CHICHESTER INSTITUTE OF HIGHER EDUCATION
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UNIVERSITY OF SOUTHAMPTON

THE USE OF SALIVARY IMMUNOGLOBULIN-A AS A MARKER OF EXERCISE-INDUCED IMMUNOMODULATION.

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Thesis submitted for the
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This thesis has been completed as a requirement for
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ABSTRACT

FACULTY OF SCIENCE

Doctor of Philosophy

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Attempts have been made to account for the increased propensity of upper respiratory tract infections (URTI) in regularly training individuals, by focusing on the effect of exercise on the primary mucosal immunoglobulin secretory immunoglobulin A (sIgA). However, the pathophysiological significance of observed changes in sIgA levels remains unclear, as indeed does the existence of increased URTI in athletes.

The aim of this work was to examine the use of sIgA as a marker of exercise-induced immunomodulation and its possible role in protection against URTI. Before these questions could be addressed the optimum method for saliva collection, and the ideal terms for expression of sIgA levels were investigated. Experimental data advocate the use of the passive dribbling method above the use of salivettes. Salivettes were found to result in an underestimation of both saliva flow rate and protein concentrations as a consequence of limited absorption (3 ± 1ml) and variable retention of the sample (49.1 ± 24.9%).

Secretory IgA levels are commonly expressed in terms of secretion rate which is the product of saliva flow rate and sIgA concentration. Examination of the relative contribution of these two factors to secretion rate revealed that exercise-induced changes in saliva flow rate (-51%) matched the changes in secretion rate (-51%), whereas changes in sIgA concentrations (+4%) did not. It was concluded that changes in saliva flow rates have an important role with the occurrence of symptoms associated with URTI whether induced by infective or inflammatory factors.

Epidemiological data from others on the incidence of symptoms associated with URTI in marathon runners have provided evidence on the incidence of URTI in athletes. However here, reported symptoms associated with URTI were most common during the race suggesting that an infective agent was not involved. Examination of the effect of marathon running revealed a non-significant decrease in saliva flow rate (-27.7 ± 15.8%).

A final study investigated the effect of increased ambient temperature, and the possibility of fluid replacement as a intervention strategy against exercise-induced decreases in saliva flow rate. This study revealed that exercise reduced saliva flow rate exercise in the heat exacerbated this and that fluid replacement tempered the exercise-induced decrease. Changes in saliva flow rate were found to be associated with changes in plasma volume.

The overall conclusions of this thesis are that innate defence mechanisms such as saliva have a role to play in conferring defence against potential pathogens, and therefore warrant further investigation. It appears from data presented in this thesis that saliva flow rate is affected by exercise, perhaps to a greater extent than sIgA concentration. Changes in saliva flow rate with exercise may have a role to play in the purported increased incidence of URTI reported by athletes, and fluid replacement may provide an effective strategy against this exercise-induced decrease.

LIST OF CONFERENCE PAPERS

Ford JA, Trevatt N, Dix CE (1997) Th effect of of fluid replacement anf heat on salivary flow rate and optical denisty at 280nm in response to exercise. *Journal of Sports Sciences* 15 p49

Hemmings B, Ford JA, Graydon J and McMorris T (1997) The effects of interval amateur boxing training on salivary immunoglobulin A. *Journal of Sports Sciences* 15 p53

Ford JA, Wood DM and Graydon JK (1998) Saliva flow rate is influenced by saliva collection method. *Proceedings of the Third Annual Congress of the European College of Sports Science.*

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After four years of reading, writing and researching the most important thing that I have learnt is the power and value of friendship.

LIST OF ACRONYMS

ACRONYM	DEFINITION
ACTH	Adrenocorticotrophin Hormone
ANS	Autonomic nervous system
CNS	Central nervous system
CRF	Corticotrophin releasing factor
CSF	Colony stimulating factor
HPAC	Hypothalamic-Pituitary-Adrenocortical axis
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
NK cells	Natural Killer cells
PNEI	Psychoneuroendocrinoimmunology
POMC	Pro-opiomelanocorticotrophin derived polypepetides
POMS	Profile of Mood States
S.E.M	Standard error of the mean
SAM	Sympathetic adrenomedullary axis
sIgA	Secretory/salivary Immunoglobulin A
SIgM	Secretory/salivary Immunoglobulin M
TNF	Tumor Necrosis Factor
URTI	Upper Respiratory Tract Infection
VIP	Vasoactive Intestinal Polypeptide

PART

I

CHAPTER 1

INTRODUCTION

Within our society there is a general belief that regular exercise improves health and well being, and participation in sports and exercise programmes is encouraged (Hickson and Boone, 1991). However, studies have suggested that athletes who are regularly training and competing may have a higher susceptibility to infections than moderately active individuals (Fitzgerald, 1988; 1991). It appears that there may be an individual threshold at which exercise ceases to be beneficial and becomes detrimental to health, possibly increasing vulnerability to pathogens.

That there are conflicting ideas about the effects of exercise on susceptibility to infection has been recognised for some time. In the 1920s it was noted that a fatigued individual was more likely to succumb to illness (Oppenheimer and Spaeth, 1922, cited in Cannon, 1993), yet conversely that exercise improved resistance to infection (Spaeth, 1925, cited in Cannon, 1993). A decade earlier, Bernard MacFadden (Encyclopaedia of Physical Culture, 1911, cited in Nash, 1994) had advocated moderation when exercising, having recognised that excess could impede health. Nearly ninety years later the effects of exercise on health remain controversial.

The influence of exercise on susceptibility to infections has been the subject of many recent studies and reviews (e.g. Brenner, Shek and Shephard, 1994; Nieman, 1994b; Weidner, 1994). It has been reported that physically active individuals perceive themselves as more resistant to infection than more sedentary individuals (Brenner et al., 1994; Mackinnon, 1992; Schouten, Verschuur and Kemper, 1988a). Yet, contrary to this belief, there actually appears to be a higher susceptibility in athletes to certain infections than in the general population (Weidner, 1994). It is apparent from both anecdotal and research data that individuals in regular training are particularly prone to develop upper respiratory tract infections (URTI) (Douglas and Hanson, 1978; Heath, Ford, Craven, Macera, Jackson and Pate, 1991; Nieman, 1995).

It has been suggested that the immune system could be a factor limiting sporting performance (Fitzgerald, 1988). Infection may limit both exercise capacity and long term adaptation to exercise training (Daniels, Vogel, Sharp, Friman, Wright, Biesel and Knapik,

1985; Friman, 1977; Woodruff, 1980). The impact of infection on training and competition has aroused a great deal of interest. The primary mechanism mediating this susceptibility to infection is believed to be stress-induced immunosuppression (Brenner et al., 1994). There have been investigations into the effects of both acute and chronic exercise on markers of specific immune functioning and susceptibility to infection (Lewicki, Tchorzewski, Majewska, Nowak and Baj, 1988; Linde, 1987; Mackinnon, 1988; Nehlsen-Canneralla et al., 1991b; Nieman et al., 1990b; Keast, Cameron, Morton, 1988). Several studies have reported changes in blood immune parameters in response to exercise (Baj, et al., 1994; Lewicki et al., 1988; Nehlsen-Cannarella, et al., 1991b), however mucosal immunity is the primary defence against URTI. The principal mucosal antibody is secretory immunogloblin A (sIgA) (Tomasi, Tan Solomon and Pendergast, 1965), and attempts to forge a link between changes in immune status, exercise and URTI have focused upon sIgA (Mackinnon, Chick, van As and Tomasi, 1989; Tharp and Barnes, 1990). A link between decreased sIgA levels and URTI has been demonstrated (Mackinnon, Ginn and Seymour, 1993c), however, there are several other mechanisms which protect the respiratory mucosa from infection including cilia, mucins, and saliva. Many authors have assumed that sIgA concentration or secretion is the most relevant measure for URTI susceptibility, without consideration of the numerous other defence mechanisms (MacDowell, Hughes, Hughes, Housh and Johnson, 1992a, b; Schouten et al., 1988a; Tharp, 1991). There is a need for other mechanisms to be taken into account along with sIgA, and for these markers to be related to the incidence of infection, before any intervention or preventative strategies can be implemented.

It has been demonstrated that stress (both physical and psychological) modulates immune functioning (McClelland, Ross and Patel, 1985; Pedersen, 1991; Pedersen, Kappel, Klokker, Nielsen and Secher, 1994; Totman, Kiff, Reed and Craig, 1980). Links between the psyche and illness have been made since ancient times; for instance Galen observed in the second century AD that "melancholy" women were more prone to illness than sanguine women (Hillhouse, Kiecolt-Glaser and Glaser, 1991). However, it is only since the early 1980's that links between the mind/psyche and physiological control systems have been established. At a similar time evidence was collated to support the argument that the immune system was not autonomous (Ader, 1981; Ader, Felten and Cohen 1991). These discoveries have given birth to the area of psychoneuroendocrinoimmunology (PNEI), which considers the complex interactions between the psyche, nervous, endocrine,

and immune systems. The study of the manifestation of these interactions in the immune system of athletes has been a very rapidly growing area within sports science. The combination of physical, psychosocial, emotional and environmental stress experienced by athletes is unique; few other people experience a combination of these stressors on a regular basis (Mackinnon and Jenkins, 1993), which may be the reason why athletes appear to be more susceptible than the general population to URTI.

However, despite the large and growing body of data resulting from investigations of the effect of exercise on PNEI interactions, few conclusions have been drawn to explain the clinical significance of these findings. This is probably because of a lack of consistency between studies regarding subjects, parameters and methods. Current literature details the effects of a variety of sporting activities on immune functioning in a very broad range of individuals (Baj et al., 1994; Nieman, Johanssen and Lee, 1989; Oshida, Yamanouchi, Haymizu and Sato 1988), but there is a lack of consideration of the effects of such factors as exercise modality, task familiarisation, training status, and health history, which may potentially mediate exercise-induced immunomodulation. Mechanisms mediating susceptibility to infection may then be elucidated by relating changes in psychological, hormonal and immune status to the incidence of infection. Inconsistency in the methods used between studies, and the lack of an holistic approach has meant that the affective interactions between exercise, immunity and infection have not yet been established.

If salivary immunoglobulin A is to be considered as the principal mucosal defence mechanism against URTI, then surely standardisation of the collection and measurement of saliva for the measurement of salivary immunoglobulin A is necessary. Currently there is very little consistency between authors in how saliva is collected; and both whole (Mackinnon and Jenkins, 1993) or parotid (Tomasi, Trudeau, Czerwinski and Erredge, 1982) saliva has been used. Stimulation of saliva flow has been used by some authors (Schouten et al., 1988a) but not by others (MacDowell et al., 1992a). The saliva collection method used has been shown to influence the results gained for sIgA levels (Aufricht et al., 1992) suggesting that results gained from samples collected by different methods are not comparable. Salivary IgA levels have been reported in terms of secretion rate (Mackinnon, Ginn and Seymour, 1993b), concentration (MacDowell et al., 1992 a, b), and corrected for total protein (Mackinnon and Hooper, 1994), the comparability of these terms remaining unclear. Salivary IgA, in terms of secretion rate, takes into account the mobility of the

immunoglobulins around the oral cavity, and is very commonly used. Calculation of secretion rate takes into account saliva flow rate equally with sIgA concentration, so that any observed change in sIgA secretion rates will be the product of changes in both saliva flow rate and sIgA concentration. There is therefore, a need to explicate the impact of exercise on saliva flow rate and the concurrent effect of that upon calculated sIgA secretion rates. Standardisation of methods for the collection and analysis of saliva, contributing to the establishment of valid protocols for exercise immunology studies, will facilitate comparison of data between studies, and the development of valid conclusions. Optimum training regimes, and possibly intervention techniques to decrease the incidence of infection may then be developed.

The aims of the research described in this thesis are:

i) To evaluate methods commonly used in saliva collection.

ii) To examine the relative contribution of salivary flow rate ($ml.min^{-1}$) and salivary immunoglobulin A concentration ($\mu g.ml^{-1}$) on the much-discussed salivary immunoglobulin A secretion rate ($\mu g.min^{-1}$).

iii) To investigate the use of salivary immunoglobulin A levels as an indicator of susceptibility to upper respiratory tract infection.

iv) To investigate the use of saliva flow rate as a marker of susceptibility to upper respiartory tract infection.

iv) To examine the effects of physical stress induced by exercise on sIgA levels and saliva flow rate.

v) To examine the potential of fluid replacement as an intervention techniques to temper exercise-induced decreases in saliva flow rate.

The hypotheses tested within this thesis are listed in appendix 1.

Part I comprises four literature review chapters: the first being this introduction. It is followed by chapter 2 which outlines the current understanding of the neuroendocrine links between the immune system, other physiological systems and the brain. It also discusses how the stress response to exercise results in immunomodulation. Chapter 3 reviews the effect of exercise on susceptibility to infection, particularly upper respiratory tract infection. Chapter 4 outlines the defence mechanisms against upper respiratory tract infection and examines the effect of exercise upon these defences.

Part II comprises one chapter (chapter 5) which addresses the methodological issues of saliva collection, and different saliva collection methods are evaluated on the basis of a experimental data.

Part III consists of three experimental chapters in which the effect of exercise on mucosal defence mechanisms is addressed. Chapter 6 addresses the relative contribution of saliva flow rate and IgA concentration on the calculated values gained for sIgA secretion rate, and uniquely addresses the idea that saliva flow rate may be a better indicator of susceptibility to URTI than sIgA levels. Chapter 7 examines the effect of a marathon run on saliva flow rate, and symptoms associated with URTI. Experiments on the effects of exercise stress, heat and hydration status on saliva flow rate and total protein levels are described in Chapter 8.

In the final part, Part IV, the findings of the research are brought together and discussed (chapter 9) and conclusions are drawn, and future directions considered.

CHAPTER 2

EXERCISE AND IMMUNITY

2.1. THE IMMUNE SYSTEM WITHOUT AUTONOMY

The immune system is responsible for defending the body against neoplastic and infectious disease. It may therefore seem obvious that a well-developed communication system with other bodily systems and tissues is necessary, yet only in the last twenty years has it been widely acknowledged that the immune system does not operate autonomously (Ader, 1981). It is now established that the nervous, endocrine and immune systems are closely integrated, sharing cell surface receptors and communication molecules (Blalock, Harbour-McMenamin and Smith, 1985; Del Rey and Besdovsky, 1987). The organs of the immune system are extensively innervated (Felten, Felten, Carlson, Olschawaka and Livnat, 1985), as are those of the endocrine system.

The hypothalamus appears to play a central co-ordinating role in several aspects of central nervous system (CNS), nervous, endocrine, and immunological function. Bi-directional communication links exist between the hypothalamus, autonomic efferent pathways, cerebral cortex, limbic nuclei, endocrine and lymphoid glands, and peripheral leucocytes (Goetzl, Alderman and Sreedharan, 1990).

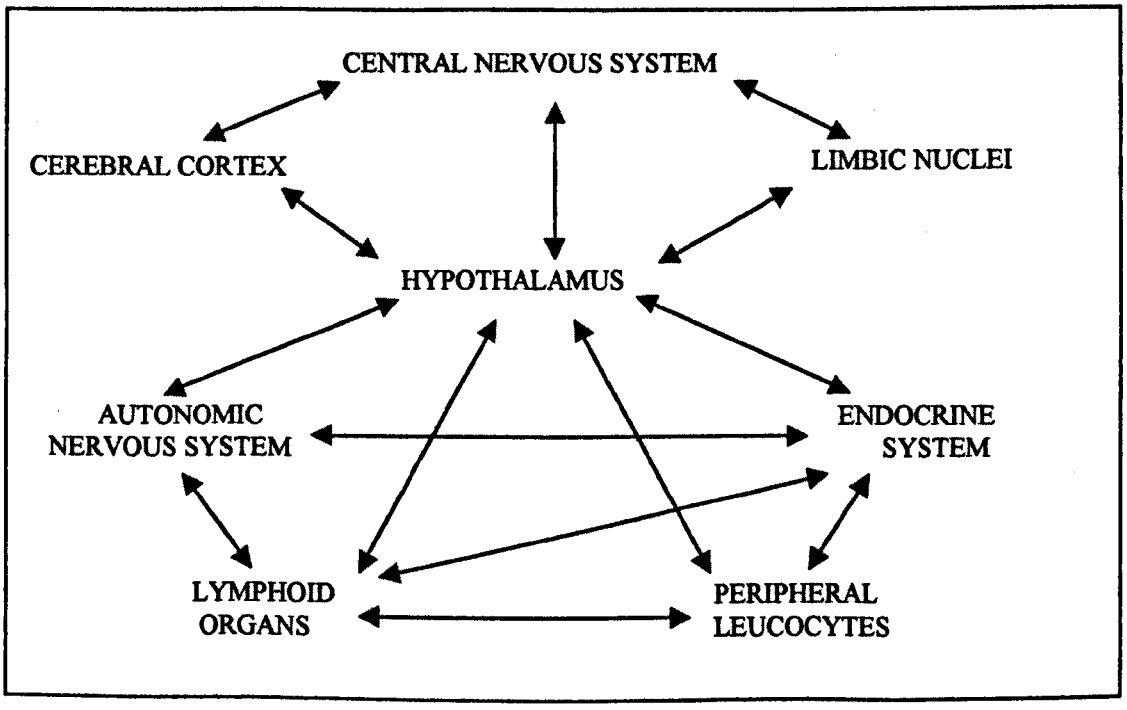

Fig 2.1. Proposed bi-directional communication links between systems.

Detection of a threat to homeostasis, be it real or perceived, by any of these systems will result in activation of some or all of the others (Ader, Felten and Cohen, 1991; Watkins, 1995).

2.1.1. *The stress response and immunomodulation.*

The term "stress" is used interchangeably for both the response to a variety of stimuli by various biological systems and the stimuli themselves, a factor which can cause confusion. The position adopted throughout this thesis is that stress is induced by a stressor, which may be a real or perceived situation to which an individual responds. Responding to a stressor results in a physiological stress response, which has been extensively studied and documented.

A link between perceived stress, homeostasis, and adaptation was identified by Walter Cannon. In 1932 he coined the expression "fight-or-flight" response, with the observation that the sympathetic branch of the autonomic nervous system prepares the body for defence against stress. It is now widely appreciated that in an attempt to maintain homeostasis the CNS, nervous, endocrine and immune systems respond to stressors in an integrated manner. The original concept of stress was defined by Hans Selye in 1936, as the reaction of an organism to some sort of outside threat. In 1956 Selye provided a description of the general pattern of response. He identified three phases of a stress response: "alarm reaction", accompanied by activation of the hypothalamic-pituitary-adrenocortical (HPAC) axis or the sympathetic adrenomedullary (SAM) axis; "resistance", whereupon the most appropriate local defence against the stress is organised; and "exhaustion", occurring if this stress is too intense or prolonged, with the result that the body's resistance declines and death follows (Selye, 1956). The emphasis in Selye's work is that 'similar' neuroendocrinological changes occur in response to any stressor, though the precise definition of the changes induced depends to some extent on the stressor.

Several studies have shown physical and psychological stress to cause immunomodulation (Baj et al., 1994; Calabrese and Wilde, 1991; Eskola et al., 1978; Jemmott et al., 1983). Even small amounts of stress are associated with nervous and endocrine perturbations. It appears that the stress response is largely a general response and is not stimulus specific. The neuroendocrine changes that have been observed are similar regardless of the modality of the stressor, be it physical, psychological or social. Features of the stressor

such as chronicity, timing, intensity, previous exposure and perceived controllability affect the ability of the individual to cope and adapt (Hillhouse et al., 1991; Nieman and Nehlsen-Canneralla, 1991).

Immunomodulation in response to stress seems to follow a pattern of phases, similar to those described for the general adaptation syndrome (Selye, 1956). After an "alarm" activation of nervous and endocrine systems, there appears to be a defensive reaction. This "resistance" phase involves increases in circulating immune cells (Nieman et al., 1989; Oshida et al., 1988), and in both secretory and serum immunoglobulins (Nehlsen-Cannarella, et al., 1991b). This apparent defensive activation has been observed in response to range of physical activities at differing intensities (Nieman et al., 1989; Nehlsen-Cannarella et al., 1991a; Oshida, Yamanouchi, Haymizu and Sato, 1988). These changes in immune functioning have been correlated with increases in plasma levels of stress hormones (Foster, Martyn, Rangno, Hogg and Pardy, 1986), and the increased number of circulating immune cells may be a result of endocrine induced changes in blood flow (Foster et al., 1986), resulting in an efflux of immature and unactivated immune cells from resting sites (Muir et al., 1984).

The immune system's response to a stressor appears to fit Selye's proposed general pattern. Just as an "alarm" and a "resistance" phase can be identified, so can a third and final "exhaustion" phase; a reduced immune response. The reduction in immune reactivity in response to intense chronic stress may be the result of an oversensitised feedback loop, an exhaustion of available immune cells, or an insufficiency of fuel reserves for the maturation and proliferation of immune cells.

The hypothalamus exerts a negative feedback control over immune activity in response to elevated blood levels of cytokines (polypepetide communication molecules released by activated immune cells), by activating immunosuppressive mechanisms, via the HPAC and the SAM axes (Goetzl et al., 1990; Watkins, 1995). The magnitude of this immunosuppression seems to be positively related to the duration or intensity of exposure to a stressor (Nieman and Nehlsen-Cannerella, 1992). This immunosuppression is believed to serve a protective function, as, without this regulation, stress-induced activation of the immune system could result in autoimmunity, which would threaten homeostasis. However, it is conceivable that on exposure to chronic or intense stress the

feedback mechanisms may become oversensitised, inducing immunosuppression in response to a small stimulus.

Alternatively the immune system may become unable to respond to any stimulus as a consequence of de-sensitisation or exhaustion of the system. Stress induced changes in blood flow result in the removal of immature immune cells from the lymphoid tissue (Foster et al., 1986). It is therefore possible that the number of immature immune cells remaining in the lymphoid tissue may be insufficient to develop an adequately rapid response to an antigen.

It is also possible that sustained physical activity could reduce the availability of fuel for immune cells. Newsholme and colleagues have provided evidence to suggest that immune cells obtain their energy from the metabolism of glutamine as well as glucose (Ardawi and Newsholme, 1985, 1983; Newsholme, Newsholme, Curi, Challoner and Ardawi, 1988). Newsholme (1994) suggested that sustained physical activity may disrupt the glutamine release process, so that it does not respond adequately to the increased glutamine requirement of the immune system.

To recapitulate: In response to any stimulus threatening homeostasis, the nervous and endocrine systems are activated via the HPAC or SAM axis, in order to maintain homeostasis. The response of these systems will depend upon the stressful stimuli imposed. It appears that the immune system's response to a stressor may fit the pattern of the general alarm reaction proposed by Selye (1956). In the early response to a stressor, immune activity appears to be increased (Oshida et al., 1988; Nehlsen-Cannarella et al., 1991b); but chronic or particularly intense acute stress results in a decrease in immune responsiveness, which may be the result of a hypothalamically controlled negative feedback system (Goetzl et al., 1990), exhaustion of the reserves of immature immune cells (Foster et al., 1986), or a lack of available energy for the proliferation and activation of immune cells (Newsholme, 1994). Therefore any stressor which is sufficiently intense or prolonged may increase an individual's susceptibility to infectious and neoplastic disease.

2.2. EXERCISE AS A STRESSOR, AND EXERCISE -INDUCED IMMUNOMODULATION.

Exercise is a combination of physiological, psychosocial and environmental stress (Cannon, 1993), which may therefore modulate immune functioning. Any exercise bout may impose one or all of these stressors on the individual, depending on the activity and how it is perceived. Exercise and competition induce neuroendocrine changes, similar to those observed in response to other stressors (Kjær, 1989). These neuroendocrinological changes induce immunomodulatory effects that may influence an individual's susceptibility to infection.

As with other stressors, the neuroendocrinogical changes are stimulated to induce changes that enable an individual to respond to the stressor in order to maintain homeostasis. In the case of aerobic exercise, the control systems facilitate movement by increasing heart rate, oxygen consumption, muscle blood flow, and by decreasing peripheral resistance. Increased activity increases the risk of injury and thus the possibility of encountering an infectious agent; therefore it is appropriate that immune activity appears to be increased concurrently with activity.

The first English language publication on exercise-induced perturbations in the immune system reported that "a violent leucocytosis" was experienced in a small group of runners after a marathon (Larabee, 1902, cited in Pedersen, 1991). It has been proposed that this apparent increase in immune activity in response to exercise may be the result of a mechanical effect of the increase in cardiac output, resulting in the demargination of leucocytes from the pulmonary circulation in exercise (Foster et al., 1986). It has also been suggested that the leucocytosis is a consequence of the increases in circulating levels of catecholamines seen with exercise (Ahlborg and Ahlborg, 1970). This idea may be supported by the fact that leucocytes are responsive to changes in the circulating levels of catecholamines through their surface receptors (Brenner, Shek, Zamecnik and Shephard, 1998). Several authors have demonstrated that adrenaline and noradrenaline induce increases in the number of circulating immune cells (Gray, Telford, Collins, Weidemann, 1993; Kappel et al., 1991; Tφnnensen, Christensen and Brinklov, 1987; Landmann, Müller, Perini, and Wesp, 1984; Muir et al., 1984). The effects of catecholamines on immune functioning are discussed further in section 2.3.2.8.

This leucocytosis fits with the changes in activity described in section 2.1.1, i.e. in response to stress the immune system initially exhibits enhanced activity, but sustained or intense stress leads to decreased immune functioning. The negative effects of exercise on the immune system have been extensively documented. Exercise, of varying durations and types, has been shown to affect immune functioning adversely (Baj et al., 1994; Eskola et al., 1978; Kendall, Hoffman-Goetz, Houston, Neil and Arumugam, 1990; Leisen and Uhlenbruck, 1992; Nieman et al., 1989). Hoffman-Goetz and Pedersen (1994), after reviewing several studies published on physical exercise and immunity, suggested that exercise-immune interactions can be viewed as a subset of stress immunology. Investigating the effects of exercise on the immune system and disease susceptibility therefore requires consideration of the mind-body interactions that take place during physical activity and athletic competition.

2.2.1. *Psychological stress and the immune system*

Training and competition are demanding on both the mind and the body. It has been demonstrated that, during preparation for competition, athletes experience considerable stress and anxiety (Hardy, Jones and Gould, 1996). The perception of the stressor (i.e. pressure to perform well) will depend upon the relative importance of the event and the personality of the athlete. Perna, Schneiderman and LaPierre (1997) suggested that while winning or exceeding performance expectations may lead to one pattern of physiological changes, increased negative mood or disappointment may lead to a different pattern. Frankenhaeuser (1990) found that the cortisol response to exercise was significantly dependent upon the cognitive appraisal of the situation. Therefore if a training programme or event is perceived to be stressful by the athlete, the effects of this psychological stress may be combined synergistically with the physical arousal to induce large perturbations in immune functioning.

It has been demonstrated that many of the characteristics of a stress response appear to be independent of the specificity of the stimuli inducing them. Psychological stress is believed to induce immunomodulation via the same mechanisms as physical stress: neuroendocrine communication links (Watkins, 1995). The limbic system of the brain, which mediates affective and cognitive behaviour, directly regulates the neurohumoral and autonomic outflow of the CNS, which in turn influences the immune system. It appears

that there is an increase in the secretion of steroid hormones only when psychological coping mechanisms have been overcome (Mason, 1968).

Non-selective adrenoreceptor blockade has been demonstrated to eliminate the immune system changes induced by psychological stress (Bachen et al. 1995). This supports the idea that SAM axis activation plays an important role in mediating the effects of psychological stress on the immune system. However, there is evidence to suggest that the effect of stress on the immune system may not solely depend upon activation of this axis. Landmann et al. (1984) found no increase in catecholamine levels in response to different types of psychological stress, even though heart rate was increased and there was evidence of immunomodulation. This immunomodulation included a reduction in the lymphocyte proliferation response and an impairment of antibody synthesis and blood leucocyte changes. It has been suggested that even though it seems that the stress response may be quite generalised, there may be some specificity in the response to a particular type of stimulus, and that this may even result in different neuroendocrine responses. The combined effects of one or more stimuli may attenuate or extenuate the stress response; for example, if stressors (physical or psychological) are not perceived as noxious or alarming, the physiological response induced may be smaller or may be in the opposite direction (Stein, Keller and Scheifer, 1985).

The influence of personality and the individual's perception of the stressor makes it very difficult to clarify the relationship between psychological state and immunity. It has been demonstrated that those individuals with a high need to control situations are more likely to show signs of sympathetic nervous system activation and become ill following a stressful situation (McClelland, Floor, Davidson and Saron, 1980). The reaction patterns to everyday life stress are probably related to the coping style of an individual (Biondi and Kotzalidis, 1990). McClelland et al. (1985) found that examination stress stimulated adrenergic activity which depressed immune function. They grouped their subjects by personality in terms of those with a high need for power, and those with a need for affiliation. The authors reported greater drops in levels of secretory immunoglobulin A (the primary mucosal immunoglobulin: see section 4.4.) for those individuals with high power scores compared with high affiliation scores.

Natural killer cells (NK) , are large granular lymphocytes and are believed to be part of the first line of defence against viral and neoplastic disease (Pedersen, 1985). It has been demonstrated in several studies that acute psychological stress causes increases in both the number and functional activity (cytotoxicity) of NK cells (Bachen et al., 1995; Benschop, Oostveen, Heijnenand, Ballieux, 1993; Brosschot et al., 1991). The same studies found decreases in the number and responsiveness of T-helper cells, and an increase in T-cytotoxic cells. T-helper cells are lymphocytes which function as effector cells to stimulate and co-ordinate an immune response to an antigen, while T-cytotoxic cells have a less specific response to an antigen. The results of this study suggest that psychological stress influences the immune system towards making the response less specific, by increasing the cytotoxic response and decreasing the ability to co-ordinate the proliferation of lymphocytes and the synthesis of antibodies.

Although the exact clinical significance of psychological stress-induced changes in immunity is not fully understood, there is evidence that both acute and chronic psychological stress can increase susceptibility to infections, and influence the progression of neoplastic disease (Rogers, Dubey and Reich, 1979). Psychological stress has been associated with increased incidence of upper respiratory tract infections (URTI) (Graham, Douglas and Ryan, 1986) (see chapter 3). The primary defence against URTI is believed to be salivary immunoglobulin A (sIgA) (Carthesy, 1997) (see section 4.3.3.). Several studies have observed changes in salivary immunoglobulin A levels in response to psychological states. Attempts have been made to quantify psychological stress and to correlate it with changes in sIgA levels; however, the data remain inconclusive. Positive mood states induced by watching a humorous film (Dillon and Minchoff, 1985) or relaxation (Green and Green, 1987; Jasnoski and Kugler, 1987) have been associated with increases in sIgA.

Negative mood states have also been associated with increased sIgA levels. Negative mood days and negative life-events were associated with high sIgA levels (Evans and Bristow, 1993). Stone, Cox, Valdimarsdottir, Neale (1987) also reported higher sIgA on highly negative mood days. These increases in sIgA may be part of a "resistance" phase to stress, i.e. part of the defence activation to a perceived threat (see section 2.1.1).

McClelland et al. (1985) found sIgA to be higher immediately after a stressful examination compared with relaxed days. The levels of sIgA decreased after the post exam

measurement to below baseline levels, and this decrease correlated with peak salivary noradrenaline levels. A plausible explanation for this response, is that it results from an increase in secretory defence mechanisms in response to a perceived stressor, followed by a suppression induced by hypothalamic controlled negative feedback. Examinations however, have frequently been shown to decrease sIgA levels (Glaser, Mehl, Penn, Speicher and Kiecolt-Glaser 1986; Jemmott et al., 1983; Kiecolt-Glaser, Garner, Speicher, Penn and Glaser 1984). Farné, Boni, Gnugoli and Corallo (1992) stated that there was a relationship between psychosocial factors and sIgA, since they demonstrated that an increase in daily hassle reporting was followed by a decrease in sIgA.

Mental stress is only one of the immunomodulating factors to which athletes are exposed. It is necessary to consider all types of stressors to which an athlete is exposed when trying to account for the influence of exercise on immunity. It is plausible that reducing the psychological stress of performance may reduce the summated stress effects on an individual's immune system. Further work is required to establish the effects of different psychological states on immunity.

2.3 THE PHYSIOLOGICAL RESPONSE TO EXERCISE AND THE IMMUNE SYSTEM

There is a vast and growing body of literature contributing to the knowledge of links between the physiological response to exercise and perturbations in immune functioning. Reviewing them all would constitute a thesis in its own right, therefore this section aims only to provide a general overview of how exercise-induced neuroendocrine changes may have an impact on the immune system.

As previously described, exercise is a stressor, and the stress which it elicits in an individual may comprise physical, psychological and environmental stressors. Although it may be possible to explicate the relative levels of psychological or physical stress an individual may be experiencing, the physiological response to these stressors is essentially the same. Therefore when considering the impact of exercise on the immune system , it is the physiological stress response that is important and not the stressor per se.

Exercise induced immunomodulation is thought to be mediated by the interplay of hormones, cytokines, neural and haematological factors. These factors display activation thresholds and a pattern of response that is related to exercise intensity and duration (Muir et al., 1984).

Physical activity (or anticipation of physical activity) results in an increased CNS and peripheral nervous activity, which operates in an integrated fashion with the endocrine system to co-ordinate the body's response and to maintain homeostasis. Nervous activity is more immediate and produces quicker effects than endocrine activity, because the communication pathways (nerves) are faster and more direct than those of the endocrine system (blood). The bi-directional communication links between the endocrine system, the ANS and the immune system have already been discussed (in section 2.1.) and therefore it seems probable that exercise which causes perturbations in both nervous and endocrine tone, could induce changes in immune functioning.

2.3.1. Neural control

The autonomic nervous system consists of two principal divisions: the sympathetic and parasympathetic; and many organs receive visceral efferent neurones from both components. Physical activity, or anticipation thereof, results in a withdrawal of

parasympathetic activity, therefore the organs respond to the sympathetic input, which facilitates movement. Sympathetic outflow results in increased heart rate, oxygen consumption, muscle blood flow and decreased peripheral resistance. Blood flow is redirected away from less active tissues, and out of resting sites, towards the active muscles.

Lymphoid organs are innervated by the sympathetic branch of the autonomic nervous system, and disruption of this innervation causes an increase in immune function (Besedovsky, Del-Rey, Sorkin , Da Prada and Keller, 1979). This suggests that immune activity is suppressed by sympathetic neural activity. Sympathetic, nor-adrenergic innervation of the thymus, spleen and lymph nodes is directed into the zones of the T-lymphocytes rather than the nodular or B-lymphocyte regions (Felten et al., 1985). This implies that activation of these nerves has greater impact on T-cells than on other types of immune cell. However, autonomic nerves have been shown to regulate activation of mast cells (Mansini et al., 1985), B-cells, T-cells and macrophages, and chemoattraction of eosinophils and neutrophils (Barnes, 1986).

As well as directly affecting systemic immune cells, sympathetic activity has a direct effect on defences in mucosal tissues. It has been demonstrated that lymphoid aggregates within the mucosae are extensively innervated (Felten et al., 1985). Within the mucosa the majority of effector immune cells are located within the lamina propria (Stead, Bienenstock, and Stanisz, 1987), where an extensive nerve plexus has been demonstrated (Davidson, 1983). Therefore it seems probable that the defences at the mucosal level are as much under the influence of the ANS as are the systemic defences of the peripheral blood. Although the clinical implications of this remain unclear, it is plausible that ANS activity could influence the susceptibility and progression of infections in the gastro-intestinal and upper respiratory tracts.

The response of the autonomic system to exercise is the fastest and the most widespread of all the physiologic systems. It can be assumed that because of the extensive innervation of lymphoid tissue (where immune cells mature and proliferate), the arousal of these nerves would be immunomodulating in some way. However, it is difficult to separate the influences of direct innervation and the effects of the sympathomimetic hormones, the

catecholamines; adrenaline and noradrenaline. The effects of these hormones on the immune system have been investigated and will be addressed later in this chapter.

2.3.2. Endocrine

As has already been discussed, there are very well established bi-directional communication links between the CNS, nervous, endocrine and immune systems. Much of this communication is facilitated by neuroendocrine messengers including catecholamines, pro-opiomelanocortin (POMC) derived peptides: adrenocorticotrophic hormone (ACTH) and β-endorphin, thyrotropin (TSH), gonadotropin, growth hormone and prolactin. It has been demonstrated that immune cells possess receptors for these messenger molecules. Cytokines, which were once thought to be an exclusive inter-leucocyte communication network are now known to be produced by, and communicate with, virtually all nucleated cells (Smith and Weidemann, 1990). There is a large body of work investigating the effects of exercise on these neuroendocrine messengers and cytokines. In recent years a link between exercise, hormone levels and immune functioning has been made by several authors.

The acute hormone response to exercise is generally believed to be important for the regulation of fuel mobilisation (Bunt, 1986). The endocrine response to exercise evolved as part of the "flight or fight" response and is therefore part of the more complex stress response. The endocrine response to the stress of exercise is complex and involves numerous feedback loops. The response is individualised with respect to stressors and may be modified by behaviour, prior exposure, and coping responses (Hillhouse et al., 1991). The release of neuroendocrine hormones into circulation is therefore affected by the intensity and duration of exercise (Galbo, 1983; Viru, 1992), and the capabilities of the individual performing. Untrained subjects may therefore exhibit a greater response than their trained counterparts, at the same relative intensity.

It has been demonstrated that the pattern of the endocrine response is dependent on an individual's ability to cope with that 'stress'. Brenner et al. (1998) described the stress hormones as being catecholamines and cortisol, both of which are catabolic hormones, and human growth hormone which is anabolic and believed to play a protective role. The same authors suggested that active coping mechanisms are accompanied predominately by

the secretion of catecholamines whereas passive coping is marked by increased secretion of neuropeptides and corticosteriods.

2.3.2.1 Prolactin

Exercise has been demonstrated to have a stimulatory effect on the secretion of neurohormones and the presentation of their receptors on cell membranes. It is thought that prolactin does not increase with exercise intensity until above 50% $\dot{V}O_{2\,max}$ (Galbo, 1983), although others have been unable to demonstrate changes in response to either submaximal or exhaustive running exercise (Rolandi et al., 1988). Its primary function in exercise is believed to be to mobilise fatty acids to be used as a fuel source (Galbo, 1983). Prolactin has been associated with several immune enhancing activities (Bernton, Melzer and Holaday, 1988). For example, increases in plasma levels of prolactin, in response to an exhaustive swim, were shown to increase the phagocytic function of macrophages (Ortega, Rodriguez, Barriga and Farmer, 1996).

2.3.2.2 Vasoactive intestinal polypeptide

In contrast, vasoactive intestinal polypeptide (VIP), which is prominent within sites involved in neuroendocrine regulation (Biondi and Kotzalidis, 1990), is immunosuppressive. VIP has been demonstrated to increase in response to prolonged exercise (Galbo, 1983). In response to a 90 minute treadmill run, plasma VIP levels increased in both endurance runners and hockey players (MacLaren, Raine, O'Connor and Buchanen, 1995). It has also been demonstrated to increase in response to submaximal and exhaustive exercise (Rolandi et al., 1988) and a 3 hour low intensity bicycle ride (Galbo, Hilstead, Fahrenkrug, Schaffalitzky De Muuckadell, 1979). These authors also observed increases in VIP with calorie deficiency that could be attenuated with carbohydrate feeding. Stead et al. (1987) demonstrated that VIP had a suppressive function on both immunoglobulin synthesis and lymphocyte proliferation. VIP has also been demonstrated to modulate natural killer cell activity (as reviewed by Wenger and O'Doriso, 1991). It is possible that VIP forms part of a negative feedback loop to control immune activity, as it has been demonstrated that receptors for VIP increase on mononuclear cells in response to a combination of strenuous physical activity and calorie deficiency (Wilk, Opstad, Kordahl and Blyume, 1988).

2.3.2.3 Substance P

Payan, Levine, and Goetzl (1984) demonstrated the existence of membrane surface receptors for the neurohormone, substance P, on human lymphocytes. However, much of the work reported on the immunomodulating effects of substance P has addressed its effects on the mucosal defences. Stead et al. (1987) reported that substance P enhanced immunoglobulin synthesis and that IgA synthesis was enhanced more than IgM while IgG was virtually unchanged. Therefore substance P can be considered to be immune-enhancing. Substance P is a vasodilatory neuropeptide, and it has been postulated that its actions may be counteregulatory to vasoconstriction induced by noradrenaline. However, Lind, Brudin, Lindholm and Edvininsson (1996) found that substance P levels remained constant during incremental exercise, but increased significantly 30 minutes after exercise. Therefore even though it has been demonstrated to have an immunomodulatory role, further work is needed to establish whether substance P has a role in exercise-mediated immunosuppression.

2.3.2.4. Somatostatin

Somatostatin has been demonstrated to increase in response to muscular exercise (Harris, 1994). Stanisz et al. (1986) found that it had the opposite effect to substance P, in that it suppressed DNA synthesis by up to 50% and inhibited the synthesis of IgA by between 30-80%. Somatostatin is a potent inhibitor of the release of growth hormone which has been shown by several authors to have immune enhancing effects.

2.3.2.5 Growth hormone

Growth hormone is released from the anterior pituitary in response to growth hormone-releasing factor from the hypothalamus. Physical and psychological stress increase the release of growth hormone (Kelley and Dantzer, 1991). Plasma levels increase with increasing exercise intensity or duration. The circulating concentration of growth hormone has been shown to be elevated after 30 minutes of very low intensity exercise (10-15% $\dot{V}O_{2\,max}$) and rises with increasing workload (Galbo, 1986). Plasma concentrations have been demonstrated to increase in proportion to intensity in response to both aerobic and resistance exercise (Deschenes, Kraemer, Maresh and Crivello, 1991). The threshold for the change is believed to be around 40% of maximal oxygen uptake (Shephard, 1983, cited by Brenner et al., 1998). Its role in exercise is to mobilise fatty acids and inhibit

carbohydrate metabolism. After exercise the actions of growth hormone promote protein synthesis and cellular adaptation.

Growth hormone has been associated with the development and maintenance of immunocompetence (Baroni, 1967; Berczi 1986 cited by Smith and Weidemann 1990). Growth hormone, like prolactin, augments many activities of lymphoid and myeloid cells (Kelley and Dantzer, 1991). These authors suggested that growth hormone and prolactin may augment many immune activities or at the very least counteract the immunosuppressive effects of glucocorticoids on cell-mediated functions, and may be integral to the resistance phase of the exercise stress. However, Brenner et al. (1998) found that intravenous infusion of growth hormone induces a marked neutrophilia , but had no other major effects on leucocyte count or immune function at the concentrations anticipated during exercise (Brenner et al., 1998). Therefore further work is required to elucidate the role of growth hormone as a mediator of exercise-induced immunomodulation.

2.3.2.6. Pro-opiomelanocorticotrophin derived polypeptides

In response to stress and exercise, corticotrophin releasing factor (CRF) is secreted from the hypothalamus (Silverman, Ho-Yu and Kelley, 1989). CRF leads to the secretion of pro-opiomelanocorticotrophin (POMC) derived peptides ACTH and β-endorphin from the anterior pituitary (Hillhouse et al., 1991). An increase in these hormones has been demonstrated in response to exhaustive graded treadmill exercise (Oleshansky, Zoltick, Hermon, Mougey and Meyerhoff, 1990). However, it has been suggested that CRF does not appear to play a major role in mediating the ACTH response to an acute episode of vigorous exercise (Wittert et al., 1991). These authors suggested that it was a more direct effect of arginine vasopressin (AVP) which is secreted from the posterior pituitary, however CRF will still have an indirect effect as one of the stimulators of AVP.

A variety of immune cells have been shown to exhibit receptors for POMC derived polypeptides, as well as being capable of synthesising the polypeptides themselves (Hughes and Kosterlitz, 1983). POMC polypeptides can suppress NK cell activity, modulate tumour growth, inhibit T-cell chemotactic factor production, augment interferon production and inhibit lymphocyte proliferation (McCain, Lamster and Bilotta, 1986 cited

by Hillhouse et al., 1991). It is difficult to postulate a role in exercise-induced immunomodulation given that these are apparently antagonist actions.

