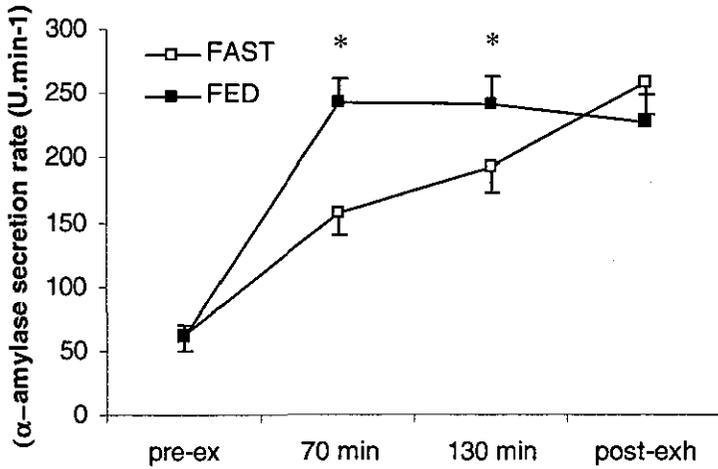


Figure 4.7. Effect of a fed (FED) or fasted (FAST) state on α -amylase secretion rate. Values are mean \pm SEM ($N = 24$). * Significantly higher than FAST at that time point ($P < 0.001$).

Salivary cortisol

Salivary cortisol increased post-exercise in the FAST trial only (interaction: $F_{2,24} = 5.023$, $P = 0.013$; Figure 4.8).

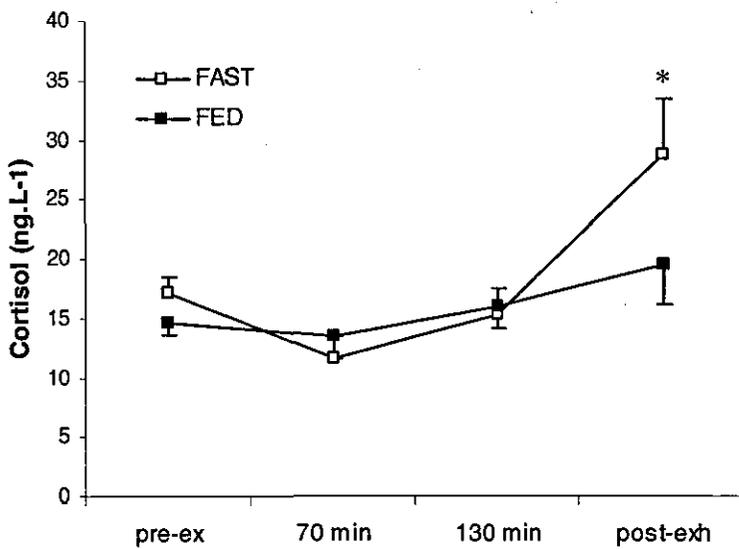


Figure 4.8. Effect of a fed (FED) or fasted (FAST) state on salivary cortisol. Values are mean \pm SEM ($N = 24$). * Significantly higher than 70 min and 130 min ($P < 0.05$).

4.4. Discussion

The main findings of the present study were that feeding a high CHO cereal bar during prolonged exhaustive cycling increased exercise capacity, attenuated the cortisol response but had no significant effect on s-IgA. In contrast, lysozyme and α -amylase were significantly higher in FED compared with FAST.

It was previously reported that fasting saliva yields higher levels of s-IgA concentration than non-fasting saliva at rest (Gleeson *et al.*, 2004a). However, the results in Chapter 4 did not support this finding since the feeding of a high CHO meal 2 h before exercise had no effect on the s-IgA response. It was speculated that the timing of the meal might explain in part these inconsistencies, and hence the present study sought to examine the effect of feeding *during* exercise on these responses. Although there was no significant main effect of treatment in s-IgA ($P = 0.091$), a definite trend can be seen in that mean s-IgA concentration was consistently higher in FAST compared with FED across all time points; a pattern similar to that reported by Gleeson and colleagues (1990). It is likely that the higher s-IgA concentration in FAST was a result of the concomitant reduction in saliva flow rate, since no differences in s-IgA secretion rate were observed between treatments. These results suggest that the timing of the meal may be an important factor when investigating the effect of nutritional status on s-IgA concentration at rest and during exercise.

The present study found no significant changes in saliva flow rate with exercise. This is in contrast to the majority of published research, where reductions have often been reported in response to prolonged exercise (Bishop *et al.*, 2000; Li and Gleeson 2005; Walsh *et al.* 2002; Sari-Sarraf *et al.*, 2007b). Although a withdrawal of parasympathetic activity is thought to be

responsible for the reduction in saliva flow rate during exercise (Proctor and Carpenter, 2007), it has been argued that it is the hydration status of the individual that has the greatest impact (Walsh *et al.*, 2004). This group found a significant reduction in saliva flow rate during exercise when fluid intake was restricted; but when fluid provision was sufficient to offset fluid losses, no differences were observed. Our results corroborate this, since 200 mL of water was provided every 20 min which resulted in only small changes in body mass (~1%), and suggests that this practice is successful in maintaining the saliva flow rate during prolonged exercise.

A significant increase in s-IgA secretion rate was observed post-exercise. This is in contrast to research in trained individuals where decreases (Laing *et al.*, 2005; Walsh *et al.*, 2002) or no change (Li and Gleeson, 2004) in s-IgA secretion rate have been previously reported in response to prolonged cycling. However, since the s-IgA concentration did not show a decrease in these studies, it is likely that the observed decrease in secretion rate post-exercise was a result of the decrease in saliva flow rate.

It has been proposed that elevated levels of the stress hormone cortisol can depress certain aspects of immune function (Nieman, 2007), and it is thought to play a role in inhibiting s-IgA mobilisation and/or production (Hucklebridge *et al.*, 1998). In the present study, salivary cortisol was measured as a surrogate marker of the levels in blood (Chicharro *et al.*, 1998). A significant increase in cortisol was observed post-exhaustion in the FAST trial only, suggesting that ingestion of the cereal bars was successful in attenuating the cortisol response. Despite the higher cortisol concentration in the FAST trial, no inhibitory effect on s-IgA levels occurred. Although there was no inhibition of s-IgA in the short-term, this does not discount an involvement of cortisol in the longer-term, since previous studies have

reported an exercise-induced fall in s-IgA concentration between 2 and 24 h following particularly long bouts of exercise (Mackinnon *et al.*, 1987; Gleeson *et al.*, 2001).

In contrast to the results for s-IgA, feeding a high CHO cereal bar during exercise resulted in increases in lysozyme concentration and secretion rate compared with fasting. Lysozyme is a cationic protein with wide antimicrobial activities (West *et al.*, 2006) and its secretion is under strong neurohormonal control (Bosch *et al.*, 2002). As noted above, cortisol concentration was similar between the two treatments during the steady state exercise and increased significantly in the FAST trial at the post-exhaustion time point only. Since differences in lysozyme levels were already apparent during the steady state exercise, an involvement of cortisol in the acute regulation of this secretory protein seems unlikely. Moreover, although catecholamines were not measured in this study, the similar heart rate and RPE responses suggest no obvious differences in sympathetic activity between the two treatments. One possible explanation for the differences is that the ingestion of the cereal bars caused activation of mechanical and/or gustatory receptors which would lead to reflex stimulation of lysozyme protein secretion via the autonomic nerves (Pedersen *et al.*, 2002). Furthermore, since higher values in lysozyme remained evident at the post-exhaustion time point (30 min after the consumption of the final cereal bar), this would suggest that these effects are relatively long lasting. Further research may seek to address the time course of these effects as the results may have implications for future studies on salivary antimicrobial proteins involving food and/or fluid ingestion.

Research investigating lysozyme in response to exercise is limited at present though lysozyme has been proposed as a potential marker of psychological stress (Perera *et al.*, 1997) and physical stress (Koutedakis *et al.*, 1996) effects on the innate immune system. The present findings show a progressive increase in both the lysozyme concentration and

secretion rate with exercise duration. As discussed previously, it is unlikely that these effects are mediated by changes in the stress hormone cortisol. It is more likely that they are initiated by increases in SNS activity occurring at the onset of exercise, since it is known that the exocytosis of secretory proteins occurs rapidly (within 15 s) upon neuronal stimulation and with β -adrenergic stimulation in particular (Carpenter *et al.*, 1998). These results are in contrast to those reported by Koutedakis *et al.* (1996) in elite swimmers and provide the first clear evidence of enhanced lysozyme levels in response to a bout of prolonged exhaustive exercise.

Significant increases in α -amylase were also observed with exercise. Moreover, higher levels were observed in FED compared with FAST during the steady state exercise, although this difference disappeared following the time to exhaustion trial. Since the role of α -amylase is to break down starch (Scannapieco *et al.*, 1994); it is not surprising that higher levels were found in FED compared with FAST during steady state exercise, as this was when the cereal bars were consumed. Although these results show a positive, stimulatory effect of feeding on α -amylase activity, it also highlights a possible limitation for those attempting to use this measure as a marker of SNS activity when other stimulatory factors are present, since previous studies have suggested that α -amylase activity can be a predictor of plasma noradrenaline under a variety of stressful conditions including exercise (Chatterton *et al.*, 1996). This becomes even more apparent since one would expect blood catecholamines (and resulting surrogate markers of SNS activity) to be blunted with feeding. In this instance, the timing of the meal must be taken into consideration to prevent any additional stimulatory effect on α -amylase.

The present study administered an artificially sweetened placebo drink to examine the additional effect of a high CHO cereal bar - intended for nutritional support of athletes - on

exercise capacity. Not surprisingly, the exercise duration was significantly longer in the high CHO (FED) trial compared with the placebo (FAST) trial. A limitation in doing this was that the participants consumed ~500 mL more fluids in the FAST trial, which may potentially confound the results, given the potential impact of additional fluids on saliva quantity and quality. Although body mass losses in the FED trial were significantly greater than in the FAST trial (1.08 vs 0.53 kg, respectively), suggesting slight differences in hydration status, this did not appear to have a significant negative effect on any of the salivary variables measured.

The fact that feeding enhanced lysozyme and α -amylase secretion but had no effect on s-IgA may be related to the way these proteins are stored and secreted into saliva. Both lysozyme and α -amylase are stored in secretory granules, which are released spontaneously upon autonomic stimulation. Unlike these proteins, s-IgA is secreted onto mucosal surfaces across epithelial cells via pIgR, which is activated by neuronal stimuli that may differ to other salivary proteins (Proctor and Carpenter 2001). Feeding during exercise appears to activate a reflex stimulation to enhance the secretion of the stored salivary proteins into saliva but has little effect on the transport and subsequent secretion of s-IgA.

In summary, the present findings suggest that prolonged exhaustive cycling can increase the secretion of salivary antimicrobial proteins. In addition, these results show that the feeding of a high CHO cereal bar during exercise can result in further increases in lysozyme and α -amylase, but has no effect on s-IgA.

Chapter 5 - Study 3: Effect of stimulating saliva flow on the changes in salivary secretion of IgA, lysozyme and α -amylase with prolonged exhaustive exercise

Abstract

A higher rate of upper respiratory tract infection experienced by some athletes may be due to an exercise-induced decrease in mucosal immune defences. Stimulating saliva flow during exercise may potentially increase oral immune protection. Therefore, the aim of the present study was to investigate the salivary secretion rates of immunoglobulin A (s-IgA), lysozyme and α -amylase in response to strenuous exercise in both stimulated and unstimulated saliva flow conditions. Twenty four fit, healthy, men (mean \pm SEM age: 23 ± 1 yr; maximal oxygen uptake, $\dot{V}O_{2\max}$: 56.6 ± 1.0 ml.kg⁻¹.min⁻¹) cycled for 2.5 h at 60% $\dot{V}O_{2\max}$ (with regular water ingestion) and then cycled to exhaustion at 75% $\dot{V}O_{2\max}$. Timed collections of whole saliva were made immediately before exercise, mid-exercise, after completion of the 2.5 hour moderate exercise bout and immediately after the exhaustive exercise bout. After each unstimulated saliva collection a stimulated saliva flow sample was collected following chewing mint-flavoured gum for one minute. Saliva was analysed for s-IgA, lysozyme and α -amylase and secretion rates were calculated. Saliva flow rate was ~3 times higher when saliva flow was stimulated. Saliva flow rate decreased with exercise for stimulated saliva flow only ($P < 0.01$). Exercise was associated with increases in lysozyme and α -amylase concentration and secretion rates in saliva ($P < 0.01$). Stimulating saliva flow caused a higher secretion rate of lysozyme (> 2-fold higher) and α -amylase (> 3-fold higher) compared with unstimulated saliva flow (both $P < 0.01$). S-IgA concentration and secretion rate increased with exercise but were both lower in stimulated saliva compared with unstimulated ($P < 0.05$). Following the exhaustive exercise bout, s-IgA secretion rates were 129 ± 92 and 165 ± 97 $\mu\text{g}\cdot\text{min}^{-1}$ in

the stimulated and unstimulated saliva flow conditions, respectively ($P < 0.05$). Stimulating saliva flow during exercise had positive effects on the quantity of saliva and on the antimicrobial proteins lysozyme and α -amylase, but resulted in a slightly lower secretion rate for s-IgA. Increases in lysozyme, α -amylase, and the saliva flow rate suggest that stimulating saliva flow during exercise by chewing gum may acutely provide increased oral immune protection.

5.1. Introduction

The study of the mucosal immune response to exercise frequently involves the collection and analysis of saliva samples. The most common method is by collecting an unstimulated sample usually by dribbling into a sterile plastic tube; however, some studies have chosen to stimulate saliva production by either by chewing gum (Horswill *et al.*, 2006; Kreiger *et al.*, 2004; Nevzesh *et al.*, 1982; Proctor and Carpenter., 2001) or sucking on flavoured mints (Gleeson *et al.*, 2003b). There is some evidence to show that these methods may result in differing salivary flow rates and composition. For example, stimulating saliva secretion by chewing can increase the flow rate by up to 3-fold compared with unstimulated saliva secretion (Hector and Linden, 1999), and this can also alter the secretion of certain salivary proteins (Proctor and Carpenter, 2001). It is therefore necessary to understand such differences between the two methods of collection and to be aware of these when collecting or interpreting results.

There have been few studies investigating the effect of stimulated versus unstimulated saliva flow on the oral immune system. Proctor and Carpenter (2001) showed that stimulated saliva secretion by chewing on a tasteless piece of polythene tube increased s-IgA secreted from the parotid saliva gland compared with unstimulated saliva secretion, most likely via increased

epithelial cell transcytosis. Similar results were found for total protein and α -amylase secretion. These findings may have important implications for the s-IgA response to exercise, as stimulating saliva flow could increase s-IgA levels and reduce the risk of URTI. Indeed, Gleeson *et al.* (2003b) examined the effects of stimulated and unstimulated salivary flow on the s-IgA response to high intensity exercise. It was found that sucking a flavoured mint for 1 min during exercise increased the s-IgA secretion rate.

Since exercise may be associated with a reduction in saliva flow rate and/or the secretion of certain antimicrobial proteins, stimulating saliva flow by chewing may function to counteract these changes by increasing the oral immune protection and reduce the risk of developing URTI. Thus, the aims of the present study were to investigate the influence of stimulated saliva flow on salivary antimicrobial proteins by chewing flavoured gum during prolonged exhaustive cycling.

5.2. Methods

5.2.1. Participants

The participants and preliminary measurements were as described previously in Chapter 4. At least 1 week following the preliminary measurements, participants visited the laboratory on one occasion for the main trial.

5.2.2. Experimental procedures

Participants arrived at the laboratory at 8:30 h following an overnight fast (10 – 12 h). They were required to sit quietly for 5 min before giving a saliva sample. The participants were then asked to empty their bladders before body mass was measured wearing their shorts only.

They then performed 2.5 h cycling at 60% $\dot{V}O_{2\max}$ on a stationary cycle ergometer. Heart rate and RPE were measured at 20 min intervals and expired gas was collected at 30 min, 90 min and 114 min of exercise using a Douglas bag. Participants were given 200 mL of water every 20 min during exercise; in addition they ingested 300 ml of flavoured water immediately before the exercise began, and again after 50 min and 110 min of exercise. Saliva samples were collected at rest, following 70 min and 130 min of exercise and immediately post-exhaustion. It was ensured that no fluid was consumed in the 10 min prior to each saliva collection.

