

# **Dose Response Effects of Caffeine Ingestion on Salivary Immunoglobulin A Following High-Intensity Exercise**

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of the requirements for the degree of Master of Sport and Exercise (MSpEx)

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## Table of Contents

List of figures	iii
List of tables	iv
List of abbreviations	v
Attestation of authorship	vi
Acknowledgements	vii
Intellectual property rights	viii
Thesis overview	ix
Abstract	x
<b>Chapter One: Introduction</b>	<b>1</b>
<b>Chapter Two: Literature review</b>	<b>6</b>
2.1 Mucosal immunity	6
2.2 Saliva	7
2.3 Immune markers in saliva	9
2.3.1 IgA	9
2.3.2 $\alpha$ -amylase	11
2.4 Are athletes more susceptible to infection?	12
2.5 Mucosal responses to acute exercise	14
2.5.1 Saliva flow rate	15
2.5.2 Saliva IgA	16
2.5.2.1 Continuous exercise	17
2.5.2.2 Intermittent exercise	21
2.5.2.3 Exercise intensity and duration	26
2.5.3 $\alpha$ -amylase	27
2.6 Methodological issues	28
2.6.1 Saliva collection methods	28
2.6.2 Result reporting	29
2.7 Potential mechanisms	30
2.8 Caffeine	32
2.9 Caffeine and sport	34
2.9.1 Dose response effects	35
2.10 Caffeine and saliva IgA at rest and during exercise	36
2.11 Summary	37
2.12 Potential effects of caffeine on saliva IgA responses to exercise	38

<b>Chapter Three: Experimental study</b>	39
3.1 Abstract	39
3.2 Introduction	39
3.3 Methods	42
3.3.1 Participants	42
3.3.2 Preliminary testing	42
3.3.3 Experimental trial procedures	43
3.3.4 Saliva collection	44
3.3.5 Saliva analysis	45
3.3.6 Statistical analysis	45
3.4 Results	46
3.5 Discussion	55
3.6 Perspectives	59
<b>Chapter Four: General Discussion</b>	60
4.1 Overview	60
4.2 Remaining issues in exercise immunology research	60
4.2.1 Mode of exercise	60
4.2.2 Participant characteristics	61
4.2.3 Individual responses to exercise, caffeine and basal s-IgA	62
4.3 Strengths and limitations	63
4.4 Future directions	65
4.5 Implications of this thesis	66
<b>References</b>	67
<b>Appendices</b>	79
A Informed consent form	80
B Health screen for study participants	81
C Physical activity questionnaire	82
D Caffeine consumption questionnaire	83

## List of Figures

Figure 1.	'J' shaped curve of infection risk. Adapted from Nieman (1994).	1
Figure 2.	'S' shaped curve of infection risk. Adapted from Teuw et al., (2006).	2
Figure 3.	Epithelial transport of IgA into saliva.	10
Figure 4.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on saliva caffeine concentration.	50
Figure 5.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on saliva IgA secretion rate.	51
Figure 6.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on saliva $\alpha$ -amylase secretion rate.	52
Figure 7.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on saliva IgA secretion rate as percent changes from pre-exercise to post-exercise for individual participants.	53
Figure 8.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on saliva IgA secretion rate for two participants.	54

## List of Tables

Table 1.	Salivary IgA responses to acute bouts of exercise of moderate durations.	18
Table 2.	Salivary IgA responses to acute bouts of exercise of longer durations.	20
Table 3.	Salivary IgA responses to endurance and ultra-endurance races.	21
Table 4.	Salivary IgA responses to repeated sprint cycle exercise.	23
Table 5.	Salivary IgA responses to a single training session.	24
Table 6.	Salivary IgA responses to sporting games or team sport exercise.	25
Table 7.	Caffeine content of common caffeine-containing beverages and foods.	33
Table 8.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on HR, RPE, fluid loss, RER, substrate oxidation and energy expenditure during exercise.	47
Table 9.	The effect of 2, 4, 6 and 8 mg·kg <sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80% $\dot{V}O_{2peak}$ on saliva caffeine concentration, IgA concentration, $\alpha$ -amylase activity, osmolality and saliva flow rate.	49

## List of Abbreviations

ANOVA	analysis of variance
BM	body mass
CAF	caffeine
CHO	carbohydrate
EE	energy expenditure
g	gram
h	hour
kg	kilogram
kJ	kilojoule
km	kilometre
L	litre
m	metre
mg	milligram
min	minute
ml	millilitre
mmol	millimole
nmol	Nanomole
pIgR	polymeric immunoglobulin receptor
PLA	placebo
RER	respiratory exchange ratio
RPE	ratings of perceived exertion
s	second
SD	standard deviation
s-IgA	salivary immunoglobulin A
URTI	upper respiratory tract infection
$VO_{2peak}$	peak oxygen uptake
WADA	World Anti-Doping Agency
°C	degrees Celsius
µg	microgram
µl	microlitre
µmol	micromole

## **Attestation of Authorship**

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed: \_\_\_\_\_

Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

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## **Thesis overview**

This thesis adheres to pathway 2, as classified by AUT University post-graduate thesis structure guidelines (AUT Post Graduate handbook 2012). It is important to note that the layout of this thesis is not conventional and consequently some information may be repeated. Briefly, this thesis is comprised of four chapters. Chapter One provides an overview of the thesis. Chapter Two (Review of Literature), introduces the reader to the concept of mucosal immunity and how exercise may affect mucosal immune function, as well as the methodological issues surrounding mucosal measures. Caffeine is then discussed in terms of its prevalence and impact amongst the athletic population, and finally the potential effects caffeine may have on mucosal immune function is considered. Chapter Three is an experimental study presented in the style of a journal article. This chapter includes the study design and methodology. Chapter Four provides an overall discussion of the study findings including limitations, applications of findings and areas of potential future research in this area.

## Abstract

Many athletes consume caffeine for its known ergogenic properties, with doses between 2-13 mg·kg<sup>-1</sup> body mass (BM) being shown to enhance performance. While the range of ergogenic doses is large, caffeine has been reported to have no dose-response effect. In addition, large doses have been associated with negative side effects and have also been reported to have immunosuppressive effects, which have been attributed to increases in adrenaline. However, despite the prevalence of caffeine consumption in both athletic and non-athletic populations, and research showing the potential for caffeine to suppress some markers of immune function, little research has examined the effects of caffeine ingestion on immune function in response to exercise in humans. Therefore the aim of this thesis was to investigate the influence of several doses of caffeine (typically used in training and competition situations) on the saliva IgA response to prolonged high-intensity exercise.

In a double-blind repeated-measures crossover design, 12 endurance-trained males ran for 70 min on a treadmill at 80%  $\dot{V}O_{2peak}$ , 60 min after ingesting 2, 4, 6 or 8 mg·kg<sup>-1</sup> body mass (BM) of anhydrous caffeine or placebo (cornflour) (PLA, 2CAF, 4CAF, 6CAF or 8CAF). Unstimulated whole saliva samples were obtained before supplementation, pre-exercise, mid-exercise, immediately post-exercise and 1 h post-exercise. Participants were habituated caffeine users and abstained from caffeine 60 h prior to trials. Trials were conducted 7 days apart at the same time of day. Saliva caffeine concentration was significantly higher in all caffeine trials than placebo at pre-exercise, mid-exercise, immediately post-exercise and 1 h post-exercise ( $P < 0.01$ ). Saliva IgA concentration, secretion rate and saliva flow rate remained unchanged following placebo and caffeine trials. In contrast saliva  $\alpha$ -amylase activity was higher in 6CAF, 4CAF and 2CAF trials when compared to PLA and 8CAF ( $P < 0.05$ ).

In conclusion, the findings of this thesis demonstrate that while ingesting caffeine doses of 2-8 mg·kg<sup>-1</sup>BM has neither a positive or negative affect on saliva IgA or flow rate, it does appear to increase  $\alpha$ -amylase activity. While the biological significance of these findings in terms of caffeine's potential to modify an individual's susceptibility to infection following prolonged high-intensity exercise is unknown, it is suggested that athletes consume the lowest beneficial dose in order to avoid potential side effects observed with higher doses of caffeine.

## Chapter One: Introduction

In recent years exercise has been shown to have both positive and negative effects on immune function depending on the intensity and duration of exercise performed (Henson et al., 2008; 1993). Research investigating the effects of exercise on immune function has often focused on upper respiratory tract infection (URTI) risk, as this is one of the leading causes of visits to general practitioners throughout the world (Graham, 1990) and is the most common medical complaint of athletes (Neville, Molloy, Brooks, Speedy, & Atkinson, 2006; Robinson & Milne, 2002). This focus has also been driven by the belief that the risk of incidence of URTI is increased in athletes following high intensity ( $> 70\% \dot{V}O_{2peak}$  (Nieman, 1997) and prolonged ( $> 90$  min (Nieman, 1997) exercise (Gleeson, Pyne, & Callister, 2004; Walsh et al., 2011) or during periods of heavy training or competition (Libicz, Mercier, Bigou, Le Gallais, & Castex, 2006; Novas, Rowbottom, & Jenkins, 2003).

The relationship between exercise intensity and risk of infection has been suggested to be “J-shaped” (Nieman, 1994; Figure 1). In this model, regular moderate physical activity reduces the risk of infection below that of sedentary individuals. In contrast, strenuous high-intensity exercise is thought to increase risk of infection compared to sedentary individuals due to the suppression of various immune markers (Nieman, 1994).

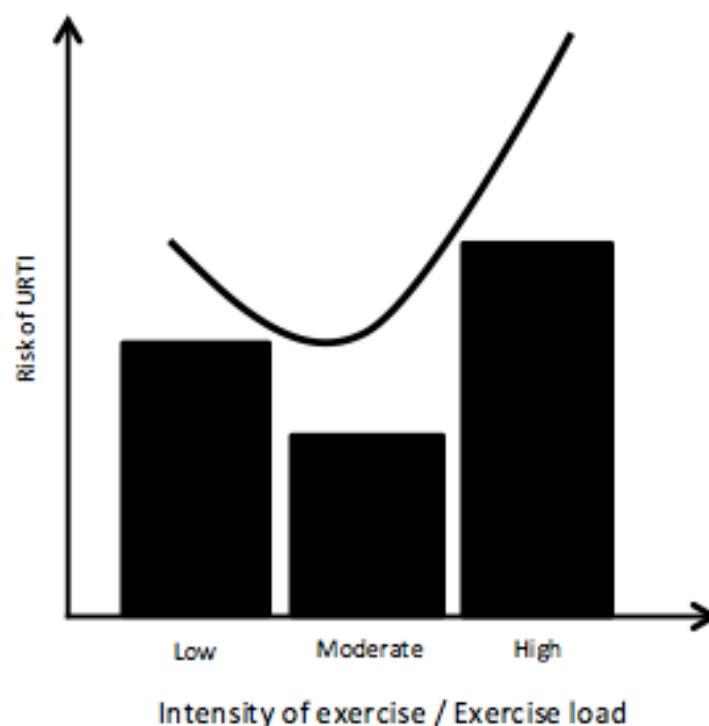


Figure 1: 'J' shaped curve of infection risk. Adapted from Nieman 1994.

While this “J-shaped” relationship has been supported by a number of studies (Foster, 1998; Matthews et al., 2002), Malm (2006) questioned this model on the basis that elite athletes would not be able to perform at such a high level if they had a greater susceptibility to infection. Malm (2006) suggested that elite athletes must possess an immune system capable of withstanding infections during severe physiological and psychological stress; otherwise they would not be able to remain at an elite level. Consequently Malm (2006) proposed an “S-shaped” curve (Figure 2) where elite athletes appear to have a reduced risk of infection compared to non-elite athletes performing high-intensity exercise.

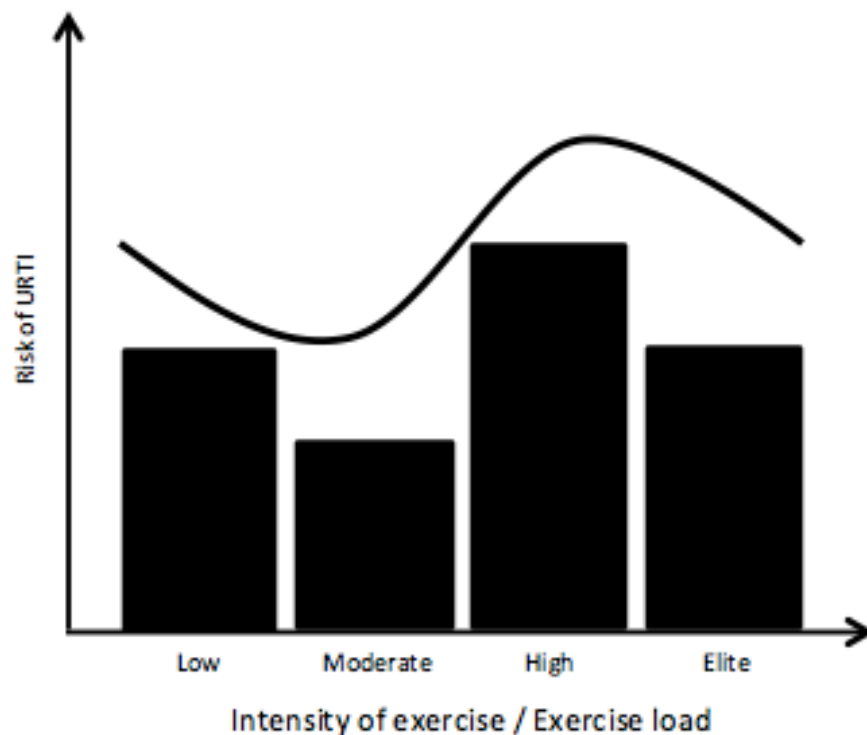


Figure 2: 'S' shaped curve of infection risk. Adapted from Malm 2006.

Studies investigating the effects of exercise on mucosal immunity often report a temporary depression in production of saliva secretory Immunoglobulin-A (s-IgA) as the major effector function, following acute bouts of intensive (70%  $\dot{V}O_{2peak}$  to maximal effort) or prolonged (2 to 16 h) exercise (Laing et al., 2005; Pacque, Booth, Ball, & Dwyer, 2007; Walsh, Bishop, Blackwell, Wierzbicki, & Montague, 2002). This depression has also been observed following short bouts of high-intensity exercise such as repeated maximal effort Wingate tests (Engels, Fahlman, Morgan, & Formolo, 2004; Fahlman, Engels, Morgan, & Kolokouri, 2001) through

to ultra-endurance running races (160km) (Nieman et al., 2006; Palmer et al., 2003) and in a range of sports (American football, swimming, cycling etc). These alterations in s-IgA following acute bouts of intensive exercise have been suggested to be related to the activation of the sympathetic nervous system and subsequent changes in stress hormone concentrations (cortisol and adrenaline) that are thought to suppress a number of immune functions for several hours following intensive exercise (as reviewed by Walsh et al., (2011).

In addition to decreased levels of immune markers, a number of studies have shown a correlation between exercise, low s-IgA secretion rate and increased incidence of upper respiratory symptoms (URS) or clinically confirmed URTI. For example, Fahlman and Engels (2005) found s-IgA secretion to decrease over the course of an American football season, which was associated with increased incidence of URS. This association was also found by Neville, Gleeson and Folland (2008) who reported a relationship between a decrease in s-IgA relative to baseline and increased incidence of URTI in professional yachtsmen over a 50-week period. A depression in immune function in the hours following intense exercise has been suggested to put athletes at greater risk of infection, as pathogens and viruses may be able to enter the body more easily (Nieman, 2000). Although this hypothesis was posed over a decade ago, a decline in host defense *in vivo* after exercise and increased infection risk is yet to be determined.

To gain an insight into variables that could affect immune responses to exercise researchers have examined the effects of commonly consumed supplements on immune responses to exercise. In particular, caffeine ingestion has been investigated due to its common use in the athletic population (Chester & Wojek, 2008). To date researchers have primarily investigated the effects of caffeine and exercise on systemic immunity, such as neutrophils and lymphocytes, (Fletcher & Bishop, 2010b; Gleeson, Bishop, Oliveira, & Tauler, 2011; Walker et al., 2008) however, only one study has examined the effects of caffeine on mucosal immune function following exercise (Bishop, Walker, Scanlon, Richards, & Rogers, 2006). Caffeine is one of the most commonly consumed and socially accepted legal psychoactive substances in the world. It is part of the methylxanthine family of drugs and despite having no nutritional benefit, is found in many types of food and drink (Graham, 2001). Caffeine is popular in a wide range of sporting populations, including runners and cyclists, due to its known ergogenic effects (as reviewed by Goldstein et al., (2010). Doses of 2-6 mg·kg<sup>-1</sup> body mass (BM) of caffeine have been found to enhance performance for a wide range of athletic activities, from short sprints to endurance events as well as for sports that are both

continuous or intermittent in nature (Goldstein et al., 2010). Furthermore, caffeine has also been recommended for use in training situations to enhance athletes' abilities to sustain high intensity exercise and maintain mental focus (Sokmen et al., 2008).

While caffeine has been found to be ergogenic (as reviewed by (Sokmen et al., 2008) it does not appear to have a dose-response effect on performance. In particular, Pasman, van Baak, Jeukendrup, and de Haan (1995), compared 3 doses (5, 9, and 13 mg·kg<sup>-1</sup> BM) of caffeine on cycling (at 80% W<sub>peak</sub>) time to exhaustion. While time to exhaustion was increased by 27% with caffeine ingestion when compared to placebo, there were no significant differences in performance between caffeine doses. Graham and Spriet (1995) also found similar results when comparing doses of 3, 6, and 9 mg·kg<sup>-1</sup> BM on running (at 85%  $\dot{V}O_{2peak}$ ) time to exhaustion. However, they found that the 9 mg·kg<sup>-1</sup> BM dose had little effect on time to exhaustion and attributed this to the dose being too high and consequently over-stimulating the central nervous system. Other researchers have also noted the potential side-effects of caffeine, especially when doses are high (> 6 mg·kg<sup>-1</sup> BM) (Burke, 2008). Caffeine ingestion has been associated with insomnia, headaches and gastro-intestinal distress (Evans & Griffiths, 1992; Leonard, Watson, & Mohs, 1987) which all have the potential to limit exercise performance. Consequently it could be suggested that athletes should use the lowest dose possible for them to enhance performance due to the potential side effects seen with higher doses of caffeine.

Despite its widespread consumption in society (approximate daily intake in New Zealand adults 3.5 mg·kg<sup>-1</sup> BM (Thomson & Schiess, 2010)) as well as in athletic populations (Chester & Wojek, 2008), only one study has investigated the effects of caffeine on mucosal immune responses following exercise (Bishop et al., 2006). This is surprising considering both exercise and caffeine have been consistently shown to increase adrenaline concentration (Graham, 2001; Zouhal, Jacob, Delamarche, & Gratas-Delamarche, 2008), which has been suggested to perhaps mediate some of the suppression seen with some immune markers (Bishop, Fitzgerald, Porter, Scanlon, & Smith, 2005; Fletcher & Bishop, 2011). Bishop et al., (2006) compared the effects of a 6 mg·kg<sup>-1</sup> BM dose of caffeine relative to placebo on s-IgA responses after a 90 min cycle at 70%  $\dot{V}O_{2peak}$ . Caffeine ingestion was associated with a 50% increase in s-IgA levels compared to placebo. However, these effects were only short-lived, with values returning to baseline by 1 h post exercise. While caffeine ingestion increased s-IgA responses following exercise, the dosage used in this study is considered quite high by some researchers, and is unlikely to be used by athletes in a typical training situation

(Chester & Wojek, 2008). Consequently this thesis set out to investigate the dose-response effects of caffeine ingestion on s-IgA responses to high-intensity exercise.

## Chapter Two: Literature Review

### 2.1 Mucosal Immunity

The mucosal immune system is the largest immune network in the body, defending the respiratory system, mouth, eyes, reproductive tract and intestine from infection. Mucosal immunity is often seen as a first line of defense against infection, as it reduces the need for involvement from systemic immunity (Gleeson, 2006a). Systemic immunity is primarily inflammatory and has the potential to damage tissues, so whilst it offers protection it can also harm the body.

The mucosal immune system comprises a large network of structures found at mucosal surfaces and can provide protection at these surfaces as well as sites distal to the original site of antigen presentation (Brandtzaeg et al., 1999). This network is comprised of many different mucosal associated lymphoid tissues (MALT), including tissues in the salivary glands, nasal passages, respiratory tract, gut, urogenital tracts, lacrimal glands, and lactating mammary glands (Williams, 2012). Whilst mucosal immunity contributes to the body's first line of defense, it is not part of the innate immune system. Instead, it provides an adaptive immune response by reacting to antigens in the body. This is achieved via a process starting with the humoral arm of the immune system where circulating B-cells differentiate to become antibody-producing B plasma cells (Gleeson, 2006a). These cells are capable of producing 5 antibody isotypes; immunoglobulin (Ig) A, G, M, D and E, depending on which antigen is presented at the cell surface. Once an antibody-producing plasma cell has differentiated to produce a specific antibody, special receptors on the cell surface (which are specific to each antibody isotype) enable it to preferentially migrate into MALT, or remain in systemic circulation. These antibodies are found in both systemic and mucosal immune systems, however, due to the specificity of cell receptors on the antibody-producing cells, the proportion of each Ig differs between these systems; with IgA being the most common Ig in MALT, and IgG in systemic immunity (Williams, 2012).

In addition to antibodies, antimicrobial enzymes such as  $\alpha$ -amylase and lysozyme also play an important role in mucosal immunity (Gleeson, 2000).  $\alpha$ -amylase, produced by acinar cells in salivary glands contributes to mucosal immunity by inhibiting bacterial adherence and growth to epithelial surfaces (Scannapieco, Solomon, & Wadenya, 1994). However lysozyme, produced by epithelial cells is thought to offer protection by killing bacteria via enzymatic and non-enzymatic mechanisms (Williams, 2012).

