SALIVARY TESTOSTERONE AND CORTISOL MEASUREMENTS IN PROFESSIONAL ELITE RUGBY UNION PLAYERS

By

Kevin Andrew Morgan

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Certificate of Research
This is to certify that the work described in this thesis is the result of my own work.

Neither this thesis, nor any part of it, has been presented, or been submitted, in candidature for any degree in any other University

Professor Bruce Davies
(Director of Studies)

Kevin Morgan
(Candidate)

Professor Julien Baker
(Supervisor)

ABSTRACT

This thesis presents three studies investigating the use of salivary measurements in monitoring plasma concentrations of testosterone (T) and cortisol (C). Study one investigated the relationship between total serum and saliva concentrations of T and C in healthy men (n = 4) under resting conditions. Blood and saliva were sampled simultaneously from 9:00 am until 5:00pm to determine the relationship between total serum and saliva concentration of T and C. Total serum and saliva C showed a strong and significant correlation ($r = 0.95$, $p<0.05$). Total serum and saliva T showed a moderate and significant correlation ($r = 0.62$, $p<0.05$). Results suggest that under resting conditions, salivary measurements of T and C and T/C ratio can reflect the concentrations of these hormones in blood.

Study two investigated the relationship between total serum and saliva concentrations of T and C in response to a treadmill exercise protocol (~ 70% $\dot{V}O_{2max}$) in nine healthy men. Blood and saliva were sampled simultaneously pre-exercise, immediately after and at 10, 20, 30, 60 and 90 min post exercise. Total serum was significantly correlated with saliva C ($r = 0.82$, $p<0.05$). No correlation was observed between total serum and saliva concentration of T ($r = 0.22$, $p>0.05$). Correction for plasma volume on total serum measurements had little effect on the relationship between both analytes. These results indicate that saliva measurements of C can reflect total serum concentrations under exercise conditions. However, further investigations are required to determine if saliva T measurements reflect the concentration of total serum T in response to exercise.

Study three investigated pre-competition hormonal and psychological states in male elite rugby union players. Hormonal response was measured using salivary T and C. In addition, psychological states were assessed using the Competitive State Anxiety Inventory-2 (CSAI-2R). Physiological measures indicated that both T and C were significantly higher before competition ($p<0.05$) than in resting conditions. Furthermore, significant differences between games were found in cortisol response ($F = 6.84$, $p<0.01$) and testosterone response ($F = 3.46$, $p<0.05$) suggesting the response of both hormones is affected by venue and/or opposition. Psychological measures indicated that players had higher somatic and cognitive anxiety ($p<0.05$) before competition than in resting

conditions. No significant change was reported in anxiety or confidence measures between games. The present results suggest that differences in pre-competition hormonal states may play a key role in subsequent performance.

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COMMON ABBREVIATIONS

1RM = 1 Repetition max

ACTH = Adrenocorticoptropic hormone

C = Cortisol

CBG = Cortisol binding globulin

CRH = Corticotropin releasing hormone

CRF = Corticotropin releasing factor

CSAI = Competitive state anxiety inventory

CV = Coefficient of variance

DNA = Deoxyribonucleic acid

EDTA = Ethylenediamene-tetra-acetic-acid

FSH = Follicle stimulating hormone

GCF = Gingival crevicular fluid

GnRH = Gonadotropin releasing hormone

HPA = Hypothalamus – pituitary – adrenal

LH = Luteinizing hormone

PV = Plasma volume

PVN = Paraventricular nucleus

RNA = Ribonuleic acid

RM = Repetition maximum

SCN = Suprachiasmatic nucleas

SD = Standard deviation

SEM = Standard error from mean

SHBG = Sex hormone binding globulin

SST = Serum separation tube

T = Testosterone

T/C = Testosterone/cortisol

VO2max = Maximum oxygen uptake

CHAPTER 1

1.0 INTRODUCTION

Since the declaration by the International Rugby Board (IRB) in 1995 that the game would become professional, the sport has changed dramatically both on and off the field (Mellalieu et al., 2008). Rugby is an intense sport, consisting of both running and contact play, which appears to impose psychological and physiological stress on players (Suzuki et al., 2004). The physical demands on players during competition and training has increased. Due to the intensity and duration of the rugby calendar, it is important to monitor the training and recovery process. This will facilitate the appropriate player management procedures which will maximise optimal performance with the absence of overtraining. Previous studies have shown that testing player hormonal status may provide coaches with an indicator of training maladapatation (Maso et al., 2004).

Cortisol is a catabolic hormone produced by the adrenal cortex. The most important function of cortisol relates to its influence on protein breakdown and energy metabolism. Stressful situations or physical activity stimulate the hypothalamic paraventricular neurons (PVN) to release corticotrophin releasing factor which in turn causes the anterior pituitary gland to release adrenocorticotrophin hormone (ACTH) (McArdle et al., 2006). ACTH subsequently causes cortisol release from the adrenal cortex (Bao et al., 2008). Aerobic, anaerobic and resistance exercise in addition to competition have been shown to elicit a change in blood and saliva levels of cortisol (McGuigan et al., 2004; Luger et al., 1987; Elloumi et al., 2003).

Testosterone is a potent anabolic hormone that stimulates muscle protein synthesis (Vingren et al., 2008). The main site of testosterone production in men is the testis. The hypothalamus releases gonadotropic-releasing hormone which stimulates the pituitary to

secrete luteinizing hormone (LH) which in turn activates the testis to produce testosterone (Hackney et al., 1995). Authors have shown that different exercise modalities affect both blood and saliva concentrations of testosterone (Hakkinen and Pakarinen, 1993; Fahrner and Hackney, 1998; Kraemer et al., 2001).

Maso et al., (2004) speculated that testosterone and cortisol are implicated with overtraining. The concentrations of these hormones as an indicator of overtraining has also been outlined in football (Filaire et al., 2003), cycling (Hoogeveen and Zonderland, 1996), swimming (Hooper et al., 1993) and rowing (Vervoorn et al., 1991) with the authors appraising any possible disturbance in the anabolic-catabolic balance, which may express itself in decreased performance.

It has been stated that the pre-competitive hormone response in individuals may provide an indicator to the eventual outcome of the contest (Salvador et al., 2003). With this in mind it may be possible that an athlete's psychophysiological response to the onset of competitive activity may have an effect on subsequent performance. Previous studies have measured testosterone and cortisol in relation to acute psychological stress (Smyth et al., 1998), territoriality (Neave and Wolfson, 2003) and anxiety (Benjamins et al., 1992). Pre-competition physiological and psychological states have also been investigated in hockey (Carre, et al. 2006), judo (Salvador et al., 2003) and golf (Doan et al., 2006) to assess measures of perceived competitive stress in relation to hormone response and its possible affect on the outcome of competition.

This investigation will attempt to categorise possible hormonal changes in an elite rugby union environment. Study 1 will investigate the validity of resting saliva measurements of T and C in relation to plasma levels. Study 2 will investigate the post exercise validity

of saliva measurements of T and C as a representation of plasma levels. The influence of exercise induced plasma volume change on the interpretation of hormonal measurements will also be investigated. Study 3 will evaluate the effect of pre-competition stress on saliva concentrations of T and C in elite rugby union. It is anticipated that this study will supplement current knowledge of exercise stress and associated changes in serum/saliva hormones. Knowledge of this in an elite rugby union environment may aid in appropriate tailoring of future training programs and establishment of fitness goals.

2.0 LITERATURE REVIEW

2.1 The Endocrine System

The functions of the body are regulated by two major control systems:

1) the nervous system
2) the hormonal, or endocrine system

In general the endocrine system is concerned principally with control of the different metabolic functions of the body, such as rates of chemical reaction in the cells and the transport of substances through cell membranes or other aspect of cell metabolism such as growth or secretion (Pocock and Richards, 2006).

A hormone is a chemical substance that is secreted into the internal bodily fluids by one cell or a group of cells and has a physiological control effect on other cells of the body (Guyton and Hall, 2000). Most of the general hormones are secreted by specific endocrine glands. The locations of the important endocrine glands of the body are shown in Figure 2.1.1:

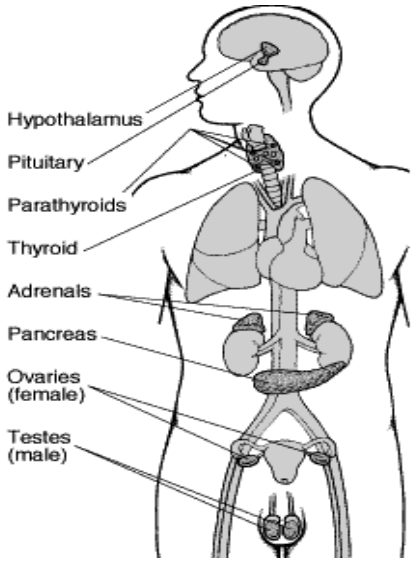

Fig 2.1.1. Anatomical loci of the principle endocrine glands of the body (male and female) (Guyton and Hall, 2000)

2.1.1 The Endocrine Glands

Listed below are the endocrine glands and a description of the hormones they secrete (Guyton and Hall, 2000):

Pituitary Gland

Anterior Pituitary Hormones

1) Growth Hormone: produces growth in almost all cells and tissues of the body including the skeleton, and plays an important role in glucose homeostasis.
2) Adrenocorticotropin: controls the activity of the adrenal glands and their production of glucocorticoids and mineralocorticoids.
3) Thyroid-stimulating hormone: stimulates the thyroid gland to secrete thyroxin and triiodothyronine.
4) Follicle-stimulating hormone: promotes the formation of sperm in the male testis and follicle growth in the female ovary.
5) Luteinizing hormone: causes secretion of testosterone by the testes, and also stimulates the production of oestrogen and progesterone by the ovaries.

Posterior Pituitary Hormones

1) Vasopressin: produces the retention of water in the kidneys, thus increasing the water content of the body. In high concentrations it results in constriction of the blood vessels throughout the body and elevates blood pressure.

Adrenal Glands

1) Cortisol: the principle glucocorticoid has multiple metabolic functions for control of the metabolism of carbohydrates, fats and proteins. It is also a vital component in the body's defence against stress.
2) Aldosterone: the principle mineralocorticoid, it reduces sodium excretion and increases potassium excretion by the kidneys.

Thyroid Gland

1) Thyroxine and Triidothyronine: increase the rates of chemical reaction in almost all cells of the body, thus increasing the general level of body metabolism.
2) Calcitonin: promotes the deposition of calcium in the bones and thereby decreases calcium blood levels.

Islets of Langerhans in the Pancreas

1) Insulin: promotes glucose entry into most cells of the body, principally muscle and liver, in this way controlling the rate of metabolism of carbohydrates.
2) Glucagon: increases the synthesis and release of glucose from the liver into circulating blood.

Parathyroid Gland

1) Parathyroid hormone: exerts effects on bone, gut and kidneys to raise plasma calcium levels and reduce phosphate levels. It is released in response to a fall in plasma calcium concentration and acts to maintain normal plasma calcium. Its

combined effects with the hormones cholecalciferol (Vitamin D), which regulates the absorption of dietary calcium by the intestine, and calcitonin result in the regulation of plasma calcium levels.

Testes

1) Testosterone: stimulates growth of the male sex organs and also promotes the development of the male secondary sex characteristics.

Ovaries

1) Estrogen: stimulates the development of the female sex organs and various secondary sex characteristics.
2) Progesterone: stimulates secretion of uterine milk.

2.1.2 Chemistry of hormones

Chemically, hormones are of three different types (Pocock and Richards, 2006);

1) Steroid hormones: These hormones all have a chemical structure based on the steroid nucleus, similar to that of cholesterol and in most instances are derived from cholesterol e.g. cortisol, aldosterone and testosterone.
2) Derivatives of the amino acid tyrosine: the two thyroid hormones, thyroxin and triiodothyronine, are tyrosine derivatives. The two catecholamine hormones of the adrenal medulla, epinephrine and norepinephrine, are also derived from tyrosine.

3) Proteins or peptides: all the remaining important endocrine hormones are either proteins or peptides or derivatives of these. The anterior and posterior pituitary hormones are examples of this hormone.

2.1.3 Storage, Secretion and Transport of Steroid Hormones

The amounts of steroid hormones stored in glandular cells in the adrenal cortex, testes and ovaries are usually quite small, but large amounts of precursor molecules, especially cholesterol and various intermediates between cholesterol and the final hormones are present in these cells. Within minutes, enzymes can cause the necessary chemical conversions to the final hormones followed by immediate secretion into the bloodstream (Guyton and Hall, 2000).

The chemical nature of hormones influences the manner in which they are transported in the bloodstream. Catecholamines and peptide hormones are hydrophilic and can travel in free solution in plasma, the steroid and thyroid hormone are hydrophobic and are carried in the bloodstream bound to a variety of plasma proteins (Pocock and Richards, 2006). The binding proteins have a high affinity for specific hormones. Examples of these include sex hormone-binding-globulin, cortisol-binding-globulin and thyroid hormone-binding-globulin. Hormones carried by proteins are referred to as 'bound' hormones and are in dynamic equilibrium with unbound (free) hormones in plasma (Davies et al., 2001). It is only the free hormone that can pass from the capillaries into tissue spaces and interact with target cells. As the amount of free hormone decreases, it is replaced by the release of bound hormone from the carrier proteins, thus restoring the bound:free ratio for that hormone in the plasma (Davies et al., 2001).