Following completion of the 2.5 hours cycling, the participants were allowed a 5 min rest before commencing the ride to exhaustion trial at 75% $\dot{V}O_{2\max}$ with no verbal encouragement and no information on time elapsed. The time to exhaustion was 897 ± 122 s. A final saliva sample was obtained immediately after completing the exercise to exhaustion and body mass was measured. Mean temperature and humidity in the lab during the trial were 23 ± 0.5 °C and 32 ± 4 % respectively.

5.2.3. Saliva collection

The saliva collection was based on the method described in Chapter 3. An unstimulated (UNSTIM) sample was collected first which was immediately followed by 1 min of chewing sugar-free mint flavoured gum (Wrigley Orbit) at an even pace and force. Immediately after removing the gum the participants provided a second saliva sample (STIM) by dribbling into the tube for a further 1 min.

5.2.4. Saliva analysis

Salivary IgA, lysozyme, α -amylase activity and cortisol were measured as detailed in Chapter 2.

5.2.5. Statistical analysis

A two-way ANOVA (2 conditions x 4 sample times) with repeated measures design was used to examine the salivary data. Significant differences were assessed using Student's paired *t*-test with Holm-Bonferroni adjustments for multiple comparisons. Differences in HR between the steady state exercise and time to exhaustion trial were assessed using Student's paired *t*-tests. Statistical significance was accepted at $P < 0.05$.

5.3. Results

5.3.1. Physiological variables and RPE

Attainment of an average of 60% $\dot{V}O_{2max}$ was achieved during the steady state exercise; where mean $\dot{V}O_2$ was $60.1 \pm 2.8\%$ of $\dot{V}O_{2max}$. Mean HR was 137 ± 11 beats.min⁻¹ and 174 ± 9 beats.min⁻¹ during the steady state exercise and the time to exhaustion trial, respectively. Mean RPE measured during the steady state exercise was 12 ± 2 and the post-exercise body mass loss was 0.53 ± 0.10 kg.

5.3.2. Salivary variables

Saliva flow rate

Saliva flow rate was significantly higher in STIM compared with UNSTIM across all time points (main effect of condition: $F_{1, 23} = 177.1$, $P < 0.001$). Saliva flow rate decreased

significantly with exercise duration in the STIM trial only (interaction: $F_{3, 56} = 10.37$, $P < 0.001$; Figure 5.1).

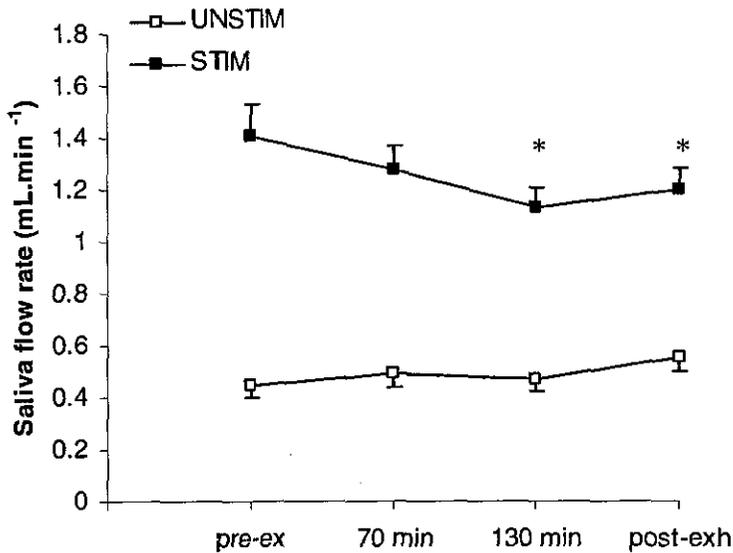


Figure 5.1. The effect of exercise on stimulated and unstimulated saliva flow rate. Values are means \pm SEM (N = 24). * Significantly lower than pre-exercise ($P < 0.01$).

Salivary IgA concentration

Salivary IgA concentration increased with exercise duration (main effect of time: $F_{3, 50} = 4.448$; $P < 0.05$) and was significantly higher in UNSTIM compared with STIM across all time points (main effect of condition: $F_{1, 23} = 44.841$; $P < 0.001$; Figure 5.2).

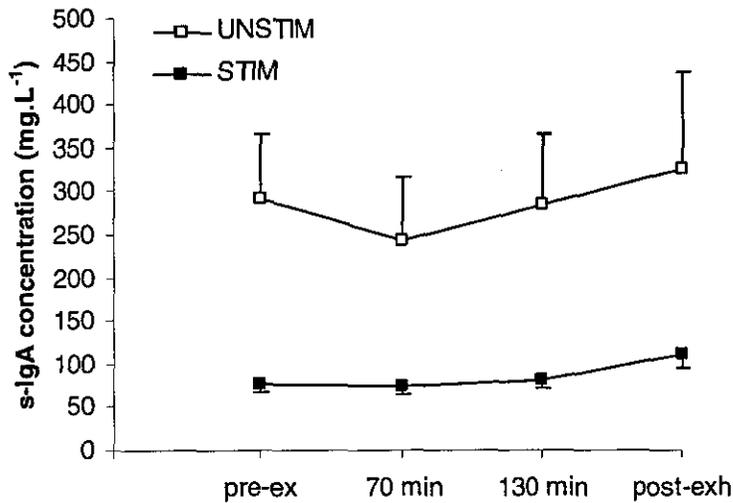


Figure 5.2. Effect of exercise on stimulated and unstimulated s-IgA concentration. Values are means \pm SEM (N = 24).

Salivary IgA secretion rate

Salivary IgA secretion rate increased post-exercise compared with baseline levels (main effect of time: $F_{3,46} = 9.810$; $P < 0.001$) and was significantly higher in UNSTIM compared with STIM (main effect of condition: $F_{1,23} = 5.154$; $P < 0.05$; Figure 5.3)

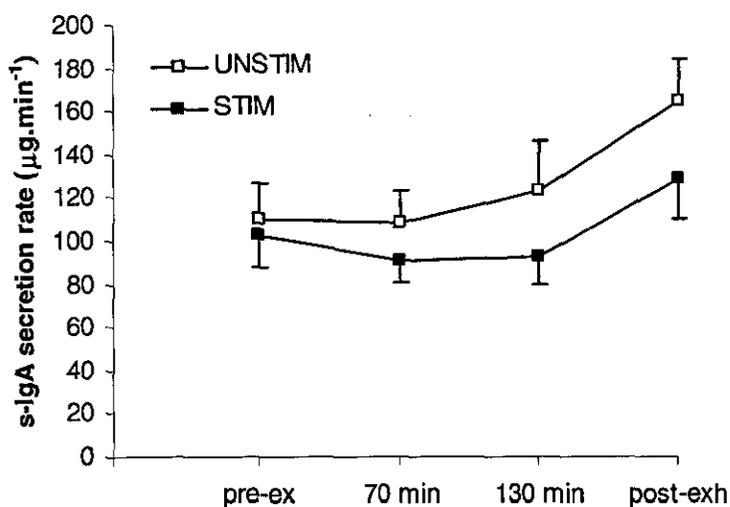


Figure 5.3. Effect of exercise on stimulated and unstimulated s-IgA secretion rate. Values are means \pm SEM (N = 24).

Salivary α -amylase activity

Salivary α -amylase activity increased with exercise duration (main effect of time: $F_{3, 69} = 107.769$; $P < 0.001$), but there were no differences between conditions (Figure 5.4).

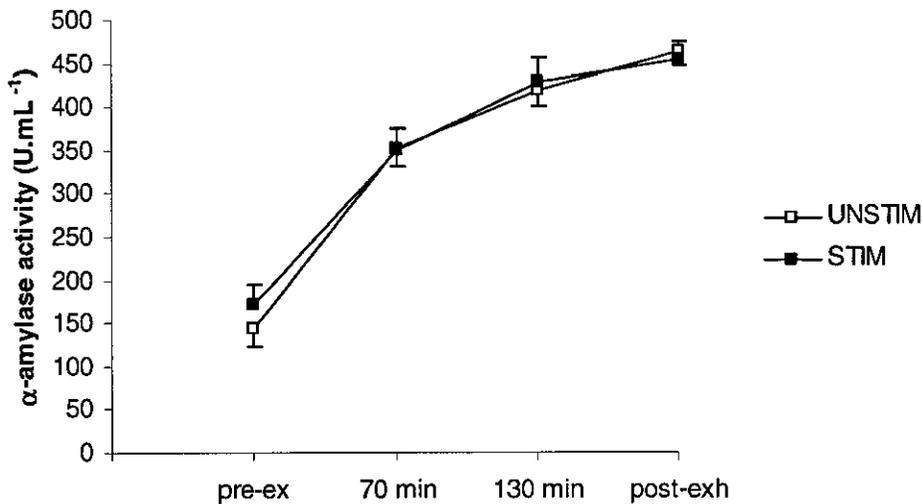


Figure 5.4. Effect of exercise on stimulated and unstimulated α -amylase activity. Values are means \pm SEM (N = 24).

Salivary α -amylase secretion rate

Salivary α -amylase secretion rate increased with exercise duration (main effect of time: $F_{3, 49} = 45.986$; $P < 0.001$). Salivary α -amylase secretion rate was significantly higher in STIM compared with UNSTIM (main effect of condition: $F_{1, 23} = 166.854$; $P < 0.001$; Figure 5.5).

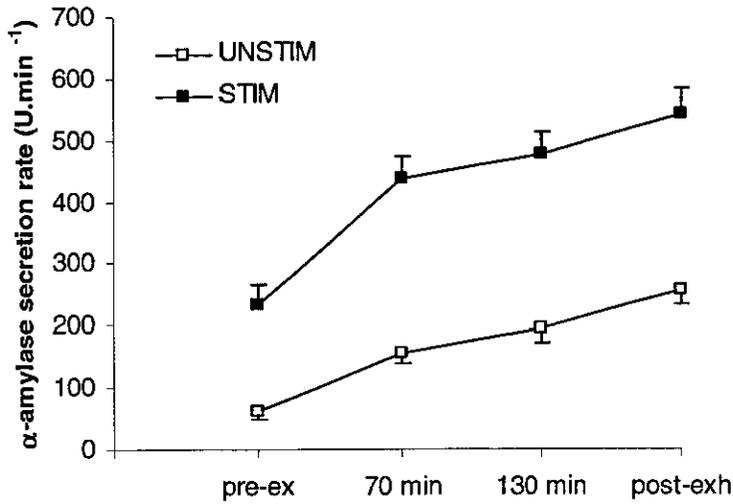


Figure 5.5. Effect of exercise on stimulated and unstimulated α -amylase secretion rate. Values are means \pm SEM (N = 24).

Salivary lysozyme concentration

Salivary lysozyme concentration increased with exercise duration (main effect of time: $F_{3,33} = 23.970$, $P < 0.001$), but there were no differences between conditions (Figure 5.6).

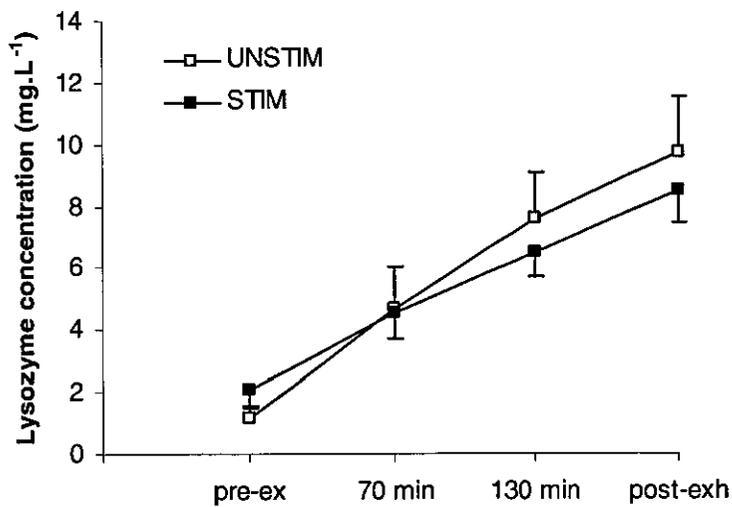


Figure 5.6. Effect of exercise on stimulated and unstimulated lysozyme concentration. Values are means \pm SEM (N = 12).

Salivary lysozyme secretion rate

Salivary lysozyme secretion rate increased with exercise duration (main effect of time: $F_{3,33} = 18.002$, $P < 0.001$) and was significantly higher in STIM compared with UNSTIM across all time points (main effect of condition: $F_{1,11} = 35.051$, $P < 0.001$; Figure 5.7).

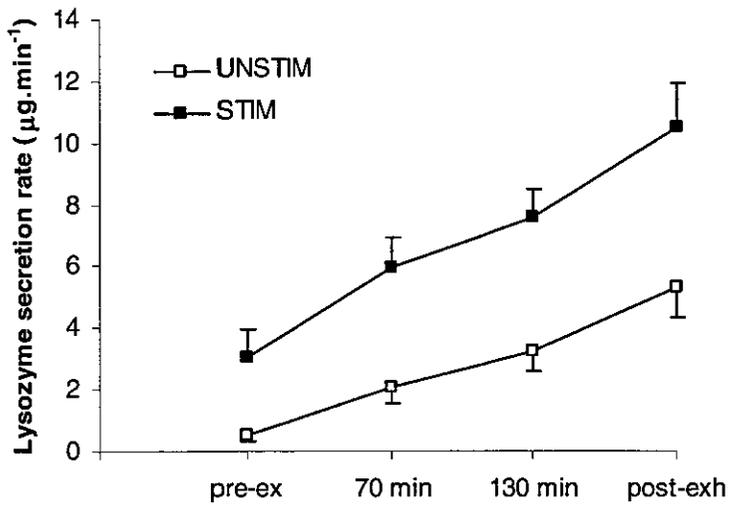


Figure 5.7. Effect of exercise on stimulated and unstimulated lysozyme secretion rate. Values are means \pm SEM(N = 12).

Cortisol

There was a significant change over time in salivary cortisol which increased post-exhaustion (main effect of time: $F_{2,19} = 12.734$, $P < 0.001$; Figure 5.8)

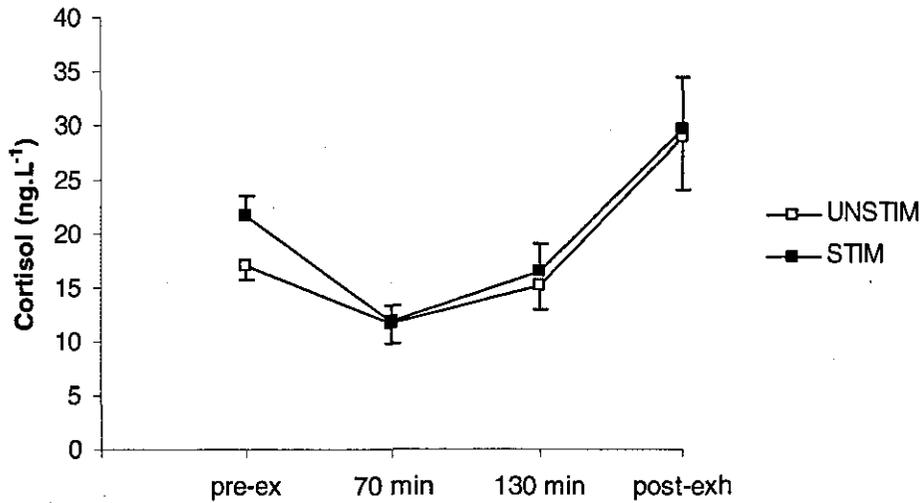


Figure 5.8. Effect of exercise on stimulated and unstimulated cortisol concentration. Values are means \pm SEM ($N = 12$).

5.4. Discussion

The main findings of the study were 1) saliva flow rate decreased with exercise in STIM only 2) saliva flow rate was ~ 3 fold higher in STIM compared with UNSTIM 3) s-IgA concentration increased post-exercise 4) s-IgA concentration and secretion rate were lower in STIM compared with UNSTIM 5) α -amylase activity and secretion rate and lysozyme concentration and secretion rate all increased with exercise 6) α -amylase and lysozyme secretion rates were higher in STIM compared with UNSTIM.