2.3.2.6.1. *Beta-Endorphin*

Beta-Endorphin is released from the anterior pituitary in response to very intense acute exercise (if sustained for longer than three minutes) or prolonged activity of intensities greater than 50% $\dot{V}O_{2\,max}$ (Farrell, Kjær, Bach and Galbo, 1987). Like other opioid peptides it promotes euphoria and analgesia, and is synthesised in neural, endocrine, and immune system tissues. Beta-Endorphin has been shown to have a variety of influences on immune functioning (Keast and Morton, 1992).

2.3.2.6.2. *Adrenocorticotrophic hormone*

ACTH is also released from the anterior pituitary in response to low cortisol levels and ACTH releasing factor from the hypothalamus. Its secretion is dependent upon relative workloads from 60% $\dot{V}O_{2\,max}$ and above (Bunt, 1986). It has been shown to increase in response to high intensity exercise (Kraemer et al., 1998; Bosco et al., 1996). ACTH stimulates the secretion of cortisol from the adrenal cortex, and both ACTH and cortisol have also been shown to increase in response to psychological stress alone (Perna et al., 1997). ACTH has been demonstrated to have an immunosuppressive effect, impairing the responsiveness of T-lymphocytes to antigenic and mitogenic stimuli (Kavelaars, Ballieux, and Heijnen, 1988); it may therefore have a role to play in stress-induced immunosuppression either directly, or indirectly via the stimulation of cortisol.

2.3.2.7 Glucocorticoids

Sufficiently intense psychological and physical stimulation results in an increased secretion of glucocorticoids from the adrenal cortex (Galbo, 1983, Viru, 1992), which in turn causes adaptive mechanisms in cardiovascular, metabolic and anti-inflammatory mechanisms. Of the three glucocorticoids, cortisol, corticosterone and cortisone, cortisol is responsible for 95% of glucocorticoid action. Cortisol is a catabolic hormone promoting the use of fatty acids and protein catabolism, and acts as a insulin antagonist, conserving blood sugar. The secretion of cortisol in response to a stressor is considered to be an adaptive mechanism; however, it has been suggested that chronically elevated levels of cortisol may be harmful. McEwen and Stellar (1993) have suggested that cortisol may

mediate the relationship between stress and illness. Cortisol has well documented immunosuppressive and anti-inflammatory properties (Miller and Tyrell, 1995).

Changes in plasma cortisol recorded during exercise, like catecholamines, are more closely related to relative rather than absolute workloads, and plasma cortisol is more likely to increase if the work period is long rather than short (McCarthy and Dale, 1988). However, a link between cortisol and exercise intensity has been established for over twenty years. The levels of serum cortisol generally increase in response to aerobic exercise at intensities more than 60% $\dot{V}O_{2\,max}$ and heavy resistance exercise (Deschenes et al., 1991).

Since 1975 it has been known that corticosteriods can induce a decrease in the number of circulating lymphocytes and a decrease in mitogen-induced lymphocyte stimulation and induce redistribution of T-cells from the circulating pool to the bone marrow (Fauci and Dale, 1975). In more recent years the effects of the levels of glucocorticoids seen in exercise, on various components of the immune system, have been further documented and quantified.

Corticosteroids impair several components of cellular immunity including T-lymphocytes (Cupps and Fauci, 1982) macrophages (Pavilidis and Chirigos, 1980) and natural killer cells, this influence being mediated via the corticosteriod receptors that leucocytes carry (Rabin, Moyna, Kusenecov, Zhou and Shurin, 1996). Cortisol appears to have a bi-phasic effect on immune cells with low levels inducing lymphocytosis and high levels, lymphopenia. Cortisol typically stimulates the migration of cells from the bone marrow into the circulation and subsequently into lymphatic glands and injured tissue. Although small increases in plasma cortisol may enhance immune responses, larger doses of this hormone have a suppressant effect (Brenner et al. 1998).

Glucocorticoids administered *in vivo* have been reported to cause neutrophilia, eosinopenia, lymphocytopenia and suppression of both NK and T-cell function (Nieman, 1997), lymphokine production, monocyte function, suppressor cells, cytotoxic response and serum immunoglobulin production (Tsokos and Balow 1986, cited by Hillhouse et al., 1991). All of these changes in immune activity have been shown to occur during recovery from prolonged high intensity aerobic exercise (Nieman, 1997). In contrast Tφnnensen et al. (1987) found that continuous infusion of cortisol for five hours induced the previously

described leucocytosis, neutrophilia and lymphopenia and reduction in the number of T-lymphocyte subsets, but no change in the activity or number of natural killer cells. However, a negative relationship between NK cell activity and plasma cortisol concentrations has been demonstrated after long endurance running (Berk et al., 1990). It may be possible that in an exercise situation the summated effects of circulating hormones including cortisol could account for the changes in NK cell activity.

It is important to understand that the observed exercise-induced immunomodulation is the result of a complex interaction of the circulating hormones with immune cells, and not the result of a single hormone acting on a single receptor. Tφnnensen et al. (1987) observed that adrenaline infusion reversed cortisol-induced lymphopenia, but not the associated neutrophilia. Therefore it appears that cortisol and catecholamines have mutually antagonistic effects regarding the movement of lymphocytes. There have been suggestions that the effects of cortisol in moving immune cells out of the blood stream and back into the tissue is partly to control the exercise-induced leucocytosis which may be a consequence of an increase in blood flow or catecholamines. Robertson et al. (1981) found an exercise-induced lymphocytosis which returned to pre-exercise levels 15 minutes post exercise. They suggested that exercise discharges intra-vasculary sequestered cells and that cortisol may be involved in the re-sequesterisation of stress-released cells. Proper functioning of the immune system depends upon the movement of immune cells between the blood and the lymphoid tissues. Therefore the movement of immune cells from the plasma into the tissues is not an immunosuppressive effect but one which confers protection to tissues and increases the probability of the immune system locating pathogens within organs.

Although there is a great deal of work detailing the effects of glucocorticoids, particularly cortisol, on the immune system, and the effect of exercise on plasma cortisol levels, there is less work documenting the effects of exercise on cortisol and the concomitant immunomodulation. However, it appears that elevations in plasma cortisol levels result in a similar pattern of immunomodulation regardless of how it is induced. The clinical implications of this cortisol-induced immunomodulation are unclear, because although circulating levels of some cells are reduced, their movement into tissues may facilitate the detection of pathogens. The actions of glucocorticoids may also be part of a self-regulatory mechanism, preventing the over sequesterisation of cells and the suppression of

inflammatory effects in order to prevent tissue damage. Therefore the idea that cortisol mediates the relationship between stress and illness requires further thought and investigation.

2.3.2.8. Catecholamines

The primary catecholamines in the peripheral circulation are adrenaline and noradrenaline, and dopamine in the central nervous system (Brenner et al., 1998). In acute stress conditions, adrenaline (primarily from the adrenal medulla) and noradrenaline (primarily from the post-ganglionic sympathetic nerve terminals) act through α- and β- adrenergic receptors to mediate adaptive cardiovascular and metabolic effects. Leucocytes, the spleen lymph nodes, the liver, and thymus all carry adrenoreceptors (Weicker and Werle, 1991) and therefore these tissues, which are central to immune system functioning, are responsive to the effects of circulating catecholamines.

The catecholamines are sympathomimetic, and therefore have the same effects on the viscera that the sympathetic nervous system does. Adrenaline increases blood pressure by increasing heart rate and constricting the blood vessels; it accelerates the rate of respiration, dilates respiratory passages, decreases the rate of digestion, increases the efficiency of skeletal muscular contractions, increases blood sugar level and stimulates cellular metabolism.

The central catecholamine release caused by physical and psychological stress can activate both the HPAC and SAM axes (Weicker and Werle, 1991). The SAM axis is more sensitive to stressors than the HPAC axis (Brenner et al., 1998). The response of the SAM is seen within a few seconds whereas a change in the level of glucocorticoids may take 20-30 minutes (Deuster et al., 1989). Increased sympathoadrenal activity is the most immediate and widespread exercise response; the catecholamines will increase proportionally to an increase in relative workload and duration (Bunt, 1986). Arterial plasma concentrations increase almost linearly with duration of dynamic exercise and exponentially with intensity, relative to an individual's maximum oxygen uptake (Kjær, 1989). Of the two amines, adrenaline is more sensitive than noradrenaline to mental stress, and in contrast, noradrenaline is more sensitive to physical stressors (McCabe and Schneiderman, 1985).

All leucocytes carry receptors for catecholamines (Brenner et al., 1998). The metabolism of catecholamines is rapid and therefore the effect on the immune system is short lived (Shephard , 1983, cited by Brenner et al., 1998). Catecholamines have been demonstrated to have a stimulatory effect on immune cells at low concentrations and an inhibitory effect at high concentrations (Felten et al., 1985).

Intense interval exercise at maximal oxygen uptake was found to induce an initial catecholamine dependent lymphocytosis, the authors suggested that this may be due to increased cardiac output, enhanced perfusion of low flow areas such as the lungs (Muir et al., 1984) and spleen, or a consequence of reducing adhesion to the vascular endothelium (Gabriel and Kindermann, 1997). Increases in plasma adrenaline or noradrenaline result in an increase in the peripheral count of leucocytes in venous blood specimens. The magnitude of the immediate exercise-induced leucocytosis is quite closely correlated to the circulating catecholamine levels (Gray et al., 1993).

Investigations have established that exercise-induced mobilisation of lymphocytes is largely a β_2-adrenoceptor and spleen dependent process, produced predominately by the actions of adrenaline rather than noradrenaline (Gray et al., 1993). Adrenaline perfusion induces a preferential mobilisation of lymphocyte subsets expressing a relatively high density of β_2-adrenoceptors (i.e. $CD8^+$ and NK cells rather than $CD4^+$). Landmann, et al. (1984) concluded that adrenaline plays an important role in the mobilisation of immunocompetent cells and might lead to a distribution pattern favouring immunosuppression during stress. This idea was supported by Kappel et al. (1991) who demonstrated that increased plasma adrenaline caused a redistribution of mononuclear subpopulations that resulted in altered function of NK cells. Tφnnensen et al. (1987) found that continuous infusion of adrenaline for one hour produced an instantaneous increase in natural killer cell activity and a selective increase in circulating NK cells along with leucocytosis, lymphocytosis and neutrophilia. All returned to pre-infusion levels fifteen minutes after completing infusion. Ahlborg and Ahlborg (1970) found that adrenaline caused a smaller increase in neutrophil count than a bout of vigorous exercise. It is possible that the exercise bout induced neural activation and the secretion of noradrenaline, and therefore the effects of the two hormones would have been summated resulting in a greater effect.

It appears therefore, that the effects of catecholamines are immune-enhancing, adrenaline having been shown to increase NK cell activity and redistribute other immune cells (Gray et al., 1993). Noradrenaline can enhance the primary antibody response and the ability of T-lymphocytes to lyse target cells (Hillhouse et al., 1991). Catecholamines inhibit the degranulation of mast cells and basophilic granulocytes, and consequently hypersensitivity reactions, which can cause non-specific tissue damage, are decreased (Weicker and Werle, 1991).

Gruchow (1979) concluded that elevated levels of catecholamine activity may increase susceptibility to disease, by interfering with the immune response, and, in the presence of an agent, lead to an infectious disease episode. However, it appears that catecholamine-induced changes may actually be immune-enhancing. Unfortunately the effects of the catecholamines are short-lived and may provide an initial protective response as part of an 'alarm/resistance' phase. As part of a 'flight or fight' response they are released to activate protective mechanisms, which may include immune defences. However, if the stress is sustained, then the release of cortisol results in the re-sequesterisation of immune cells, and growth hormone augments immune cell activity, possibly to prevent exhaustion of the system. If the stress imposed was sufficient to maintain secretion of catecholamine, then there would be, therefore, a possibility of reaching an 'exhaustion phase', which would increase the possibility of disease.

2.3.2.9. Cytokines

Cytokines are produced by, and effect, nearly all nucleated cells and have autocrine, paracrine and endocrine effects. The cytokines include interleukins (IL) interferons (IF), tumour necrosis factors (TNF), colony stimulating factors (CSF) and growth factors. It was originally thought that they formed an exclusive communication system within the immune system, and although they do influence all immune system activities, they communicate with all other nucleated cells. However their presence and co-ordinating activity is necessary for the growth, differentiation, and functional activation of all cells of the immune system (Northoff, Weinstock and Berg, 1994). Cytokines initiate the inflammatory cascade, orchestrate the various activities of different immune cells to a pathogen, and communicate the heightened immune activity to the CNS. There are pro-inflammatory and anti-inflammatory cytokines which will be released and co-ordinated in order to ensure that an adequate but not damaging response is mounted.

Changes in the plasma levels of cytokines have been found after various types of exercise. In response to a bout of intense running exercise, IL-2 decreased immediately after exercise and returned to baseline within twenty-four hours (Espersen et al., 1990). The same authors found increases in TNF-α two hours after completion of an intensive run, which again returned to baseline after 24 hours. Prolonged endurance exercise causes an increase in plasma levels of IL-1, possibly in response to muscle tissue injury although the same exercise induced a decrease in IL-2 levels (Shephard, Rind and Shek, 1994). No changes in plasma cytokine levels were seen after a competitive run or swim by elite runners or swimmers; however, significantly lower baseline values for IL-6 were seen for the swimmers (Espersen et al., 1996).

Cytokines communicate with all immune cells in a sophisticated and complex manner and therefore to describe the effects of exercise-induced changes would involve detailing their numerous immunomodulating effects at rest. Although the understanding of the cytokine response to exercise is increasing, as the body of descriptive data grows, the role that cytokines have in mediating the observed altered susceptibility to infection in athletes is unclear. It has been suggested that a suppressed ability to elicit a pro-inflammatory cytokine response after exercise may relate to an increased incidence of infection after intense exercise or training (Bagby, Crouch and Shepherd, 1996).

Cytokines are known not only to have a direct effect on immune functioning but they also influence the secretion of immunomodulating hormones, possibly as part of a self-regulatory feedback loop. Receptors have been found in the brain for IL-1, one of the actions of which is to stimulate the secretion of glucocorticoids which suppress the synthesis of IL-1 (Kelley and Dantzer, 1991). IL-1 mediates various endocrine changes including increased release of insulin, glucagon, growth hormone, prolactin, ACTH, TSH and vasopressin (Dinerarello, 1984, cited in Kennedy and Jones, 1991): hormones which are known to have an effect on immune functioning; and to suppress the secretion of cytokines (Kelley and Dantzer, 1991). There is evidence to suggest that IL-1 and IL-6 can induce the pituitary gland to release ACTH, which stimulates levels of cortisone sufficient to sustain an acute-phase response (Woloski, Smith, Meyer, Fuller and Blalock, 1985).

2.3.3. The acute-phase response

As previously described there is evidence to suggest that exercise causes large perturbations in immune functioning, and that this is probably mediated through exercise-induced neuroendocrine changes. Exercise has been shown to induce an acute-phase response, which in turn may place demands on the immune system that are sufficient to impair an individual's ability to mount a response to a pathogen. The acute-phase response is a stereotyped sequence of reactions by the immune system (Evans and Cannon, 1991). The acute-phase response is part of an immediate host-defence reaction (much quicker than a specific response) and involves the mobilisation and activation of phagocytic cells, production of immunomodulating factors, cytokines and complement, as well as an altered concentration of trace metals, production of binding proteins, and development of a fever (Evans and Cannon 1991).

The acute-phase response to exercise is believed to be part of a muscle repair and inflammation process. It has been demonstrated that the greater the muscle damage induced, the greater the acute-phase response. Increases in pro-inflammatory cytokines IL-1, IL-6 and TNF (Evans, Meredith and Cannon, 1986), have been particularly associated with eccentric exercise which is known to induce greater muscle damage than other types of exercise (Armstrong, 1992). It has been suggested that IL-6 is probably the major systemic cytokine after strenuous exercise, causing the acute-phase response (Northoff and Berg, 1991). Authors have expressed concern that the enmeshment of a large part of the immune system in this process will mean that protection from pathogens is compromised (Nieman and Nehlsen-Cannarella, 1992).

The acute-phase response involves many mature and immature immune cells, and therefore a considerable amount of energy is required for their growth and proliferation. It is conceivable, therefore that the acute-phase response could decrease the energy available for mounting an immune response to a pathogen. Frisina, Gaudieri, Cable, Keast and Palmer (1994) suggested that the exercise-mediated changes in metabolic capacity involve the re-orientation of lymphocyte metabolism towards increasing dependence on glutaminolysis as opposed to glycolysis. Ardawi and Newsholme (1983) found evidence that glutamine was an essential fuel source for lymphocytes and macrophages. Newsholme and colleagues have questioned the availability of glutamine after exercise and have suggested that sustained physical activity may disrupt the process of releasing

glutamine to the immune system (Newsholme, 1994). Castell, Poortmans and Newsholme (1996) reported that a drink containing glutamine, administered after exercise, reduced the incidence of infections compared with a group that consumed a placebo drink. This study suggests that there is a deficiency in the availability of glutamine and that this deficiency does result in immunosuppression.

Therefore it is conceivable that the acute-phase response to damaging exercise may exacerbate the effects of the neuroendocrine-induced perturbations in two-ways: by involving large numbers of mature and immature immune cells; and by decreasing the energy available for the immune system to mount a response to a pathogen. However, in one of the few studies that has looked at the effect of plasma glutamine levels *in vitro*, Mackinnon and Hooper (1996) concluded that the appearance of URTI's was not related to changes in plasma glutamine in overtrained swimmers.

Although the clinical significance of many of the exercise-induced immune function changes needs to be established, a link has been made between exercise and increased susceptibility to URTI (see chapter 3). It is conceivable that some of the symptoms that have been reported by the subjects in epidemiological studies, noting a high URTI incidence, could be part of an inflammatory reaction in the upper respiratory tract, and that some of the perceived problems are a direct consequence of an acute-phase response.

2.4. SUMMARY

It has been extensively documented that a bi-directional communication system exists between the organs of the CNS, nervous, endocrine and immune systems. This enables these systems to react in a co-ordinated and integrated fashion to anything threatening homeostasis. As suggested by Selye it appears that after initial alarm and resistance phases, upon exposure to intense or prolonged stress these systems may become exhausted and homeostasis may be threatened. One manifestation of the suggested exhaustion phase is a decrease in immune functioning.

Exercise poses a threat to homeostasis and therefore induces the same neuroendocrinoimmunological changes that other stressors do. There is a large and growing body of evidence demonstrating that exercise of varying types, intensities and durations induces perturbations in immune functioning. The details of the mechanisms mediating these changes have not yet been fully elucidated. However, it is known that immune functioning is highly sensitive to changes in nervous and endocrine tone. Exercise induces changes in activation of neural pathways and the levels of circulating hormones, in order to facilitate movement. The effects of these exercise-induced changes on immune functioning are not only numerous but also involve complicated feedback loops. There has been an explosion of literature investigating the various communication molecules, pathways, and receptors involved; however, the exact pathophysiological significance of exercise-induced immunomodulation remains unclear. It is probable that the negative immunomodulation induced by exercise increases an individual's susceptibility to disease. The next chapter explores the reported effects of exercise on susceptibility to infection.

CHAPTER 3

UPPER RESPIRATORY TRACT INFECTIONS

3.1. UPPER RESPIRATORY TRACT INFECTIONS IN ATHLETES.

There is large and growing body of both anecdotal and research-based evidence which suggests that there is a higher susceptibility in athletes to certain infections, particularly infections of the upper respiratory tract, than in the general population (Weidner, 1994). Berglund and Hemmingson (1990) reported that 92.3% of infectious episodes in elite skiers were upper respiratory tract infections.

Anatomically the upper respiratory tract is the extra-thoracic part of the respiratory system (nasopharynx, sinuses, throat, larynx, and trachea). The two most frequent types of upper respiratory tract infections (URTI) are commonly known as cold and flu. URTI's are most commonly virally induced; and at least 120 viruses, 1 mycoplasma and numerous bacteria cause acute URTI in humans (Reynolds and Merril, 1981). The symptoms are diverse, including runny or blocked nose, sore throat, cough, sneezing, headache, malaise, chills, shakes, fever, laryngitis, and aching joints or muscles (Nieman et al., 1990b). Transmission of these pathogens is most commonly through hand to hand, or object to hand, contact (Weidner, 1994).

The general population averages 1.5 to 2 episodes of URTI per year (Brenner et al., 1994). Heath et al., (1991) stated that an average of 1.2 incidences were self reported by athletes in a 12 month period, a value that is relatively low when compared to the mean population score. In contrast, Linde (1987) showed that experienced elite orienteers experienced significantly more infectious episodes than non-athlete controls (2.5 versus 1.7 over a year). It seems that although the infection most commonly suffered by athletes is an URTI, the claim that athletes suffer a higher incidence of URTI than the general public may require further substantiation.

Nieman and Nehlsen-Cannarella (1992) have described a model of susceptibility to infection in athletes. This model can be diagrammatically represented as a J-shaped curve and has generally been accepted as an accurate description of the relationship between susceptibility to URTI and exercise.

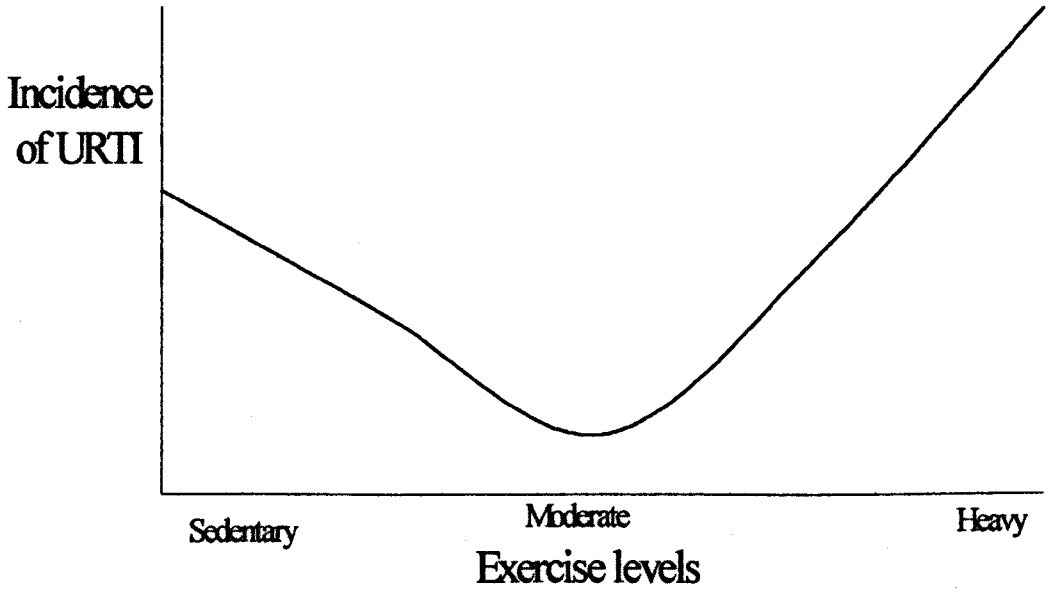

Fig. 3.1 A model of the effect of exercise on the susceptibility to URTI.

(Nieman and Nehlsen-Cannarella, 1992)

From the literature available it seems apparent that moderate exercise decreases the risk of an URTI to below that of a sedentary individual. Nieman et al. (1990b) demonstrated a reduction in upper respiratory tract infection symptoms in 36 mildly obese women after a 15 week low-intensity exercise programme. However, very intense, very prolonged, or excessively frequent exercise has been associated with negatively altered immune function and an increase in infection symptomology (Nieman, 1994b). It is important to recognise that the perceived intensity of exercise is relative to an individual; an exercise bout which one individual may find moderate may be excessive to another. Therefore all measures of exercise and infection incidence must be corrected for the capabilities of the individuals involved.

Many studies base diagnosis of URTI on the identification by subjects of some or all the symptoms listed above, rather than on a professional clinical diagnosis. Graham et al. (1986) suggested that symptoms not induced by infectious agents may be perceived as illness and may be a factor influencing the results of epidemiological studies. Allergies, particularly allergic rhinitis, would induce some similar symptoms, the incidence of which

would not be a consequence of immunosuppression, but conversely of hyperactivity. Current data are difficult to interpret because non-infection induced symptomology has not been considered. It is conceivable that athletes may experience more airway irritation than the general population because of the high rates of ventilation they sustain during exercise. There is a need for clinical diagnosis of an infection or measures of changes in the immune system. This is necessary so that conclusions can be drawn about the purportedly high incidence of URTI symptomology reported in athletes, and concurrently the effect of exercise on susceptibility to infection.

3.2. EXERCISE AND UPPER RESPIRATORY TRACT INFECTION.

Sport and exercise participation has been associated with an increased incidence of URTI in several scenarios (Brenner et al., 1994; Nieman, 1994; Weidner, 1994). However, the suggested increased incidence of infection has not been supported by all studies. For example, a retrospective study of 175 young adults showed no relationship between weekly exercise energy expenditure and URTI in males and a weak negative relationship in females (Schouten et al., 1988b). In 1989, 'Runners World' conducted a subscriber survey which revealed that 61% of 700 runners reported fewer colds since beginning to run while only 4% felt they had experienced more (cited by Nieman, 1997). The contrasting results in this area are probably due to inconsistency between studies in subject and protocol choice, and a lack of control of exposure to pathogens. A major problem with questionnaire data is that the results can be misleading due to confusion over the symptoms; they may be symptomatic of something other than URTI, such as inflammation of the upper respiratory tract. Shephard, Verde, Thomas and Shek (1991) identified intensity, duration of exercise and the training status of the individual as probable factors affecting the occurrence or magnitude of immunosuppression. There is, in fact, a body of evidence demonstrating that susceptibility to infection is modulated by the duration, intensity, and volume of training (Heath et al., 1991; Mackinnon and Hooper, 1994; Peters and Bateman, 1983).

3.2.1. *Exercise duration.*

Marathon running has repeatedly been used to investigate the effects of long-duration exercise on the incidence of infection. Peters and Bateman (1983) reported a significantly higher incidence of URTI symptoms in runners (33.3%) than controls (15.3%) in the 2 weeks after a marathon. Nieman et al. (1990a) recorded that 12.9% of 2311 people who participated in the Los Angeles marathon experienced infectious episodes within a week of the race as compared to 2.2% of non-participating runners (runners who trained for but did not run the marathon). Nieman, Johanssen and Lee (1989) found that there was no increased incidence of URTI in 1200 runners in the 2 months before, or in the first week after a 5k, a 10k, or a half marathon race. Nieman (1995) investigated a broad range of training habits in runners, and suggested that risk of URTI is relative to the training distance covered. There is a lack of data on the effect of other long-duration activities on the incidence of URTI. The data that are available on the effects of prolonged exercise on markers of immune activity suggest that susceptibility to URTI will be increased in response to any type of prolonged exercise (Mackinnon, Chick, van As, Tomasi, 1989).

Peters and Bateman (1983) suggested the increased risk of URTI following a marathon is attributable to the stress and fatigue of marathon running. It was also suggested that drying and cooling of the upper respiratory tract mucous membrane during mouth breathing may result in local damage. Exercise duration is probably a factor in the occurrence of URTI symptomology because of prolonged increases in minute ventilation. This may result in drying of the airways, and possibly airway irritation, or increased chances of inhaling pathogens or allergens. Levels of stress hormones have also been shown to increase with exercise duration (Deuster et al., 1989). Hickson and Boone (1991) commented that long term exercise may predispose an individual to more of an immunosuppressed state than short-term exercise. Increased susceptibility to URTI after marathon running may be (as noted above) a consequence of an increased exposure to pathogens, either through contact with large numbers of people, or by increasing the volumes of inspired air possibly containing pathogens. An exercise-induced decrease in the effectiveness of specific or non-specific defence mechanisms may be another factor.

3.2.2. *Exercise intensity.*

There are limited data available on the effects of short-duration intense exercise on susceptibility to URTI, although the relative intensity of physical activity has been shown

to be an important determinant of the stress response evoked (Nieman and Nehlsen-Cannarella, 1992). Mackinnon and Jenkins (1993) found changes in mucosal immunity to be an effect of exercise intensity but not duration. Mackinnon and Hooper (1994) reported a cumulative suppressive effect of daily intense exercise on mucosal immunity. These findings suggest that intense exercise would increase susceptibility to URTI by negative modulation of the mucosal immune system which protects the respiratory tract.

There is evidence suggesting that exercise duration is a factor in the incidence of URTI. However, Peters and Bateman (1983) found that runners who completed the marathon fastest had a significantly higher incidence of URTI in the 2 weeks following the race than the control group of non-runners, but that the slow runners had no more infections than the control group. This implies that the intensity at which the race was run may be a more important factor than its duration, in influencing susceptibility to URTI. In order to gain a faster time these runners may have run the race harder (i.e. at a greater intensity) and therefore experienced more physiological and psychological stress, which has been demonstrated to cause greater perturbations in immune functioning. Elite marathon runners may experience more psychosocial pressure than slower runners because they have potentially more to gain by achieving a faster time (e.g. prize money), and are therefore possibly experiencing a greater 'intensity' of psychological stress.

3.2.3. *Training volume.*

A final possibility is that the faster runners may have trained more heavily than the slower runners in the build up to the race and not allowed themselves sufficient time to recover: the greater the time spent training, the less time they are able to spend recovering. Increased training volume would also mean that breathing rate is increased for longer periods of time, therefore increasing the exposure to airborne pathogens. Greater training volumes could also increase perturbations in nervous and endocrine tone, factors believed to cause immunomodulation.

Heath et al. (1991) showed that an average runner who ran 32km.wk^{-1} exhibited 1.2 URTI per year, but the lowest incidence of infection was found in individuals who ran less than 16km.wk^{-1}. The incidence of URTI in both these groups of athletes is below the common population average reported by Brenner et al. (1994) of 1.5 to 2 incidences per year. It may be that running 32km.wk^{-1} conferred some increased resistance while 16km.wk^{-1} was

even more beneficial. Nieman, Tan, Lee and Berk (1989) suggested that, as compared with a sedentary lifestyle, running an average of 42km.wk^{-1} was associated with a slight reduction in the incidence of URTI, while Nieman et al. (1990a) demonstrated that runners training more than 97km.wk^{-1} reported more infectious episodes than those undertaking less training. These studies support the J-shaped model proposed by Nieman and Nehlsen-Cannerella (1992).

3.3. SUMMARY

It is apparent that athletes suffer a high incidence of URTI symptoms (Peters and Bateman, 1983; Nieman et al., 1990a). However, before intervention strategies can be introduced, there is a need to establish that reported symptoms are the result of an infection and not of airway irritation or stress-induced psychosomatic symptomology. To date only one study (Mackinnon, Ginn and Seymour, 1993c) has demonstrated links between exercise, depressed immune functioning (decreased salivary immunoglobulin A levels) and clinically diagnosed URTI.

There is a body of evidence to support the idea that mucosal immunity is affected by both physical and psychological stress. Further investigation is required to establish the mechanisms linking this immunomodulation with changes in the susceptibility to infection, particularly URTI. Attempts to explicate the mechanisms mediating URTI susceptibility have focused on the effects of exercise and sports participation on the principal mucosal antibody, secretory immunoglobulin A (sIgA). Relatively few studies have considered the effect of exercise on mechanisms of mucosal immunity other than sIgA (Tomasi et al., 1982; Peters and Bateman, 1983; Shepherd and Shek, 1993).

The high ventilatory rates sustained during exercise result in oral breathing, and this means that the nasal filter is bypassed. Additionally exercise may result in cooling and drying of the airways, damage to cilia lining the upper respiratory tract, and slowing of mucociliary clearance (Shepherd and Shek, 1993). Further investigation is required into the impact of exercise-induced inhibition of innate defence mechanisms on susceptibility to URTI. There is a need to examine the effects of exercise on several aspects of mucosal immunity before a temporal relationship between exercise, immunity and susceptibility to URTI can be established. In the next chapter (chapter 4), the mechanisms by which humans defend against URTI and the effect of exercise upon those defence mechanisms will be reviewed, and the potential mechanisms for the purported increase in susceptibility to URTI in athletes will be discussed.

CHAPTER 4

UPPER RESPIRATORY TRACT IMMUNITY AND THE EFFECT OF EXERCISE.

The first interaction of an individual with any pathogen is at the external surfaces of the body. Although internal, much of the mucosal surface is in continuity with the external environment, and it is here that microbial pathogens and toxic agents make their first contact with the host and potentially cause local or systemic disease (MacNabb and Tomasi, 1981). The mucosal surface area is enormous, probably more than 100 times that of the skin (Brandtzaeg, 1995), and therefore there is an ample surface for pathogens to find 'space' either to colonise or to gain entry to the bloodstream. Defence mechanisms at the mucosal surfaces are not only responsible for the protection of those tissues, but must also perform the role of preventing the entry of pathogens into the internal environment. The immune system is just one of the homeostatic network mechanisms in place within the body to maintain both structural and functional integrity, and within the respiratory tract there are a number of mechanisms in place to protect its primary function, air exchange.

The occurrence of an infection within an individual is highly dependent upon exposure to a virulent pathogen and the effectiveness of defence mechanisms against that infectious agent. There are a number of biochemical, physical and immunological barriers that confer protection against the entry of and colonisation by pathogens and therefore provide a defence against infectious diseases. The salivary glands, oral cavity and the nasal mucosa are particularly important to the immunological defence of the upper respiratory tract. (Reynolds and Merril, 1991). The local mucosal immune system is of primary importance to the defence against certain infections, particularly those which are non-invasive and confined to the mucous surfaces. Peters and Bateman (1983) speculated that increased susceptibility to URTI in athletes was a consequence of functional changes in the mechanisms that normally defend against URTI: impaired mucociliary clearance, macrophage function, and changes in the amounts of other substances protecting the mucosal surfaces e.g. lysozyme and IgA. All of these mechanisms are components of a local mucosal immune system.

4.1 EVIDENCE FOR A LOCAL IMMUNE SYSTEM

In 1875 Klein described lymphoid tissue within the wall of the bronchus in a number of species including humans. This confirmed the fact that immune cells and specific immune defences were not confined to the blood. In 1965 Tomasi, Tan, Solomon, and Pendergast characterised mucosal immunity as being separate from that of systemic immunity,

> "There appears to be an immunological system which is characteristic of certain external secretions. Its properties including the production of a distinctive type of antibody separate from the "systemic" system responsible for the production of circulating antibody. This system may play a significant role in the body's defence mechanisms against allergens and micro-organisms" (page 121).

Thirty years later Brandtzaeg described activation of the mucosal immune system as being at least partially independent of the systemic immune system (Brandtzaeg, 1995). Further substantiation of the existence of a common mucosal immune system has centred around the fact that specific/adaptive mucosal immunity is primarily humorally mediated and that the immunoglobulins found in external secretions have different characteristics from those found in serum.

The lamina propria (loose connective tissue subjacent to the epithelial layer) of the respiratory and gastrointestinal tracts has been shown to contain large numbers of immunoglobulin secreting plasma cells (Brandtzaeg, Fjellander and Geruldsen, 1967; Tourville, Adler, Bienenstock and Tomasi, 1969). However, unlike serum plasma cells where IgG is the predominant immunoglobulin secreted (Lamm, 1976; Remington, Vosti and Zimmerman, 1964), the principal immunoglobulin secreted by plasma cells situated in the mucosa is IgA (Tomasi et al., 1965). IgA forms a minor component (10-20%) in the serum, compared to its levels in external secretions (60-100 %) (Tomasi et al., 1965).

However, it is not only the predominance of IgA over other immunoglobulins in the mucosa that characterises mucosal humoral immunity. Secretory IgA has been demonstrated to differ in several of its physicochemical characteristics from immunoglobulin A in serum. Tomasi et al. (1965) demonstrated that salivary and colostrum IgA were alike, and had a sedimentation coefficient of 11s, larger than the

monomer (7s) found in serum. In the same paper Tomasi et al. (1965) also reported that the IgA in external secretions was synthesised locally, dispelling ideas that humoral immunity is the result of antibodies transported from the serum.

The local mucosal immune system may be immunised against a pathogen without any involvement of the systemic immune system; for instance, the respiratory tract may be directly immunised to act against upper respiratory tract pathogens like influenza viruses (Jurgensen et al., 1973). One of the markers of the existence of a local immunity is that recovery from viral respiratory diseases is better correlated with levels of local antibody than serum antibody (Tomasi and Bienenstock 1968), thus the mucosal immune system has evolved to respond to a pathogen without any involvement of the systemic immune system.

There has been a frequent finding of a dissociation between both the levels and the class of antibody activity in serum and secretion of individuals following infections and active immunisation (South, Warwick, Wollheim and Good, 1967). The separate synthesis and regulation of mucosal and systemic antibodies is particularly relevant to athletes and the problem of increased susceptibility to infections of the upper respiratory tract. It has been demonstrated that serum and mucosal immunoglobulins often respond differently to exercise (Mackinnon, 1996). Therefore in order to understand the effect of exercise on susceptibility to upper respiratory tract infections it is important to focus upon the effect that exercise and training may have on the defence mechanisms that exist to protect against upper respiratory tract infections.

A large body of data exists investigating the effect of exercise on sIgA (see section 4.3.3.3). Yet secretory IgA levels may not provide a good indication of susceptibility to infection, as it appears that some individuals are capable of protecting themselves by some other mechanism (Cannon, 1993). Tomasi and Grey (1987) commented that viral infections were relatively rare in IgA deficient patients, and hypothesised that resistance to viral infections at the mucosal surface can be mediated by mechanisms other than those involving the secretory immunoglobulins. Goldberg, Barnet and Fudinberg (1968) reported the existence of individuals without any IgA who remain completely asymptomatic, even though it may be assumed that they come into contact with pathogens in their day to day living. There is a possibility that in response to IgA deficiency

(congenital or acquired), individuals may adapt by developing other mechanisms to protect the mucosal surfaces against infection. Stanley and Cole (1985) found that patients with recurrent infections had nasal sIgA (11s) concentrations that were no different from those of normal healthy individuals. The high incidence of infection in these patients was clearly a consequence of some other factor. Although it is commonly used, the value of sIgA as an indicator of immune status may be questionable. All the factors which may confer protection from URTI must be investigated in order to explicate the mechanisms by which athletes appear to have an increased susceptibility to URTI.

Protection against infection is not only conferred by a well-developed sophisticated immune system and the humoral antibodies that are so often referred to in the context of defence against upper respiratory tract infections, but there are too a number of physical and mechanical barriers that also contribute to minimising the entry and establishment of infections. This is commonly known as non-specific immunity.

4.2 NON-SPECIFIC IMMUNITY

Non-specific immunity has mechanical, physical, biochemical and cellular components, with its categorising feature being that none of the factors have the capacity to recognise pathogens, mount a specific response or store a memory of the pathogen, unlike the more highly evolved specific immunity.

Within the upper respiratory tract, non-specific immunity is conferred by anatomic structures, cilia, epithelial cells, mucosal fluids, innate proteins, enzymes and cellular components with a non-specific action.

4.2.1. *Epithelial cells*

The epithelial cells lining the respiratory tract provide a weak mechanical barrier (Reynolds and Merril, 1981).

4.2.2 *Cilia*

Ciliated epithelial cells possess approximately 200 cilia each which beat 300-600 times a minute and propel mucous and other debris up the airways, so that they are removed from the body (Reynolds and Merril, 1981).

4.2.3. *Mucosal Fluids*

The capacity of human nasal secretions to inactivate viruses was described in 1917 by Amos and Taylor (Shvartsman and Zykov, 1976). That such activity was due to specific antibody was first demonstrated in 1943 (Francis, 1943 cited Tomasi et al., 1943). Although the antibodies have been credited with the neutralising activity of the secretions, the fluids themselves do confer some protection by forming a physical layer over the mucosal surfaces, having a washing effect, and by moving the anti-pathogenic proteins contained within the mucosal fluids over the mucosa. Immunoglobulins can only be effective in their anti-pathogenic role if physical contact is made with the pathogen. The probability of secretory IgA interacting with invading pathogens may be dependent upon the extent of mobilisation by the secretory fluid bathing the mucosal surfaces. In the case of the upper respiratory tract this is primarily saliva and mucus.

Saliva is the main fluid in the upper respiratory tract. It has a non-specific, mechanical washing effect preventing the adherence of potential pathogens, propelling them to the anti-pathogenic environment of the stomach. This is probably an important factor in the prevention of primary infection of the oral mucosa (Mason and Chisholm, 1975).

4.2.3.1. Properties of saliva.

Saliva is a complex fluid containing an array of inorganic (electrolytes) and organic (enzymes, mucous, immunoglobulins, other proteins, excretory products, vitamins) substances (MacFarlane and Mason, 1975). A thin film of saliva bathes the surface of the mucosa. Many of the anti-pathogenic characteristics of saliva can be attributed to its constituent immunoglobulins (IgG, IgM, and IgA) although other salivary proteins have been shown to have a non-specific anti-pathogenic effect. Long before the properties of sIgA were characterised in the 1960s, it was recognised that saliva had bacterostatic, bactericidal, agglutinative, transformative and mutative properties and was able to attract leucocytes to an area of infection (Appleton, 1945, cited in Alfonsky, 1961). Appleton

also noted that these properties of saliva may influence the occurrence, severity and progress of infections of the oral, pharyngeal, nasal, and respiratory mucosa (areas which are now generally termed the upper respiratory tract).

Saliva has several protective functions within the oral cavity; its contains both specific and non-specific anti-pathogenic proteins, and reduces the adherence of pathogens to the mucous membranes by creating a physical barrier and having a mechanical washing effect. Protection is afforded by basal secretion, the on-going, low-level salivary flow in the absence of stimulation (Suddick and Dowd, 1980). The physical flow of saliva effectively removes a lot of potentially harmful bacteria from the mucosal surfaces (Mandel, 1987) to the hostile environment of the stomach.

4.2.3.2. Saliva production

Human saliva is produced by three pairs of major salivary glands: parotid, submandibular and sublingual (Mandel and Wotman, 1976). Parotid saliva enters the oral cavity via Stensen's ducts. The parotid glands have the most serous and therefore least viscous secretion, while the secretions of the sublingual gland are more viscous due to a higher mucin content (see section 4.2.4.2). The submandibular glands, located at the midpoint of the mandible, secrete both serous and mucous saliva (Mason and Chisholm, 1975). The sublingual glands are located on the floor of the mouth under the tongue. Saliva from both the sublingual and submandibular glands enters the oral cavity via Wharton's ducts. There are also several minor salivary glands: (interior and posterior lingual, labial, buccal, molar, incisive and palatine glands) which collectively secrete approximately 8% of the whole mixed saliva that bathes the oral mucosa. (Saracco and Crabbins, 1993).

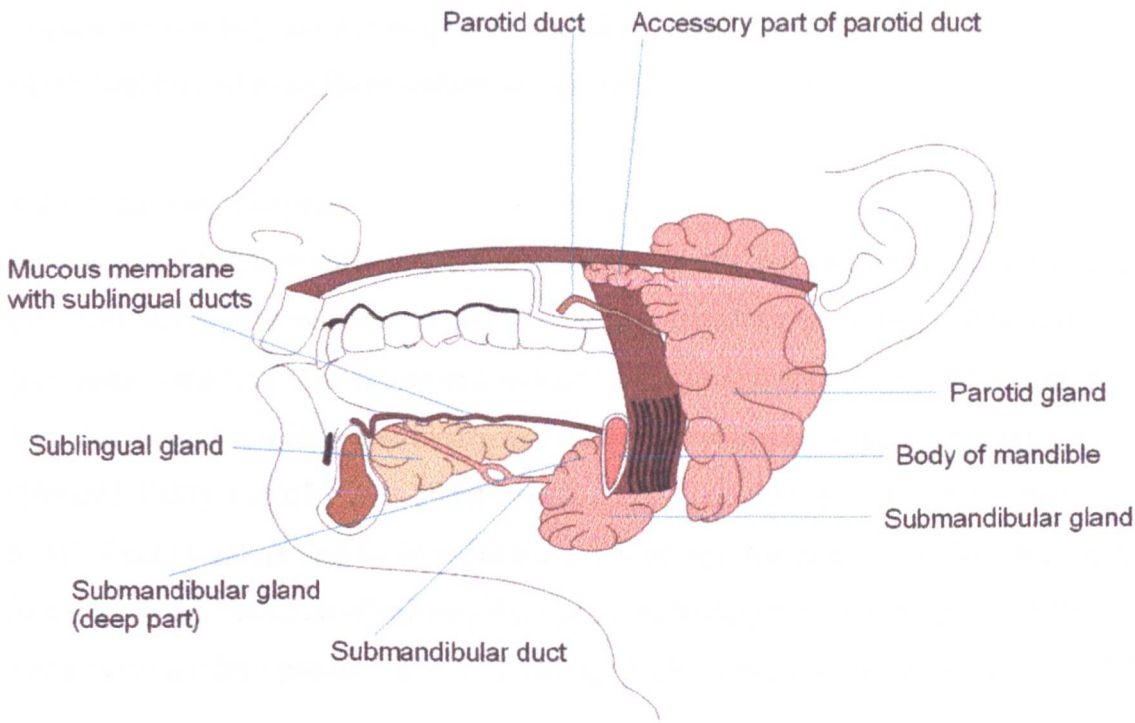

Fig. 4.1. Diagram of the three major saliva glands.