Together these antibodies and enzymes work as first line defenders to protect the body against pathogens, antigens and allergens presented at mucosal surfaces (Gleeson et al., 2000). This protection is achieved via the activation of an immune response that aims to prevent pathogen adherence, neutralize viruses within epithelial cells and excrete immune complexes (antigen bound to an antibody) across mucosal epithelial cells to the cell surface (Lamm, 1998).

## **2.2 Saliva**

Saliva is a key component of the mucosal immune system, with approximately 750-1500 ml secreted each day (Bishop & Gleeson, 2009). Saliva is a colourless, dilute fluid composed primarily of water, has a density ranging from 1002 to 1012 g·L (Schneyer, Young, & Schneyer, 1972) and a pH of approximately 6.64 which varies depending on blood CO<sub>2</sub> levels (Kreusser, Heidland, Hennemann, Wigand, & Knauf, 1972). The composition of saliva includes a number of antibodies, enzymes, peptides, hormones, mucus and antibacterial compounds (Chicharro, Lucia, Perez, Vaquero, & Urena, 1998). Typically, concentrations of these compounds are lower in saliva than in the blood (Schneyer et al., 1972), however research has shown that some compounds, such as cortisol, testosterone, dehydroepiandrosterone (DHEA) and aldosterone, provide a reliable reference for their respective blood concentrations (Cadore et al., 2008; Dawes, 1974).

Sub-maxillary, parotid, sub-lingual and numerous minor mucous glands found in the oral cavity are responsible for saliva secretion in humans (Dawes, 1974). Saliva secretion is primarily regulated by the autonomic nervous system (ANS), with salivary glands being innervated by parasympathetic cholinergic and sympathetic adrenergic nerves, which affect both saliva flow rate and composition (Baum, 1987; Denniss, Schneyer, Sucanthapree, & Young, 1978). Sub-maxillary glands are responsible for ~65% of total un-stimulated saliva secretion with parotid glands contributing ~23%, sublingual glands ~4% and minor mucous glands ~8% (Dawes, 1974). Innervation of the different salivary glands also varies, with sub-maxillary and minor mucous glands being innervated by sympathetic nerves, and parotid and sublingual glands by parasympathetic nerves (Baum, 1987). While normal saliva secretion is achieved by a combination of both parasympathetic and sympathetic innervation, parasympathetic activity provides the primary stimulus for increased saliva secretion (Schneyer et al., 1972).

Sympathetic activity is associated with vasoconstriction of blood vessels and has been suggested to result in decreased blood flow to salivary glands and thereby reduce saliva flow rate (Chicharro et al., 1998; Lavelle, 1988). Sympathetic activation has been shown to result in low saliva flow rates, but saliva that is high in organic and inorganic compounds including  $\alpha$ -amylase (Bishop & Gleeson, 2009).  $\alpha$ -amylase in particular is often seen to be elevated with sympathetic activation and has been suggested as a potential indicator of increased sympathetic activity (Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007). In contrast, parasympathetic activity results in the opposite response with increased blood flow to glands leading to increased flow rate and saliva that is low in organic and inorganic compounds (Baum, 1987).

Saliva flow rate and composition can also be altered via other mechanisms. Stimulation of saliva glands has been shown to modify saliva flow rate as well as composition by chewing polythene tubes (Proctor & Carpenter, 2001) or paraffin film (Engelen, de Wijk, Prinz, van der Bilt, & Bosman, 2003), sucking mints (Gleeson, Crampton, Strachan, Mundel, & Till, 2003) or sour lozenges (Ljungberg, Ericson, Ekblom, & Birkhed, 1997) and holding citric acid, or salt on the tongue (Noble, 2000; Watanabe & Dawes, 1988). It is suggested that stimulation of saliva flow rate and consequent alterations in composition is likely due to changes in gland secretions, with studies showing parotid secretion contributing more than 50% of stimulated saliva secretion (Humphrey & Williamson, 2001; Sreebny, 2000). Composition of saliva may also be affected by preferential stimulation of salivary glands as a result of differences in nerve innervation between glands and some proteins and antibodies being primarily secreted by different glands (Crawford, Taubman, & Smith, 1975; Speirs, Herring, Cooper, Hardy, & Hind, 1974). Consequently it is important that researchers state whether saliva was stimulated or not and if saliva collected was 'whole-mixed' (saliva from all glands) or from specific glands, to allow for accurate comparisons between studies.

Saliva flow rate may also be affected by other factors including fasting, dehydration, age, gender and circadian rhythm (Chicharro et al., 1998). For example, 24 h of fasting with no food or water has been shown to decrease resting parotid saliva flow rate and increase saliva osmolality which has been suggested as an indicator of dehydration (Ship & Fischer, 1997). In addition, un-stimulated saliva flow rate was seen to decrease in elderly subjects compared to younger adults (Ben-Aryeh, Miron, Szargel, & Gutman, 1984; Gutman & Ben-Aryeh, 1974), despite age appearing to have no effect on stimulated parotid saliva flow rates (Baum, 1981). Gender differences have also been investigated, with females showing lower

un-stimulated whole-saliva flow rates compared to males (Inoue et al., 2006). This was attributed to differences in saliva gland size, resulting from smaller body sizes in females compared to males (Inoue et al., 2006). Lastly, circadian rhythm is thought to possibly affect saliva flow rate with some studies showing a decrease in saliva flow rate over the day, with highest rates seen early in the morning and lowest in the evening (Palmai & Blackwell, 1965; Palmai, Blackwell, Maxwell, & Morgenstern, 1967). In contrast, a recent study by Nehlsen-Cannarella et al., (2000) found increases in saliva flow rate over the course of a morning in resting participants, supporting findings of Dawes (1972), who also reported increases in saliva flow rate over the course of the day. Currently it is unclear why these discrepancies exist and as such it appears that further research in this area should be conducted

## **2.3 Immune markers in saliva**

### **2.3.1 IgA**

Secretory IgA is the most abundant immunoglobulin found in mucosal secretions, including saliva (Proctor & Carpenter, 2001). It is a polymeric molecule comprising two IgA monomers, a joining chain (J-chain) and a secretory component (Teeuw, Bosch, Veerman, & Amerongen, 2004). Immunoglobulin A is synthesised by antibody producing plasma cells that have migrated to mucosal tissues, and predominates over other immunoglobulins in salivary glands (Michalek & Childers, 1990). The formation and secretion of IgA is a multi step process (Figure 3). First, two IgA monomers secreted from the plasma cell attach to the J-chain to produce polymeric IgA (pIgA). pIgA then attaches to a polymeric immunoglobulin receptor (pIgR) due to the J-chain's affinity for the pIgR. The pIgR is located at the basolateral (inside) membrane of a cell surface and once bound to the pIgA, is endocytosed into a vesicle and transcytosed to the apical (outside) surface of the cell. At the apical surface the pIgA-pIgR complex is proteolytically cleaved, leaving the extracellular component of pIgR, a secretory component (SC), attached to the pIgA. The secretory component acts to stabilise pIgA, protecting it from proteolysis (Brandtzaeg, 1995).

Immunoglobulin A has multiple protective functions, and defends the body's mucosal surfaces via inhibiting pathogen adherence, virus neutralization and excretion of immune complexes (Lamm, 1997). Pathogen adherence is generally considered the most important function of IgA as it inhibits bacteria from penetrating mucosal epithelium at the apical surface (Teeuw et al., 2004). This is achieved when IgA binds to antigen making it too large to penetrate cells, or by blocking the ability of antigen to bind to cells (Williams, 2012). Immunoglobulin A also has the ability to neutralize viruses intracellularly when it is being

transcytosed as the pIgA-pIgR complex to the apical cell surface. In this instance IgA binds to the viral protein inhibiting viral replication with the immune complex being excreted once it reaches the apical surface (Mazanec, Kaetzel, Lamm, Fletcher, & Nedrud, 1992). Immunoglobulin A protection also occurs at the basolateral surface when antigen have crossed through epithelia or if they have been produced in the submucosa. This is also achieved via the attachment of antigen to IgA and transcytosis to the apical cell surface (Kaetzel, Robinson, Chintalacharuvu, Vaerman, & Lamm, 1991).

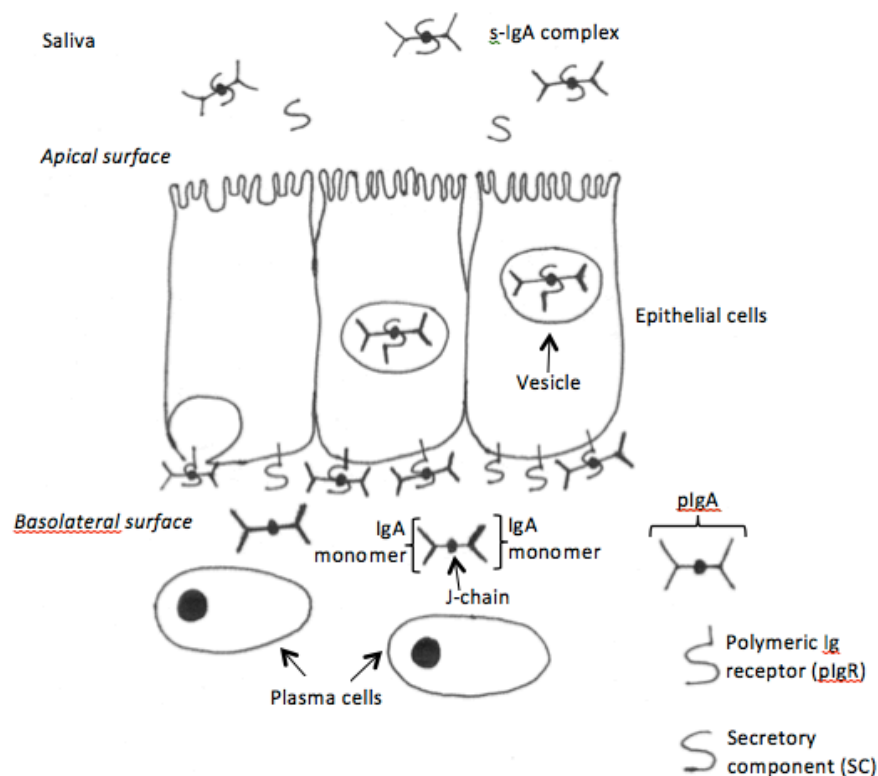


Figure 3: Epithelial transport of IgA into Saliva. Adapted from Teuw et al., 2004.

In addition to antigenic stimulation, neuroendocrine regulation appears to play an important role in IgA synthesis and secretion (as will be discussed in section 2.10). Studies in rats have demonstrated that the secretion of IgA can be increased with both sympathetic and parasympathetic nerve stimulation and that adrenaline appears to increase the transport of human IgA into rat saliva via salivary cells (Carpenter, Garrett, Hartley, & Proctor, 1998; Carpenter, Proctor, Ebersole, & Garrett, 2004). Secretion of s-IgA may also be affected by other factors such as stimulation from chewing or food. Stimulation from chewing a polythene tube has been shown to increase the concentration of s-IgA secreted from the

parotid gland (Proctor & Carpenter, 2001), and stimulation from sucking on a mint also resulted in higher s-IgA secretion rates compared to un-stimulated saliva (Gleeson et al., 2003). In addition, differences in s-IgA levels may also be reported if saliva is collected from a particular gland, or area of the mouth as s-IgA is primarily secreted by minor salivary glands, which produce 30-35% of total s-IgA and is 4 times greater than the secretion of s-IgA from parotid glands (Crawford et al., 1975). Consequently it appears that there may be several mechanisms regulating the secretion and transport of IgA.

### **2.3.2 $\alpha$ -amylase**

Saliva contains a number of enzymes and proteins with antimicrobial action that contribute to mucosal immunity (Papacosta & Nassis, 2011).  $\alpha$ -amylase is one of these antimicrobial enzymes and is the most dominant enzyme found in both pancreatic and salivary fluids (Papacosta & Nassis, 2011).  $\alpha$ -amylase contributes to innate mucosal immunity by inhibiting bacterial adherence and growth to epithelial surfaces (Scannapieco et al., 1994) and is seen to comprise part of the body's first line of defense against infection (Scannapieco, 1993).  $\alpha$ -amylase is secreted primarily by the parotid glands (Speirs et al., 1974), but more specifically, is secreted from acinar cells in salivary glands innervated by both sympathetic and parasympathetic nerves and is primarily secreted by parotid glands. As a result  $\alpha$ -amylase, like IgA, is largely regulated by neuronal pathways (Chatterton Jr, Vogelsong, Lu, Ellman, & A, 1996).  $\alpha$ -amylase activity has also been shown to be affected by physiological and psychological mechanisms. For example, chewing has been shown to stimulate  $\alpha$ -amylase activity, in order to help to break down food (Mackie & Pangborn, 1990). In addition, it has been proposed that the increase in  $\alpha$ -amylase activity may be due to increases in saliva flow rate that occur with chewing, with greater volumes of saliva helping to flush  $\alpha$ -amylase out from ducts (Fuller & Gallacher, 1984).  $\alpha$ -amylase activity has also been shown to be affected by food, with both lemon juice and salt resulting in increased levels of  $\alpha$ -amylase (Noble, 2000).

Psychological stress has also been shown to increase  $\alpha$ -amylase activity through interactions with the autonomic nervous system (Chatterton Jr et al., 1996). Chatterton Jr et al., (1996) investigated  $\alpha$ -amylase activity before and after a written examination and found  $\alpha$ -amylase activity as well as norepinephrine to increase. Consequently they suggested that  $\alpha$ -amylase activity may be predictive of catecholamine levels (Chatterton Jr et al., 1996). These findings are also supported by Takai et al., (2007) who examined the effects of a stressful video (corneal surgery) on  $\alpha$ -amylase activity and saliva cortisol concentration. They reported  $\alpha$ -

amylase activity to increase following the video which was also associated with increases in salivary cortisol. Takai et al., (2007) also recommended  $\alpha$ -amylase activity for its use as a marker of stress. In addition, a recent review by Nater and Rohleder (2009) concluded that  $\alpha$ -amylase was appropriate for use as a non-invasive marker of biological stress and autonomic nervous system activity. Although studies have consistently shown  $\alpha$ -amylase activity to increase with psychological stress, reasons as to how or why increases in  $\alpha$ -amylase may be useful to the body are yet to be determined. One suggestion is that increased availability of  $\alpha$ -amylase could help to speed up digestion and result in more energy being available to the body (Nater & Rohleder, 2009), however no suggestions were given concerning potential antibacterial implications.

## **2.4 Are athletes more susceptible to infection?**

It has been proposed that athletes may be more susceptible to infection, particularly URTI in the hours following intensive exercise (Gleeson, 2007). Researchers have suggested that this could be due to a depression in a number of immune measures, including the antibody IgA, predominantly found in mucosal secretions (Mackinnon & Jenkins, 1993; Nieman et al., 2003; Pacque et al., 2007). Approximately 95% of all infections are initiated at mucosal surfaces (Bosch, Ring, de Geus, Veerman, & Nieuw Amerongen, 2002), which are protected by antibodies and antimicrobial enzymes. Salivary secretory IgA is the most abundant antibody in saliva (Brandtzaeg, 2003) and is important in mucosal immunity as it acts to defend the body against viral pathogens, allergens and antigens (Lamm, 1998) that are primarily responsible for URTI (Mazanec, Nedrud, Kaetzel, & Lamm, 1993). Research has shown that s-IgA levels may be linked to an athlete's potential risk of URTI, with low levels of s-IgA associated with increased risk of URTI and high levels with reduced risk (Fahlman & Engels, 2005; Hanson, Rkander, & Oxelius, 1983; Neville et al., 2008).

A number of studies have shown a correlation between exercise, s-IgA and incidence of URTI (Fahlman & Engels, 2005; Gleeson, Bishop, Oliveira, McCauley, Tauler, et al., 2011; Nieman et al., 2003; Nieman et al., 2006). For example, a 160 km endurance running race was associated with a ~50% decrease in s-IgA secretion rate (rate of s-IgA secreted per minute) from pre- to post-race. In addition, decreases in s-IgA secretion rate were related to incidence of URTI in the two weeks following the event, with those experiencing URTI showing the greatest decreases in s-IgA secretion rate (Nieman et al., 2003; Nieman et al., 2006). Participant characteristics in these studies were heterogeneous with both male and female runners included and ages ranging from 19 and 68 years. Despite this, no significant

differences were found between gender or age and the pattern of change of s-IgA secretion rate or incidence of URTI.

Several researchers have also conducted longitudinal studies investigating the effects of training on s-IgA concentration and secretion and incidence of URTI (Fahlman & Engels, 2005; Gleeson et al., 1999; Neville et al., 2008). The effects of a 7-month training period on s-IgA levels and incidence of URTI were investigated in 26 elite Australian swimmers (Gleeson et al., 1999). Resting s-IgA concentrations (absolute values of s-IgA at the mucosal surface) were measured and found to decrease by 4% after each additional month of training. In addition, an inverse correlation was found between pre-training measures of s-IgA concentration and incidence of URTI (Gleeson et al., 1999). Pre-season resting s-IgA levels were also identified as another possible indicator of infection risk with those athletes showing lower pre-season s-IgA levels as having a greater incidence of infection. Similar results were also seen in a study monitoring s-IgA levels in 75 American college football players over a 12-month period (Fahlman & Engels, 2005). Both s-IgA concentration and secretion rate were measured, and both were found to decrease over the course of the season. Incidence of URTI increased over the season and were found to be best associated with decreases in s-IgA secretion rate rather than concentration (Fahlman & Engels, 2005). Secretion rate has been suggested to be the strongest predictor of URTI due to saliva flow rate being taken into account as it identifies the rate at which IgA is being secreted into saliva (Nieman et al., 2003; Nieman et al., 2002). A recent study in professional America's Cup yachtsmen over a 50-week period also investigated the effects of training on s-IgA and incidence of URTI (Neville et al., 2008). Saliva samples were collected weekly and incidence of URTI confirmed by the team physician. In contrast to previous studies, s-IgA was reported as a relative percentage to baseline when athletes were free of URTI (in addition to reporting concentration) and was found to be the stronger predictor of risk of URTI. A decrease in s-IgA of 40% below relative baseline levels was found to indicate a one in two chance of contracting URTI. It must be highlighted that Gleeson et al., (1999) and Neville et al., (2008) clinically confirmed URTI in participants rather than using self-reporting techniques like most s-IgA and URS studies (Fahlman & Engels, 2005; Nieman et al., 2003; Nieman et al., 2006), which may make their findings more accurate and reliable. Lastly, a recent study by Gleeson, Bishop, Oliviera, McCauley et al., (2011) investigated s-IgA secretion rate and incidence of URTI in 54 athletes completing endurance training over a 4-month period. In this study participants followed their own individual training programs (not prescribed or standardised by the researchers), recording details for each training session

they completed. Researchers then compared incidence of URTI with the different training loads and intensities completed by participants. Athletes experiencing 3 or more weeks of URTI were found to have higher training loads and lower rates of s-IgA secretion compared to athletes who did not experience URTI. This study in particular highlights a potential link between intensity and duration of exercise and URTI incidence, which is often reported in acute exercise and s-IgA studies.

Although a number of researchers have investigated s-IgA responses to exercise and incidence of URTI, no studies have been able to show a direct link between s-IgA secretion rate or concentration and URTI. This is likely due to ethical issues, as such a study would require participants to be purposefully infected with a virus and could lead to significant negative health outcomes such as myocarditis (a viral infection of the heart muscle (Gleeson, 2006a)). With this issue in mind, further research in this area could be strengthened to enable more confident associations between s-IgA and incidence of URTI. This could be achieved by making sure sound study protocols are used/employed to ensure reports of incidence of URTI, and quantification of exercise frequency, mode, duration and intensity are accurate.

Overall, although only a handful of studies have investigated s-IgA levels and risk of URTI, researchers generally report an association between a depression of s-IgA and increased risk of URTI. Consequently, it has been proposed that athletes undertaking prolonged intensive training in conjunction with insufficient recovery may be more susceptible to URTI (Gleeson et al., 2004; Gleeson & Walsh, 2011; Pyne & Gleeson, 1998), and as such, it could be suggested that monitoring of s-IgA levels could be a useful, easy and non-invasive measure for predicting risk of infection in athletes.

## **2.5 Mucosal responses to acute exercise**

Exercise is a potent stimulus of sympathetic activity and catecholamine secretion (Kastello, Sothmann, & Murthy, 1993), and as such is thought to have a considerable affect on salivary gland activity (Nater & Rohleder, 2009). Exercise is often shown to affect both saliva flow rate and composition, which is generally attributed to sympathetic stimulation and parasympathetic withdrawal in salivary glands (Bishop & Gleeson, 2009). Saliva flow rate is generally seen to decrease following strenuous or prolonged exercise (Hall, Fahlman, & Engels, 2007; Nieman et al., 2003). In contrast,  $\alpha$ -amylase activity is generally seen to increase with exercise, with increases suggested to be dependent upon exercise intensity

(Bishop, Blannin, Armstrong, Rickman, & Gleeson, 2000). Saliva IgA responses however are more varied. Saliva IgA has been found to increase (Allgrove, Gomes, Hough, & Gleeson, 2008), remain unchanged (Moreira et al., 2009) or decrease (Palmer et al., 2003) following exercise, however the wide variations in findings are often attributed to differences in exercise intensity and duration as well as methods used to collect saliva and the way findings are reported.