A significant reduction in the saliva flow rate post-exercise was observed in the STIM trial only. Although this has been a commonly reported response to prolonged exercise (Bishop *et al.*, 2000, Li and Gleeson 2005, Walsh *et al.* 2002; Sari-Sarraf *et al.*, 2007b), other studies have found no effect of exercise on the saliva flow rate (Chapters 3 and 4). It has been previously suggested that dehydration plays a role in the reduction in saliva flow rate during exercise (Chicharro *et al.*, 1998; Walsh *et al.*, 2004). In the present study the participants

were provided with 200 mL of fluid to consume every 20 min which resulted in a small net mean body loss of 0.53 ± 0.11 kg. This suggests that the participants were only marginally hypohydrated and is unlikely to have influenced the saliva flow rate (Walsh *et al.*, 2004). It is possible that the decreased parasympathetic nervous system activity during exercise, causing a removal of vasodilatory influences (Procter and Carpenter, 2007), may limit the increase in flow rate with chewing. A further explanation is that the exhaustive nature of the exercise affected the force with which the participants chewed the gum. Although participants were instructed to chew at a regular rate and force for each saliva collection, it cannot be overlooked that the fatiguing nature of the exercise may have affected this. Since salivary production is directly related to the applied chewing force (Hector and Linden, 1999), a reduction in the amount of saliva produced could occur. This is an obvious limitation with a stimulated saliva flow collection.

Salivary IgA concentration increased with exercise in both trials which is consistent with previous studies (Chapters 4 and 5). Furthermore, significantly lower values in STIM compared with UNSTIM were observed throughout the exercise protocol. This is in accordance with Procter and Carpenter (2001), who reported a lower s-IgA concentration in whole-mouth saliva following chewing. These changes are likely to be a result of the increased saliva flow rate with chewing which acts to dilute the saliva.

A significant increase in the s-IgA secretion rate was observed with exercise. This is in agreement with Blannin *et al.* (1998) who also reported increases following both short duration and long duration exercise to exhaustion. S-IgA secretion rate showed little change during the steady state exercise at $60\% \dot{V}O_{2\max}$. However, following the ride to exhaustion at $75\% \dot{V}O_{2\max}$ the s-IgA secretion rate increased by 50% in the UNSTIM trial and 26% in the STIM trial. This suggests that the effect of exercise on s-IgA secretion rate may be intensity

dependent. Indeed, Blannin *et al.* (1998) found a greater increase in s-IgA secretion rate following a higher intensity cycle to exhaustion (80% versus 55% $\dot{V}O_{2max}$). However, other researchers have found no effect of intensity on s-IgA measures (McDowell *et al.*, 1991). The transient increase in s-IgA secretion rate post-exercise is likely to be related to the increased SNS activity with exercise resulting in an increased secretion of s-IgA into saliva via transcytosis (Carpenter *et al.*, 2000). An involvement of SNS activity with exercise can be identified by the increase in α -amylase activity since this has been proposed as a potential marker of SNS activity during stressful conditions (Chatterton *et al.*, 1996). These findings suggest that increases in s-IgA secretion during exercise occur only in response to high levels of sympathetic nerve stimulation, a finding that has been previously confirmed in the rat model (Carpenter *et al.*, 2000).

The present results show that s-IgA secretion rate was significantly lower in STIM compared with UNSTIM. These findings do not support Proctor and Carpenter (2001) who found that stimulating saliva flow by chewing increased the secretion rate of s-IgA from the parotid gland into saliva at rest. The differences in methods may account for these discrepancies. Proctor and colleagues stimulated saliva by chewing on a piece of polythene tube whereas the present study administered a commercially available flavoured chewing gum. The resulting differences between masticatory stimulation only, from chewing the polythene tube, and gustatory and masticatory stimulation combined when chewing the flavoured gum, may account for these inconsistencies. However, Gleeson *et al.* (2003b) also reported an elevated s-IgA secretion rate during exercise when sucking on a mint (gustatory stimulation only) for 1 min prior to saliva collection.

In addition to the different types of stimulation, it is possible that the secretion rate of s-IgA in STIM during exercise may have been affected by the reduction in saliva flow rate which

occurred in this trial. Such potential variances highlight a further limitation of using a stimulated saliva collection in comparison to unstimulated saliva collection (Navazesh and Christensen, 1982). Thus, further research is required to determine the effects of the different methods of stimulating saliva on s-IgA secretion rate at rest and during exercise to clarify these inconsistencies. Although stimulating saliva flow appeared to have a detrimental effect on the rate of s-IgA secretion during exercise, the differences compared with the unstimulated condition were small and the impact of this in terms of URTI risk may have little consequence.

Lysozyme concentration and secretion rate were increased with exercise duration. These findings are in accordance with those in Chapter 4 and suggest a temporary enhancement in this aspect of mucosal immunity with exercise. Stimulating saliva flow did not affect lysozyme concentration. This is in agreement with Rudney (1989) who reported that salivary lysozyme is unaffected by the flow rate. However, when lysozyme was expressed as a secretion rate, significantly higher values in STIM compared with UNSTIM were observed.

Alpha-amylase activity and secretion rate were higher during STIM compared with UNSTIM and this confirms findings by Proctor and Carpenter (2001) who also found an increased secretion of α -amylase following chewing. Exercise also resulted in increases in this enzyme. This is a common finding amongst studies (Chatterton *et al.*, 1996, Walsh *et al.*, 1999, Bishop *et al.*, 2000), and can be attributed to the increase in SNS activity with exercise (Chatterton *et al.*, 1996). Both lysozyme and α -amylase have antimicrobial effects (Tenovuo, 1998); thus, the finding of an increase in their secretion rate with exercise which is further enhanced by stimulating saliva flow suggests these factors have a beneficial effect on the oral immune system

It was speculated in Chapter 4 that the differences between the secretion of these proteins with feeding compared with fasting may be related to the way these proteins are stored and secreted into saliva. Lysozyme and α -amylase are stored in secretory granules which are released spontaneously upon autonomic stimulation (Bosch *et al.*, 2002). However, s-IgA is secreted onto mucosal surfaces across epithelial cells via the polymeric immunoglobulin receptor (pIgR), (Proctor and Carpenter 2001). These findings show that the combination of masticatory and gustatory stimuli through chewing flavoured gum activate the secretion of stored salivary proteins into saliva (lysozyme and α -amylase) but do not enhance the receptor mediated secretion of s-IgA.

A further explanation for the findings may be related to the relative contributions of the different salivary glands during unstimulated and stimulated saliva flow, since specific salivary glands have been shown to be activated by some stimuli more than others (Noble, 2000). For example, mastication predominantly activates the parotid glands, which produce large amounts of α -amylase. In contrast, strong taste stimuli activate the submandibular and sublingual glands (from which lysozyme is mainly produced) more than the parotid gland. Thus, the increase in α -amylase and lysozyme by chewing flavoured gum may be explained by the increase of salivary secretion from these specific glands. The relative contribution of salivary glands for s-IgA secretion is not clear. Crawford *et al.* (1975) reported s-IgA concentration to be four times higher in the minor salivary glands than parotid glands although other data suggest a low parotid s-IgA secretion rate is associated with high susceptibility to dental caries, suggesting a greater role of the parotid gland in s-IgA secretion (Brandtzeag, 1976). These uncertainties make it difficult to relate the changes in s-IgA secretion in stimulated and unstimulated saliva flow to the stimulation of specific glands.

In conclusion, these results suggest that prolonged exhaustive exercise at 60% and 75% $\dot{V}O_{2max}$ in trained men can result in increases in salivary antimicrobial proteins, which may be beneficial to oral immune status. Moreover, stimulating saliva flow during exercise has a further enhancing effect on α -amylase and lysozyme secretion but has little effect on s-IgA secretion rate. Although these findings highlight a possible benefit of chewing gum while performing strenuous exercise, the potential risk of choking while doing this should be considered.

Chapter 6 - Study 4: Effect of exercise intensity on salivary antimicrobial proteins and markers of stress in active men

Abstract

The present study investigated the effects of exercise intensity on salivary immunoglobulin A (s-IgA) and salivary lysozyme and examined how these responses were associated with salivary markers of adrenal activation. Using a randomised design, 10 healthy active men participated in 3 experimental cycling trials: 50% $\dot{V}O_{2max}$, 75% $\dot{V}O_{2max}$ and an incremental test to exhaustion (EXH). The durations of the trials were the same as a preliminary incremental test to exhaustion (22.3 ± 0.8 min; mean \pm SEM). Timed, unstimulated saliva samples were collected at pre-exercise, post-exercise and 1 h post-exercise. The EXH trial significantly increased the secretion rates of both s-IgA and lysozyme. Increases in lysozyme secretion rate also occurred at 75% $\dot{V}O_{2max}$. No significant changes in saliva flow rate were observed in any trial. Cycling at 75% $\dot{V}O_{2max}$ and EXH significantly increased the secretion of α -amylase and Chromogranin A immediately post-exercise but higher values of cortisol at 75% $\dot{V}O_{2max}$ and EXH compared to 50% $\dot{V}O_{2max}$ were observed at 1 h post-exercise only. These findings suggest that short duration, high intensity exercise increases the secretion rate of s-IgA and lysozyme despite no change in the saliva flow rate. These effects appear to be associated with changes in sympathetic activity and not the hypothalamic-pituitary-adrenal axis.

6.1. Introduction

To date, several studies have investigated the effects of exercise on s-IgA with many reporting reductions following intense exercise (Tomasi *et al.*, 1982; Steerenberg *et al.*, 1997; Fahlman *et al.*, 2001), and increases following more moderate or lower intensity exercise

(Dorrington *et al.*, 2003; Reid *et al.*, 2001). However, results from the previous chapters in this thesis do not support these findings. In chapters 4 and 5 significant increases in s-IgA levels were observed post-exercise and these effects appeared to be greater following the higher intensity exercise bout ($75\% \dot{V}O_{2max}$) compared with the lower intensity exercise bout ($60\% \dot{V}O_{2max}$). Furthermore, preliminary findings suggest that lysozyme is also increased with high intensity exercise (Chapters 5 and 6). Thus, it appears that the relationship between exercise intensity and mucosal immunity remains controversial.

The responses of saliva flow rate and composition during exercise are influenced by sympathetic nervous system activity and the hypothalamic-pituitary-adrenal (HPA) axis. Generally it is considered that sympathetic stimulation (via noradrenaline) leads to higher salivary protein concentrations (e.g. α -amylase), whereas increased rates of fluid output occur in response to parasympathetic stimulation (Chicharro *et al.*, 1998). In rodents, the secretion of s-IgA can be increased by both parasympathetic and sympathetic stimulation (Carpenter *et al.*, 2000) and adrenaline has been recently shown to increase the entry of human IgA into saliva by rat salivary cells via increased mobilisation of the polymeric Ig receptor (Carpenter *et al.*, 2004). Since the levels of catecholamines appear to increase in direct relation to exercise intensity (McMurray *et al.*, 1987), it may be speculated that this would have an impact on the salivary responses to exercise.

Alpha-amylase has been previously identified as a marker of SNS activity during exercise (Chatterton *et al.*, 1996), and its secretion appears to be dependent on the exercise intensity (Bishop *et al.*, 2000; Ljunberg *et al.*, 1997; Walsh *et al.*, 1999). However, the response of α -amylase to exercise has been questioned since it can also be affected by parasympathetic activity (Bosch *et al.*, 2002), and as demonstrated in Chapters 4 and 5, by prior food ingestion and/or mastication during exercise. Another potential marker of SNS activity is salivary

Chromogranin A (CgA). It is co-stored and co-released with catecholamines from the adrenal medulla and neuronal vesicles during exocytosis (Banks and Helle, 1965). Salivary CgA has been shown to increase rapidly under psychological stress (Nakane *et al.*, 1998); however, its response to exercise has yet to be determined. Since catecholamines measured in saliva are regarded as a poor index of sympathetic nervous activity (Kennedy *et al.*, 2001), α -amylase and CgA may serve as potential non-invasive tools for evaluating the relationship between the sympathetic nervous system and mucosal immunity following exercise at different intensities.

Exercise also stimulates the release of cortisol from the adrenal cortex and salivary cortisol is considered a reliable index of HPA activity (Chicharro *et al.*, 1998). Cortisol has been suggested to play an important role in inhibiting s-IgA mobilisation and/or production (Hucklebridge *et al.*, 1998). Moreover, it has been implicated in inhibiting lysozyme production and secretion (Perera *et al.*, 1997). Circulating cortisol is also dependent on the exercise intensity, where elevations occur following intensities above approximately 60% $\dot{V}O_{2\max}$ (Virtanen, 1996).

The effect of exercise on the mucosal immune system appears to be influenced by the release of stress hormones such as catecholamines and cortisol, yet how these hormones relate to changes in mucosal immunity following exercise at different intensities remains unclear. Therefore, the primary aim of the present study was to investigate the s-IgA and lysozyme responses to constant duration exercise at 3 different intensities: 50% $\dot{V}O_{2\max}$, 75% $\dot{V}O_{2\max}$ and an incremental exercise test to exhaustion. A secondary aim was to assess the response of non-invasive salivary markers of adrenal activation during exercise at different intensities and to examine how these relate to the responses of salivary antimicrobial proteins.

6.2. Methods

6.2.1. Participants

Ten healthy males (mean \pm SEM: age 23 ± 1 yr; height: 183 ± 2 cm; body mass 76.2 ± 2.2 kg; $\dot{V}O_{2\max}$ 51.4 ± 1.9 mL.kg⁻¹.min⁻¹; HR_{max} 190 ± 2 beats.min⁻¹) volunteered to participate in the study. Following preliminary measurements, the participants completed 3 main trials in a randomised order separated by at least 3 days.

6.2.2. Experimental procedures

Participants reported to the laboratory at 11:45 h. They were requested to abstain from drinking alcohol and taking medication in the 24 h preceding each experimental session. They were instructed to consume their normal breakfast including 500 mL of water at 9:00 h prior to each of the trials, and then repeat this breakfast for each of the subsequent trials. They were then asked to consume a further 500 mL of water 90 min before exercise in order to standardise hydration status. Upon arrival at the laboratory, participants were instructed to empty their bladder before body mass was measured. They then sat quietly for 5 min before an initial pre-exercise saliva sample was collected. The collection time for each participant was designed to ensure that a minimum volume of 1.5 mL was obtained. They were then randomly assigned to one of 3 exercise trials: Trial 1 was performed at a work rate equivalent to 50% $\dot{V}O_{2\max}$, Trial 2 was performed at a work rate equivalent to 75% $\dot{V}O_{2\max}$ and Trial 3 was a repetition of the incremental test to exhaustion (EXH). Each trial was for the same duration as the initial preliminary $\dot{V}O_{2\max}$ test. The mean duration (\pm SEM) for the trials was 22.3 ± 0.8 min. The participants began cycling at 12:00 h. Heart rate and RPE were recorded every 3 min during exercise and during the final minute before cessation of exercise. A saliva sample was collected immediately post-exercise and a final sample was obtained at 1 h post-

exercise. A fingertip capillary blood sample was also obtained post-exercise to determine blood lactate concentration using an Analox PGM7 analyser (Analox, Stokesley, North Yorkshire, UK) and body mass was measured immediately after the post-exercise saliva sample had been collected. Water consumption was not permitted during exercise; however, participants were given 200 mL of water to consume immediately after the post-exercise saliva sample. No other fluid or food was permitted until after the final 1 h post-exercise saliva sample had been collected. The laboratory temperature and relative humidity were 23.4 ± 0.5 °C and 41 ± 2 %, respectively.

6.2.3. Saliva collection

Methods of saliva collection and analysis are detailed in Chapter 2.

6.2.4. Statistical analysis

Salivary data, HR and RPE were analysed using a 3 (trials) x 3 (times of measurement) repeated measures ANOVA. Body mass losses and blood lactate concentration were examined using a one-factor repeated measure ANOVA. Significant differences were assessed using Student's paired t-test with Holm-Bonferroni adjustments for multiple comparisons. Statistical significance was accepted at $P < 0.05$.