The average volume of saliva produced per day in a 70kg man is approximately 1000-1500ml (Best and Taylor, 1966). Ninety percent of this is from the parotid and submandibular glands in approximately similar proportions and the rest from the sub-lingual and minor saliva glands (Mandel and Wotman, 1976). At rest (without stimulation) the major salivary glands will produce approximately 0.05ml/min/gland but, because the salivary glands are almost constantly stimulated, (primarily by gustatory and masticatory stimuli), flow rate is typically 0.5ml/min/gland (Mandel and Wotman, 1976).

4.2.4. *Innate proteins*

Saliva contains several proteins, including enzymes, mucins, serum proteins (e.g. albumin and immunoglobulins), locally produced immunoglobulins and indigenous mucosal microflora. The indigenous microflora present within the mucosa make it ecologically difficult for other micro-organisms to colonise. The immunoglobulins present in the saliva will have a specific immunological action, however, the majority of the proteins present in saliva and linings of the upper respiratory tract are not capable of mounting a specific response. Nevertheless, the presence of any non harmful substances will form a physical barrier to pathogens by limiting the availability of 'space' within the oral cavity and particularly on the epithelial surface for colonisation. Some proteins within the upper

respiratory mucosa have a non-specific chemical effect upon pathogens, resulting in their neutralisation and inability to induce an infection.

4.2.4.1. Salivary Enzymes

Proteases and amylase are primarily to aid digestion of food, but their non-specific action can result in the destruction of pathogens with carbohydrate (amylase) and protein (proteases) moieties. Lysozyme is a mucolytic enzyme; it splits bacterial cell walls by hydrolysing glycoproteins containing muramic acid. It can also aggregate bacteria (Mandel, 1987). Lactoferrin is effective against bacteria that require iron for their metabolism by competing for iron-chelating molecules (Masson, 1966). Lactoperoxidase is involved in the oxidation of salivary thiocynate by hydrogen peroxide (produced by bacteria) to produce products that will damage both viruses and bacteria (Mandel, 1987).

4.2.4.2. Mucins

Mucins are complex hydrated gels composed of a variable group of glycoproteins, which form a protective layer over much of the respiratory tract, inhibiting the adherence of particles to the epithelial wall of the mucosa (Gibbons, Spinell and Skobe, 1976, cited in MacNabb and Tomasi, 1981), and give saliva its visco-elastic properties. They have also been demonstrated to neutralise (non specifically) influenza viruses and inhibit their haemagglutin activity (Shvartsman and Zykov, 1976), conferring extra protection from upper respiratory tract infections. The partial confluent film formed by the mucins in the respiratory tract is steadily moved along by ciliary action or fluid dynamics (MacNabb and Tomasi, 1981), therefore any particles larger than 2-3μm retained within the mucin layer are eventually removed by either cough, sneeze or ciliary action. However, if none of these removal mechanisms is effective, the particles may still enter the body (Bienenstock, 1984).

4.2.5. *Cellular*

In the lamina propria reside a number of phagocytic cells including eosinophils, granulocytes, and macrophages, along with natural killer cells which are capable of recognising, attaching to and destroying virally-infected cells. Within the lamina propria there are also the specifically acting T lymphocytes and a large number of plasma cells (Ernst, Befus and Bienenstock, 1985). Natural killer cells are believed to be a first line response until the antigen-specific immunity responds (Welsh, 1986 cited by Nieman et

al., 1990a). They have spontaneous cytolytic activity against virus-infected cells and therefore may be the most relevant non-specific activity against URTI, which are commonly virally induced (Reynolds and Merril, 1981).

4.3. SPECIFIC IMMUNITY

Unlike non-specific immune mechanisms, specific immunity is acquired following exposure to an antigen. After an initial interaction with an antigen, the immune system develops memory cells which instigate fast and specific defence mechanisms upon re-exposure, resulting in a quick and efficient disabling of pathogenic particles (Kuby, 1992).

4.3.1. *T- lymphoctyes*

At the mucosal surfaces, adaptive immunity is exerted mainly by locally produced and actively externalised sIgA and sIgM antibodies which after infancy quantitatively represent the most important humoral immune system of the body (Brandtzaeg, Baklien, Bjerke, Rognum, Scott and Valnes, 1987). However, if a virus is successful in infiltrating the initial barriers, locally situated T-cells (in the lamina propria) function to recognise and lyse virus infected cells (Eichmann, 1991). The T-lymphocytes are also important in activating the maturation of B-lymphocytes into immunoglobulin secreting plasma cells.

4.3.2. *Plasma cells*

The major production of the antibodies that appear in the external secretions occurs in the plasma cells located in the submucosal tissues (Crabbe, Carbonara, and Heremans, 1965). The plasma cells of the respiratory tract are located in the mucosa and the submucosa of the nose, trachea and bronchus, and are present in the greatest concentrations in the main bronchus and the upper trachea (Shvartsman and Zykov, 1986). A plasma cell will only secrete immunoglobulins of one isotype, and all plasma cells derived from a given B cell will only secrete antibody molecules with the same antigen-binding specificity. Predominately IgA producing plasma cells are found in the mucosa (Tomasi et al., 1965), hence the fact that sIgA is the predominant mucosal immunoglobulin, though there also are plasma cells committed to the production of immunoglobulins of other isotypes (sIgM and sIgG) (Selby, Janossy, and Jewell, 1971). Mucosal plasma cells are apparently unable to produce long-term memory cells, and therefore the immune protection afforded is short (Shvartsman and Zykov, 1976).

4.3.3. *Immunoglobulins*

Immunoglobulins are produced by plasma cells in the lamina propria of the mucosal tissue (Tourville et al., 1969). Antibodies are immunoglobulins that have a particular antigen binding capacity, and are believed to operate by agglutinating potential invaders and facilitating their removal via mucociliary clearance. In the respiratory tract, antibodies for a number of viruses known to cause upper respiratory tract infections have been identified: for influenza, parainfluenza, adenoviruses, rhinoviruses and poliovirus (Artenstein, Bellanti and Buescher, 1964). Immunoglobulins have been demonstrated to inhibit bacterial adherence (Williams and Gibbons, 1972) and to neutralise viruses directly without the help of complement (MacNabb and Tomasi, 1981).

Immunoglobulins were first identified in human saliva by Ellison, Mashimo and Mandel in 1960. Mucosal immunity is primarily humorally mediated by three immunoglobulins, IgA, IgM, IgG. IgG is present as a consequence of serum leakage and IgM and IgA are actively secreted in polymeric forms to protect them from destruction by salivary proteases.

Secretory antibodies are removed from the surface of the respiratory tract soon after they are formed. The latent period between immunisation and the appearance of the detectable secretory antibody is variable, but it tends to be longer than for a circulating antibody. This may be a consequence of the complexing antibody with its eliciting pathogen, therefore making it undetectable. (Shvartsman and Zykov, 1986).

4.3.3.1. Immunoglobulin G

Fluid obtained from the gingival surfaces of the oral mucosa is rich in IgG, which has been transported from serum (Reynolds and Merrill, 1981).

4.3.3.2. Secretory Immunoglobulin M

Like sIgA, sIgM is synthesised and secreted by plasma cells located in the mucosal lamina propria and is transported across the epithelial cell membrane by the same polymeric immunoglobulin receptor as is sIgA, the secretory component (Tomasi and Plaut, 1985).

4.3.3.3. Secretory immunoglobulin A

Immunoglobulin A is the major class of antibody in mucosal secretions. The prevalence of IgA in human parotid saliva, colostrum and lacrimal secretions was demonstrated by Chodirker and Tomasi in 1963, and a year later its predominance in nasal and bronchial fluids was also confirmed (Remington et al., 1964). Since then there have been many more investigations concerning isotypes, structure, function and the origin of salivary immunoglobulin. In 1981 MacNabb and Tomasi described the physical characteristics of the secretory IgA molecule. The secretory component is synthesised in the submucosal epithelial cells and binds to the polymeric IgA while it is being actively transported to the mucosal surface (Eskeland and Brandtzaeg, 1974). J-chain, synthesised within the plasma cell, remains attached to the penultimate cysteine residues of the α-chains (Mestecky, Schrohenler, Kulhavy, Wright and Tomana, 1974) and becomes incorporated into IgA just prior to secretion of the dimer from the plasma cell.

IgA exists in many polymeric forms. Two main subclasses of human IgA have been identified on the basis of antigenic determinants found on the α-chain (Delacroix, Dive, Rambaud, and Vaerman, 1982). IgA_1 predominates in the serum whereas IgA_2 predominates in mucosal secretions (Grey, Abel, Yount and Kunkel, 1968). However, in saliva IgA_1 constitutes 67% and IgA_2 33%. IgA_2 is more resistant to proteolysis by bacterial proteases (Mestecky and Russell, 1986). Although it is understood that the majority of salivary IgA is synthesised locally, the proportional contribution of serum IgA has not been established and is probably highly variable. Tomasi and Grey (1987) suggested that approximately 96% of IgA in saliva is synthesised locally while some may transudate. Sheldrake, Husband, Watson and Cripps (1984) suggested an inverse relationship between the extent of local production and the selective transport of IgA from the serum. Parkhouse and Corte (1973) reported that the major factor that limited the availability of polymer IgA was the availability of J-chain, and that if there was insufficient J-chain then monomer IgA would be secreted. Secretory IgA constitutes 32-34% of the salivary protein from unstimulated parotid and submandibular glands (Stone Cox, Valdimarsdottir and Neale, 1987).

4.4 THE EFFECT OF EXERCISE ON DEFENCE AGAINST URTI

The autonomic nervous system controls many aspects of airway function, influencing the secretion of mucous from the submucosal glands, transport of fluid across the airway epithelium, permeability of blood flow in the bronchial circulation and release of mediators from mast cells and other inflammatory cells (Barnes, 1986). Therefore, it is probable that the withdrawal of parasympathetic arousal and the concomitant increased sympathetic arousal during exercise would result in perturbations of immune defences in the upper respiratory tract.

4.4.1. Innate protection

4.4.1.1 Mucociliary clearance

Exercise results in an increase in the circulating levels of adrenergic and cholinergic hormones (Kjær, 1989), which increase cilia beat frequency (Olseni and Wollmer, 1992, cited in Müns, Singer, Wolk and Rubinstein, 1995) as part of a defence reaction to a perceived stressor. However, as previously described, particularly acute or chronic stress can impair defence mechanisms (see section 2.1.1), for instance strenuous exercise has been demonstrated to impair nasomucociliary clearance (Müns et al., 1995). This impairment was most prominent 24 hours after the exercise and took several days to return to baseline. They also observed an increased number of ciliated cells with non-motile cilia and dead ciliated cells up to 5 days after a race. These changes may have been caused by an increase and redirection in airflow, cooling of respiratory mucosa or altered mucosal blood flow. Beta-antagonists have been demonstrated to have no significant effect on mucociliary clearance in humans, suggesting an absence of sympathetic tonic secretion, at least via β-receptors (Pavia, Bateman, and Clarke, 1980). A change in ambient humidity at 23°C has been shown to have no effect on nasal mucociliary clearance, but any change in ambient temperature from 23°C was followed by a reduction in clearance (Proctor, Adams and Anderson, 1978). A decrease in mucociliary clearance would increase vulnerability to a pathogen.

Submucosal glands are supplied by both cholinergic nerves (Baker, Peatfield, Richardson, 1985) and adrenergic nerve fibres, with stimulation of either resulting in increased mucus secretion. Gallagher et al. (1975) demonstrated that vagal stimulation enhanced the volume

of respiratory mucus and the secretion of mucins. However, stimulation of adrenergic nerves results in a more viscous secretion probably because of selective stimulation of the mucus, rather than the serous, cells of the submucosal glands (Basbaum, Ueki, Berzina and Nadel, 1981). Barnes (1986) reported that β-agonists may increase mucus and water secretion into the airways, which should result in increased mucociliary clearance, although few studies have shown an improvement.

4.4.1.2. Saliva flow

Hydration, diet, antigenic stimuli, exercise (Edgar and O'Mullane, 1990), and circadian rhythms (Dawes, 1972), along with a variety of pathologic, psychological, and situational conditions, have been shown to cause variation in saliva flow rates and, as a consequence, the concentrations of salivary constituents including sIgA (Brandtzaeg, 1971).

Salivary flow and composition depends on neural and endocrine control (Levin and Khaikina, 1987). The cells of the salivary glands are supplied by portal circulation and are thus susceptible to the influence of circulating hormones. However, saliva secretion is believed to be principally controlled by the autonomic nervous system (Mason and Chisholm, 1975). There is extensive innervation of the salivary glands; and cholinergic, α- and β-adrenergic fibres have all been demonstrated to have a primary role in the regulation of secretion (Quissell, 1993). The roles of the sympathetic and parasympathetic nervous systems remain controversial (Saracco and Crabbins, 1993), although control of saliva flow does seem to be primarily parasympathetic. In the brain stem the superior and inferior salivatory nuclei are parasympathetic, and parasympathetic stimulation has a stronger and longer lasting effect than sympathetic outflow, increasing blood flow to the salivary glands by vasodilatation.

Sympathetic nerves innervate the blood vessels of the saliva glands rather than the glandular elements. Sympathetic stimulation causes contraction of the myoepithelial cells, reducing blood flow and subsequently saliva flow rate. The saliva secreted in response to parasympathetic stimulation is serous and contains few organic components and a high proportion of water, whereas under sympathetic stimulation the saliva secreted is more viscous and contains many organic components (Quissell, 1993). It thus seems possible that sympathetic control of saliva-gland function is principally aimed at controlling saliva composition, while parasympathetic control appears to be important in the control of saliva

flow. The secretion of many salivary proteins is stimulated by adrenaline and noradrenaline and inhibited by acetylcholine and substance P (Edgar and O'Mullane, 1990).

The exercise-induced increase in sympathetic and decrease in parasympathetic arousal is very well documented (Powers and Howley, 1990). It is therefore possible that physical activity would result in an increased production of viscous saliva and a decrease in saliva flow rate. Decreased saliva production has been observed in response to muscular activity, fatigue, sweating and dehydration (Alfonsky, 1961); states which often occur in exercise.

Bogdonoff, Bosnoff and Wolfe (1961) concluded that personality and the way in which a person responds to stress influences whether flow rate is increased or decreased in response to psychological stress. The sensation of a dry mouth when anxious is something that is familiar to many, yet Edgar and O'Mullane (1990) suggested that mental stress did not exert an important effect on saliva flow rate and composition. In an exercise scenario it is difficult to separate the effects of physical stress and dehydration from the effects of psychological stress, but it is probably the combination of them all that results in the oral drying that occurs with exercise.

During physical activity minute ventilation increases in order to satisfy the oxygen requirement of the active muscles; this requires a shift from nasal to oral breathing to reduce resistance to the flow of air. Mouth breathing results in oral drying despite normal saliva gland function (Quissell, 1993). It seems likely that exercise may induce the production of viscous saliva, and oral drying. A decrease in saliva flow or an increase in saliva viscosity would reduce the normal mechanical washing effect that saliva has. The likelihood of salivary proteins coming into contact with invading pathogens would also be decreased because the proteins would be trapped in a viscous layer, therefore being rendered relatively immobile.

Evans and Bristow (1993) investigated the effects of mood state and life-events on saliva flow and sIgA levels. A positive correlation between net positive events and sIgA secretion was found. SIgA concentration was inversely related to the total volume of saliva ($r = -0.47$). This suggests that saliva flow is an important consideration in studies assaying saliva constituents.

4.4.1.3. Cellular

The submucosal tissue cannot be observed without the use of invasive techniques and therefore all investigations into the effect of exercise on the cellular compartment of the immune system have addressed changes in the number and activity of cells circulating in the blood. Many of the changes in circulating cell number have been the result of changes in blood flow, and are therefore unlikely to reflect the cellular response in the mucosa. However, changes in functional activity may provide some indication of the effect of exercise on submucosal cellular activity. There is evidence to suggest that exercise increases cellular cytotoxic activity by increasing the number of natural killer cell and by a change of ratio of T-lymphocytes (see section 4.5.2.1). This increase in cytotoxic activity increases the capacity of the immune system to destroy virus infected cells and therefore may be vital if pathogenic particles successfully infiltrate the primary mucosal barriers.

4.4.1.3.1. Natural killer cells

Both maximal (Deuster et al., 1988; Lewicki et al., 1988) and submaximal (Berk et al 1990; Edwards et al 1984; Pedersen, Tvede and Christensen, 1989) exercise has been demonstrated to affect both the number and activity of natural killer cells. It appears that moderate exercise enhances defence mechanisms while chronic, or particularly intense, exercise results in a reduction in numbers or the functional activity of NK cells.

Targan, Britvan and Dorey (1981) demonstrated that after a bout of moderate exercise, NK cells have a transitory increased ability to kill target cells. Exercise has been demonstrated to increase NK activity. Crist, Mackinnon, Thompson, Atterbom and Egan, (1989) demonstrated an increase in NK cell activity in response to three months of exercise training, and Nieman et al. (1990a) reported that NK activity was increased after 6 weeks of exercise training but not after 15 weeks, however, NK activity rose in the control subjects over the 15 week duration of the study. Nieman et al.(1990) found that the increased NK cell activity after 6 weeks of training correlated with decreased URTI symptomatology during the entire 15 week study. The authors suggested that the decreased URTI symptomatology may be the result of a combination of effects of exercise on the immune system. Although moderate activity has been demonstrated to increase NK cell activity, exhaustive long endurance exercise has been demonstrated to result in a decreased NK number and activity (Mackinnon and Tomasi, 1986). This may go some way to

explaining the increased URTI seen in runners, although it does require further substantiation.

Many of the changes seen in NK cells are short-lived and little research has been carried out on the long term effects, although one cross sectional study by Pedersen, Tvede and Christensen, (1989) suggested that trained individuals have significantly higher NK levels than their untrained counterparts.

4.4.1.3.2. Phagocytes

Müns (1994) found that there was an increase in the number of neutrophils in the nasal lavage of runners after a long distance race, however, it was found that the ability of these cells to phagocytose was impaired. In 1996 Müns, Rubinstein and Singer found that, after a race, runners exhibited increased neutrophil, albumin, and total protein levels for up to three days into recovery, and suggested that this was part of an ongoing inflammatory process. This inflammatory process may result in symptoms that could be mistaken for URTI and it could increase susceptibility to URTI by impairing defence mechanisms through relocation of resources.

4.4.2. *Specific immunity*

Mucosal specific immunity is under the same influence as the non-specific defence mechanisms are: neural and endocrine control. As previously described, the function of the salivary glands is under autonomic (primarily parasympathetic) control, therefore the presence of humoral factors in saliva will be influenced by this even though secretory immunoglobulins are not directly secretory dependent proteins (Brandtzaeg, 1971). If there is very little saliva available or it is particularly viscous, immunoglobulins will be difficult to detect because they will remain attached to the mucosal surfaces.

Blood flow to the mucosal surfaces is also under autonomic control; it is probable that circulating levels of hormones and cytokines will influence the activity of submucosal cells but this is difficult to assess because of the invasive techniques required.

4.4.2.1 T-lymphocytes

There have been several studies that have reported a decrease in the ratio of T helper cells to T suppressor cells after both submaximal and maximal exercise (Berk et al., 1988; Espersen et al., 1989; Landmann et al., 1984; Lewicki et al., 1988). This may be indicative of some cellular controlling mechanism, where there is a reduction of cells that stimulate an immune response and an increase in those that suppress it. MacNeil, Hoffman-Goetz, Kendall, Houston and Argumugam (1991) found that T-cell mitogenesis was decreased after an ergometer ride, which would correspond with the findings of other authors that exercise down-regulates T lymphocyte responsiveness. The direct implications of these findings for T lymphocytes in the upper respiratory mucosa is unknown and therefore the exact clinical significance of this remains unclear.

4.4.2.2 Immunoglobulins

The immunoglobulins of the upper respiratory tract are much easier to measure because they are externalised from the mucosal tissue into the mucosal fluids. The diluent effect of the mucosal fluid must be considered when assessing concentrations of immunoglobulins; Secondly immunoglobulins may become trapped in particularly viscous fluids and therefore removed by mucociliary clearance. A tertiary consideration may be that if there is very little mucosal fluid available then the immunoglobulins may remain on the mucosal surface and remain undetectable.

Data available on the effect of exercise on salivary immunoglobulins suggests that their effect is specific to the secretory immunoglobulins and may therefore be the result of exercise-induced changes across the submucosal epithelium. Mackinnon et al. (1993a) measured the response of other secretory immunoglobulins, IgG and IgM, to intense interval exercise. The exercise had an IgA specific influence; no changes in the other secretory immunoglobulins were found. Mackinnon et al. (1989) demonstrated a decrease in secretory immunoglobulins (sIgM and sIgA) after two hours of cycle ergometry; no change in salivary IgG or serum antibodies was found. This suggests a mechanism of suppression specific to the secretory immunoglobulins. Similar data were produced when sIgA and sIgM were reduced after brief, supramaximal interval exercise, whereas IgG had not changed relative to total protein (relative to total protein is believed to correct for oral drying) (Mackinnon and Jenkins, 1993). In addition to this they also found that there

appeared to be a specific effect of intense exercise on sIgA concentration, greater than that due to decreased saliva flow alone.

Much of the work available investigating the increased susceptibility of athletes to URTI has focused upon sIgA, because it is the primary mucosal antibody and because its levels have been demonstrated to decrease in response to all types of stress including exercise.

4.4.2.2.1 Immunoglobulin A

Secretory IgA in the nose and oropharynx is often studied because it the primary mucosal immunoglobulin and because of its purported role in combating viral infections. IgA has been shown to correlate more closely with resistance to URTI than serum antibodies (Pedersen and Bruunsgaard, 1995).

The effect of participation in a variety of sports on sIgA levels has been investigated (Mackinnon et al., 1993a, b; Tharp, 1991; Tharp and Barnes, 1990; Tomasi et al., 1982). In 1982 Tomasi et al. investigated the effects of cross-country skiing competitions on sIgA levels. They demonstrated that the elite athletes had significantly lower sIgA levels than age-matched controls, and that levels of sIgA were shown to decrease even further after competition. It was suggested that these decreases were a consequence of the loss of mucosal fluid, and possibly a malfunction of mucosal immune cells as a result of the low temperatures. Since then several studies have reported decreases in sIgA after exercise (MacDowell et al., 1992a; Mackinnon et al., 1989; Tomasi et al., 1982).

Swim training has also been shown to result in reduced sIgA levels. Tharp and Barnes (1990) observed in swimmers that acute bouts of exercise can reduce sIgA levels, the largest decrease being seen after moderate training as opposed to light or heavy training. Chronic high-intensity swim training was also reported to reduce resting levels of sIgA. Mackinnon and Hooper (1994) commented that sIgA was lower in overtrained swimmers as opposed to well-trained swimmers over a season. Mackinnon et al. (1993b) demonstrated a decrease of 27% to 38% in sIgA levels of elite kayakers after three intense interval training sessions.

Increases in sIgA levels have also been observed with sports participation. Tharp (1991) found an increase in sIgA in junior basketball players after practice sessions and games, in comparison to their pre-exercise levels. Mackinnon et al. (1993c) demonstrated an

increase in sIgA levels after exercise in squash players. Not all of the squash team responded in the same way, and those who exhibited decreased sIgA were clinically diagnosed with an URTI within two days. This is possibly the first study to demonstrate a temporal relationship between changes in sIgA and URTI. The same study also observed hockey players and noted a decrease in sIgA. Individuals who developed an URTI had the greatest decreases in sIgA two days prior to symptom onset.

In order to try to quantify the amount of physical stress experienced, laboratory-based tests have been carried out. Schouten et al. (1988a) investigated the effects of a treadmill $\dot{V}O_{2\,max}$ test on stimulated salivary IgA. They observed a decrease in sIgA in women and an increase in men. MacDowell et al. (1992a) demonstrated a mean decrease of 24.4% in unstimulated sIgA levels after a treadmill $\dot{V}O_{2\,max}$ test. The depressed sIgA levels were still apparent one hour post exercise cessation (16.9%). However, five of the twenty-nine subjects in this study actually had increased sIgA levels after the test, ranging from 1.3-54.3%. Although the authors focussed upon the depressed sIgA levels, it is important to note that exercise does not always result in a decrease in sIgA levels in all individuals. Exhaustive exercise theoretically ensures that individuals are exposed to the same amount of physical stress, so that the differences in the immune response to the same task seen between individuals is possibly a consequence of psychological influences or other factors. The level of familiarity with the task may influence perceived stress, and consequently the type and extent of immunomodulation, therefore it is important to take such factors into account when investigating the effect of exercise on sIgA.

Mackinnon and Hooper (1994) compared the effects of a 40 minute run at 55% and 75% $\dot{V}O_{2\,max}$ on recreational joggers, and running at the same intensity but for 90 minutes on competitive runners. The sIgA secretion rate did not change significantly for either group after any bout of exercise. They also tested the competitive runners on three consecutive days running at 75% $\dot{V}O_{2\,max}$ for 90 minutes. The secretion rate of sIgA decreased after each bout, and decreased further after the second and third bouts, demonstrating a cumulative effect of the stress. Heath et al. (1991) attempted to address the effect of environmental stress and exercise on sIgA. It was found that running at 80% $\dot{V}O_{2\,max}$ at 6°C, 19°C or 34 °C did not alter sIgA levels. Similar temperatures have, however, been found to have an impact on other aspects of mucosal immunity (Proctor et al., 1978).

There is a shortage of laboratory-based data on anything other than running. Mackinnon et al. (1989) demonstrated that after cycling for two hours at 90% of anaerobic threshold, salivary IgA (relative to total salivary protein) was decreased one hour post exercise, but was above baseline levels 24 hours post. Nasal IgA, which was collected by washing of the mucosal surfaces, remained unchanged. This may have been a consequence of the dilutional effects of collection. This task was intended to eradicate psychological stress by removing the factor of competition. It is, however, conceivable that 2 hours of ergometer cycling in a laboratory environment induced some psychological stress.

Mackinnon and Jenkins (1993) investigated the effects of five 60 second bouts of supramaximal interval cycling. Salivary IgA and IgM (relative to total protein) decreased after each bout; sIgG did not change. This protocol was repeated 3 times a week for eight weeks and no changes in the resting levels in any of the measured immune parameters was observed. These authors concluded that apparent decreases observed in sIgA and sIgM were due in part to decreases in saliva flow rate.

4.5 SUMMARY

There are a number of defence mechanisms protecting the upper respiratory tract from infection. These include both specific and non-specific factors. It has been demonstrated that many of these factors are affected by exercise (see section 4.5), and mechanisms for these exercise-induced perturbations have been proposed. For example, Müns Singer, Wolk and Rubinstein (1995) suggested that an observed impairment in mucociliary clearance in response to exercise was probably the result of a redirection of air flow, cooling of the respiratory mucosa, or an altered mucosal blood flow. The increased ventilatory rates and the evaporation of fluid from the mucosa probably also have a role to play.

For many of the exercise-induced changes observed, the mechanisms are poorly understood although changes in mucosal blood flow, circulating hormones and cytokines, and an increased sympathetic outflow (all of which occur during exercise) are probable factors. However, regarding the increased incidence of infection within the athletic population, perhaps the primary consideration should be the clinical significance of these changes and whether or not they increase susceptibility to URTI. There has been little work done to address this question. Mackinnon et al. (1993c) found a temporal relationship between exercise-induced decreases in sIgA and have demonstrated the appearance of URTI in athletes. Absolute IgA concentration was decreased in athletes who developed URTI within two days. Rossen et al. (1970) demonstrated that volunteers with high initial concentrations of IgA synthesised secretory antibodies more quickly in response to a challenge and therefore those athletes found to have chronically suppressed levels may be slower to respond to an antigenic challenge, thus allowing enough time for an infection to become established.

Secretory immunoglobulin A has been the most extensively-studied upper respiratory tract defence mechanism, the reasons being that mucosal immunity is primarily humorally mediated, and secretory IgA is the principal mucosal immunoglobulin. It is also easy to monitor compared to other defence mechanisms, because of its accessibility via the mucosal fluids. There are also a number of commercially available assays for sIgA quantification. Unlike other defence mechanisms a link between changes in sIgA levels

and incidence of URTI has been found, but this could be a consequence of its popularity as a postulated marker of exercise -induced immunomodulation.

Despite the large number of studies that have been carried out, there is still broad disagreement regarding the reported responses of sIgA to exercise. This is probably a consequence of the fact that the saliva has been collected by a variety of different techniques, over a broad spectrum of times, and a range of quantitative techniques have been used, analysing either whole saliva or saliva collected directly from a saliva gland. Therefore these and other methodological issues need to be addressed before any conformity of opinion can be expected.

Levels of sIgA have been reported in terms of concentration and in terms of secretion rate (the product of concentration and saliva flow rate). Both methods have their problems: concentration does not account for the diluent effects of increased saliva flow, and secretion rate takes into account changes in saliva flow rate to the same extent as changes in sIgA concentration. Reporting sIgA in terms of secretion rate may provide a more complete picture of the defences in the oral mucosa. However, it may not provide a good indicator of changes in specific immune function [i.e. in the production of sIgA by submucosal plasma cells]. There is a need to investigate further the relative impact of saliva collection method and saliva flow rate on calculated sIgA levels before conclusions can be made regarding the role of sIgA in terms of an increased susceptibility to URTI. As reviewed in this chapter there are also many other factors that have a role in the protection of the upper respiratory tract which warrant further consideration.

PART

II

CHAPTER 5

THE COLLECTION OF SALIVA: PROBLEMS AND ISSUES

5.1 INTRODUCTION

Saliva is increasingly being used as a sample for biochemical assessment (Miller, 1993). Collection of saliva has the advantage of being non-invasive and it contains several substances that may be detected or quantified in the saliva as an alternative to blood. Several methods have been used for saliva collection, and it has been reported that the method employed can influence the assessment of salivary IgA levels (Aufricht et al., 1992). If saliva is to be used as a valid clinical and research tool then it is imperative that it is collected reliably. If meaningful comparisons are to be made then there must be standardisation of collection method. There are four primary considerations when choosing an effective saliva collection method:

* whether to collect whole saliva or the saliva of a particular gland.
* whether to stimulate saliva flow.
* how to measure saliva flow rate.
* how to minimise the debris in the sample.

5.1.1. *Whole saliva?*

Whole saliva is a mixture of the secretions of several salivary glands, gingival fluid, cellular debris, and micro-organisms. Stone et al. (1987) suggested that for more accurate estimates of concentrations of IgA, saliva should be collected directly from the major salivary glands (e.g. the parotid glands). Some authors have justified their use of parotid saliva for sIgA analysis because, unlike whole saliva, it has little proteolytic activity and will not break down the proteins to be assayed (Mackinnon et al., 1989). Parotid saliva is a serous secretion, containing little mucus and therefore does not interfere with the absorbance readings on which many sIgA assays rely.

Collection of parotid saliva has been carried out using the Curby cap (Curby, 1953) to collect saliva from the parotid gland (Atkinson et al., 1990; Mackinnon et al., 1989; Tomasi et al., 1982). The saliva from the submandibular and sublingual glands can also be collected separately, although immunoglobulins are secreted in smaller quantities from

these glands. The oral mucosa is not protected exclusively by sIgA from one type of gland, as immunoglobulins from all glands contribute to the mucosal immune system. Jemmott and McClelland (1989) argue for the collection of whole saliva to gain a complete picture of mucosal immunity.

Collection of whole saliva by asking subjects to dribble passively into a collection vial has been commonly used (Green, 1988; Housh et al., 1991; MacDowell et al., 1992a, b; Mackinnon et al., 1993a; Tomasi et al., 1982). However, there have been reports of subjects being reluctant to do this, finding it embarrassing. A fairly new, more discreet and increasingly popular method is to place a cotton wool or polyester swab in the oral cavity to absorb the saliva. This has been developed by Starstedt (Leicester, UK.) to produce the salivette, which is a swab in a container. The containers are sectioned so that when centrifuged the saliva collects in the bottom and is completely separated from the swab (see fig 5.1).

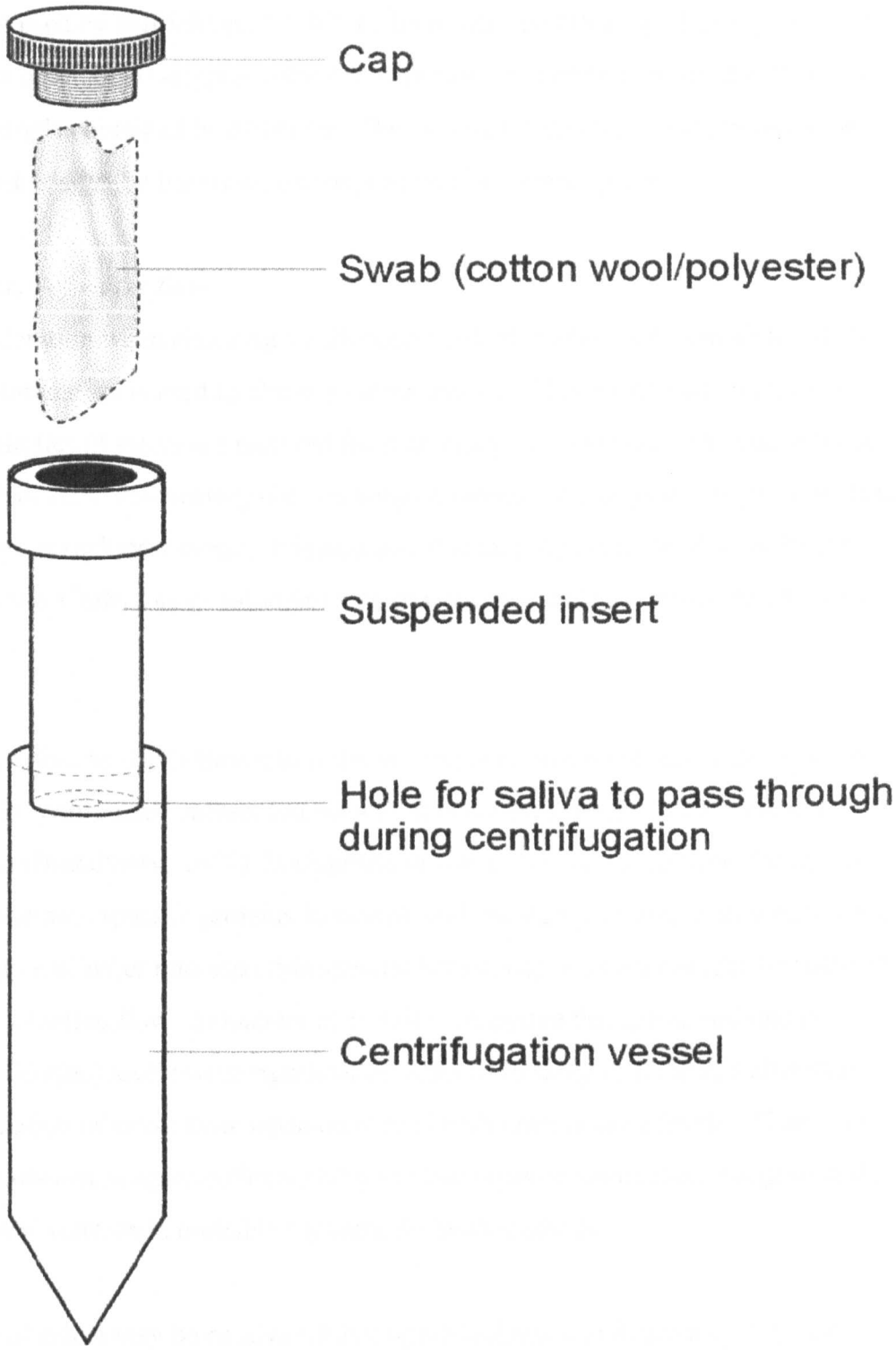

Fig 5.1 Diagrammatic representation of a salivette

Salivettes were used by Aufricht et al. (1992) who commented that significantly lower IgA levels had been obtained in samples collected with salivettes when compared with results gained from samples obtained by dribbling. This is possibly a consequence of retention of some saliva and/or sIgA by the swab, and requires further investigation.

5.1.2. *Stimulation of saliva flow?*

Another consideration when choosing a collection method is whether to stimulate saliva flow. Saliva stimulation is used to obtain a larger sample. This is often unnecessary as very small quantities of saliva are required for sIgA analysis. There are two main ways of stimulating saliva flow: masticatory (i.e. chewing of rubber, wax or gum); or gustatory (i.e. citric acid drops or sucking sweets). It is also possible that the presence of something in the oral cavity (i.e. Curby cap or salivette) may provide mechanical stimulation of saliva flow.

Stimulation of saliva has been shown to influence the composition of saliva, and it seems that sIgA may be particularly influenced because it is not transported like a secretion specific protein (Brandtzaeg, 1971). In response to stimulation of saliva flow, the content of water and secretion specific proteins increases; and the sIgA concentration is decreased. The concentration of other non-secretion specific substances in saliva can also be reduced by stimulation of saliva flow. Schneider et al. (1997) reported that saliva cotinine (a metabolite of nicotine) levels were significantly reduced in samples collected after sugar and wax stimulation of saliva flow when compared with unstimulated levels. There was no difference between samples collected after the two types of stimulation, suggesting that the mechanism of dilution is probably the same for both methods.

Expectoration of saliva may be passive (dribbling) (Mackinnon and Jenkins, 1993) or forced (spitting) (Schouten et al., 1988). It is possible that forced expectoration might mechanically stimulate the saliva glands and therefore increase water flow and therefore the transport of some secretion specific proteins but not sIgA. Samples collected by spitting have been found to have the highest salivary flow rates and the lowest sIgA levels (Aufricht et al., 1992).

To avoid the interference of stimulation with sIgA levels, it may be important to collect unstimulated samples. This may require asking subjects to refrain from eating or drinking

anything for a time before testing (Rudney, Krig, Neuvar, Soberay and Iverson, 1991). Salivary flow is influenced by a number of factors including food ingestion, sensory stimulation, drugs, smoking, body positioning, stress and an individual's hydration status (Dawes, 1993; Navaesh, 1993). Additionally the sIgA concentration in saliva may be affected by dietary factors, daily mood and intense physical activity (Stone et al., 1987). All of these possible variables need to be controlled when collecting samples for biochemical analysis, to avoid any confounding influence on the results.

5.1.3. *Measurement of saliva flow rate*

Whole unstimulated saliva is most commonly collected by asking subjects to expectorate until a certain volume has been obtained. The volume required has varied: from 2 ml (Olness et al., 1989), 3 ml (McClelland et al., 1985; Tharp and Barnes, 1991) 5ml (Rudney, 1991) to 8 ml (Jasnoski and Kluger, 1987). Saliva flow rate is calculated by dividing the volume produced by the time taken to produce the required volume. However, saliva is not produced nor secreted in aliquots and therefore it is unlikely that the exact volume is expectorated, which would result in erroneous calculation of saliva flow rate. Determination of flow rate by fixing the time of collection, and weighing the collection vessel pre and post sampling, may improve the accuracy. Mackinnon et al. (1993 a, b, c) and Green and Green (1987) have used four minute collection times, Jemmott et al. (1983) and MacDowell et al. (1993) used five minute collection times. Providing sufficient saliva is collected for analysis, the length of time is not vital but collection times should ensure that sufficient saliva is collected from individuals with particularly low flow rates. The reduction in saliva flow rate that occurs in response to dehydration and sympathetic arousal should also be taken into account.

5.1.4. *Reduction of sample debris.*

Assays for sIgA often rely on an absorbance measurement, and therefore the presence of any debris in the saliva could produce falsely-high absorbance readings. Some authors have attempted to minimise the problem of excessive debris in whole saliva by asking the subjects to rinse their mouths out with water prior to the onset of collection (Housh et al., 1991; MacDowell et al., 1993; Tharp and Barnes, 1991; Schouten et al., 1988a). Navazesh (1993) strongly advocated rinsing of the mouth with deionized water before the collection, to void the mouth of saliva. This is intended to remove any food particles and large protein particles that may be in the mouth. However, swilling the mouth out, particularly

if the water is cold, may result in some gustatory stimulation, and retention of some of the water in the mouth may result in dilution of any proteins present.

Removal of debris after the sample has been collected is often done by centrifugation (Bennet and Reade, 1982; MacDowell et al., 1992a; McClelland et al., 1985), with the resulting clear supernatant being used for analysis. Mandel and Wotman (1976) insisted that centrifugation was essential to remove extraneous material from the sample, and even then the sample may remain contaminated with soluble non-salivary components. Filtering of the sample would serve the same purpose and has been used by some authors (Tomasi et al., 1982; Mackinnon et al. 1989; 1993a). These methods may introduce variable loss of IgA, though opinions of the impact of this loss on sIgA levels differ. Akerlund, Hanson, Alstedt and Carlsson. (1977) reported that centrifugation resulted in a 14 to 30% loss of salivary IgA, but others have reported it to have negligible impact (Kraus and Sirinha 1962). Centrifugation may result in warming of the sample, resulting in increased protein degradation; however, it is possible that this could be minimised with the use of a refrigerated centrifuge.

5.1.5. *Issues to be addressed.*

Collection of whole unstimulated saliva has been demonstrated to have the least influence on concentration of salivary proteins (Aufricht et al., 1992), and has been used by authors of a wide range of studies on sIgA (Jasnoski and Kluger, 1987; Jemmott et al., 1983; Mackinnon and Jenkins, 1993; Mackinnon et al., 1993 a, b, c; Olness et al., 1989). The most simple method for collection of whole unstimulated saliva is passive dribbling. Salivettes were initially only available in cotton wool but, after concerns over proteins being trapped in the wool fibres, polyester salivettes are now available. It is necessary to establish the relative merits and limitations of the dribbling and salivette collection methods before an informed choice of saliva collection method can be made.

The chapter describes the investigations to determine:

* whether the variability in saliva flow rate and saliva protein concentration is affected by the collection method used;
* the maximum saliva absorption capacity of salivettes, and what maximum collection time this dictates;
* whether the salivettes retain saliva after centrifugation.

If the collection method affects the sample contents, particularly those which are to be assayed, then this must be established along with the regions of error introduced and the possible impact which this may have upon the sample. Studies examining sIgA concentration or secretion rate often collect saliva across a time course which may be within one day or across a few days. Therefore this study will also investigate the variability in saliva flow rate and saliva protein concentration within one day and across a period of ten days. For the purposes of this study whole saliva, without gustatory stimuli, will be collected in order to avoid any effect that these stimulants may have on either saliva flow rate or protein concentration.

5.2. THE VARIABILITY IN SALIVA FLOW RATE AND TOTAL PROTEIN LEVELS OF SALIVA SAMPLES COLLECTED BY TWO METHODS.

The hypotheses tested within this section (1-9) are listed in appendix 1.

5.2.1 Method

5.2.1.1 Subjects

Informed consent was obtained from twelve subjects (9 male, 3 female; age (mean ± SD) 30.0 ± 5.8 years), all of whom believed themselves to be free from oral cavity and upper respiratory tract infections. For the duration of the experiment subjects were asked to maintain hydration status, to drink a pint of water before going to bed each night, and to refrain from consuming alcohol. All were non-smokers.