### **2.5.1 Saliva flow rate**

Increased sympathetic activity is associated with the vasoconstriction of blood vessels (Siemionow & Sonmez, 2011) and has been suggested as the cause of reduced saliva flow rates seen with exercise (Bishop et al., 2006; Moreira, Arsati, de Oliveira Lima-Arsati, de Freitas, & de Araújo, 2011) due to presumed reduced blood flow to salivary glands. However, it has been proposed that changes in saliva flow rate after exercise may be primarily due to parasympathetic withdrawal rather than sympathetic stimulation (Bishop & Gleeson, 2009) as sensations of 'dry mouth' associated with psychological stress have been related to parasympathetic withdrawal rather than sympathetic stimulation (Buckworth & Dishman, 2002). Decreases in saliva flow rate are most often reported following intermittent, short-term high-intensity exercise such as repeated Wingate sprints (Engels, Fahlman, & Wirth, 2003; Hall et al., 2007) and continuous prolonged exercise such as endurance running races (Henson et al., 2008; Nieman et al., 2006; Nieman et al., 2002). Despite a general trend for saliva flow rate to decrease following exercise (Dimitriou, Sharp, & Doherty, 2002; Ljungberg et al., 1997) some authors have found no effect of exercise on saliva flow rate (Davison, 2011; Li & Gleeson, 2004). In addition, understanding of saliva flow rate responses to exercise is further limited by a number of authors choosing to exclude saliva flow rate data from their studies.

Continuous exercise of longer durations (> 45 min) has also been reported to decrease saliva flow rate following a range of different exercise protocols. Sixty min of cycling at 75%  $\dot{V}O_{2peak}$  (Usui et al., 2011) provided enough stimulus to decrease saliva flow rate compared to resting individuals, as was a 2 h cycle at 60%  $\dot{V}O_{2peak}$  (Li & Gleeson, 2004). In addition, 90 min of cycle exercise at 70%  $\dot{V}O_{2peak}$  (Bishop et al., 2006), and 90 min of treadmill exercise at 9.7 km·h<sup>-1</sup> (Sari-Sarraf, Reilly, & Doran, 2006) also resulted in decreased saliva flow rate. In contrast, Krzykowski et al., (2001) and Davison et al., (2009) found no change to saliva flow rate following a 2 h cycle at 75% and 55-65%  $\dot{V}O_{2peak}$  respectively. Bishop et al., (2000) also found no change in saliva flow rate following a 2 h cycle at 60% in participants in a control

group, but saw decreases in saliva flow rate in participants who had restricted fluid intake, indicating that hydration status may affect saliva flow rate. Overall, whilst there is some variability in saliva responses to exercise it appears that saliva flow rate is likely to decrease or remain unchanged following exercise of varying durations and intensities. Consequently it is suggested that researchers should be more consistent in reporting variables that may help explain a change, or lack of change in saliva flow rate, such as body mass changes or measures of osmolality (an indicator of electrolyte concentration, primarily affected by hydration).

### **2.5.2 Saliva IgA**

The s-IgA responses to acute exercise are variable and have been suggested to be influenced by a number of possible factors. Many authors have found decreases in s-IgA concentration and secretion rate following exercise (Moreira et al., 2011; Tomasi, Trudeau, Czerwinski, & Erredge, 1982), however some authors have found no change (Davison, Allgrove, & Gleeson, 2009; McDowell, Chaloa, Housh, Tharp, & Johnson, 1991) or even increases (Allgrove et al., 2008; Davison, 2011) in both s-IgA concentration and secretion rate. Differences in methodologies employed have been suggested to explain the disparity in findings, in particular exercise protocols employed, duration, intensity, mode of exercise used and saliva collection methods (Bishop & Gleeson, 2009; Gleeson et al., 2004). Participant fitness levels are also thought to possibly affect results with suggestions that elite athletes respond differently to non-elite athletes (Gleeson & Pyne, 2000). To further complicate matters, comparison between study findings can also be difficult due to differences in the way data is often reported (Bishop & Gleeson, 2009).

There are several types of studies that have been used to investigate s-IgA responses to acute exercise and usually involve either continuous or intermittent exercise. Continuous exercise protocols have investigated the effects of different durations and intensities on s-IgA; whilst intermittent protocols often examine s-IgA responses to repeated sprints, the acute effects of a single training session, or team sport games. The effects of these types of exercise on s-IgA are discussed below.

### **2.5.2.1 Continuous Exercise**

Saliva IgA levels are often reported to decrease after high-intensity prolonged exercise (Pacque, Booth, & Dwyer, 2002; Walsh et al., 2002), however research findings vary considerably in experimental studies. A number of studies support increases (Blannin et al., 1998), decreases (Krzywkowski et al., 2001) or no change (Li & Gleeson, 2004) in s-IgA secretion rate following exercise. While there is no consensus as to how continuous exercise affects s-IgA, a large proportion of studies support the concept of an exercise induced suppression of s-IgA, especially if s-IgA is reported as a secretion rate rather than a concentration.

#### ***Moderate Duration Exercise***

Continuous exercise of moderate durations (< 45 min) is often shown to have little effect on s-IgA concentration or secretion rate (Table 1). For example, Allgrove et al., (2008) investigated the effect of ~22 min of cycling at different intensities (50% and 75%  $\dot{V}O_{2peak}$ ) as well as cycling to exhaustion. They found that exercise at 50 and 75%  $\dot{V}O_{2peak}$  had no effect on s-IgA, while exercise to exhaustion increased s-IgA concentration and secretion rate. Despite an increase in s-IgA secretion rate following the exhaustion trial, this measure returned to baseline by 1 h post exercise, so whilst exhausting exercise had a positive effect on s-IgA, the short lived nature of this may have little effect on modifying infection risk. A study by McDowell et al., (1991) also investigated the effects of treadmill running at different exercise intensities and durations on s-IgA. Exercising for 15, 30 or 45 min at 60%  $\dot{V}O_{2peak}$  and 20 min of exercise at 50, 65 and 80%  $\dot{V}O_{2peak}$  had no effect on s-IgA concentration. These findings were supported by Housh, Johnson, Housh, Evans and Tharp (1991) and Mylona, Fahlman, Morgan, Boardley and Tsivitse (2002) who also reported no change in s-IgA concentration from baseline following a 30 min treadmill run at 80%  $\dot{V}O_{2peak}$  and 70% heart rate reserve respectively. While these findings could indicate no alteration to immune function it is difficult to know if the absence of change in s-IgA concentration was due to exercise not being sufficiently stressful on the body. Since saliva flow rate was not reported it is possible that no change in s-IgA concentration could have also been associated with decreased s-IgA secretion rate as saliva flow rate is often decreased with exercise. However, overall these studies demonstrate that neither a low-moderate exercise intensity nor short duration exercise up to 45 min has any substantial effect on s-IgA responses.

Table 1. Salivary IgA responses to acute bouts of exercise of moderate durations

Study	Participants	Exercise protocol	Salivary IgA response	
			Concentration	Secretion rate
Allgrove et al., (2008)	10m healthy	1) ~22 min cycle at 50% $\dot{V}O_{2peak}$	↑	NC
		2) ~22 min cycle at 75% $\dot{V}O_{2peak}$	↑	NC
		3) Incremental $\dot{V}O_{2max}$ test	↑	↑
Housh et al., (1991)	9m	30 min treadmill run at 80% $\dot{V}O_{2peak}$	NC	-
Hubner-Wozniak et al., (1997)	11 P.E. students	1) Incremental cycle exercise until exhaustion	NC	-
		2) 30 min cycle at power output equivalent to 3mM lactate	NC	-
Hubner-Wozniak et al., (1998)	7 elite wrestlers	1) 30 s cycle exercise at 0.075kp/kg	NC	-
		2) 30 s upper limb exercise on cycle at 0.065kp/kg	NC	-
McDowell et al., (1991)	9m	15 min, 30 min, and 45 min treadmill run at HR at 60% $\dot{V}O_{2peak}$ 20 min trials at HR for 50, 65 and 80% $\dot{V}O_{2peak}$	NC for all	-
McDowell et al., (1992)	29m active	Incremental treadmill test to exhaustion	↓	-
Mylona et al., (2002)	16f active	30 min run on a track at 70% HR reserve	NC	NC

m = male, f = female, NC = no change, - = measure not reported.

### **Longer Duration Exercise**

Saliva IgA responses to longer durations of continuous exercise, unlike shorter durations, appear to be more varied (Table 2). Typically s-IgA concentration shows the greatest variation, with some studies showing decreases (Mackinnon, Chick, Van As, & Tomasi, 1989), no change (Pacque et al., 2002), or increases (Blannin et al., 1998). Saliva IgA secretion rate however is a little more consistent with results usually showing no change (Bishop et al., 2000), or a decrease (Krzykowski et al., 2001) following exercise. For example, 2.5 h of cycling at 55-65%  $\dot{V}O_{2peak}$  resulted in no change to s-IgA concentration or secretion (Davison et al., 2009) whereas a 2 h cycle at 70%  $\dot{V}O_{2peak}$  increased IgA concentration, but decreased IgA secretion rate (Walsh et al., 2002). Laing et al., (2005) also reported similar findings when investigating s-IgA responses to a 2 h cycle at 55% peak power output with increases in s-IgA concentration but decreases in s-IgA secretion rate of up to 34%. Despite conflicting findings, there appears to be no discernible differences between these study's protocols. In addition, saliva collection methods and as such saliva sample technique could be responsible for differences with Davison et al., (2009) using a passive dribble but Laing et al., (2005) and Walsh et al., (2002) using a polyester swab under the tongue which could have altered the saliva sample composition. It is however, possible that the seemingly small differences in

intensity (55-65% v 70%  $\dot{V}O_{2peak}$ ) between studies, may have been enough to stress the body to a greater extent and result in an immune suppression of s-IgA, although this does not explain findings by Pacque et al., (2002), discussed below. Additionally, although a number of studies have reported decreases in IgA secretion rate the exercise protocols employed have been wide ranging. For example, a 2 h walk at 5 km·h with a 20-kg backpack in male and female army participants resulted in decreased secretion of IgA (Pacque et al., 2002), and this was also seen following a 2 h cycle at 75%  $\dot{V}O_{2peak}$  in endurance trained males (Krzywkowski et al., 2001). This is interesting considering the vast differences in the protocols employed, and subsequently it seems that there may be quite a range of factors influencing s-IgA responses if such different exercise protocols are capable of eliciting the same response. To add further confusion, one group of researchers found that cycle exercise to exhaustion (at 55% and 80%  $\dot{V}O_{2peak}$ ) increased s-IgA secretion rate (Blannin et al., 1998).

Since the s-IgA responses to continuous exercise of longer durations are so variable, further research is needed to better elucidate the true effects of these types of exercise on s-IgA. As such, it could be suggested that researchers should investigate the test-retest reliability of their protocols to ensure that the protocols they use are reliable. In addition, s-IgA responses to exercise may be better explained if researchers used more consistent methodological protocols.

Table 2. Salivary IgA responses to acute bouts of exercise of longer durations.

Study	Participants	Exercise protocol	Salivary IgA response	
			Concentration	Secretion rate
Allgrove et al., (2009)	16 (8m, 8f) active	2 h cycle at 65% $\dot{V}O_{2peak}$	↑	NC
Bishop et al., (2000)	15m active	2 h at 60% $\dot{V}O_{2peak}$		
		Placebo	NC	NC
		Restricted Fluid Intake	↑	NC
Bishop et al., (2006)	11m endurance trained	90 min cycle at 70% $\dot{V}O_{2peak}$	NC	NC
Blannin et al., (1998)	18m active	1: Cycle at 80% $\dot{V}O_{2peak}$ to exhaustion	↑	↑
		2: Cycle at 55% $\dot{V}O_{2peak}$ to exhaustion or 3 h	↑	↑
Davison et al., (2009)	12m active	2.5 h cycle at 55-65% $\dot{V}O_{2peak}$	NC	NC
Krzywkowski et al., (2001)	11m endurance trained	2 h cycle at 75% $\dot{V}O_{2peak}$	↓	↓
Laing et al., (2005)	12m cyclists	2 h cycle at 55% peak power output	↑	↓
Li & Gleeson (2004)	8m active	Single: 2pm – 2 h cycle at 60% $\dot{V}O_{2peak}$	↑	NC
		Repeated: 9am – 2 h cycle at 60% $\dot{V}O_{2peak}$ + 2pm 60% $\dot{V}O_{2peak}$ to fatigue	↑	NC
Luna Jr et al., (2011)	25m marathon runners	Treadmill run at 85% $\dot{V}O_{2peak}$ to exhaustion	↓	-
Mackinnon et al., (1989)	8m cyclists	2 h cycle at 70-75% $\dot{V}O_{2peak}$	↓	-
Pacque et al., (2002)	38 (27m, 11f) army	2 h treadmill walk at 5 km·h <sup>-1</sup>	NC	↓
Sari-Sarraf et al., (2006)	8m active	90 min run at 9.7 km·h <sup>-1</sup>	NC	NC
Usui et al., (2011)	10m active	1 h recumbent cycle @ 75% $\dot{V}O_{2peak}$	NC	NC
Walsh et al., (2002)	15m cyclists	2 h cycle at 70% $\dot{V}O_{2peak}$	↑	↓

### ***Endurance and Ultra Endurance Exercise***

A number of authors have investigated the effects of competitive endurance events on s-IgA. While reports of s-IgA concentration are varied, s-IgA secretion rate is more consistently reported to decrease from pre- to post race (Table 3). Several researchers have observed the effects of endurance running races on s-IgA responses in male and female participants aged between 20 and 70 years (Nieman et al., 2006; Nieman et al., 2002; Palmer et al., 2003). Saliva IgA secretion rate has been reported to decrease by 30-50% from pre- to post-race after 42.2 km (Nieman et al., 2002), 80 and 82 km (Palmer et al., 2003; Pacque et al., 2007) and 160 km races (Henson et al., 2008; Nieman et al., 2003, 2006). Reports of s-IgA

concentration however have been more varied, with some researchers finding a decrease (Nieman et al., 2002; Palmer et al., 2003) and others no change (Henson et al., 2008; Nieman et al., 2003, 2006; Steerenberg et al., 1997). Similarly, s-IgA concentration (the only saliva measure reported) has also been shown to decrease following 20 and 50km Nordic ski races in nationally ranked female and male skiers (Tomasi et al., 1982). In addition, an Olympic distance triathlon was found to decrease s-IgA secretion rate by 65% while s-IgA concentration remained unchanged (Steerenberg et al., 1997). While reports of s-IgA concentration are varied this may be explained by accompanied decreases in saliva flow rate which could act to have a concentrating effect on IgA and result in increases or no change in s-IgA. Thus, once s-IgA is reported relative to saliva flow rate, the findings of these studies show a more consistent decrease in s-IgA secretion rate.

Table 3. Salivary IgA responses to endurance and ultra-endurance races.

Study	Participants	Race length	Salivary IgA response	
			Concentration	Secretion rate
Henson et al., (2008)	39 m + f runners	160 km ultra-marathon	NC	↓
Ljungberg et al., (1997)	20 runners (17m, 3f)	42.2 km marathon	NC	NC
Nieman et al., (2002)	98 runners (86m, 12f)	42.2 km marathon	↑	↓
Nieman et al., (2003)	31 runners (22m, 9f)	160 km ultra-marathon	↓	↓
Nieman et al., (2006)	155 m + f runners	160 km ultra-marathon	↓	↓
Pacque et al., (2007)	17 runners (13m, 4f)	82 km ultra-marathon	↓	↓
Palmer et al., (2003)	28 runners	80 km ultra-marathon	↓	↓
Steerenberg et al., (1997)	42 m + f triathletes	Olympic distance triathlon	NC	↓
Tomasi et al., (1982)	8 national Nordic skiers (5m, 3f)	20 km (f) or 50km (m) Nordic ski	↓	-

### 2.5.2.2 Intermittent Exercise

A wide range of protocols involving intermittent exercise have been employed in research investigating s-IgA responses to exercise. Generally it appears that there is a trend for s-IgA concentration to increase or remain unchanged following intermittent exercise (Davison 2011; Engels et al., 2003), while s-IgA secretion rate is often reported to decrease or remain unchanged (Nieman et al., 2004; Ricardo et al., 2009). These differences in responses between s-IgA concentration and secretion however, are most likely attributed to anticipated exercise induced decreases in saliva flow rate discussed previously.

### ***Repeated sprint exercise***

Saliva IgA has been measured before and after repeated sprint exercise in several studies (Table 4). Studies by Engels et al., (2003, 2004), Fahlman et al., (2001) and Hall et al., (2007) had participants complete 3 x 30 s Wingate tests with 3 min recovery between tests. In these studies s-IgA secretion rate was found to decrease by 40-55% from pre-exercise to immediately post-exercise, while the effect on s-IgA concentration was not consistent; saliva IgA concentration was unaffected in studies by Engels et al., (2003) and Fahlman et al., (2001), yet was decreased by up to 69% in Hall et al., (2007). Similarly, Mackinnon and Jenkins (1993) found 5 x 60 s of maximal cycling with 5 min rest between efforts significantly decreased IgA secretion rate by 52%. Despite such a large decrease in secretion, s-IgA concentration increased pre- to post-exercise by up to 22%. As saliva flow rate was not reported, it is expected that participants would have experienced a significant decrease in saliva flow rate as seen in Engels et al., (2003, 2004) studies, which would better explain Mackinnon and Jenkins' findings. A study by Davison (2011) also used a similar protocol to Engels et al., (2003, 2004), Fahlman et al., (2001) and Hall et al., (2007) in which participants completed 4 x 30 s Wingate tests with 4 min rest between. In contrast to the above findings Davison (2011) reported s-IgA concentration to increase from pre- to post-exercise, but no effect on s-IgA secretion rate. Lastly, Walsh (1999) also used a protocol involving short sprints. Participants in this study completed 20 x 1 min cycle at 100%  $\dot{V}O_{2peak}$  with 2 min active recovery between sprints at 30%  $\dot{V}O_{2peak}$ . While no change was seen in s-IgA concentration, this was also the case for s-IgA secretion rates. As such, it is possible that the 2 min period of active recovery may have been sufficient enough for participants' immune function to recover to baseline values therefore avoiding a depression in s-IgA. Overall these findings suggest that maximal effort exercise is likely to reduce s-IgA secretion rates, despite inconsistent responses with s-IgA concentration.

Table 4. Salivary IgA responses to repeated sprint cycle exercise.

Study	Participants	Exercise protocol	Salivary IgA response	
			Concentration	Secretion rate
Davison (2011)	9m active with Wingate experience	4 x (30 s Wingate test + 4 min active recovery)	↑	NC
Engels et al., (2003)	38 active students	3 x (30 s Wingate + 3 min recovery as 90 s active, 90 s passive)	NC	↓
Engels et al., (2004)	35 active (19f, 16m)	3 x (30 s Wingate + 3 min passive recovery)	-	↓
Fahlman et al., (2001)	26f active	3 x (30 s Wingate + 3 min recovery as 90 s active, 90 s passive)	NC	↓
Hall et al., (2007)	32 active	3 x (30 s Wingate + 3 min recovery as 90 s active, 90 s passive)	↓	↓
Walsh (1999)	8m well trained games players	60 min of cycle exercise as: 20 x (1 min 100% $\dot{V}O_{2peak}$ + 2 min 30% $\dot{V}O_{2peak}$ )	NC	NC

### ***Measures before and after a single training session***

Studies investigating the effects of a single typical training session on s-IgA responses vary with some reporting no change (Tharp, 1991; Tharp & Barnes, 1990) or decreases (Gleeson et al., 1995; Novas et al., 2003) in s-IgA concentration and/or secretion for training sessions of varying intensities (Table 5). Differences in findings could be attributed to a lack of motivation, requirement for higher intensity exercise, or due to rest intervals between exercise possibly allowing athletes' immune function to start to recover. Both Gleeson et al., (1995) and Tharp and Barnes (1990) found s-IgA concentration to decrease after swimming training. However Tharp and Barnes' findings only applied to training sessions of moderate intensity, with training intensities classified as light, heavy and taper showing no effect on s-IgA concentration. These unique findings could be attributed to participant characteristics, as compared to Gleeson et al., (1995) who used elite swimmers; Tharp and Barnes' (1990) participants were only stated to be 'university swimmers' who may have been less able to cope with the requirements of training. In contrast, 60 min of tennis training had no effect on s-IgA concentration, but reduced s-IgA secretion rate in nationally ranked tennis players (Novas et al., 2003). Findings by Novas et al., (2003) are also supported by Mackinnon, Ginn and Seymour (1993), who showed that 30-40 min of kayak training in elite athletes had no effect on s-IgA concentration, but decreased s-IgA secretion by up to 38%. While these studies help to give an insight into the effects of training on s-IgA, it is very difficult to compare findings due to the wide range of possible exercise tasks that athletes could

complete in any one training session. In addition, it is difficult to quantify the total amount of work and/or effort completed in a training session, which further adds to issues in comparing findings.

Table 5. Salivary IgA responses to a single training session.