2.1.4 Hormone receptors and their effect on target cells

The endocrine hormones seldom affect the intracellular machinery to control the final cellular chemical reactions, instead they usually first combine with receptors on the surface of cells or inside the cell cytoplasm. The combination of hormone and receptor then initiates a cascade effect of reactions inside the cell, with each stage of reactions more powerful than the previous stage (Pocock and Richards, 2006). The steroid hormone first crosses the plasma membrane by first diffusing through the lipid layer of the cell and then binds to its cytoplasmic receptor. The receptor hormone complex migrates into the cell nucleus where it increases transcription of DNA into the appropriate messenger RNA. The new messenger RNA is then used as a template for protein synthesis. Each receptor is usually highly specific for a single hormone. The target tissues that are affected by a hormone are those that contain its specific receptor.

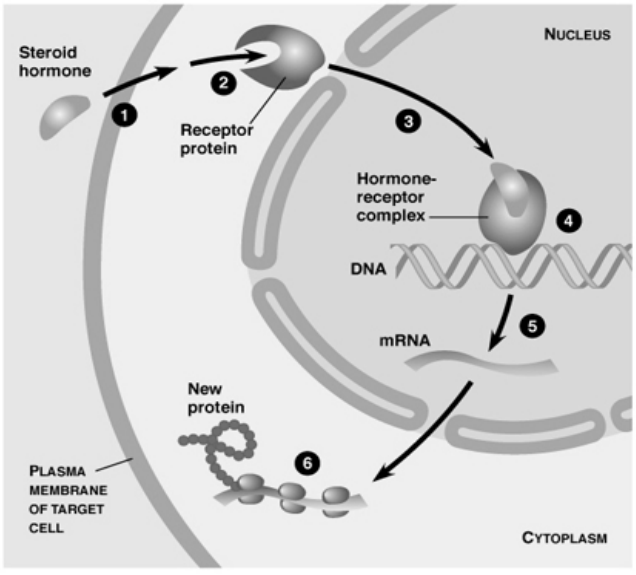

Fig 2.1.2: A simplified diagram of how steroid hormones regulate gene transcription in target cells (Pocock and Richards, 2006)

2.2 Cortisol

Cortisol is a catabolic hormone produced by the adrenal cortex. Stressful situations or physical activity stimulate the hypothalamus to release corticotrophin releasing factor (CRF) into the hypophysiol portal blood vessels. CRF in turn stimulates the cells in the anterior pituitary gland to secrete adrenocorticotrophin hormone (ACTH) into the blood stream (McArdle et al., 2006). ACTH regulates the synthesis and secretion of corticosteroid hormones secreted by the adrenal cortex. These steroid hormones can be categorised into three groups: mineralocorticoids, glucocorticoids, and androgens. Cortisol is the major glucocorticoid secreted by the adrenal cortex. Cortisol has a direct negative feedback effect on the hypothalamus to decrease the formation of CRF, in addition, the anterior pituitary gland is stimulated to decrease the formation of ACTH. Both of these feedbacks help regulate plasma concentrations of cortisol (Guyton and Hall, 2000).

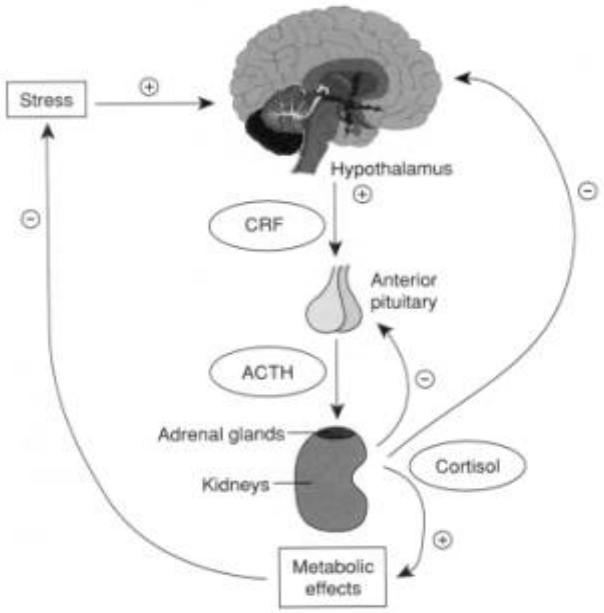

Fig 2.2.1. Mechanism for regulation of cortisol secretion (Davies et al, 2001)

In circulating blood, about 94% of cortisol is bound, mainly to corticosteroid binding globulin (CBG) and to a lesser extent with albumin, the free fraction constitutes around 6% (Obminski and Stupnicki, 1997). The most important function of cortisol relates to its effects on protein, carbohydrate and fat metabolism. Cortisol effects energy metabolism by:

- Stimulating gluconeogenesis: the most important effect of cortisol is its ability to stimulate gluconeogenesis (formation of carbohydrate from proteins and other substances) by the liver. It does this by increasing all enzymes required to convert amino acids into glucose.
- Reducing cellular protein: one of the principle effects of cortisol is to reduce protein synthesis in all cells of the body except the liver.
- Mobilizing fatty acids: it mobilizes fatty acids from adipose tissue. This increases the concentration of fatty free acids in plasma, which also increases their utilisation for energy (Guyton and Hall, 2000).

Cortisol also plays a crucial role in the response of the body to stressful stimuli. It has a global effect in reducing all aspect of the inflammatory process, as well as being immunosuppressive and anti-allergic (Pocock and Richards, 2006). Cortisol also acts as an antagonist for insulin by inhibiting glucose uptake and oxidation (Hatfield, 2003).

Cortisol is a known marker for physiological and psychological stress and therefore its use as an index of global stress has been documented in previous investigations (Kirchbaum and Hellhammer, 1994; Neary et al., 2002). As mentioned above, stressful situations or physical activity influence blood levels of cortisol (Paccotti et al., 2004). Past studies in exercise science literature have used cortisol to monitor an athlete's

response to testing, training and competition, and have found cortisol as a valid and reliable index linked to physical effort (Neary et al., 2002). Cortisol is also a powerful natural immuno-suppressant (Petrovsky et al., 1998), making it a hormone of interest in studies investigating immune function. Therefore, some investigators have suggested cortisol as an indicator of overtraining, overreaching and reduced performance (Hoogeveen & Zonderland 1996; Hooper et al., 1993).

Acute psychological stress has been associated with an increase in cortisol (Kirchbaum and Hellhammer, 1994). Competition is a challenging situation which normally stimulates an intense psychological response in participants. Numerous studies have investigated cortisol response in anticipation of competition (Salvador et al., 2003; Doan et al., 2006) and some have suggested it may be used as an indicator of competitive outcome (Salvador et al., 2003).

2.2.1 Circadian Variation of Cortisol

The suprachiasmatic nucleus (SCN) is the major circadian pacemaker of the central nervous system. This small ovoid structure, situated at the base of the hypothalamus, receives light information as the main input to synchronize its circadian activity to the day-night cycle (Buijs et al., 1999). Cortisol is a corticosteroid produced by the adrenal cortex. The endogenous pacemaker also generates a circadian rhythm in this hypothalamic-pituitary-adrenal secretion via the SCN pathway (Hofstra & Weerd, 2008). Cortisol is therefore highly rhythmical, with declining levels throughout the day, a nocturnal quiescence, and a sharp rise in the second half of the night toward a morning

maximum, in the hours around waking up. The nadir is reached within approximately 2 hours after beginning sleep (Hofstra & Weerd, 2008).

There have been many studies investigating the possibility of plasma cortisol (C) displaying a circadian rhythm. Sharma et al. (1989) measured plasma C over a 24hr period in subjects with an age range of 19-89 years. It was shown that plasma C displayed a circadian rhythm in all subjects. Levi et al. (1988) measured plasma total and free C in healthy young males over a 24hr period. A circadian rhythm was statistically validated for both plasma free and total C. Individual mean total C ranged from 0.338 ηg/dl to 0.116 ηg/dl with free C ranging from 0.017 ηg/dl to 0.008 ηg/dl. Salivary C has also been shown to display a circadian rhythm in a study by Nater et al. (2008). Mean saliva cortisol peaked at 08:00 hrs (4.2 ηg/ml) with a nadir occurring at 22:00 hrs (1.1 ηg/ml). Authors in these investigations showed C peaked in early morning, approximately 60 min after awakening, with levels then gradually reducing through the day.

2.2.2 Postprandial Cortisol response

It has already been shown that cortisol (C) exhibits a circadian variation with peak levels during early morning and then gradually reducing throughout the day. However, studies have shown that C secretion can be affected by meals (Gibson et al., 1999) and that the affect on C is dependant on the composition of the meals consumed (Follenius et al., 1982). In particular, studies have shown that high protein meals produce a greater C response than meals with a high carbohydrate or fat level meal (Slag et al., 1981). The mechanism behind the influence of a protein based meal on the hypothalamus-pituitary-

adrenal (HPA) system is unclear. Benedict et al. (2005) suggested that the increase in cortisol originates from an amino acid dependant activation of the gastrointestinal mucosa. How this signal is reported to the HPA system to stimulate cortisol release is not clear. Slag et al. (1981) found that a high protein diet (4 gm protein/kg body weight) induced significant increases in plasma C at 30 and 60 minutes after a 1200 hr meal and 30 min after a 1600 hr meal. Increases in C were also observed after both the 1200 hr and 1600 hr meals in the standard, high carbohydrate and high fat diet (all 1 gm protein/kg bodyweight), but in a much smaller magnitude. Gibson et al. (1999) observed that salivary C levels were significantly higher after a high protein meal compared to a low protein meal. Mean C levels were higher at 30 and 60 minutes post-meal but no longer differed by 120 minutes post-meal. In summary, these studies have shown that consumption of a meal leads to an increase in cortisol with protein having the greatest stimulatory effect on C.

2.2.3 Hydration status and its effect on cortisol

There has been little research into the effect of hydration alone on circulating concentrations of cortisol. Maresh et al. (2006) examined the effect of hydration status on cortisol response pre and post exercise at intensities of 70% and 85% $\dot{V}O_{2max}$. It was shown that before both exercise protocols, cortisol levels in subjects were significantly higher when dehydrated (~5% body mass loss) compared to euhydrated. The mechanism for this response of the hypothalamus-pituitary-adrenal (HPA) system is unclear. Batmanghelidj, (2001) stated that in dehydration, cell membranes become less permeable, hampering the flow of hormones and nutrients into the cell and preventing

waste products such as oxidants that cause cellular damage from flowing out. It is possible that dehydration induces cellular stress mechanisms capable of elevating basal cortisol levels. Judelson et al. (2008) also suggested the resultant increase in core temperature associated with dehydration could possibly stimulate the increase in cortisol levels.

It has been documented that exercising while dehydrated elicits an exaggerated stress hormone response compared with that observed during exercise in a euhydrated state (Mitchell et al., 2002; Bishop et al., 2004; Francesconi et al., 1985). Again the mechanisms for this exaggerated cortisol response to exercise when dehydrated remains unclear. Judelson et al. (2008) and Mitchell et al. (2002) suggested that the increase in cardiovascular demand due to the reduction in plasma volume with dehydration is the likely cause of increases in cortisol. Francesconi et al. (1985) clearly demonstrated that cortisol (C) responses prior to and during exercise were influenced by the level of dehydration. In this study six subjects were asked to perform four consecutive 25 min walks interspersed by four 10 min rest periods. These were performed once during a euhydrated state and once during dehydration (3, 5 and 7% of base line body weight). It was shown that circulating plasma C concentrations increased with exercise at each level of dehydration compared with exercise in a euhydrated state with a significant increase occurring at the 5% and 7% dehydration level.

Bishop et al. (2004) investigated the effect of fluid intake versus no fluid intake on C response in subjects cycling for 2 hr at 65% $\dot{V}O_{2max}$. It was shown that immediately after exercise plasma C concentrations increased significantly from pre-exercise values in both trials but was significantly higher in the no fluid trial. The post exercise plasma C

concentration in the no fluid trial was calculated at ~13% higher than in the fluid trial. Mitchell et al. (2002) have also assessed the combined effect of hydration status and heat stress on C response after exercise. In this study subjects were asked to perform exercise for 75 min at a target intensity of 55% $\dot{V}O_{2max}$ under four different conditions: (1) euhydrated neutral, (2) euhydrated hot, (3) dehydrated neutral and (4) dehydrated hot. Authors showed that serum C levels in (2) and (4) were significantly greater than those in (1) and (3) at the post-exercise time point.