5.2.1.2 Timing of samples

Subjects were asked to give saliva samples by two collection methods for ten consecutive days. This was done on waking in the morning, to avoid circadian variation. The method used first for saliva collection was rotated every other day (i.e. there were five days when each method was used first), to avoid order effects.

Another ten samples were also collected consecutively within one day during the ten day collection period. This was done twice (once for each collection method) at the same time of day one week apart, thus hopefully reducing circadian variation. Subjects were given a two minute break between samples, and asked to rinse their mouths out before each sample. Subjects were asked not to eat or drink anything except water for an hour prior to commencing sampling. When requested, drinks of water were given during the two minute rest periods.

5.2.1.3 Saliva collection methods

Two methods were compared; passive dribbling and salivettes [cotton wool salivettes (n=4), and polyester salivettes (n=8)]. Saliva was collected for four minutes by each method. Before giving a sample, subjects were asked to rinse their mouths out with water, and having spat this out, to swallow all of the remaining fluid in their mouth. At this point the saliva collection began.

For the passive dribbling method, subjects were asked to eject any fluid in their mouth into a pre-weighed 5ml plastic vial (Medfor, UK). At the end of 4 minutes any fluid present in the oral cavity was expectorated into the vial, and the vial was then stored at -20°C.

For the salivette method the salivettes (Starstedt, Leicester, UK) were weighed with Ohaus E120 weighing scales (cv=0.09%), to gain a pre-sample mass for both the whole salivette and the swab. Subjects were asked to place the swabs on the left hand side of the oral cavity (to maintain consistency of position) at the start of the four minute collection period. The swabs were removed from the mouth at the end of four minutes, and placed back in the salivette container. The salivettes were stored at -20°C.

5.2.1.4 Determination of saliva flow rate

The saliva samples were defrosted at room temperature. The vials containing the samples were weighed on Ohaus weighing scales. The pre-sample mass was subtracted from the post-sample mass in order to obtain the mass of the saliva collected. A specific gravity of 1 was assumed (Kerr, 1961; Schouten et al., 1988) and therefore a mass increase of 1 gram was considered to be equivalent to 1ml of saliva collected.

The salivettes were centrifuged for 15 minutes at 2000 r.p.m. in a refrigerated centrifuge (@5°C) (800 series, Centurion Scientific Ltd., UK). The mass of the saliva sample was calculated by subtracting the pre-sample mass of the whole salivette.

5.2.1.5. Determination of total salivary protein concentration

The total protein concentration of the saliva was determined by optical density at 280nm using a Shimadzu spectrophotometer. Saliva was poured into a microcuvette (Hughes & Hughes, UK) and the optical density was read against a blank of distilled water.

5.2.1.6. Statistical analysis

5.2.1.6.1 Saliva volume

Mean saliva flow rates were calculated for each subject; for the saliva collected by the dribbling method; for the saliva absorbed by the salivettes; and for the saliva collected after centrifugation. Each of these saliva volumes was calculated twice per subject; the mean for the samples collected within one day (intra) and the mean of the samples collected across ten days (inter). The standard deviations around the saliva volume means were also calculated.

In order to gain an index of variability, the standard deviations of the individual means for saliva flow rate were averaged, to produce a group mean for standard deviation in saliva flow rate for samples collected by dribbling, saliva absorbed by salivettes and the saliva collected after centrifugation of the salivettes were calculated.

A two-way repeated measures ANOVA (method x day) was carried out to investigate if there was any difference in the volume of saliva collected by dribbling method, that which was absorbed by the salivette and that which was collected from the salivette after centrifugation (method 3 levels). The second factor, day, was to compare between the volume of saliva in samples collected within a day (intra) and those collected across ten days (inter). This two-way ANOVA also investigated if there was any interaction between these two factors.

Another two-way repeated measures ANOVA (method x day) was carried to see if the variability in the volume of saliva collected was different between collection methods (as described above) or between samples collected within a day(intra) or over ten days (inter), and to see if there was an interaction effect of method and day on variability of the volume collected.

The appropriateness of coefficient of variation as an expression of variability for saliva volumes collected, was tested by plotting saliva volume against the standard deviations for pooled data. Coefficient of variation assumes that the variability is proportional to the mean. Therefore lack of a positive relationship between the mean and the standard

deviation would indicate that it is not appropriate to use coefficient of variation as an expression of variability.

In order to investigate whether variation in saliva flow rate was as high within a subject as between subjects, a Levene's homogeneity of variance test was carried out on randomly selected samples from ten subjects on one day, and ten samples collected from one subject on one day.

A Pearson's product moment correlation was carried out to investigate the relationship between the volume of saliva collected by the dribbling method and the saliva absorbed by the salivettes during the 4 minute collection time (data were pooled for samples collected within and between days, n=24).

5.2.1.6.2 Total protein levels

The group mean and the standard deviation around this mean were calculated for the optical density values achieved for analysis of the total protein levels of the samples. These values were calculated for samples collected both within one day (intra) and over ten days by both the dribbling and the salivette method. A Student's paired t-test was carried out on the available paired data to investigate the difference in the total protein levels in samples collected by either dribbling or salivettes.

5.2.2 Results

5.2.2.1. Saliva flow rates.

The saliva flow rates for the dribbling and salivette methods, both within one day and across ten days, are presented in Table 5.1.

Table. 5.1. Mean Saliva flow rates and variability in saliva flow rate for samples collected by two methods, within one day (intra) and across 10 days (inter).

METHOD	SALIVA FLOW RATE (ml per 4 min)		VARIABILITY IN SALIVA FLOW RATE (ml per 4 min)	
	Intra	Inter	Intra	Inter
Passive dribbling	1.67 ± 0.75	1.1 ± 0.65	0.450 ± 0.19	0.432 ± 0.43
Salivettes (Saliva absorbed)	1.05 ± 0.65	0.73 ± 0.29	0.280 ± 0.28	0.341 ± 0.25
Salivettes (Saliva collected)	0.56 ± 0.49	0.47 ± 0.23	0.250 ± 0.10	0.324 ± 0.22

Data are mean ± SD.

A two-way repeated measures ANOVA (method x day) revealed that there was a significant interaction between the method of collection (3 levels; saliva collected by dribbling, saliva absorbed by the salivette, saliva collected after centrifugation of the salivette) and whether collected on one day or across ten days (2 levels; inter and intra day) ($F_{(2,14)}$=18.72, $p<0.001$). There was no main effect for (day) whether the saliva was collected on one day (intra) or across ten days (inter) (($F_{(1,7)}$=1.93, $p>0.05$). There was a however, a main effect for how the saliva was collected (method) ($F_{(1,7)}$=12.75, $p<0.01$). It is evident from table 5.1. that the volume of saliva collected was largest for passive dribbling, while the amount of saliva absorbed by salivettes was lower, suggesting that the salivettes limit the amount of saliva collected (possibly by a limiting absorption rate or maximal absorption capacity); this is discussed further in section 5.3. The smallest

volumes of saliva were collected after centrifugation of the salivette, suggesting that the salivettes are retaining some of the sample. This retention is discussed further in section 5.4.

A second two-way repeated measures ANOVA (method x day) for variability in the volume of saliva collected revealed that there was no significant interaction between the way in which the samples were collected and when they were collected ($F_{(2,16)}$=0.55, $p>0.05$). Nor was there any significant main effect for the method of collection ($F_{(2,13)}$=2.75, $p>0.05$). No main effect for day was found either ($F_{(1,8)}$=1.25, $p>0.05$). Therefore the variability in the volume saliva collected was the same regardless of how it was collected and whether it was collected over ten days or within one day.

It appears from the results presented in table 5.1. that if the variability was reported in terms of coefficient of variation (standard deviation as a percentage of the mean) then the difference between the methods would be more apparent. However, the use of coefficient of variation as an of expression of variability assumes that variability is proportional to the mean. This assumption was tested by plotting the saliva volumes (pooled data, n=72) against the standard deviations. The relationship was found to be negative with an r^2= 0.073. Therefore no relationship between saliva volumes and the standard deviations around their means exists for this data, and coefficient of variation cannot be used to express variability for this data.

A Levene's homogeneity of variance test revealed that there was no difference in the variability of ten samples collected from one subject on one day compared with randomly selected samples from ten subjects collected on one day ($F_{(1,8)}$=2.39, $p>0.05$); both sets of samples being collected by the dribbling method. This suggests that the variability in saliva flow rate is the same within subjects as it is between subjects.

Both the dribbling and salivette method were used to collect saliva for the same period of time, the same time of day, in the same subjects. Therefore a very strong positive relationship between the results would be expected. This relationship was tested with a Pearson's product moment correlation. The results are plotted in figure 5.2. Surprisingly no relationship was found to exist between the dribbling and salivette saliva flow rates.

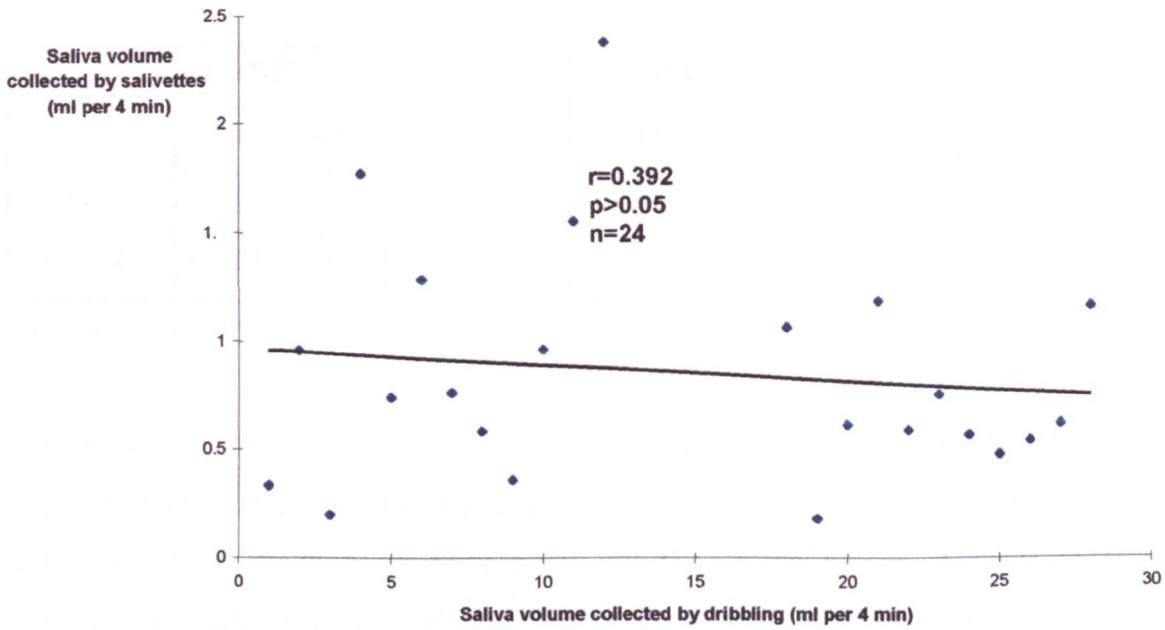

Fig. 5.2. The relationship between the saliva flow rates of samples collected by passive dribbling and those collected by salivettes

5.2.2.2. Salivary total protein.

Total saliva protein was measured using optical density. Not all of the samples were large enough for total protein levels analysis; this was particularly true of the samples collected by salivettes. The mean optical density readings are presented in Table 5.2 below. The number of samples assayed per method is supplied with the results; this is out of a potential 120 (i.e. 10 samples per subject for 12 subjects).

Table 5.2. Total protein levels of saliva samples collected by two methods, within one day (intra) and across ten days (inter).

	DRIBBLING		SALIVETTES	
	Mean ± SD	n	Mean ± SD	n
INTRA	0.24 ± 0.13	88	0.12 ± 0.13	47
INTER	0.274 ± 0.13	49	0.06 ± 0.06	40

Data are ± SD.

Because a lot of the samples were too small for the analysis of optical density, only 58 sets of paired data were gained. The mean optical density values for samples collected by the dribbling method, the mean of samples collected by salivettes and the standard deviation around these means were also calculated and are presented in table 5.3. The difference between the optical densities of saliva collected by the two methods was calculated and the mean and standard deviation around this mean are also presented in table 5.3.

Table 5.3. The mean total protein levels (as measured by optical density) for samples collected by two methods (n=58).

	DRIBBLING (OD@280nm)	SALIVETTES (OD@280nm)	DIFFERENCE (dribbling-salivette) (OD@280nm)
MEAN	0.24 ± 0.11	0.11± 0.13	0.13 ± 0.19

Data are mean ± SD.

A Student's paired t-test was carried out on the available paired data (n=58) and revealed the total protein concentration of the saliva collected by the dribbling method was significantly higher ($T_{(1,57)}=5.047$, $p<0.0001$) than that which was collected by the salivette method.

5.2.3. *Discussion*

A two-way repeated measures ANOVA (method x day) revealed that the volume of saliva collected using the dribbling method, that which was absorbed by salivettes, and that which was returned after centrifugation of the salivettes were all different from one another. The mean volume values (table 5.1.) demonstrate that the greatest volume of saliva was collected by the dribbling method (hypotheses 4& 5, appendix 1). The difference between the amount of saliva collected by the dribbling method and that which is absorbed into the salivette is probably a consequence of the salivettes not absorbing all the saliva in the oral cavity. This may be the result of a limiting absorption rate of the salivettes allowing saliva to pool without being absorbed during the collection time. It may also be the outcome of the salivettes having maximal absorption capacity; so that after a certain point of saturation they cease to absorb any more saliva. These issues of a limiting absorption rate or capacity of salivettes and the impact on this saliva analysis required further investigation and more extensive studies are reported in section 5.3.

The lower volume of saliva collected with salivettes could also be a problem of the positioning of the salivette. In order to maintain a consistency of position the subjects were asked to place the salivette in the side of the mouth. This would mean that saliva forming in the other side of the oral cavity and under the tongue would not be readily absorbed. There is a need for subjects to keep the salivette still in order to reduce the danger of any saliva being squeezed out of the swab. There is a need to maintain a consistency of position in order to reduce positional effects on the type of saliva collected. If the swab were positioned in the cheek for the first sample and under the tongue for the second, then the first sample would be a more serous secretion. Additionally, if the swab were moved around the mouth and kept in different positions in the mouth for different periods of time, different types of secretion may be collected, making intra as well as inter subject samples non-comparable. When using salivettes, it is difficult to collect a sample

that is representative of whole saliva, and it is also difficult to collect all of the saliva which may form in the mouth during the collection time. This problem could be reduced by using two salivettes, one either side of the mouth, however, this would double expense, might be uncomfortable, and would probably stimulate the saliva glands, and fails to solve the problems with the type of saliva collected. Moreover the position of the salivettes will still mean that it is likely that predominately parotid saliva would be collected.

The saliva volumes collected after centrifugation were found to be lower than the volumes absorbed (table 5.1.) (hypothesis 6, appendix 1); which suggests that the saliva has been 'lost' somewhere and the only logical explanation is that the salivette swabs retains some of the sample even after centrifugation. Although optical density is not an absolute quantitative measure, it does allow relative comparisons between samples. Total protein levels in samples collected by salivettes were found to be significantly lower than those collected by the dribbling method (table 5.3.) (hypothesis 9, appendix 1). There are a few potential reasons for this: some of the protein in the absorbed saliva (sIgA included) may be trapped in the fibres of the salivette; or the saliva collected by dribbling might contain some protein debris that would be too large to be absorbed by the salivettes. It is also possible that the salivettes may have stimulated saliva flow producing a more serous secretion, or a more serous secretion may have been collected because the salivettes were positioned next to a parotid gland (between the teeth and the cheek). The retention of saliva by salivettes will be further addressed in section 5.4.

On occasion both methods resulted in insufficient saliva for the total protein assay, in order to overcome this saliva collection times would have to be increased. The majority of assays require less than 500µl (the minimum amount required for optical density measurement) although it is necessary to consider the need for keeping some sample spare for duplicates and repeat assays. If problems with quantities arose, it would be possible to dilute the sample and multiply the concentration value gained by the dilution factor. This does however, introduce error, and assay variability, expressed in absolute terms, would be multiplied by the same factor.

A two-way repeated measures ANOVA (method x day) revealed that there was no main effect for day (whether samples were collected over ten days or within one day) (hypothesis 1, appendix 1); this suggests that samples collected in time course experiments can be compared with one another whether they are collected over one or more days.

Another two-way repeated measures ANOVA (method x day) on the variability in the saliva volume collected revealed that there was no difference in the variability of samples collected by either method or within or between days (hypotheses 2 & 7, appendix 1). The mean volume of saliva collected differed across the three methods though the standard deviation stayed approximately the same, therefore variability expressed as a coefficient of variation (standard deviation as a percentage of the mean) would have probably demonstrated a difference between methods. However, the standard deviation was not proportional to the mean in this case and therefore coefficient of variation would have been an inappropriate expression of variability.

Levene's homogeneity of variance of test revealed that there was no difference in the saliva flow rates (samples collected by dribbling) collected from one subject across ten days compared with samples taken from ten subjects on the same day (hypothesis 3, appendix 1), suggesting that inter subject comparisons of flow rate are valid.

Studies investigating saliva constituents and flow rate have used the dribbling (Green, 1988; Mackinnon et al. 1993a) or salivette (Aufricht et al., 1992) saliva collection methods. For the data gained from these studies to be comparable, a strong relationship between the results obtained by the two methods is required. In the present study no correlation between saliva flow rate from samples collected by salivettes and those collected by dribbling was found ($r= 0.392$, $p>0.05$) (figure 5.2) (hypothesis 8, appendix 1). This finding means that there was no agreement in the saliva flow rate values gained by the two methods, which means that there was no comparability between results collected by the two methods. This lack of agreement was surprising as both methods were being used to collect samples from the same subjects at the same time of day.

Salivettes are often favoured over the passive dribbling method because they are discrete and simple. However, the results presented here suggest that their use may result in not only a smaller sample for analysis, but an underestimation of saliva flow rate and possibly also of the constituent protein concentrations. It is possible that the differences between the samples collected by passive dribbling and those collected by salivettes are a consequence of limited absorption of the sample and retention of the sample after centrifugation. These issues will be addressed in the rest of this chapter.

5.3. ABSORPTION OF SALIVA BY SALIVETTES.

5.3.1. *A comparison of the rate of absorption of saliva by two types of salivette.*

The results described in this chapter so far suggest that there may be several problems with salivettes. There are two main points during the collection at which salivettes may confound the results: the way in which the sample is absorbed, and in the return from centrifugation. The different retention properties of the cotton wool and polyester salivettes suggest that they need to be investigated separately. Before an optimal collection time can be established, it is necessary to know the maximum capacity of the salivettes, and whether saliva absorption remains linear as the swab becomes more saturated. This study sets out to address these questions for salivettes made from two different fibre types, cotton wool and polyester (hypothesis 10, appendix 1).

5.3.1.1. Method

5.3.1.1.1. Subjects

Twelve adults consented to be subjects for this study. Individuals were randomly assigned to two groups of six such that each group contained 3 males and 3 females [Group one (polyester), ages 25.3 ± 2.6 years. Group two (cotton wool); ages 27.5 ± 4.7 years]. All subjects believed themselves to be free from upper respiratory and oral cavity infection. All were non-smokers. The groups were randomly assigned to a salivette type.

5.3.1.1.2. Saliva collection

Subjects were asked to place a salivette swab in the centre of their mouth on top of the tongue, in order to try and collect whole saliva as opposed to just parotid gland secretion, which may occur with salivettes positioned in the side of the mouth. The salivettes were left in this position for 30, 60, 90, 120, 150, 180, 210, 240, or 270 seconds. The order of the duration that the salivettes were left in the oral cavity was varied; 3 subjects within each group started with the shortest holding duration and the time was progressively increased for the following eight salivettes. This timing order was reversed for the other 3 subjects.

5.3.1.1.3 Storage and analysis of samples

Upon removal from the oral cavity the swabs were placed in the salivette vial, and immediately stored at -20°C, in order to replicate the normal treatment of samples (Bennet and Reade, 1982; McClelland et al., 1985; MacDowell et al., 1992; Tharp and Barnes, 1991).

Prior to further analysis the samples were allowed to defrost at room temperature. The swabs containing the saliva were weighed and their pre-sample weight subtracted to establish the volume of saliva collected. The salivettes were then centrifuged at 5°C for 15 minutes at 2000 rpm (800 series, Centurion Scientific Ltd., UK). The volume of the saliva extracted was measured by weighing. The amount of saliva retained was calculated by subtracting this volume from the pre-centrifugation weight of the swab.

5.3.1.1.4. Statistical analysis

A two-way ANOVA (material x time), with one repeated measure for time, was carried out to investigate if there was a difference in the absorbance of saliva by the two materials, and if a significant amount of saliva was absorbed with time, and if there was any interaction between these two factors. The ANOVA F test was modified when data lacked sphericity. This was done according to the true value of epsilon, if epsilon ≤ 0.75 then Greenhouse-Geiser correction factor was used, if epsilon ≥ 0.75 then Huyn-felt correction factor was used. The reasoning behind this being that Huyn-felt becomes too liberal below 0.75 and Greenhouse-Geiser too strict (Huyn and Felt, 1976).

5.3.1.2. Results

The mean mass increase in polyester salivettes held in the oral cavity for increasing periods of time show that there is a reasonably linear increase, up to a holding time of 210 seconds. The plateau in weight increase seen after this point may be indicative of salivette saturation (fig 5.3.). The cotton wool salivettes appear to be slower to absorb saliva than the polyester salivettes. However the mean mass increase in the cotton wool salivettes appears to be linear after it has plateaued for the polyester salivettes. This suggests that the cotton wool salivettes can continue to absorb saliva after those made of polyester have become saturated.

One subject had to be excluded from the polyester group because of an incomplete data set.

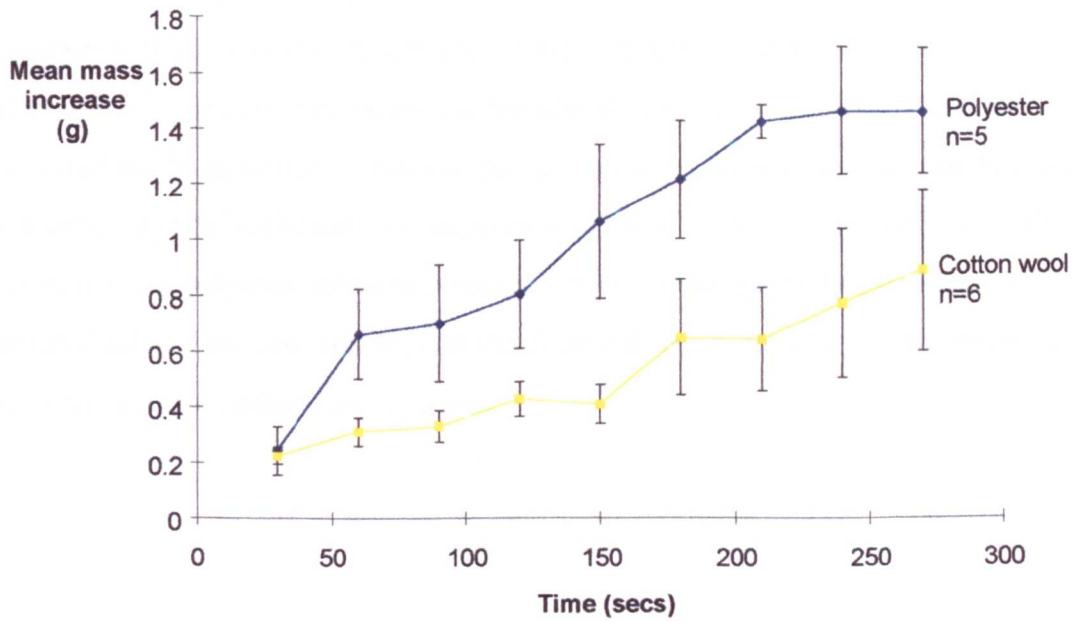

Fig.5.3. The increase in salivette mass (mean± S.E.M) as a function of the time for which the salivette was held in the oral cavity.

A two-way ANOVA (material x time) revealed that there was no significant interaction between the two factors ($F_{(1.53,6.11)} = 1.47$, $p>0.05$). A main effect for material was found ($F_{(1,4)} = 7.69$, $p=0.05$), therefore the way in which the two materials absorb the saliva is different, from fig 5.3 it is apparent that the cotton wool salivettes have a lower absorption rate than those made of polyester. As would be expected there was a significant main effect for time ($F_{(1.68,6.72)} = 15.48$, $p<0.005$); there was a increase in mass of the salivettes with time as they absorbed the saliva.

5.3.1.3. Discussion

No interaction was found between material and time however, a difference was found between the two material types with the cotton wool salivettes absorbing less than those made of polyester (hypothesis 10, appendix 1). It is possible that the polyester salivettes simulated flow rate, subjects complained that the polyester salivettes were uncomfortable, and scratched the inside of their mouths. It has been reported that gustatory stimulation increases the flow of serous rather than mucosal saliva. This would result in an increase in the water content of the saliva and a decrease in the concentration of non-secretion stimulated proteins, including sIgA. If the polyester salivettes do increase flow rate in this way, then it would result in erroneous calculation of sIgA secretion rate; flow rate would be falsely high and the sIgA concentration low.

Alternatively, this difference in the increase in mass, between the two materials, may be a consequence of a lower absorption rate for the cotton wool salivettes. If this is the case, then flow rate would be underestimated because the saliva produced over the collection time would not be absorbed. However, all subjects reported a greater accumulation of saliva with polyester salivettes as compared with those made from cotton wool. This suggests that the polyester salivettes were saturated earlier, possibly a consequence of stimulated saliva flow rate or a smaller maximal holding capacity than the cotton wool type. This requires further investigation.

5.3.2. Comparison of the maximal absorption capacity of two types of salivette for saliva and water

Absorption of saliva from the oral cavity is probably not an ideal way of assessing the absorption rate and maximum capacity of the salivettes. Salivette absorption capacity was investigated in the oral cavity as opposed to in an artificial situation in order to take into account any possible impact the different salivette materials may have on saliva flow rate. The accumulation of saliva in the oral cavity reported by all subjects within a collection time of two minutes may suggest that the polyester salivettes had reached their maximal capacity. However, to assess the maximal capacity of the salivette there needs to be guaranteed saturation that can only be achieved in an artificial environment. This study sets out to examine the maximal absorption capacity of two different types of salivette, in an artificial situation (in a beaker with water) and in the oral cavity absorbing saliva (hypotheses 11&12, appendix 1).

5.3.2.1. Method

Five polyester salivettes were saturated with water, by placing them individually in a beaker containing 10ml of distilled water for ten minutes. This was repeated for 5 cotton wool salivettes.

Five polyester and five cotton wool salivette swabs were saturated with saliva, by placing a salivette of either type alternately in the cheek of a 24 year old female subject for ten minutes.

5.3.5.1.1. Statistical analysis

The mean mass increases in the swab and the standard deviation around this mean were calculated for both types of salivette when saturated with both water and saliva. The results are displayed in table 5.4. A two-way General factorial ANOVA (material x fluid) was carried out to investigate if either fluid (saliva or water) were absorbed differently by the salivettes, or whether the material of the salivette had an effect on the maximal absorption capacity of the swab (polyester or cotton wool), and also to investigate if there is any interaction between these two factors; i.e. if the maximal absorption capacity of a material is dependent upon the fluid.

5.3.2.2. Results

The mean increases in mass after the swabs were saturated with either water or saliva are presented below (table 5.4). A two-way general factorial ANOVA (fluid x material) revealed that there was no significant interaction between the two factors ($F_{(1,19)}$=3.682, $p>0.05$). No main effect was found for fluid ($F_{(1,19)}$=0.055, $p>0.05$). There was a main effect for material ($F_{(1,19)}$=53.25, $p<0.0001$); the way in which the fluid is absorbed is different for the two types of swab, indicating that the results achieved in saliva studies with salivettes are dependent upon the type of swab used.

Table 5.4. Mean mass increases for salivettes saturated with saliva and distilled water

	WATER	**SALIVA**
	mean ± SD (g)	**mean ± SD (g)**
Polyester	**2.59 ± 0.80**	**2.02 ± 0.62**
Cotton wool	**4.04 ± 0.64**	**4.49 ± 80**

Data are mean ± SD.

5.3.2.3. Discussion

Both types of salivette absorbed saliva and water to the same extent (hypothesis 12, appendix 1), therefore it would be possible to carry out further studies on the salivettes using water.

The results suggest that the maximal absorption capacity of polyester salivettes is approximately 2mls while cotton wool salivettes appear capable of absorbing approximately twice this amount (hypothesis 11 appendix 1);. These results, along with those presented in fig 5.3, would suggest that within a collection time of 210 seconds the polyester salivettes were approaching saturation, and that after this point the rate of absorption decreased. It would therefore be inappropriate to use polyester salivettes for a period longer than this, which brings into question the commonly used collection time of 4mins/240secs (Green and Green, 1987; Mackinnon et al., 1993a).

The higher maximal capacity of the cotton wool salivettes (table 5.4.) would suggest that the cotton wool salivettes were not saturated within 270 seconds (fig 5.3); they may potentially be more useful for experiments involving collection times longer than 210 seconds. For both types of salivette the manufacturer (Starstedt UK) recommends a collection time of just 15 seconds; which seems to be too short to gain a representative sample, or a sample which is large enough for most biological assays.

The fact that the salivettes have different absorption capacities implies that comparisons cannot be made between samples that are collected by the different types of salivette, nor would it be valid to make comparisons between studies that have used salivettes with different fibre types.

5.3.3. Summary

The results from investigations examining the absorption of saliva by salivettes demonstrates that there is a difference in the maximal absorption capacities between the two types of salivette available (fig 5.3). It appears that the absorption capacity of the polyester salivettes is approximately half that of the cotton wool salivettes. It is also possible that the plastic coating over the salivette not only makes the swab uncomfortable for the subject but may also stimulate saliva flow rate. It is probable that the rate of absorption of saliva by the salivettes decreases as the swab approaches saturation, and that therefore the polyester salivettes are useful for a shorter period of time than the cotton wool type. A generic optimal collection time for the two types of salivette is, therefore, unlikely to be established. The fact that saliva absorption does not remain linear as it approaches saturation (fig 5.3.) suggests that only very short collection times should be used. The use of salivettes for 4 minutes may result in loss of sample, variable loss of sIgA and miscalculation of saliva flow rate. The use of salivettes would therefore dictate the use of a shorter sample collection time; however, optimal collection times would be highly dependent upon individual flow rates. The rest of this chapter examines the second point during collection at which salivettes may confound the results gained for saliva flow rates and protein secretion rates: the return of the sample after absorption.

5.4. RETENTION OF SALIVA BY SALIVETTES

5.4.1 *Introduction*

The results described earlier in this chapter (see section 5.2) demonstrate that salivettes collect both less saliva and less total protein than samples collected by passive dribbling, probably because of the limiting absorption capacity of the salivettes (section 5.3.). Originally all salivette swabs were cotton wool, with concerns over proteins being retained in the fibres leading to the development of the polyester salivettes. The aim of this section is to address the question of whether saliva is being retained in salivettes after centrifugation, and if so, if retention is greater in one type of salivette as compared to another (hypotheses 13-20, appendix 1).

5.4.2. *Method*

Salivettes from sections 5.2. and 5.3. were examined for the amount of sample they retained. This involved 130 cotton wool swabs and 178 which were made of polyester. In order to calculate the amount of saliva retained, the pre sample mass of the swab in the suspended insert was subtracted from the mass of the swab after centrifugation. The increase in mass of the swab was assumed to be the result of saliva being retained in the swab. The amount of saliva retained was recorded as a volume and a percentage of the initial collected saliva volume (see table 5.5).

5.4.2.1. Statistical analysis

The mean and standard deviation around the means for the volume absorbed, the volume retained, and the percentage (of the absorbed sample) retained were calculated and are presented in table 5.5.

Two one-way ANOVA's (material) were carried out to investigate if one type of salivette retained more saliva after centrifugation than the other.; the first looked at the volume of saliva retained and the second at the percentage of the initial sample that was retained. A third one-way ANOVA compared the amount of saliva absorbed by the two types of salivette.

Four Pearson's product moment correlations were carried out to investigate the relationship between the type of salivette and the amount of saliva retained; these

comparisons were between: the amount of saliva absorbed and the amount of saliva (mls) for cotton wool (fig 5.4) and then again for polyester salivettes (fig 5.6); and between the amount of saliva retained, expressed as a percentage of the initial swab weight for cotton wool (fig 5.5) and then polyester salivettes (fig 5.7).

A fifth Pearson's product moment correlation was also carried out to investigate if there was any relationship between the amount (mls) of saliva retained by the polyester salivettes and the pre sample swab mass (fig 5.8).

5.4.3. *Results*

A one-way ANOVA (material) comparing the absorption of saliva by the two materials found that the cotton wool salivettes absorbed significantly more saliva than those made of polyester($F_{(1,242)}=7.85$, $p<0.001$). The salivettes always retained some saliva. A one-way ANOVA (material) revealed that the cotton wool salivettes retained a higher volume of saliva ($F_{(1,242)}=31.32$, $p<0.0001$). It is possible that a higher volume was retained as a consequence of a higher volume being absorbed, however, a third one-way ANOVA (material) revealed that the cotton wool salivettes also retained a greater percentage of the initial sample than the polyester salivettes ($F_{(1,242)}=23.02$,$p<0.0001$).

Table 5.5. Mean volume and percentage values for the saliva absorbed and retained by cotton wool and polyester salivettes

	Saliva absorbed Mean ± SD (ml)	**Volume retained Mean ± SD (ml)**	**Percentage Mean ± SD (%)**
COTTON WOOL	0.87± 0.63	0.47 ± 0.27	62.98 ± 20.16
POLYESTER	0.77± 0.47	0.22 ± 0.21	35.29 ± 29.7

Data are mean ± SD.

A Pearson's product moment correlation between the amount of saliva absorbed and the amount of saliva retained for the cotton wool salivettes revealed a positive relationship (see fig 5.4.). The greater the volume of saliva absorbed, the greater the amount of saliva retained.

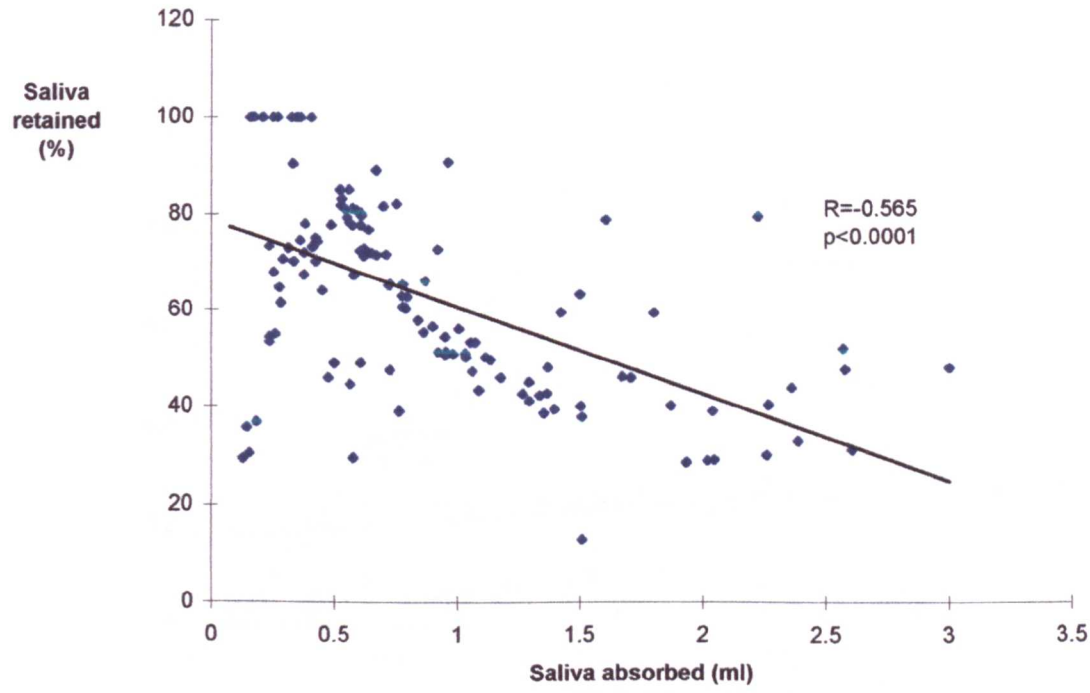

Fig.5.4. The relationship between the amount of saliva absorbed and the amount retained for cotton wool salivettes.

A significant negative relationship was found between the amount of saliva absorbed and the percentage retained. Therefore the smaller the sample the greater the percentage retained; however, even in the largest samples (approximately 2.5ml) between 30 and 55% of the sample was retained (see fig. 5.5.).

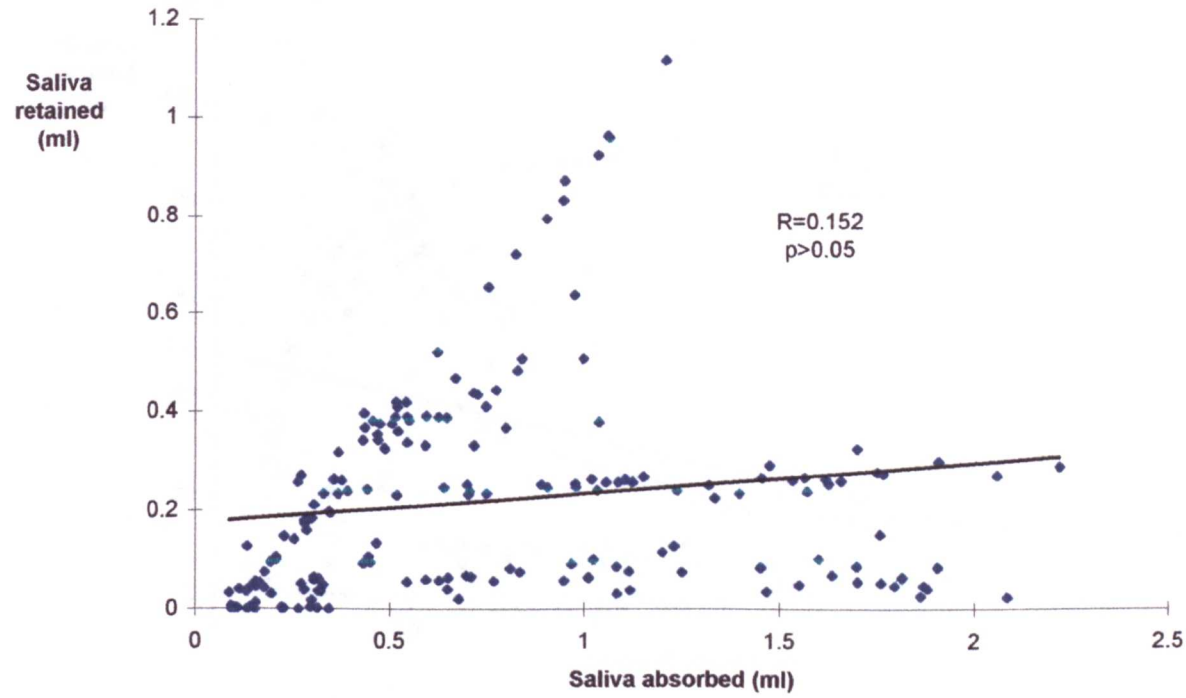

Fig. 5.5. The relationship between the amount of saliva absorbed and the percentage retained for cotton wool salivettes

Unlike the cotton wool salivettes, no relationship was found using a Pearson's product moment correlation, between the amount of saliva absorbed and retained for the polyester salivettes (see fig. 5.6.)

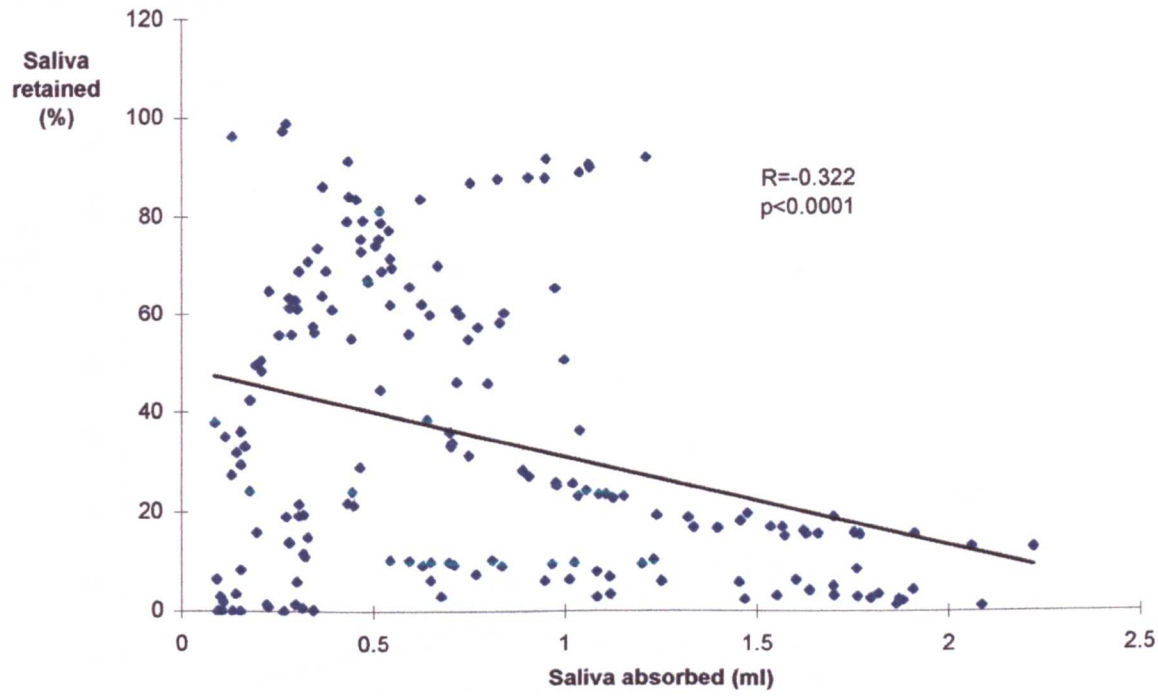

Fig. 5.6. The relationship between the amount of saliva absorbed and the amount retained for polyester salivettes.

A significant negative correlation was identified using Pearson's product moment, between the amount of saliva absorbed and the percentage retained (see fig 5.7). As with the cotton wool salivettes the smaller the sample the greater the percentage of the sample retained (see fig 5.5. and fig 5.7)

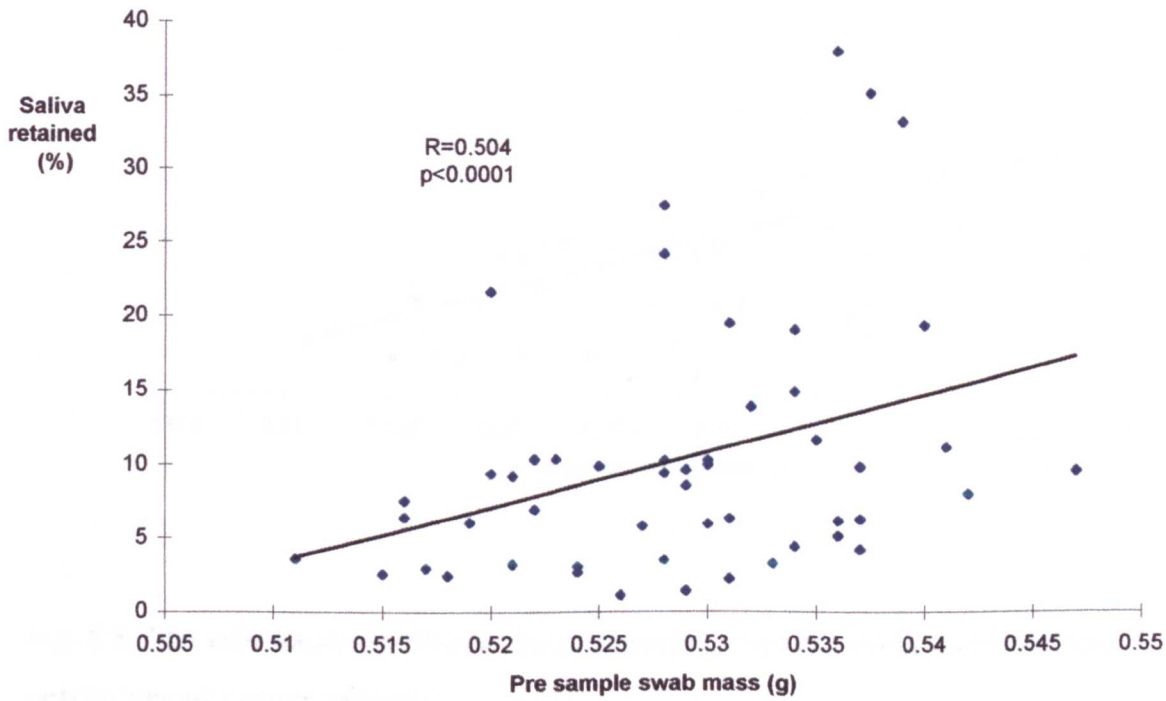

Fig. 5.7. The relationship between the amount of saliva absorbed and the percentage retained for polyester salivettes

Pearson's Product Moment correlation revealed a positive relationship between the polyester salivette swab mass and the percentage of saliva retained (see fig. 5.8.)