Study	Participants	Training session	Salivary IgA response	
			Concentration	Secretion rate
Gleeson et al., (1995)	26 (15m, 11f) elite swimmers	Training duration not stated, intensity not quantified	↓	-
Mackinnon et al., (1993)	8m elite kayakers	1: 30 min 7.5/10 intensity	↓	↓
		2: 25 min 8/10 intensity	↓	↓
		3: 40 min 8.5/10 intensity	↓	↓
Novas et al., (2003)	17f nationally ranked tennis players	60 min, intensity not quantified	NC	↓
Tharp & Barnes (1990)	21m swimmers	Training duration not stated		
		Moderate intensity	↓	-
		High intensity	NC	-
		Taper	NC	-
Tharp (1991)	27 pre-pubescent (10-12yr)	Basketball training duration not stated, intensity not quantified	NC	-
	23 post-pubescent (16-18yr)		NC	-

### ***Team sport and field-testing***

The s-IgA response to participation in team or combat sport is wide-ranging (Table 6). Moreira et al., (2009) and Moreira, Arsati, Lima-Arsati, Franchini and de Araújo, (2010) found s-IgA concentration and secretion rate to remain unchanged following a friendly football game (70 min game) and a kickboxing match (3 x 4 min rounds with 1 min rest). However, two highly competitive futsal games consisting of 4 x 10 min quarters (with 5 min recovery intervals) resulted in decreases to both s-IgA concentration and secretion rates (Moreira et al., 2011). Since these exercise protocols are clearly different it is difficult to compare findings. Consequently more research needs to be conducted in this area to further elucidate the effects of these types of exercise on s-IgA. One group of researchers has taken a different approach to investigating s-IgA in team sport athletes by employing an experimental protocol simulating the exercise requirements of soccer (Sari-Sarraf et al., 2006). They used a 90 min soccer specific treadmill protocol to examine s-IgA responses to soccer-simulated exercise. Despite using exactly the same exercise protocol Sari-Sarraf et al., 2006 found no changes in s-IgA concentration or secretion rate from pre- to post-exercise, while Sari-Sarraf, Reilly, Doran and Atkinson (2007) showed increases in both s-IgA

concentration and secretion rate. This is interesting considering there were no discernible differences in either study's participants or protocols. Both studies involved healthy male participants of similar fitness levels ( $\dot{V}O_{2peak}$  56-58ml·kg·min<sup>-1</sup>) and testing was conducted at the same time of day for all trials. The findings of these researchers are further intriguing as Sari-Sarraf et al., (2007) do not discuss any potential reasons as to why their results differed from their earlier study (Sari-Sarraf et al., 2006). As such, it could be hypothesised that these differences in findings may be due to variability in between or within subject responses. In addition, it would be interesting to know if the s-IgA responses to experimental soccer-specific exercise protocols would be similar to those experienced following an actual soccer game. It is possible that experimental protocols for team sport exercise may not be applicable to game situations due to likely differences in psychological motivation for exercise. As such, a potential area for future investigations would be to compare s-IgA responses to experimental sport-simulation protocols to responses to actual games.

Table 6. Salivary IgA responses to sporting games or team sport exercise.

Study	Participants	Exercise protocol	Salivary IgA response	
			Concentration	Secretion rate
Moreira et al., (2009)	24m professional soccer players	70 min regulation friendly soccer game as: 2 x 35 min with 10 min rest at half time	NC	NC
Moreira et al., (2010)	20m kick-boxers	Kickboxing fight (3 x 4 min round + 1 min rest)	NC	NC
Moreira et al., (2011)	10m professional futsal players	2 x Highly competitive Futsal games 4 x 10 min with 5 min between quarters	↓	↓
Sari-Sarraf et al., (2006)	8m active	90 min soccer specific ex as: 2 x 45 min = 7 static pauses, 40 x walking, 30 x jogging, 22 x cruising, 16 x sprints (15 min between 'halves')	NC	NC
Sari-Sarraf et al., (2007)	10m active	90 min soccer specific ex as above		
		Single (PM) or Repeated (AM + PM) bouts of exercise	↑ ↑	↑ ↑
Tharp, 1991	27 pre-pubescent (10-12yr)	Basketball game duration not stated	↑	-
	23 post-pubescent (16-18yr)		↑	-

### 2.5.2.3 Intensity and Duration

Although there is a lack of agreement in exercise s-IgA literature, a common conclusion is that s-IgA is more likely to be depressed following prolonged and high intensity exercise compared to shorter duration and lower intensity exercise (Bishop et al., 2006). Intensities in experimental protocols have ranged from 50% to 85%  $\dot{V}O_{2peak}$ , with the majority of authors using an intensity between 65-70%  $\dot{V}O_{2peak}$ . In addition, a wide range of exercise durations have also been used with experimental protocols ranging from ~5 min – 2.5 h (Davison et al., 2009; Mackinnon & Jenkins, 1993) and races from 2 - 27 h (Henson et al., 2008; Libicz et al., 2006).

Despite the hypothesis of a relationship between exercise intensity and s-IgA responses, experimental studies, which have often used protocols of similar intensities fail to support this with a lack of agreement in findings. Continuous exercise protocols using intensities of 80-85%  $\dot{V}O_{2peak}$  have shown s-IgA secretion to increase (Blannin et al., 1998) and s-IgA concentration to remain unchanged (Housh et al., 1991) or decreased (Luna Jr et al., 2011). Similarly, continuous exercise studies using intensities between 70-75% also show a wide range of findings of decreased s-IgA concentration and secretion (Krzywkowski et al., 2001) as well as increased and decreased s-IgA concentration, with no change to s-IgA secretion (Walsh et al., 2002). In contrast, exercise requiring maximal effort such as that in repeated sprints, or exercise that is particularly taxing on the body, such as endurance running races, generally appear to result in decreased s-IgA secretion rate. In particular repeated sprint cycling has been shown to decrease s-IgA secretion rate from pre- to post-exercise by up to 55%, as well as endurance-running races (42.2 – 160 km) also showing s-IgA secretion rate to decrease from pre- to post-race by up to 50% (Nieman et al., 2003; Pacque et al., 2007). Thus, it may be that exercise intensity needs to be near maximal or extremely taxing in order to depress s-IgA secretion.

On the other hand, prolonged exercise is generally thought to have greater effects on immune function, as it is more likely to elicit a stress response capable of affecting s-IgA. This is supported by findings of (Nieman et al., 2002, 2003, 2006; Pacque et al., 2007; Palmer et al., 2003) who found decreased s-IgA secretion following endurance running races, but is not seen in experimental studies with durations of up to 2.5 h. Consequently, it appears that non-competitive prolonged exercise may not have a negative effect on s-IgA even at intensities of 80%  $\dot{V}O_{2peak}$  (Blannin et al., 1998). Saliva IgA is more commonly reported to remain unchanged or even enhanced following exercise ranging from 1 - 2.5 h at intensities

of 60-80%  $\dot{V}O_{2peak}$  (Bishop et al., 2006; Usui et al., 2011). As such, it may be that prolonged exercise must be performed at a high intensity (such as that in a running race) in order to reduce secretion of s-IgA. Overall these findings suggest that s-IgA responses may be more influenced by exercise intensity rather than duration.

### 2.5.3 $\alpha$ -amylase

$\alpha$ -amylase responses to exercise are possibly the most consistent in exercise and mucosal immunology literature.  $\alpha$ -amylase activity is largely affected by sympathetic stimulation, and as such is frequently reported to increase following exercise of varying durations and intensities (Allgrove et al., 2008; Bishop et al., 2006; Walsh, 1999).

Marathon running has been shown to increase  $\alpha$ -amylase secretion rate by 60% from pre- to post-exercise, with  $\alpha$ -amylase activity remaining high (20% above baseline) even 1 h after the race (Ljungberg et al., 1997). Similar results were also found following 60 min of intermittent sprint cycle exercise with  $\alpha$ -amylase activity increasing almost 6 fold (Walsh, 1999). In addition, 2 h of cycling at 60%  $\dot{V}O_{2peak}$ , as well as cycling at 60%  $\dot{V}O_{2peak}$  to exhaustion, have been shown to increase  $\alpha$ -amylase secretion rate almost 3 fold. However, 3 h of rest between morning and afternoon exercise protocols was enough time for  $\alpha$ -amylase to return to baseline levels (Li & Gleeson, 2004). In a more recent study,  $\alpha$ -amylase responses to exercise were also investigated following ingestion of a 6 mg·kg<sup>-1</sup> BM dose of caffeine (Bishop et al., 2006). As with other studies,  $\alpha$ -amylase was increased from pre- to post-exercise following 90 min cycling at 70%  $\dot{V}O_{2peak}$ , however caffeine ingestion was found to increase  $\alpha$ -amylase by 3 fold from pre- to post-exercise compared to placebo trials. Exercise of shorter durations has also been shown to increase  $\alpha$ -amylase activity. Allgrove et al., (2008) compared the effects of ~22 min of exercise at 50% and 75%  $\dot{V}O_{2peak}$  with an incremental exercise test to exhaustion. While  $\alpha$ -amylase secretion rate was found to increase by up to 70% with exhaustive exercise and exercise at 75%  $\dot{V}O_{2peak}$ ,  $\alpha$ -amylase was unaffected by exercise at 50%  $\dot{V}O_{2peak}$ . Findings by Chatterton Jr et al., (1996) were also similar, with  $\alpha$ -amylase activity increasing with jogging (48%) and running exercise (158%) but not walking. As such, it may be that a particular level of stimulation or exercise intensity is required to elicit increases in  $\alpha$ -amylase. Overall these findings suggest that exercise and associated sympathetic activity has a marked effect on  $\alpha$ -amylase activity, further supporting suggestions for its use as a surrogate marker for sympathetic activity.

## **2.6 Methodological issues**

A number of reviews on mucosal immune responses to exercise have highlighted issues with previous research (Bishop & Gleeson, 2009; Gleeson & Pyne, 2000; Gleeson et al., 2004; Walsh et al., 2011), though, despite this many studies still employ a wide range of protocols making comparison difficult. Key issues discussed were methods used to collect saliva (un-stimulated versus stimulated, or whether any material was used to aid collection) and the ways in which data is reported in literature (as concentrations, secretion rates or ratios).

### **2.6.1 Saliva collection methods**

Researchers have used a wide range of methods to collect saliva, which has made comparisons between study findings difficult. Since saliva composition is known to change under stimulation, as well as differ from gland to gland, it may be inappropriate to compare data between studies if their collection methods are too varied.

The majority of studies reviewed have collected whole saliva samples using an un-stimulated passive dribble method (Allgrove et al., 2009; Tomasi et al., 1982; Walsh, 1999). However, some researchers have used other collection methods including placing cotton swabs under the tongue (Bishop et al., 2000, 2006; Walsh et al., 2002), chewing a cotton swab (Usui et al., 2011) or parafilm (Ljungberg et al., 1997; Steerenberg et al., 1997) or sucking a sour lolly (Mackinnon et al., 1989; Ljungberg et al., 1997). This is problematic when the composition of stimulated saliva is known to differ in comparison to un-stimulated saliva (Bishop & Gleeson 2009). Gleeson et al., (2003) investigated this in a study comparing stimulated to un-stimulated saliva following cycling exercise at 85%  $\dot{V}O_{2peak}$  to exhaustion. Stimulation from sucking a mint resulted in a 3 fold increase in saliva flow rate and s-IgA concentration lower than un-stimulated values. However, once flow rate was accounted for, s-IgA secretion rate was reported to be higher in stimulated compared to un-stimulated samples. Another method that has been used in saliva collection is to have participants place cotton or polyester swabs in their mouth to soak up saliva. While this method has been used to minimize the risk of gingival bleeding and for their comfort ratings, they have also been shown to increase sample acidity (Granger, Weisz, McCracken, Kauneckis, & Ikeda, 1994) and to give false concentrations of saliva components (Strazdins et al., 2005). In addition, collection methods involving the placement of swabs in the mouth could also result in preferential stimulation of salivary glands and therefore modify saliva composition (Chicharro et al., 1998). Despite these limitations cotton swabs may be appropriate for researchers wanting to collect saliva samples whilst participants are still exercising (for example a mid-

exercise sample). In addition, cotton swabs could also be useful when exercise is strenuous as it may be difficult to obtain a sample due to the potential for evaporative loss of saliva when breathing, especially if participants were to use a passive dribble method (Walsh, 1999). However, overall, passive dribble is seen to be the most reliable method in saliva collection due to its known effects on saliva composition, as well as allowing for the measurement of saliva flow rate by timed collection in a pre-weighed vial (Papacosta & Nassis, 2011).

## **2.6.2 Result reporting**

To date a number of reporting methods have been employed in exercise and s-IgA studies, making it difficult to compare findings. s-IgA has been reported as a concentration (Bishop et al., 2000; Davison, 2011), secretion rate (Moreira et al., 2011; Walsh 1999) or as a ratio to either total protein (Henson et al., 2008; Usui et al., 2011), or osmolality (Allgrove et al., 2009; Pacque et al., 2007). Recently Bishop and Gleeson (2009) evaluated the usefulness of these reporting methods, concluding that s-IgA secretion rate is likely to be the most appropriate due to the fact that it takes any changes in saliva volume into account. Secretion rate was also recommended, as both saliva flow rate and s-IgA concentration are influential factors in host defense (Gleeson, 2006b), and it has also been shown to be a better predictor of URTI risk than other measures (as reviewed by Nieman et al., 2002).

Saliva IgA concentration is commonly reported and has been suggested to be important as it represents the actual amount of s-IgA available at mucosal surfaces (Mackinnon et al., 1991). However, this method has been argued to be misleading because it does not take saliva flow rate into account and any increases could actually reflect a concentrating effect of low saliva flow rates (Bishop & Gleeson, 2009). Reporting of s-IgA as a concentration has been suggested to be a less useful reporting technique due to a lack of correlation with URTI, compared to s-IgA secretion rates that have been shown to be a better predictor of URTI (Fahlman & Engels, 2005).

Another common approach is to report s-IgA concentration as a ratio to total protein concentration; with the assumption that total protein secretion rates do not change in response to exercise (Mackinnon et al., 1989; Tomasi et al., 1982). This method however has been questioned as several studies have shown protein concentration (Ljungberg et al., 1997; Sari-Sarraf et al., 2007; Walsh, 1999) and secretion rate (Krzywkowski et al., 2001; Steerenberg et al., 1997) to increase with exercise, which has been suggested to be due to

increased  $\alpha$ -amylase secretion resulting from sympathetic stimulation ((Gleeson, 2006b). Consequently, it is possible that total protein may be better as a standalone measure.

Another alternative method that has been used is the expression of s-IgA concentration as a ratio to osmolality. This method was suggested as it was thought that osmolality fell in proportion to the fall in saliva flow rate (Bishop & Gleeson, 2009). However, research has shown osmolality to increase with exercise (Allgrove et al., 2008; Davison et al., 2009) making the reasoning for this method flawed. Osmolality provides an indication of electrolyte concentration, and is largely affected by hydration status (Walsh, Montague, Callow, & Rowlands, 2004). As such, osmolality concentration has been seen to increase as participants become dehydrated and saliva flow rate decreases (Mahdivand, Askari, Askari, & Barzegari, 2011). Similarly other studies measuring osmolality have found concentrations to increase (Allgrove et al., 2008, 2009; Davison et al., 2009; Laing et al., 2005; Mahdivand et al., 2011; Sari-Sarraf et al., 2006, 2007; Walsh et al., 2002), thus it may be more appropriate as a standalone measure of hydration status, than a ratio to s-IgA.

Overall it seems reporting s-IgA as a ratio to protein or osmolality may be misleading and inappropriate (Bishop & Gleeson, 2009). Accordingly reporting s-IgA as a secretion rate may be more suitable due to its correlation with risk of URTI, and because it takes saliva flow rate into account, which has been reported to change with exercise.

## **2.7 Potential mechanisms**

Mechanisms for alterations in salivary IgA responses to exercise are yet to be fully explained. Recent studies of saliva gland control and regulation in animal models has provided some insight into possible mechanisms, which may be involved during exercise. Currently researchers suggest that mechanisms are likely related to autonomic regulation of salivary glands, however this also needs investigation in humans.

As discussed earlier, researchers suggest that changes in saliva flow rate are most likely due to parasympathetic withdrawal, rather than sympathetic stimulation (Bosch et al., 2002). Previously it was thought that sympathetic stimulation and its associated vasoconstriction may result in reduced blood flow to saliva glands, and therefore reduce saliva flow (Chicharro et al., 1998). Under this hypothesis, it was anticipated that caffeine ingestion and assumed increases in sympathetic activity might exacerbate any decreases in saliva flow rate, however this was not seen in a recent study by Bishop et al., (2006) who investigated the effects of a 6 mg·kg<sup>-1</sup> BM dose of caffeine on mucosal measures at rest. These findings

further support the suggestion that parasympathetic rather than sympathetic control of saliva glands has a greater influence on saliva secretion (Bosch et al., 2002).

*In vivo* stimulation of salivary glands in rats has shown s-IgA secretion to respond differently to saliva flow rate (Carpenter et al., 1998). Unlike saliva flow rate, s-IgA, which is secreted predominantly by sublingual glands, has been shown to be primarily affected by sympathetic activation rather than parasympathetic withdrawal (Carpenter et al., 1998; Proctor, Carpenter, & Garrett, 2000). Researchers have investigated the effects high frequency sympathetic and parasympathetic nerve stimulation, as would be experienced with high-intensity exercise, with bi-polar electrodes in rats, on salivary gland secretion of s-IgA. While parasympathetic stimulation resulted in increased IgA secretion in both sublingual and parotid glands almost 3 fold, sympathetic stimulation increased IgA secretion 6 fold in sublingual glands, but only 3 fold in parotid, compared to un-stimulated saliva (Carpenter et al., 1998 Proctor et al., 2000), supporting the above suggestion that perhaps the increase in s-IgA secretion in responses to exercise is due to sympathetic activation as opposed to parasympathetic withdrawal.

Recent research has suggested that s-IgA secretion rate could be most affected by alterations in the availability of pIgR and therefore transport of s-IgA into saliva rather than production of s-IgA from plasma cells (Carpenter et al., 2004; Proctor, Garrett, Carpenter, & Ebersole, 2003). Due to the time-course (minutes) of alterations in s-IgA often seen in response to acute exercise (Engels et al., 2003; Krzywkowski et al., 2001; Moreira et al., 2011; Pacque et al., 2002) it seems logical to suggest that the availability of pIgR could be the principal mechanism responsible for alterations in s-IgA secretion in response to acute strenuous exercise. In support of this, a study by Proctor et al., (2003) demonstrated that injecting autonomic mimetics in anaesthetised rats to acutely stimulate beta-adrenoreceptors increased s-IgA secretion up to 7 fold as a result of increased transcytosis in a dose dependent manner above a certain threshold of stimulation. Carpenter et al., (2004) investigated this further and demonstrated that the increased IgA transcytosis may have been the result of an increased availability of pIgR as stimulation with adrenaline was shown to increase the uptake of human IgA in rat salivary cells as a result of increased mobilisation of pIgR. Although this mechanism has not been investigated in humans, a recent study by Bishop et al., (2006), comparing the effects of caffeine ingestion on s-IgA responses to exercise, supports findings by Carpenter et al., (2004) reporting increases in s-IgA to be accompanied by increases in plasma adrenaline. While this mechanism can be used to

explain increases in s-IgA observed following exercise, it does not help to explain findings of either no change (Davison, 2011; Ljungberg et al., 1997) or decreases (Novas et al., 2003; Walsh et al., 2002) in s-IgA secretion seen following intense physical activity. It is possible that the critical threshold for increased plgR mobilization, observed by Proctor et al., (2003) may explain why some studies have seen no change in s-IgA secretion following exercise of moderate intensities (Allgrove et al., 2009 Davison et al., 2009).

Decreases in s-IgA secretion following intense exercise are more difficult to elucidate. However, findings from a recent study by Kimura et al., (2008) may help to explain decreases in s-IgA observed following exercise in the rat model. Unlike previous rat studies using high frequency stimulation to investigate s-IgA mechanisms, Kimura et al., (2008) examined the effects of running to exhaustion on s-IgA responses in rats. They found that exhaustive exercise (treadmill running for ~ 60 min) induced a significant depression of s-IgA concentration, which was accompanied with decreases in plgR mRNA expression. These findings of decreased plgR mRNA expression support those of Carpenter et al., (2004) and Proctor et al., (2003) suggesting that secretion of s-IgA is most likely limited by the availability of plgR. As such, Bishop and Gleeson (2009) have tentatively speculated that there could be a second critical threshold or duration of stimulation above which plgR expression becomes down-regulated. However, to date there has been limited research in either animal models or humans to support these speculations.

## **2.8 Caffeine**

The effect of caffeine ingestion on s-IgA responses in humans either at rest or following exercise has not been extensively studied. Caffeine ingestion however has been consistently shown to induce sympathetic nervous system activation and increase plasma adrenaline concentration (Graham & Spriet 1995; Graham, 2001; Bishop et al., 2005). Furthermore, the main mechanism proposed to alter s-IgA secretion rate in response to exercise has been suggested to be mediated predominantly via sympathetic activation (Carpenter et al., 1998). Therefore, caffeine has the potential to modulate s-IgA responses possibly via indirect stimulation of adrenaline release.

Caffeine is a member of the methylxanthine family of drugs and, due to its presence in numerous foods and fluids, is probably the most widely consumed psychoactive substance known to man (Fredholm, Zvartau, Battig, Nehlig, & Holmen, 1999). Caffeine is a chemical substance that is an odourless crystalline powder found in numerous plants (Debry, 1994).

Although caffeine is found and consumed in many different foods and fluids, it is not considered a nutrient or essential for health (Graham, 2001). It is estimated that the mean daily intake of caffeine in New Zealand is  $3.5 \text{ mg}\cdot\text{kg}^{-1} \text{ BM day}$ , which is equivalent to approximately 2-4 cups of coffee (Thomson & Schiess 2010). However caffeine intake has been found to vary widely between individuals as well as geographically. Thomson and Schiess (2010) recently compared New Zealand intakes to that in other countries. New Zealand intake was found to be similar to that in the UK ( $3.6 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$ ), but lower than intakes in South America ( $4.0 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$ ) and higher than the US ( $2.5 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$ ). Common sources of caffeine in a person's diet are found in the form of coffee, tea, chocolate drinks and energy drinks, as well as in chocolate foods (Mandel, 2002). The levels of caffeine in foods vary greatly, particularly in coffee or tea due to different preparation methods. The caffeine content in common caffeine-containing products are presented in (Table 7).