6.3. Results

6.3.1. Physiological variables and RPE

Results for HR, RPE, body mass losses and blood lactate are presented in Table 6.1. Mean HR was significantly higher in the 75% $\dot{V}O_{2max}$ and EXH trials compared with the 50% $\dot{V}O_{2max}$ trial (main effect of trial; $F_{1, 12} = 77.4$, $P < 0.001$). Only during the final stage of

exercise was HR significantly higher in the EXH trial compared with the 75% $\dot{V}O_{2\max}$ trial ($P < 0.05$). Similarly, mean RPE was significantly higher with the 75% $\dot{V}O_{2\max}$ and EXH trials compared with the 50% $\dot{V}O_{2\max}$ trial (main effect of trial; $F_{2,14} = 48.3$, $P < 0.001$). Only during the final stage of exercise was RPE significantly higher in the EXH trial compared with the 75% $\dot{V}O_{2\max}$ trial ($P < 0.05$). Body mass losses were similar between trials. Post-exercise blood lactate concentration increased significantly with exercise intensity (main effect of trial; $F_{1,13} = 64.0$ $P < 0.001$).

Table 6.1. Effect of exercise intensity on heart rate, RPE, body mass loss and blood lactate concentration.

Variable	50% $\dot{V}O_{2\max}$		75% $\dot{V}O_{2\max}$		EXH	
	Mean	End	Mean	End	Mean	End
Heart Rate (beats.min ⁻¹)	124 (1)	128 (4)	162 (1)*	170 (3)*	144 (1)* [†]	186 (2)* [†]
RPE	10 (1)	10 (1)	14 (1)*	16 (1)*	13 (1)* [†]	19 (1)* [†]
Body mass loss (kg)		0.3 (0.1)		0.4 (0.1)		182 ± 1
Blood Lactate (mmol.L ⁻¹)		1.8 (0.3)		7.5 (0.6)*		0.3 (0.1)

Values are means (\pm SEM) ($N = 10$). * Significantly different from 50% $\dot{V}O_{2\max}$ ($P < 0.05$).

[†] Significantly different from 75% $\dot{V}O_{2\max}$ ($P < 0.05$).

6.4. Salivary variables

Saliva flow rate

Saliva flow rate did not change significantly throughout the experimental protocol (Table 6.2).

Table 6.2. The effect of exercise intensity on saliva flow rate, salivary osmolality, S-IgA:Osmolality and lysozyme:Osmolality.

	Pre-exercise	Post-exercise	1 h Post-exercise
Saliva flow rate (mL·min ⁻¹)			
50% $\dot{V}O_{2max}$	0.48 (0.09)	0.40 (0.07)	0.45 (0.07)
75% $\dot{V}O_{2max}$	0.41 (0.08)	0.43 (0.09)	0.50 (0.09)
EXH	0.41 (0.10)	0.42 (0.09)	0.45 (0.09)
Osmolality (mOsmol·kg ⁻¹)			
50% $\dot{V}O_{2max}$	69 (6)	80 (7) **	65 (6)
75% $\dot{V}O_{2max}$	68 (9)	108 (16) *	67 (7)
EXH	72 (9)	129 (14) ** †	64 (6)
s-IgA:Osmolality			
50% $\dot{V}O_{2max}$	3.5 (0.4)	3.4 (0.4)	3.8 (0.4)
75% $\dot{V}O_{2max}$	3.3 (0.3)	2.8 (0.5)	3.4 (0.4)
EXH	3.4 (0.4)	2.8 (0.4)	3.2 (0.3)
lysozyme:Osmolality			
50% $\dot{V}O_{2max}$	0.07 (0.02)	0.08 (0.02)	0.08 (0.02)
75% $\dot{V}O_{2max}$	0.07 (0.02)	0.11 (0.01)	0.09 (0.02)
EXH	0.06 (0.01)	0.08 (0.01)	0.08 (0.02)

Values are means (\pm SEM) ($N = 10$). * Significantly higher than pre-exercise (within trial), $P < 0.05$; ** $P < 0.01$. † Significantly higher than 50% $\dot{V}O_{2max}$ at that time point, $P < 0.01$.

Salivary IgA

Salivary IgA concentration increased post-exercise which was independent of exercise intensity (main effect of time; $F_{1, 10} = 7.7$, $P = 0.018$; Figure 6.1). Salivary IgA secretion rate increased by 50% post-exercise in the EXH trial but returned to baseline at 1 h post-exercise (interaction; $F_{3, 25} = 3.2$, $P = 0.042$; Figure 6.2).

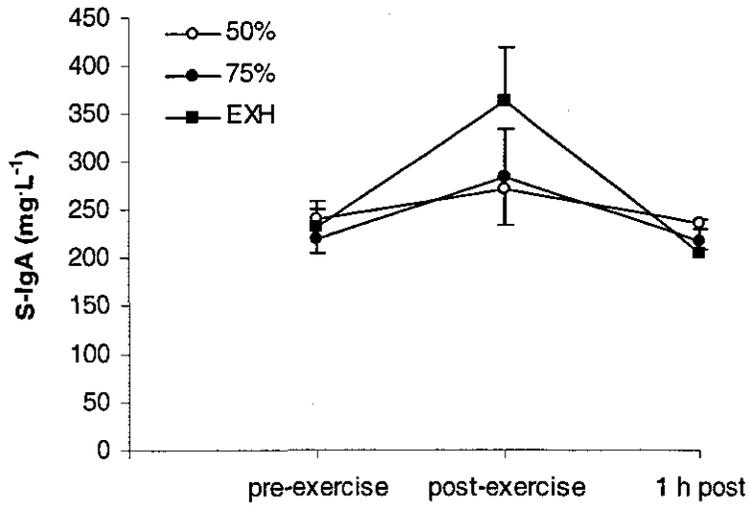


Figure 6.1. The effect of exercise intensity on s-IgA concentration. Values are means \pm SEM ($N = 10$).

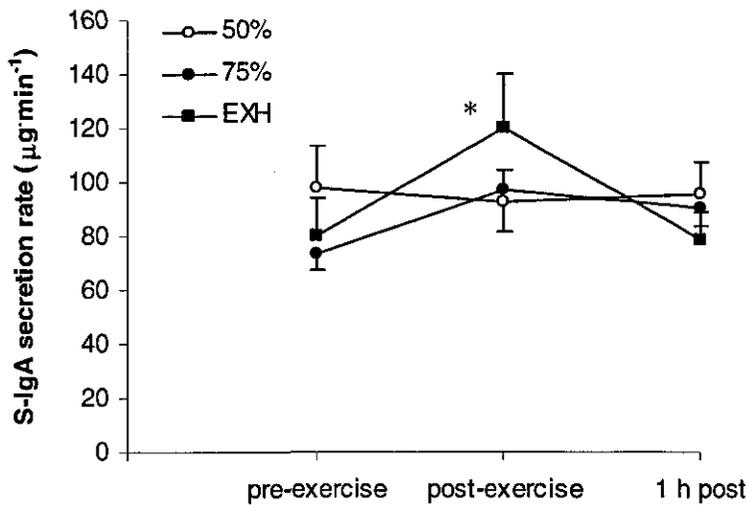


Figure 6.2. The effect of exercise intensity on s-IgA secretion rate. Values are means \pm SEM ($N = 10$). * Significantly higher than pre-exercise (EXH trial), $P < 0.05$.

Salivary lysozyme

Salivary lysozyme concentration increased post-exercise which was independent of exercise intensity (main effect of time; $F_{2, 17} = 25.6$, $P < 0.001$; Figure 6.3). Salivary lysozyme

secretion rate increased by 160% post-exercise in the 75% $\dot{V}O_{2\max}$ and EXH trials returning to baseline at 1 h post-exercise (interaction; $F_{3,25} = 4.9$, $P = 0.01$), but there was no change in the 50% $\dot{V}O_{2\max}$ trial (Figure 6.4).

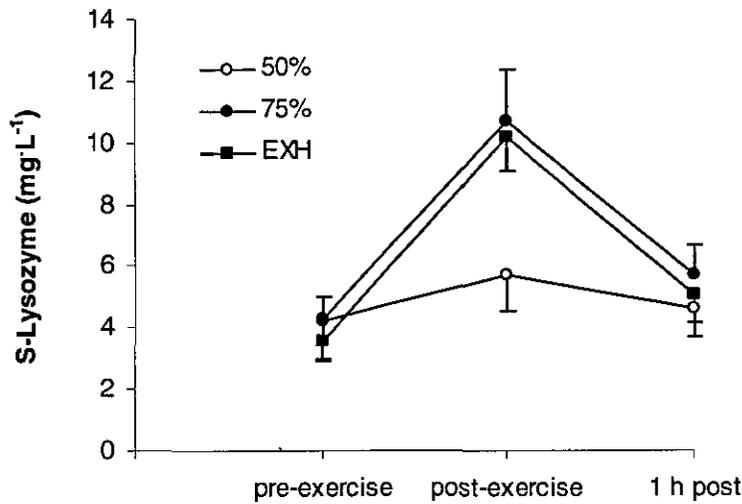


Figure 6.3. The effect of exercise intensity on lysozyme concentration. Values are means \pm SEM ($N = 10$).

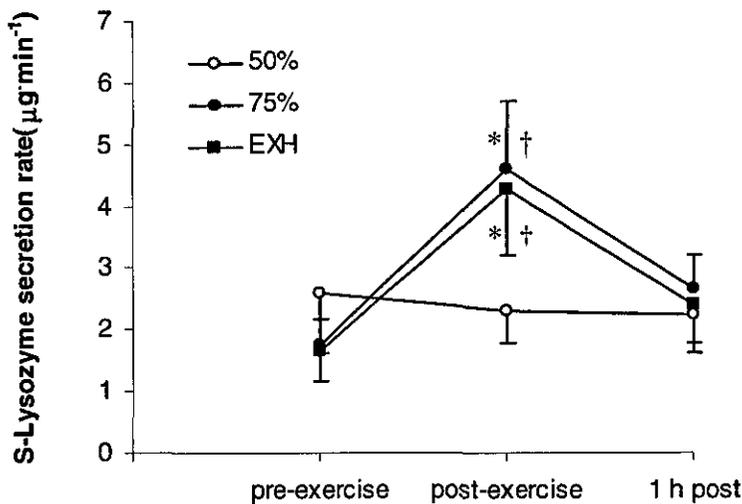


Figure 6.4. The effect of exercise intensity on lysozyme secretion rate. Values are means \pm SEM ($N = 10$). * Significantly higher than pre-exercise, $P < 0.01$. † Significantly higher than 50% $\dot{V}O_{2\max}$ trial at that time point, $P < 0.05$.

Salivary osmolality

Salivary osmolality (Table 6.2) increased post-exercise in all trials (main effect of time; $F_{1,11} = 54.3$, $P < 0.001$) and was significantly higher at post-exercise in the EXH trial compared with the 50% $\dot{V}O_{2\max}$ trial (interaction; $F_{2,22} = 7.5$, $P = 0.002$). Salivary IgA to osmolality ratio did not change significantly during the experimental protocol. Salivary lysozyme to osmolality ratio increased post-exercise independent of exercise intensity (main effect of time; $F_{2,15} = 8.0$, $P = 0.006$).

Stress markers

Alpha-amylase activity (Table 6.3) increased post-exercise which was independent of exercise intensity (main effect of time; $F_{1,11} = 24.2$, $P < 0.001$). Alpha-amylase secretion rate increased by about 60% post-exercise in the 75% $\dot{V}O_{2\max}$ and EXH trials returning to baseline at 1 h post-exercise (interaction $F_{3,27} = 3.9$, $P = 0.019$), but there was no change in the 50% $\dot{V}O_{2\max}$ trial. Salivary cortisol was unchanged immediately post-exercise but was significantly higher at 1 h post-exercise in the 75% $\dot{V}O_{2\max}$ and EXH trials compared with the 50% $\dot{V}O_{2\max}$ trial (interaction; $F_{2,17} = 9.0$, $P = 0.003$; Table 6.3).

Table 6.3. The effect of exercise intensity on α -amylase activity, α -amylase secretion rate and salivary cortisol.

	Pre-exercise	Post-exercise	1 h Post-exercise
α -amylase activity (U mL^{-1})			
50% $\dot{V}\text{O}_{2\text{max}}$	450 (54)	552 (77)	385 (61)
75% $\dot{V}\text{O}_{2\text{max}}$	372 (65)	674 (77)	418 (63)
EXH	456 (65)	710 (41)	370 (55)
α -amylase secretion rate (U min^{-1})			
50% $\dot{V}\text{O}_{2\text{max}}$	226 (55)	200 (34)	176 (35)
75% $\dot{V}\text{O}_{2\text{max}}$	150(47)	264 (47) *	196 (42)
EXH	191 (60)	272 (46) *	170 (51)
Cortisol (nmol L^{-1})			
50%	37.8 (4.5)	49.3 (2.8)	40.6 (2.8)
75%	51.5 (2.8)	54.9 (4.5)	54.1 (4.1) †
EXH	52.6 (5.3)	53.8 (6.0)	61.7 (6.4) †

Values are means (\pm SEM) ($N = 10$). * Significantly higher than pre-exercise (within trial), $P < 0.05$.

† Significantly higher than 50% $\dot{V}\text{O}_{2\text{max}}$ at that time point, $P < 0.01$.

Chromogranin A concentration increased by about 350% post-exercise in the 75% $\dot{V}\text{O}_{2\text{max}}$ and EXH trials returning to baseline at 1 h post-exercise (interaction; $F_{3, 18} = 4.1$, $P = 0.025$; Figure 6.5), but there was no change in the 50% $\dot{V}\text{O}_{2\text{max}}$ trial. Similarly, CgA secretion rate increased by about 350% post-exercise in the 75% $\dot{V}\text{O}_{2\text{max}}$ and EXH trials returning to baseline at 1 h post-exercise (interaction; $F_{2, 16} = 7.9$, $P = 0.019$; Figure 6.6), but there was no change in the 50% $\dot{V}\text{O}_{2\text{max}}$ trial.

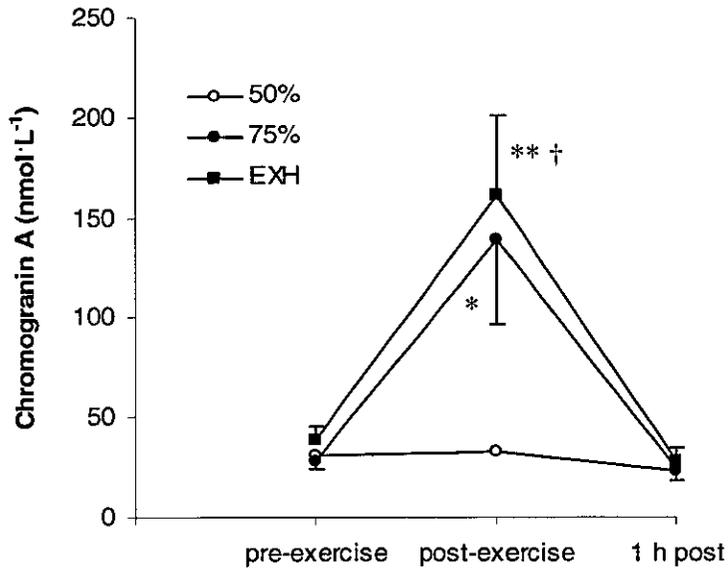


Figure 6.5. The effect of exercise intensity on CgA concentration. Values are means \pm SEM ($N = 8$). * Significantly higher than pre-exercise (within trial), $P < 0.05$; ** $P < 0.01$. † Significantly higher than 50% $\dot{V}O_{2\max}$ at that time, $P < 0.01$.

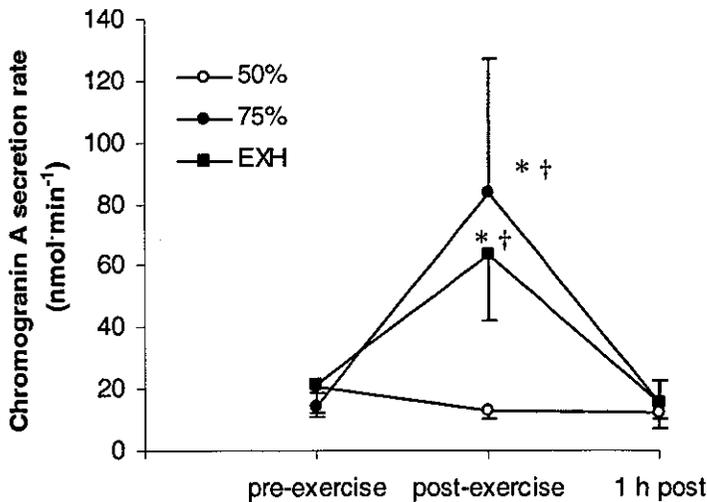


Figure 6.6. The effect of exercise intensity on CgA secretion rate. Values are means \pm SEM ($N = 8$). * Significantly higher than pre-exercise (within trial), $P < 0.01$. † Significantly higher than 50% $\dot{V}O_{2\max}$ at that time, $P < 0.05$.