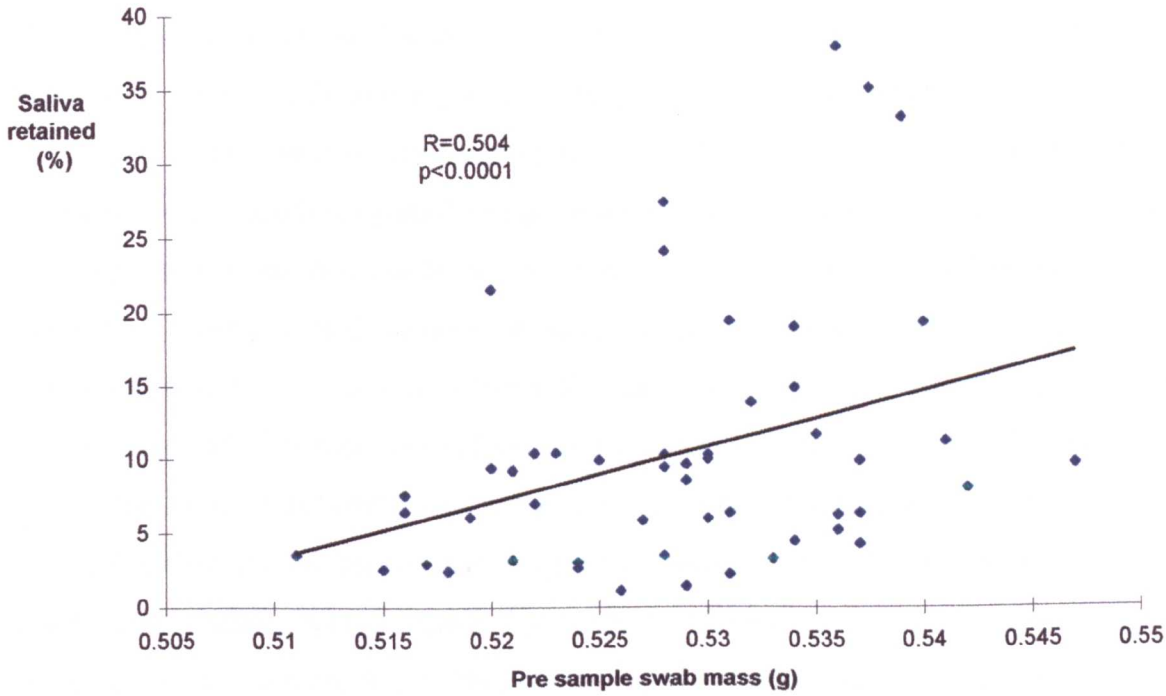

Fig. 5.8. The relationship between the pre sample polyester swab mass and the percentage of sample retained.

5.4.4. *Discussion*

The salivette swabs always retained some saliva after centrifugation (hypothesis 13, appendix 1). The cotton wool salivettes retained a greater volume of saliva than the polyester swabs (table 5.5) (hypothesis 14, appendix 1), which may be a consequence of the fact that they were also found to absorb more. However, the cotton wool swabs were found to retain a significantly higher percentage of the absorbed sample (hypothesis 15, appendix 1). The pattern of sample retention by the two types of salivette was further explored, and a significant positive relationship between the amount of saliva collected and that which is retained was found for cotton wool salivettes (fig 5.4) (hypothesis 16, appendix 1) but not for those made of polyester (fig 5.6) (hypothesis 18, appendix 1), suggesting that the 'interaction' between the material and the saliva may be different for the two materials. For both types of salivette a significant negative relationship was found between the size of the sample and the percentage of the sample retained, therefore the less saliva absorbed, the greater the percentage of the sample that is retained, 100% of some of the smallest samples being retained (figs 5.5 & 5.7) (hypotheses 17 & 19, appendix 1). The problems of retention are thus further compounded in individuals with low flow rates. It is possible that the high retention of small samples could lead investigators to believe that flow rates had decreased further than they had after exercise or other stressful events and therefore report an effect greater than, that , which actually happened. This underestimation of saliva flow would be further amplified if investigators only reported the amount of saliva collected after centrifugation (which has the combined problems of both low absorption and high retention) and not the amount of the saliva absorbed by the salivette.

A positive correlation between the pre sample swab mass and the percentage of saliva retained was found for the polyester salivettes (fig. 5.8) (hypothesis 20, appendix 1). This suggests that the retention of saliva by salivettes is the direct result of saliva and salivary proteins becoming trapped within the fibres of the swab; the more fibres the greater the retention. The manufacturers (Starstedt UK) developed polyester salivettes to avoid the retention of saliva and salivary proteins that had been observed with the cotton swabs; the manufacturers are therefore already aware of retention as a potential problem of sample collection. However, despite the polyester salivettes being an attempted improvement on the cotton wool swab, the results of this study would suggest that they still retain a mean

percentage of 35%, which is a significant amount of the sample, and even greater proportions for smaller than average samples.

It was hoped that there would be sufficient saliva for the analysis of total salivary protein to assess if the fibres trapped the proteins within themselves. If this is the case then both saliva flow rates and salivary protein concentrations will be underestimated. Unfortunately insufficient amounts of sample were available and so it was not possible to assay for total saliva protein levels. It has been demonstrated by this study that the retention of saliva by salivettes is not consistent and therefore it is not possible to calculate a correction factor for retention of the sample. The percentage of the sample retained is approximately inversely proportional to the volume of the sample absorbed; it appears therefore that the extent of the retention is also dependent upon the initial size of the salivette swab, which, like the size of the sample produced, is variable.

The results of this investigation demonstrate that some saliva is always retained after centrifugation of swab in accordance with manufacturer's guidelines. It appears that cotton wool salivettes retain a greater percentage of the sample than do polyester salivettes. The findings also suggest that the smaller the sample collected the greater the retention percentage of that sample by either type of salivette fibre. Although not fully investigated, it is possible that salivary proteins are trapped within the fibres of the salivette swab. The retention of saliva by salivettes would thus confound the calculation of sIgA secretion rate two-fold, by underestimation of both saliva flow rate and sIgA concentration.

5.5. SUMMARY

The results of the studies reported in this chapter do not support the use of salivettes for the collection of saliva when the volume gained or the concentrations of the contents of the saliva are important. The use of salivettes results in a smaller sample, as a result of a limiting absorption capacity and a variable retention of the sample. This reduces the number of assays that can be carried out on a sample. The lower total protein levels found in samples collected by salivettes suggests that, as well as collecting a smaller sample, the resulting sample is different from that collected by passive dribbling. Therefore comparing samples collected by the two methods would be problematic. This was confirmed by correlating the saliva flow rate values of samples collected by the two methods (fig.5.2); the results gained by one method were unrelated to those of the other method.

The results from section 5.3. suggest that both polyester and cotton wool salivettes have a limiting absorption capacity, (approximately 2ml and 4ml respectively) and that as the salivettes approach saturation the rate of absorption decreases, resulting in variable loss of sample and an underestimation of saliva flow rate. The difference in the absorption capacity of the two different types of salivette means that a generic optimal collection time cannot be established, and the fact that absorption of saliva does not remain linear as the salivette approaches saturation suggests that only very short collection times may be used, calling into question the validity of results gained using a four minute collection period.

Although cotton wool salivettes absorbed more saliva than the polyester type, the retention studies (section 5.4) revealed that they also retained more of the sample, in terms of both volume and percentage (of the sample absorbed). Salivettes of both types always retained some saliva: cotton wool $62.89 \pm 20.16\%$ and polyester $35.29 \pm 29.7\%$. The percentage of the sample retained is high in both cases. A strong positive relationship exists between the volume of saliva absorbed and that retained for cotton wool salivettes. No such relationship exists for polyester salivettes suggesting that the relationship between the absorbance and retention of saliva is different for the two types of salivette. This means that results obtained with the different salivettes are not comparable. However, there was a positive relationship between the pre sample mass of the polyester swab and the amount of saliva retained, suggesting that in the case of polyester salivettes, retention is a function of the size of the swab.

Saliva retention by the salivette will have an impact on sample size by increasing the size difference between large and small samples and either exacerbating or disguising changes in saliva flow rate. Interpretations of changes in saliva flow rate could also be skewed by the use of salivettes because of variable loss of sample due to a decreased absorption rate, at, or close to, saturation of the salivette. The use of salivettes would therefore make it difficult to detect accurately changes in saliva flow rate, and may result in variable loss of sIgA.

The results obtained suggest that comparisons made between results from the dribbling method cannot be compared with those gained from salivettes, nor can results from samples collected by one type of salivette be compared with those collected by another type. There is thus a need for the consistency between studies of the method used to collect saliva, before any conformity of opinion can be reached. The conclusions of this chapter are that even though it is a less discreet method, collection of saliva by passive dribbling is recommended over the use of salivettes because the latter results in the variable loss of both saliva and salivary proteins. This is both a novel and important finding, with the growth of studies investigating salivary components an increasing number of investigator are using salivettes and assuming that their findings are comparable with those who have collected saliva by passive dribbling, when it appears that they are not. Studies in part III of this thesis collect whole saliva by the passive dribbling method.

PART III

CHAPTER 6

AN ANALYSIS OF THE RELATIVE CONTRIBUTION OF SALIVA FLOW RATE AND SALIVARY IMMUNOGLOBULIN A CONCENTRATION TO SALIVARY IMMUNOGLOBULIN A SECRETION RATE.

6.1. INTRODUCTION

As previously described in chapter 4, saliva has a mechanical washing effect and contains several anti-pathogenic proteins which may contribute to defence against URTI. It may therefore be prudent to monitor saliva flow rate with the intention of taking these innate defence mechanisms into consideration. Several authors have emphasised the importance of considering saliva flow rate when expressing sIgA levels (Evans and Bristow, 1993; Mandel and Khurana, 1969; Stone et al., 1987). Evans and Bristow (1993) argued for the expression of sIgA levels in terms encompassing saliva flow rate, because they found an inverse relationship between the volume of saliva produced and sIgA concentration, therefore high saliva flow rates would result in low sIgA concentrations and vice-versa, because of the diluent effect of saliva on sIgA.

It is necessary to establish the relative contribution of changes in saliva flow rate and sIgA concentration to the change in secretion rate induced by exercise. It is possible that authors expressing sIgA levels in terms of secretion rate may be unwittingly giving greater consideration to the effect of exercise on saliva flow rate than on the production of sIgA.

Salivary immunoglobulin A levels have been adjusted for saliva flow rate in several studies (Green, 1988; Jemmott et al., 1983; Jemmott and McClelland, 1989; Mackinnon et al., 1993a; Mackinnon and Jenkins, 1993; Stone et al., 1987) by multiplying the sIgA concentration ($\mu g.ml.^{-1}$) by the saliva flow rate ($ml.min.^{-1}$) to obtain a secretion rate ($\mu g.min.^{-1}$). Stone et al. (1987) calculated secretion rate as above but referred to the value as being representative of rate of synthesis of sIgA. As both the synthesis and transport of sIgA are regulated by mechanisms other than those which regulate the control of saliva flow rate, [demonstrated by studies using saliva stimulation (Brandtzaeg, 1971)] it may be inaccurate to consider this sIgA secretion rate an indicator of sIgA synthesis. It may

indicate sIgA release from the luminal surfaces; however, it does not seem reasonable to assume exercise-induced immunsuppression from a decreased sIgA secretion rate, an idea which unfortunately forms the basis of a large number of studies investigating the effect of exercise on susceptibility to URTI.

Salivary IgA concentration has been demonstrated to decrease when saliva flow has been increased through stimulation (Stone et al., 1987; Brandtzaeg, 1971), quite simply because of a dilution effect of the saliva. Therefore if the increase in saliva flow rate increases beyond the decrease in sIgA concentration, then the calculated secretion rate is increased. Brandtzaeg (1971) demonstrated IgA concentration 3 to 4 times higher in unstimulated samples than stimulated samples, but the levels of sIgA were lower in unstimulated samples in terms of secretion rate. It is therefore clear that the terms of expression for sIgA levels are critical to the subsequent interpretation of results.

Jemmott et al. (1983) reported that the relationship between sIgA and academic stress was the same whether sIgA levels were reported in terms of concentration or secretion rate. Evans and Bristow (1993) also reported that their findings of a correlation between life events and sIgA levels were comparable regardless of terms of expression. Schouten et al. (1988a, b) reported exercise to have no effect on the volume of saliva produced, however flow was stimulated and therefore there may have been a ceiling effect with saliva flow at its maximum rate both pre and post exercise.

Salivary IgA concentration may give a better indication of the activity of sub-mucosal plasma cells than sIgA measured in terms of secretion rate. However, sIgA secretion rate may be a better indicator of defence at the mucosa because it considers the amount of sIgA in the oral cavity per unit time, particularly when one considers the several anti-pathogenic functions of saliva. Secretion rate could also be important when considering sIgA levels relative to defence against URTI because sIgA on the oral surfaces, although important, may be less likely to encounter a pathogen than sIgA being swilled around the oral cavity.

Mackinnon and Jenkins (1993) and Mackinnon et al. (1993a) reported sIgA levels in terms of mg of total protein and secretion rate. Regardless of the terms of expression, sIgA levels decreased after exercise in both studies; sIgA levels decreased to a greater extent in terms of secretion rate. In both cases, sIgA levels could have decreased without a change

in sIgA production, purely as a consequence of changes in saliva flow rate. The representation of sIgA levels in terms of total protein involves expressing the sIgA concentration relative to a reference total protein concentration of 100 $mg.ml^{-1}$. The actual sIgA concentration obtained is divided by the total protein concentration and multiplied by 100 to achieve a value relative to $100mg.ml^{-1}$ of the total protein concentration. Total protein concentration may have increased because of a decrease in the volume of fluid in which it is diluted (saliva). The correction for this increase in total protein concentration would result in a decrease in reported sIgA levels, independent of any change in sIgA levels. A decrease in saliva flow rate would result in decrease in secretion rate more directly, because secretion rate is the product of flow rate and concentration. A decrease in saliva flow rate in response to exercise may be expected as a consequence of sympathetic arousal or dehydration (see chapter 8).

The aim of this study is to assess the relative contribution of saliva flow rate and sIgA concentration to calculated sIgA secretion rate. The hypotheses tested within this chapter (21-28) are listed in appendix 1.

6.2 METHOD

6.2.1 *Subjects*

Seven sub-elite amateur boxers aged 23 ± 2.6 years consented to give saliva samples immediately before and after three interval training sessions, over a seven day period.

6.2.2 *Collection of saliva and measurement of saliva flow rate.*

Whole unstimulated saliva samples were collected by the passive dribbling method, in 5ml plastic vials (Medfor, UK), as described in chapter 5. Training took place at the same time each day and lasted between 45 and 60 minutes, and the saliva samples were collected immediately pre and post. Samples were immediately stored at -20°C.

The volume of saliva produced was determined by weighing the defrosted saliva, with Ohaus E120 scales. After the mass of the collection vial had been subtracted, the mass in grams was divided by the collection time to determine flow rate. The samples were then centrifuged at 2000rpm for 10 minutes in a Sanyo (2-15) centrifuge. The supernatant was drawn off for sIgA analysis with enzyme-linked immunosorbant assay.

6.2.3 *Enzyme-linked immunosorbant assay*

Salivary Immunoglobulin A (sIgA) has been quantified using a number of techniques including laser nephletometry, radial-immunodiffusion (Brandtzaeg, Fjellander and Gjerulden 1970) and enzyme-linked immunosorbant assay (ELISA) (Akerlund et al., 1977). The use of ELISA is becoming increasingly common because it has several advantages over other methods: visual tests can be developed for mass screening, safety is improved; the reagents of the assay are stable and easily stored; the equipment and techniques make it accessible for a wide range of laboratories; and there are a wide range of commercially available conjugate enzyme products making a wide range of assays possible. It has also been credited with being the most sensitive and economical assay for IgA (Evans and Bristow, 1993), and has been recommended above other methods (Stone et al., 1987). ELISA has been used in a number of studies quantifying sIgA in athletes (Mackinnon, 1993 a, b, c; McDowell, 1992 a, b; Tharp, 1991). Of the several types of ELISA in use, sandwich ELISA, [an immunoenzymetric assay developed by Engvall and Perlmann (1972)], is credited with being the most sensitive. Butler, Peterman, Suter and

Dierks (1987) reported sandwich assays to be 10-100 fold more sensitive than competitive assays for immunoglobulins.

Sandwich ELISA is carried out by a process of sequential addition of the assay components into wells in a plastic microtitre plate. Each stage is incubated at a pre-determined optimal temperature for a given time to allow the required interactions to occur. For the measurement of immunoglobulins, a capture antibody, specific for the isotype of the immunoglobulin being measured, is physically adsorbed onto the plastic microtitre plate. Unabsorbed capture antibody is removed by emptying the wells of the microtitre plate, and then washing the plate with a saline solution containing non-ionic detergent. Any plastic not coated with capture antibody then has an inert blocking protein adsorbed onto it, to ensure that the following phases of the assay bind only to the capture antibody and not to the plate. Again the unbound fraction is washed off. After the blocking protein has been added, the dilute biological sample is added to the wells, and the plate is incubated again. If present, the immunoglobulin for which the capture (primary) antibody is specific is bound. Any unbound sample is washed off. A detector antibody is then added, which is also specific for the isotype of the immunoglobulin. This antibody is conjugated to an enzyme. Again the unbound fraction is washed off.

In the final stage the substrate conjugated enzyme [i.e. Hydrogen Peroxide (substrate) Horseradish Peroxidase (enzyme)] is added. The ensuing reaction generates a coloured product, the optical density of which is quantified by photometry. The absorbance reading obtained is proportional to the amount of bound immunoglobulin. Sandwich ELISA is typically sensitive for the measurement of immunoglobulins in the range of 0.1 to 50 ng/ml (Butler, 1988). A physiological range for salivary immunoglobulin A has not been established, but Brandtzaeg et al. (1970) reported an observed sIgA range of 87- 615μgml^{-1}, which means that for an accurate assay of sIgA, the sample will have to be considerably diluted.

6.2.3.1 Quantification of IgA

(see appendix 3 for chemical composition of buffers and materials list)

A 96-well polystyrene plate (Greiner Laboratechnik, UK) was coated with anti-IgA antibody (Sigma I-8760), diluted 1:800 in coating buffer, by adding 100µl to each well. The plate was then incubated for 1 hour at 37°C. After this point disposable face masks (Hospital management supplies, UK) were worn whenever handling the plate to avoid contamination of the plates with aspirated saliva.

After incubation the plates were then blotted and washed five times with PBS-Tween-80 (washing buffer). The 'blocking' solution was made (2% solution of dried skimmed milk, in phosphate buffered saline), and 100µl added to the coated wells (except control wells). The plate was again incubated for 1 hour at 37°C, in a humid chamber (a plastic container with wet paper towels at the bottom).

The plate was again blotted and washed five times with PBS-Tween-80 (washing buffer). Serially diluted human serum IgA (Sigma), in the following concentrations, 10, 6.5, 5, 2.5, 1, 0.5µgml^{-1} was added in duplicate to the desired wells. Saliva samples were diluted 1:100 with distilled and deionised water and 100µl was added in duplicate to the desired wells. After addition of the samples the plates were incubated for 1 hour at 37°C.

The blotting and washing procedure was repeated using PBS-Tween-80 (washing buffer). Anti-human IgA horseradish peroxidase conjugate was diluted 1:1000 in 'blocking' solution and 100µl of this secondary antibody solution was added to each well. The plates were incubated again for 1 hour at 37°C.

The washing procedure was repeated again with PBS-Tween-80 (washing buffer). The enzyme substrate solution was made by dissolving a 10mg 2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) tablet (Sigma) in 20ml of citrate buffer, and adding 5µl of 30% w/v hydrogen peroxide. The enzyme substrate solution was added to the plate by pipetting 50µl into the wells across the plate (2-11) and then in reverse order (11-2) to avoid any timing effects on the intensity of the colour developed. The plate was then incubated at room temperature for fifteen minutes before the absorbance was read at a

wavelength of 414nm using a Multiscan 310 plate reader (Biological Instrumentation Service Ltd., UK).

The linear regression equation obtained from a log-log plot of IgA concentration (serial dilution of IgA standard) versus absorbance (at a wavelength of 414nm) was used to convert the means of the two absorbance readings for each sample into a concentration value.

6.2.4 *Statistical analysis*

Group means for saliva flow rate, IgA concentration and IgA secretion rate were calculated for pre and post exercise, and the difference between each of the pre and post values was also calculated. A two-way repeated measures ANOVA (exercise x time) was carried out to investigate if there was any interaction between the exercise session (3) and time (pre to post), and also to see if there was a significant main effect for either exercise session or time. The ANOVA F test was modified when data lacked sphericity, as described in section 5.3.1.1.4.

One-way repeated measures ANOVA's (Session) were carried out to investigate if there were any differences between the pre and then the post session values for saliva flow rate, sIgA concentration and sIgA secretion rate.

Data for the three exercise sessions were pooled and seven Pearson's product moment correlation's were carried out. One to investigate the relationship between the change in saliva flow rate and the change in sIgA concentration induced by exercise (change = post - pre training session). Three correlations were carried out to investigate the relationship between pre exercise values and the change induced by exercise: for saliva flow rate; IgA concentration; and IgA secretion rate. Another three correlations were carried out to investigate the relationship between pre exercise values and the percentage change (relative to pre-exercise values) with exercise for saliva flow rate, IgA concentration and IgA secretion rate.

The direction and magnitude of the change in the three variables were analysed in terms of percentage change from pre exercise values.

Group means for saliva flow rate (table 6.1), IgA concentration (table 6.2) and IgA secretion rate (table 6.3) were calculated pre and post the three exercise sessions and are presented in the tables below.

Table 6.1. Saliva flow rates pre and post exercise for three sessions ($ml.min^{-1}$)

SESSION	PRE	POST	DIFFERENCE
1	0.36 ± 0.23	0.177 ± 0.134	-0.19 ± 0.12
2	0.26 ± 0.11	0.11 ± 0.04	-0.15 ± 0.09
3	0.2 ± 0.12	0.11 ±0.06	-0.09 ± 0.67

Data are mean ± SD. (n=7)

A two-way repeated measures ANOVA (time x session) revealed a significant interaction between time and exercise session for saliva flow rate ($F_{(2,12)}$=4.29, $p<0.05$). This interaction was further investigated using paired t-tests with Bonferroni correction which confirmed that there was a significant decrease in flow rate pre to post exercise for every session (for all sessions $p<0.05$). There was a main effect for time ($F_{(1.7,6)}$= 21.83, $p<0.005$); saliva flow rate always decreased with exercise; there was no main effect for session ($F_{(1.3,7.6)}$= 4.116, $p>0.05$). Therefore there was a difference in the flow rates between each session, which is possibly a factor of the differences in pre exercise flow rates between each session.

A one-way ANOVA (Session) revealed that there was no difference in the post exercise valuse for saliva flow rate between the three sessions. A second one-way ANOVA revealed that there was a difference in flow rate between the pre-session samples ($F_{(1.7,10.26)}$= 4.09, $p>0.05$). A Newman-Kuels post hoc test revealed that the pre-session differences were between session 1 and 3. These session differences for pre exercise flow rate are further highlighed in fig 6.1.

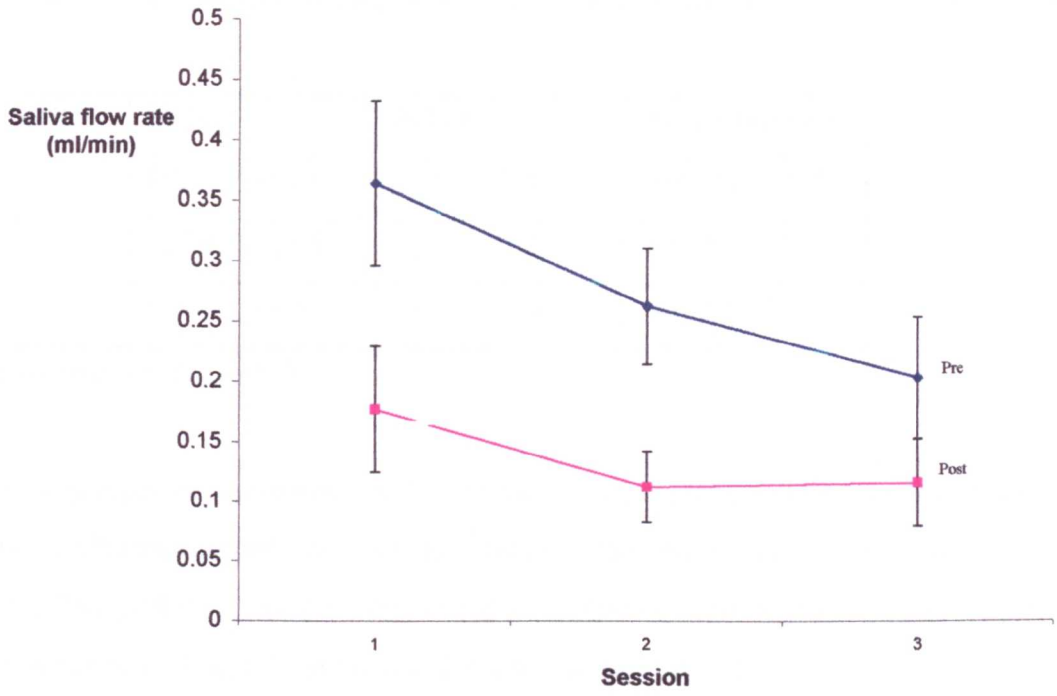

Fig. 6.1. The mean (±S.E.) saliva flow rate pre and post each exercise session.

It appears from fig 6.1. that the differences in flow rate between the sessions is decreasing, as a factor of a decreasing pre-exercise flow rate rather than a difference in the post exercise values.

Table 6.2. SIgA concentrations pre and post exercise for three sessions ($\mu g.ml^{-1}$).

SESSION	PRE	POST	DIFFERENCE
1	577.3 ± 189.9	608.4 ± 222.4	+31.1 ± 287.9
2	595.9 ± 317.8	569.3 ± 236.0	-25.5 ± 183.2
3	551.4 ± 128.9	621.7 ±267.5	+70.3 ± 209.6

Data are mean ± SD. (n=7)

A two-way repeated measures ANOVA (time x session) revealed that there was no significant interaction between time and exercise session for IgA concentration ($F_{(1.2,7.5)}$=0.325, p>0.05). Nor was there any main effect for either time ($F_{(1,6)}$=0.225, p>0.05) or exercise session ($F_{2,12)}$=0.006, p>0.05). Therefore sIgA concentration did not change significantly with exercise in any session.

Table 6.3. SIgA secretion rates pre and post exercise for three sessions ($\mu g.min^{-1}$)

SESSION	PRE	POST	DIFFERENCE
1	176.0 ± 159.6	85.5 ± 68.4	-109.2 ± 135.4
2	177.9 ± 163.8	62.1 ± 27.3	-155.8 ± 147.8
3	119.9 ± 84.5	73.2 ± 51.4	-46.7 ± 65.6

Data are mean ± SD. (n=7)

A two-way repeated measures ANOVA (time x session) revealed that there was no significant interaction between time and the exercise session for IgA secretion rate ($F_{(1,7)}$=0.748, $p>0.05$), therefore the decrease in secretion rate followed the same pattern for each session. A significant main effect was found for IgA secretion rate with exercise ($F_{(1,6)}$=9.926, $p<0.05$): IgA secretion rate was found always to decrease with exercise session. However, there was no significant main effect for session ($F_{(1,9)}$=1.597, $p>0.05$). SIgA secretion rate decreased with exercise and there was no significant difference between sessions , even though there appears to be less of a decrease after the third training session.

The fact that a significant decrease was found to occur for both saliva flow rate and IgA secretion rate, suggests that the relationship between these two variables is stronger than the relationship between IgA concentration and IgA secretion rate. This was further examined by calculating the percentage change in saliva flow rate, IgA concentration and secretion rate after exercise, on the pooled data from all three sessions (table 6.4).

Table 6.4. The percentage change from pre to post exercise for saliva flow rate, sIgA concentration and secretion rate.

	PRE	POST	% CHANGE
Saliva flow rate ($ml.min^{-1}$)	0.28 ± 0.17	0.14 ± 0.09	**-51**
sIgA concentration ($\mu g.min^{-1}$)	574.9 ± 215.5	599.8 ± 231.4	**+4**
sIgA secretion rate ($\mu g.ml^{-1}$)	176.0 ± 159.6	85.5 ± 68.4	**-51**

Data are mean ± SD. (n=21).

Students paired t-tests on mean data revealed significant decreases in saliva flow rate ($T_{(1,20)}$= 6.46, $p<0.0001$) and IgA secretion rate ($T_{(1,20)}$=3.46, $p<0.005$) pre to post exercise. The extent of these decreases are clear from the percentage changes values reported (table 6.4.). No significant change was found for IgA concentration, although there was a small percentage increase pre to post exercise (table 6.4.).

A two-tailed Pearson's product moment correlation revealed a very weak positive relationship between changes in saliva flow rate (pooled data) and changes in sIgA concentration (pooled data) pre to post exercise (see fig. 6.1.); however, there was no significant correlation between the two, suggesting that changes in IgA concentration are independent of changes in flow rate.

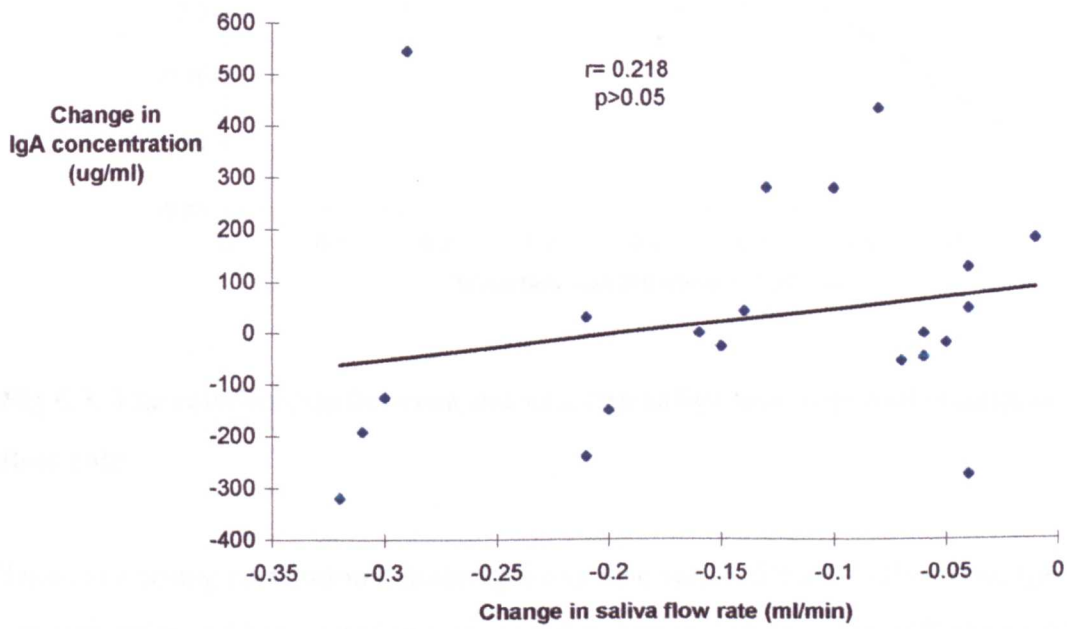

Fig. 6.2. The relationship between the change in saliva flow rate and the change in sIgA concentration pre to post exercise.

The results for saliva flow rate (table 6.1.) suggest that the change in flow rate observed with exercise is a function of the pre-exercise value, and that there may be a floor effect where all post exercise values are the same regardless of the pre exercise values. This relationship was examined by a series of two-tailed Pearson's product moment correlations between the pre exercise values for saliva flow rate, IgA concentration and IgA secretion rate, and the change that occurred with exercise (Figs 6.3, 6.4 and 6.5).

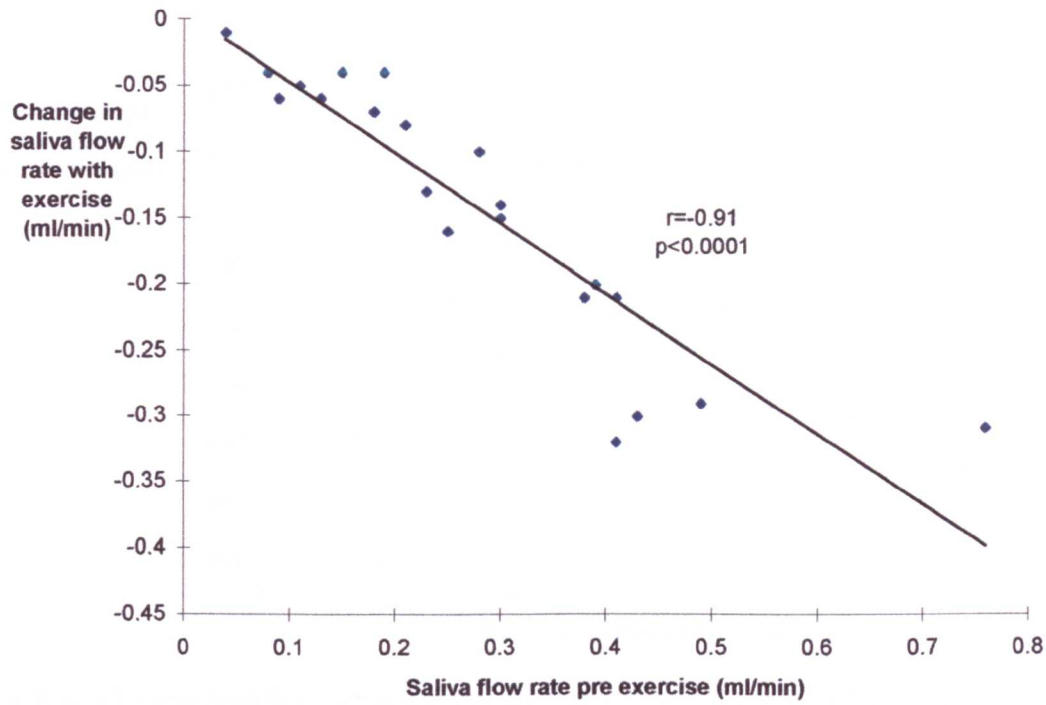

Fig 6.3. The relationship between pre exercise saliva flow rate and change in saliva flow rate.

There is a strong correlation between pre exercise values for saliva flow rate, IgA concentration and IgA secretion rate, and the change that occurred with exercise (Figs 6.3, 6.4 and 6.5). This suggests that there is a floor effect; exercise appears to have the effect of reducing both saliva flow and IgA concentration to the same level post exercise, therefore the larger the pre exercise value, the larger the exercise-induced difference.

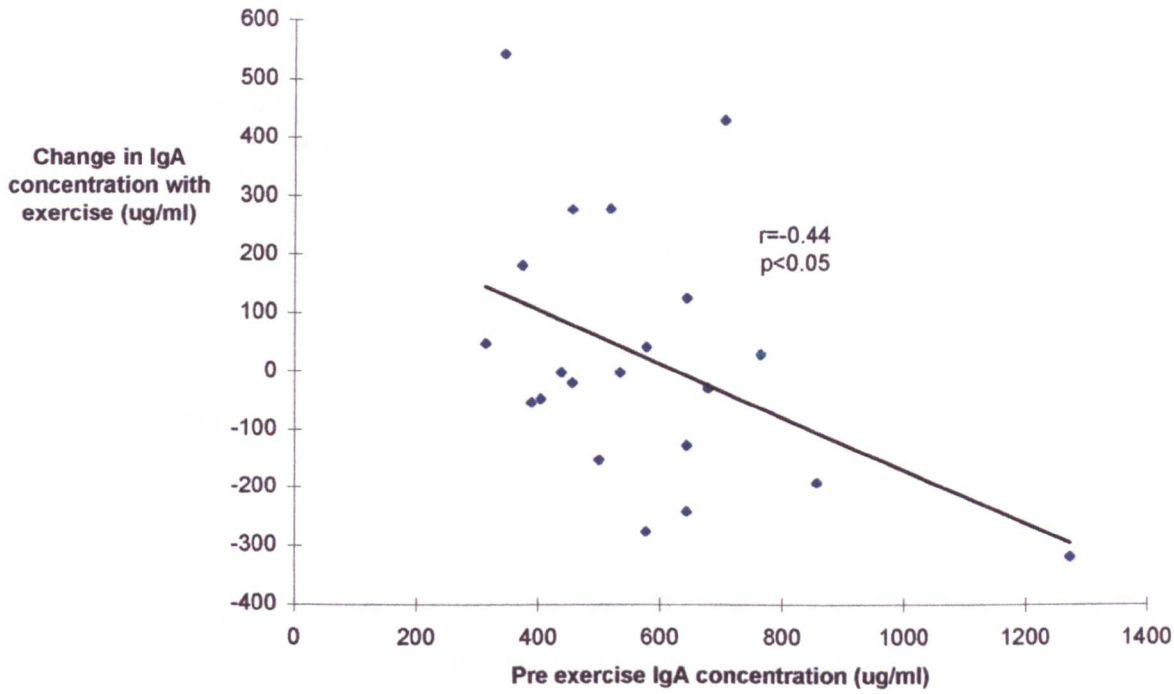

Fig 6.4. The relationship between pre exercise sIgA concentration and the change in sIgA concentration.

Even though the relationship is weaker than for change in saliva flow rate and change in sIgA secretion rate there is a significant correlation between the change sIgA concentration and pre exercise values. This finding indicates that individuals with the highest sIgA levels would suffer the greatest decreases in levels with exercise.

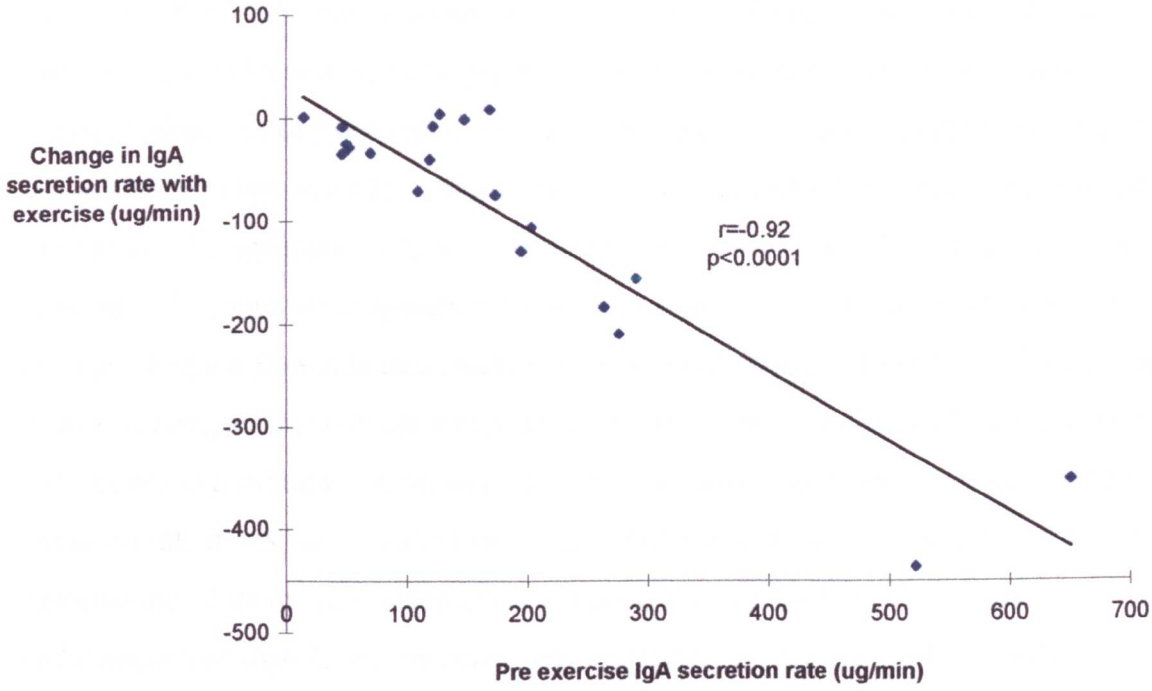

Fig 6.5. The relationship between pre exercise saliva flow rates and change in saliva flow rate (μg/min)

The change in sIgA secretion rate appears to have a relationship with its pre exercise values as strong as that observed for the change in saliva flow rate and its pre exercise values.

6.4 DISCUSSION

The aim of this study was to investigate the relative contribution of changes in saliva flow rate and sIgA concentration to calculated sIgA secretion rate. In this investigation, interval session boxing training resulted in no significant change in sIgA concentration (hypothesis 22, appendix 1) but saliva flow rate (hypothesis 21, appendix 1) and sIgA secretion rate (hypothesis 23, appendix 1); both decreased to the same extent (51%) (hypothesis 24, appendix 1). Therefore it appears that calculated sIgA secretion rate is influenced more by changes in saliva flow rate than changes in sIgA concentration (table 6.4). This pattern of change is comparable with other reports of change of secretion rate and sIgA concentration in response to a stressor. Jemmott et al. (1983) reported significant decreases in sIgA secretion rate in response to academic stress, while McClelland, Ross and Patel (1985) reported increases in sIgA concentration in response to a very similar stressor. It is unfortunate that sIgA levels are commonly expressed in only one term or another.

The results suggest that exercise-induced changes in saliva flow rates may have a greater influence on calculated changes in secretion rate than exercise-induced changes in IgA concentrations. Therefore it may not be appropriate to report changes in sIgA secretion rate as a result of decreased sIgA production signifying a decrease in specific immune activity, which is unfortunately common practice. In principle, secretion rate does provide a good indicator of defence against URTI because it takes into account the presence of the specifically acting IgA and its functional distribution, along with the innate protection provided by saliva. However, this study found that IgA concentration did not significantly change and that any changes in IgA secretion rate were a function of changes in saliva flow rate. The problem with using IgA secretion rate as a marker of susceptibility to URTI is that it combines both innate and specific defence mechanisms, and although this might seem advantageous, giving an holistic approach, it actually confounds investigations into why athletes appear to suffer URTI symptoms because the saliva flow rate and sIgA production are controlled separately.

Little consideration has previously been given to saliva flow rate and other innate defence mechanisms despite the fact that not only is saliva flow rate cheap and easy to measure, it seems logical that it would be compromised during exercise with high minute ventilation

causing evaporation of mucosal fluids, sympathetic arousal directing blood away from the saliva glands and dehydration, reducing the amount of fluid available for saliva production.

No relationship was found between change in saliva flow rate and change in sIgA concentration (fig 6.1.) (hypothesis 25, appendix 1). This supports the idea that these two factors are independent of one another, that sIgA is not a secretion specific protein and that its presence in the oral cavity is not dependent upon saliva carrying it from the saliva gland into the oral cavity (Brandtzaeg, 1971). Some studies have found a negative relationship between saliva flow rate and sIgA concentration; however, this is probably because they stimulated saliva flow which stimulates saliva water content but has no effect on immunoglobulin production, thus producing a dilution effect (Evans and Bristow, 1993; Rudney, 1991). The lack of relationship between change in saliva flow rate and sIgA concentration reinforces the idea that IgA secretion rate is an inappropriate way of expressing immune activity. Reporting merely sIgA secretion rate masks the actual changes in both sIgA concentration and saliva flow rate. In this investigation the changes in saliva flow rate were so marked that the lack of change in IgA concentration would not have been recognised if just secretion rates had been reported.