**Table 7.** Caffeine content of common caffeine-containing beverages and foods

Source (serving size)	Caffeine content (mg)
Coffee (150 ml) *	50-135
Tea (150 ml) *	25-50
Coca-cola (330 ml)	30
Hot chocolate (250 ml)	5
Energy drinks (250 ml)	85
Chocolate bar (50 g)	20
NoDoz (1 tablet)	100

Caffeine content obtained from Harland 2000. \* Large variation in caffeine content due to source and preparation of product.

Caffeine is rapidly absorbed through the gastrointestinal tract (Fredholm, 1995) and moves efficiently through cellular membranes to tissue (Fredholm et al., 1999). Elevated levels of caffeine have been seen in the bloodstream within 15-45 min of ingestion, with concentrations peaking approximately an hour after ingestion (McArdle, Katch, & Katch, 2007; Robertson et al., 1978). However, some researchers have reported participants to reach peak plasma caffeine concentrations in as little as 15 min or as long as 120 min, which suggests that there may be large inter-individual differences in response to caffeine ingestion (Desbrow, Barrett, Minahan, Grant, & Leveritt, 2009; Fredholm et al., 1999). Caffeine is metabolised in the liver, resulting in three metabolites: paraxanthine (80%), theobromine (4%), and theophylline (11%) (Graham & Spriet, 1995). Once metabolized,

caffeine and its metabolites are excreted by the kidneys, and up to 10% of ingested caffeine may be expelled from the body unchanged in urine (Tang-Liu, Williams, & Riegelman, 1983). Clearance from the blood stream appears to be similar to the rate at which caffeine is absorbed and metabolised, with studies showing concentrations (for ingestion of doses under  $10 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$ ) to be decreased by 50-75% within 3-6 h of consumption (Sokmen et al., 2008). However, this too has been shown to vary between individuals, with some researchers reporting the half-life to range from 2-12 h in healthy adults (Benowitz, 1990). Despite potential inter-individual differences in caffeine metabolism, it appears that caffeine-pharmacokinetics are not affected by exercise, dehydration or gender (as reviewed in Goldstein et al., 2010).

## **2.9 Caffeine and sport**

Many researchers have shown caffeine to be ergogenic with doses between  $3\text{-}6 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  improving performance in events lasting as little as 30 s or as long as 2 h (Graham, 2001; Goldstein et al., 2010). For example, Paton, Lower, and Irvine (2010) investigated the effects of a 240 mg dose of caffeine on repeated sprint cycling. Participants completed 4 sets of 5 x 30 s sprints and maintained a higher mean power output with caffeine ingestion (5.4%) compared to placebo. Additionally, caffeine ingestion has also been reported to increase the distance cycled in a 60 min 'time trial' (McNaughton et al., 2008). In this study,  $6 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  of caffeine was associated with an increase in cycle distance of  $\sim 1 \text{ km}$ . This may explain why caffeine consumption is common amongst a wide range of athletes from different sporting disciplines and why consumption has increased since being removed from the World Anti Doping Association (WADA) prohibited substance list in 2004 (Chester & Wojek, 2008). In addition to ergogenic effects on exercise performance, caffeine has also been suggested for use due to its stimulatory properties of enhanced cognition, alertness and mood (Davis et al., 2003; Evans & Griffiths, 1992; Yeomans, Ripley, Davies, Rusted, & Rogers, 2002). Furthermore, researchers have also recommended caffeine to be a useful tool in training situations to help athletes sustain high exercise intensities during heavy endurance training, as well as for its ability to reduce pain perception (Sokmen et al., 2008).

### 2.9.1 Dose response effects on performance

To date research shows no dose-response effect of caffeine ingestion on performance (Graham & Spriet, 1995; Motl, O'Connor, Tubandt, Puetz, & Ely, 2006; Pasman et al., 1995). Since ingestion of high doses of caffeine ( $>6 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$ ) have been associated with possible adverse affects such as insomnia, headaches, dizziness and gastro-intestinal distress (Evans & Griffiths, 1992; Leonard et al., 1987) it could be recommended that athletes consume the lowest dose beneficial for them in order to avoid any possible negative outcomes. This idea is further supported, with some researchers suggesting a link between higher doses of caffeine and decreased exercise and cognitive performance in some athletes (Evans & Griffiths, 1992; Leonard et al., 1987; Lieberman, 2003).

Doses as small as  $1 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  and as large as  $13 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  have been shown to have positive effects on time to fatigue, endurance performance and sprint or power events (Sokmen et al., 2008). Since researchers examining caffeine and exercise have used such a wide range of doses several studies have been conducted to investigate whether a dose-response on exercise performance exists. For example, Pasman et al., (1995) investigated the effect of three doses of caffeine (5, 9 and  $13 \text{ mg}\cdot\text{kg}^{-1}$ ) on time to exhaustion with athletes cycling at  $80\% W_{\text{max}}$ . While results showed an average improvement in time to fatigue of 27% with caffeine ingestion compared to placebo, there was no significant difference between caffeine doses. Similarly, Graham and Spriet (1995) compared the effects of 3, 6 and  $9 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  doses of caffeine on an endurance run (at  $85\% \dot{V}O_{2\text{peak}}$ ) to exhaustion. Like Pasman et al., (1995), they found increased time to exhaustion with caffeine ingestion compared to placebo, however this was only seen with 3 and  $6 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  doses. Interestingly, Graham and Spriet (1995) suggested that the  $9 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  dose may not have been ergogenic due to excessive stimulation of the central nervous system, resulting in some subjects complaining of 'mental confusion' and an inability to focus. In addition, they also found that while the  $3 \text{ mg}\cdot\text{kg}^{-1} \text{ BM}$  dose improved performance, it was not associated with increases in adrenaline. This is important considering some researchers have suggested that high levels of adrenaline may cause some of the perturbations in immune markers observed following intensive exercise (Fletcher & Bishop, 2010a; Gleeson & Pyne, 2000). As such, using a dose of caffeine for ergogenic purposes that does not increase adrenaline concentration above that with exercise alone could be suggested by some to be more beneficial from an immune perspective (Fletcher & Bishop, 2010a).

Dose-responses were also investigated in athletes completing a 2000 m row (Bruce et al., 2000). Six and 9 mg·kg<sup>-1</sup> BM doses were compared to placebo, with results showing significant improvement in rowing time (1.2%) with caffeine but not placebo ingestion. Likewise, no differences in performance were seen between 6 and 9 mg·kg<sup>-1</sup> BM trials. Dose-response effects of caffeine have also been investigated in a study looking at caffeine and ratings of leg muscle pain during cycle exercise (Motl, et al., 2006). In this study females ingested either 5 or 10 mg·kg<sup>-1</sup> BM caffeine and then cycled for 30 min at 60%  $\dot{V}O_{2peak}$ . Caffeine ingestion was associated with significant reductions in leg muscle pain compared to placebo, but again no dose response was seen. Overall these findings indicate that caffeine is unlikely to exert dose-response effects on exercise performance or cognition, and as such athletes should consume the lowest beneficial dose (2-3 mg·kg<sup>-1</sup> BM) to avoid any potential negative side effects on performance.

## **2.10 Caffeine and saliva IgA at rest and during exercise**

To the author's knowledge only one study has investigated the effects of caffeine ingestion on s-IgA at rest and following exercise (Bishop et al., 2006). A pilot study by Bishop et al., (2006) investigated the effects of caffeine on s-IgA and  $\alpha$ -amylase at rest. Participants ingested either 6 mg·kg<sup>-1</sup> BM of caffeine or placebo at 10:00 h and then rested in a laboratory for 3.5 h while providing saliva samples at specified time points (11:00 h, 11:45 h, 12:30 h, and 13:30 h). Saliva flow rate, s-IgA concentration and secretion rate were unaffected by caffeine ingestion, however  $\alpha$ -amylase secretion was over four times higher 1 h after caffeine ingestion compared with placebo. Subsequently, it can be suggested that despite caffeine increasing  $\alpha$ -amylase secretion rate, its effects alone did not appear to elicit a strong enough stimulus so as to alter s-IgA responses at rest.

However, when Bishop et al., (2006) investigated the effects of a 6 mg·kg<sup>-1</sup> BM dose of caffeine compared to placebo on s-IgA responses after 90 min cycle at 70%  $\dot{V}O_{2peak}$ , they found that s-IgA concentration increased from baseline to mid-exercise by 50% with caffeine ingestion compared to placebo, and still remained 40% higher than placebo post-exercise. Similar results were found for s-IgA secretion rates with caffeine being almost double that of placebo, although no differences were seen post-exercise. As expected,  $\alpha$ -amylase secretion increased in the caffeine trial from baseline to post-exercise and was almost 2 fold higher than that in the resting trial.  $\alpha$ -amylase secretion also increased 2 fold from baseline to post-exercise in the placebo trial, however the increase was 30% lower than with caffeine ingestion suggesting that exercise also stimulates sympathetic activity. In contrast, saliva

flow rate was unaffected by caffeine ingestion, but was reduced with exercise, decreasing by 30% from baseline to post-exercise. In summary these findings suggest that caffeine ingestion 1 h before exercise has no effect on saliva flow rate, but has the potential to elevate s-IgA, even though this is short lived.

## **2.11 Summary**

Saliva IgA is the most abundant antibody in mucosal secretions and is often reported to be affected by exercise. Mechanisms for alterations in the rate of s-IgA secretion following acute intensive exercise have as yet not been fully elucidated. However, evidence from rat *in vivo* studies suggests that sympathetic stimulation appears to exert the greatest effects on s-IgA, usually resulting in increases. Exercise is a potent stimulator of sympathetic activity and is known to increase adrenaline, which has been shown to inhibit a number of immune functions that are often suppressed for several hours following exercise. Since caffeine consumption is known to increase adrenaline, it is thought that ingestion may exacerbate any exercise-induced immunosuppression. However, since several researchers have reported doses of 2-3 mg·kg<sup>-1</sup> BM caffeine to have no affect on adrenaline, it is possible that smaller doses of caffeine may have lesser effects on immune function. Considering the only known caffeine, exercise and s-IgA study examined the effects of a single dose of caffeine (6 mg·kg<sup>-1</sup> BM), which is considered quite high by some researchers, and the typical reported caffeine doses for athletes looking to use caffeine as an ergogenic aid is ~2-3 mg·kg<sup>-1</sup> BM, it seems that it would be useful to investigate the effects of a range of caffeine doses on s-IgA responses following high-intensity exercise.

## **2.12 Potential effect of caffeine on s-IgA responses following exercise**

The aim of this thesis was to determine the effects of different doses of caffeine on saliva IgA and  $\alpha$ -amylase responses to prolonged high-intensity exercise.

Based on the reviewed literature it was hypothesised that:

- Caffeine at any dose could exacerbate or negate any depression in saliva IgA caused by exercise and the response could be dose-dependent.
- Caffeine at any dose in combination with exercise would increase  $\alpha$ -amylase secretion above that seen with exercise alone and the response would likely be dose-dependent.
- Caffeine at any dose would have no affect on saliva flow rate at rest or during exercise.
- Exercise alone will likely cause saliva flow rate to decrease.
- Exercise alone may be strenuous enough to cause a depression in saliva IgA secretion rate.

## Chapter Three: Dose response effects of caffeine ingestion on salivary Immunoglobulin A following high-intensity exercise

### 3.1 Abstract

The purpose of this study was to examine the dose response effects of caffeine ingestion on salivary IgA responses to prolonged high-intensity running. In a double blind randomised crossover design, 12 endurance trained male runners (age:  $29 \pm 3$ ,  $\dot{V}O_{2peak}$   $62.7 \pm 5.1$  mL·kg<sup>-1</sup>·min<sup>-1</sup>, mean  $\pm$  SD) ran for 70 min at 80%  $\dot{V}O_{2peak}$  60 min after ingesting 2 (2CAF), 4 (4CAF), 6 (6CAF) or 8 mg·kg<sup>-1</sup> BM (8CAF) of caffeine or placebo (PLA = cornflour). Unstimulated whole saliva samples were obtained before supplementation, pre-exercise, after 35 min of exercise, immediately post-exercise and 1 h post-exercise. Saliva caffeine concentrations were significantly increased at all time points (pre-, mid-, post- and 1 h post-exercise) following caffeine ingestion in a dose-dependent manner ( $P < 0.001$ ). Saliva IgA concentration and secretion rates were unaffected by exercise or caffeine ingestion. Saliva  $\alpha$ -amylase activity appeared to follow a dose-response effect, with activity increasing with each caffeine dose up to 6CAF (main effect for trial,  $P < 0.01$ ; PLA (687), 2CAF (720), 4CAF (817) and 6CAF (837 U·mL<sup>-1</sup>)). Interestingly activity on 8CAF was no different to that on PLA (8CAF:  $P > 0.05$ ). Saliva  $\alpha$ -amylase secretion rates were both higher than pre-supplement at all time points (main effect for time,  $P < 0.01$ ). Caffeine ingestion regardless of dose did not affect saliva flow rate. In summary, these findings suggest that caffeine ingestion 60 min prior to prolonged high-intensity treadmill running has no effect on s-IgA responses to exercise.

**Keywords:** Saliva, IgA, Immune, Treadmill, Methlyxanthine

### 3.2 Introduction

Caffeine is a member of the methylxanthine family of drugs, and due to its presence in numerous foods and fluids is probably the most widely consumed psychoactive substance known to man (Fredholm et al., 1999). Many athletes consume caffeine for its known ergogenic properties, with doses between 2-6 mg·kg<sup>-1</sup> BM being shown to enhance performance for events lasting from 5 min to 2 h (as reviewed by Graham, 2001; Goldstein et al., 2010). This may explain why caffeine consumption has increased since being removed from the World Anti Doping Association (WADA) prohibited substance list in 2004 (Chester &

Wojek, 2008). Caffeine has also been suggested to be a useful tool for training to help athletes sustain high exercise intensities during heavy endurance training, as well as for its ability to reduce pain perception (Sokmen et al., 2008). Numerous studies have shown caffeine to improve exercise performance using a range of caffeine doses, from as small as 2 mg·kg<sup>-1</sup> BM to as large as 13 mg·kg<sup>-1</sup> BM (as reviewed in Goldstein et al., 2010), however research to date shows no dose response effect of caffeine on sports performance (Graham & Spriet, 1995; Pasman et al., 1995). Since ingestion of high doses of caffeine (>6 mg·kg<sup>-1</sup> BM) have been associated with possible adverse affects such as insomnia, headaches, dizziness and gastro-intestinal distress (Evans & Griffiths, 1992; Leonard et al., 1987) it could be suggested that athletes consume the lowest beneficial dose in order to avoid any potential side effects.

Immunoglobulin A (IgA), the most abundant antibody in mucosal secretions is often seen to decrease both in concentration and secretion rate following prolonged or intense exercise and has consequently been suggested as a potential indicator of risk of upper respiratory tract infection (URTI) (Neville et al., 2008). Saliva IgA (s-IgA) is thought to provide an indicator of URTI risk; as individuals with selective s-IgA deficiency show a high incidence of URTI, while high levels of s-IgA are often associated with low incidence of URTI (Hanson et al., 1983; Rossen, Butler, & Waldman, 1970). s-IgA is produced by plasma cells that have migrated to mucosal tissues (Michalek & Childers, 1990), and is transported to the epithelial cell surface via attachment to a polymeric immunoglobulin receptor (pIgR) (Brandtzaeg, 1995). s-IgA contributes to the body's first line of defence against infection by inhibiting pathogen adherence and penetration, neutralizing viruses within epithelial cells, and binding pathogens for transport to the epithelial cell surface (Lamm, 1998).

Chronic effects of exercise seen over a competitive season in football and yachting have reported s-IgA concentration and secretion rates to display a cumulative decline from month to month and also reported correlations between decreased s-IgA and risk of URTI (Fahlman & Engels, 2005; Neville et al., 2008). In addition acute bouts of prolonged or high intensity exercise have also been shown to decrease s-IgA secretion and saliva flow rate (Engels et al., 2004, Nieman et al., 2002, Walsh et al., 2002), and consequently it has been proposed that this depression may make athletes more susceptible to infection (Gleeson, 2007).

Saliva secretion and composition are regulated by the autonomic nervous system with salivary glands being innervated by both parasympathetic and sympathetic nerves (Dennis et al., 1978). Parasympathetic stimulation results in high saliva flow rates with low protein

content, while sympathetic stimulation elicits low saliva flow rates high in protein content (Baum, 1987). Stimulation of salivary glands via autonomic nerve stimulation and injections of autonomimetics in the rat model have been shown to increase IgA transport via increased mobilisation of the pIgR (Carpenter et al., 2004; Proctor et al., 2003) in a dose-dependent manner above a certain threshold of stimulation (Proctor et al., 2003). Since caffeine ingestion is associated with increased sympathetic activity it has been suggested that ingestion may further affect IgA transport (Bishop et al., 2006). Although caffeine ingestion has not demonstrated a dose-response effect upon exercise performance (Graham & Spriet, 1995), because rat studies have demonstrated a critical threshold for alterations in s-IgA levels (Proctor et al., 2003), it is possible that IgA transcytosis may also exhibit a dose response to caffeine ingestion via the potential increase in adrenaline concentration with each dose of caffeine. In contrast, a recent *in vivo* s-IgA and exercise study in rats reported s-IgA and pIgR mRNA expression of decrease following exhaustive exercise (Kimura et al., 2008), leading a recent review (Bishop & Gleeson, 2009) to tentatively speculate that there could be a second critical threshold or duration of stimulation above which pIgR expression becomes down-regulated. However, to date there has been limited research in either animal models or humans to support these speculations.

Additionally  $\alpha$ -amylase, a key antimicrobial protein found in saliva has also been shown to play an important role in mucosal immunity by inhibiting pathogen adherence and growth.  $\alpha$ -amylase has been proposed as a biomarker of sympathetic activity (Nater & Rohleder, 2009) and has been shown to increase with both psychological and physiological stress (Chatterton et al., 1996; Nater et al., 2005; Takai et al., 2007; Walsh, 1999). Because caffeine stimulates sympathetic activity it is expected that caffeine ingestion would result in further increases in  $\alpha$ -amylase activity as observed by Bishop et al., (2006).

The potential of caffeine ingestion to alter sympathetic activity and key immune function markers are intriguing. To the author's knowledge, only one study has investigated the effects of caffeine ingestion on s-IgA responses to exercise (Bishop et al., 2006). This study examined the effects of a single dose of caffeine ( $6 \text{ mg} \cdot \text{kg}^{-1} \text{ BM}$ ) ingested 1 h before a 90 min cycle at 70%  $\dot{V}\text{O}_{2\text{peak}}$ . While s-IgA concentration and secretion rate were significantly increased from pre- to mid-exercise, these increases were short-lived with measures returning to baseline by 1 h post-exercise. Since a  $6 \text{ mg} \cdot \text{kg}^{-1} \text{ BM}$  dose of caffeine is considered quite high by some researchers, and is unlikely to be used by athletes in a typical training situation (Chester & Wojek, 2008) it would be interesting to know what effects

smaller or larger doses have on mucosal immune responses to exercise and whether a dose-response exists. Therefore the aim of this study was to determine the dose response effects of caffeine ingestion on saliva IgA and flow rate responses to a 70 min run at 80%  $\dot{V}O_{2peak}$ . In addition the effect of caffeine on  $\alpha$ -amylase activity was assessed.

### **3.3 Methods**

#### **3.3.1 Participants**

Twelve endurance trained male runners [mean (SD): 29 (3) years; body mass 74.2 (6.4) kg;  $\dot{V}O_{2peak}$  62.7 (5.1) mL·kg<sup>-1</sup>·min] volunteered to participate in this study. All participants were fully informed about the rationale and experimental protocols of the study. Participants completed an extensive health screening and physical activity questionnaire to determine their suitability for the study. Participants then provided written informed consent prior to participating in the study, which had earlier received the approval from the Auckland University of Technology Ethics Committee. At each subsequent visit participants also completed a health-screening questionnaire. Any participants that were currently on medication or had reported symptoms of infection in the 4 weeks prior to the study were excluded. Participants' habitual caffeine consumption was recorded using a caffeine consumption questionnaire administered during their initial visit. Daily caffeine intake among participants ranged from 25 to 444 mg·day<sup>-1</sup>. Four participants were characterized as high users (>250 mg·day<sup>-1</sup>), 7 as moderate users (50-250 mg·day<sup>-1</sup>) and 1 as a light user (<50 mg·day<sup>-1</sup>).

#### **3.3.2 Preliminary testing**

Approximately 1 week before the beginning of experimental trials, each participant performed a continuous incremental exercise test to volitional exhaustion on a treadmill (h/p/Cosmos Saturn 250/100, Germany) to determine their peak oxygen consumption ( $\dot{V}O_{2peak}$ ). Participants started running at a speed of 10 - 12 km·h<sup>-1</sup> and the treadmill gradient was set at 1.0 %. Gradient remained constant throughout the test while speed was increased by 1 km·h<sup>-1</sup> every 3 min, continuing until participants reached volitional exhaustion. To cater for a range of fitness levels the starting speed was adjusted to ensure each participant completed at least 6 but no more than 9 stages. Verbal encouragement was given to each participant to ensure maximal effort. Heart rate was measured throughout the test using short-range radio telemetry (Polar RS800, Polar Electro, Finland) and minute ventilation, O<sub>2</sub> consumption and CO<sub>2</sub> production were determined from expired samples using a metabolic system (Parvo Medics TrueOne 2400, Sandy, UT), which was calibrated

before each test. A work-rate equivalent to 80%  $\dot{V}O_{2peak}$  for each participant was interpolated from the  $\dot{V}O_2$  (l·min) – speed (v) relationship.