2.2.4 Vitamin C Supplementation and Cortisol Response

It has been postulated that vitamin C supplementation results in the reduction of post exercise serum cortisol levels in trained athletes (Peters et al., 2001(a); Peters et al., 2001(b)). However, the mechanism by which vitamin C supplementation attenuates the post exercise increase in cortisol has yet to be established. Plasma C concentration is elevated after exercise, suggesting an increased release from the adrenal glands (Davison & Gleeson 2005). Mobilization of vitamin C from the adrenals as a component of an adaptive response to oxidative stress may therefore be coupled with the increase in cortisol that results from exercise (Peters et al., 2001(a)). Hence, these events may be attenuated with the supplementation of the vitamin. In a study by Peters et al. (2001(b)), supplementation of vitamin C at 1000 mg/day for 7 days prior to participation in a 90 kilometre ultra marathon was shown to significantly reduce (30%) post race levels of serum cortisol compared to a control (placebo). Serum cortisol subsided to baseline values 24 hours after completion of race. The same author (Peters et al., 2001(a)) also assessed the effect of different doses of vitamin C on the levels of serum cortisol after

ultra marathon running. It was shown that the administration of 1500 mg/day for 7 days, but not 500 mg/day, significantly attenuated the immediate post race increase in serum cortisol (average decrease of 34.7% relative to placebo group). Serum cortisol values were also shown to subside to baseline values by 24 hours post race. Authors speculated that a threshold value of approximately 1000 mg vitamin C may represent the an adequate dosage in attenuating exercise induced hypercortisolemia. Conversely, Davison and Gleeson, (2005) also investigated the effect of a single dose of vitamin C on post exercise plasma concentration of cortisol. Participants were asked to consume a beverage containing an approximate total of 3400 mg of vitamin C before and during exercise. It was shown that an acute dose of vitamin C did not significantly affect the values of post exercise plasma cortisol.

2.2.5 Cortisol response to Acute Exercise

Stress is a term defining a physiological response to events perceived as potentially or actually threatening the body's integrity. Strenuous exercise is a form of physical stress. Among the major effectors of this response are the neurons of the paraventricular nucleus in the hypothalamus, which secrete corticotropin-releasing hormone (CRH) and vasopressin, hence activating the HPA axis and eventually increasing the cortisol output from the adrenal glands (Paccotti et al., 2005). There has been a plethora of studies investigating the response of cortisol (C) to acute exercise and competition in sport. Values have been shown to increase in a variety of exercise models. McGuigan et al. (2004) investigated the response of two trials of acute resistance exercise on salivary C. The high intensity resistance exercise (6x10 @ 75% 1RM bench press) resulted in a

significant rise (97%) in mean salivary C values post exercise compared to pre exercise C values. In contrast, low resistance exercise (3x10 @ 30% 1RM bench press) did not result in any significant changes in C levels.

Luger et al. (1987) examined the effect of three different exercise intensities on plasma C. Subjects were asked to exercise on a treadmill at 50, 70 and 90% $\dot{V}O_{2max}$ on three separate occasions. The 50% $\dot{V}O_{2max}$ protocol showed no significant increase in serum C post exercise, whereas exercise intensities of 70% and 90% $\dot{V}O_{2max}$ produced significant increases in serum C during and post exercise with the latter protocol producing the greater increase. Peak mean C levels of 11.6 μg/dl and 21.8 μg/dl occurred at 35 min post exercise in both the 70% and 90% $\dot{V}O_{2max}$ exercise intensities respectively.

Hackney et al. (1995) investigated the effect of aerobic (65% $\dot{V}O_{2max}$ for 60 min) and anaerobic (interval exercise consisting of 2 min at 110% $\dot{V}O_{2max}$ followed by 2 min at 40% $\dot{V}O_{2max}$) exercise on serum C response. The investigators reported a significant increase ($p<0.05$) post exercise in C compared to pre exercise concentrations (16.1 μg/dl) in both the aerobic and anaerobic protocols. Mean post exercise serum C values were 28.2 μg/dl and 23.7 μg/dl for the anaerobic and aerobic protocols respectively.

Elloumi et al. (2003) investigated the saliva C response of participants competing in an amateur international rugby union match. Salivary C was collected immediately post game. Results showed an approximate 148% increase ($p<0.001$) in salivary C post game compared to baseline levels recorded at the same time on a control day. Individual levels varied in a range of 17.1ηmol/l - 22.3 ηmol/l post game compared to control day values ranging from 6.8 ηmol/l to 9.7 ηmol/l.

The effect of multiple wrestling matches on circulating C in university wrestlers has also been investigated (Kraemer et al., 2001). Pre and post match serum C was measured over 5 matches during the tournament. Cortisol was elevated post match for all matches with a significant increase for matches 1, 2 and 5. Mean values ranged from 245 ηmol/l to 353 ηmol/l at pre match, with post match concentrations ranging from 317 ηmol/l to 472 ηmol/l.

2.2.6 Psychological Stress: Effects on Cortisol

Pfister and Muir, (1992) have described stress as the physical or emotional influences that disturb homeostasis of the organism and produce psychological and physiological changes in the organism. Acute psychological stress has been reported to increase the activity of the hypothalamus-pituitary-adrenal (HPA) axis (Kirchbaum et al., 1994). Activity of the corticotrophin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVN) forms the basis of the activity of the HPA-axis. The CRH neurons induce adrenocorticotropin (ACTH) release from the pituitary, which subsequently causes cortisol release from the adrenal cortex (Bao et al., 2008). The C response to the onset of an acute psychological stressor is thought to peak after 20-30 min (Kirchbaum et al., 1989). Benjamins et al. (1992) reported on salivary free C values in response to severe dental anxiety. Patients reporting high dental anxiety were shown to have nearly twice the C levels of controls. The C response of dental undergraduates undertaking academic assessment was investigated by Vivian et al. (2003). The mean C level before the test was 6.32 ηmol/l. This was reported as significantly higher than the post test mean value of 5.16 ηmol/l ($p<0.05$).

In addition to acute psychological stress, changes is mood state have also been shown to influence cortisol response. Smyth et al. (1998) examined the response of salivary C to positive and negative stressors. The results showed that a positive stressor affect was significantly associated with lower salivary C levels and a negative affect was significantly associated with higher levels. Berk et al. (1989) examined the role of laughter on changes in stress hormones. Participants viewed a humorous film on one occasion and on another occasion indulged in a protocol of neutral stimuli. Blood samples were taken every 10 min for 30 min prior to each protocol, during the protocol (60 min) and for 30 min post. It was found that when subjects viewed the humorous film, their C levels decreased at a more rapid rate ($p<0.05$) than a control group who indulged in the protocol of neutral stimuli.

The cortisol response in anticipation of competition has been investigated by Salvador et al. (2003) who studied the anticipatory cortisol C response to judo competition in a group of young men. The study reported that levels of C were significantly higher pre contest than in resting conditions. Carre et al. (2006) investigated home and away game response on salivary C. No statistical differences were reported between pre-game salivary C concentration for home games compared to away. Mean saliva C was measured at 3.73 ± 0.35 ηg/ml for homes games and 2.91 ± 0.38 ηg/ml for away games.

2.3 Testosterone

Testosterone (T) is an anabolic hormone produced by the testes in males and by the adrenal glands and ovaries in females (Brownlee et al., 2005). The control of the production of testosterone begins with the secretion of gonadotropin releasing hormone

(GnRH) by the hypothalamus. This hormone in turn stimulates the anterior pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). In males, FSH stimulates germinal epithelium growth in the testes to promote sperm development. LH stimulates the Leydig cells of the testes to secrete the sex hormone testosterone. High levels of testosterone inhibit the release of FSH and LH indicating that T production is mediated by a negative feedback mechanism. After secretion by the testes, around 97% of testosterone binds with plasma proteins, either loosely bound to plasma albumin or more tightly bound with sex hormone binding globulin (SHBG), with a small fraction bound to cortisol binding globulin (CBG), and circulates in the blood for 30 min to 1 hr (Obminski et al., 1997). By that time the testosterone either becomes fixed to target tissues or is degraded into inactive products that are subsequently excreted (Guyton and Hall, 2000). As with other steroid hormones, the levels of binding globulins and therefore the concentration of bound T is dependant on many physiological and pathological situations in humans.

All or most of the effects of testosterone relate to an increased rate of protein formation in target cells. Therefore, testosterone stimulates production of proteins virtually everywhere in the body, although more specifically those proteins in target organs or tissues (Guyton and Hall, 2000). Testosterone has many functions in the body that include, the development of primary and secondary sexual characteristics, bone growth, sperm production, red blood cell production, muscular development, fat lipolysis and protein synthesis (Zitzmann et al., 2001).

Testosterone concentration is commonly used as a marker for physiological stress and as an index of anabolic status due to its influence on increasing lean body mass and

muscular strength in athletes (Tremblay et al., 2003). Previous investigations have documented the influence of physical activity on blood levels of testosterone (Hakkinen et al., 1993; Fahrner et al., 1998) and have used the hormone to monitor an athlete's response to training and competition (Kraemer et al., 2004; Hoffman et al., 2002).

Territoriality and aggression, and their relationship to testosterone, has been documented in numerous studies (Archer, 1991; Book et al., 2000). In particular, this relationship has been studied in a pre-competitive environment (Salvador et al., 2003; Mazur et al., 1992), and some have suggested that testosterone levels may have an influence on athletic performance (Doan et al., 2007).

2.3.1 Circadian Variation of Testosterone

The suprachiasmatic nucleus (SCN) of the hypothalamus is the locus of a master circadian clock controlling behavioural and physiological rhythms, including rhythmic secretion of gonadal hormones via the testis (Karatsoreos et al., 2007). Past studies have investigated the circadian rhythm of testosterone (T) in normal men. Leymarie et al. (1974) measured plasma T in four young adult males every 30 minutes for 24 h. Results showed that all four participants exhibited a circadian rhythm in plasma T, with peak levels upon awakening and then gradually reducing throughout the day. Mean plasma T levels varied from 2.78 ηg/ml to 4.00 ηg/ml amongst participants. T levels were shown to be extremely variable from one subject to the other. Faiman et al. (1971) also investigated the presence of a circadian rhythm in men. Plasma samples were obtained every 4hrs over 24hrs on two separate days, one week apart. Results again showed a significant circadian rhythm over a 24 hr period with highest levels in the morning. The individual

cycles for T showed considerable intersubject variation as well as differences in some subjects between the two test days, although the mean value in each subject was similar for both days (Faiman and Winter, 1971). Umehara et al. (1991) have also shown the presence of a circadian rhythm in salivary T. Saliva T was measured in normal males (n = 21) from 08:30 hrs until 16:00 hrs. Mean saliva T peaked at 08:00 hrs (0.28 ηg/ml) with a nadir reached at 13:30 hrs (0.16 ηg/ml).

2.3.2 Postprandial testosterone response

Testosterone (T) response to meals and their composition has been investigated in many studies. Volek et al. (2001) examined the response of total (T) and free (T) to a high fat meal (86% fat, 11% carbohydrate, 3% protein) in eleven men. Concentrations of total T and free T were significantly reduced by 22% and 23% respectively at 1h postprandial compared to pre meal values. The authors did not compare the results obtained to a control condition without a meal. This could have controlled for the possible effects of the circadian rhythm of T. Meikle et al. (1990) investigated postprandial response of plasma T and free T after meals of different nutrient composition. Subjects consumed beverages consisting of either a non-nutritive sweetener (<1 kcal), or isocaloric sources with either high fat (9% protein, 57% fat, 34% carbohydrate) or mixed carbohydrate and protein (25.5% protein, 1.2% fat, 73.3% carbohydrate) content. Results showed a significant decrease in postprandial total and free T after consumption of the fat containing meal at 2, 3 and 4 hours post meal, compared to pre-meal values. No changes in total or free T post meal in either the non-nutritive of mixed carbohydrate and protein meals were shown. In summary, consumption of a meal with a high fat content leads to a

decrease in T concentrations. The mechanism(s) by which a high-fat containing meal decreases testosterone response are uncertain. Habito et al. (2001) suggested this may be due to increased hepatic clearance, resulting from increased splanchnic blood flow after eating. Meikle et al. (1990) postulated that elevated chylomicrons after a high fat meal may inhibit luteinizing hormone (LH)-stimulated testosterone production.

2.3.3 Testosterone response to Acute Exercise

The response of testosterone (T) to exercise has been investigated in many studies. A variety of exercise protocols have been shown to elicit an increase in post exercise testosterone (Hakkinen et al., 1993; Fahrner et al., 1998; Kraemer et al., 2001). However, the physiological mechanism inducing the increase in testosterone to exercise remains unclear. In resting conditions, the testes are primarily under the influence of the gonadotropins (LH, FSH). However, no relationship has been found between the levels of the gonadotropins and the increase in testosterone with exercise (Raastad et al., 2000). An increase in free testosterone due to alterations in the binding affinity of sex hormone binding globulin (SHGB) has also been discounted by Fahrner et al. (1998). It has been suggested that the increase in testosterone in due to a reduced metabolic clearance rate (MCR) (Cadoux-Hudson et al., 1985), alternatively Cumming et al. (1986) postulated that the increase may be mediated by sympathetic stimulation of the testes via the adrenal gland.