The changes induced by exercise in saliva flow rate, sIgA concentration, and secretion rate, were all found to have a strong relationship with their pre exercise values (hypotheses 26, 27 & 28, appendix 1). The cognisance of a floor effect in either sIgA levels or saliva flow rate has not been observed in the current literature. A significant change in sIgA concentration was not observed in this study however, there was a significant change in sIgA secretion rates which is probably a consequence of changes in saliva flow rates. The relationship between pre exercise levels and the changes induced (fig 6.3) is very interesting because it suggests that any intervention to prevent such low post exercise flow rates would only work after exercise, because the larger the pre exercise flow rate the greater the exercise-induced decrease. This 'floor' effect may be a basic protection mechanism designed to maintain minimal mucosal protection. There was no relationship between any of the post exercise values for the three variables and percentage change with exercise, the percentage change being dependent only upon the pre exercise value.

In conclusion, the terms in which sIgA levels are expressed are critical to the interpretation of the results, and the different terms of expression used do not appear to be synonymous.

Further consideration of the terms of expression used for sIgA levels relative to defence against URTI and as a marker of immune activity must be given. It appears that there is a large decrease in saliva flow rate with exercise, and it is likely that this will go some way to explain the occurrence of symptoms related to URTI in athletes and regularly training individuals. Much of the epidemiological work suggesting that athletes and these groups of regularly training individuals are more susceptible to URTI has been carried out on marathon runners (e.g. Nieman et al., 1990a; Peters and Bateman, 1983). Chapter 7 begins to investigate saliva flow rate as an URT defence mechanism , reporting an investigation into the relationship between marathon running, changes in saliva flow rate and the report of symptoms associated with URTI.

CHAPTER 7

THE EFFECT OF MARATHON RUNNING ON SALIVA FLOW RATE

7.1. INTRODUCTION

Nieman and Nehlsen-Cannerella (1992) presented epidemiological data to suggest an increased risk of URTI during the recovery period from exhaustive prolonged exercise. It has been demonstrated that prolonged endurance exercise (longer than 2 hours) leads to transient but significant perturbations in immunity and host defence. It is possible that this data may provide a possible physiologic rationale for the apparent prevalence of URTI. Nieman et al. (1989) found that experienced marathoners who ran for 3 hours to exhaustion in a laboratory setting demonstrated significant leucocytosis, granulocytosis, neutrophilia, monocytosis and eosinopenia during recovery. Another study by Nieman and colleagues found that 2.5 hours of running at 75% $\dot{V}O_{2\,max}$ by experienced marathoners was associated with a decline in lymphocyte proliferative response and a decrease in T cell concentrations (Nieman et al., 1995). As reviewed in section 2.1.1., prolonged exercise may lead to an 'exhaustion' of the defences possibly through an exhaustion of available immune cells, or an insufficiency of available fuel reserves. The clinical significance of the perturbations in immune activity remain unclear.

Data supporting the anecdotal evidence that athletes are more susceptible to URTI have been derived from questionnaires handed out to marathon runners. Peters and Bateman (1983) and Nieman et al. (1990b) have reported significantly higher incidences of URTI symptoms in individuals after a marathon race as compared with age-matched controls (see section 3.2.1.). Both studies used questionnaires to gather information on demographic data, training habits, race results, and the incidence of symptoms of URTI in themselves and in nominated controls with whom they had regular contact, in an attempt to correct for exposure to pathogens.

As has previously been described, mucosal surfaces are the first-line of defence against URTI, and these defences may be compromised during exercise (section 4.5). The increased metabolic requirement during exercise results in an increase in oxygen uptake; concurrently, in order to minimise airflow resistance, air is taken in through the mouth instead of the nose. The large volumes of air passing over the mucosa of the upper

respiratory tract during exercise means that air is not 'processed' in the same way that it is during rest; it bypasses the nasal filter and is not 'warmed' and is therefore more likely to cause airway irritation. The increase in ventilation not only increases the probability of inhaling a pathogenic particle, but it also results in a drying of the airways (by increasing the rate of evaporation of mucosal fluids), increased viscosity of the mucus, and decreased ciliary action, resulting in increased susceptibility to invading pathogens (Shephard et al., 1994). Anti-pathogenic proteins only function when in physical contact with the invading pathogens, and the likelihood of interaction is reduced with a decrease in the volume, or an increase in the viscosity, of mucosal fluid. The large volumes of air moving rapidly over the mucosal surfaces may not only affect upper respiratory tract innate immune defences but also alter some specific immune functioning. It has been reported, for instance, that the epithelial transport of sIgA or the function of sIgA secreting cells residing beneath the oral mucosa may be reduced (Mackinnon et al., 1989).

Although many studies have focused upon salivary IgA levels in order to account for the purported increase in susceptibility to URTI in athletes, it has been demonstrated in chapter 6 that saliva flow decreases considerably with exercise. Saliva flow rate may decrease through increased sympathetic outflow, decreased water availability, or evaporation of moisture from the mucosal surfaces (see section 4.5.1.3.). Ljunberg, Ericson, Ekblom and Birkhed (1997) demonstrated a decrease in whole unstimulated saliva flow rate from 0.32 ± 0.06ml/min to 0.23 ± 0.06 ml/min after a marathon race. Since saliva confers protection to the upper respiratory tract by forming a physical barrier, having a mechanical washing effect and by carrying both specific and non-specific proteins around the mucosa, it is possible that exercise-induced decreases in saliva flow rate may have a simple but important role in the purported susceptibility to URTI.

The aim of this study is investigate whether there is a relationship between changes in saliva flow rate induced by a marathon race and the report of symptoms associated with URTI, such as a runny nose, a sore throat and a fever. The hypotheses tested within this chapter are listed in appendix 1 (26 & 29-31).

7.2. METHOD

7.2.1. *Subjects*

Eight members (7 male; 1 female) of the 100 marathon club aged 49.5 ± 10.0 years, mass 78.7 ± 12.1 kg, and height 174.0 ± 7.9cm (mean ± SD) consented to give saliva samples and complete questionnaires before and after a marathon run. All subjects were very experienced, having run competitively for 16.25 ± 7.0 years and having completed 167.25 ± 45.0 marathons each. Subjects reported that they suffered an average of 1.3 (range 0 to 4.5) infections per year. This demographic data was collected by questionnaire (appendix 2) completed before the marathon. The questionnaire also asked for expected completion time.

7.2.2. *Collection of saliva samples.*

Whole unstimulated saliva samples were collected by the passive dribbling method, as described in chapter 5, for four minutes. Samples were collected on waking for two mornings before the race day and on waking the morning of the race. The values of these three samples were averaged to produce a mean baseline value. Samples were also collected immediately prior to, immediately after (i.e. within half an hour), and the morning following the marathon.

7.2.3 *Calculation of saliva flow rate*

The volume of saliva produced was determined by weighing, with Ohaus E120 scales and subtracting the weight of the collection vial.

7.2.4. *Symptom Checklist*

A symptom checklist (see appendix 2) was given to subjects after completion of the marathon. It asked subjects to note the occurrence and to scale (1 being very slight and 5 being very severe) the severity of six symptoms commonly associated with upper respiratory tract infections, at six time points, 2 before the run, one during and three after the marathon.

7.2.5 *Statistical analysis*

A mean baseline saliva flow rate was obtained for each individual from samples collected on three mornings prior to the race. The group mean and standard deviations calculated for baseline samples and samples collected immediately prior to the marathon, immediately after, and the next morning, are presented in table 7.1.

A repeated measures one-way ANOVA (time) was carried out to compare the saliva flow rate values at four time points: baseline; immediately pre; immediately post; and the morning following the marathon.

A Pearson's product moment correlation was carried out to investigate the relationship between pre exercise flow rates and the change in flow rate with exercise.

The reported symptoms were analysed by a two-way repeated measures ANOVA (time x symptom) in order to investigate if any one symptom had a higher report score (i.e. was more frequent or more severe) or if any one time point had a higher report score (i.e. the severity of the symptoms were higher or more symptoms were reported), and to investigate if there was an interaction of the effect of these two factors on symptom report score. The ANOVA F test was modified when data lacked sphericity, as described in section 5.3.1.1.4.

7.3. RESULTS

The mean time to complete the marathon (4hrs 45 mins ± 41 mins) matched the time predicted by the subjects (4hrs 43 mins ± 47 mins).

Saliva flow rate decreased with exercise in every case.

Table 7.1. The group mean values and differences relative to a mean three day baseline value for saliva flow rate.

	Mean (ml/min)	**Difference in saliva flow rate (ml/min).** (relative to immediately before marathon)
BASELINE	1.76 ± 1.46	0.01 ± 1.17
PRE	1.78 ± 1.51	0.00 ± 0.00
IMMEDIATELY POST	1.35 ± 1.26	-1.03 ± 1.86
1-DAY POST	1.95 ± 1.72	-0.49 ± 2.14

Data are mean ± SD. (n=8)

A one-way repeated measures ANOVA (time) revealed that there was no main effect ($F_{(3,28)}$=0.525, $p>0.05$) of time on saliva flow rate.

The group means for change in saliva flow rate (as compared with the immediately pre exercise) were plotted for immediately before a marathon, immediately after a marathon, and the day after a marathon in figure 7.1. Although saliva flow rate values did not differ significantly between the four time periods, it is evident that some change in flow rate was occurring with exercise.

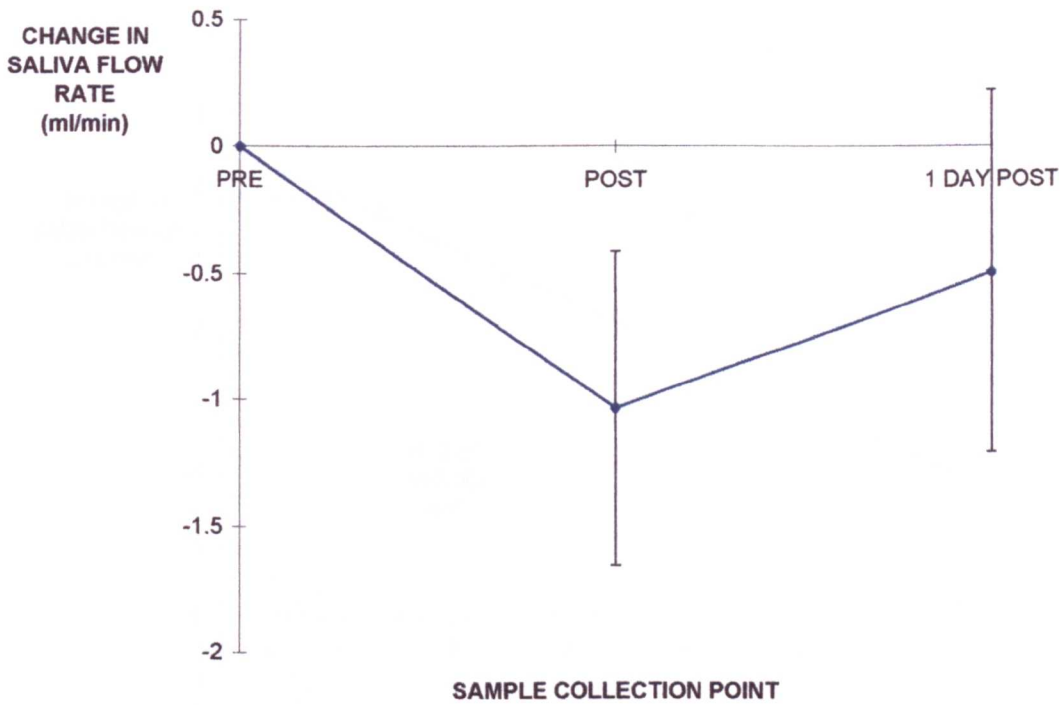

Fig 7.1. Change in saliva flow rate relative to the mean baseline value (mean ± SE.)

Results reported in chapter 6 suggested that there was a strong relationship between pre exercise saliva flow rates and the change in saliva flow rate induced by exercise (fig 6.3 $r=-0.91$, $p<0.0001$). A Pearson's product moment correlation revealed that, although the alpha value was just above the 5% level, there was still a moderate relationship between pre exercise saliva flow and the negative change induced by exercise (Fig 7.2.). It is possible that the correlation here is weaker than in chapter 6 because significant change in flow rate was not observed for this data.

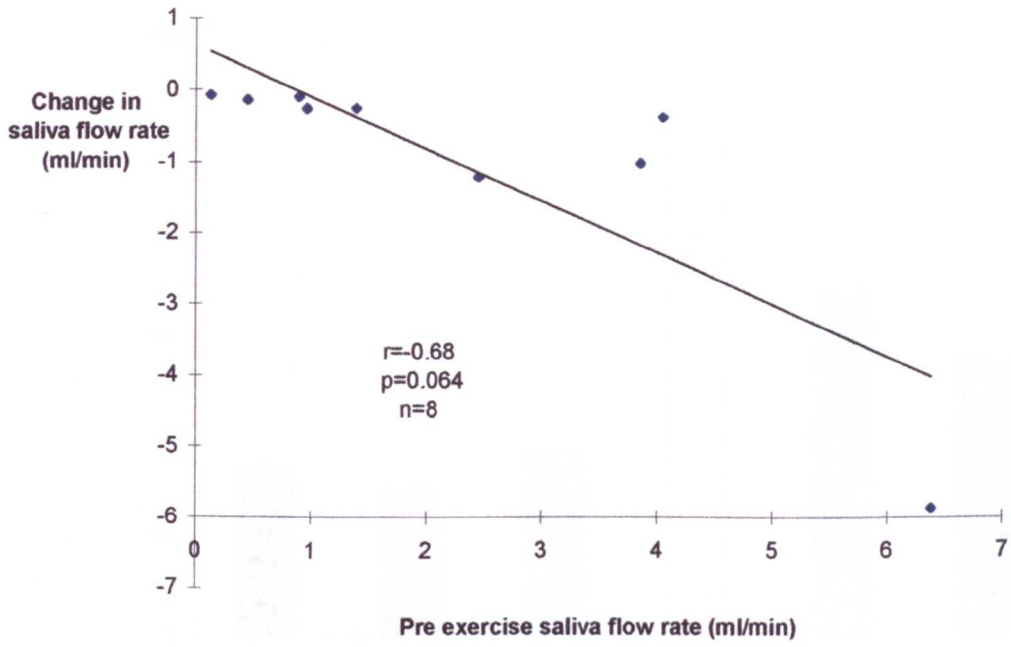

Fig 7.2. The relationship between pre exercise saliva flow rates and change in saliva flow rate with exercise.

A two-way repeated measures ANOVA (time x symptoms) revealed that there was no significant interaction ($F_{(3,23)}$=1.17, p>0.05) between the time and the symptoms reported. No main effect was found for symptom ($F_{(2,14)}$=1.32, p>0.05). Nor was there any main effect for time ($F_{(2,13)}$= 0.39 p>0.05), symptoms were not reported to be any worse at any one particular time point.

The sum of the scores for each symptom at all time points, and the sum of all symptoms at each time points, were calculated and are presented in figs 7.3. and 7.4.

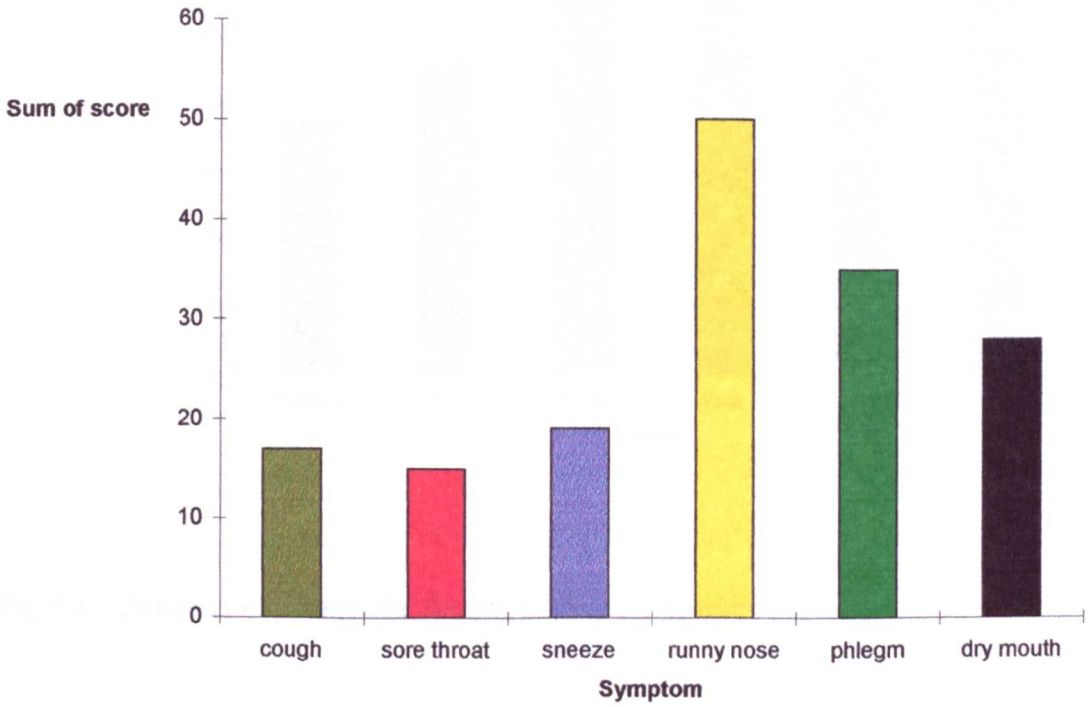

Fig 7.3. The sum score for each symptom

Although no significant difference was found between the occurrence of symptoms, it is clear from fig. 7.3. that the most prominent symptom was a runny nose. It is interesting that the occurrence of sore throat is "at the bottom of the list". This may be the only symptom on the list which would be the result of either inflammation or infection, whereas all other symptoms could be the result of a defensive reaction.

Although no significant difference was found between the time points, the sum of scores presented below demonstrate that more symptoms were reported during the marathon and numbers were still high immediately afterwards. The level of symptom-reporting appeared to remain elevated as compared with the three day baseline the day after the marathon, however this was not found to be significantly different.

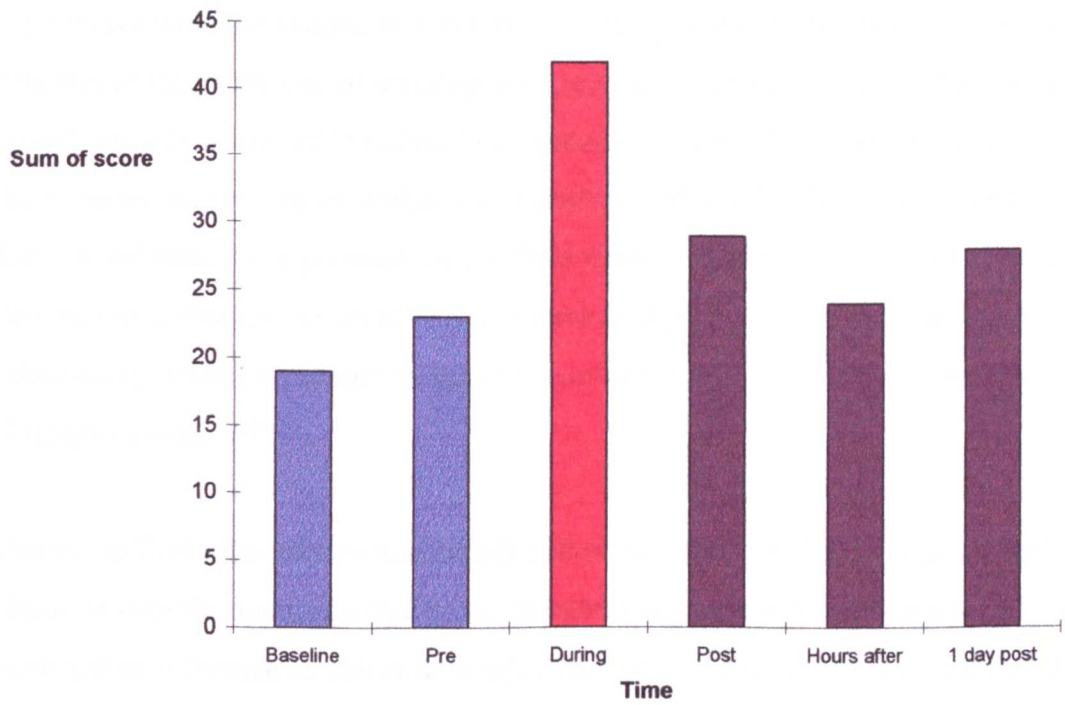

Fig 7.4. The sum of scores for the time when symptoms occur

7.4. DISCUSSION

Saliva flow rate was decreased after the marathon as compared with the samples collected immediately before the race (fig. 7.1.). However, these changes were not found to be significant (hypothesis 29, appendix 1), primarily due to a lack of statistical power as a consequence of high variability and low subject numbers. It is possible that fluid intake during the race tempered the exercise-induced decrease in flow rate. However, details of fluid intake were not sought, in order to avoid influencing the behaviour of the runners. The aim of this study was to investigate whether running a marathon under normal conditions was sufficient to induce large changes in saliva flow rate, and to investigate the time course and severity of symptoms associated with URTI, therefore it was important that normal patterns of preparation and fluid intake were followed. It had previously been demonstrated that the variation in fluid intake throughout a marathon showed no relationship between changes in salivary flow or concentrations of components (Ljungberg et al., 1997).

The saliva flow rates taken immediately before the race were found to be virtually identical with the mean baseline value, therefore suggesting that rhythms which might have had an influence on saliva flow rates, had not. Dawes (1972) reported that the flow rates of whole unstimulated saliva have powerful circadian rhythms, with saliva flow rate peaking in the afternoon at 15:26 hours. The post race sample may thus have been influenced by circadian variation, and if saliva flow rates were naturally increased by circadian rhythms then it is possible that some of the marathon-induced decrease was masked. The only way of knowing if a circadian variation in flow rate tempered the exercise-induced decrease would be to have a matched control group providing samples at the same time points. Alternatively the baseline sampling could be extended and samples could be collected at the same time points as the race day for a few days before the race. This would provide an idea of any decreases and increases in flow rate that may occur naturally. If the decreases in flow rate were against the direction of variation then the effect of the marathon would be larger than the effect found here.

It is interesting that a relationship between pre exercise saliva flow rate and the change in flow rate induced by exercise was found in these samples collected round a marathon, as in

samples collected before and after boxing training demonstrated in chapter 6 (hypothesis 26, appendix 1),. This finding suggests that the greater an individual's flow rate before exercise then the greater the impact of that exercise. As described in chapter 6, there appears to be a floor effect, a point at which saliva flow rate appears to stop decreasing. It would be interesting to investigate if the same effect was seen when intervention (i.e. fluid ingestion) was given prior to exercise, to increase pre exercise flow rate. If a floor effect does exist, then the reduction in flow rate induced by exercise would be increased (because of the higher pre exercise flow rate). If this was the case, then it would only be useful to use intervention strategies to improve saliva flow rates after, or possibly during, rather than before the exercise.

It was found that none of the symptoms were reported to be significantly more severe and frequent than any of the others (hypotheses 30 & 31, appendix 1),; however from figure 7.3 it is possible to see that the highest report score was for a runny nose. A runny nose is probably part of an innate defence reaction, as the body attempts to prevent the entry of any particles through the nose. The symptoms of phlegm and a dry mouth are probably a consequence of evaporation of fluid from the mucosal fluid. Increased sympathetic arousal, [which occurs during exercise to facilitate movement (see section 2.2.3.2.8) and has been demonstrated to occur in response to marathon running (Nieman et al., 1989)], has been demonstrated to decrease the water and increase the glycoprotein content of mucosal fluids, making them more viscous, resulting in the production of phlegm (see section 4.5.1.1). The most prominent time for symptom reporting was during the race (fig 7.4), which supports the idea that the symptoms were a consequence of inhaling large volumes of air and the exercise state itself. The symptom that was least likely to occur as part of a defensive reaction, (a sore throat), had the lowest incidence, and could have also been a consequence of airway drying and damage caused by the friction of large volumes of air passing along the URT. It is possible that none of the symptoms experienced were the result of an infective agent, but a consequence of either a protective reaction to remove any inhaled particles (runny nose, cough and sneeze) or the result of the exercise state (sympathetic arousal, drying of the airways).

It is feasible that other symptom reports could have mistaken the results of a defensive reaction or the exercise state as the consequence of an infection. Peters and Bateman (1983) collected symptom data both two weeks before and two weeks after a race.

Symptoms included sore throat, nasal symptoms, cough, and fever plus URTI. Of the 33.3% of subjects that reported post race symptoms, the majority reported that the symptoms lasted longer than 4-7 days (33%) or more than 7 days (47%) and the worst symptom was sore throat, with nasal symptoms (running nose/ sneezing) being very close. Only 3 subjects suffered from a fever, which would almost certainly confirm an infective origin. As previously described, it is possible that the incidence of a sore throat was a consequence of friction caused by inhaling large volumes of air, and the nasal symptoms the consequence of a defensive reaction. However, if this was the case then it is unlikely to persist for up to four days and longer, unless the subjects continued to train hard never allowing themselves to recover. The authors themselves wrote that they wondered if some of the reported symptoms were primarily the result of the athletes' preoccupation with their health.

In another commonly cited marathon study (Nieman et al., 1990a) the incidence of URTI was established by questionnaire, subjects were asked to report on their incidence of cold, flu or sore throat 2 months before and 7 days after the race. They found that 43.2% subjects reported symptoms in the 2 months before the race, and 12.9% became sick in the week following the race, compared with 2.2% of the controls. Although it is feasible that the subjects were accurate in their symptom reporting, the data could be influenced by the fact that the subjects were being asked to focus upon how they were feeling and their possible preoccupation with this.

There is a lack of studies which have identified the existence of a pathogen or even a clinical diagnosis of an URTI. Much of the epidemiological data available is based on the presence of symptoms commonly associated with URTI. Graham, Douglas and Ryan (1986) have suggested that symptoms not actually induced by an infectious agent may be perceived as a illness and this may be a factor in biasing the results of epidemiological studies. It was not possible for this study to carry out the necessary microbiological tests, however, there is a need to establish conclusively if athletes do suffer from an increased incidence of infection rather than just an increased incidence of symptoms associated with URTI. It is feasible that the symptoms often regarded as associated with the occurrence of URTI, may be the result of inhaling large volumes of air and sympathetic arousal. However, regardless of the cause, symptoms which have been associated with prolonged or

intense exercise would not facilitate maximum performance and are therefore still an issue for athletes.

There is a need not only to establish the cause of the symptoms that athletes and regularly-training individuals are reporting, but also to develop intervention strategies that would prevent this discomfort and avoid any possible physical or psychological impact that it may have on performance. If airway irritation is an important factor, then it is feasible that saliva would afford some protection, coating the mucosal surfaces and protecting them from frictional damage.

The next chapter further questions the effect of exercise on saliva flow rate, monitors the level exercise-induced physical stress and investigates the use of fluid-replacement strategies to temper any exercise-induced decreases in saliva flow rate.

CHAPTER 8

THE EFFECT OF FLUID REPLACEMENT AND HEAT ON SALIVA FLOW RATE AND SALIVA TOTAL PROTEIN LEVELS IN RESPONSE TO EXERCISE.

8.1 INTRODUCTION

It has so far been discussed that the mucosal surfaces and the immune defences at these surfaces provide the primary defence against potential URTI pathogens. To date the majority of studies focussing on reports of increased URTI symptoms in athletes has focussed upon exercise-induced changes in sIgA levels. However, findings contained within this thesis (chapter 6) suggest that exercise induces large reduction in saliva flow rate and concomitantly a reduction in sIgA secretion rate and that sIgA concentration is affected to a much lesser extent. The importance of saliva in defence against pathogens has been described in section 4.2.3. It is also likely that the physical barrier afforded by a lining of saliva would provide some protection against frictional damage induced by inhaling large volumes of cold dry air during exercise. Therefore if the saliva flow was compromised by exercise then the possibility both of pathogenic particles gaining entry to the mucosa and the chances of an inflammatory response induced by frictional damage would be increased, placing previously unrecognised importance on saliva flow rate and the changes induced by exercise.

It has already been described that saliva flow and composition depend upon neural and endocrine control and are influenced by a number of factors including hydration status, exercise, and a variety of psychological and situational conditions. Although it has been proposed that this observed exercise-induced decrease in flow rate may be a consequence of dehydration and sympathetic outflow decreasing saliva production, it requires further investigation.

Exercise may decrease saliva flow rate through a variety of mechanisms; however, dehydration would exacerbate this, both water deficiency (Gantt, 1929) and dehydration (Kerr, 1961) having been associated with depressed saliva flow rate. It has been reported that an 8% reduction in body water content can cause a 100% reduction in salivary flow rate (Holmes, 1964). As previously described (chapter 4), saliva flow is principally

controlled by the autonomic nervous system. Dehydration and heat stress (Hales, 1986), both of which are likely to occur during exercise, affect saliva flow rate on two levels, primarily decreasing fluid availability and secondarily via sympathetic arousal which decreases blood flow to the salivary glands and subsequently decreases saliva production (Carlson, 1986, cited Green and Green, 1987).

There is a need to investigate the extent to which dehydration will reduce saliva flow rate. It has been demonstrated that increases in ambient temperature decrease saliva flow rate (Shannon, 1966), possibly because increases in environmental temperature are likely to result in greater fluid loss through increased sweating, and possibly by increased evaporation from the oral mucosa.

There was a need to further investigate changes in saliva flow rate and salivary protein levels in controlled laboratory conditions after attempts to demonstrate changes 'in the field ' proved inconclusive (chapter7), along with the time course of changes in saliva flow rate and changes in indices of physical stress and hydration status in an attempt to account for any observed changes in saliva flow rate.

This part of the study aims to investigate the effect of exercise and of exercise in the heat on saliva flow rate and total protein levels, while monitoring indices of hydration status (change in body mass, blood volume and plasma volume) and sympathetic outflow and physical stress (heart rates, perceived exertion scores and blood lactate levels), and to map the period of recovery. The potential value of fluid replacement before and during exercise as an intervention strategy against exercise-induced decreases in saliva flow rate was also be investigated. A number of hypotheses were tested are listed in appendix 1 (32-61).

8.2 METHOD

8.2.1 *Subjects*

The volunteers for this study were twelve healthy male college students: physical characteristics (mean ± SD) age 23.1 ± 4.1 years; height 181.1 ± 5.8 cm; mass 76.3 ± 6.0kg; maximum power output 331.3 ± 11.9 Watts). The subjects were in good health. Informed consent was obtained prior to testing. Subjects arrived at the laboratory normally hydrated and in a rested state.

8.2.2 *Preliminary testing*

Each subject performed an incremental test to exhaustion to determine peak power output (MAP) on a Monark cycle ergometer (E814): following a 5 minute warm up, subjects cycled continuously at 60revs.min.$^{-1}$. Every 90 seconds the resistive load was increased by 0.5kg (30 watts) until volitional exhaustion. Pedal revolutions, heart rate and rating of perceived exertion (RPE), (Borg, 1975) were recorded for the last 60 seconds of each stage. MAP was determined from maximum power sustained over any 60 second period.

8.2.3 *Exercise testing*

Each subject completed three exercise trials in a counterbalanced order, consisting of 30-min bouts on a cycle ergometer at 50 % MAP (as determined by preliminary testing) in an environmental chamber with relative humidity 40-50%. The experimental conditions were; 35°C with no fluid replacement; 21°C with no fluid replacement; 21°C with fluid replacement. The tests were performed at the same time of day, 7 days apart, to avoid circadian variation.

Subjects were asked to arrive at the laboratory having not eaten for an hour, to ensure the saliva flow was unstimulated. Prior to the bout nude body mass was recorded. Subjects were seated on the cycle ergometer for 15 minutes to avoid positional factors influencing blood sample results. During this time the polar heart rate monitors (Polar Electro, Finland) were positioned as were the tympanic temperature probe (Edale, UK) and insulating ear pads. For the hydrated trial subjects consumed 3ml.kg.$^{-1}$ of body mass of water, and for the non-hydrated trials the subject rinsed their mouth with water. A saliva

sample was collected during the last 4 minutes of the 15. Heart rate, thirst scale and capillary blood samples were collected during the last minute, before exercise began.

During each bout, heart rate, RPE and thirst were recorded every five minutes. Finger prick capillary blood samples were taken every 10 minutes. In the hydrated trial 2ml.kg^{-1} of body mass of water were given; in the other trials the mouth was rinsed with water every 10 minutes.

Nude body mass was recorded immediately post exercise. Subjects were monitored for the first hour of recovery, a saliva sample was collected immediately post and then every 15 minutes, and water was administered as during the trial. Capillary blood samples were taken at 30 and 60 minutes of recovery.

8.2.4. *Saliva collection and quantification*

The whole unstimulated saliva samples collected in 5ml plastic vials (Medfor, UK), as described in chapter 5, immediately pre and post as well as 15, 30, 45, and 60mins post cessation of exercise bout, were immediately placed on ice and stored at -20°C within 30 minutes of collection. Baseline saliva samples were collected on waking for 4 days prior to the first exercise bout.

The volume of saliva produced was determined by weighing the defrosted saliva, with Ohaus E120 scales. After the mass of the collection vial had been subtracted, the mass in grams was divided by the collection time to determine flow rate. The samples were then centrifuged at 2000rpm for 10 minutes in a Sanyo (2-15) centrifuge. The supernatant was drawn off using a Pasteur pipette into a cuvette (Hughes and Hughes Ltd. UK). Total salivary protein can be estimated by optical density. Tomasi et al. (1982) demonstrated optical density to be as reliable as the commonly used buiret method. The optical density of the saliva was determined using a Shimadzu spectrophotometer at a wavelength of 280nm.

8.2.5 *Blood collection and analysis.*

Three finger prick capillary blood samples were collected in volumes of 47µl and 44.7µl in graded heparinised capillary tubes (Corning, USA), and 0.4µl non-heparinised graded Reckman capillary tubes, immediately prior to and at 10, 20, 30 mins during the 30 minute

exercise bouts, as well as 30 and 60 minutes post. Postural effects on blood flow were avoided by seating the subjects on the ergometer for 15 minutes prior to the first sample, post exercise they were seated in a chair and asked not to walk about. Samples were used to determine blood lactate concentration (47μl) using YSI 2300 stat; mean haemoglobin concentration (44.7μl) was determined by the cyanmethaemoglobin method (Boehringer Mannheim). Haematocrit samples were collected in a 0.4μl graded Reckman tube, sealed using a cristaseal and left to clot for at least 10 minutes. Samples were then centrifuged at 2000rpm for 15 minutes using a Sanyo MSE Haemo Centaur. The haematocrit was determined using a Hawksley microhaematocrit reader (Hawksley and Sons Ltd., UK). Percentage changes in volumes of blood, plasma, and erythrocytes were calculated using the Dill and Costill (1974) method.

8.2.6. *Measurement of physiological and perceived stress.*

Heart rate (b.p.m) was recorded by polar heart rate monitors. Rating of perceived exertion was recorded according to the Borg scale (Borg, 1975) (appendix 2). Perceived thirst was established with a thirst scale (Fallowfield, Jackson, Wilkinson and Harrison, 1996) (appendix 2). A tympanic thermometer was used to establish body temperature. Rate of glycolysis was monitored by blood lactate levels. Changes in blood and plasma volume, along with changes in body mass, were used as indicators of hydration status.

8.2.7 *Statistical Analysis*

A series of two-way repeated measures ANOVA's (time x condition) was carried out for; saliva flow rate; changes in saliva flow rate; total protein levels; changes in total protein levels; changes in plasma and blood volume; heart rate; perceived exertion rating; change in body mass; blood lactate levels; tympanic temperature; and thirst scores.

Two one-way ANOVA's with four repeated measures were carried out between the mean baseline value and the three pre-exercise values (for each condition) for saliva flow rate and total protein levels, in order to establish that these variables were the same immediately before each bout, and that this was not different from the baseline values.

Two-tailed Pearson's product moment correlations were carried out to elucidate if there was any relationship between change in saliva flow rate and any of the following: pre

exercise saliva flow rate; change in body mass; change in plasma volume; change in blood volume; perceived thirst score during exercise and the perceived thirst score post exercise.

A Pearson's product moment correlation was also carried out between saliva flow rate and saliva total protein levels. The relationships between temperature and heart rate, temperature and perceived exertion scores, heart rate and perceived exertion scores; and heart rate and change in plasma volume were also examined using Pearson's product moment correlations.

8.3 RESULTS

Data are means from all twelve subjects unless otherwise stated. A one-way repeated measures ANOVA (time) revealed no differences in saliva flow rate between the mean of four baseline samples and any of the immediately pre bout samples ($F_{(3,31)}$ =0.53, p >0.05).

The mean change in saliva flow rate, relative to pre-exercise values was plotted for each condition (figure 8.1).

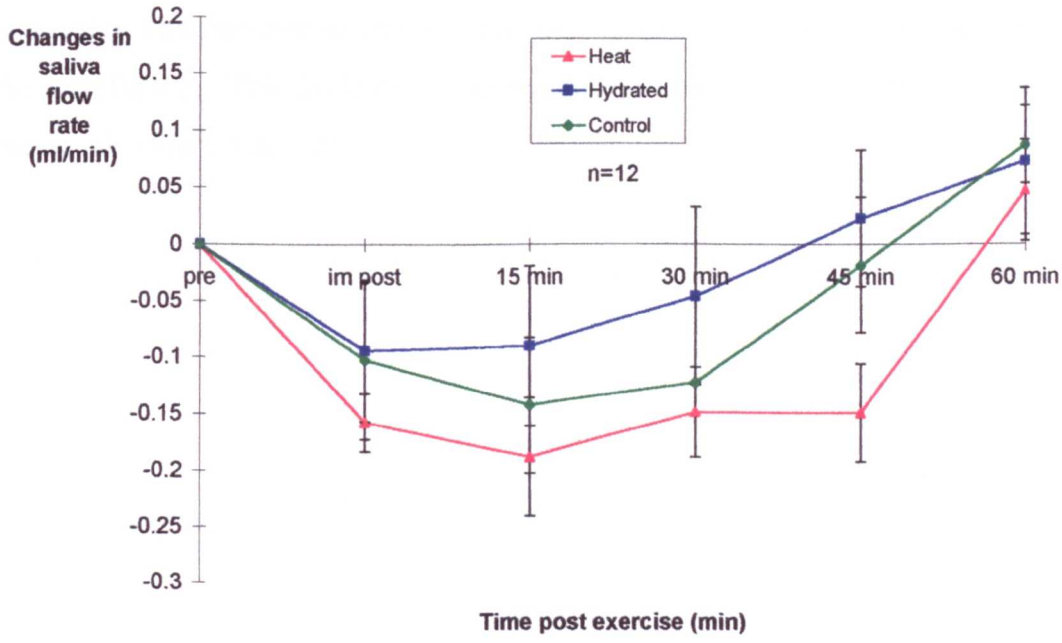

Fig. 8.1. Mean changes in saliva flow rate from the pre exercise value after exercise (mean ± S.E.).

Exercise reduced saliva flow rate, and exercise in the heat exacerbated this. All flow rates increased above pre exercise values after 60 minutes recovery, however fluid replacement facilitated a faster recovery of flow rate increasing above pre exercise values by 45 minutes (fig. 8.1). A two-way repeated measures ANOVA (time x condition) showed that there was no significant interaction between time and condition for saliva flow rate ($F_{(10,90)}$=1.37, p>0.05). However, there was a main effect for both condition ($F_{(2,18)}$=6.7, p<0.05) and time ($F_{(3,33)}$=6.3, p<0.05) for saliva flow rate.

There was a decrease in saliva flow rate immediately after exercise followed by a gradual increase with time for all conditions (fig. 8.1). A two-way repeated measures ANOVA (time x condition) for the change in saliva flow rate, (relative to pre-exercise values) revealed that there was no significant interaction ($F_{(4,41)}$=0.343, $p>0.05$). There was no significant main effect for condition ($F_{(2,21)}$=1.13, $p>0.05$), indicating that the pattern of change was the same for every condition. However, a significant main effect for time was found for change in saliva flow rate ($F_{(2,24)}$=9.2, $p<0.05$): the change in saliva flow rate was different between different time points.

A Pearson's product moment correlation between pre exercise flow rates and the change induced by exercise (pre to immediately post) revealed a significant correlation between the two (fig 8.2.). This finding is in accordance with what had been demonstrated previously (figs 6.3 & 7.2).

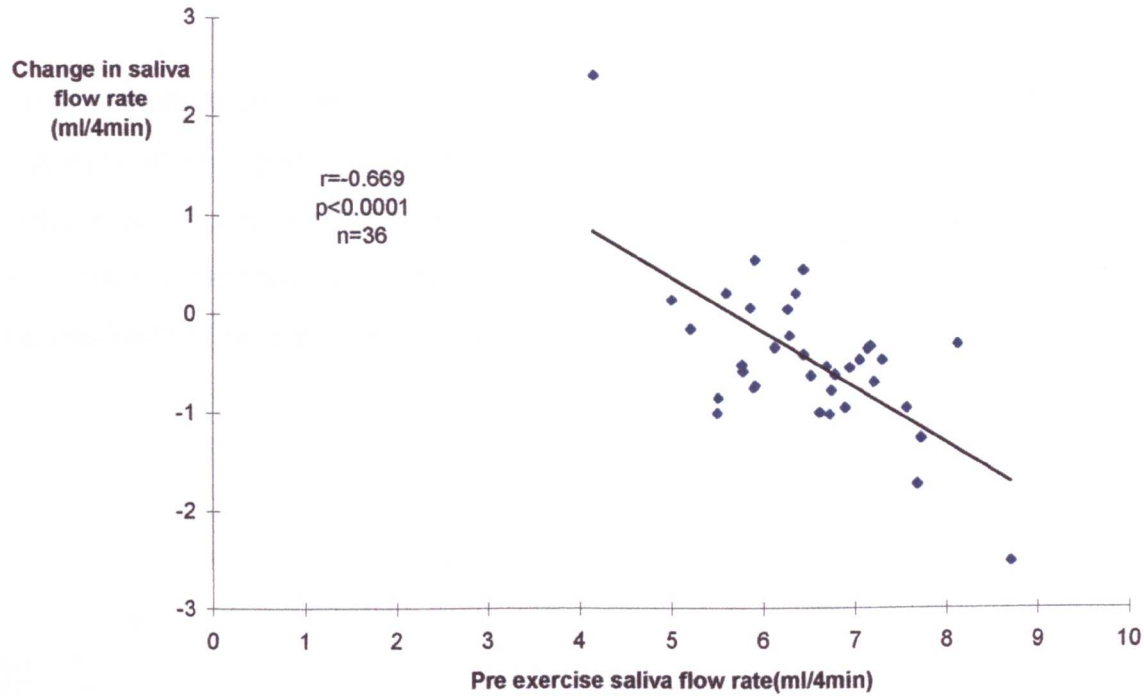

Fig 8.2. The relationship between pre exercise saliva flow rate and the change induced by exercise.

A one-way repeated measures ANOVA (time) revealed that the total protein levels (as determined by optical density) of samples taken immediately pre bout were not significantly different from the baselines ($F_{(3,31)} = 2.4$, $p>0.05$).

Two two-way repeated measures ANOVA's (time x condition) revealed that there were no significant interactions between time and condition for total protein levels ($F_{(3,14)}=0.66$, $p>0.05$) or change in total protein levels (relative to baseline) ($F_{(8,88)} = 0.26$, $p>0.05$). Nor was there a main effect for time on total protein levels ($F_{(2,25)}=0.94$, $p>0.05$), or for the change in total protein levels relative to baseline ($F_{(2,27)}=1.22$, $p>0.05$). There was no main effect for condition on either total protein levels ($F_{(1,5)}=3.41$, $p>0.05$) or change in saliva total protein levels with exercise ($F_{(2,22)}=0.284$, $p>0.05$). In summary neither exercise or condition had any effect on saliva total protein levels.