6.5. Discussion

The main findings of the present study were that a short duration incremental exercise test to exhaustion resulted in a temporary increase in the secretion rate of both s-IgA and lysozyme but did not affect the saliva flow rate. In addition, the lysozyme secretion rate was also elevated following exercise at 75% $\dot{V}O_{2max}$. These changes appeared to be associated with increases in the levels of the stress markers α -amylase and CgA but not cortisol.

The effect of exercise on saliva flow rate remains controversial. In Chapters 4 and 5 saliva flow rate remained unchanged with exercise and the results from the present study corroborate this. However, others have reported a marked decrease in saliva flow rate in response to 3 consecutive maximal Wingate tests (less than 8 min duration) (Fahlman *et al.*, 2001; Engels *et al.*, 2003) or in response to prolonged (> 90 min) endurance exercise (Chapter 4; Bishop *et al.*, 2000; Li and Gleeson 2005). The reasons for the discrepant findings may be two-fold. Firstly, there may be a threshold level of parasympathetic nervous system activity below which, the saliva flow rate decreases. Taking this into consideration, it is possible that the duration and/or intensities of exercise performed in this study were not sufficient to achieve this threshold. Secondly, Walsh and colleagues (2004) reported that dehydration had a greater involvement in the reduction in saliva flow rate than neuroendocrine regulation following prolonged strenuous exercise and a significant reduction in saliva flow rate was only observed when dehydration of at least 2% body mass loss occurred. Although salivary osmolality increased post-exercise in the present study, body mass losses were small and similar between trials (~ 0.3 kg equating to 0.4% body mass). Therefore, based on the findings by Walsh and colleagues, it would seem that the trials did not induce dehydration sufficient to affect saliva flow rate. Moreover, the increases in salivary osmolality without a reduction in saliva flow rate or large body mass losses indicate

a greater effect of neural innervation of saliva electrolyte secretion rather than dehydration *per se*. This may question the reliability of salivary osmolality as an indicator of hydration status post-exercise.

Previous research conducted in athletes suffering from recurrent infections has shown that exercise of equal duration performed at 75% $\dot{V}O_{2max}$ and 100% $\dot{V}O_{2max}$ were associated with a trend for a lower s-IgA concentration post-exercise while levels increased slightly after exercise at 50% $\dot{V}O_{2max}$ (Williams *et al.*, 2001). A similar pattern was observed in children (8 – 12 yr) when s-IgA was expressed as a ratio to albumin (Dorrington *et al.*, 2003). From this work these researchers concluded that there exists a positive association between the degree of immune suppression post-exercise and the exercise intensity level. The present study sought to re-examine these findings in a cohort of healthy active males. Contrary to the above findings, significant increases in s-IgA concentration and secretion rate were observed post-exercise following the EXH trial. Due to the incremental nature of this trial, peak HR was only higher than the 75% $\dot{V}O_{2max}$ trial at the end of exercise. Furthermore, overall mean HR was significantly lower in EXH compared with 75% $\dot{V}O_{2max}$. This suggests that it could be the peak intensity rather than the overall intensity which is important in influencing s-IgA secretion.

An elevation in the s-IgA secretion rate following exercise indicates an increased availability of s-IgA present on the mucosal surfaces and not just a concentrating effect of the saliva as a result of a reduction in saliva flow rate. The regulation of s-IgA is via a short-term (minutes) mobilisation (transcytosis) modulated by sympathetic nerves and/or a long-term (days) modification of s-IgA synthesis (Goodrich and McGee, 1998). Proctor *et al.* (2003) reported that acute stimulation of β -adrenoreceptors in anaesthetised rats increased s-IgA secretion via

elevated transcytosis from the glandular pool in a dose-independent manner above a certain threshold. Therefore, it could be argued that in the short term, it is only very high intensity exercise that will induce sympathetic stimulation sufficient to increase the transportation of s-IgA into saliva as was observed in the EXH trial. However, when this stimulus becomes more prolonged (as would occur in longer duration - though less intense - exercise) the amount of IgA available for transport might become depleted and result in a reduction in the secretion of IgA (Proctor *et al.*, 2003). Indeed, the different durations, intensities and types of adrenergic stimulation could help to explain the inconsistencies in the literature previously cited. Despite demonstrating significant changes in s-IgA concentration and secretion rate post-exercise, no significant changes at any intensity were observed when s-IgA was expressed relative to osmolality. This highlights once more the uncertainty of which is the most appropriate method for expressing s-IgA.

The present study measured α -amylase and CgA as surrogate markers of sympathetic activity. Dawes (1981) reported that an elevated α -amylase activity could be induced by increases in plasma catecholamines and sympathetic nervous activity with exercise. Furthermore, a previous study found a significant correlation between salivary α -amylase and plasma noradrenaline concentration following exercise (Chatterton *et al.*, 1996), although others have failed to confirm this (Nater *et al.*, 2006). Significant increases in both α -amylase activity and α -amylase secretion rate were observed post-exercise although only the secretion rate was significantly affected by the exercise intensity. A significant increase in both the concentration and secretion rate of CgA was found post-exercise and both were influenced by the exercise intensity. These findings lend further support to an increased involvement of sympathetic activity in the high intensity trials, which may function to increase the mobilisation of IgA into saliva. What these results also suggest is that both the concentration

and secretion rate of salivary CgA warrant further investigation as a potential index of sympathetic nervous activity during exercise.

Although the levels of cortisol were affected by the exercise intensity, differences occurred only at the 1-hour post-exercise time point. This is not surprising since the appearance of cortisol in the circulation normally occurs after a lag-period that in some cases may be more than 1 h (Virus and Virus, 2004). Since cortisol concentration did not change immediately post-exercise an involvement of the HPA axis in the regulation of s-IgA immediately after exercise seems unlikely. However, the effects on s-IgA may be more delayed and occur between 2 - 24 h following exercise (Mackinnon *et al.*, 1987; Gleeson *et al.*, 2001).

Lysozyme increased post-exercise in the 75% $\dot{V}O_{2max}$ and EXH trials. These findings are in accordance with Chapters 4 and 5 but contrast those reported by Koutedakis and colleagues (1996) where significant decreases in the lysozyme concentration and secretion rate following an intense training session in elite swimmers were found. The differences in participant training status and the absolute intensity and duration of the training session performed might account at least in part for these contrasting findings.

The regulatory factors governing salivary gland secretion of lysozyme are largely unknown at present. Previous authors have attributed reductions in lysozyme levels in response to psychological stress to an increased secretion of glucocorticoids. These may inhibit the production and secretion of lysozyme leading to lower concentrations present in saliva (Perera *et al.*, 1997). Since the observed changes in the present study are unlikely to be attributable to glucocorticoids, it seems plausible that the elevations in lysozyme following short duration high intensity exercise may be mediated in a similar manner to IgA. Hence, increases could be related to perturbations in sympathetic nervous activity (Bosch *et al.*,

2002), resulting in a transient increase in mobilisation of lysozyme into saliva. Moreover, since similar elevations in lysozyme were also observed in the 75% $\dot{V}O_{2\max}$ trial, it is possible that the threshold of sympathetic nervous activity to increase mobilisation may be lower for that of lysozyme than s-IgA. Although increases in salivary antimicrobial proteins may suggest a temporary enhancement of immune function, the significance of this in terms of infection risk is currently unclear and thus, the clinical relevance of these findings remains unknown.

In conclusion, the data from the present study indicate that a short duration incremental exercise test to exhaustion can result in temporary increases in the secretion rate of both s-IgA and lysozyme without affecting saliva flow rate. Furthermore, an increase in the lysozyme secretion rate also occurred post-exercise at 75% $\dot{V}O_{2\max}$. These results appear to be associated with increases in the secretion rate of CgA and α -amylase. Collectively, these findings suggest that sympathetic stimulation during intensive exercise appears to be great enough to increase s-IgA and lysozyme transport into saliva without affecting the saliva flow rate.

Chapter 7 - Study 5: Salivary IgA and respiratory illness in elite male and female swimmers during a 6-month period of training and competition

Abstract

The purpose of the present study was to examine the impact of a 6-month period of training and competition on mucosal immunity and respiratory illness in a cohort of 12 elite male and female swimmers. Unstimulated, timed saliva samples were collected at rest on 8 occasions over a 6-month period leading up to and during an international swimming competition for the analysis of salivary immunoglobulin A (s-IgA) and salivary osmolality. In addition, symptoms of respiratory illness were recorded on a weekly basis. There were no significant changes in saliva flow rate, s-IgA concentration or the ratio of s-IgA to osmolality ($P > 0.05$) throughout the training study period. There was a significant main effect of time for s-IgA secretion rate ($P < 0.05$), which was significantly lower at week 22 (pre-competition) compared with week 26 (post-competition). Lower levels of s-IgA secretion rate were also observed at week 7 (pre-trials) and week 17 (post-intensified training) compared with week 26, although these did not quite reach significance ($P = 0.054$). Significant differences in gender were found in all the salivary measures with females demonstrating lower levels than males. However, the responses of these measures to the training period did not differ between genders. Symptoms of respiratory illness were highest during week 8 of the study (post-trials) where 7 swimmers (3 male and 4 female) reported ill. However, this did not appear to be directly related to lower s-IgA levels. These results suggest that a 6-month season of swim training and competition may result in significant changes in the s-IgA secretion rate but not the s-IgA concentration. Significant differences in gender exist in resting salivary composition but these are not differently affected by swim training.

7.1. Introduction

Over the past decade there has been a proliferation of research investigating the effects of exercise on immune function and infection risk in athletes. The perceptions remain that athletes report a higher incidence of upper respiratory tract infections (URTI) during periods of heavy training and competition and this is thought to be related to perturbations in certain aspects of immune function (Nieman, 1994, Nieman *et al.*, 1990a, Peters, 1997). It is now widely accepted that the immune system exhibits change following both acute exercise and chronic training, but a direct link between these changes and infection risk has yet to be convincingly established.

Secretory immunoglobulin A (IgA) represents the first line of defence against infectious agents related to URTI. IgA has the capacity to inhibit the colonisation of pathogens, bind antigens for transport across the epithelial barrier and neutralise viruses (Lamm, 1998). It is well known that individuals with IgA deficiency experience a higher incidence of URTI and a significant relationship between salivary IgA (s-IgA) concentration and the incidence of URTI in the general population has been reported (Jemmott and McClelland, 1989). Numerous studies have investigated how the levels of s-IgA are affected by exercise although the results have been inconsistent to date. Following acute exercise, some have reported that s-IgA concentration was depressed (Tomasi *et al.*, Mackinnon *et al.*, 1987, Tharp and Barnes, 1990), whereas others have reported either no change (McDowell *et al.*, 1991, Walsh *et al.*, 1999) or even elevations (Blannin *et al.*, 1998; Tharp, 1991).

Longitudinal studies have shown that the changes may be cumulative over time since resting s-IgA concentration was shown to fall with each additional month of training in elite swimmers (Gleeson *et al.*, 1999). Similar findings were reported in American college

footballers: during a 12-month football season reductions in both the concentration and the secretion rate of s-IgA were observed during the most intense periods of training. In contrast, Gleeson *et al* (2000) reported small but significant increases in s-IgA during a 12-week training cycle leading up to competition in elite swimmers.

Some studies have successfully linked changes in s-IgA to the reported incidence of URTI in athletes. A significant inverse association between pre-training s-IgA concentration and the numbers of reported infections was found in elite swimmers (Gleeson *et al.*, 1999). Furthermore, Fahlman and Engels (2005) found an inverse correlation with the s-IgA secretion rate and respiratory illness in American footballers. However, others have not been able to repeat these findings. No correlation between URTI and s-IgA concentration was found in a different cohort of swimmers over 12 weeks (Gleeson *et al.*, 2000), in a group of elite female rowers over 2 months (Nehlsen-Cannarella *et al.*, 2000) or in elite tennis players studied over 12 weeks (Novas *et al.*, 2003). The differences in these findings could be related to the limited duration of these studies or the method of expressing s-IgA. In the earlier studies, s-IgA was expressed as a concentration. However, more recently researchers have chosen to represent it as a secretion rate or as a ratio to osmolality. Expressing s-IgA relative to osmolality may be a valid measure when significant variations in saliva flow rate occur (Blannin *et al.*, 1998). However, it is thought that the secretion rate of s-IgA may be more appropriate since it represents the amount of s-IgA present on the mucosal surface as both the saliva flow rate and IgA concentration are important for host defence (Mackinnon *et al.*, 1993a). This was supported by Fahlman and Engels (2005) who concluded that the secretion rate may be the most useful clinical biomarker to predict the incidence of URTI. However, further research into this area is required before any general consensus can be arrived at.

The majority of published research into the effect of exercise on the immune system has mainly focused on male athletes overlooking potential differences in gender. Indeed, significant gender differences in s-IgA concentration have been reported in elite swimmers with females exhibiting lower values than males both at rest and post-exercise (Gleeson *et al.*, 1999). It is not known whether the same can be said for other methods of expressing s-IgA or whether the s-IgA response to training is the same. Whether this factor might explain some of the inconsistencies in the literature remains to be investigated. Therefore, this study sought to extend previous findings to determine a) the effects of a 6-month season of training and competition on measures of mucosal immunity in elite male and female swimmers and b) whether the selected markers of mucosal immunity were related to reported episodes of URTI.

7.2. Methods

7.2.1. Participants

Twelve elite swimmers (7 male and 5 female) were recruited to participate in the present study. All were members of the British Swimming National team, ranked in the top 3 in their event nationally and the top 100 internationally. Their physical characteristics are outlined in Table 7.1.

Table 7.1. Participant Characteristics (N = 12)

Variable	Mean (\pm SEM)	
	Males (N = 7)	Females (N = 5)
Age (yr)	22 \pm 1	21 \pm 1
Mass (kg)	79.4 \pm 2.4	62.5 \pm 3.0*
Height (m)	1.90 \pm 0.01	1.70 \pm 0.01*

* Significantly different to Males ($P < 0.05$).

7.2.2. Study design

The swimmers were monitored on 8 occasions during a 6-month period of training and competition leading up to and during the Commonwealth Games in Melbourne, Australia in March 2006. At the beginning and end of each training cycle a timed, unstimulated saliva sample was collected from each swimmer. The collections were made at rest, between 7:30 h and 8:00 h, at least 1-h post-prandially and a minimum of 18 h after the previous training session. At the beginning of each training week, a log recording symptoms of URTI (Appendix E) was completed. This referred to any symptoms exhibited during the previous week. A positive episode of URTI was recorded when two or more symptoms were exhibited on two consecutive days, at least 1-week apart from a previous episode.