Hakkinen and Pakarinen, (1993) investigated the acute response of serum total and free T to two different heavy resistance protocols in 10 male athletes. Subjects were asked to perform two different resistance training sessions, the first (Session A) containing 20 sets

of 1RM of barbell squats and the second (Session B) containing 10 sets of 10 repetitions at 70% of 1RM. Serum samples were taken pre and post exercise from all subjects and analysed for total and free T. No significant change was observed in total or free T after session A, but session B produced a significant increase in both free and total T post exercise with concentration reaching basal levels one hour after exercise. Mean serum total testosterone concentration increased to 28.3 ηmol/l post exercise compared to pre exercise concentrations (22.9 ηmol/l). Mean serum free testosterone concentration increased from 66.2 pmol/l pre exercise to 80.9 pmol/l post exercise. Fahrner et al. (1998) studied the effects of endurance exercise on plasma total and free T in physically active males. Subjects were asked to perform a 45 min treadmill run at 70% of VO_{2max}. Significant increases in both total (+32.0%) and free (+39.6%) T was observed compared to pre exercise values.

The effect of aerobic (65% $\dot{V}O_{2max}$ for 60 min) and anaerobic exercise (interval exercise consisting of 2 min at 110% $\dot{V}O_{2max}$ followed by 2 min at 40% $\dot{V}O_{2max}$) on serum T was investigated by Hackney et al. (1995). In this study it was shown that both aerobic and anaerobic exercise produced significant increases in serum T immediately post exercise compared with pre exercise values. Mean serum T concentrations increased to 7.1 ηg/ml and 6.8 ηg/ml immediately post exercise for aerobic and anaerobic protocols respectively. Testosterone response has also been measured in a competitive environment. Kraemer et al. (2001) investigated the influence of multiple wrestling matches on circulating T in university wrestlers. Pre and post match measures of T showed a significant elevation ($p<0.05$) in post match T for all matches. Mean post match serum T varied in a range of 17.3 ηmol/l - 24.9 ηmol/l over the course of the tournament.

Hoffman et al. (2002) and Kraemer et al. (2002) examined T response to a football and rugby match, respectively. Both studies reported no change in post match T compared to pre match values.

2.3.4 Psychological stress: Effects on Testosterone

Many studies confirm the role of psychological stress on hormone response. It has been reported that chronic stress can produce decreases in T levels (Aakvaag et al., 1978; Matsumoto et al., 1970). It has been suggested that the suppression in steroidogenesis in the testes is due to reduced synthesis of testicular androgens, caused by the inhibitory effect of high adrenocorticotropic hormones (ACTH) levels that accompany chronic stress (Chichinadze et al., 2008). Conversely, there are data that suggest that a bout of acute mental stress can cause an initial increase in circulating T (Chichinadze et al., 2008; Rivier C et al., 1991). The mechanism for the increase in testosterone to acute stress remains unclear, however, Chichinadze et al. (2008) suggested it is due to increased sensitivity of the testes to LH. This increase of sensitivity is a result of the activation of the sympathetic nervous system by norepinephrine, which has been shown to stimulate production of LH in both men and women (Chrousos et al., 1998).

In selected non-human mammals the relationship between aggression and T is well established (Brain, 1977, 1979). However both Archer, (1991) and Book et al. (2001) found only a weak positive relationship between aggression and T in humans. In another study by Hellhammer et al. (1985), young males were asked to view five different films containing erotic, sexual, stressful, aggressive and neutral material. Salivary T levels had increased 15 min after the beginning of the sexual and erotic films and had decreased at

the same time during the stressful film. There were no changes in T during the neutral or aggressive films. Bernhardt et al. (1998) investigated the T response of football fans watching their teams win or lose. The study found that the elation the winning team's fans felt was accompanied by a rise in post game T levels compared to pre game. The despondency felt by the losing team's fans was accompanied by a decrease in T.

Physiological T responses in anticipation of competition have been reported in many studies. Salvador et al. (2003) studied anticipatory salivary T responses to judo competition in a group of young men (n = 17) compared to resting concentrations on numerous control days. Saliva T levels measured on control days varied in a range of 128.56 – 311.20 pmol/l (202.88 ± 34.32 pmol/l). Authors showed that anticipatory T was increased but not significant for the whole group. Mazur et al. (1992) examined the response of T to non-physical competition. The investigation studied the salivary T response of Chess players during regional and national tournaments. Results showed a significant pre-competition rise in T amongst the winners, but not in the players who lost.

The home advantage in sport is well documented in virtually every team game. One aspect that has been proposed to account for this effect is territoriality and territorial aggression has been related to circulating levels of T (Archer, 1991). Territoriality is prevalent amongst many animal species, which typically display agonistic behaviour and attack more readily and with greater vigour when defending a home territory (Neave et al., 2003). Several studies have suggested that agonistic behaviour in male animals is related to circulating levels of testosterone (Lincoln et al., 1972; Brain & Nowell, 1969).

The influence of game location on the response of T has been investigated by Neave et al. (2003). In this study salivary T was measured in professional footballers before (-1h) a

home and away game against the same opposing team. Results revealed that T was significantly higher before the home game (mean = 9.93 ηg/dl, S.E. = 1.80) than the away game (mean = 5.79 ηg/dl, S.E. = 1.39). In the same study, mean salivary T levels were recorded for home and away game against moderate and extreme rivals. Again T levels were statistically higher for the home matches compared to the away matches. It was also noted that T was higher against an extreme rival (mean = 14.52 ηg/dl) than against a moderate rival (mean = 11.65 ηg/dl) although with the home games occurring in October and the away games taking place the following spring, the observed differences could be represented by circannual variations. Carre et al. (2006) similarly investigated home and away game response on saliva T. Again pre-game T was statistically higher ($p<0.05$) for the home games (176.8 ± 24.6 pg/ml) compared to away (129.4 ± 18.3 pg/ml).

2.4 T/C Ratio

Testosterone is considered a key anabolic hormone with multiple physiological functions in the human body (Brownlee et al., 2005). Cortisol, the main glucocorticoid in humans, is a catabolic hormone secreted by adrenal cortex in response to physical and psychological stress. Decreased levels of testosterone and increased levels of cortisol are suggested to be indicative of a disturbance in the anabolic-catabolic balance, which may express itself in decreased performance (Hoogeveen and Zonderland, 1996). The ratio between total testosterone and cortisol and free testosterone and cortisol has been used to evaluate training responses and help obtain optimal performance in athletes (Kraemer et al., 2004; Elloumi et al., 2003; Hoogeveen and Zonderland, 1996; Banfi et al., 1993). This ratio (T/C) is considered to reflect states of anabolism and tapering off when it is

high, and inversely, states of catabolism and overtraining when it falls by 30% or more (Maso et al., 2004).

2.5 Effect of Travel on Circulating Testosterone and Cortisol

Elite athletes competing in both individual and team sports may undertake multiple journeys for both competition and training. Travelling to an away game in team sports can involve either a bus journey or flight or both, this can result in a whole day of travelling.

Studies (Bullock et al., 2008, Bullock et al., 2007) into the effect of long haul travel across multiple time zones have revealed a depression in salivary cortisol in the day following travel. Psychological responses to short haul travel in team games have been investigated (Pace & Carron, 1992; Snyder & Purdy, 1985). To the authors knowledge there have been no studies on short haul travel and its effect on circulating hormones in athletes.

2.6 Saliva

Saliva is a biological fluid secreted in abundant quantity and at a relatively regular rate. Saliva is produced by glands in various locations in and around the mouth. Three primary glands occur in pairs located symmetrically on both sides of the head: the parotids, the submandibulars (also known as the submaxillarys), and the sublinguals (Fig 2.6.1). In addition to the primary glands, there are also hundreds of smaller glands located in the lips, cheeks, tongue and palate. Although the parotid glands are the largest in size, they produce only about 25% of the total saliva in the unstimulated rest state, and the minor

glands and the sublinguals together contribute only about an additional 5%. The submandibulars are by far the most active glands in the unstimulated state, and they are estimated to produce about 70% of the total rest volume.

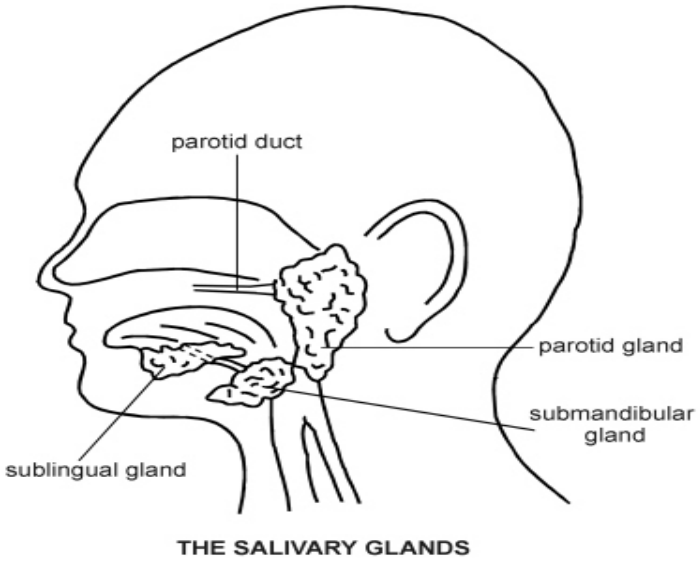

Fig 2.6.1: Locations of primary saliva glands (Boron and Boulpaep, 2003)

Saliva contains a number of elements (which are common to other bodily fluids, blood and urine) in sufficiently large concentrations to make them available for biological analysis. The convenient stress free, non-invasive collection of saliva makes it one of the most accessible body fluids and is potentially of value in studying human physiology (Vining et al., 1983(a)).

2.6.1 Salivary Gland Structure

The primary salivary glands are composed of numerous clusters of 15 to 100 secretory cells arranged in globular or tubular configurations (Fig 2.6.2). These clusters are called

acini (singular acinus.) The acini open into ducts, which merge to carry the saliva towards the mouth. Duct cells also transport electrolytes in and out of saliva, and they can participate in secretory activity to a limited degree. The acini and ducts are surrounded by myoepithelial cells, which can contract to help accelerate saliva flow (Boron and Boupaep, 2003).

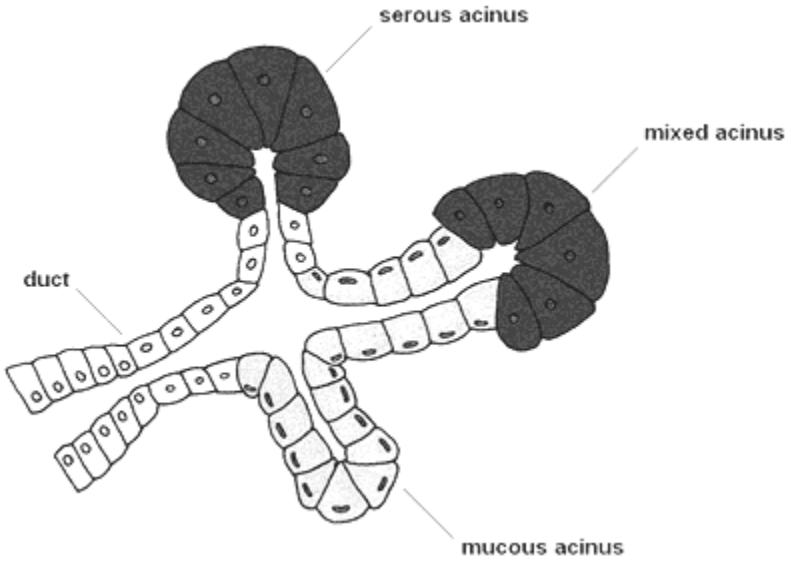

Fig 2.6.2: Cross section of a saliva gland, showing 3 types of acini (Boron and Boulpaep, 2003)

Acini are composed principally of two types of secretory cells, serous and mucous, which are both specialized for the production of large quantities of proteins. Serous cells produce a thin, watery saliva containing the digestive enzyme α-amylase. Mucous cells produce a thicker saliva rich in mucins–a type of glycoprotein–which help lubricate food for swallowing (Boron and Boupaep, 2003).

2.6.2 Main components of saliva

I. **Water:** this is the most abundant element in saliva (99%). Water determines the volume of saliva secreted per unit of time (500ml-1500ml/day) (Lac, 2001). In the case of hyposialia (saliva flow under 0.1-0.2 ml/min) arising from pathology or post-exercise dehydration, salivary flow can be amplified using chewing gum to assist with sampling. However previous studies have shown that normal physiological secretion allows saliva to be collected within one to three minutes after exercise without discomfort.(Lac, 2001)

II. **Electrolytes:** the presence of the main plasma electrolytes (Na^+, K^+, Ca^{2+}, Cl^-, HCO^{3-}) has been confirmed in previous studies (Bloomfield et al 1976).

III. **Lipids:** saliva contains neutral lipids. Their concentrations are maximum in mixed saliva (1.3 mg/dl). Cholesterol and its esters, the mono, di and triglycerides and the fatty free acids represent 99% of these lipids (Lac, 2001)

IV. **Proteins:** proteins in saliva can be of secretory, serum or mixed variety. They can be grouped into three categories (Lac, 2001):

- **Glycoproteins:** confer to the saliva its viscosity, its protective role towards the epithelium and its role in the palatability of food. By their abundance they can hinder the measurement of other compounds, in particular in radioimmunassays. However, centrifugation after the freezing/thawing process makes it possible to precipitate them and thereby obtain a clear and limpid saliva.