A Pearson's moment correlation was carried out (on pooled data) in order to investigate if there is any relationship between total protein levels and saliva flow rate. The two variables were found to be independent of one another (Fig 8.3). A second Pearson's product moment correlation revealed that there was no relationship between changes in saliva total protein levels and saliva flow rate ($r=-0.6$, $p>0.05$).

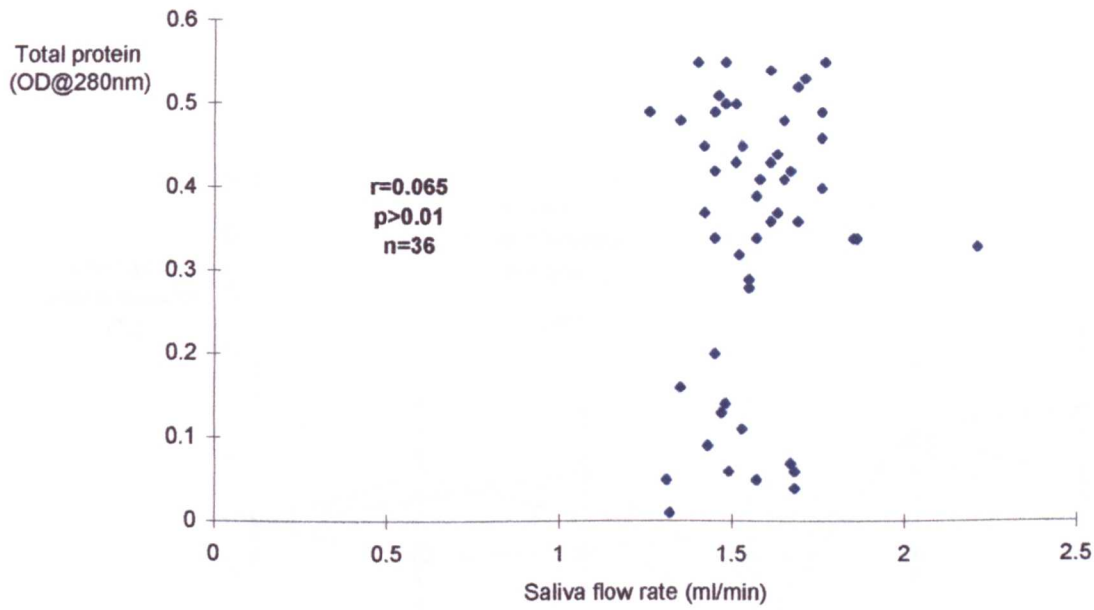

Fig. 8.3. The relationship between total protein levels against flow rate for saliva samples (mean ± S.E.).

In an attempt to account for the exercise-induced decrease in saliva flow rate, blood volume, plasma volume and body mass were monitored as markers of hydration status. Each of these makers was analysed using a two-way repeated measures ANOVA (time x condition).

A two-way repeated measures ANOVA (time x condition) for change in blood volume (relative to pre exercise) revealed that there was no significant interaction between condition and time ($F_{(2,20)}=0.47$, $p>0.05$). No significant main effect for condition

($F_{(1,2)}$=0.05, p>0.05) was found, nor was there any significant main effect for time ($F_{(3,24)}$=1.86, p>0.05) for change in blood volume.

The mean change in blood volume for the three conditions was plotted against time (Fig 8.4). Although not significantly different, the trends in the graph (fig 8.4) show that blood volume decreased with exercise, and recovered by 30 minutes after exercise. Blood volume decreased below pre exercise values in the heated trial faster than in the hydrated and control conditions. A Pearson's product moment correlation revealed that there was no relationship between change in saliva flow rate and change in blood volume at 30 minutes (only time point when data was collected for both variables) post exercise (r=-0.07, p>0.05).

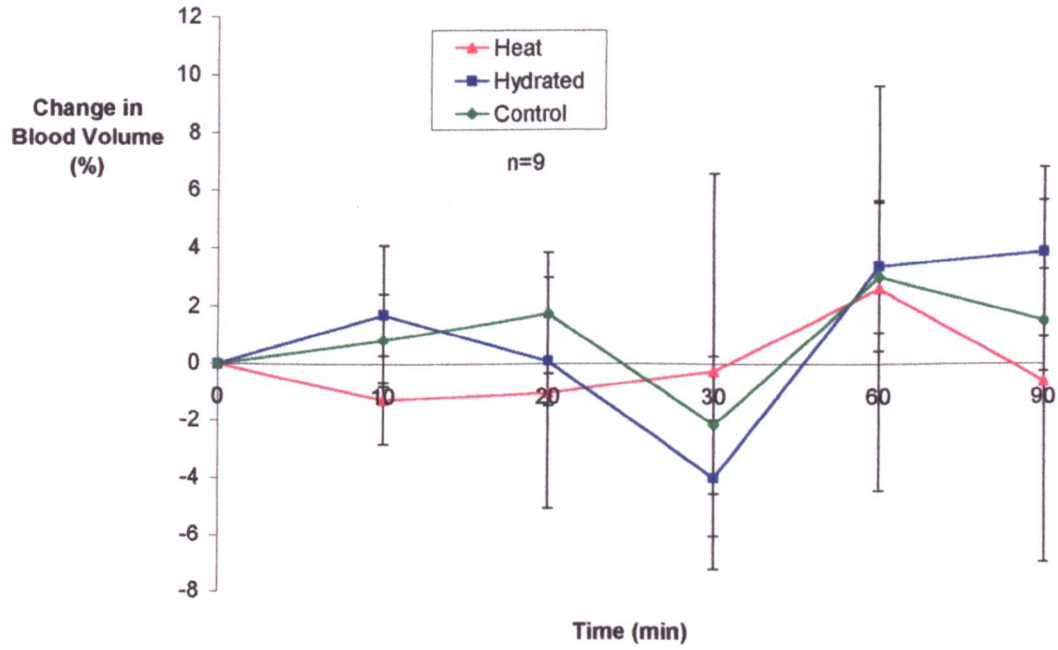

Fig. 8.4. Mean change in blood volume during and post exercise (mean ± S.E.).

A two-way repeated measures ANOVA (time x condition) revealed that there was an interaction for change in plasma volume ($F_{(4,20)}$ =2.85, p=0.05), the change in plasma volume was different between conditions for time points during exercise. Exercise induced a slight variance in plasma volume. Exercise in the heat caused a severe reduction in plasma volume that had not returned to basal levels at the end of the recovery period. Fluid replacement caused an increase in plasma volume. There was no main effect for condition and change in plasma volume ($F_{(2,10)}$=2.33, p>0.05), nor was there a main effect of time ($F_{(2,10)}$=3.17, p>0.05). There was a gradual plasma expansion during recovery in each condition which increased plasma volume beyond baseline levels by the end of recovery in the control and hydrated conditions (figure 8.5).

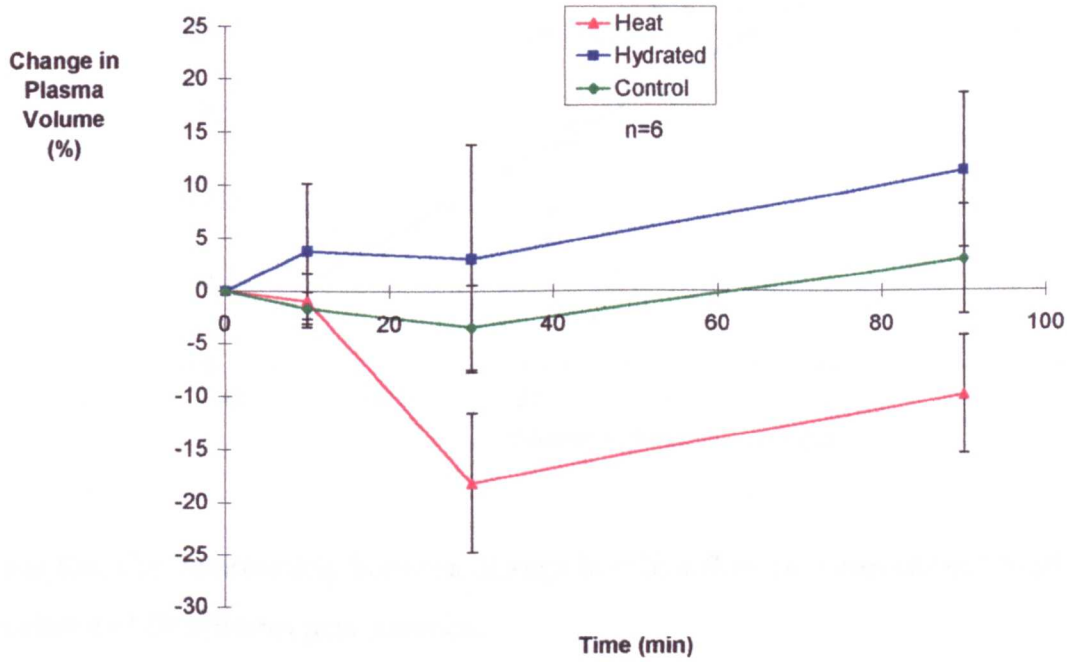

Fig. 8.5. Mean change in plasma volume during and post exercise (mean ± S.E.).

A Pearson's product moment correlation revealed a significantly positive relationship between change in saliva flow rate [relative to pre at 30 minutes post exercise (this is the only time point when measurements were taken for both variables)] and change in plasma volume (at 30 minutes post exercise) (Fig 8.6.).

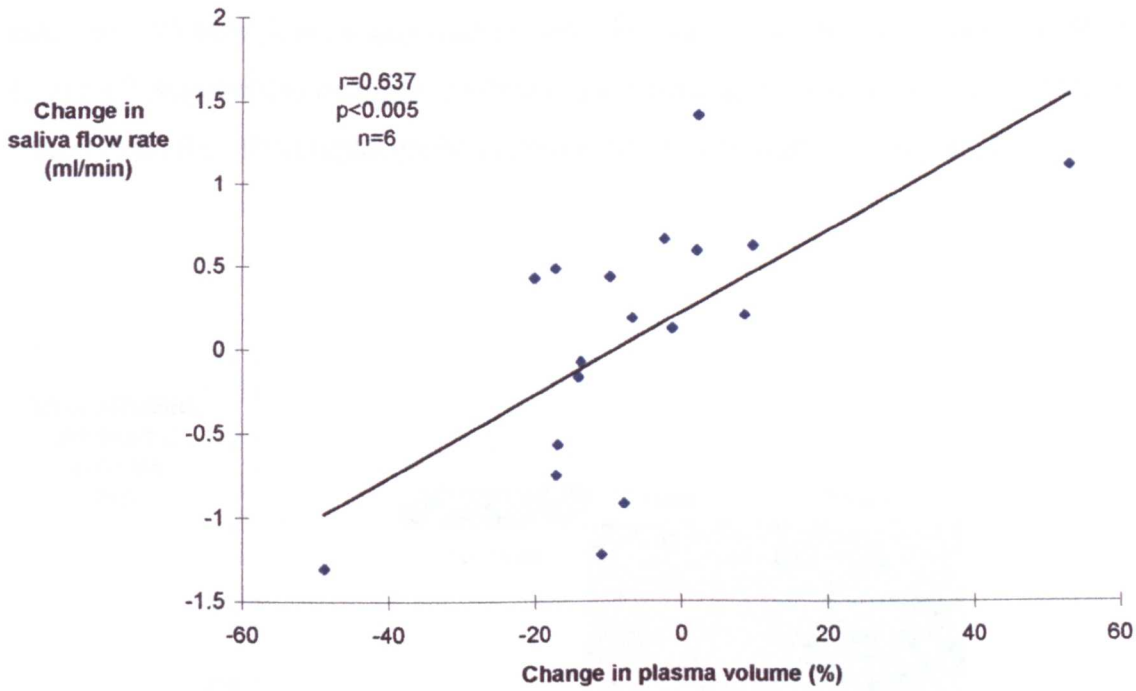

Fig 8.6. The relationship between change in saliva flow rate and change in plasma volume at 30 minutes post exercise.

Even though plasma volume was not found to be statistically different with exercise compared with baseline measures, this correlation (fig. 8.6) suggests that any change in plasma volume is mirrored by a change in the same direction in saliva flow rate.

Changes in blood volume (fig 8.4.) and plasma volume (fig 8.5.) did not reveal that there was a significant change in hydration status, possibly because of high variability in the results and the concurrent low statistical power. A two-way repeated measures ANOVA did reveal a significant interaction (time x condition) for change in body mass ($F_{(2,14)}$ =36.97, $p<0.001$). There was a significant main effect for change in body mass with time ($F_{(1,8)}$ =71.15, $p<0.001$), suggesting that a significant amount of fluid had been lost with exercise. No significant main effect of condition was found, but the results plotted in figure 8.7. suggest that exercise resulted in a decrease in mass while exercise in the heat exacerbated this; fluid replacement caused a pre-post increase in mass (fig 8.7.).

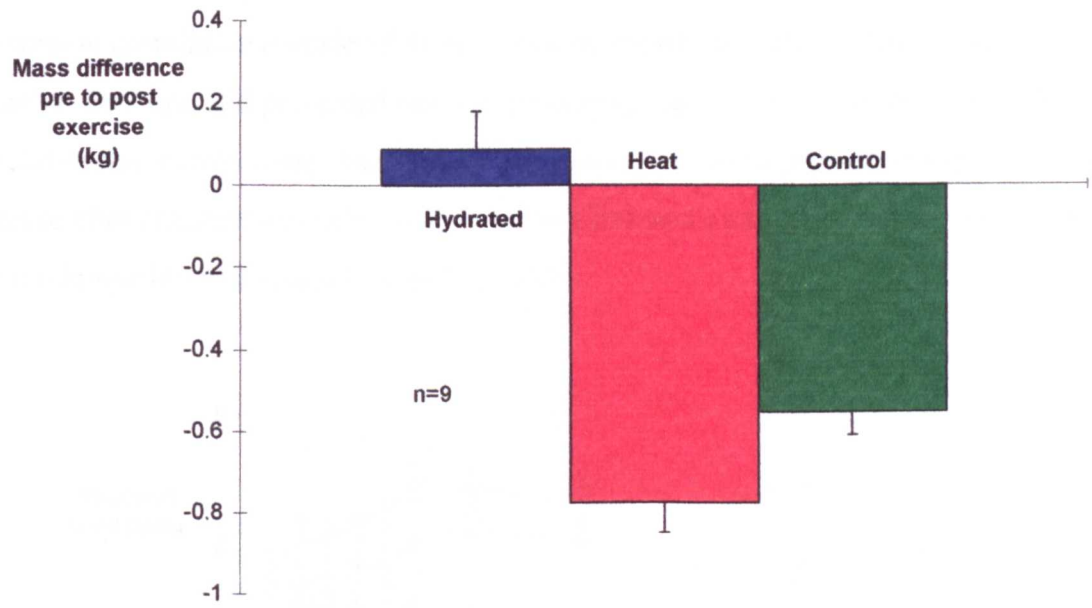

Fig 8.7. Mean mass differences pre to post exercise (mean ± S.E.)

A two-tailed Pearson's product moment correlation revealed that there was no relationship between changes in body mass and changes in saliva flow rate (pre to post exercise) ($r=0.115$, $p>0.05$).

The effect of the three trials on perceived thirst were also examined using a two-way repeated measures ANOVA (time x condition) and was found to have highly significant interaction ($F_{(5,56)} = p<0.001$). A main effect for condition was also found ($F_{(1,17)} =32.71$, $p<0.001$), but there was no significant main effect for time for perception of thirst score ($F_{(3,29)} =3.88$, $p>0.05$). The pattern of perceived thirst over time was plotted for the three conditions (fig 8.8.). Fluid replacement decreased the perception of thirst post exercise. Exercise increased it and exercise in the heat exacerbated this. Thirst in the heated and control conditions peaked towards the end of exercise (fig. 8.8). A Pearson's product moment correlation revealed that there was no significant relationship between change in saliva flow rate and perceived thirst score during exercise ($r=0.092$, $p>0.05$). The relationship between the change in saliva flow rate with exercise and the perceived thirst score after exercise were also examined with Pearson's product moment correlation; again no relationship was found ($r=0.207$, $p>0.05$).

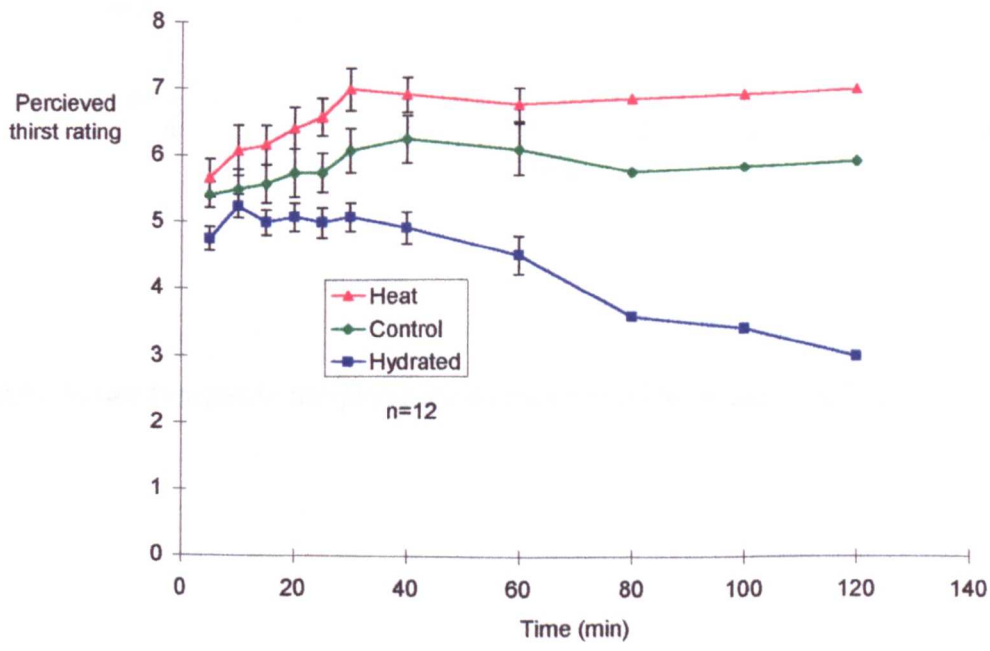

Fig. 8.8. Mean thirst scores during and post exercise (mean ± S.E.)

Core temperature is very closely regulated and therefore little change in tympanic temperature was expected; the results gained supported this theory. A two-way repeated measures ANOVA revealed that there was no significant interaction (time x condition) for tympanic temperature ($F_{(1,17)}$ =1.09, p>0.05). There was no main effect for condition ($F_{(1,17)}$ =0.35, p>0.05). However, there was a main effect for time ($F_{(1,17)}$ =8.46, p=0.005). Mean tympanic temperatures were plotted against time for each condition (fig. 8.9.).

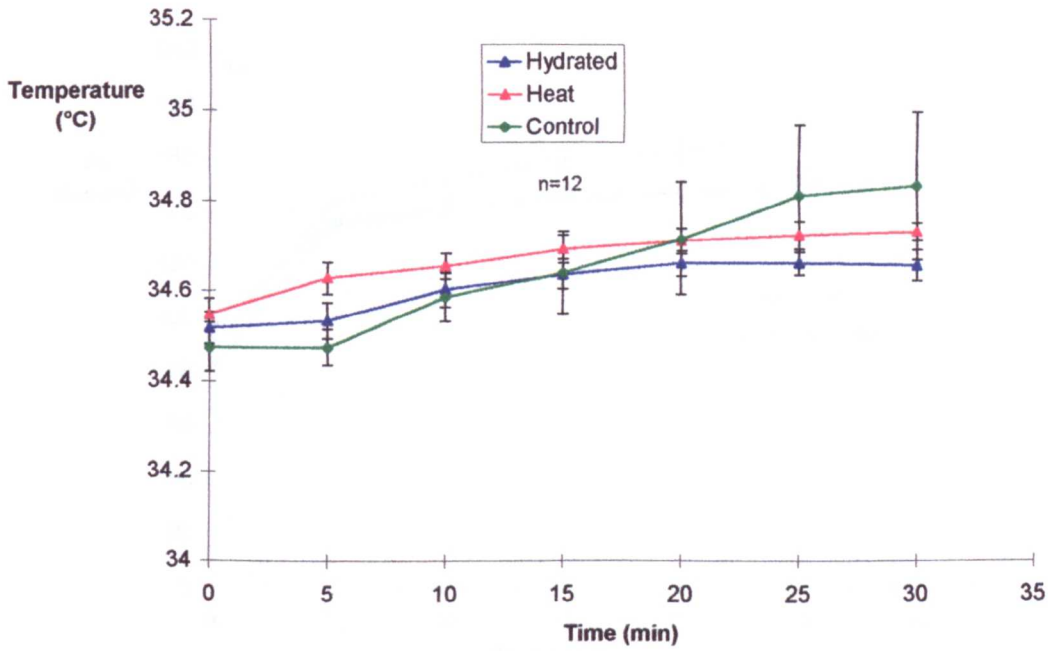

Fig. 8.9. Mean tympanic temperature during exercise (mean ± S.E.)

The relative physical and perceived exertion were monitored with heart rates and ratings of perceived exertion, respectively. Exercise with and without fluid replacement induced increases in heart rate of a similar magnitude, and quickly plateaued. Exercise in the heat induced a steady increase throughout exercise (figure 8.10). A two-way repeated measures ANOVA (time x condition) revealed a significant interaction for heart rates ($F_{(12,20)}$ =5.88, $p<0.001$), therefore indicating that the pattern of the heart rate response to exercise was different for each of the three conditions. There was a significant main effect for both condition ($F_{(2,18)}$=21.15, $p<0.0001$), and time ($F_{(1,10)}$=99.85, $p<0.0001$) with heart rates rising to the highest levels during the heated condition.

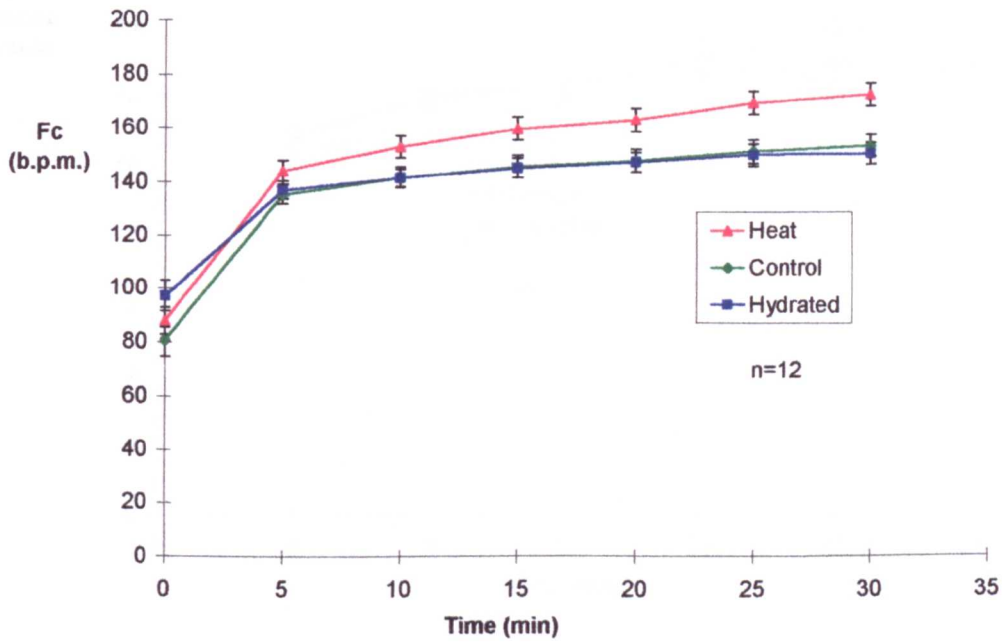

Fig. 8.10. Mean heart rates during exercise in three conditions (mean± S.E.).

Pearson's product moment correlations revealed that there was no relationship between heart rates and change in plasma volume ($r=0.158$, $p>0.05$). There was a relationship between tympanic temperature and heart rate ($r=0.9$, $p>0.05$).

The pattern for the perceived exertion scores is very similar to that of heart rate with the heated condition resulting in the highest scores (figure 8.11). A significant interaction (time x condition) was revealed by a two-way repeated measures ANOVA for rating of perceived exertion scores ($F_{(3,37)}$ =3.09, p <0.05). There was a significant main effect for time ($F_{(1,15)}$ =33.5, p<0.001), but there was no main effect for condition ($F_{(1,14)}$ =2.45, p>0.05).

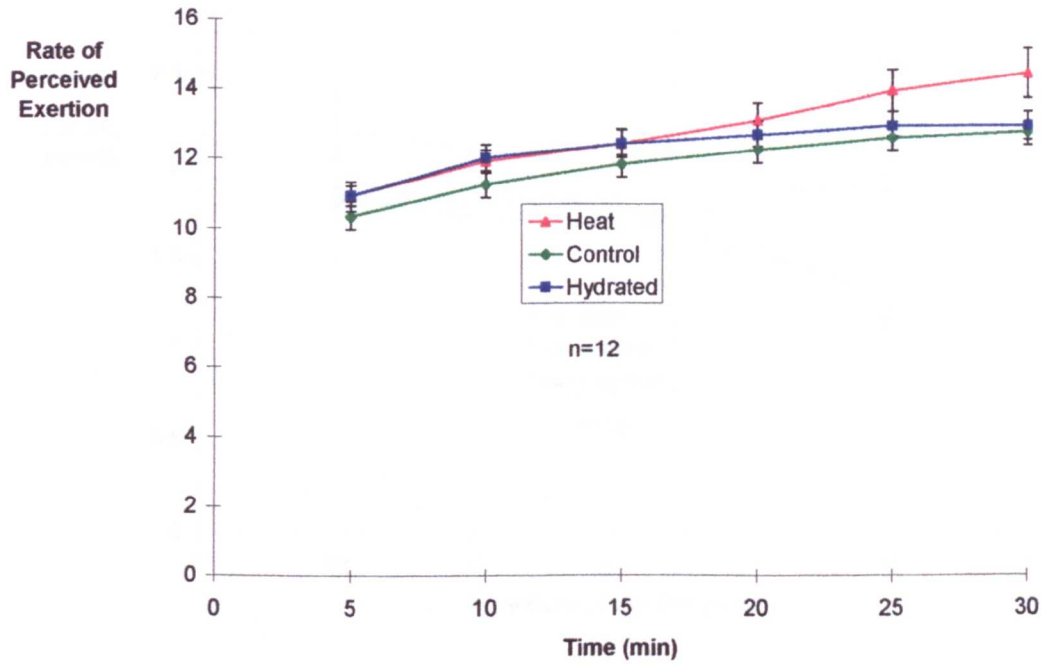

Fig. 8.11. Mean scores for rate of perceived exertion (mean ± S.E.).

A Pearson's product moment correlation revealed that there was a highly significant relationship between heart rates and the rate of perceived exertion scores (r=0.608, p>0.0001).

Another product moment correlation revealed that there was no significant relationship between tympanic temperature and perceived exertion scores (r=0.131, p=0.055).

The patterns of lactate levels were different for the three conditions; in the heated condition levels gradually decreased; the control condition induced a rise after 20 mins, the hydrated condition resulted in a decrease at the same point (figure 8.12). A two-way repeated measures ANOVA revealed there was no significant interaction (time x condition) for blood lactate levels ($F_{(4,32)}$ =2.65, p>0.05). However, there was a main effect for condition ($F_{(2,16)}$=5.38, p<0.05), suggesting that the physical effort between the three conditions was different. No main effect for time was found ($F_{(2,16)}$=2.15, p>0.05).

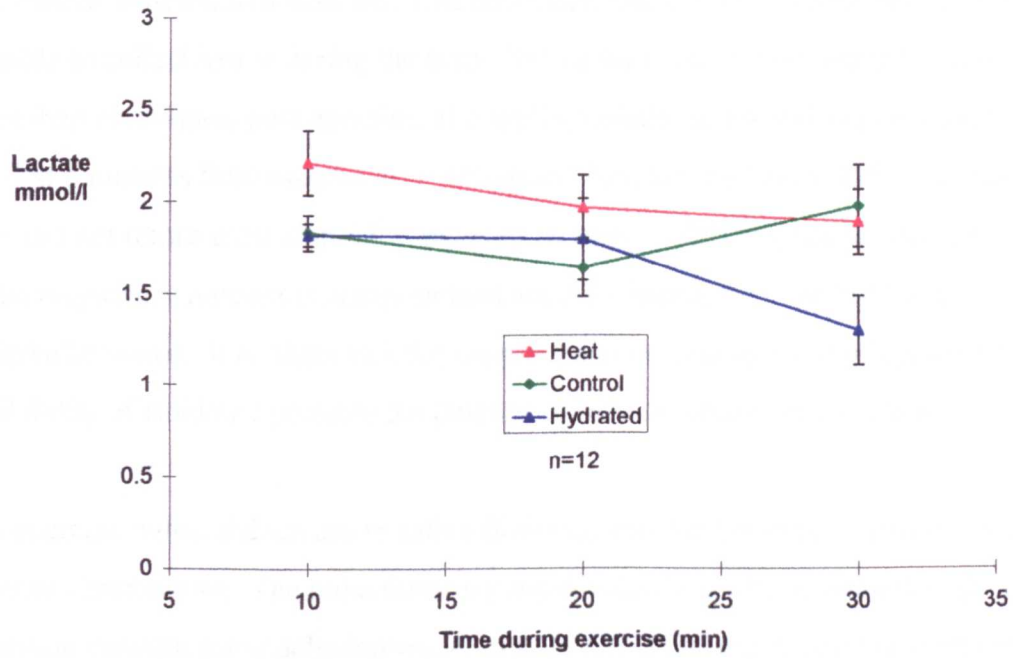

Fig. 8.12 Mean blood lactate levels during exercise (mean ± S.E.)

8.4 DISCUSSION

The exercise bout induced a decrease in salivary flow rate (hypothesis 32, appendix 1) which was exacerbated by exercise in the heat and tempered with fluid replacement (hypotheses 33 & 34, appendix 1). There was no difference found between baseline and pre exercise samples, so any differences found between conditions cannot be attributed to circadian variation, nor is there any reason to expect that there was an order effect as a random cross over design was implemented.

The time at which saliva flow rate first decreased could not be established as it was not possible to collect saliva during the bout. Saliva flow rate did not begin to recover until more than 30 minutes post exercise, although sympathetic activation is withdrawn within the first 5 minutes following exercise (Nieman, Tan, Lee and Berk, 1989). Baseline flow rates did not return until after 45 minutes of recovery. This may mean that the protection of the respiratory mucosa is compromised not only during exercise but for a considerable period afterwards. It is likely that the suppression is a consequence of decreased availability of fluid and possibly the delayed effects of sympathetic outflow.

The exercise-induced decrease in saliva flow rate was further exacerbated by increases in ambient temperature. The reductions in plasma volume and body mass during the heated condition indicate some dehydration. A strong correlation was found between changes in plasma volume and changes in saliva flow rate (figure 8.6.) (hypothesis 41, appendix 1); this indicates that fluid availability is a powerful factor in the production of saliva, and that exercise-induced decreases in plasma volume may go some way to explaining the observed decrease in saliva flow rate. Higher heart rates and high blood lactates were also seen in this condition. Increased heart rate (Febbraio et al., 1994) (hypothesis 53, appendix 1), and significantly increased blood lactate levels (Young, Sawka, Levine, Cadarette and Pandolf, 1985) are typical findings for exercise in a hot environment. The increase in heart rate is thought to reflect an increase in cardiac output, required to meet the increased need for skin blood flow in the face of a reduced plasma volume, and is mediated via increased sympathetic tone (Hales, 1986). The higher levels of blood lactate seen in the heated trial may reflect increased muscle glycolysis (Febbraio et al., 1994), as a consequence of increased sympathetic outflow.

Increased sympathetic outflow during exercise may account for some of the decrease in saliva flow rate seen in exercise. Sympathetic outflow was not measured directly but heart rate and blood lactate levels were measured and these variables might give some insight into the relative degree of sympathetic activation. The heart rate response in the exercise and hydrated conditions was very similar and heart rate barely increased after 5 minutes of exercise (hypothesis 52, appendix 1). Lactate levels in the hydrated trial decreased in the final 10 minutes of exercise (hypothesis 61, appendix 1). This may be a consequence of lower sympathetic outflow (Ward and Mefford, 1985) in this condition but may more probably be the result of a relatively higher plasma volume.

Fluid intake did not appear to temper the exercise-induced decrease in saliva flow rate, but allowed a quicker recovery of salivary flow rate post exercise. Baseline flow rates were not reached until after 30 minutes of recovery. The intake of fluid would have a mechanical washing effect, and perhaps afford some artificial protection. The changes in plasma volume and the increase in body mass which were observed for the hydrated trial suggest that fluid availability was highest in this condition. Greater fluid availability may have facilitated the faster recovery in flow rate, which was supported by the significant correlation between changes in plasma volume and changes in saliva flow rate (fig 8.6).

Hydration status was monitored by change in blood and plasma volumes. Unfortunately not all of the results for these measures were gained, due to technical difficulties. Blood volume did not change significantly in response to any of the three conditions (fig 8.4) (hypothesis 36, appendix 1), therefore no relationship was found with change in saliva flow rate (hypothesis 37, appendix 1). Although only six sets of data were complete for changes in plasma volume, a time by condition interaction ($p=0.05$) was revealed; exercise per se had very little effect on plasma volume while exercise in the heat decreased it and fluid replacement resulted in an expansion of plasma volume (hypotheses38, 39 & 40, appendix 1),

The changes in plasma volume with exercise were slight and can be accounted for by movement of fluid into metabolising tissues to maintain osmotic balance. A gradual increase post exercise would be expected due to movement of excretory products into the blood followed by water to maintain osmotic balance. No decrease during exercise was

seen in the hydrated trial suggesting the fluid ingested was sufficient to offset the usual exercise-induced decrease in plasma volume. The large decrease in plasma volume in the heated trial, is probably a consequence of dehydration, due to increased sweating. The decrease in body mass would support this. The failure of the plasma volume to return to baseline by the end of the recovery period supports the idea that the decrease in plasma volume is not just a consequence of the movement of fluid, but is probably influenced by hydration status.

Change in body mass was intended to indicate fluid gain or loss. An interaction between time and condition was found for change in body mass ($p<0.001$): a decrease in body mass with exercise, and body mass was further decreased by exercise in the heat, whereas an increase in body mass was seen in the fluid replacement trial (figure 8.7) (hypotheses 42, 43 & 44, appendix 1). No correlation was found between change in body mass and change in saliva flow rate (hypothesis 45, appendix 1).

Perceived physical stress was measured with the Borg scale . Ratings of perceived exertion increased steadily during the first 15 - 20 minutes of exercise and tended to plateau towards the end of exercise (figure 8.11) (hypothesis 57, appendix 1). No difference was found for RPE between the three trials (hypothesis 58, appendix 1). The hydrated trial produced higher perceptions of effort initially, which also plateaued. Exercise in the heat resulted in higher levels of perceived exertion which continued to increase until the end of exercise. It is conceivable that a greater degree of stress was felt in the heated condition. This would be supported by the heart rate and lactate data however, this was not demonstrated with the RPE data. This stress may not be solely physical exertion; the scores given may have a large psychological component.

Monitoring tympanic temperature was intended to give an indication of heat stress. No interaction was found between the conditions (figure 8.9). The pattern of results is close to those expected, with the heated condition being slightly raised above the other two conditions. The higher temperatures seen at the end of the control condition cannot be explained (hypotheses 50 & 51, appendix 1). Rectal thermometers may have given a better indication of core temperature.

Perception of thirst was as expected and matched changes in flow rate. The heated condition induced the highest feelings of thirst (hypothesis 46, appendix 1), and the hydrated condition the lowest (figure 8.8) (hypothesis 47, appendix 1). However, no significant correlation was found between changes in the perception of thirst and changes in saliva flow rate either during (hypothesis 48, appendix 1), or after exercise (hypothesis 49, appendix 1). It would not seem to be possible to use the thirst scale as an indicator of changes in flow rate.

The saliva optical densities in the control condition were tonically high relative to baseline. After the hydrated trial no large change in optical density was observed. After the heated bout, optical density was lower than in the other conditions (figure 8.3). The lack of relationship between saliva flow rate and optical density supported the findings of chapter 6: it appears that the presence of proteins in the oral cavity is not dependent on saliva flow rate, and changes in protein levels are independent of changes in flow rate (hypothesis 35, appendix 1). The variability in the measured values for optical density was high. Photometry only gives a relative rather than an absolute protein concentration. Absolute concentrations of the functional proteins can only be obtained by assaying for each separately.

It is interesting to note that a significantly positive relationship was found again between pre exercise saliva flow rate and the change in flow rate induced by exercise (figure 8.2.), as described in chapters 6 and 7. This supports the ideas proposed in chapter 6 that there may be a floor effect with saliva flow rate and exercise; there is a limit to how far flow rate can decrease and therefore the magnitude of decrease is dependent upon the rate pre exercise. It was suggested in chapter 6 that intervention strategies with fluid replacement may only work after exercise, because the exercise-induced decrease in flow rate appeared to be greater in individuals with high flow rates. However, in this study fluid was given both before and during the hydrated trial; this appeared to attenuate the decrease in flow rate seen with exercise and allowed a faster recovery, therefore fluid replacement before and during exercise may temper the drying of the airways that occurs during exercise, thus keeping levels of oral pathogens low and reducing the vulnerability of the upper respiratory mucosa to infection. Further investigation is required to explicate the effect of fluid replacement in hot environments.

PART IV

CHAPTER 9

DISCUSSION AND CONCLUSIONS

9.1. DISCUSSION

It is generally believed that regularly training individuals suffer an increased incidence of upper respiratory tract infections as compared with the general population (Nieman and Nehlsen-Cannarella, 1992; Weidner 1994). Attempts to address this issue have focused on exercise-induced changes in salivary immunoglobulin A levels. Research for this thesis was originally intended to consider further the effect of exercise on sIgA levels and individuals' propensity to URTI.

This research began by addressing methodological issues regarding the collection of saliva for sIgA quantification. Within the available literature there is wide variability in the methods used: stimulated collection by either gustatory or masticatory methods, saliva collected directly from the parotid gland or whole saliva. The comparability of results gained from studies using such variable methods is unclear. Despite the potential problem with using whole saliva (collection of debris that can interfere with quantitative analysis) and small sample sizes collected when flow is not stimulated, whole saliva was collected without stimulus for all studies in this thesis. The choice to use this method was based upon arguments presented in the literature suggesting that analysis of whole saliva rather than saliva from an isolated gland will give a more complete picture of mucosal immunity (Jemmott and McClelland, 1989), and that stimulation of saliva flow results in an underestimation of sIgA levels (Brandtzaeg, 1971).

The only two methods described in recent literature for the collection of whole unstimulated saliva are salivettes and passive dribbling. A comparative study on passive dribbling and salivettes as methods for the collection of whole unstimulated saliva was carried out and is described in chapter 5. It was found that less saliva was collected using salivettes than by the dribbling method. The lack of relationship between the volumes of saliva collected by these two methods suggested that results gained from the use of one method would not be comparable with those collected by the other method. Further investigation into the absorbance of the sample by salivette swabs revealed that the salivette swabs have a limiting absorption rate and that variable volumes of saliva are not

absorbed and are therefore 'lost', possibly because the saliva flow rate is faster than the rate of absorption, resulting in a pooling of saliva in the mouth which is then swallowed instead of being collected. This is probably particularly true as the swab approaches saturation and absorption rate decreases.

The salivettes were also found to have a limiting maximal absorption capacity which would therefore limit the volumes of samples collected. Unlike the rate of absorption, the maximal absorption capacity of the salivettes was found to differ between the two types of swab. The polyester salivettes were found to have approximately half the maximal absorption capacity of the cotton wool salivettes (table 5.4.), thus are only appropriate for shorter collection times. The results gained suggest that they should not be used for longer than three minutes, or to collect more than 1ml of saliva (whichever occurs first).

It was also found that the salivettes always retained some of the sample after centrifugation. The retention of the sample in the two types of salivette was investigated, and the amount of sample retained was found to be highly variable. An inverse relationship was found between the percentage of the initial sample retained and the amount absorbed, therefore the problems of retention would have greatest impact on the smallest samples. Any decrease in saliva flow rate would thus be magnified by the effect of sample retention.

Polyester salivettes were developed by the manufacturer's (Starstedt) in order to reduce the problems of retention that had been discovered with swabs made of cotton wool. The results gained would suggest that the problem of retention was smaller in the polyester salivettes, however, a mean of $35.3 \pm 29.7\%$ of the sample was still found to be retained. Furthermore, the high variability in the amount that was retained means that there is no way of correcting for retained sample when calculating saliva flow rate.

Lower total protein levels were found in samples collected by salivettes than by the dribbling method (table 5.3.), suggesting that salivary proteins may have become trapped in the fibres in the swab. The combined effect of limited absorption and retention of the sample would result in an underestimation of both saliva flow rate and saliva protein concentrations. Although the cotton wool salivettes have a greater absorption capacity, they also retain more saliva. Therefore it was concluded that the passive dribbling method

would have less impact on sample volumes and protein concentrations and was the preferred method for investigations contained within this thesis.

The lack of uniformity in studies investigating the effect of exercise on sIgA is evident not only in the way that samples are collected but also the methods by which the samples are assayed. The two most popular methods for assaying sIgA concentrations have been radial immuno-diffusion (Brandtzaeg et al., 1970; Green and Green, 1988; Schouten et al., 1988) and enzyme-linked immunosorbant assay (ELISA) (Akerlund, Hanson, Ahlstedt and Carlsson, 1977; Mackinnon, 1992; MacDowell et al., 1992a, b).

Enzyme-linked immunosorbant assay (ELISA) was used for the assay of sIgA levels in this thesis (chapter 6) because it has been advocated over the use of other methods for several reasons; for instance it is credited with being the most sensitive and economical (Evans and Bristow, 1993), and has been used in a number of studies quantifying sIgA in athletes (Mackinnon, 1993a, b, c; McDowell, 1992a, b; Gomez et al., 1991; Tharp, 1991). The validity of inter-study comparisons of sIgA concentrations is questionable because although the basic principles of the ELISA assay are always employed; (a specific antibody sandwich with an enzyme-conjugate to detect and quantify bound sIgA), the way in which the assay is carried out varies markedly between studies. The two most important factors besides validity for the assay of sIgA are sensitivity and reliability. It is because of problems caused by varying sensitivity of different batches of antibody detection systems and variability in the binding capacities of different microtitre plates that authors have used different techniques in order to maximise assay sensitivity. The variability in assay components used, their concentrations, and the times and temperatures of incubations between studies, is so extensive that it is difficult to suppose that the results are comparable. It also means that it is impossible for any researcher wanting to set up an ELISA assay for sIgA to assume that any of those in the published data are producing valid (despite the reported sensitivity and reliability) results which will be comparable in absolute terms to other data sets. It is also virtually impossible to carry out a comparative study of the variations in the assay reported in the literature because the concentrations of the assay reagents are not always reported. Therefore it is difficult for anyone wishing to set up an ELISA for sIgA to know which is the most appropriate method to begin with. Concentrations for assay reagents have been recommended, e.g. for capture antibody ranges of 1-10$\mu g.ml^{-1}$ (Engvall, 1980) and 5-15$\mu g.ml^{-1}$ (Kemeny and Challacombe, 1988).

However, concentrations outside these ranges are regularly used in reported assays [e.g. Miletic et al. (1996) used 1mg.ml^{-1}]. Although there are guidelines available on what techniques and methods should be employed in order to optimise the assay, there is little evidence that these have been followed in many of the published studies on assays employing sandwich ELISA to quantify sIgA. There is also little or no comment on the relative benefits of the variants of the assay.