7.2.3. Training loads

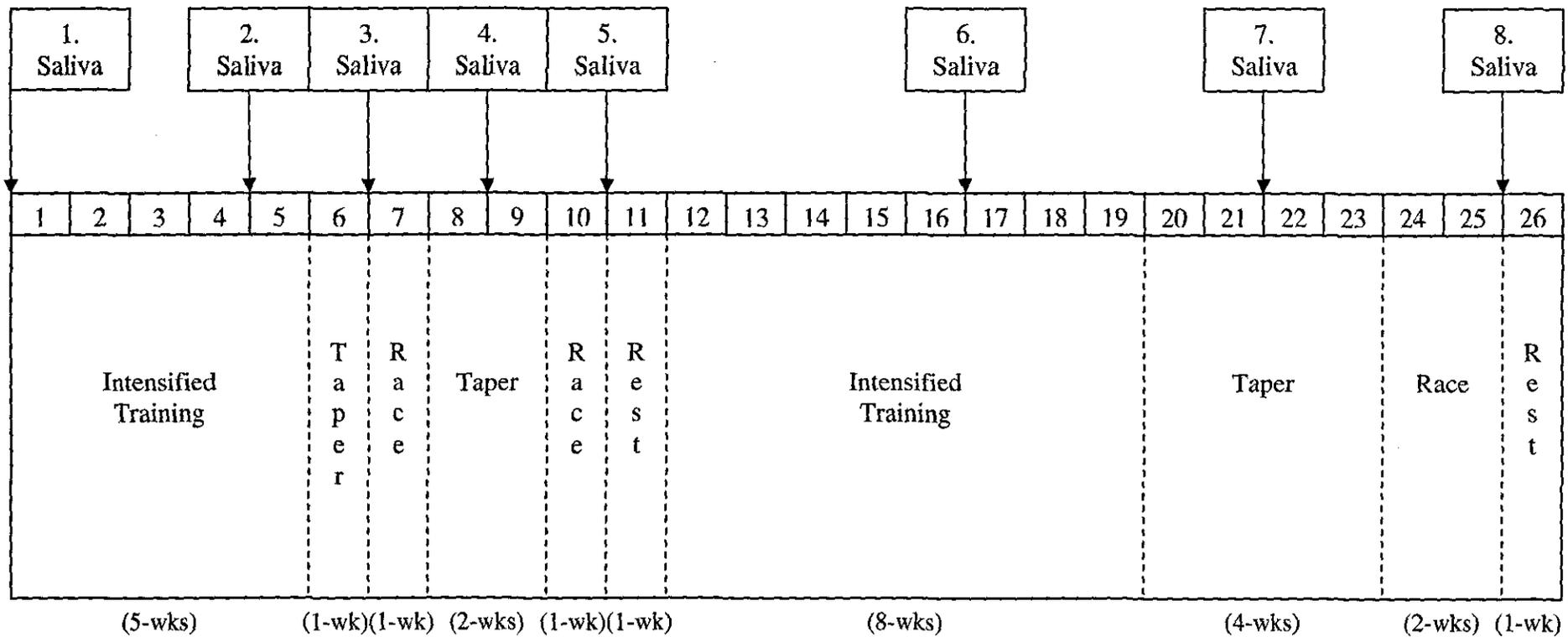
All types of training were incorporated into the study. A typical week consisted of 20 - 25 h of pool training and 6 - 8 h of dry-land training involving core strength and flexibility work. The subjective rating for the training sessions was defined and controlled by the British Swimming Head Coach and Team Physiologist. Individual training intensities were based on the swimmers' personal best times and maximum heart rate recorded during an incremental

swim test performed one-month previously. A schematic illustration of the experimental protocol is displayed in Figure 7.1. The sample time point and the type of training performed are described below:

- 1) Week 1: Baseline values at the beginning of October before swimmers commenced intensified training phase
- 2) Week 5: Data point at the end of October following 4 weeks of intensified training
- 3) Week 7: Data point at mid-November following 1 week of taper prior to the Commonwealth Games Trials 2005
- 4) Week 9: Data point at the end of November. This was in-between the Commonwealth Games Trials and a second National Swim Meet
- 5) Week 11: Data point at mid-December. This was post-competition and prior to the next phase of intensified training
- 6) Week 17: Data point at the end of January following 5 weeks of intensified training
- 7) Week 22: Data point at the end of February 2 weeks prior to the Commonwealth Games 2006
- 8) Week 26: Data point at the end of March 1 week after the Commonwealth Games 2006

Training Volume

The data for training volumes (average distances swum) and training intensity are presented in Figure 7.2. The mean training volume throughout the study period was similar between genders; $56 \pm 6 \text{ km.wk}^{-1}$ and $54 \pm 5 \text{ km.wk}^{-1}$ for males and females, respectively.



(Symptoms of URTI recorded weekly)

Figure 7.1. Schematic illustration of the experimental protocol.

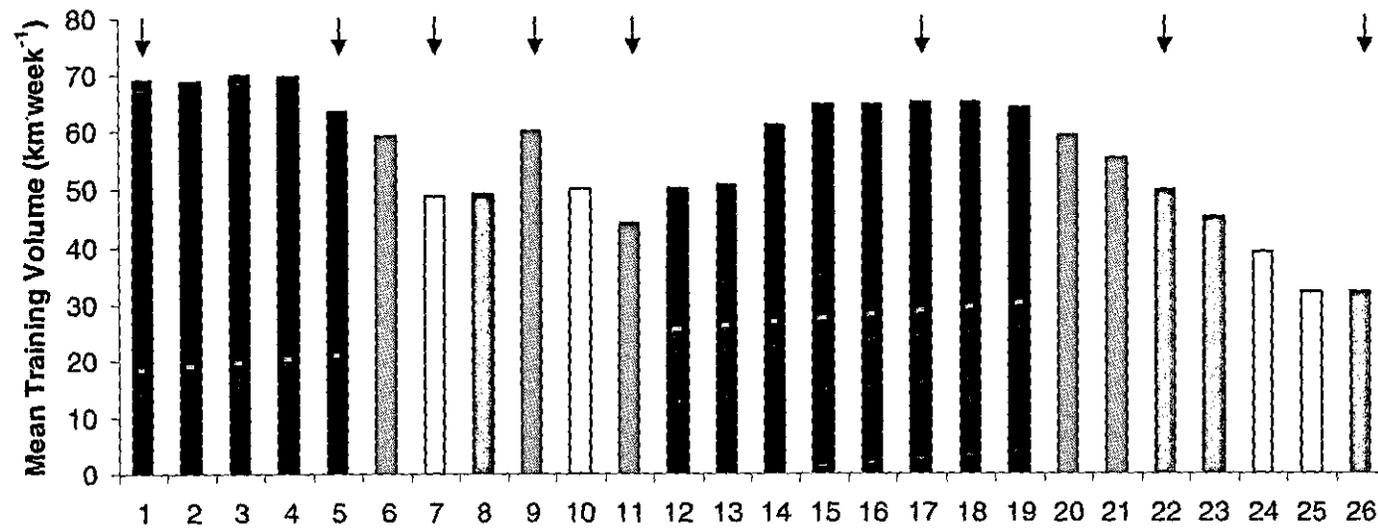


Figure 7.2. The weekly training programme undertaken during the study indicating the type of training and mean distances swum (km.week⁻¹) each week.

■ High intensity training, ▒ Moderate intensity training, ▨ Low intensity training, □ Competition, ↓ Saliva sampling.

7.2.4. Saliva collection and analysis

All saliva collection and analysis were performed as described previously in Chapter 2.

7.2.5. Statistical Analysis

The group data were analysed using a one factor repeated measures ANOVA and for the effects of gender, a two factor repeated measures ANOVA was employed. *Post hoc t*-tests with Holm-Bonferroni correction were applied where appropriate. Correlations between s-IgA secretion rate and symptoms of URTI were assessed using Pearson correlation analysis. Statistical significance was accepted at $P < 0.05$.

7.3. Results

7.3.1. Reported symptoms of URTI

Symptoms of URTI were highest during week 8, immediately after the Commonwealth Games Trials, where 7 swimmers exhibited symptoms (Figure 7.3). The mean number of reported episodes of URTI per swimmer over the 6-month study period was similar between genders, 3 ± 1 and 4 ± 2 for males and females, respectively.

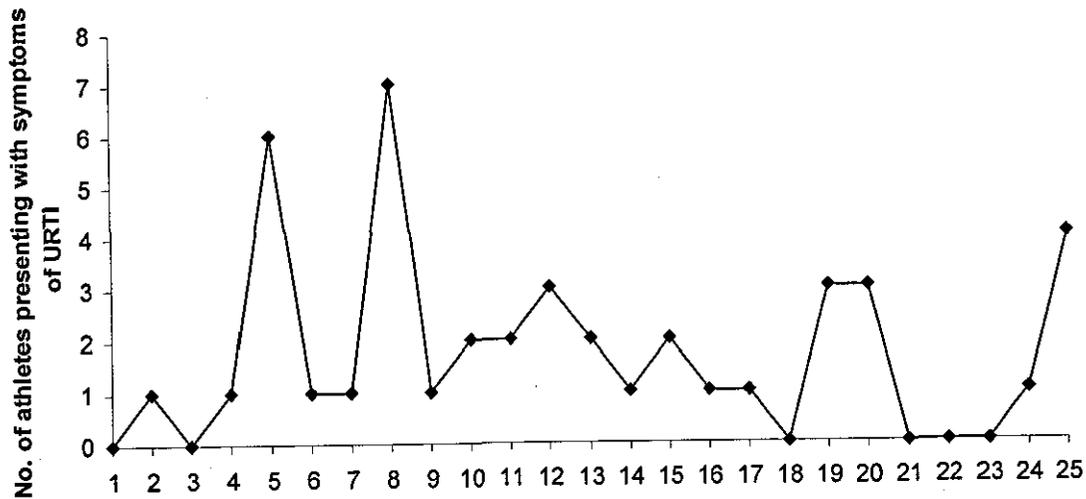


Figure 7.3. The incidence of reported symptoms of URTI during the 6-month training study ($N = 12$).

7.3.2. Salivary variables

Saliva flow rate

There were no significant changes in saliva flow rate throughout the study period (Figure 7.4). There was a significant main effect of gender, saliva flow rate was lower in females compared with males ($F_{1, 80} = 11.268$, $P = 0.001$).

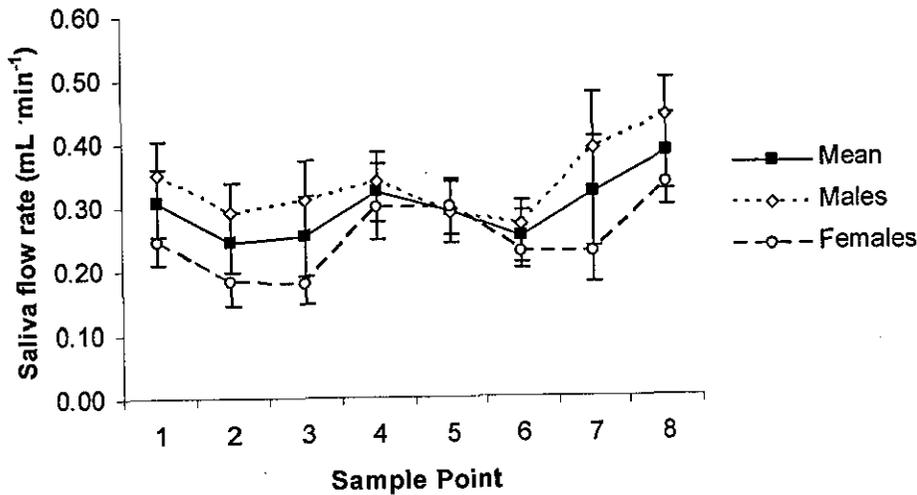


Figure 7.4. Mean (\pm SEM) values for saliva flow rate during the training study (Mean $N = 12$, Males $N = 7$, Females $N = 5$).

S-IgA concentration

S-IgA concentration did not change significantly during the study period (Figure 7.5). There was a significant main effect of gender; s-IgA concentration was significantly lower in females compared with males ($F_{1, 80} = 62.829$, $P = 0.000$).

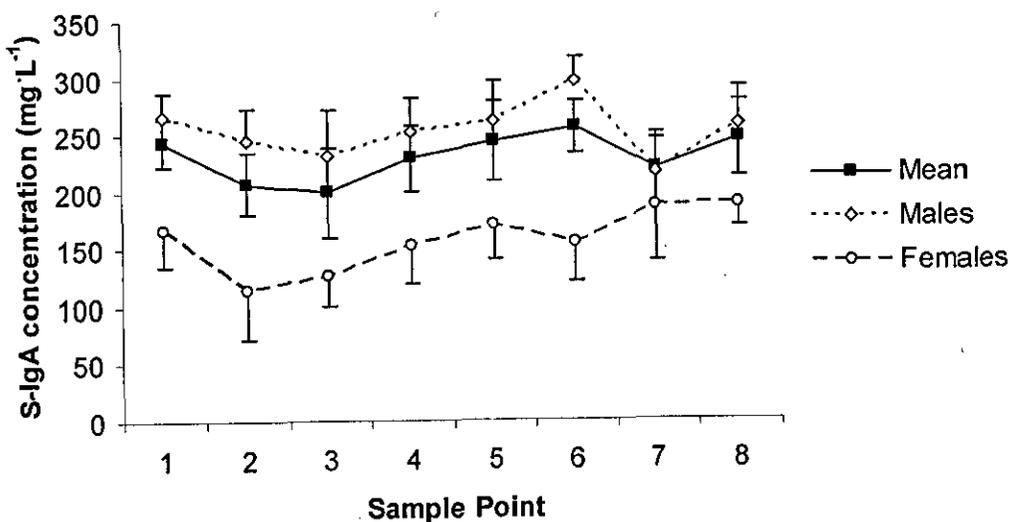


Figure 7.5. Mean (\pm SEM) values for s-IgA concentration during the training study (Mean $N = 12$, Males $N = 7$, Females $N = 5$).

S-IgA secretion rate

There was a significant main effect of time for s-IgA secretion rate ($F_{4, 43} = 3.395$, $P = 0.018$) (Figure 7.6). It was significantly lower at time point 7 (pre-competition) compared with time point 8 (post-competition; $P = 0.028$). Lower values at time point 3 (pre-trials) and 6 (after 5 weeks of intensive training) compared with time point 8 approached significance ($P = 0.054$). There was a significant main effect of gender for the secretion rate which was significantly lower in females compared with males ($F_{1, 80} = 59.159$, $P = 0.000$).

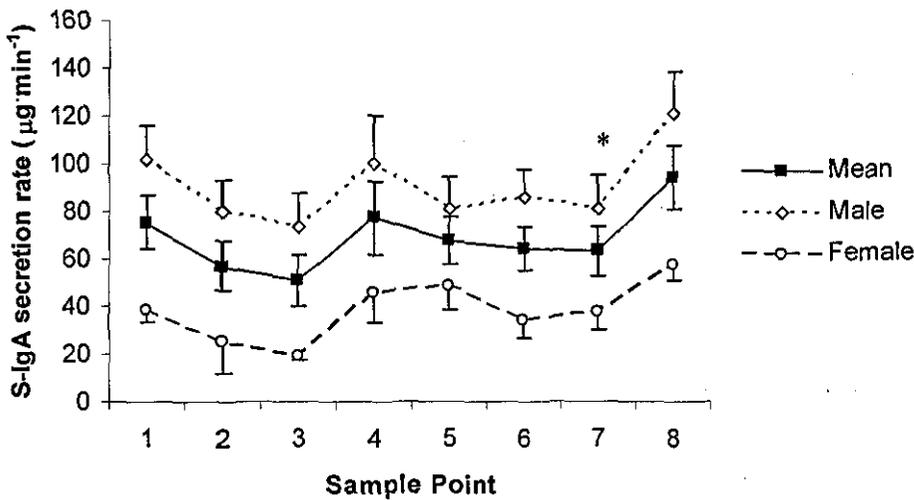


Figure 7.6. Mean (\pm SEM) values for s-IgA secretion rate during the training study (Mean $N = 12$, Males $N = 7$, Females $N = 5$). * Significantly lower than sample point 8, $P < 0.05$.

Salivary osmolality

Salivary osmolality did not change significantly throughout the study period (Figure 7.7). There was a significant main effect of gender for osmolality which was lower in females compared with males ($F_{1, 80} = 27.852$, $P = 0.000$). Similarly, there were no significant changes over time in the s-IgA:Osmolality but there was a significant main effect of gender (Figure 7.8). S-IgA:Osmolality was significantly lower in females compared with males ($F_{1, 80} = 5.592$, $P = 0.017$).