- **Albumin:** this protein is of plasma origin. Because of its molecular size it enters saliva via the gingival fluid and its concentration is relatively low (saliva/plasma ratio 1/500)
- **Protein Enzymes:** amylase, lysosyme and antiproteases.

2.6.3 Modes of entry into the oral cavity

There are several ways for molecules to enter into the oral cavity (Lac, 2001):

I. **Secretion by the salivary glands**: this is the case for the main compounds found in saliva, namely water, proteins and secretory immunoglobulin's (IgA's).

II. **Filtration through the tight junctions of the saliva secretory epithelium cells**: this involves the small size molecules dissolved in plasma or interstitial fluid, which can be of hormonal nature or steroid esters. The molecules which use this mode of transfer have very low concentration when compared to plasmatic concentrations (from 1/300 to 1/3000).

III. **Passive diffusion**: this mode consists of the transfer of liposoluble molecules from the extra-cellular fluids, through the cell membrane of the salivary epithelium. The capillaries surrounding the salivary glands are quite porous for many substances. Materials can pass from the blood system into the space surrounding the glands, and then make their way directly through the membranes of acinus or duct cells (Fig 2.6.3). The ability of a molecule to diffuse passively through cell membranes depends partly on its size, and partly on how much electrical charge it carries. Steroid hormones are relatively small in size, and most of them are fatty, non-polar compounds, so they tend to pass relatively easily by

diffusion. Other molecules such as the large protein hormones, or drugs that are bound to large carrier proteins while in the bloodstream, are too big to enter by this route. The non esterified and unbound (free) steroid hormones enter saliva via this process (Vining et al., 1983a). Therefore, a balance exists between the plasma free concentration and the saliva concentration, with a concentration of generally 10 to 100 times weaker in saliva than in plasma. Due to a relatively fast transfer the balance between the salivary concentration and the plasma free concentration is quite constant. The measurement of the saliva has been suggested as an acute representation of the plasma free concentration (Vining et al., 1983a, 1983b; Obminski and Stupnicki, 1991).

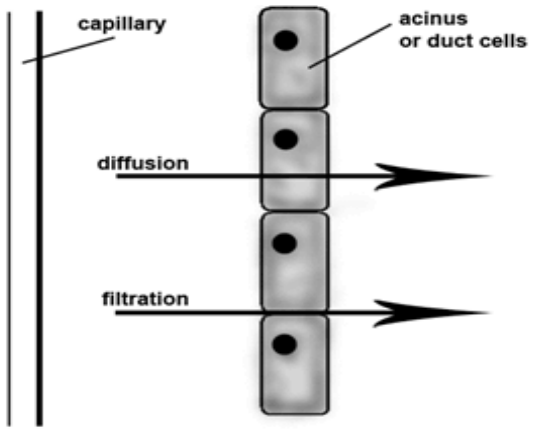

Fig 2.6.3: Movement of compounds through membrane in saliva via filtration and passive diffusion (Boron and Boulpaep, 2003)

IV. **Exudation of plasma in the oral cavity**: Blood components can also gain entry into saliva from the outflow of the serum-like gingival crevicular fluid (GCF) from the gums, or from small injuries or burns in the mouth. GCF is believed to be a major route by which certain molecules, which would ordinarily be too large

to pass by either diffusion or filtration, can find their way from serum into saliva (Vining et al., 1983a).

2.6.4 Steroid Hormones in Saliva

The presence of steroid hormones in saliva has been reported since 1966 (Shannon et al.), but measuring hormones in saliva has only become commonplace since 1980. Due to their mode of entry in saliva, correlations between salivary and serum levels are shown to be very high (Kirchbaum C et al., 1989; Luisi M et al., 1984; Vining et al., 1983b; Lac et al., 1993). In addition, for steroid hormones, the concentrations found in saliva are representative of the total serum and serum free concentrations. The equilibrium between the salivary and the plasma free concentration is relatively rapid, approximately 60 secs for cortisol (Vining et al., 1983a). The permeability constant for cortisol is the lowest among all the steroids (Vining et al., 1987). This demonstrates that the salivary levels of a steroid hormone are representative of the plasma concentrations, even in situations where hormonal levels change rapidly (Lac, 2001).

2.6.5 Cortisol in Saliva

The saliva/plasma ratio for cortisol (C) is estimated at 1:30 (Lac et al., 1993). The correlations between salivary C and serum C are high (up to $r = 0.9$) (Lac et al., 1993). They are also highly correlated with the serum free concentration (up to 0.97) (Vining et al., 1983b). Furthermore, salivary levels of C are not altered by variations in salivary flow (Vining et al., 1983a). In this study salivary C concentration was measured at high and low salivary flow in normal adults. There were no significant differences in the C

concentrations in saliva collected at the two flow rates. However, a study by Obminski and Stupnicki, (1990) has shown that when concentrations of total serum C exceed 500nmol/l, the free fraction of cortisol in serum disproportionally increases due to the saturation of cortisol binding globulin (CBG) binding sites in blood. As a consequence, the relationship between serum C and saliva becomes non-linear beyond this point. Salivary C has been used for analysis of psychological disorders (Kirchbaum et al., 1994), Cushing's syndrome (Shannon et al., 1966), and for situations of stress (Rystedt et al., 2008). It has also been widely used in the field of sports physiology due to the stress free and non-invasive collection procedures (Maso et al., 2004; Elloumi et al., 2003). However, an earlier study (del Corral et al., 1994) has questioned the use of salivary C as a reliable indicator of glucocorticoid response to exercise. This study reported no significant correlation between salivary and plasma C with exercise in children.

The increase in clinical studies using salivary C as a measure of adrenocortical function has led to numerous investigations into the methodology of saliva collection, storage and its effect on measured C levels.

The use of passive drool compared to 'Salivette' as a tool for collection of saliva was investigated by Poll et al. (2007). Collection by 'Salivette' (a saliva collection device manufactured by Sarstedt®) involves subjects gently chewing on a cotton roll for 60 secs, the cotton roll is then transferred to a container for future analysis. The passive drool method involved subjects allowing saliva to pool in their mouth naturally before dribbling the saliva into collection tubes. Salivary C values for both methods were compared to serum total (TSC) and calculated free serum cortisol (FSC) in 10 healthy adults. Stronger overall correlation was observed in Salivette-salivary cortisol vs. TSC (r

= 0.813) and FSC (r = 0.836) as compared to passive drooling-salivary cortisol vs. TSC (r = 0.735) and FSC (r = 0.751). However, it has been suggested by Shiltcliff et al. (2001) and Salimetrics®, who produce a salivary C assay kit, that either method is efficient when collecting saliva for C analysis. Clements et al. (1998) investigated the stability of salivary C and compared frozen to unfrozen saliva samples. Samples from seventeen adults were each divided into two with one half being frozen immediately and the other half being subjected to varying temperature (60-100℉) and movement over 5 days. A statistically significant positive correlation was found between C concentrations in the frozen and non-frozen saliva samples ($r = 0.96$, $p<0.001$).

The long term stability of salivary C has been studied by Garde et al. (2005). Samples were stored at -20C and -80C and it was found that there were no effects on C concentration after 1 year storage. Samples were also repeatedly frozen and thawed to investigate this effect on C concentration. Repeated thawing and freezing (-20℃) up to four times before analysis did not alter the concentration of salivary C.

In summary, collection of saliva by passive drool or by Salivette was suggested when analysing for C and the saliva samples are fairly stable and robust to temperature change between analysis. Saliva can be stored at -20℃ or below for future analysis.

2.6.6 Testosterone in Saliva

The presence of sex steroids in saliva has been documented in previous studies. In the plasma, a large percentage (around 97%) of circulating testosterone (T) is bound to either sex-hormone-binding globulin or loosely bound to albumin with the rest being in a non-protein bound (free) state (Johnson et al., 1987; Forest et al., 1970). The high correlation

between salivary T and serum total T ($r = 0.92$, $p<0.01$) and serum free T ($r = 0.83$, $p<0.01$) at rest has been reported by Johnson et al. (1987). Wang et al. (1981) has shown a direct correlation between salivary T and serum total T at rest ($r = 0.94$). Furthermore, in a study by Arregger et al. (2007), salivary T was shown to be independent of flow. However, a recent study by Cadore et al. (2008) found no significant correlation between salivary and serum free T after a session of heavy resistance exercises ($r = 0.26$, $p>0.05$). The authors could give no explanation as to why there was no relationship between T concentrations in both fluids post exercise.

Salivary T has been widely used as a physiological marker in the analysis of sport (Kraemer et al., 2001 & 2004; Elloumi et al., 2003). As the number of studies using salivary T as an indicator of the activity of the hypothalamic-pituitary-gonadal axis has increased, so has the reported information regarding the circumstances capable of compromising the data gathered been documented. Granger et al. (2004) investigated the effects of different sample collection techniques on the measurement of salivary T. In this study, T values using cotton dental roll, cotton swab and sugar free gum were compared to un-stimulated saliva collection. Compared to un-stimulated saliva (mean=10.06 pg/ml), T levels were twofold higher (mean=20.66 pg/ml) using dental cotton roll, and almost threefold higher (28.28 pg/ml) after using a cotton swab. Chewing sugar free dental gum resulted in an increase in T levels after 1 min (mean 48.58 pg/ml versus 39.82 pg/ml) but thereafter no significant differences were seen. The same study also investigated the method of storing saliva samples on the stability of salivary T. Saliva samples were stored at 4°C and were assayed for T on a weekly basis for 4 consecutive

The long term stability of salivary T when frozen was also investigated. Samples were frozen at -20℃, -40℃ and -80℃. Both the -20℃ and -40℃ freezing protocols resulted in decreases over time. After 6 months at -20℃, T values were 18.3% below baseline and by 24 months there was a 28.1% decrease. At the -40℃ protocol, T measured 6.5% below at 6 months and 23.1% below at 24 months compared to baseline values. At -80℃ there was no significant change in measured T across time and was true even when the study was extended to 30 and 36 months.

In summary, when analysing for T, saliva should be collected using an un-stimulated passive drool method, where saliva is allowed to accumulate in the floor of the mouth and then spat into collection tubes. Samples should be frozen immediately and stored at -80C until future analysis.

2.6.7 Blood Contamination of Saliva

Another factor that can compromise the validity of hormone analysis in saliva is blood leakage into the oral mucosa. Previous literature has warned that when blood is present is saliva, estimation of salivary hormones can be compromised (Granger et al., 2004; Kivlighan et al., 2004). Blood can leak into saliva as a result of micro-injuries such as burns, cuts or abrasions. In sport, the use of gum shields and facial injuries can lead to the presence of blood in saliva.

With this in mind Kivlighan et al. (2004) investigated the effect of blood leakage on values of salivary testosterone (T) and cortisol (C). Saliva samples were taken before,

immediately after, and every 15 min for 1 hr following vigorous tooth brushing and the same protocol without tooth brushing was performed by a control group. Results showed there was a significant increase in T at 15, 30 and 45 min post micro-injury ($p<0.001$) compared to control. On the other hand cortisol levels showed no difference between the micro-injury and the C conditions suggesting that cortisol is unaffected by blood contamination in saliva. Other studies have confirmed these findings (Granger et al., 2004). In conclusion, the authors suggested that when measuring T in saliva, samples visibly contaminated with blood should be excluded. Given that the presence of blood contamination is not always visible then an immunoassay for salivary transferrin may be a useful screening tool (Granger et al., 2004). Shirtcliff et al. (2002) discarded any samples with transferrin levels greater than 1.2 mg/dl when analysing saliva for T. In another investigation, Gleeson et al. (2007) rejected saliva samples with transferrin levels greater than 2.0 mg/dl when analysis for T and C.

2.6.8 Saliva Osmolality

Osmolality is defined as the concentration of a solution in terms of osmoles of solutes per kilogram of solvent. Values are expressed as milliosmols per kilogram (mOsmol/kg) of water. Plasma osmolality is a widely accepted hydration index and is known to quantitatively reflect changes in hydration during dehydration evoked by exercise and heat stress (Walsh et al., 2004). Saliva osmolality has been shown to correlate strongly ($r = 0.87$, $p<0.01$) with plasma osmolality during dehydration (Walsh et al., 2004). The authors showed that body water losses equivalent to 3% body mass resulted in a saliva

osmolality of 105 ± 41 (range 55-200) mOsmol·kg^{-1}. Saliva osmolality in euhydrated subjects measured 50 ± 11 (range 38-71) mOsmol·kg^{-1}.

2.7 Factors Involved in Inducing Plasma Volume Changes

A number of studies have demonstrated considerable plasma volume changes during and after exposure to a variety of physiological and environmental conditions. These changes are thought to be the result of fluid shifts in and out of the intravascular space. There are a number of factors where there is a potential for haemodilution (fluid shift into) or haemoconcentration (fluid shift out) of the intravascular space.