Participants visited the laboratory on a separate occasion to undertake a familiarization trial. This required participants to run on a treadmill for 70 min at 80%  $\dot{V}O_{2peak}$ . Heart rate was monitored continuously during the familiarization trial and 1 min expired gas samples were collected at 20 min intervals to ensure participants were exercising at the correct intensity. If  $\dot{V}O_2$  was below 78%  $\dot{V}O_{2peak}$  or above 82%  $\dot{V}O_{2peak}$  the treadmill speed was adjusted and expired gases were monitored until the correct intensity was reached. This session was also used to familiarize participants with saliva collection procedures and to establish the participants' individual saliva flow rates. This information was used to determine an appropriate collection time during experimental trials to ensure an adequate volume (ml·g<sup>-1</sup>) of saliva was collected for subsequent analysis.

### **3.3.3 Experimental trial procedures:**

Participants were given an information sheet detailing pre-trial conditions that they were required to follow. This included a list of caffeine containing foods and beverages that participants were to abstain from in the 60 h prior to each experimental trial. Participants were also asked to avoid alcohol and strenuous exercise in the 24 h preceding each experimental trial. In order to standardize nutritional status, participants were asked to complete a 24 h food diary the day before the first experimental trial and were asked to follow this during the 24 h preceding subsequent trials. Nutritional status on the day of each experimental trial was also standardized, with participants consuming a breakfast of known composition at 08:00 hours. Each breakfast was designed to give participants approximately 2 g·kg<sup>-1</sup> body mass (BM) carbohydrate, 0.5 g·kg<sup>-1</sup> BM protein, 0.1 g·kg<sup>-1</sup> BM fat and 45 kJ·kg<sup>-1</sup> BM energy which is in line with general pre-exercise meal recommendations (American College of Sports Medicine, American Dietetic Association, & Dietitians of Canada, 2009; Kreider et al., 2010). Participants were required to eat this same breakfast prior to all subsequent trials. Following their breakfast at 08:00 hours participants were asked to refrain from eating and to only consume water until the completion of the days testing.

In a double-blind repeated-measures crossover design, participants completed 5 experimental trials separated by approximately 1 week. Double blinding was achieved by having a laboratory technician prepare participant capsules. Participants were randomly assigned to PLA, 2CAF, 4CAF, 6CAF or 8CAF and acted as their own controls. Participants reported to the lab at 11:45 hours and rested until 12:00 hours, at which time a pre-

supplement saliva sample was collected. Immediately after this participants ingested 0, 2, 4, 6 or 8 mg·kg<sup>-1</sup> BM of caffeine powder (Acros Organics, Belgium) taken in the form of cellulose capsules (Skybright NZ Ltd., Christchurch, NZ) with 5 ml·kg<sup>-1</sup> BM of plain water. For the 0 (PLA) caffeine dose participants consumed 8 mg·kg<sup>-1</sup> BM of cornflour (Edmonds, Auckland, NZ) in the same cellulose capsules. Participants then rested quietly in the lab for 1 h before a further (pre-exercise) saliva sample was collected, after which pre-exercise body mass (in shorts only) was recorded. Immediately after this participants began running on the treadmill for 70 min at a work rate equivalent to 80%  $\dot{V}O_{2peak}$ . No warm up was permitted prior to starting exercise, however participants were given 3 min to reach their 80% intensity speed in an attempt to prevent risk of injury. During the 70 min run participants' heart rate and subjective ratings of perceived exertion (RPE) (Borg, 1982) were recorded every 15 min. Participants also consumed 2 ml·kg<sup>-1</sup> BM water every 15 min in order to standardize fluid intake. At 20, 40 and 60 min of exercise, 1 min expired gas samples were collected to determine  $\dot{V}O_2$  and  $\dot{V}CO_2$  to ensure that participants were exercising at the correct intensity. Gas sampling also allowed for estimation of fat and carbohydrate oxidation as well as energy expenditure using stoichiometric equations (Peronnet & Massicotte, 1991). Thirty-five min into the run a mid-exercise saliva sample was collected during a 4 min non-exercising measurement period during which participants stopped running, provided a saliva sample and recommenced running. A further saliva sample was obtained immediately after exercise cessation before post exercise BM (in shorts only) was recorded. Participants then rested quietly in the lab for a further hour before a final (1 h post-exercise) saliva sample was collected. During this time participants were given 5 ml·kg<sup>-1</sup> BM plain water to consume and no additional fluid or food was permitted. At the end of the testing session participants completed a questionnaire to determine their perception of supplement order and to report any side effects that they attributed to the supplement ingestion. Laboratory conditions throughout were  $21.9 \pm 1.1^\circ \text{C}$  and  $59 \pm 5\%$  relative humidity.

### **3.3.4 Saliva collection**

All saliva collections were made with participants seated, leaning forward, and with their heads tilted down. Participants were instructed to swallow in order to empty their mouth of saliva before an un-stimulated whole saliva sample was collected over a pre-determined time into a pre-weighed sterile bijoux tube (7 ml-capacity with screw top, Labserve, Auckland, NZ). Care was taken to allow saliva to dribble into the collection vial with minimal orofacial movement. All saliva collections were obtained at least 5 min after any scheduled drink ingestion. Samples were frozen and stored at  $-80^\circ \text{C}$  until analysis.

### 3.3.5 Saliva Analysis

Following collection saliva volume was estimated by weighing to the nearest milligram assuming saliva density to be  $1.0\text{g}\cdot\text{ml}^{-1}$  (Cole & Eastoe, 1988). Saliva flow rate ( $\text{ml}\cdot\text{min}^{-1}$ ) was calculated by dividing the volume of saliva by collection time. For analysis samples were defrosted. After thawing, samples were then spun at 13,400 rpm for 2 min and the saliva was subsequently analyzed for s-IgA and  $\alpha$ -amylase using spectrophotometric methods. The concentration of s-IgA ( $\mu\text{g}\cdot\text{L}^{-1}$ ) was determined by an enzyme linked immunosorbent assay (ELISA) method using a commercially available kit (DRG SLV-4636, DRG Instruments, Marburg, Germany).  $\alpha$ -amylase activity was measured using a commercially available kit (Infinity™  $\alpha$ -Amylase Liquid Stable Reagent, Thermo Scientific, UK), with proportional reduction of volumes so that the assay could be carried out in a microtitration (96-well) plate. Briefly, sample analysis was performed in duplicate using 20  $\mu\text{L}$  of saliva diluted 1:100 with 1.0mM  $\text{CaCl}_2$  which was then mixed with 180  $\mu\text{L}$  of Infinity reagent. The plate was incubated at  $20^\circ\text{C}$  for 1 min and the increase in absorbance at 405 nm was recorded for minutes 1 and 3 on an automated plate reader (Multiskan\* GO Microplate Spectrophotometer, Thermo Scientific, UK). The difference in absorbance per minute was multiplied by 2515, which is a reagent and temperature specific factor provided by the manufacturer of the amylase reagent. Additionally osmolality was determined using a freeze point depression osmometer (Model 3320 Micro-Osmometer, Advanced Instruments, Massachusetts, USA) with 20  $\mu\text{L}$  of saliva. Saliva caffeine concentrations were determined by high-performance liquid chromatography with UV detection as previously described (Perera, Gross, & McLachlan, 2010).

The secretion rates of s-IgA ( $\mu\text{g}\cdot\text{min}^{-1}$ ) and  $\alpha$ -amylase ( $\text{U}\cdot\text{min}^{-1}$ ) were calculated by multiplying saliva flow rate ( $\mu\text{L}\cdot\text{min}^{-1}$ ) by the concentration of the measured analyte ( $\text{mg}\cdot\text{L}^{-1}$  and  $\text{U}\cdot\text{ml}^{-1}$ , respectively) and dividing by 1000. All samples from one participant were analyzed on the same microplate. The intra assay coefficients of variation for the analytical methods were 3.0%, 1.3% and 1.5% for saliva IgA,  $\alpha$ -amylase and osmolality assays respectively.

### 3.3.6 Statistical analysis

Data are presented as mean values and the standard deviation. Data were examined using a 5 (trial) x 5 (time of measurement) ANOVA with repeated measures design, after first checking for normality of distribution. If a data set was not normally distributed, statistical analysis was performed on the logarithmic transformation of the data. Assumptions of

homogeneity and sphericity in the data were also checked and, where appropriate, adjustments in the degrees of freedom for the ANOVA were made using the Huynh-Feldt method of correction. Any significant data were assessed post-hoc using paired samples *t*-tests, with Holm-Bonferroni adjustments for multiple comparisons applied to the unadjusted *P* value. Single comparisons between trials for overall exercise intensity, fat and carbohydrate oxidation rates, rate of energy expenditure were made using Student's paired *t*-tests. Statistical significance was accepted at  $P < 0.05$ . The observed powers of the reported main and interaction effects are all  $>0.8$ .

### **3.4 Results**

#### *Exercise intensity, heart rate, indirect calorimetry and changes in body mass*

Mean %  $\dot{V}O_{2peak}$ , heart rate and RPE were similar between exercise trials (Table 8). Likewise, RER, energy expenditure and rates of CHO and fat oxidation did not differ amongst trials (Table 8). Post-exercise changes in BM (corrected for fluid intake) were similar across trials.

Table 8. The effect of 2, 4, 6 and 8 mg·kg<sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80%  $\dot{V}O_{2peak}$  on HR, RPE, fluid loss, RER, substrate oxidation and energy expenditure during exercise.

	PLA	2CAF	4CAF	6CAF	8CAF
% $\dot{V}O_{2peak}$ (ml·kg·min <sup>-1</sup> )	79.5 (0.9)	79.6 (0.7)	79.8 (0.8)	80.3 (0.7)	79.9 (0.7)
Heart rate (bpm)	164 (15)	166 (12)	165 (12)	168 (14)	170(9)
RPE	15 (2)	14 (2)	14 (2)	14 (2)	14 (2)
Fluid loss (kg)	1.8 (0.3)	1.8 (0.3)	1.8 (0.3)	1.8 (0.4)	1.8 (0.4)
Fluid loss as % of BM	2.4 (0.5)	2.5 (0.5)	2.5 (0.5)	2.5 (0.6)	2.5 (0.6)
RER	0.91 (0.03)	0.90 (0.03)	0.91 (0.03)	0.91 (0.03)	0.92 (0.03)
Fat oxidation (g·min <sup>-1</sup> )	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.6 (0.1)	0.6 (0.2)
Carbohydrate oxidation (g·min <sup>-1</sup> )	3.1 (0.5)	2.9 (0.5)	3.1 (0.5)	3.7 (0.6)	3.2 (0.6)
% Fat oxidation	33 (9)	31 (9)	29 (9)	30 (10)	28 (11)
% Carbohydrate oxidation	67 (9)	69 (9)	71 (9)	70 (10)	72 (11)
Energy expenditure (kJ·min <sup>-1</sup> )	74 (7)	74 (8)	75 (8)	75 (7)	75 (8)
Values are mean of all recordings throughout exercise (SD)					

### Saliva

*Saliva caffeine concentration:* A significant time x trial interaction was found for saliva caffeine concentration ( $P < 0.01$ ) with higher concentrations pre-, mid-, post- and 1 h post-exercise with 8CAF when compared with 6CAF, 4CAF, 2CAF, and PLA ( $P < 0.01$ ). At the same time points concentrations with 6CAF were significantly higher than 4CAF, 2CAF and PLA ( $P < 0.01$ ). Similarly concentrations with 4CAF were also significantly higher at these time points than 2CAF and PLA ( $P < 0.01$ ), with concentrations on 2CAF significantly higher than PLA ( $P < 0.01$ ) (Figure 4).

*Saliva flow rate:* Saliva flow rate did was not influenced by either exercise or caffeine ingestion as no significant trial x time interaction or time/trial effects were found (Table 9).

*Saliva IgA:* No significant time x trial interaction was found for s-IgA concentration (Table 9) or secretion rate (Figure 5). There was also no significant time x trial interaction or main effects when s-IgA concentration and secretion rate were expressed as a percentage relative to pre-supplement values due to large inter-individual differences (Figure 7).

*$\alpha$ -amylase activity:* There were no time x trial interactions for  $\alpha$ -amylase activity, however there was a main effect for trial and time (both  $P = 0.01$ ).  $\alpha$ -amylase activity was higher in 6CAF compared to PLA, 2CAF, 4CAF and 8CAF (all  $P < 0.05$ ).  $\alpha$ -amylase activity was higher in 4CAF compared to PLA, 2CAF, and 8CAF (all  $P < 0.01$ ).  $\alpha$ -amylase activity was also higher again in 2CAF, but only when compared to PLA ( $P = 0.01$ ).  $\alpha$ -amylase activity appeared to increase over time throughout all trials and was significantly higher than pre-supplement for all time points (pre-, mid- and post-exercise  $P < 0.01$  and 1 h post-exercise  $P < 0.05$ ).  $\alpha$ -amylase activity was also significantly higher at mid- and post-exercise when compared to pre-exercise and 1 h post-exercise ( $P < 0.001$ ), however 1 h post-exercise values were still significantly higher than pre-supplement ( $P < 0.05$ ) (Table 9).

*$\alpha$ -amylase secretion rate:* There were no significant interaction effects for  $\alpha$ -amylase secretion rate, however there was a main effect for time ( $P < 0.01$ ).  $\alpha$ -amylase secretion rate increased over time throughout trials and was significantly higher than pre-supplement at all time points (pre-, mid- and post-exercise  $P < 0.01$  and 1 h post-exercise  $P < 0.05$ ).  $\alpha$ -amylase secretion rate was also higher at mid- and post-exercise compared to pre-exercise and 1 h post-exercise ( $P < 0.001$ ). Again, as with  $\alpha$ -amylase activity, 1 h post-exercise values remained significantly higher than pre-supplement ( $P < 0.05$ ) (Figure 6).

*Saliva osmolality:* Saliva osmolality increased over time and was independent of trial (main effect for time;  $P < 0.01$ ). Saliva osmolality was higher at mid- and post-exercise compared to pre-supplement, pre-exercise and 1 h post-exercise ( $P < 0.001$ ). In addition saliva osmolality was significantly higher at post-exercise compared to mid-exercise ( $P < 0.001$ ). Saliva osmolality decreased from post-exercise to 1 h post-exercise to return to pre-supplement levels (Table 9).

Table 9. The effect of 2, 4, 6 and 8 mg·kg<sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80%  $\dot{V}O_{2peak}$  on saliva caffeine concentration, IgA concentration,  $\alpha$ -amylase activity, osmolality and saliva flow rate.

	Pre-supplement	Pre-exercise	Mid-exercise	Post-exercise	1 h Post-exercise
	(12:00 h)	(1:00 h)	(1:35 h)	(2:10 h)	(3:10 h)
Saliva caffeine concentration ( $\mu$ M)					
PLA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2CAF	0 (0)	10 (3)†‡	9 (2) †‡	8 (3) †‡	7 (3) †‡
4CAF	0 (0)	18 (6)§‡	16 (7) §‡	17 (6) §‡	15 (7) §‡
6CAF	0 (0)	33 (11) ◊‡	32 (10) ◊‡	33 (5) ◊‡	27 (7) ◊‡
8CAF	0 (0)	43 (10)‡‡	45 (8)‡‡	43 (7)‡‡	35 (6)‡‡
s-IgA concentration (mg·L <sup>-1</sup> )					
PLA	125 (238)	118 (133)	94 (147)	85 (29)	114 (109)
2CAF	137 (117)	119 (118)	67 (63)	89 (82)	110 (82)
4CAF	168 (215)	99 (66)	117 (175)	99 (120)	117 (112)
6CAF	114 (94)	92 (60)	90 (153)	93 (131)	118 (151)
8CAF	154 (239)	85 (75)	69 (88)	89 (60)	117 (99)
$\alpha$ -amylase activity (U·mL <sup>-1</sup> )					
PLA	458 (402)	525 (379)	900 (445)	1088 (707)	466 (354)
2CAF	455 (226)	623 (348)	907 (511)	1097 (688)	520 (216)
4CAF	590 (553)	767 (800)	924 (533)	1219 (709)	586 (375)
6CAF	459 (258)	712 (474)	971 (628)	1300 (805)	743 (336)
8CAF	512 (489)	725 (709)	1108 (1051)	1155 (1155)	722 (708)
Osmolality (mOsmol·kg <sup>-1</sup> ) * N = 8					
PLA	49 (16)	57 (9)	77 (20)	90 (5)	54 (7)
2CAF	57 (13)	50 (13)	72 (28)	74 (14)	52 (15)
4CAF	60 (19)	52 (14)	75 (21)	90 (16)	53 (11)
6CAF	55 (10)	51 (8)	77 (23)	89 (16)	58 (10)
8CAF	58 (20)	52 (16)	80 (30)	88 (17)	56 (16)
Saliva flow rate ( $\mu$ L·min <sup>-1</sup> )					
PLA	475 (219)	506 (273)	585 (156)	594 (251)	540 (176)
2CAF	369 (35)	459 (139)	641 (255)	506 (291)	441 (134)
4CAF	435 (163)	569 (109)	569 (218)	563 (196)	611 (232)
6CAF	391 (170)	482 (92)	634 (253)	524 (205)	421 (157)
8CAF	557 (97)	606 (164)	618 (124)	521 (149)	473 (125)

Values are means  $\pm$  SD.

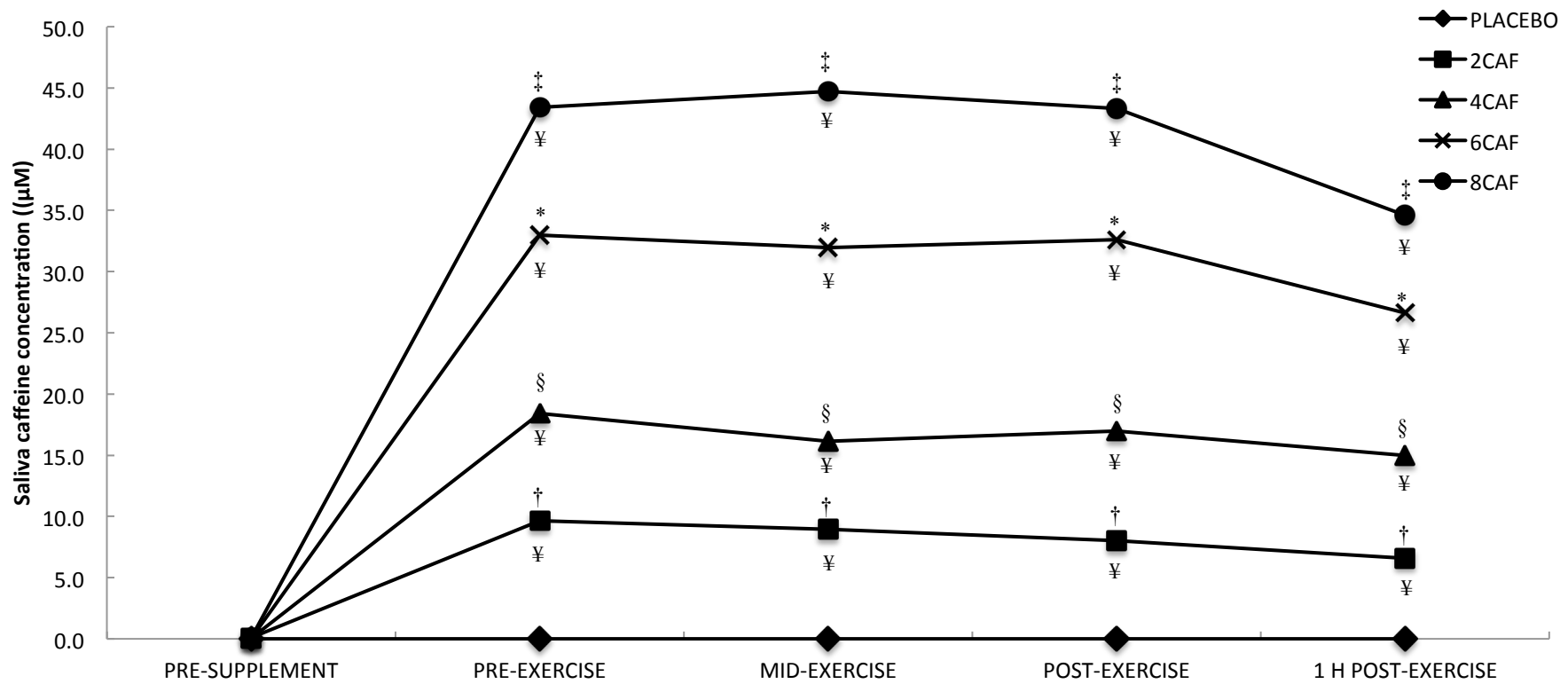
‡ significantly higher than PLA, 2CAF, 4CAF and 6CAF at all time points except pre-supplement:  $P < 0.01$

◊ significantly higher than PLA, 2CAF and 4CAF at all time points except pre-supplement:  $P < 0.01$

§ significantly higher than PLA and 2CAF at all time points except pre-supplement:  $P < 0.01$

† significantly higher than PLA at all time points except pre-supplement:  $P < 0.01$

‡ significantly higher at all time points within trial:  $P < 0.01$



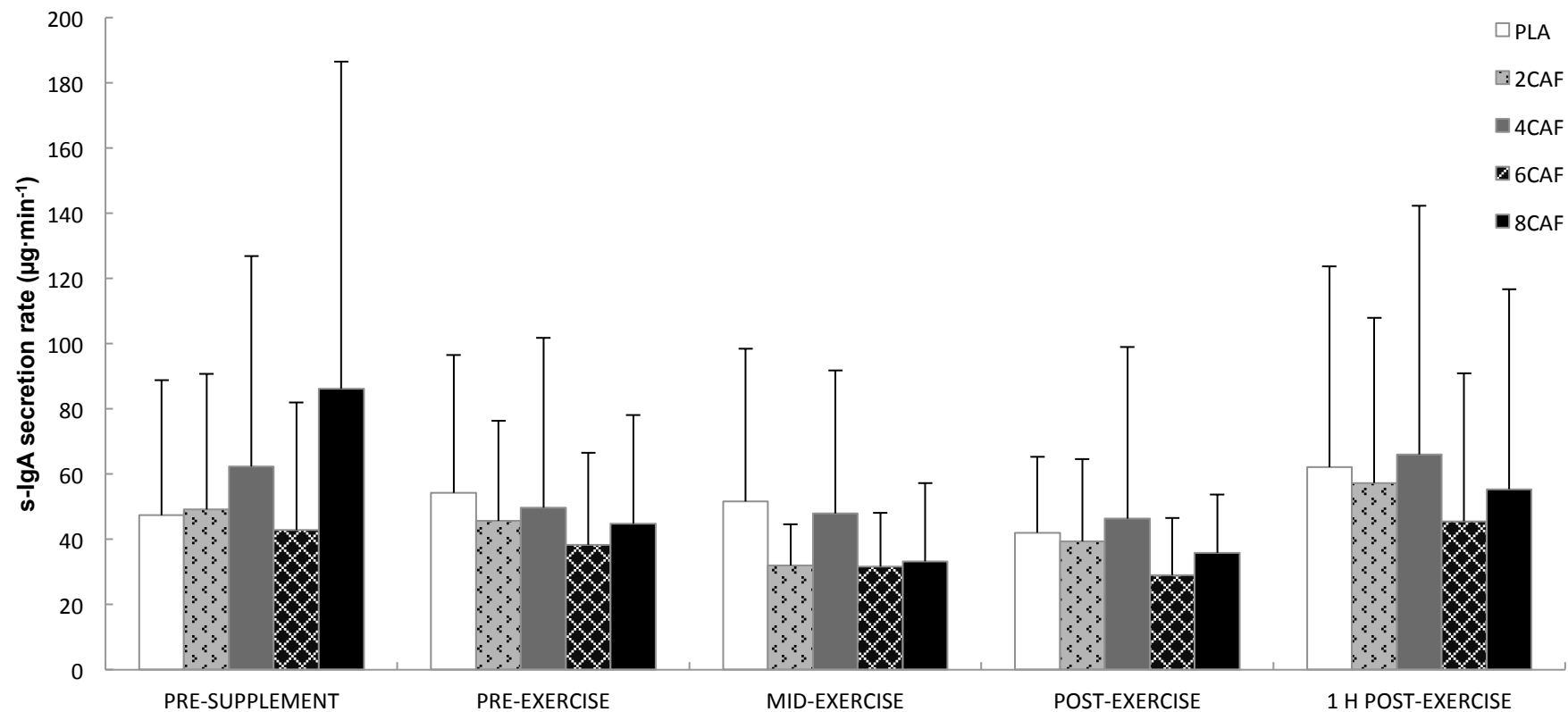


Figure 5. The effect of 2, 4, 6 and 8 mg·kg<sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80%  $\dot{V}O_{2peak}$  on saliva IgA secretion rate.