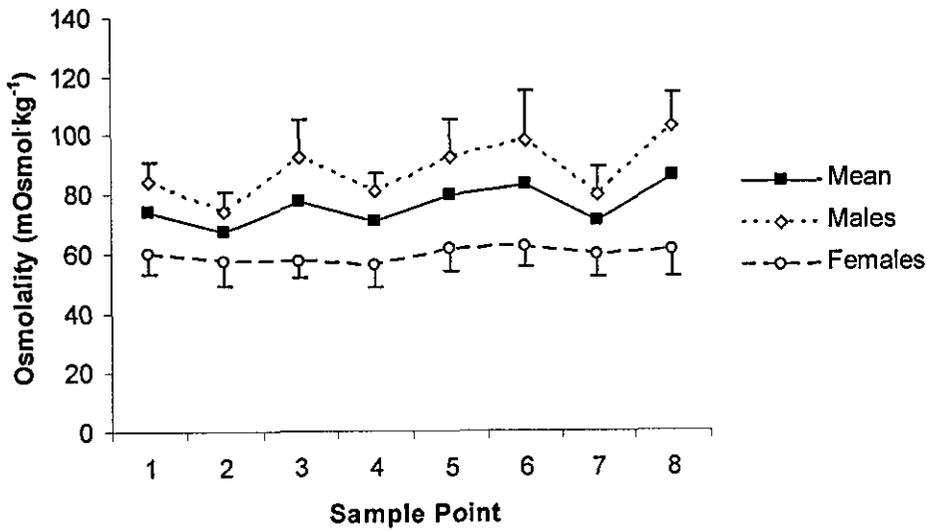


Figure 7.7. Mean (\pm SEM) values for salivary osmolality during the training study (Mean $N = 12$, Males $N = 7$, Females $N = 5$).

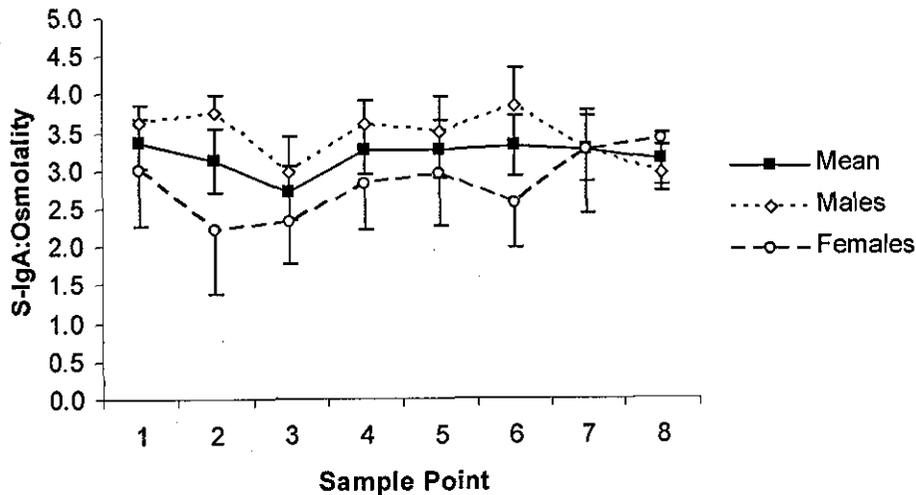


Figure 7.8. Mean (\pm SEM) values for s-IgA:Osmolality during the training study (Mean $N = 12$, Males $N = 7$, Females $N = 5$).

7.3.3. Predictors of infection

The initial baseline s-IgA secretion rate for each swimmer did not predict the number of reported URTI episodes during the training period ($r = 0.028$, $P > 0.05$). Furthermore, there was no association between the mean the s-IgA secretion rate for individuals over the training period and URTI episodes ($r = 0.38$, $P > 0.05$).

7.4. Discussion

The present investigation was a longitudinal study designed to investigate the changes in s-IgA over a 6-month period of training and competition in preparation for and during the Commonwealth Games 2006 in elite male and female swimmers. A further aim was to examine the possible relationship between s-IgA and reported episodes of URTI.

The findings show significant changes in s-IgA secretion rate but not s-IgA concentration during the 6-month period, which were lower following intensified training periods prior to competition compared with post-competition. Significant gender differences were also observed in all the selected measures of saliva composition with females exhibiting lower values compared with males. Despite these differences, the salivary responses to the training and competition were the same between genders. The highest number of reported URTI episodes were reported during week 8 of the study which followed the lowest measured levels of s-IgA secretion rate. However, the initial baseline levels of s-IgA were not predictive of reported URTI episodes.

There is little published research examining the effects of a training season on s-IgA secretion rate, since most researchers in the past have reported values of s-IgA concentration alone (Gleeson *et al.*, 1999, Mackinnon *et al.*, 1987; Tharp and Barnes, 1990). Fahlman and Engels

(2005) reported lower levels of s-IgA secretion rate in a cohort of 75 male American college footballers following two 6-week periods of high intensity training and competition, but which recovered during periods of recovery and rest. However, Whitham *et al.* (2006) reported no significant change in s-IgA secretion rate during a 20 week training study in Parachute Regiment trainees, despite decreased saliva flow rate at week 20 (June) compared with baseline (January). The results of the present study followed a similar pattern to Fahlman and Engels, (2005), where values tended to be lower following periods of intensified training, prior to competition compared with post-competition, where they seemed to recover. Indeed, previous research on acute exercise has suggested that the mucosal immune response may be affected by the exercise intensity (Fahlman *et al.*, 2001; Mackinnon *et al.*, 1987; Mackinnon and Jenkins, 1992; Tomasi *et al.*, 1982), but the relationship between s-IgA and chronic training is less clear. Significant increases in s-IgA concentration have been reported following 12-weeks of moderate intensity training in previously sedentary adults (Klentrou *et al.*, 2002) and after a 12-week training period in elite swimmers (Gleeson *et al.*, 2000). In contrast, studies of a longer duration have reported decreases in s-IgA concentration after a 4-month training period in elite swimmers and a downward trend during a 7-month season in another group of elite male and female swimmers (Gleeson *et al.*, 1999). However, none of these groups measured s-IgA levels post-competition or reported s-IgA secretion rate. Clearly further work is required in this area which examines all the available methods of expressing s-IgA before a general conclusion can be drawn.

It is noteworthy that in the present study significant differences in s-IgA secretion rate were only observed between the pre-Commonwealth Games (week 22) and post-Commonwealth Games (week 26) time points and not with the initial baseline values. One possible factor to account for this finding was that the levels may have been slightly suppressed at the beginning of the training study by psychological stress and anxiety of the training and

competition that lay ahead. Indeed, chronic psychological stress and anxiety have been repeatedly associated with reductions in s-IgA (Deinzer and Schuller, 1998; Jemmott and Magloire, 1988), and this may also have partly accounted for the lower levels in s-IgA secretion rate observed prior to competition. In addition to this, the majority of the swimmers had been involved in some basic endurance training before joining the squad at the beginning of the study, which may also have affected their baseline s-IgA levels. Thus, this data point may not represent a true baseline level, a finding that has also been reported previously in swimmers (Gleeson *et al.*, 2000).

The main limitation of the present study was that no control group was examined. Thus, one cannot be sure that the measures were not influenced by seasonal fluctuations. For example, saliva flow rate is known to be lower during the warm summer months (Whitham *et al.*, 2006), and this could in turn affect the s-IgA concentration and/or secretion rate. Furthermore, there exists a seasonal variation in URTI incidence, which is generally higher during the winter (Matthews *et al.*, 2002). The present study was conducted in the winter months when saliva flow rate was unlikely to be affected. However, it is not known whether the increased reported symptoms of URTI during week 8 of the study were a result of the training intervention or representative of a seasonal variation.

The present study found no significant changes in the concentration of s-IgA throughout the training period. This is in contrast to previous reports which found consistent changes in the concentration following a period of training in elite swimmers (Gleeson *et al.*, 1999; Gleeson *et al.*, 2000; Tharp and Barnes, 1990). Since the s-IgA secretion rate was not measured by these groups, a direct comparison cannot be made. However, our findings show that it was only the secretion rate of s-IgA (and not the concentration) that was sensitive to the training stimulus. A change in the s-IgA secretion rate may be a significant finding since it represents

the amount of s-IgA available on the mucosal surfaces for protection against infectious agents and takes into account the concentration of s-IgA and the saliva flow rate, both of which are important for host defence (Mackinnon *et al.*, 1993a). In addition to s-IgA concentration, no differences were observed when s-IgA was expressed relative to osmolality. Taken together, these findings highlight the disparities that occur when measuring these aspects of mucosal immunity in response to training, and raise the question over their utility as markers of URTI risk in athletes.

A further significant finding in the present investigation was the striking differences found between genders. In all of the salivary measures females consistently exhibited lower values when compared with males. However, their responses to the training stimulus were the same. Gleeson and colleagues (1999) reported a significantly lower s-IgA concentration in elite female swimmers compared with their male counterparts with no apparent difference in URTI incidence, and the present results extend these findings. A lower saliva flow rate and indeed s-IgA secretion rate measured in females may not be surprising since it has been shown that females possess smaller salivary glands than males (Inoue *et al.*, 2006). The lower values in osmolality and s-IgA concentration may initially suggest that the females produced a more dilute saliva which could be related to their hydration status. However, the s-IgA relative to osmolality values were also lower in females, which seems to suggest a lower level of mucosal immunity in females compared with males within this cohort of swimmers.

The reasons for the observed gender differences are unknown at present. It is known that sex hormones play a role in modulating certain aspects of immune function at rest (Timmons *et al.*, 2005). However, Burrows *et al.* (2002) found no differences in mucosal immune status in female endurance athletes over three consecutive menstrual cycles. Moreover, no relationship was found between s-IgA concentration and progesterone levels. It is possible that the

absolute training status of the swimmers (men being faster than women) may have influenced the results, since Francis *et al.* (2005) reported increased values of s-IgA concentration in higher trained athletes compared with moderately trained athletes. On the other hand, the differences may be related to the relative training load performed. Although the training intensity was different and relative to each individual based on personal best times and maximum heart rates, the absolute volume performed was similar. Since immune function is affected by high volumes of training (Nieman, 2000), it is possible that this volume of training placed a higher demand on the female athlete, resulting in an increased physiological and/or psychological stress which may in turn impact negatively on s-IgA. Future investigations designed to measure the levels of stress (quantitatively or qualitatively) during a training period may provide more of an insight into this idea since negative associations between the levels of stress hormones and s-IgA have been previously found (Hucklebridge *et al.*, 1998). One important point arising from these findings is that such differences should be considered if normative levels for the prediction of infection risk in elite athletes are to be established. The importance of this finding in terms of infection risk cannot be determined from such a small sample size and although the females did report a higher number of reported URTI episodes on average, this difference was not significant.

A negative association between s-IgA levels and URTI symptoms has been previously established in elite swimmers (Gleeson *et al.*, 1999; Tharp and Barnes, 1990) and American footballers (Fahlman and Engels, 2005). However, others have been unable to find an association (Gleeson *et al.*, 2000; Nehlsen-Cannarella *et al.*, 2000; Novas, 2003), and the results from the present study corroborate this. The failure to link perturbations in immune function to infection incidence following heavy training and/or competition may largely be due to the difficulty of measuring immunity in groups of athletes large enough to have sufficient statistical power to detect an effect. This is something that is often outside of the

control of the researcher. During the study period, the highest number of URTI symptoms was reported in the middle of the training period following the first competition. This occurred two weeks after the lowest values in s-IgA secretion were recorded. Since s-IgA was not measured in the week in between these two time points, one cannot exclude the possibility that the levels of s-IgA may have remained low or dropped even further during this time leading to an increased susceptibility of URTI as was observed. Mackinnon *et al.* (1993b) found that lower levels of s-IgA preceded infection by 48 h and this highlights the necessity of regular sampling if a direct link between these two factors is to be established. Although s-IgA secretion rate tended to be lower following periods of intense training, without a control group, it is difficult to ascertain whether these levels were below a critical 'healthy' level, which would subsequently leave them more susceptible to URTI. Moreover, every attempt was made to accurately classify symptoms of URTI by questionnaire, however, viral load was not measured directly and hence, one cannot be certain that the reported symptoms were entirely representative of an infectious challenge.

Although a significantly lower s-IgA secretion rate was found prior to the Commonwealth Games compared with afterwards, only one swimmer reported an episode of URTI during the meet. This may indicate that the levels recorded as a group were not at a critical level, which would leave them more susceptible to illness. This can be reassuring for the coaches since URTI has been shown to have a negative impact on performance (Pyne *et al.*, 2001).

In summary, the present data demonstrate that s-IgA secretion rate tends to be lower after a period of high intensity training leading up to competition and recovers post-competition. However, s-IgA concentration and s-IgA to osmolality ratio remain unaffected. Females exhibit lower values of saliva flow rate, osmolality, s-IgA concentration and secretion rate at rest compared with males but their response to the training stimulus appears to be the same.

Reported symptoms of respiratory illness were highest during week 8 of the study - following the period when s-IgA secretion rate was lowest; but the initial baseline values of s-IgA secretion rate did not directly predict the number of infections for each individual swimmer.

Chapter 8 - General Discussion

Despite the abundance of research conducted into the effect of exercise on mucosal immunity the consensus remains unclear. Much of the inconsistencies arise from the design of the protocols, the participants studied and their nutritional status, and methodological and analytical differences. The purpose of this thesis was to address some of these factors and to investigate potential methods of enhancing the immune response to exercise.

8.1. Nutritional status

Gleeson and colleagues reported that the nutritional status of an individual can affect resting s-IgA levels with fasting saliva yielding higher and more variable s-IgA concentrations than non-fasting saliva (Gleeson *et al.*, 1990; Gleeson *et al.*, 2004a). This was examined further in this thesis where firstly the effects of a fed or fasted state prior to exercise were compared. The results showed that the ingestion of a high CHO breakfast 2 h before exercise had no effect on saliva flow rate, s-IgA concentration and secretion rate and it was concluded that exercise performed in either a fed or fasted state (of approximately 10 h) would have little influence on the absolute levels or the response pattern of s-IgA. It was speculated that the differences in the findings could be related to the timing of the meal and/or hydration status of the individual. Thus, in Chapter 4 the influence of feeding versus fasting *during* exercise on s-IgA was investigated; with the additional measurement of the salivary antimicrobial proteins lysozyme and α -amylase. No significant effects of nutritional status on the s-IgA response to exercise were found. In contrast, feeding the high CHO cereal bars during exercise resulted in a significant increase in lysozyme and α -amylase compared with fasting. Carbohydrate is known to blunt the cortisol response during exercise and appears to limit the degree of immunodepression (Nieman, 2007). However, since higher values of cortisol in the

fasted state compared with the fed state were only observed at the post-exhaustion time point, it was unlikely that cortisol played a role in these immune responses. The salivary glands are innervated by parasympathetic and sympathetic nerves (Chicharro *et al.*, 1998), and it is possible that the ingestion of cereal bars during exercise activates mechanical and/or gustatory receptors leading to a reflex stimulation of protein secretion via the autonomic nerves. Moreover, since higher values in lysozyme and α -amylase remained evident post-exhaustion, it suggests that these effects are relatively long lasting.

The design of the study was such that it prevented the effects of feeding on the salivary responses to be separated from masticatory stimuli alone or in combination with gustatory (flavoured) stimuli. Although both trials would have elicited a certain amount of gustatory stimuli, the fact that the flavours were not identically matched, may have confounded the responses. Thus, future research should attempt isolate these effects as well as from the effects of CHO, to examine which may have the most potentially beneficial effects during exercise.

8.2. Exercise and saliva flow rate

The results in this thesis show that an acute bout of exercise can differently affect the saliva flow rate. In Chapter 3 a significant drop in the flow rate was observed with exercise which recovered to baseline levels at 1 h post-exercise and these findings are consistent with many studies (Bishop *et al.*, 2000; Li and Gleeson 2005; Sari-Sarraf *et al.*, 2007b; Walsh *et al.*, 2002). In contrast, saliva flow rate was unaffected by exercise in Chapters 4, 5 and 6, which is in accordance with other studies (Bishop *et al.*, 2000; Walsh *et al.*, 2004).

It has been previously demonstrated that dehydration has the greatest impact on saliva flow rate during exercise (Walsh *et al.*, 2004), which seems to occur after losses of ~3% body mass. However, in the present studies, body mass losses were small (< 1.5%), indicating that the participants consumed sufficient fluids to offset most of their body mass losses. This suggests that the reduction in saliva flow rate observed in Chapter 3 was a result of changes in autonomic nervous system activity and removal of parasympathetic vasodilatory influences, rather than dehydration *per se*. Indeed, the saliva flow rate was at its lowest point during exercise when parasympathetic nervous system activity would have been inhibited, and this started to recover upon cessation of exercise when parasympathetic activity would have returned.