Several investigations have reported changes in plasma volume with movement between supine and standing positions. It is now well established that movement from an initial standing position to a supine position resulted in significant haemodilution (Hagan et al., 1978; Thompson et al., 1982). Similar investigations have also reported that a significant haemoconcentration ($p<0.05$) was associated with movement from supine to standing, with an apparent stability was achieved after 20 min of standing. Thompson et al. (1982) also confirmed these findings which indicate a rapid increase in haematocrit with upright posture which decreased when the individual reclined.

These observations led Harrison, (1985) to recommend that, prior to exercise, athletes should rest in the specific exercise posture for at least 20 min to reduce the effect of plasma volume shifts on baseline measurements of plasma constituent levels.

Plasma volume has also been shown to be influenced by hydration status. Senay, (1978) suggested that fluid status prior to exercise may influence body fluid adjustments during exercise. Dehydration, singularly and concurrently with exercise, has been shown to

result in haemoconcentration in blood due to plasma volume reduction (Costill & Fink, 1974). To prevent any potential hydration related variability in plasma volume, Kargotich et al. (1998) recommended that the hydration status of the individual prior to exercise be considered.

2.7.1 Plasma Volume Changes with Acute Exercise

Acute exercise has been associated with ionic disturbance in blood and muscle which are due, in part to a redistribution in water and ions between body fluid compartments (Kargotich et al., 1998). Moderate to intense exercise results in a reduction in plasma volume as water moves from the plasma compartment into both the interstitial and intracellular fluid compartments of contracting muscle (Kargotich et al., 1998).

Starling's Law states that fluid filtration through capillary membranes is dependant on the balance between the colloid osmotic pressure and hydrostatic pressure in the plasma flowing along the length of the capillary. The rate and direction of flow is determined by the net effect of the opposing pressures. A number of reasons are responsible for post exercise haemoconcentration. Firstly, increased arterial pressure and contraction on venules boosts capillary hydrostatic pressure forcing an ultrafiltrate of plasma into the extravascular compartments (Lundvall et al., 1972). Secondly, plasma volume shifts are influenced by osmotic gradients. Working muscles accrue metabolites (e.g. Lactate), thereby increasing tissue osmolarity and promoting the movement of an ultrafiltrate of plasma into myocytes and the interstitium (van Beaumont et al., 1981). In addition, perspiration and increased insensible fluid losses during exercise further reduce plasma volume (Kargotich et al. 1998). Several studies have documented a reduction in plasma

volume during and after exercise (Kargotich et al., 1998; van Beaumont et al., 1981; El Sayed et al., 1990). Haemoconcentration was observed during short term, high intensity exercise with a linear relationship between the amount of plasma lost from the vasculature and exercise intensity (van Beaumont et al., 1981; El Sayed et al., 1990). It was shown that a reduction in plasma volume occurred immediately following high intensity exercise. Studies monitoring the course of plasma volume changes in the hours after high intensity exercise found that the immediate post exercise haemoconcentration lasted approximately 30 to 60 minutes (van Beaumont et al., 1981; Boulay et al., 1995) and was then followed by a haemodilution (Boulay et al., 1995).

Haemoconcentration has also been shown to occur during and immediately after prolonged exercise (Fellman, 1992; Fellman et al., 1989). Again a haemodilution occurred post exercise with the increase in plasma volume being directly related to the intensity of the prolonged exercise (Fellman, 1992).

In recent years there have been several studies documenting the changes in plasma hormone levels with exercise. Some have considered the influence of plasma volume changes (Kargotich et al., 1997), others have either not mentioned or not corrected for the changes in plasma volume (Kuoppsalmi et al., 1980; Cummings et al., 1986). If the levels of plasma hormone values are to be measured for research purposes then it is important to consider the influence of plasma volume changes on the results. It was also stated that blood samples monitored over a trial should adopt a standard sampling posture for each sample (Kargotich et al., 1998).

2.8 Measuring Competitive Anxiety

Anxiety is one of the most commonly measured constructs in sports psychology, with at least 22 published scales devoted to its measurement (Ostrow, 1996). Competitive state anxiety is generally viewed as a situation-specific multidimensional construct with both a cognitive and a somatic component, which affects an athlete's cognitive and motor performance during competition (Lundqvist and Hassmen, 2005). Cognitive anxiety refers to negative self evaluations and self doubts about an athlete's ability to perform. Somatic anxiety is characterized by the athlete's perception of physiological elements of anxiety, such as muscle tension and increased heart rate (Gould et al., 2002). Some of the more common scales for measurement are the CSAI-2, POMS and BRUMS. The Competitive State Anxiety Inventory-2 (CSAI-2) has been instrumental in developing knowledge regarding changes in self-confidence and anxiety in the period immediately preceding athletic competition (Martens et al., 1990). The use of CSAI-2 has been published in a plethora of articles investigating anxiety in sport (Ostrow, 1996) and is perhaps the most widely used anxiety instrument used in sports psychology research.

The CSAI-2 was constructed to measure 3 subscales: cognitive anxiety, somatic anxiety and self confidence, each of which consisted of nine items. Several studies have now been published which raised concerns about the factorial of the CSAI-2 (Cox et al., 2003) Recently Cox et al. (2003) have recommended a revised version of the CSAI-2 (CSAI-2R) be used in place of the original. Ten problematic CSAI-2 items were deleted to create the revised CSAI-2R version and this study recommends that the CSAI-2R be used by both researchers and clinicians.

Numerous studies have used CSAI-2R to measure anxiety in a competitive environment. Carre et al. (2006) recorded pre-competition anxiety scores using the CSAI-2R in elite hockey players. Authors showed that players were more self confident when playing at their home venue [mean scores: home = 25.45, away = 24.25] ($p<0.01$) and also had higher somatic [home = 15.07, away = 16.61] ($p<0.05$) and cognitive anxiety [home = 18.31, away = 19.64] ($p<0.05$) when playing in their opponents' venue.

Doan et al. (2006) investigated participant's psychological and physiological responses to a 36-hole golf competition. Psychological anxiety was measured (using CSAI-2R) in relation to salivary testosterone and cortisol before, during and after competition. Authors showed that CSAI-2R somatic anxiety was correlated to pre-round cortisol ($r = 0.81$) and testosterone response ($r = -0.8$).

2.9 Summary

Acute exercise for prolonged intense periods has been shown to elicit a hormonal response in humans. Plasma testosterone (T) and cortisol (C) have been used in numerous studies to investigate the effects of acute exercise on the anabolic/catabolic status in individuals. Recent studies have shown salivary hormone values correlate favourably to plasma values and this has led to salivary T and C being used as means to investigate the response of these hormones to acute exercise and the stressors of participation in the sporting field. However, few studies have investigated the correlation between both plasma and the appearance of these hormones in saliva after acute exercise. There is an obvious need to compare plasma versus saliva values of T and C at rest and following acute exercise in recreational trainers. The effect of plasma volume shifts would also be an interesting consideration.

Pre-competition stress has been shown to stimulate physiological responses in athletes. Anticipation, anxiety and mood state are known to elicit a response in T and C preceding a contest. This investigation will compare the pre-game hormone and psychological responses of elite rugby players and will present a unique opportunity to evaluate pre-competitive stress via saliva hormone analysis in an elite rugby union environment.

2.10 Objective and Aims of the Study

Objective

The objective of this study was to investigate the effect of pre-competition stress on levels of testosterone and cortisol in elite rugby union.

Aims

More specifically, this study had the following aims:

1. To evaluate the relationship between plasma T and C and saliva T and C in individuals at rest.
2. To evaluate the relationship between plasma T and C and saliva T and C in individuals following acute exercise.
3. To investigate if the change in plasma volume with exercise affects the validity of the relationship between saliva T and C.
4. To evaluate the effect of pre-competition stress on the responses of salivary T and C in elite rugby union.

CHAPTER 3

3.0 GENERAL METHODOLOGY

3.1 Selection of Subjects

Study 1 and 2 involved the recruitment of healthy male volunteers. All subjects were recruited by either word of mouth or via a volunteer request poster placed on university notice boards.

Study 3 involved rugby union players contracted to a local professional rugby team.

3.2 Health Screening and Consent

Approval for all studies in this thesis was obtained from the University of Glamorgan Ethics Committee. All participants were provided with and read an information sheet, which contained details of the experimental protocol. Subjects fully understood the purpose of the study and the risks involved. They were informed that they were free to withdraw from the investigation at any time. All subjects provided written consent prior to the commencement of the study and were asked to complete a health screen questionnaire prior to participation in the investigation (Appendix A and Appendix B).

3.3 Blood Sampling

A butterfly cannula was inserted into the antecubital vein of the forearm of each subject at the beginning of the biological variance and the exercise trials. The cannula was kept patent with an infusion of 2ml of heparinised-saline after each sample collection. Blood was drawn at each sampling point into an SST (serum separation tube) vaccutainer [6ml].

For the exercise trial an additional sample was drawn into an EDTA (ethylenediamene-tetra-acetic acid) vaccutainer [3ml] at each sampling point. A standard sampling posture was adopted for each sample taken over a trial. SST vaccutainors were inverted before being left to sit for 10 minutes. EDTA vaccutainors were immediately analysed for potential changes in blood plasma volume. The SST vaccutainors were centrifuged at 3000 rpm x 10 min and serum was aspirated into pre labelled eppendorfs. All samples were subsequently refrigerated at -80°C for future analysis.

3.4 Blood Analysis

All analyses were undertaken in a quality controlled registered laboratory (University of Wales Institute Cardiff).

3.4.1 Cortisol

On the day of analysis the selected frozen serum samples to be analysed were thawed and brought up to room temperature. All samples were analysed in duplicate using a commercial ELISA (DRG Diagnostics, Germany) according to manufacturer's instructions. Appropriate volume (20 µl) of standards, control and samples were dispensed into appropriate wells (in duplicate) followed by addition of 200 µl of enzyme conjugate (anti-cortisol antiserum conjugated to horseradish peroxidise) as competition for binding site. After mixing and incubation (60 minutes), the unbound conjugate was washed off by rinsing the wells three times with provided wash solution. After washing, 100 µl of substrate solution (Tetramethylbezidine; TMB) was added to wells before incubation for 15 minutes. The colourimetric enzymatic reaction was then stopped via

addition of 100 µl of stop solution 0.5-M H_2S0_4 (Sulphuric acid). Wells were then read at 450 ηm within 10 minutes of stopping the reaction. The lower and upper limit for detection in this assay was 0-800 ηg/ml (sensitivity 2.5 ηg/ml).

3.4.2 Testosterone

On the day of analysis the selected frozen serum samples to be analysed were thawed and brought up to room temperature. All samples were analysed in duplicate using a commercial ELISA (DRG Diagnostics, Germany) according to manufacturer's instructions. Appropriate volume (25 µl) of standards, control and samples were dispensed into appropriate wells (in duplicate) following by addition of 200 µl of enzyme conjugate (testosterone conjugated to horseradish peroxidise) as competition for binding site. After mixing and incubation (60 minutes), the unbound conjugate was washed off by rinsing the wells three times with provided wash solution. After washing, 200 µl of substrate solution (Tetramethylbezidine; TMB) was added to wells before incubation for 15 minutes. The colourimetric enzymatic reaction was then stopped via addition of 100 µl of stop solution 0.5-M H_2S0_4 (Sulphuric acid). Wells were then read at 450 ηm within 10 minutes of stopping the reaction. The lower and upper limit for detection in this assay was 0-16 ηg/ml (sensitivity 0.083 ηg/ml).

3.4.3 Plasma Volume Change

Blood haemoglobin was analysed using a HeamoCue-B® Haemoglobin Analyser. All samples were analysed in duplicate. Micro centrifugation was used for determination of haematocrit. Whole blood (20 µl) was transferred into plain microhaematocrit capillary

tubes before being centrifuged for 5 min at 1200rpm using a Hawksley® microhaematocrit centrifuge. All blood samples taken were analyzed in duplicate and read using a microhaematocrit reader (Hawksley & Sons, Sussex, England) for packed cell volume (PCV). Once the haemoglobin and haematocrit values were known, percentage changes in plasma volume during exercise were calculated using the method of Dill & Costill, (1974).

3.5 Saliva Sampling

Before each saliva collection the subjects were instructed to rinse their mouth well with water and wait for 2 min before depositing 2-3 ml of saliva into pre labelled eppendorfs. The subjects were asked to provide the sample via a passive drool method. This procedure involved subjects allowing saliva to collect naturally in the mouth for 60 seconds before dribbling the saliva into containers. All samples were subsequently refrigerated at -80°C for future analysis.

3.6 Saliva Analysis

All analyses were undertaken in a quality controlled registered laboratory (University of Wales Institute Cardiff).

3.6.1 Cortisol

A Salimetrics® high sensitivity salivary cortisol enzyme immunoassay kit was used for analysis of salivary cortisol.

On day of analysis the selected frozen samples to be analysed were thawed and centrifuged at 1500 g x 15 min, the supernatant was subsequently aspirated for cortisol analysis. All samples were analysed according to manufacturer's instructions and in duplicate along with appropriate standard controls from the same company.