Values are mean + SD

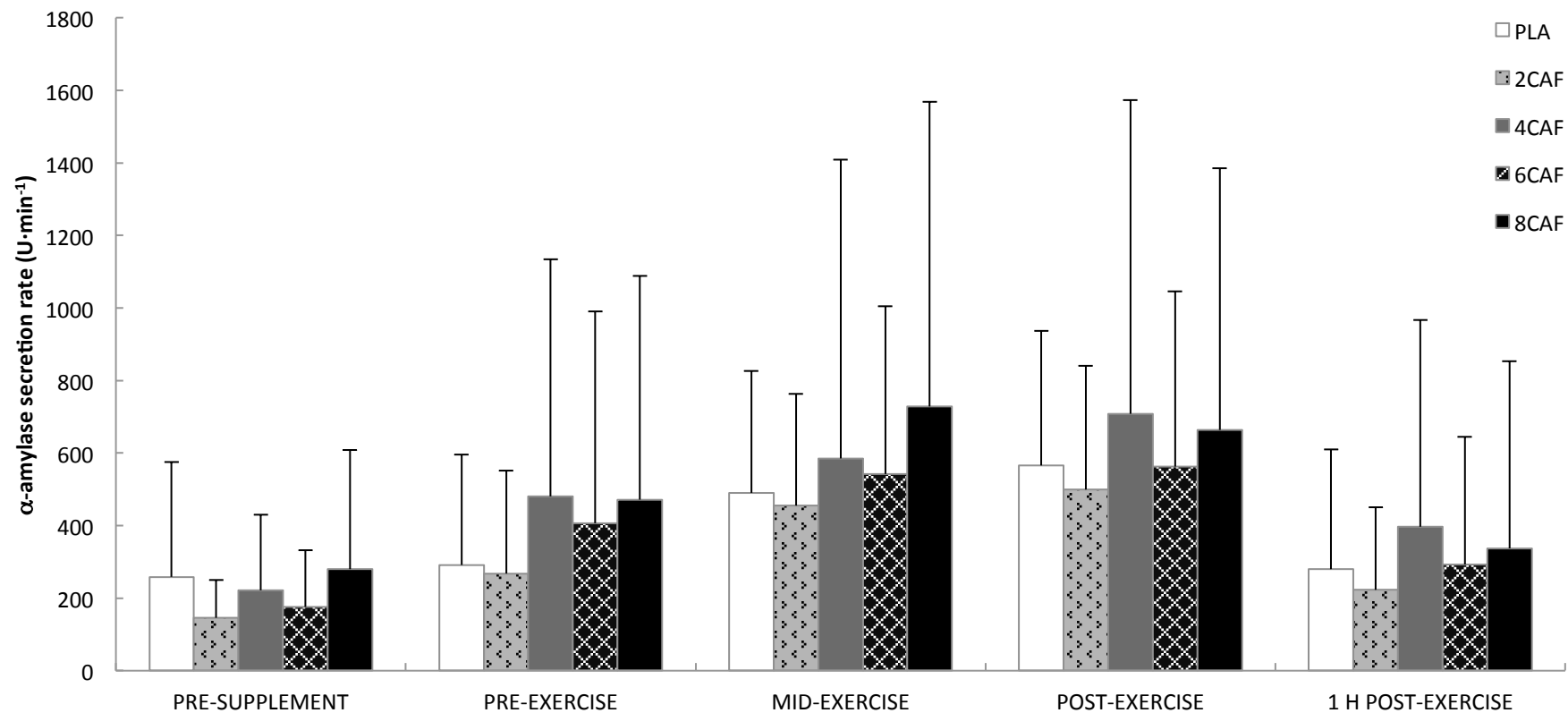


Figure 6. The effect of 2, 4, 6 and 8 mg·kg<sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80%  $\dot{V}O_{2peak}$  on saliva  $\alpha$ -amylase secretion rate. Values are mean + SD.

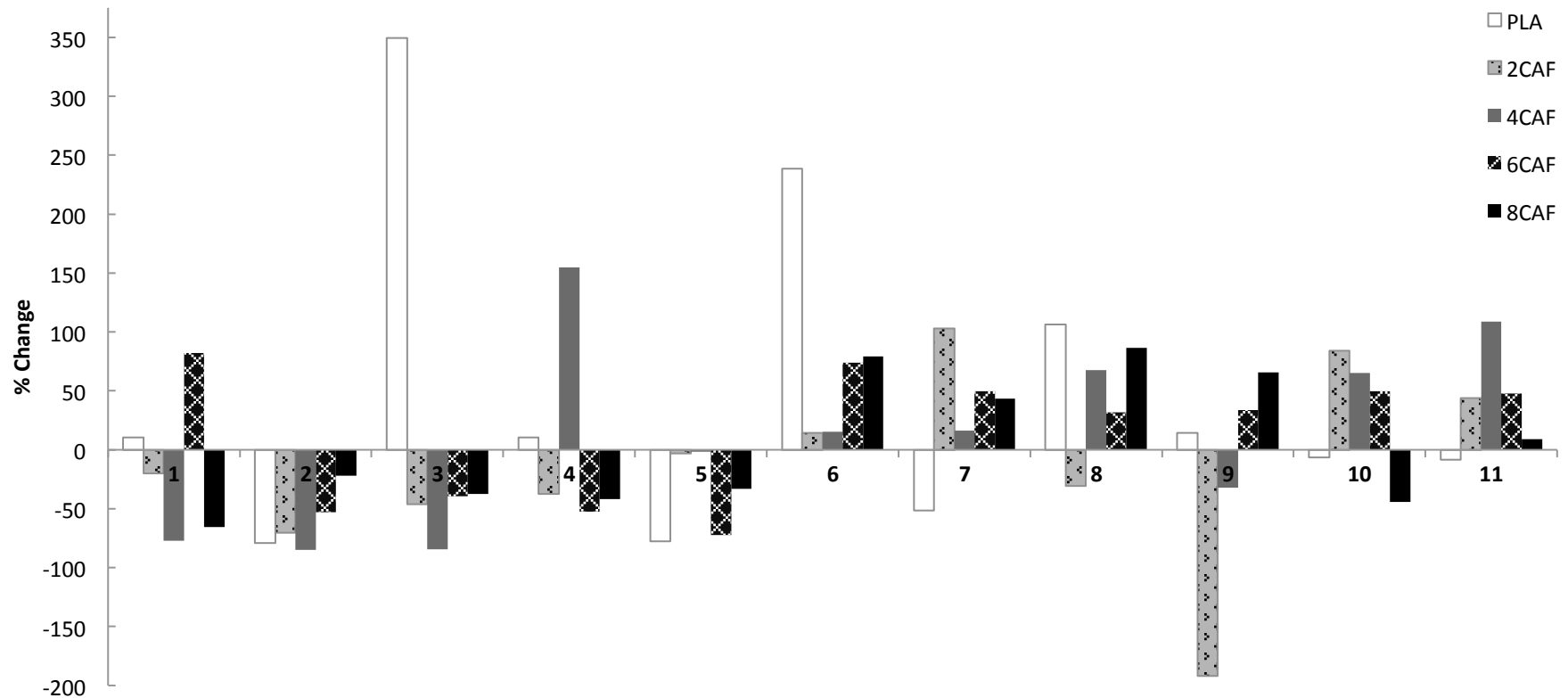


Figure 7. The effect of 2, 4, 6 and 8 mg·kg<sup>-1</sup> BM caffeine or placebo ingestion 1 h before running for 70 min at 80%  $\dot{V}O_{2peak}$  on saliva IgA secretion rate as percent changes from pre-exercise to post-exercise for individual participants.

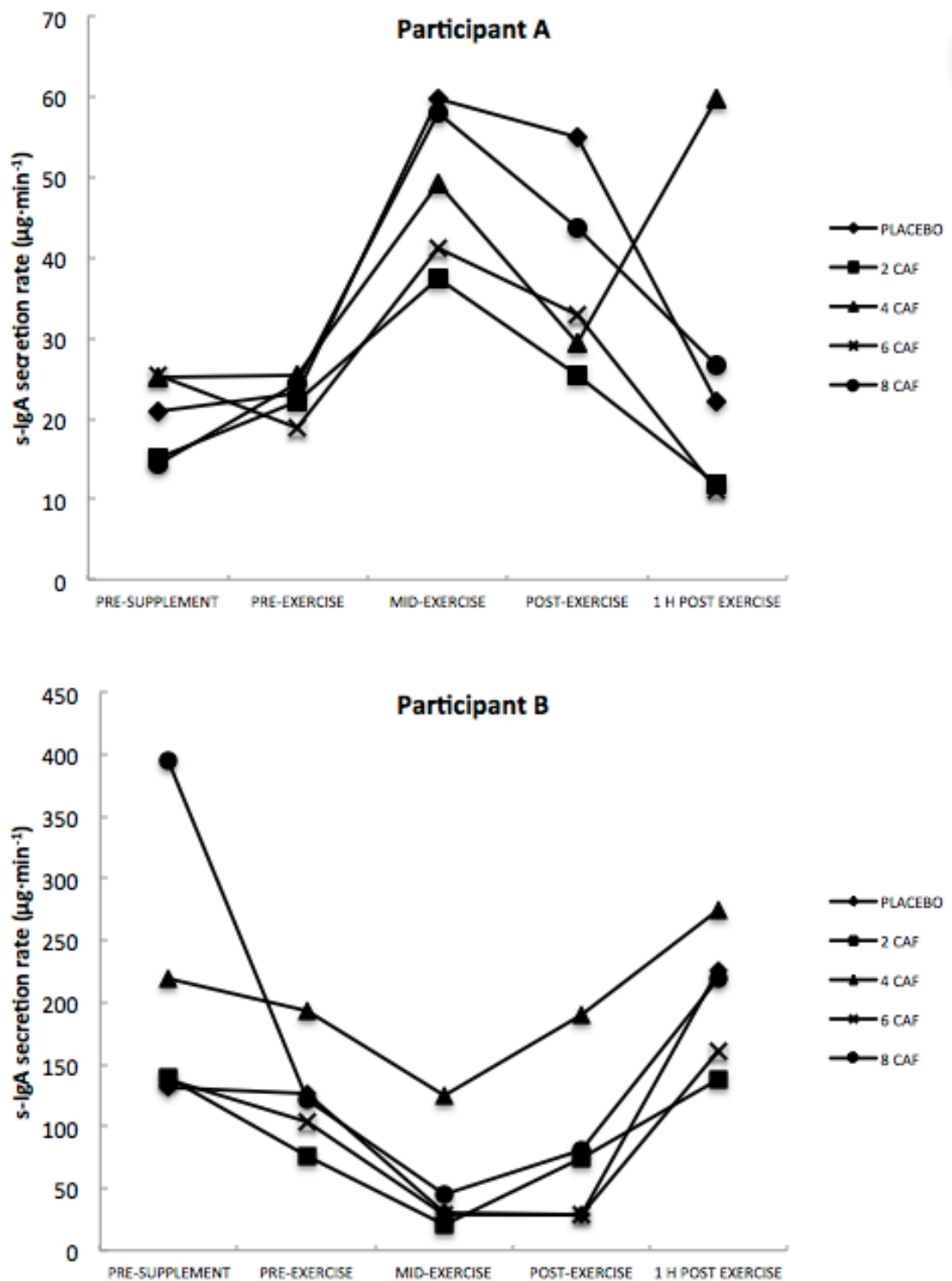


Figure 8. The effect of 2, 4, 6 and 8  $\text{mg}\cdot\text{kg}^{-1}$  BM caffeine or placebo ingestion 1 h before running for 70 min at 80%  $\dot{V}\text{O}_{2\text{peak}}$  on saliva IgA secretion rate for two participants.

### 3.5 Discussion

This is the first study to investigate the dose-response effects of caffeine ingestion on salivary IgA responses to prolonged high-intensity exercise. The main findings suggest that while caffeine ingestion increased saliva caffeine concentration in a dose-dependent manner, s-IgA concentration, secretion rate and saliva flow rate remained largely unaffected by 2-8 mg·kg<sup>-1</sup> BM doses of caffeine ingested 1 h both at rest and after a strenuous 70 min bout of running at 80%  $\dot{V}O_{2peak}$ . In addition, salivary  $\alpha$ -amylase activity appeared to follow a dose-response effect, increasing with each caffeine dose up to 6CAF, above which 8CAF was no different to PLA.

**Saliva IgA:** Secretion of s-IgA in rats is reported to be significantly affected by sympathetic stimulation (Carpenter et al., 1998, 2000), and that effects of stimulation may be dose dependent, with higher frequencies of nerve stimulation resulting in higher secretion rates of s-IgA (Proctor et al., 2003). Using this hypothesis, it was anticipated that caffeine ingestion, in conjunction with exercise might further enhance secretion of s-IgA. In addition, it was also expected that caffeine might exert a dose-response effect on s-IgA regulation, with higher doses resulting in greater increases in s-IgA.

Despite exercise and caffeine being potent stimulators of sympathetic activity (Kastello et al., 1993) findings from the present study however do not support these reports since s-IgA concentration and secretion rates were unaffected by exercise as well as caffeine ingestion (Table 9), with no dose-response effect apparent. Findings from the present study however, support those of Graham and Spriet, (1995) and Pasman et al., (1995) who reported no dose-response effect of caffeine on sports performance, and it appears that this may be the same for immune responses. Findings by Fletcher and Bishop (2011) support this suggestion, reporting no dose-response effects of caffeine ingestion of 2 and 6 mg·kg<sup>-1</sup> BM on antigen-stimulated NK cell activation following 90 min cycling at 70%  $\dot{V}O_{2peak}$ . They found that caffeine doses of both 2 and 6 mg·kg<sup>-1</sup> BM elicited similar increases in antigen-stimulated NK cell activation 1 h post-exercise. In addition, whilst studies in the rat model consistently show *in vivo* sympathetic stimulation to increase s-IgA secretion (Carpenter et al., 1998; Proctor et al., 2003), a recent exercise study in rats by Kimura et al., (2008) found exercise to exhaustion to decrease s-IgA secretion by up to 70% in rats, bringing to question the appropriateness of artificial stimulation even when administered at frequencies likely to occur during acute high-intensity exercise. Consequently it may be

that s-IgA responses in the rat model, in particular responses to *in vitro* stimulation, may not be appropriate for comparisons in humans.

The findings of this study are also in contrast to those from the only other caffeine, exercise and s-IgA study in humans (Bishop et al., 2006) despite saliva caffeine concentrations being similar. It is possible however that findings from the present study were different to Bishop et al., due to testing being conducted at different times of the day (12:00 h compared to 10:00 h in Bishop et al., (2006)). As we did not conduct a resting trial, timings were chosen upon recommendations by Gleeson et al., (2001) who reported that s-IgA was most consistent from 12:00 h onwards. However, this is an inherent limitation to our study. Bishop et al., (2006) found that a 6 mg·kg<sup>-1</sup> BM dose of caffeine ingested 1 h before 90 min of cycle exercise at 70%  $\dot{V}O_{2peak}$  increased s-IgA concentration from pre- to post-exercise, but only increased s-IgA secretion from pre- to mid-exercise, with values returning to baseline by the end of exercise. Whilst Bishop et al., (2006) only showed a transient increase in s-IgA, it is possible that findings from our study were different due to disparities in exercise protocols employed. The exercise protocols used by Bishop et al., (2006) were quite different to that employed in the present study, with a different mode (cycling v running), duration (90 min v 70 min), and intensity (70% v 80%  $\dot{V}O_{2peak}$ ) of exercise. Physiologically, mean heart rates were 10 bpm higher (166 v 157) and mean energy expenditure also 10 kJ·min<sup>-1</sup> higher (75 v 67) in our study compared to Bishop et al., (2006) (Table 1). Likewise other studies using similar exercise protocols to Bishop et al., (2006) (2 h cycle at 60-70%  $\dot{V}O_{2peak}$ ) have reported lower mean heart rates (~140 bpm) in conjunction with either no change, or increases s-IgA concentration and secretion (Allgrove et al., 2009; Bishop et al., 2000; Li & Gleeson, 2004). However, it is difficult to compare effects of exercise protocols between studies as most report limited physiological and perceptual data (most often HR, % $\dot{V}O_{2peak}$  and RPE). Comparisons between findings from Bishop et al., (2006) study may also be limited in that a cotton swab under the tongue was used for saliva collection which 1) has the potential to increase saliva acidity and give false concentrations of saliva components (Granger et al., 1994; Strazdins et al., 2005) and 2) has the potential to modify saliva composition by either preferentially stimulating specific saliva glands, or resulting in the saliva collected being primarily from the nearby glands. Despite these limitations, the findings from the present study indicate that 70 min of treadmill running at 80%  $\dot{V}O_{2peak}$  has little effect on s-IgA, and consequently, it seems that exercise must be of a higher intensity or longer duration to induce a suppression of s-IgA.

***α-amylase:*** Numerous researchers have shown *α*-amylase activity to increase during both psychological (Bosch et al., 1998; Nater et al., 2005; Takai et al., 2007) and physiological (Bishop et al., 2006; Chatterton et al., 1996; Walsh, 1999) states of stress, and as such it is considered a good indicator of enhanced sympathetic activity (Nater & Rohleder, 2009). So, although plasma adrenaline concentrations were not directly determined in the present study, we measured *α*-amylase as a surrogate marker for sympathetic nervous system activity. Likewise, *α*-amylase was found to increase in the present study and was affected by both exercise and caffeine ingestion. At rest, caffeine doses of 2-6 mg·kg<sup>-1</sup> BM appear to result in considerable but non-significant increases (30-55%, Table 9) in *α*-amylase activity. In addition, exercise appeared to further augment *α*-amylase activity, with post-exercise measures being up to 2 fold higher than pre-exercise (main effect of time *P* < 0.01). Changes in *α*-amylase activity with exercise however were similar between trials suggesting that exercise may have exerted greater effects on *α*-amylase activity than caffeine ingestion alone. These findings are supported by Bishop et al., (2006) who reported increases in *α*-amylase activity at rest with caffeine ingestion of 6 mg·kg<sup>-1</sup> BM, and further increases following 90 min cycling at 70%  $\dot{V}O_{2peak}$ . Interestingly, while doses of 2-6 mg·kg<sup>-1</sup> BM caffeine in the present study increased *α*-amylase activity above that of placebo they were also significantly higher than values found for the 8 mg·kg<sup>-1</sup> BM dose which might suggest that a dose-response only occurs up to 6 mg·kg<sup>-1</sup> BM, above which *α*-amylase activity may be down-regulated. These findings may be explained by Graham and Spriet (1995), who suggested that hepatic caffeine metabolism may be saturated with doses higher than 6 mg·kg<sup>-1</sup> BM, which may have limited any further effects of an 8 mg·kg<sup>-1</sup> BM caffeine dose on *α*-amylase activity. It is important to note however that the effects observed in the present study were short-lived (close to pre-supplement by 1 h post for PLA, 2CAF and 4CAF) and suggests that while both exercise and caffeine ingestion increase *α*-amylase activity separately, these increases are further augmented when caffeine and exercise are combined

***Saliva flow rate and osmolality:*** Saliva flow rate is often reported to decrease following exercise of 1-2 h, and intensities ranging from 60-75%  $\dot{V}O_{2peak}$  (Bishop et al., 2006; Laing et al., 2005; Li & Gleeson, 2004; Usui et al., 2011; Walsh et al., 2002), however this was not seen in the present study. Saliva secretion is primarily under the control of the autonomic nervous system, with saliva glands innervated by both sympathetic and parasympathetic nerves (Chicharro et al., 1998). While saliva secretion is principally regulated by parasympathetic mechanisms (Proctor & Carpenter, 2007), sympathetic activation is also

thought to affect saliva secretion via vasoconstrictive mechanisms, which would help to explain decreases in saliva flow rate with exercise. Using this hypothesis it could be speculated that caffeine ingestion might have exacerbated any decreases in saliva flow rate with exercise, however this was not seen in the present study, or by Bishop et al, (2006) who used a  $6 \text{ mg}\cdot\text{kg}^{-1}$  BM dose. Consequently, it is suggested that researchers investigate parasympathetic responses to exercise to directly determine if or how parasympathetic withdrawal affects saliva responses with exercise.