The problems with ELISA appear to stem not only from a lack of consistency between studies, but there do also appear to be some innate problems with the assay that would be difficult to resolve even with the greatest care and caution. Butler et al. (1986) reported that when the capture antibody is in functional excess, the percentage of bound IgA is constant below a concentration of 0.025μg.ml^{-1}, but one hundred percent of the sample IgA is never bound. Therefore comparisons would be valid only in situations where the investigator was confident that the capture antibody was in functional excess of the sIgA and that the concentration of sIgA was below 0.025μg.ml^{-1}. This would require extensive dilution of the sample with no guarantee that sufficient dilution had been achieved. In order to convert the final result to an absolute antibody concentration, the result for the diluted samples would have to be multiplied which may result in a multiplication of any errors. A very wide range of IgA concentrations has been seen in biological samples (i.e. 87-615μg/ml, Dorian et al., 1982). Since the possible range is so large, titrated dilutions of the sample are necessary (Butler, 1986). The fact that one hundred percent of the sample is never bound and that above concentrations of 0.025μg.ml^{-1} the percentage bound is variable, means that this 'loss' cannot be corrected for, and therefore absolute concentrations of sIgA cannot be achieved.

Another fundamental problem with the assay is with batch variation in the capacity of the plates to bind the capture antibody (Wreghitt and Nagington, 1983). The microtitre plates used for ELISA are usually only controlled for optical density and not their ability to bind protein (Oliver, Saunders, Hogg and Hellman, 1981). Therefore there is inconsistent binding of capture antibody both between and within plates, making it impossible to guarantee that the assay is equally sensitive between or even within plates. The problems of inconsistent binding between batches of plates and possibly even between individual cells, calls into question the validity of inter-plate comparison, and threatens assay reliability. In order to avoid any possible errors caused by inconsistent binding it may be

necessary to assay all samples from an individual on one plate, which then limits the number of samples that can be assayed. Moreover, this intra plate comparison does not resolve problems of inconsistent binding within a plate.

Despite these apparent problems with ELISA it is still credited with being the most sensitive and reliable method for the quantification of sIgA. The other popular method for the quantification of sIgA is radial immunodiffusion (RID), which is a passive gel-based diffusion method that was developed by Mancini, Carbonara and Heremans (1965). Despite the fact that it is accused of being less sensitive than ELISA (Stone et al., 1987) it has become popular because it requires minimal technical expertise and yields precise and reproducible results for sIgA (Kubitz, Peavey and Moore, 1986), the whole process being made even simpler by the availability of many commercial kits.

As with ELISA there is a large variation in the way that the RID assays are carried out between different authors. Different incubation times and temperatures are used in an attempt to optimise the assay, presumably because no two plates are ever identical, however the variation in the method used is vast even between plates produced in the same laboratory.

It is not surprising that investigations concerning changes in sIgA levels have not led to any conclusive evidence regarding observed exercise-induced changes and the incidence of URTI, because of lack of consistency not only in the way in which samples are collected but also the wide variability in the way they are assayed. There appear to be several problems with both the ELISA and RID assays, and although attempts have been made to overcome them by manipulating assay characteristics the comparability of the sIgA levels generated by these methods requires further investigation. It is probable that results achieved with the ELISA method are comparable within a plate using the same batch of assay reagents. This does however limit the number of assays that can be carried out within a study and is very restrictive in terms of the number of samples that can be compared. Between study comparisons could only be made in terms of percentage change in sIgA concentrations. The high reproducibility in the assay used in chapter 6 suggests that the results gained can be compared within the study; however, it is difficult to compare them with reported physiological ranges because of lack of confidence in the 'absolute' concentrations.

There is a third issue besides collection of the sample and its assay for sIgA levels that explains the lack of consistency in studies investigating sIgA, and that is the terms in which sIgA is expressed. Some authors have reported sIgA levels just in terms of concentration, relative to total salivary protein levels and in terms of secretion rate. The relative contribution of saliva flow rate and sIgA concentration were investigated (chapter 6) using samples collected from boxers before and after three training sessions. It was found that sIgA concentration did not change, but saliva flow rate decreased significantly with each training session, the mean of the three sessions being a decrease of 51%. The calculated sIgA secretion rate decreased to the same extent, again the mean of the three sessions equalling -51%. Therefore in this case changes in sIgA secretion did not reflect a change in mucosal plasma cell activity but rather was just a report of the change in saliva flow rate.

From studies in which changes in sIgA secretion rate are reported alone there is no way of knowing whether those changes are a factor of a change in saliva flow rate or a function of the change in immune activity. Saliva flow rate is highly likely to change during exercise through decreased fluid availability, through evaporation of fluid direct from the oral cavity through oral breathing, and dehydration. Sympathetic arousal, which is known to occur during exercise in order to facilitate movement, has been demonstrated to decrease the water content of saliva, thus decreasing saliva flow. It is emphasised, then, that reports of a decrease in sIgA secretion rate may not be reporting the change in mucosal plasma cell activity but a change in saliva flow rate.

Saliva confers innate protection of the URT by forming a physical barrier and also has a mechanical washing effect, sweeping pathogens down to the uninhabitable environment of the stomach. Saliva mobilises several specific and non-specific acting anti-pathogenic proteins around the oral cavity, thus increasing their probability of counteracting a pathogen. A decrease in saliva flow rate may thus decrease both specific and non-specific defence against URT pathogens. Therefore monitoring changes in saliva flow rate may provide more information about immune status in the upper respiratory tract than a single measure of sIgA levels. Innate immune defences have been largely ignored until recently when Müns et al. (1996) have investigated the effect of exercise on nasal mucociliary clearance and Ljunberg et al. (1997) investigated the effect of a marathon run on saliva

flow rate. Both studies found that exercise decreased the effectiveness of these innate defences. If saliva flow rate can provide some indication of defence against URTI then it is considerably cheaper and easier to monitor than IgA. The only difficulty in monitoring saliva flow rate is that care is required in the choice of method (i.e. collection by dribbling or salivettes), timing needs to be accurate and large pots may have to be used in order to decrease the chances of any of the sample being lost over the sides of the pot. With an accurate balance the assay is not only simple but also reliable and reproducible.

In an attempt to explicate a link between changes in innate defence mechanisms and the report of symptoms associated with URTI, saliva flow rate was monitored and a symptom questionnaire was given before and after a marathon run (chapter 7). Although decreases in saliva flow rate were found, the difference was not significant possibly because of the small size of the subject group. The symptom report did not show the duration of symptoms that had been reported in previous studies (Peters and Bateman, 1983; Nieman et al., 1990a).

Much of the data reporting an increased propensity to URTI have been based upon questionnaire data, asking for the occurrence and severity of symptoms associated with URTI: sneezing, blocked or runny nose, coughing and sore throat. It is possible that the symptoms reported are in fact a consequence of a defensive reaction, since sneezing, coughing or a runny nose could be accounted for on the basis that they would occur in order to remove particles that had been inhaled during the run. Phlegm may be increased as a consequence of increased viscosity of mucosal fluids, induced by sympathetic arousal and evaporation of moisture from the mucosal surfaces. An increased incidence of a sore throat could be a consequence of inhaling large volumes of cold air. It is possible that the increased incidence of symptoms associated with URTI may be much more the consequence of an inflammatory reaction as a result of breathing in large volumes of cold air (which bypasses the nasal filter) during training or competition. Attempts have been made to account for this: Peters and Bateman (1983) asked whether subjects experienced a fever as well as symptoms associated with URTI in an attempt to confirm an infection: only three subjects could confirm that this was the case. The ideas that the symptoms could be a consequence of a defensive or inflammatory reaction does not explain those that lasted for more than a day, although if the competitors had not eased up on their training at all after competition then the symptoms could be prolonged. It is, however, possible that

an inflammatory reaction would increase vulnerability to pathogens because of an inappropriately active immune system and perhaps physical damage to the mucosal surface. There is a need for the purported increased incidence in URTI in regularly training individuals to be confirmed by clinical diagnosis and analysis of swabs taken from the URTI to identify pathogens. Until the real causes of the problems suffered by athletes are confirmed, it will be impossible to determine an appropriate intervention strategy.
It is possible that no significant decrease was seen in saliva flow rate during the marathon run because the subjects were allowed to drink ad libitum, in an attempt not to interfere with their normal pattern of behaviour. Regular drinking during the marathon could have meant that dehydration did not occur to an extent sufficient to have an impact on saliva flow rate. Further work was therefore carried out in a laboratory environment to investigate the effect of exercise, exercise in the heat and exercise with fluid replacement, on saliva flow rate (chapter 8). It was found that exercise decreased saliva flow rate, exercise in the heat exacerbated this and fluid replacement allowed faster recovery of the exercise-induced decreases. The changes in saliva flow rate were found to be related to changes in plasma volume, suggesting that hydration status is the major factor in exercise-induced changes in saliva flow rate. The faster recovery of saliva flow with fluid replacement during exercise suggests that drinking may be an effective strategy against exercise-induced decreases in saliva flow rate. Drinking may also have an artificial protective effect by washing any inhaled pathogens away from the oral mucosa.

In all the current studies where saliva flow rate was investigated (chapters 6,7 &8) a relationship between the change in saliva flow rate and pre-exercise saliva flow rates was found. The greater the pre-exercise flow rates the greater the decrease in response to exercise. It is possible that there is a floor effect, a point after which saliva flow rate can decrease no further. This may be a defensive mechanism, providing a limit to how low saliva flow rate can go. However, it does imply that the observed effect of exercise on flow rate is a consequence of the pre-exercise flow rate, rather than a varying magnitude of effect. This has implications for intervention strategies (e.g. fluid replacement) that increase saliva flow rate. It is possible that nothing will be gained by increasing saliva flow rate before exercise and the important time to apply an intervention may be during or shortly after exercise. This requires further investigation.

9.2 CONCLUSIONS

Exercise imposes a number of stresses on the body, and the physiological response mounted to facilitate movement and maintain homeostasis induces changes in immune functioning and activity. The clinical significance of this is unclear, particularly with regard to URTI. There currently exists a lack of conformity in the reports of the effect of exercise on sIgA, probably because of a lack of consistency in the methods employed. It is possible that assays used to quantify IgA may be fundamentally flawed and the comparability of sIgA concentration values between studies questionable. Data contained within this thesis suggest that the use of salivettes to collect saliva samples will result in an underestimation of both saliva flow rate and salivary proteins including sIgA; reporting sIgA in terms of secretion rate is an ineffective indicator of sIgA levels, and reflects changes in saliva flow rate to a greater extent than changes in sIgA levels; exercise decreases saliva flow rate; the extent of decreases in flow rate with exercise are a factor of the pre-exercise flow rate; and that fluid replacement may provide an effective strategy against exercise-induced decreases in saliva flow rate. Before effective intervention strategies against the upper respiratory symptoms said to be suffered by regularly training individuals can be elucidated, there is a need to further investigate other mechanisms of defence of the upper respiratory tract. There is also a need for clinical proof that the reported symptoms are URTI, i.e. the result of an infection and not the result of an inflammatory reaction to regularly breathing large volumes of cold dry air. Despite the fact that there is a considerable and growing body of data on the effect of exercise on the sIgA levels, there is a need to co-ordinate the methodologies used, in order to facilitate inter-study comparisons. It is unlikely however, that monitoring levels of just a single marker (sIgA) of upper respiratory tract defence will provide the answer as to why athletes seem to suffer an increased incidence of upper respiratory tract infection as compared with the general population.

The work detailed in this thesis is original in that it critiques and 'moves away' from the use of salivary immunoglobulin A as a marker of exercise-induced immunomodulation, and suggests that if the focus is to be "why do athletes suffer an increased incidence of symptoms associated with URT?" It then there are many more factors that should be taken into consideration.

The first concerns what it is that is inducing the reported symptoms, what provides defence against it, and thirdly are there any appropriate intervention strategies? I have suggested that some of the symptoms may be a consequence of an inflammatory reaction, and an important move forward would be to investigate the cause of the increased URTI symptom report in athletes, to investigate the occurrence of infective agents and the possibility of an exercise-induced inflammatory reaction. The confirmation of the existence of a causative infective agent would be quite simple via the monitoring of antibody titre levels to URTI pathogens and taking swabs of the URT for microbiological analysis. The occurrence of an URT inflammation without evidence of the existence of such an infective agent may be assumed to be induced by frictional damage.

Consideration of other future directions for this work suggests a number of areas for further investigation. For instance, I have demonstrated that exercise has a large impact on saliva flow rates in the laboratory. However, this requires further investigation in a competition setting. I intend to repeat the experiment described in chapter 7 with a larger subject group, with a wide variety of marathon running experience, as the subjects used in the study described here were very experienced marathon runners and therefore did not perceive the race as stressful. Body mass, as an indicator of fluid loss during the marathon, and fluid intake would also be recorded, to account for any observed changes.

It would be advantageous, too, if future saliva flow rate studies also monitored indices of hydration status (plasma volume was shown to be influential, chapter 8), and levels of stress hormones, such as adrenaline and nor-adrenaline, in an attempt to explain the mechanisms behind any observed changes.

It was found (chapters 6,7 & 8) that the impact of exercise on saliva flow rate was influenced by the pre exercise flow rate. This is a novel finding and warrants greater consideration. It suggests that the timing of intervention strategies is very important and therefore this too requires further investigation. Fluid replacement does increase the recovery of saliva flow rates after exercise, however more work is required to establish the ideal types of fluids, volumes and the timing to minimise the reduction and maximise the recovery in saliva flow rate.

PART V

Appendices

Appendix 1

SUMMARY OF HYPOTHESES TESTED

SUMMARY OF HYPOTHESES TESTED

No.	SECTION	HYPOTHESIS	Hypothesis supported Y Hypothesis rejected N
1	5.2	There is a difference in mean saliva flow rate for samples collected within one day compared with those collected across ten days.	N
2	5.2	There is a difference in the variability in saliva flow rate in samples collected within one day as compared with those collected across ten days.	N
3	5.2	There is a difference in the variability in salvia flow rate between samples collected from one subject over ten days and samples from ten subjects on one day.	N
4	5.2	There is more saliva collected by the passive dribbling method and that which is absorbed by salivettes.	Y
5	5.2	There is more saliva collected by the passive dribbling method and that which is collected after centrifugation of salivettes.	Y
6	5.2	Less saliva is collected after centrifugation of salivettes than is initially absorbed into the swab.	Y
7	5.2	The variability in saliva flow rate is different between the passive dribbling and salivette methods.	N
8	5.2	There is a relationship in the volume of saliva collected between the samples collected by two methods (dribbling and salivettes).	N
9	5.2	The total protein levels of samples collected by passive dribbling are greater than of samples collected by the salivette method.	Y
10	5.3.1	The absorption of saliva is different between the cotton wool and polyester salivette swabs.	Y
11	5.3.2	The cotton wool salivettes have a greater maximal absorption capacity than the polyester salivettes.	Y

No.	SECTION	HYPOTHESIS	Hypothesis supported Y Hypothesis rejected N
12	5.3.2	The salivette swabs did not absorb saliva differently from water.	Y
13	5.4	The salivette swabs always retained some saliva after centrifugation.	Y
14	5.4	The cotton wool swabs retained a greater volume of the sample than the polyester swabs.	Y
15	5.4	The cotton wool swabs retained a greater percentage of the sample than the polyester swabs.	Y
16	5.4	There is a positive relationship between the volume of saliva absorbed and the volume of saliva retained for cotton wool salivettes.	Y
17	5.4	There is a negative relationship between the volume of saliva absorbed and the percentage of the sample retained for cotton wool salivettes.	Y
18	5.4	There is a positive relationship between the volume of saliva absorbed and the volume of saliva retained for polyester salivettes.	N
19	5.4	There is a negative relationship between the volume of saliva absorbed and the percentage of the sample retained for cotton wool salivettes.	Y
20	5.4	There is a positive relationship between the pre sample mass of the polyester swab and the percentage of the sample retained after centrifugation.	Y
21	6.0	There is a decrease in saliva flow rate after an interval training session	Y
22	6.0	There is a decrease in sIgA concentration after an interval training session.	N
23	6.0	There is a decrease in sIgA secretion rate after an interval training session.	Y
24	6.0	SIgA secretion rates decreased to the same extent as saliva flow rates.	Y

No.	SECTION	HYPOTHESIS	Hypothesis supported Y Hypothesis rejected N
25	6.0	There is a no relationship between the change in IgA concentration and the change in saliva flow rate with exercise.	Y
26	6.0, 7.0, 8.0	There is a relationship between the change in saliva flow rate induced by exercise and the pre-exercise saliva flow rate.	Y
27	6.0	There is a relationship between the change in sIgA concentration induced by exercise and the pre-exercise sIgA concentration.	Y
28	6.0	There is a relationship between the change in sIgA secretion rate induced by exercise and the pre-exercise sIgA secretion rate.	Y
29	7.0	Saliva flow rate decreased significantly after a marathon run	N
30	7.0	Symptoms associated with upper respiratory tract infection were more prominent after a marathon run than before or during the race.	N
31	7.0	There is a difference in the severity of symptoms associated with a marathon run.	N
32	8.0	Moderate intensity prolonged exercise decreases saliva flow rate.	Y
33	8.0	Moderate intensity prolonged exercise in the heat exacerbated exercise-induced decreases in saliva flow rate.	Y
34	8.0	Fluid replacement during moderate intensity prolonged exercise resulted in a faster recovery of exercise-induced decreases in saliva flow rate.	Y
35	8.0	There is a relationship between saliva total protein levels and saliva flow rate.	N
36	8.0	There is a change in blood volume with moderate intensity prolonged exercise.	N
37	8.0	There is a relationship between change in blood volume and change in saliva flow rate.	N
38	8.0	There is a change in plasma volume with moderate intensity prolonged exercise.	Y

No.	SECTION	HYPOTHESIS	Hypothesis supported Y Hypothesis rejected N
39	8.0	Moderate intensity prolonged exercise in the heat results in a reduction in plasma volume.	Y
40	8.0	Moderate intensity prolonged exercise with fluid replacement results in an expansion of plasma volume.	Y
41	8.0	There is a relationship between change in saliva flow rate and change in plasma volume.	Y
42	8.0	Moderate intensity prolonged exercise results in a decrease in body mass (pre to post exercise)	Y
43	8.0	Moderate intensity prolonged exercise in the heat exacerbates the exercise-induced decrease in body mass.	Y
44	8.0	Moderate intensity prolonged exercise with fluid replacement results in an increase in body mass (pre to post exercise).	Y
45	8.0	There is a relationship between change in saliva flow rate and change in body mass.	N
46	8.0	There is an increase in perceived thirst rating when exercise is performed in an environment with increased ambient temperature.	Y
47	8.0	There is a decrease in perceived thirst rating when fluid replacement is given during exercise.	Y
48	8.0	There is a relationship between change in saliva flow rate and perceived thirst score during exercise.	N
49	8.0	There is a relationship between change in saliva flow rate and perceived thirst score after exercise.	N
50	8.0	There is an increase in tympanic temperature with moderate intensity prolonged exercise.	Y
51	8.0	There is a greater increase in tympanic temperature with moderate intensity prolonged in the heat as compared with normal ambient temperature.	N
52	8.0	There is an increase in heart rate with prolonged moderate intensity exercise	Y
53	8.0	There is a greater increase in heart rate with moderate intensity prolonged in the heat as compared with normal ambient temperature.	Y

No.	SECTION	HYPOTHESIS	Hypothesis supported Y Hypothesis rejected N
54	8.0	There is a relationship between change in heart rate and change in saliva flow rate induced by prolonged moderate intensity exercise.	N
55	8.0	There is a relationship between change in plasma volume and heart rate with prolonged moderate intensity exercise	N
56	8.0	There is a relationship between tympanic temperature and heart rate with prolonged moderate intensity exercise.	Y
57	8.0	Prolonged moderate intensity exercise results in an increase in perceived exertion scores.	Y
58	8.0	There is a difference between perceived exertion scores between 3 conditions: prolonged moderate intensity exercise; prolonged moderate intensity exercise in the heat, and prolonged moderate intensity exercise with fluid replacement.	N
59	8.0	There is a relationship between the rate of perceived exertion and heart rate during prolonged moderate intensity exercise.	Y
60	8.0	There is a relationship between tympanic temperature and perceived exertion scores.	N
61	8.0	There is a difference between blood lactate levels in response to 3 conditions: prolonged moderate intensity exercise; prolonged moderate intensity exercise in the heat, and prolonged moderate intensity exercise with fluid replacement.	Y

Appendix 2

INSTRUCTIONS & QUESTIONNAIRES

Informed consent and Health Check forms

CHICHESTER
INSTITUTE of
HIGHER EDUCATION

A College of the University of Southampton

BISHOP OTTER CAMPUS, COLLEGE LANE
CHICHESTER, WEST SUSSEX PO19 4PE
TEL: 01243 816000; FAX: 01243 816080

INFORMED CONSENT FOR PHYSIOLOGICAL TESTING PROCEDURES

I (print name and date)_____

hereby give my consent to participate in the exercise test(s) explained to me. I am satisfied that I understand the procedures involved and accept the possible health risks due to the nature of strenuous exercise testing.

In particular I am aware of the possible dangers of certain blood borne diseases (H.I.V., HEPATITIS B etc) associated with blood sampling. Also I recognise that I am at liberty to withdraw my involvement at any stage of the work.

Subject's full signature: _____

Experimenter's signature: _____

Supervisor's signature: _____

Head of section's signature: _____

Before we can carry out any physiological tests on you we have to check that you are in a satisfactory condition to undergo strenuous exercise. We would therefore like you to fill in the following questionnaire about yourself. All information given will be treated as strictly confidential.

CHICHESTER INSTITUTE of HIGHER EDUCATION

A College of the University of Southampton

BISHOP OTTER CAMPUS, COLLEGE LANE
CHICHESTER, WEST SUSSEX PO19 4PE
TEL: 01243 816000; FAX: 01243 816080

Name Date of Birth

Specialist sport Sex (M/F) ☐ Age ☐

1. How would you describe your present level of activity in both your work and recreation?

Sedentary ☐ Moderately active ☐ Active ☐ Highly active ☐

2. In terms of fitness how would you describe your present level of fitness?

Very unfit ☐ Moderately fit ☐ Trained ☐ Highly trained ☐

3. How do you view your current body weight? Are you:

Underweight ☐ Ideal Weight ☐ Slightly overweight ☐ Very overweight ☐

4. Are you, or have you ever been a smoker? Yes ☐ No ☐

If yes how many did/or do you smoke a day? ☐

5. Do you drink alcohol? Yes ☐ No ☐

If you do, do you consider yourself to be a:

V. Light drinker ☐ Light drinker ☐ Heavy drinker ☐ V. Heavy drinker ☐

6. Have you had to consult your doctor during the last six months? If so, briefly say why

7. Have you suffered from a bacterial or viral infection in the last two weeks? Yes ☐ No ☐

If so, briefly say why:

8. Are you presently taking any form of medication? Yes ☐ No ☐

If yes, give details

9. Do you suffer or have you suffered from Diabetes? Yes ☐ No ☐

If yes, give details

10. Do you suffer or have you suffered from Asthma? Yes ☐ No ☐

If yes, give details

11. Do you suffer or have you suffered from Bronchitis? Yes ☐ No ☐

If yes, give details

12. Have you ever suffered from any form of heart complaint? Yes ☐ No ☐

If yes, give details

13. Is there a history of heart disease in your family? Yes ☐ No ☐

If yes, give details

14. Do you currently have any form of muscular or joint injury? Yes ☐ No ☐

If yes, give details

15. Have you ever suffered from Hepatitis? Yes ☐ No ☐

16. Have you ever had a blood transfusion? Yes ☐ No ☐

17. Are you a member of a social grouping which is considered to be particularly at risk from Acquired Immune Deficiency Syndrome? Yes ☐ No ☐

18. Have you had, for any reason, to suspend your normal training for the past two weeks prior to this test? Yes ☐ No ☐
If yes, give details

19. Lastly, is there anything to your knowledge that may prevent you from successfully completing the tests that have been outlined to you Yes ☐ No ☐
If yes, please state reasons:

Please sign:

Date:

Chapter 5
Instruction sheet

Saliva Collection Study

First and foremost thank you very much for volunteering to take part in this study. You may be aware that athletes are believed to be more susceptible to upper respiratory tract infections (colds and flu) than the general population. For the last few years studies investigating this phenomenon have focused upon antibodies found in saliva. In order to quantify salivary antibodies the saliva must be collected carefully and over a set period of time so that a rate of saliva production can be calculated. Saliva collection has been carried out in variety of ways. This study aims to investigate two of the most popular methods.

Subjects for this study must

- have been free of symptoms of a cough or cold for at least 1 week
- be non-smokers
- and for the duration of the study not drink alcohol
- and have 10 minutes to spare when they first wake up

Protocol

1. Before going to bed at night, drink 1 pint of water and clean your teeth (order in which you do this is irrelevant). Ensure that there is a glass of water and the saliva collection vessels beside your bed.
2. On waking rinse your mouth out thoroughly with water for at least 30 seconds (you may swallow this water if you wish)
3. Sit comfortably on the edge of the bed, lean forward slightly with the head bent down.
4. Wait for another 30 seconds before swallowing all remaining liquid in you mouth and begin the first collection **(Salivettes)**
5. Remove the swab from the container and place in the most comfortable position in your mouth. **The swab must always be placed in the same position in your mouth. Do not move the swab around the mouth or move your tongue over it. Do not swallow any saliva that may be collecting in your mouth.**
6. After four minutes remove the swab and place it immediately into its container
7. Repeat the rinsing procedure, swallow all remaining liquid and begin the second collection method **(Dribbling)**
8. Collect any saliva that forms in your mouth in the container. **Do not try and force saliva production in any way.**
9. At the end of the four minutes spit out all remaining fluid into your mouth.
10. Label the collection vessels with your name and the date and store in the freezer.

REVERSE THE ORDER IN WHICH YOU CARRY OUT THESE TWO METHODS DAY BY DAY (i.e. day 1 salivettes first, day 2 dribbling first, day 3 salivettes first)

Chapter 7 Instruction sheet

INSTRUCTION SHEET

First and foremost thank you very much for volunteering to help me. This study is the last that I have to complete for my PhD. thesis. My research so far has investigated the immune system in the upper respiratory tract in an attempt to elucidate why runners are particularly susceptible to upper respiratory tract infections. I would be happy to discuss any questions you may have or the results of this study with you at anytime.

Telephone: 01243 816328
E-mail : jford@chihe.ac.uk

This study involves the collection of six saliva samples: Before the race, Friday morning (when you wake, before you have got out of bed, or had a drink) and the same again on Saturday and then Sunday, the race day, and Monday morning. these samples will need to be stored in the freezer .
At the race venue, within half an hour before the race and immediately after the race, after you have produced these samples I will collect them for cold storage.

SALIVA COLLECTION

- Friday, Saturday, Sunday and Monday morning samples should be completed upon waking, before getting out of bed and before drinking.
- All saliva samples should be collected while you are sitting in a vertical position with your feet on the floor.
- Swill your mouth out with water prior to any saliva collection.
- Swallow any saliva/fluid remaining in your mouth.
- Allow the saliva to collect in your mouth without trying to force it (Don't suck your cheeks).
- Expectorate the saliva into the container, labelled with your name and the date. Continue this until the four minute collection time is up.
- After the four minutes, place the sample container into the freezer immediately.
- Samples collected on at the race venue will follow exactly the same procedure only please give the samples to me so that they may be put into cold storage. Please do not have a drink immediately after the race until you have provided your post race sample.

MASS

When you have completed your pre and post race saliva samples, can you please jump on the scales that I will have me, in order to gain your pre and post race mass.

QUESTIONNAIRES

Please complete questionnaire 1 before the race, and if possible give to me on the race day. Questionnaire 2 will be given to you on the race day and should be posted to me with the saliva samples.

AFTER THE FINAL SAMPLE ON MONDAY PLEASE SEND ME THE SAMPLES AND THE COMPLETED QUESTIONNAIRE IN THE SAE ENCLOSED WITH YOUR SAMPLE POTS.
THANK YOU VERY MUCH FOR YOUR HELP

Chapter 7 Questionnaires

QUESTIONNAIRE

NAME: **DoB:**

MASS: **Kg** **HEIGHT:** **cm**

When did you start running? .....

Number of previous marathons? .....

In what time do you expect to complete todays marathon?

.....

What is your best previous marathon time?.....

Number of races per month?.....

TRAINING LOAD?

Average number of sessions per week? .....

Average number of steady runs per week ? .....

Average number of interval sessions per week? .....

Average number of Tempo/Threshold runs per week? .....

Average number of miles per week? .....

INFECTIONS?

How many upper respiratory tract infections (cough/cold/flu) have you had in the last 12months?.....

Is this more or less than usual? .....

Have any of these infections been severe enough to stop you from,

a) trainingYES/NO

b) competing YES/NO

Do you suffer from hayfever or asthma?.....

Are you currently taking any medication?.....

RACE DAY QUESTIONNAIRE

NAME:

Pre race mass: Kg

Post race mass: Kg

Actual completion time? .....

Are you happy with your performance? YES/NO

Symptoms Checklist

Could you please indicate whether you have any of the following symptoms either before during or after the race.

Please indicate the severity of these symptoms on the 5 point scale with 5 being very severe and 1 being very slight

	Since Friday	Immediately before race	During race	Immediately after race	Hours after	Next morning
	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
Cough						
Sore throat						
Sneeze						
Runny nose						
Phlegm						
Dry mouth						

Chapter 8 Questionnaires

Rating of perceived exertion

(The Borg Scale)

6	
7	Very, very light
8	
9	Very light
10	
11	Fairly light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Very, very hard
20	

The thirst scale

10	v.v. Bloated
9	
8	Bloated
7	
6	
5	Comfortable
4	
3	Thirsty
2	
1	v.v. Thirsty

Appendix 3

ELISA

MATERIALS AND REAGENTS

MATERIALS

Buffers

Buffer Capsules (Fisons)

Latex Gloves (Hospital Management Supplies)

Finntip disposable pipettes (Fisons)

Volac (glass) pipettes (BDH)

Volumetric Flasks (Fisons)

Winchester bottle strirrer (Radley's)

1litre Schott glass Duran bottles (Fisons/BDH)

Weighing Scales

Spatulas

Weighing Boats

Distilled Water

Coating plates :

96 Flat-welled Polystyrene Microtitre Plate (Greiner Labs.)

Seal Plate film (Greiner Labs.)

Disposable face masks (Hospital Management Supplies)

Anti-Human IgA (α-chain specific) developed in Rabbit (Sigma A4165)

Coating Buffer (pH 9.6)

Volac (glass) pipette (BDH)

25ml Schott glass Duran bottles (Fison/BDH)

Finnpipettes

Refrigerator @ 4°C

BUFFERS

Coating Buffer : 0.16% Na_2CO_3, 0.29% $NaHCO_3$ pH 9.6

1litre requires 1.6g Di-Sodium Carbonate (Na_2CO_3) , 2.9g Sodium Hydrogencarbonate ($NaHCO_3$), made up to 1 litre by addition of Distilled and Deionised H_2O.

Washing Buffer : 0.8% NaCl, 0.29% Na_2HPO_4, 0.02% KCl, 0.02% KH_2PO_4, 0.1% Tween-80 pH 7.2

1 litre requires 8g Sodium Chloride (NaCl), 2.9g Di-Sodium Orthophosphate (Na_2HPO_4), 0.2g Potassium Chloride (KCl), 0.2g Potassium Di-Hydrogen Orthophosphate(KH_2PO_4) 1ml Tween-80, made up to 1 litre by addition of Distilled and Deionised H_2O.

Phosphate Buffered Saline:0.8% NaCl, 0.29% Na_2HPO_4, 0.02% KCl, 0.02% KH_2PO_4

pH 7.2

1 litre requires 8g Sodium Chloride (NaCl), 2.9g Di-Sodium Orthophosphate (Na_2HPO_4), 0.2g Potassium Chloride (KCl), 0.2g Potassium Di-Hydrogen Orthophosphate (KH_2PO_4) made up to 1 litre by addition of Distilled and Deionised H_2O.

Citrate Buffer : 0.1 Molar pH 4.0

1 Litre requires 12.4g Citric acid , 12.05g Tri-Sodium Citrate , made up to 1 litre by addition of Distilled and Deionised H_2O.

Appendix 4
Statistical Tables

Method

	SS	df	MS	F	p
Within+ Residual	4.58	7.36	0.54		
				12.75	0.008
Method	8.34	1.05	4.17		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Method	0.646	0.986

Day

	SS	df	MS	F	p
Within+ Residual	0.88	7	0.13		
				1.93	0.208
Day	0.24	1	0.24		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Day	0.216	0.225

Method X Day

	SS	df	MS	F	p
Within+ Residual	0.52	12.69	0.04		
				18.72	0.000
Method Day	1.4	2	0.7		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Method X Day	0.728	0.999

Method X Day RM ANOVA on Variability in saliva volume collected. Section 5.2.2.

Method

	SS	df	MS	F	p
Within+ Residual	0.62	12.91	0.04		
				2.75	0.108
Method	0.21	1.61	0.11		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Method	0.246	0.466

Day

	SS	df	MS	F	p
Within+ Residual	0.23	8	0.3		
				1.26	0.294
Day	0.04	1	0.04		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Day	0.136	0.168

Method X Day

	SS	df	MS	F	p
Within+ Residual	0.57	16	0.04		
				0.55	0.589
Method Day	1.04	2	0.02		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Method X Day	0.064	0.127

Levene's Test for Homogeneity **Section 5.2.2**

Statistic	df1	df2	2-tailed
2.39	1	18	0.14

Material

	SS	df	MS	F	p
Within+ Residual	2.02	4	0.5		
Material	3.88	1	3.88	7.69	0.05

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Material		

Time

	SS	df	MS	F	p
Within+ Residual	2.37	6.72	0.07		
Time	9.17	2.13	1.16	15.48	0.004

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		

Material X Time

	SS	df	MS	F	p
Within+ Residual	2.35	6.11	0.07		
Material Time	0.86	1.53	0.11	1.47	0.292

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Material X Time		

Fluid X Material General factorial ANOVA for Mass increase Section 5.3.1.2.

	SS	df	MS	F	p
Within+ Residual	5.77	16	0.36		
Fluid	0.02	1	0.02	0.05	0.818

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Fluid	0.003	0.5

Material

	SS	df	MS	F	p
Material	19.21	1	19.21	53.25	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Material	0.769	1.000

Fluid X Material

	SS	df	MS	F	p
Fluid Material	1.33	1	1.33	3.68	0.073

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
] Fluid X Material	0.187	0.437

One-way ANOVA on Saliva Absorbed **Section 5.4.3**

Variable Saliva absorbed
By Variable Material

Source	D.f.	SS	MS	F Ratio	F Prob.
Between Groups	1	2.59	2.59	7.85	0.0055
Within Groups	242	79.77	0.33		
Total	243	82.36			

One-way ANOVA on Saliva Retained (volume) **Section 5.4.3**

Variable Saliva retained (volume)
By Variable Material

Source	D.f.	SS	MS	F Ratio	F Prob.
Between Groups	1	2.10	2.10	33.32	0.000
Within Groups	242	15.25	0.063		
Total	243	17.35			

One-way ANOVA on Saliva Retained (percentage) **Section 5.4.3**

Variable Saliva retained (percentage)
By Variable Material

Source	D.f.	SS	MS	F Ratio	F Prob.
Between Groups	1	14992.1	14992.1	23.02	0.000
Within Groups	242	157621.2	651.33		
Total	243	172614.1			

Time X Session RM ANOVA on Saliva flow rate **Section 6.3**

Time

	SS	df	MS	F	p
Within+ Residual	0.06	6	0.01		
Time	0.21	1	0.21	21.83	0.003

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time	0.784	0.97

Session

	SS	df	MS	F	p
Within+ Residual	0.14	7.65	0.01		
				4.12	0.073
Session	0.09	1.27	0.05		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Session	0.407	0.612

Time X Session

	SS	df	MS	F	p
Within+ Residual	0.03	12	0.00		
				4.29	0.039
Time Session	0.02	2	0.001		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Session	0.417	0.631

Pre

	SS	df	MS	F	p
Within+ Residual	0.114	10.3	0.011	4.903	0.036
Session	0.093	1.71	0.055		

Post

	SS	df	MS	F	p
Within+ Residual	0.048	7.74	0.006	2.324	0.167
Session	0.018	1.29	0.014		

Time X Session RM ANOVA on SIgA concentration **Section 6.3**

Time

	SS	df	MS	F	p
Within+ Residual	174410	6	29068		
				0.22	0.652
Time	6530.04	1	6530.04		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time	0.036	0.07

Session

	SS	df	MS	F	p
Within+ Residual	776288	12	64690		
				0.01	0.994
Session	744.7	2	372.35		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Session	0.001	0.051

Time X Session

	SS	df	MS	F	p
Within+ Residual	306628	7.52	2552.38		
				0.32	0.635
Time Session	16605	1.25	8302.55		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Session	0.051	0.092

Time X Session RM ANOVA on SIgA secretion rate **Section 6.3**

Time

	SS	df	MS	F	p
Within+ Residual	5197	6	8661		
				9.93	0.2
Time	85970	1	85970		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time	0.623	0.747

Session

	SS	df	MS	F	p
Within+ Residual	174194	9.16	14516		
				1.6	0.25
Session	46374	1.53	23187		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Session	0.21	0.272

Time X Session

	SS	df	MS	F	p
Within+ Residual	81451	7.07	6788		
				0.75	0.44
Time Session	10155	1.18	5078		

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Session	0.11	0.15

Students Paired T test for Saliva flow rate pre and post exercise

Section 6.3

Paired Differences

	Mean	SD	SE of Mean	t-value	2-tailed Sig
SFRPOST	0.135	0.09	0.020	-6.46	0.000
SFRPRE	0.277	0.17	0.037		

Students Paired T test for SIgA concentration rate pre and post exercise

Section 6.3

Paired Differences

	Mean	SD	SE of Mean	t-value	2-tailed Sig
CONCPRE	574.86	215.5	47.04	-0.51	0.614
CONCPOST	599.79	231.4	50.50		

Students Paired T test for SIgA secretion rate pre and post exercise

Section 6.3

Paired Differences

	Mean	SD	SE of Mean	t-value	2-tailed Sig
SECPRE	175.99	159.56	34.82	3.46	0.002
SECPOST	85.50	68.38	14.92		

One-way ANOVA on Saliva Flow Rate **Section 7.3**

Variable Saliva flow rate
By Variable Time

Source	D.f.	SS	MS	F Ratio	F Prob.
Between Groups	3	1.57	0.52	0.234	0.87
Within Groups	28	62.84	2.24		
Total	31	64.41			

Symptom X Time RM ANOVA on Symptom Scores **Section 7.3**

Symptom

	SS	df	MS	F	p
Within+ Residual	69.35	16.56	1.73		
Symptom	11.54	2.07	2.31	1.33	0.292

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Symptom	0.143	0.42

Time

	SS	df	MS	F	p
Within+ Residual	18.23	15.74	0.46		
Time	0.99	1.97	0.2	0.43	0.65

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time	0.051	0.15

Symptom X Time

	SS	df	MS	F	p
Within+ Residual	80.36	27.11	0.4		
Symptom XTime	11.75	3.39	0.47	1.17	0.34

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Symptom X Time	0.128	0.88

Time

	SS	df	MS	F	p
Within+ Residual	11.26	33.99	0.33		
Time	7.91	3.78	2.10	6.32	0.001

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.973

Condition

	SS	df	MS	F	p
Within+ Residual	27.86	18	1.55		
Condition	20.76	2	10.4	6.71	0.007

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.862

Time X Condition

	SS	df	MS	F	p
Within+ Residual	23.24	90	0.26		
Time Xcondition	3.53	10	0.35	1.37	0.209

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.657

Time

	SS	df	MS	F	p
Within+ Residual	0.32	25	0.001		
Time	0.006	5	0.001	0.94	0.474

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.278

Condition

	SS	df	MS	F	p
Within+ Residual	0.39	5.45	0.007		
Condition	0.27	1.09	0.249	3.407	0.119

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.342

Time X Condition

	SS	df	MS	F	p
Within+ Residual	0.52	13.70	0.004		
Time Xcondition	0.007	2.74	0.002	0.66	0.578

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.15

Time

	SS	df	MS	F	p
Within+ Residual	1329.3	23.13	57.48		
Time	1084.2	3.30	328.2	5.71	0.004

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.922

Condition

	SS	df	MS	F	p
Within+ Residual	334.17	7.54	44.34		
Condition	28.04	1.08	26.03	0.59	0.479

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.105

Time X Condition

	SS	df	MS	F	p
Within+ Residual	4306.5	55.66	77.37		
Time Xcondition	387.2	7.95	48.69	0.63	0.749

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.261

Time

	SS	df	MS	F	p
Within+ Residual	326.87	1	326.87		
Time	481.65	1	481.65	1.474	0.439

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.083

Condition

	SS	df	MS	F	p
Within+ Residual	31.31	1	31.31		
Condition	8.75	1	8.75	0.28	0.690

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.057

Time X Condition

	SS	df	MS	F	p
Within+ Residual	528.95	1	528.95		
Time Xcondition	643.13	1	643.13	1.216	0.469

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.078

Time

	SS	df	MS	F	p
Within+ Residual	0.26	8	0.003		
Time	2.32	1	2.32	71.15	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		1.000

Condition

	SS	df	MS	F	p
Within+ Residual	3.71	1.24	3.00		
Condition	9.60	9.89	0.97	3.09	0.105

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.385

Time X Condition

	SS	df	MS	F	p
Within+ Residual	0.40	14.3	0.003		
Time Xcondition	1.82	1.79	1.02	36.97	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		1.000

Time

	SS	df	MS	F	p
Within+ Residual	76.13	29.27	2.60		
Time	26.84	2.66	10.09	3.88	0.022

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.736

Condition

	SS	df	MS	F	p
Within+ Residual	97.09	16.98	5.72		
Condition	288.67	1.54	187.03	32.71	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		1.000

Time X Condition

	SS	df	MS	F	p
Within+ Residual	74.08	55.77	1.33		
Time Xcondition	79.50	5.07	15.68	11.8	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		1.000

Time

	SS	df	MS	F	p
Within+ Residual	2.26	17.07	0.13		
Time	1.74	1.55	1.12	8.46	0.005

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.885

Condition

	SS	df	MS	F	p
Within+ Residual	4.83	15.28	0.32		
Condition	0.16	1.39	0.11	0.35	0.631

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.091

Time X Condition

	SS	df	MS	F	p
Within+ Residual	4.20	16.92	0.25		
Time Xcondition	0.42	1.54	0.27	1.09	0.341

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.192

Time X Condition RM ANOVA on Heart Rates **Section 8.3**

Time

	SS	df	MS	F	p
Within+ Residual	8952.6	10.4	861.03		
Time	99326.4	1.16	85975.5	99.85	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		1.000

Condition

	SS	df	MS	F	p
Within+ Residual	3432.9	18	190.7		
Condition	8068.3	2	4034.15	21.15	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		1.000

Time X Condition

	SS	df	MS	F	p
Within+ Residual	6806.7	19.87	342.6		
Time Xcondition	4443.4	2.21	2012.9	5.88	0.008

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.842

Time

	SS	df	MS	F	p
Within+ Residual	56.67	15.3	3.71		
Time	172.56	1.39	124.27	33.50	0.000

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		1.000

Condition

	SS	df	MS	F	p
Within+ Residual	144.44	14.50	9.96		
Condition	32.11	1.32	24.35	2.45	1.34

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.346

Time X Condition

	SS	df	MS	F	p
Within+ Residual	47.44	37.10	1.28		
Time Xcondition	13.33	3.37	3.95	3.09	0.034

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.709

Time X Condition RM ANOVA on Lactate **Section 8.3**

Time

	SS	df	MS	F	p
Within+ Residual	11.25	15.65	0.72		
Time	2.68	1.74	15.43	2.15	0.154

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time		0.351

Condition

	SS	df	MS	F	p
Within+ Residual	15.80	16.35	0.97		
Condition	9.445	1.82	5.2	5.4	0.018

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Condition		0.74

Time X Condition

	SS	df	MS	F	p
Within+ Residual	16.13	32.28	0.50		
Time Xcondition	4.74	3.59	1.32	2.65	0.057

Effect Size Measures and Observed Power at the 0.050 Level

	Partial Eta Squared	Power
Time X Condition		0.641

PART VI

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