The fact that saliva flow rate decreased in Chapter 3 and did not change in Chapters 4, 5 and 6 may be related to the different exercise intensities and durations employed between studies, as well as the timing of the samples. This is evident as the largest drop in saliva flow rate was found during the final 5 min of the 2 h bout of exercise at 65% $\dot{V}O_{2max}$, rather than immediately post-exercise when it started to recover (Chapter 3). This suggests that there may be a threshold level and/or duration of parasympathetic innervation below which, saliva flow rate decreases.

A further interesting finding was that salivary osmolality increased post-exercise without a significant change in body mass (Chapters 3 and 6) or a change in saliva flow rate (Chapter 6). This suggests that in addition to the saliva flow rate, the autonomic nervous system exerts a control on electrolyte secretion during exercise in the absence of dehydration. These results contrast those by Bishop *et al.* (2000) and Walsh *et al.* (2004) where fluid intake (to match pre-determined sweat losses in the latter study) prevented the decrease in saliva flow rate and increase in osmolality. Such a finding may question the reliability of salivary osmolality as a

marker of hydration status (or in relation to s-IgA concentration) post-exercise. Clearly, the regulatory factors governing saliva flow rate during exercise remain controversial and further research is warranted to examine these factors before a consensus can be drawn.

The maintenance of saliva flow rate during exercise is important since it has been suggested to be the single salivary defensive factor that significantly affects oral health (Rantonen and Meurman, 2000). The finding that individuals suffering from dry mouth syndrome have an increased incidence of URTI (Fox et al., 1985) supports this notion.

8.3. Acute exercise and salivary antimicrobial proteins

The s-IgA response to acute exercise was fairly consistent across studies. The results showed an increase in s-IgA concentration following exercise. Since s-IgA concentration is affected by the saliva flow rate, it is generally thought that these increases represent a drying of the mouth with dehydration rather than genuine alterations in the mucosal immune response. Hence, this factor was accounted for by expressing s-IgA as a secretion rate (saliva flow rate x concentration) and thus reflected the total amount of s-IgA available at the mucosal surface. With the exception of Chapter 3, significant increases were found in the s-IgA secretion rate post-exercise which returned to baseline levels at 1 h post-exercise. The increases in s-IgA secretion rate can be explained by the increase in SNS activity during high intensity exercise as this has been shown to increase the mobilisation of IgA into saliva by transcytosis (Proctor *et al.*, 2003). S-IgA secretion rate did not change significantly in Chapter 4, although a decrease of 25% during exercise compared with pre-exercise was observed. Furthermore, a significant decrease in the s-IgA:osmolality ratio was found post-exercise. In this study, significant decreases in the saliva flow rate occurred with exercise. Taken together these results suggest that decreases in s-IgA secretion rate occurring with exercise are a result of

the reduction in saliva flow rate rather than s-IgA secretion *per se*. However, when the saliva flow rate is maintained (Chapters 5, 6 and 7); the secretion rate tends to increase.

Salivary lysozyme and α -amylase appeared to follow the same pattern as s-IgA. Increases were consistently observed with exercise in both their concentration and secretion rate (Chapters 5, 6 and 7). Although the regulatory mechanism(s) governing secretion of these proteins is less clear, it is assumed that these changes are mediated in the same manner as s-IgA, resulting from increases in SNS activity with exercise (Carpenter *et al.*, 1998).

Collectively, these findings suggest that an acute bout of exercise, with regular fluid ingestion can result in a temporary enhancement of mucosal immune status. These findings challenge previous reports of a depressed mucosal immunity following intense exercise (Fahlman *et al.*, 2001; Mackinnon & Jenkins, 1993; Steerenberg *et al.*, 1997; Tomasi *et al.*, 1982) and highlight the complexity of this measure of immunity in response to exercise.

8.4. Exercise intensity

The responses of antimicrobial proteins to exercise appear to be influenced by the exercise intensity. This was initially demonstrated in Chapter 6 where s-IgA secretion rate showed little change during the moderate exercise intensity ($60\% \dot{V}O_{2\max}$), but increased by $\sim 35\%$ following exercise at the higher intensity ($75\% \dot{V}O_{2\max}$). These effects were examined in more detail in Chapter 7. The results showed significant increases in both s-IgA and lysozyme post-exercise following an incremental test to exhaustion, which returned to baseline at 1 h post-exercise. Moreover, lysozyme was also elevated following exercise at $75\% \dot{V}O_{2\max}$, whereas s-IgA remained unchanged. The increases in salivary proteins following the higher intensity trials reflect the elevated SNS activity, and also suggest that the

threshold of SNS activity to increase lysosyme may be lower than that for s-IgA. Taken together, these findings suggest that mucosal immunity may be temporarily enhanced following exercise at high intensities but does not change following more moderate short duration exercise.

8.5. Stress markers

The regulation of mucosal immunity during exercise is thought to be influenced by the release of stress hormones such as catecholamines and cortisol. The measurement of stress hormones in saliva aims to provide an easy non-invasive measure of SNS activity and the HPA axis activation without inducing further stress associated with blood drawing. Salivary cortisol has been shown to correlate highly with circulating levels (Chicharro *et al.*, 1998); however, catecholamines measured in saliva are regarded as less reliable (Kennedy *et al.*, 2001). Thus, this thesis measured α -amylase and salivary chromogranin A as surrogate markers of SNS activity. In Chapter 7 these markers of adrenal activation were specifically compared in response to short duration exercise at 3 different intensities. Alpha-amylase activity and secretion rate increased with exercise but only the secretion rate was affected by the intensity. However, both the concentration and secretion rate of chromogranin A was significantly affected by the exercise intensity.

The increases in these stress markers occurred in relation to increases in lysozyme and s-IgA and hence support the idea that the changes in salivary antimicrobial proteins during exercise are mediated by changes in SNS activity. These findings also suggest that both the concentration and secretion rate of chromogranin A warrant further investigation as potential indexes of SNS activity during exercise, which should compare them to circulating levels of blood catecholamines.

Increases in cortisol with exercise occurred between 90 – 120 min after the onset of exercise and at intensities of 65% $\dot{V}O_{2max}$ and above (chapters 4, 5, 6 and 7). This is not surprising since the appearance of cortisol in the circulation is reported to occur after a lag-period that in some cases may be more than 1 h (Virus and Virus, 2004), and following intensities above 60% $\dot{V}O_{2max}$. Since the changes in salivary antimicrobial proteins occurred more rapidly, within 25 min of exercise (Chapters 5, 6 and 7), an involvement of the HPA axis in the regulation of their secretion seems unlikely. However, the effects on s-IgA may be more delayed and occur between 2 – 24 h following exercise (Mackinnon *et al.*, 1987; Gleeson *et al.*, 2001).

8.6. Chronic exercise and s-IgA

The present findings show that chronic exercise training and competition in a squad of elite male and female swimmers can affect the s-IgA secretion rate but have little impact on the s-IgA concentration. Clear trends were found in the s-IgA secretion rate which was lower following periods of intense training and higher post-competition. These findings are in accordance to those reported by Fahlman and Engels (2005) who suggested that the s-IgA secretion rate is the most useful clinical biomarker of immune status in athletes. However, these findings are in contrast to the results following acute exercise presented in this thesis where significant increases were observed post-exercise. As discussed previously, the immediate effects of exercise on s-IgA are likely to be mediated by increased SNS activity with exercise. However, stimulation of the HPA axis by exercise (the end product being cortisol) may exhibit a more delayed response. Indeed, the synthetic glucocorticoid dexamethasone has been shown to cause a decline in the mobilisation of s-IgA 24 h after a single injection (Wira *et al.*, 1990), and in the longer term cortisol may inhibit IgA synthesis by B cells in the submucosa (Saxon *et al.*, 1978). Thus, the lower levels in s-IgA secretion

rate found following intensive training could be explained by a chronic elevation of cortisol levels.

Although there was no significant correlation between s-IgA and URTI symptoms, it was observed that the highest number of reported symptoms was preceded by the lowest s-IgA secretion rate. The failure to link perturbations in s-IgA to URTI may be due to the limited number of elite athletes in the squad, and is a major limitation in conducting research in this group of individuals. Moreover, the differences between genders in the measurements would also add to the variability making an association difficult to detect. Finally, the lack of control group in this study prevents us from ascertaining whether the changes were a result of the exercise training or simply reflecting a seasonal variation. Nevertheless, the results lend some support to a chronic effect of exercise training on mucosal immunity and highlight the need for further well-controlled studies that should look to examine other salivary antimicrobial proteins.

8.7. Gender differences

Significant gender differences in s-IgA, males being higher than females, in the general population have been previously reported (Evans *et al.*, 2000). Furthermore, lower s-IgA concentrations in females compared with males were observed in elite swimmers at rest (Gleeson *et al.*, 1999). Thus, in both Chapters 4 and 8 a female group was incorporated into the study design. The results showed significantly lower levels of s-IgA concentration, secretion rate and osmolality in females compared with males (Chapters 3 and 7). In addition, in Chapter 7 the saliva flow rate was also found to be lower in females. Although these differences were apparent at rest, they did not appear to influence the salivary responses to exercise, or exercise training.

The precise reason for these gender differences is unknown at present. Sex hormones appear play an important role in some aspects of the immune function (Timmons *et al.*, 2005), however, their significance in modulating mucosal immunity is less certain (Burrows *et al.*, 2002). Other possible explanations may include the absolute training status (Francis *et al.*, 2005), training volume (Nieman, 2000) and nutritional status (Chandra, 1997; Gleeson *et al.*, 2004b). Despite the uncertainty surrounding these mechanisms, such a finding is of practical importance since it may have implications on the future design of studies, and should be considered if clinically normal ranges are to be identified. Moreover, whether these findings are important in infection risk needs to be evaluated since they contrast with reports that women are generally more resistant to viral infections than men (Beery, 2003).

8.8. Stimulating saliva flow

Stimulating saliva flow either by eating food or chewing flavoured gum during exercise was shown to enhance certain aspects of mucosal immunity (Chapters 5 and 6). In Chapter 5 it was demonstrated that eating a commercially available cereal bar every 60 min during exercise increased the secretion of lysozyme and α -amylase into saliva but did not affect s-IgA. These findings were extended in Chapter 6 where it was shown that chewing flavoured gum during exercise enhanced lysozyme and α -amylase secretion rates but resulted in a small reduction in the s-IgA secretion rate.

Eating food and/or chewing gum causes the activation of masticatory and gustatory receptors which initiates a reflex stimulation of protein secretion via the autonomic nerves (Pedersen *et al.*, 2002). The fact that lysozyme and α -amylase were affected but s-IgA showed little or no change may depend on their different secretory pathways. Lysozyme and α -amylase are

stored in membrane secretory granules and are released spontaneously upon neuronal stimulation (Bosch *et al.*, 2002). However, s-IgA is secreted onto mucosal surfaces via transport across the epithelial cells by the polymeric Ig receptor, which is activated by neuronal stimuli that may differ to those of other salivary proteins (Proctor and Carpenter 2001).

In addition, the increase in lysozyme and α -amylase by chewing may be explained by the increase in salivary secretion from specific glands. For example, mastication particularly activates the parotid glands which produce large amounts of α -amylase, whereas strong taste stimuli activate the submandibular and sublingual glands from which lysozyme is mainly produced.

As with Chapter 4, a limitation of these findings is that it is not possible to distinguish whether the differences between stimulated and unstimulated saliva flow were due to masticatory or gustatory stimuli alone or in combination. In order to separate these mechanisms, it would be interesting to compare the effects of chewing flavoured gum compared with chewing tasteless polythene tube on the salivary responses to exercise.

The finding of an increase in the secretion rates of lysozyme and α -amylase with exercise which is further enhanced by stimulating saliva flow suggests these factors have a beneficial effect on the oral immune system. At present, there have been no studies that have directly related the levels of these proteins in saliva to a reduced risk of URTI and given the present findings, this may be of interest.

8.9. Conclusions

The main conclusions arising from this thesis are:

- 1) The prior nutritional status (fed versus fasted) of an individual does not affect resting s-IgA levels or the s-IgA response to prolonged exercise.
- 2) Ingestion of cereal bars *during* exercise can increase the secretion of lysozyme and α -amylase into saliva but does not affect s-IgA.
- 3) Stimulating saliva production during exercise by chewing flavoured gum enhances lysozyme and α -amylase secretion but results in a small decrease in s-IgA secretion.
- 4) The responses of salivary antimicrobial proteins to exercise are affected by the relative exercise intensity. S-IgA secretion increases following short-duration incremental exhaustive exercise but remains unchanged following exercise at 75% and 50% $\dot{V}O_{2max}$. Lysozyme increases following exercise at both 75% $\dot{V}O_{2max}$ and to exhaustion. These responses appear to be associated with increases in SNS activation but not cortisol.
- 5) S-IgA secretion rate is affected by chronic exercise training resulting in lower values prior to competition compared with post-competition, whereas s-IgA concentration and s-IgA to osmolality remain unchanged. S-IgA secretion rate does not appear to be directly linked to reported symptoms of URTI.
- 6) Females exhibit lower values of saliva flow rate, osmolality, s-IgA concentration and secretion rate at rest but this does not affect the response to an acute bout of exercise or chronic exercise training.

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Appendix A – Participant Consent Form

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence.

I agree to participate in this study.

Your name.....

Your signature.....

Signature of investigator.....

Date

Appendix B - Health Screening Questionnaire

HEALTH SCREEN FOR STUDY VOLUNTEERS

Name.....

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 <input type="checkbox"/> | NO <input type="checkbox"/> |
| (b) | attending your general practitioner | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (c) | on a hospital waiting list | YES <input type="checkbox"/> | NO <input type="checkbox"/> |

2. **In the past two years**, have you had any illness which require you to:

- | | | | |
|-----|---|------------------------------|-----------------------------|
| (a) | consult your GP | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (b) | attend a hospital outpatient department | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (c) | be admitted to hospital | YES <input type="checkbox"/> | NO <input type="checkbox"/> |

3. **Have you ever** had any of the following:

- | | | | |
|-----|-------------------------------|------------------------------|-----------------------------|
| (a) | Convulsions/epilepsy | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (b) | Asthma | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (c) | Eczema | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (d) | Diabetes | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (e) | A blood disorder | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (f) | Head injury | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (g) | Digestive problems | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (h) | Heart problems | YES <input type="checkbox"/> | NO <input type="checkbox"/> |
| (i) | Problems with bones or joints | YES <input type="checkbox"/> | NO <input type="checkbox"/> |

- (j) Disturbance of balance/coordination 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.)

.....

.....

.....

.....

Thank you for your cooperation!

Appendix C - Physical Activity Questionnaire

PHYSICAL ACTIVITY QUESTIONNAIRE

Name:

Date of birth:

Email address:

Telephone number:

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

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?

Which type of exercise do you perform (ex: running, cycling)?

.....

Are you currently doing WEIGHT TRAINING? YES NO

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

How many minutes does each session last?

Appendix D - Health Questionnaire

Loughborough University

School of Sport and Exercise Sciences

HEALTH QUESTIONNAIRE

Please complete the following brief questions to confirm your fitness to participate in today's session:

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

- 1) On medication, prescribed or otherwise? YES NO
- 2) Seeing your general practitioner? YES NO

Do you have 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 further details below:

.....

.....

.....

.....

Would you like to take part in today's experiment? YES NO

Signature.....

Date.....

Appendix E - URTI Symptoms Questionnaire

Weekly illness log

Name:..... Date:

Note if you have felt *any* of the symptoms, no matter how insignificant they may appear.

Place a tick in the corresponding box if a symptom is present. If a symptom is *not present* please leave the rest of the line blank.

If the symptom was light, moderate or severe write "L", "M" or "S" in the corresponding box.

If a symptom is present, write the number of days that it persisted.

Symptoms	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	No. days that symptoms persisted.
Fever			
Persistent muscle soreness or tenderness (> than 8h)			
Sore throat			
Scratchy throat			
Catarrh (runny or viscous fluid) in the throat			
Runny nose			
Plugged nose			
Cough			
Repetitive sneezing			
Joint aches and pains			
Weakness/fatigue			
Loss of appetite			
Loss of sleep			
Inability to train/compete			
Headache			

In the past 7 days have you (please tick as appropriate):

a) Taken any medication? YES NO

If yes, please specify

b) Seen your doctor/GP? YES NO

Other illness not on form, please report.....