Saliva secretion has also been reported to be negatively affected by dehydration which is said to occur after losses of 2-3% BM (Walsh et al., 2004). While participants in the present study lost  $\sim 2.5\%$  BM, which could indicate mild dehydration (Saltin & Costill, 1988), saliva flow rate was unaffected by the protocols in this study. Saliva osmolality has also been proposed as an indicator of dehydration, with studies reporting increases in osmolality in conjunction with decreases in BM and decreased saliva flow rate following strenuous exercise protocols (Allgrove et al., 2009; Davison et al., 2009; Laing et al., 2005; Walsh et al., 2002). However, whilst saliva osmolality increased in this study (regardless of trial), this was not accompanied with a typical decrease in saliva flow rate. It is possible that the fluid ingested by participants during exercise ( $2 \text{ ml}\cdot\text{kg}^{-1}$  BM every 15 min) was enough to prevent a decrease in saliva flow rate, which is similar to findings by Bishop et al., (2000) and Walsh et al., (2004) who also controlled fluid intake during exercise. However this does not explain why osmolality increased with exercise, or why Allgrove et al., (2009) found decreases in saliva flow rate in conjunction with increased osmolality in the absence of any significant loss in body mass (0.5%). Consequently it appears that further research is needed to elucidate saliva responses to exercise and relationships with dehydration and osmolality.

**Individual variability:** While data presented in this study are representative of the group mean it is apparent that mucosal responses to exercise are quite variable. This is highlighted in Figures 7 and 8, where two participants' individual s-IgA secretion rates for each trial are illustrated. If longitudinal studies are considered, variability in individual s-IgA responses is evident. Recently, Neville et al., (2008) measured weekly resting s-IgA in professional yachtsmen over a period of 50 weeks and highlighted the variability of s-IgA, reporting within subject variation to be 48% (CV) and between subject variations to be 71%. Francis, Gleeson, Pyne, Callister and Clancy (2005) also highlighted this variability when comparing the variations in s-IgA between elite swimmers and active and sedentary

participants. Francis found s-IgA measures to be more variable in elite athletes (47%) compared to active (23%) and sedentary (27%) individuals. Consequently it is recommended that any future studies investigating mucosal immune function/responses to exercise in highly trained athletes should ensure adequate measurement of resting s-IgA to establish baseline s-IgA levels for each individual. In addition, the test-retest reliability for any protocol should also be established.

In summary the findings of the present study suggest that ingesting doses of  $2\text{--}8\text{ mg}\cdot\text{kg}^{-1}$  BM of caffeine 1 h before prolonged high-intensity exercise has no effect on saliva IgA concentration and secretion rate, or saliva flow rate. Caffeine ingestion of  $2\text{--}6\text{ mg}\cdot\text{kg}^{-1}$  BM but not  $8\text{ mg}\cdot\text{kg}^{-1}$  BM however, was associated with increases in  $\alpha$ -amylase activity, suggesting a dose-response effect of caffeine on  $\alpha$ -amylase, and possibly an inhibitory effect with doses above  $6\text{ mg}\cdot\text{kg}^{-1}$  BM.

### **3.6 Perspectives**

The present study indicates that ingestion of  $2\text{--}8\text{ mg}\cdot\text{kg}^{-1}$  BM caffeine has neither a positive nor detrimental effect on saliva IgA or saliva flow rate, whilst simultaneously increasing  $\alpha$ -amylase activity. Based on this it is suggested that athletes could ingest up to  $8\text{ mg}\cdot\text{kg}^{-1}$  BM of caffeine with no detrimental effect on s-IgA. However, since higher doses of caffeine have been associated with negative side effects on health and performance, as well as increases in adrenaline, which may have immunosuppressive effects on other immune markers, we recommend that athletes consume the smallest beneficial dose so as to avoid these potential side-effects.

## **Chapter Four: General Discussion**

### **4.1 Overview**

Since the key findings of this study and their implications were discussed in the previous chapter the General Discussion will explore some remaining issues in exercise and s-IgA research that may be limiting advancement in our understanding of this area. Consequently the General Discussion will examine the potential impact exercise mode, participant characteristics and individual responses may have on findings in mucosal exercise immunology research. In addition, strengths and limitations of the present study will be discussed, and recommendations for future research will also be made.

### **4.2 Remaining issues in exercise immunology research**

#### **4.2.1 Mode of exercise**

To date the majority of experimental research has looked at the effects of cycle exercise on s-IgA, with only nine studies using treadmill or running exercise (Housh et al., 1991; Luna Jr et al., 2011; McDowell et al., 1991; McDowell et al., 1992; Mylona et al., 2002; Pacque et al., 2002; Ricardo et al., 2009; Sari-Sarraf et al., 2006; Sari-Sarraf et al., 2007). While it is perhaps understandable as to why researchers would choose cycle exercise over running (ease of collection of physiological data, less chance of injury or risk with cycling compared to running) it is interesting that this mode of exercise is used so frequently considering findings from running would be more applicable to a wider range of sports, both individual and team, compared to cycling, which we feel is a strength of our study.

However, issues pertaining to the effect of exercise mode on s-IgA responses to exercise possibly lie in the differences in physiological demands of different modes of exercise. While cycling is primarily a lower body, non-weight bearing exercise that contains almost no eccentric component, running is whole body, weight-bearing exercise and involves both concentric and eccentric contractions. Consequently it is possible that running exercise may result in different stress and inflammatory responses to exercise, which could also affect immune responses to exercise. Indeed, exercise mode (cycle v treadmill) has been shown to affect substrate utilisation, with fat oxidation rates being up to 28% higher in treadmill exercise compared to cycling (Achten, Venables, & Jeukendrup, 2003; Knechtle et al., 2004). Subsequently, it is possible that using a different mode of exercise in our study could have affected participants' metabolism and therefore metabolism of caffeine. This

may explain why Bishop et al., (2006) found a transient increase in s-IgA with a 6 mg·kg<sup>-1</sup> BM dose, and why we saw no change in s-IgA regardless of dose. However, as no research to date has directly compared the effects of different modes of exercise on immune responses this is purely speculative.

Consequently, a potential limitation of this study is not knowing how stressful/demanding a 70 min run at 80%  $\dot{V}O_{2peak}$  is on the body in comparison to a 90 min cycle at 70%  $\dot{V}O_{2peak}$  as used by Bishop et al., (2006), or 2 h of cycling at intensities between 55-75%  $\dot{V}O_{2peak}$  used in other studies (Davison et al., 2009; Mackinnon et al., 1989) and it may therefore be inappropriate to compare our findings to those from other studies. The comparability of findings is further limited as previous studies have usually only reported HR, RPE and %  $\dot{V}O_{2peak}$ , which does little to illustrate the physiological demands of their exercise protocols. Subsequently it is recommended that future studies include more detailed information (such as energy expenditure, fat and carbohydrate oxidation) regarding the physiological demands of their exercise protocols to allow for easier comparison. In addition, it would be interesting if future studies were to compare the effects of different exercise protocols on both cycle and treadmill exercise to establish whether s-IgA responses differ with mode of exercise.

#### **4.2.2 Participant characteristics**

Differences in participant characteristics used in studies may also contribute to disparities in research findings. A review by Gleeson (2004) has highlighted this, discussing the potential for different outcomes to be related to age, gender or fitness level.

It is generally accepted that immune function changes with age (Makinodan & Kay, 1980; Mazzeo, 2000; Miller, 1991); as such, comparing studies that have a heterogeneity in participant ages may be inappropriate. Unfortunately the effects of a wide range of ages on immune function are yet to be determined, as research in this area has usually been conducted in either young (adolescent) or elderly populations (Fiatarone et al., 1989; Nieman et al., 1993). One group however have suggested that immune function may be less affected by age if participants maintain a healthy and active lifestyle (Kastello et al., 1993), although this has yet to be confirmed. Thus, it is possible that findings from the present study may be less comparable to those in other studies due to the mean age in this study being approximately ~5 years older than that in many experimental studies (Allgrove et al., 2009; Bishop et al., 2000, 2006; Usui et al., 2011).

Gender differences in immune function, or the immune response to exercise has had little attention in research. To the author's knowledge only two studies have investigated gender differences in exercise and mucosal immunity studies (Allgrove, Geneen, Latif, & Gleeson, 2009; Gleeson, Bishop, Oliveira, McCauley, & Tauler, 2011). Currently their findings suggest that females exhibit lower s-IgA concentration and secretion rates compared to males. Gender effects on saliva flow rate however are debatable, with Allgrove et al., (2009) finding no differences in flow rate, but Gleeson et al., (2011) finding flow rate to be lower in females than males. Accordingly it appears that it may be inappropriate to generalise findings from this study to the female population.

Differences in participant fitness levels have also been suggested to be responsible for some discrepant findings in s-IgA and exercise studies (Gleeson, 2004). Indeed if Malm's (2006) model for risk of infection is considered (Figure 2), then it is likely that elite or very well trained athletes may exhibit different immune responses to exercise. In addition, it is also anticipated that athletes of a higher fitness level may be better able to cope with strenuous demands of some exercise protocols employed, and may therefore have a dampened stress and/or immune response to exercise compared to less fit individuals (Malm, 2006; Mastorakos, Pavlatou, Diamanti-Kandarakis, & Chrousos, 2005). Based on this hypothesis, it is possible that the higher fitness level of the participants in the present study may explain why s-IgA remained unchanged following a 70 min run at 80%  $\dot{V}O_{2peak}$  which was expected to stress participants physiologically. Since most experimental studies use a homogenous population of similar ages and fitness levels, it would be interesting to investigate the s-IgA responses to exercise in participants of a wide range of fitness levels.

#### **4.2.3 Individual responses to exercise, caffeine and basal s-IgA**

The need for reporting or measuring individual immune responses to exercise has been highlighted in the present study with a number of measures, when expressed as a group mean, exhibiting standard deviations greater than the mean. This issue has been highlighted in recent research by Neville et al., (2008), who reported large within (48%) and between (71%) subject variations in resting s-IgA in professional yachtsmen, as well as Francis et al., (2005) who found s-IgA variability to be greater in elite swimmers (47%) compared to active (23%) and sedentary (28%) participants. This is disconcerting, considering a number of studies in exercise immunology literature have used elite or well-trained participants, which could bring into question the reliability of their data. As such, it may be that larger sample sizes are needed for acute exercise studies, or, that additional

measures or trials should be taken to ensure resting or baseline s-IgA values determined are actually representative, and that the re-test reliability of protocols is appropriate.

Individual differences were also highlighted in the present study with some participants' s-IgA responses following a specific pattern of change, and others showing the opposite (Figure 7). It is therefore important to consider not only within- and between-subject variation in measures, but also the possibility of differences in the ways in which participants respond to exercise (which could relate to their training status, age, gender etc) or caffeine ingestion. Recent research has indicated that genetic make-up may play a role in individual variability in caffeine consumption and responses to caffeine (Yang, Palmer, & de Wit, 2010). Consequently, it is possible that differences in patterns of change of immune measures (Figure 7) could be related to differences in individual pharmacodynamics (drug receptor) and pharmacokinetic (metabolism) responses to caffeine ingestion.

#### **4.3 Strengths and Limitations**

In the present study numerous measures were taken to attempt to control any possible variables that could affect s-IgA responses to testing. Nutritional status was controlled as much as possible with participants consuming the same foods in the 24 h prior to testing, as well as eating the same breakfast before each trial, of which the nutritional content was also standardised for all participants. We feel that this is a strength of our study as a lack of reporting or controlling of nutritional status of participants has been criticised in the past (Gleeson 2004). While it is ideal to control variables as much as possible, this can also limit the applicability of study findings to real life. However, we feel that this study was quite realistic due to the range of caffeine doses trialed, as well as time of the fasting period, which may be similar to what athletes may use in real life. There are, however, several limitations to this study that could be addressed in future research:

- The absence of a resting trial could be considered a weakness of the present study. Some researchers have reported diurnal variations in s-IgA and saliva flow rate (Chicharro et al., 1998; Dimitriou et al., 2002; Gleeson, Bishop, Sterne, & Hawkins, 2001), however a more recent study by Bishop et al., (2006) found no diurnal variation in s-IgA, amylase or saliva flow rate in resting participants. Since we had our participants exercise after a small fasting period, rather than an overnight fast employed by numerous researchers, we decided that it would be impractical to

conduct testing at the same time points as Bishop et al., (2006). Consequently, we chose our testing time points by following recommendations by Gleeson et al., (2001), who reported s-IgA to be stable from 12:00 h onwards.

- With no resting trial, we were unable to determine the effects of caffeine ingestion on our mucosal measures at rest. Consequently we were unable to demonstrate whether caffeine ingestion of a range of doses had the same effects on mucosal secretions at rest as those reported by Bishop et al., (2006).
- $\alpha$ -amylase was used as a surrogate marker of adrenaline. It is therefore not possible to know if increases in amylase actually reflected increases in adrenaline as well as whether adrenaline was affected in a dose-response manner. This also means we were unable to determine if adrenaline remained unaffected by the smallest dose (2 mg·kg<sup>-1</sup> BM), as has been demonstrated in previous research (Graham & Spriet, 1995), or if there was a limit in dosing, above which adrenaline was no longer increased.
- A review by Gleeson (2004) has criticized studies for not measuring any potential psychological or environmental influences on immune function. As these were not addressed in the present study, it is not possible to tell if there were any external influences that may have affected immune responses.

#### **4.4 Future directions**

The mechanisms underpinning how caffeine affects mucosal responses to exercise remain unclear. It is speculated that caffeine may exert its effects via stimulation of the sympathetic nervous system and increases in adrenaline, however this has not yet been investigated directly. In addition, whilst caffeine appeared to have no positive or negative effects on saliva IgA or flow rate, it is not clear if the same response would be seen in other mucosal immune markers. In addition, it is possible that caffeine at doses used in this study could have substantial effects on immune markers in systemic immunity, which could dampen any positive or negative responses in mucosal immunity. This brings to question the appropriateness of investigating the effect of exercise or supplements on one or two immune markers, because the immune response to infection is multifactorial. Consequently it is recommended that future research takes a multi-factorial approach and examines several markers of immune function in order to provide an overall indication of immune responses to exercise and interventions. In addition, the biological significance of temporary decreases in immune markers is also yet to be determined, and subsequently research is needed in this area to determine whether a short-term depression of immune function has any impact of susceptibility to infection. Future research also needs to explore immune responses to a range of individual and team sport activities so that findings may be more transferable to a wider range of sports and population groups.

#### **4.5 Implications of this thesis**

The findings of this thesis suggest that ingesting 2-8 mg·kg<sup>-1</sup> BM caffeine 1 h before prolonged intensive exercise had no dose-response effect on s-IgA or saliva flow rate. Whilst caffeine appeared to have no influence on s-IgA following exercise, other studies discussed in this thesis have demonstrated that doses of 2 and 6 mg·kg<sup>-1</sup> BM may be immunomodulatory, with some systemic immune cells showing an immunosuppression, while others were immunostimulatory.

While longitudinal studies have found associations between decreases in secretion of s-IgA and incidence of URTI, the clinical significance of any short-term suppression in s-IgA experienced following acute bouts of exercise is unknown. In addition, although studies investigating the effects of exercise on s-IgA often report a decrease in s-IgA concentration or secretion rate following intensive or prolonged exercise the findings of this thesis do not support these findings, again demonstrating the complex issue of mucosal immunity in exercise.

Based on the findings of this thesis, athletes may take doses of caffeine up to 8 mg·kg<sup>-1</sup> BM caffeine 1 h prior to training or competition with no adverse effects on the main effector measure of the mucosal immune system (saliva IgA). However, due to the potential side effects of ingesting large doses of caffeine and possible decrements in performance discussed in this thesis, and the known caffeine habits of athletes, it is suggested that athletes consume the smallest dose of caffeine possible that will still enhance performance.

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## Appendices

## Consent to Participation in Research

**Project Title:** Dose responses to caffeine ingestion on salivary IgA following prolonged exercise.

**Project Supervisors:** Dr. Deborah Fletcher, and Associate Professor Andrew Kilding.

**Researcher:** Chloe Gibson

- 
- I have read and understood the information provided about this research project (Information Sheet dated 31<sup>st</sup> May 2011). Yes/No
  - I have had an opportunity to ask questions and to have them answered. Yes/No
  - I am in good health and am not currently suffering from any injury or illness which may impair my physical performance Yes/No
  - I agree to provide saliva samples Yes/No
  - I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way. Yes/No
  - If I withdraw, I understand that all relevant information will be destroyed. Yes/No
  - I agree to take part in this research. Yes/No
  - I wish to have all samples of saliva belonging to me, returned to me at the end of the study:  
tick one: Yes ☐ No ☐
  - I wish to receive a copy of the report from the research: tick one: Yes ☐ No ☐

Participant signature: \_\_\_\_\_

Participant name: \_\_\_\_\_

Date: \_\_\_\_\_

Participant's Contact Details:

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**Project Supervisor Contact Details:**

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**Approved by the Auckland University of Technology Ethics Committee Date 17.05.11**

## Appendix B

### 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 ☐ NO ☐
  - (b) attending your general practitioner YES ☐ NO ☐
  - (c) on a hospital waiting list YES ☐ NO ☐
  
2. **In the past two years**, have you had any illness which require you to:
  - (a) consult your GP YES ☐ NO ☐
  - (b) attend a hospital outpatient department YES ☐ NO ☐
  - (c) be admitted to hospital YES ☐ NO ☐
  
3. **Have you ever** had any of the following:
  - (a) Convulsions/epilepsy YES ☐ NO ☐
  - (b) Asthma YES ☐ NO ☐
  - (c) Eczema YES ☐ NO ☐
  - (d) Diabetes YES ☐ NO ☐
  - (e) A Blood disorder YES ☐ NO ☐
  - (f) Head injury YES ☐ NO ☐
  - (g) Digestive problems YES ☐ NO ☐
  - (h) Heart problems YES ☐ NO ☐
  - (i) Problems with bones or joints YES ☐ NO ☐
  - (j) Disturbance of balance/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)** \_\_\_\_\_

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5. **Have you** had a cold or feverish illness in the past month? YES ☐ NO ☐
6. **Are you** accustomed to vigorous exercise (1-3 hours per week)? YES ☐ NO ☐

**Thank you for your participation.**

## Physical Activity Questionnaire

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

Name: \_\_\_\_\_

Date: \_\_\_\_\_

Are you currently **Endurance Training**? YES ☐ NO ☐

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

How many training sessions do you have a day? \_\_\_\_\_

How many minutes does each session last? \_\_\_\_\_

What is your weekly mileage? \_\_\_\_\_

Please break this down according to discipline. E.g. cycle, swim, run, other: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Are you involved in any of the following training programmes?

Weight training ☐

Interval training ☐

Skills training ☐

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

Weight training: \_\_\_\_\_

Interval training: \_\_\_\_\_

Skills training: \_\_\_\_\_

How many minutes does each session last?

Weight training: \_\_\_\_\_

Interval training: \_\_\_\_\_

Skills training: \_\_\_\_\_

## Caffeine Consumption Questionnaire

Please complete the questionnaire concerning your caffeine usage.

List the number of times you consume the following substances during a typical day. Please also specify the product you use, E.G. Tea – PG tips.

	Morning	Midday	Afternoon	Evening
<b>COFFEE</b> (Regular 250 ml. Mugs)				
Regular				
Brewed				
Percolated				
Instant				
Decaffeinated				
Espresso (60 ml. Serving)				
<b>TEA</b> (Regular 250 ml. mugs)				
Hot Chocolate / Mocha (Regular 250 ml. mugs)				
Chocolate (Regular Bar)				
Chocolate or Coffee Biscuits /Cakes / Cereals				
Chocolate / Coffee Dairy Products				
<b>SOFT DRINKS</b> (330ml Can Size)				
Coke				
Pepsi				
Energy Drinks (Please state which ones)				
<b>OVER THE COUNTER DRUGS</b>				
Pain Killers e.g. Panadol Extra (Please state type)				
Pro Plus				

Do you take caffeine prior to exercise? \_\_\_\_\_

If **Yes**, How much? \_\_\_\_\_

What do you take? \_\_\_\_\_

Why? \_\_\_\_\_