For analysis, 25 µl of standards and clear samples were added to a pre coated 96-well microlitre plate. All samples and standards were added in duplicate while 25 µl of assay diluent was also added to selected wells acting as zero and non-specific binding (NSB) wells. Assay controls were bought separately from the same supplier (Cortisol control, 4-COO1; Salimetrics USA). Cortisol standards (0.012-3.0µg/dL) were provided within the assay kits.

Upon re constitution of enzyme conjugate (horseradish peroxidise), 200 µl of diluted solution (1:1600, 15 µl conjugate to 24 ml diluent, cortisol) was added to all wells. The plate was mixed for 5 min (500 rpm) before incubating for 55 minutes. After incubation the plate was washed (x 4) with wash buffer. Tetramethylbezidine (TMB) was then added (200 µl) to each well as substrate and the plate was mixed (5 min, 500 rpm) and incubated (in dark) for 25 minutes. After incubation, the colourimetric reaction was stopped via addition of 50 µl of stop solution (2-M sulphuric acid). The plate was mixed again for 3 minutes before been read at 450 ηm (corrected at 620 ηm) within 10 minutes of adding stop solution.

3.6.2 Testosterone

A Salimetrics® high sensitivity salivary testosterone enzyme immunoassay kit was used for analysis of salivary testosterone.

On day of analysis the selected samples to be analysed were thawed and centrifuged at 1500 g x 15 min, the supernatant was subsequently aspirated for testosterone analysis. All samples were analysed according to manufacturer's instructions and in duplicate along with appropriate standard controls from the same company. As kits were of 'expanded range', the need to pre dilute samples was avoided.

For analysis, 25 µl of standards and clear samples were added to an already pre coated 96-well microtitre plate. All samples and standards were added in duplicate while 25 µl of assay diluent was also added to selected wells acting as zero and non-specific binding (NSB) wells. Assay controls were provided with each kit. Testosterone standards (6.1-600 pg/dl) were made up via serial dilution.

Upon re constitution of enzyme conjugate (horseradish peroxidise), 200 µl of diluted solution (1:1000, 18 µl conjugate to 18 ml diluent, testosterone) was added to all wells. The plate was mixed for 5 min (500 rpm) before incubating for 55 minutes. After incubation the plate was washed (x 4) with wash buffer. Tetramethylbezidine (TMB) was then added (200 µl) to each well as substrate and the plate was mixed (5 min, 500 rpm) and incubated (in dark) for 25 minutes. After incubation, the colourimetric reaction was stopped via addition of 50 µl of stop solution (2-M sulphuric acid). The plate was mixed again for 3 minutes before been read at 450 ηm (corrected at 620 ηm) within 10 minutes of adding stop solution.

3.6.3 Transferrin

A Salimetrics® blood contamination EIA kit was used for the analysis of transferrin in saliva (Salimetrics LLC, PA, USA). On day of analysis, thawed saliva samples were

vortexed and centrifuged. 20 µl of standards (made up via serial dilution) and clear samples (at room temperature) were added to an already pre coated 96-well microtitre plate. All standards were added in duplicate while samples were added in singleton. Assay diluent was also added to selected wells acting as zero (20 µl) and non-specific binding (NSB; 70µl) wells in duplicate. Assay positive controls were also provided and added in duplicate.

Upon re constitution of enzyme conjugate (horseradish peroxidise), 50 µl of diluted solution (1:400, 20 µl conjugate to 8 ml diluent) was added to all wells. 50 µl of antiserum (rabbit anti-human transferrin antibody) was then added to all wells except NSB wells before mixing the plate for 5 min (500 rpm) and incubating for 55 minutes. After incubation, the plate was washed (x 4) with wash buffer. Tetramethylbezidine (TMB) was then added (100 µl) to each well as substrate and the plate was mixed (5 min, 500 rpm) and incubated (in dark) for 10 minutes. After incubation, the colourimetric reaction was stopped via addition of 100 µl of stop solution (2-M sulphuric acid). The plate was mixed again for 3 minutes before been read at 450 ηm (corrected at 620 ηm) within 10 minutes of adding stop solution.

Transferrin values greater than 2.5 SD from the mean were considered the cut off score for exclusion from analysis of testosterone. This was based on published literature (Shirtcliff et al., 2002).

3.6.4 Osmolality

Saliva osmolality was measured using freezing point depression method via an Osmometer (Model 3300 MO, Advanced Instruments, Massachusetts). Prior to analysis,

the instrument was calibrated with 3 calibration standards and control of known concentrations (NaCL 50-800 mOsm-kg H2O). On the day of analysis saliva samples were allowed to thaw at room temperature. Samples (20 µl) were then analysed in duplicate. Intra and inter sample CV's were 4% and 5% respectively.

3.7 Measurement of VO_{2max}

3.7.1 Gas Collection and Analysis

The collection of expired air during all testing was carried out using the open circuit method. Subjects breathed through a two-way respiratory valve and mouthpiece. Samples were collected in a Douglas bags (150 L) and analysed for O_2 and CO_2 content using an automated gas analysis system (Servomex® 1440 Gas Analyser). Gas analysers were calibrated against known O_2 and CO_2 gas mixtures prior to each analysis (4.09% O_2, 16.1% CO_2) and also checked against atmospheric air and nitrogen. A dry gas meter (Harvard Ltd, Kent) and a thermometer were used to measure the volume and temperature of each air sample. Expired air was analysed for 1 min. FE O_2 and FE CO_2 measurements were noted and these measurements in combination with the above were used to calculate $\dot{V}O_2$ for each bag sample via indirect calorimetry.

3.7.2 Test Protocol

On the day of testing subjects were asked to complete an incremental exercise test until exhaustion to determine their $\dot{V}O_{2max}$. After reporting to the laboratory (9am – 10am), each subject was made familiar with the protocol of the test. Body mass (nearest 0.1kg) and height (nearest 0.01m) were recorded using a balanced beam scale (Seca®,

$\dot{V}O_{2max}$

1. A respiratory exchange ratio (R) greater than 1.15.
2. Voluntary exhaustion.
3. A levelling off of $\dot{V}O_2$ with increasing intensity (an increase of no more than 2ml.kg^{-1}.min^{-1}).
4. A heart rate of within 10 beats.min-1 of age predicted maximum (220 minus age in years).

$\dot{V}O_{2max}$ was calculated as the average $\dot{V}O_2$ over the last 60s of the test.

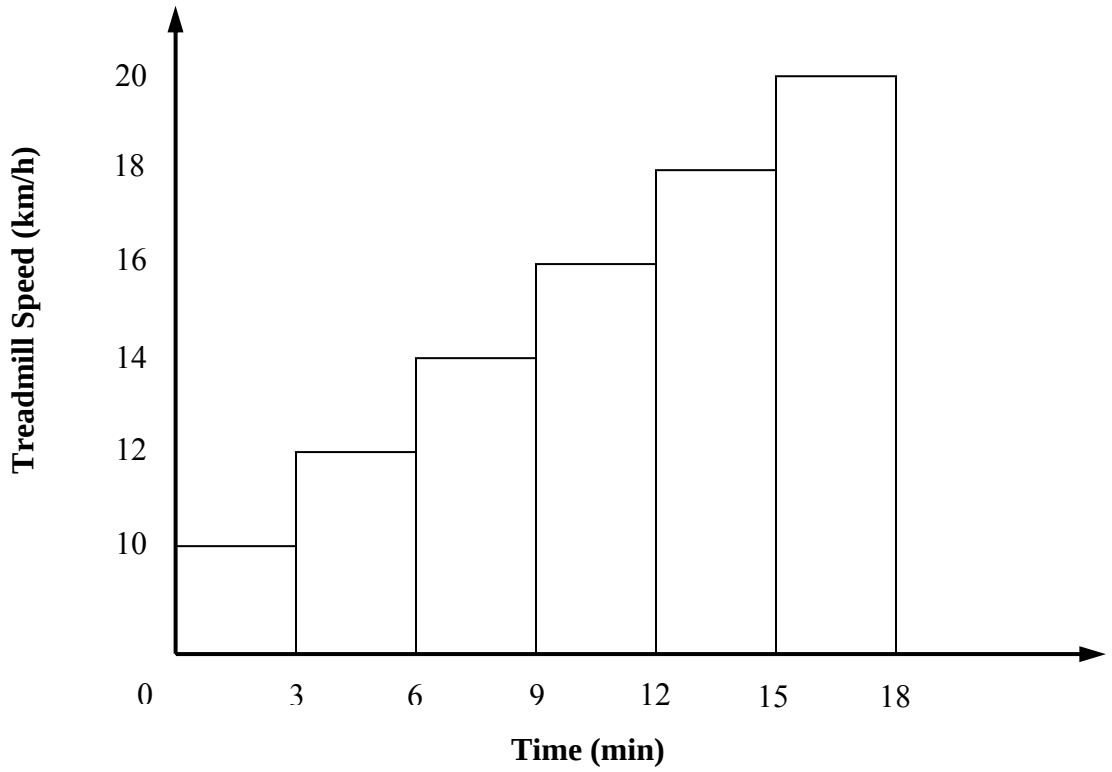

Fig 3.7.1: Incremental Exercise Test

3.8 70% VO_{2max} treadmill exercise

With the results obtained from $\dot{V}O_{2max}$ test a plot of treadmill speed (km/h) against $\dot{V}O_2$ was drawn for each subject. A calculation for 70% $\dot{V}O_{2max}$ was made and a treadmill speed corresponding to 70% $\dot{V}O_{2max}$ was then established from the plot for use in the acute exercise trial.

On the morning of the exercise trial the subjects were asked to consume 500ml of water on awakening and refrain from eating for two hours and to prior to testing. Subjects entered the lab at 8am on the day of the experiment and their height and weight were recorded.

The subjects were asked to perform a 30 min treadmill run at 70% of their $\dot{V}O_{2max}$.

During the exercise subjects provided expired air samples at 5 min, 15 min and 25min via the Douglas bag method explained in section 3.7.1. $\dot{V}O_2$ was calculated at these time points and treadmill speed was increased or decreased if needed to maintain the subject at approximately 70% $\dot{V}O_{2max}$.

3.9 Psychological Questionnaires

The CSAI-2R psychological questionnaire was chosen to assess pre-competition anxiety in study 3 (Appendix C). The CSAI-2R is a 17 item questionnaire that measures 3 subscales: cognitive anxiety, somatic anxiety and self confidence. Each item is set to a 4-point Likert scale.

Scoring key:

Somatic anxiety: 1,4,6,9,12,15,17

Cognitive anxiety: 2,5,8,11,14

Self-confidence: 3,7,10,13,16

Subscale score is obtained by summing scores for each item, dividing by the number of items, and multiplying by 10. Score range is 10 to 40 for each subscale. If a subject fails to respond to an item, merely divide by the number of items answered.

3.10 Statistical Analysis

All analyses were performed using SSPS 16.0 (SSPS inc., Chicago, IL). Statistical Significance was set at $p<0.05$. Detailed discussions of each statistical procedure are outlined in the relevant studies.

CHAPTER FOUR

STUDY ONE

4.1 BIOLOGICAL VARIANCE OF CORTISOL AND TESTOSTERONE AT REST

4.1.1 Introduction

Cortisol (C) and testosterone (T) are known to display a circadian rhythm. Studies have shown that serum C (Sharma et al., 1989) and T (Leymarie et al., 1974; Faiman and Winter, 1971) as well as saliva C (Nater et al., 2008) and T (Umehara et al., 1991) all display circadian rhythms. The circadian rhythm of both these hormones can be affected by the consumption of food and its constituents (Follenius et al., 1982; Slag et al., 1981; Volek et al., 2001; Miekle et al., 1990). While the majority of studies have investigated the biological variation of C and T independently in serum and saliva, to the authors knowledge, no studies have investigated both serum C and T and saliva C and T concurrently in a rested state.

The aim of this study was to investigate the possibility of a correlation between serum and saliva C, and serum and saliva T.

4.1.2 Methodology

Four recreationally active male subjects were recruited to take part in this study. Their characteristics are listed below.

Age (years)	27.3 ± 4.4
Height (cm)	178.8 ± 8.3
Weight (kg)	80.9 ± 12.2

Table 4.1.1: Mean ± SD subject characteristics (n = 4)

On the morning of the trial the subjects were asked to consume 500 ml of water on awakening and refrain from eating for two hours prior to testing. Subjects were asked to

enter the lab at 8 am on the day of the experiment. A cannulla was then inserted into each participant's antecubital vein (see section 3.3) to allow for easier extraction of blood samples throughout the trial. The subject was then asked to remain in the seated position for 30 min prior to the start of the trial.

Testing began at 9am after which both blood (see section 2.3) and saliva (2.4) samples were taken on the hour, every hour, throughout the morning and afternoon until 5pm. For 30 min prior to each sample point the participant was asked to remain in a seated position, to standardize the effect of plasma volume shifts on the measurements of plasma hormone levels. At 12 pm subjects were allowed a standardised meal (Table 4.1.2) and 500 ml of water. Nutrient values of consumed meal are outlined below:

Nutrient	Weight (g)
Protein	41.2
Carbohydrate	79.4
Fat	61.8

Table 4.1.2: Nutritional value of standardized meal

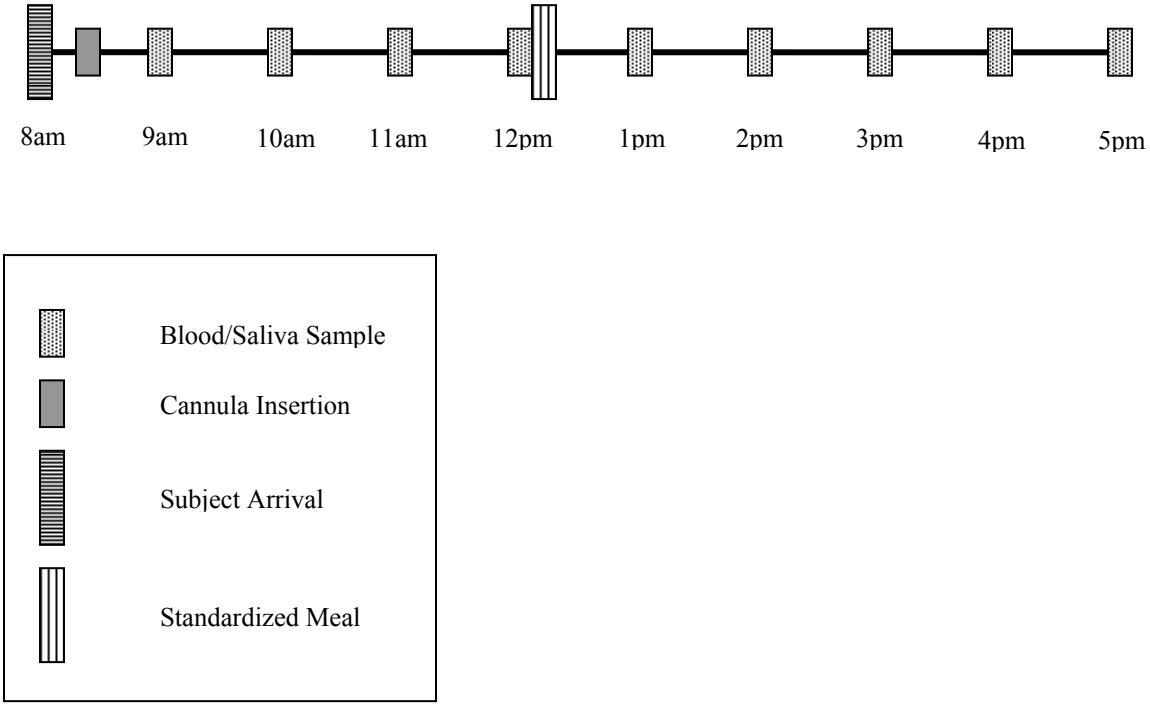

Fig 4.1.1: Experimental Timeline (cannula insertion and blood collection was as described in section 3.3; saliva collection was as described in section 3.4)

Analysis of saliva cortisol and testosterone is described in section 3.6.1 and 3.6.2, respectively. Intra-assay coefficients of variance for the above mentioned assays were 2.8% for cortisol and 2.5% for testosterone.

Analysis of total serum cortisol and testosterone is described in section 3.5.1 and 3.5.2, respectively. Intra-assay coefficients of variance for the above mentioned assays were 2.8% cortisol and 3.4% for testosterone.

Analysis of saliva transferrin is described in section 3.6.3. Intra-assay coefficients of variance was 1.6%.

4.1.3 Statistical Analysis

Normal distribution of the data was tested using the Kolmogorov-Smirnov test. A repeated measures ANOVA was used to determine if there was a significant difference over time between samples of total serum C, total serum T and salivary T. Fisher LSD post hoc procedures were used to locate significant differences among means following a significant ANOVA ($p<0.05$). Because saliva C data were not normally distributed the non-parametric Friedman test was used to determine any differences for time between samples. Wilcoxon matched pairs tests were used post hoc where relevant. Due to the small sample size, differences between matched pairs were unable to reach significance ($p<0.05$) even when a Friedman test showed significant differences over time. The relationship between total serum and saliva concentrations of cortisol, testosterone and T/C ratio was evaluated by means of Spearman rank correlation analysis.

4.1.4 Results

After analysis of saliva for transferrin levels, one sample was found to be contaminated with blood (transferrin levels > 1.2 mg/dl, Shirtcliff et al. 2002) and was omitted from testosterone analysis.

The mean (n = 4) total serum cortisol and saliva cortisol response is shown in Fig 4.1.2. Total serum cortisol (80.74 ηg/ml ± 14.92) [mean ± SEM] was highest in early morning (09:00 hrs) with a gradual reduction throughout the day until an evening nadir (17:00 hrs) [serum: 39.33 ηg/ml ± 11.00]. Similarly, peak saliva cortisol (3.02 ηg/ml ± 0.94) occurred at 09:00 hrs with levels mirroring serum cortisol by gradually reducing throughout the day until 17:00 hrs (0.96 ηg/ml ± 0.19). All total serum cortisol sample

points were significantly lower compared to samples collected at 09:00 hrs ($p < 0.05$) apart from samples collected at 13:00 hrs. No saliva cortisol sample points were significantly different over time. Friedman analysis of saliva cortisol over time was unable to reach statistical significance ($p < 0.05$) due to the small sample size ($n = 4$).

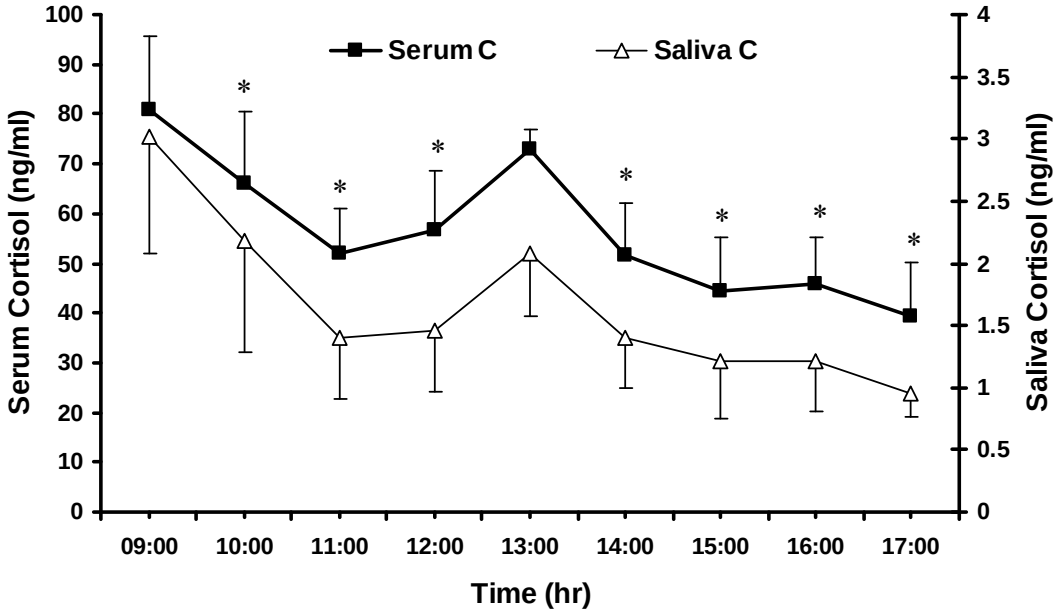

Fig 4.1.2: Mean (± SEM) biological variance of total serum and saliva cortisol in 4 male subjects.
* Significantly different ($p<0.05$) from corresponding 09:00 values

The correlation between total serum and saliva C samples concentrations taken from 09:00 to 17:00 is shown in Fig 4.1.3. Saliva C levels showed a strong correlation ($r = 0.95$, $p<0.01$, $n = 36$) with total serum C.

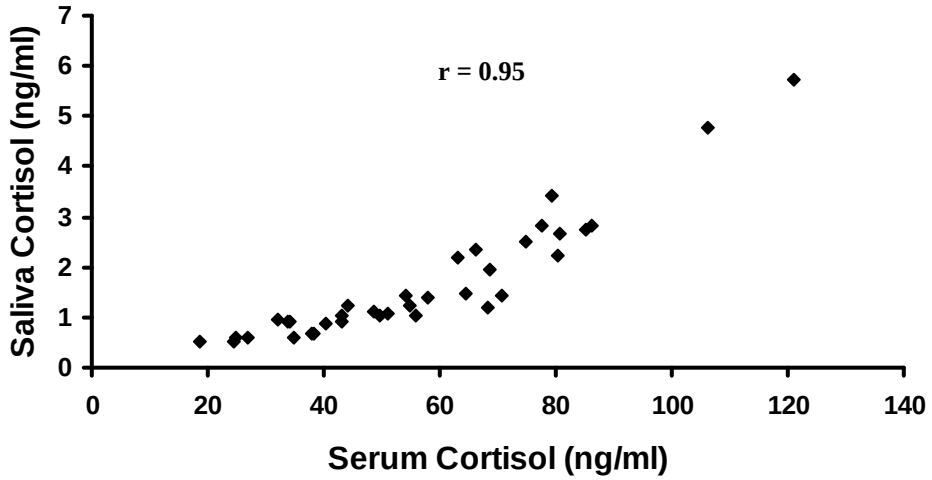

Fig 4.1.3: Correlation between saliva and total serum cortisol concentrations (n=36).

The mean (n = 4) total serum testosterone and saliva testosterone response is shown in Fig 4.1.4. Peak total serum testosterone (4.71 ηg/ml ± 0.49) levels were recorded at 09:00 hrs with a nadir (3.21 ηg/ml ± 0.4) at 15:00 hrs. Samples collected at 13:00, 15:00 and 16:00 hrs were significantly lower ($p<0.05$) when compared to concentrations at 09:00 hrs. Saliva testosterone showed a similar circadian pattern throughout the day with peak levels (0.13 ηg/ml ± 0.007) occurring at 9:00 hrs and a nadir (0.10 ηg/ml ± 0.01) at 13:00 hrs. Samples collected at 16:00 hrs were lower ($p<0.05$) when compared to concentrations collected at 09:00 hrs. Total serum T showed a significant decrease ($p<0.01$) at 13:00 hrs in response to the midday (12:00 hrs) meal. Similarly, a significant decrease ($p<0.05$) was evident in saliva T post meal.

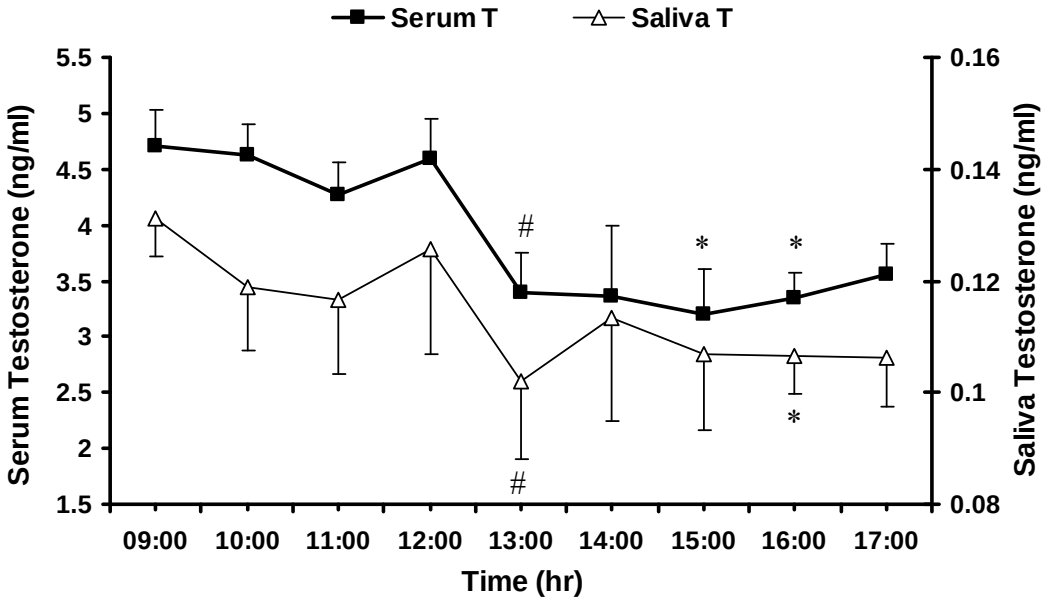

Fig 4.1.4: Mean (± SEM) biological variance of total serum and saliva testosterone in 4 male subjects
* Significantly different ($p<0.05$) from corresponding 09:00 hrs values
# Significantly different ($p<0.05$) from corresponding pre-meal (12:00 hrs) values

The correlation between total serum T and saliva T concentrations taken from 09:00 to 17:00 is shown in Fig 4.1.5. Saliva T showed a moderate but significant correlation ($r = 0.62$, $p<0.01$, $n = 35$) with total serum T.

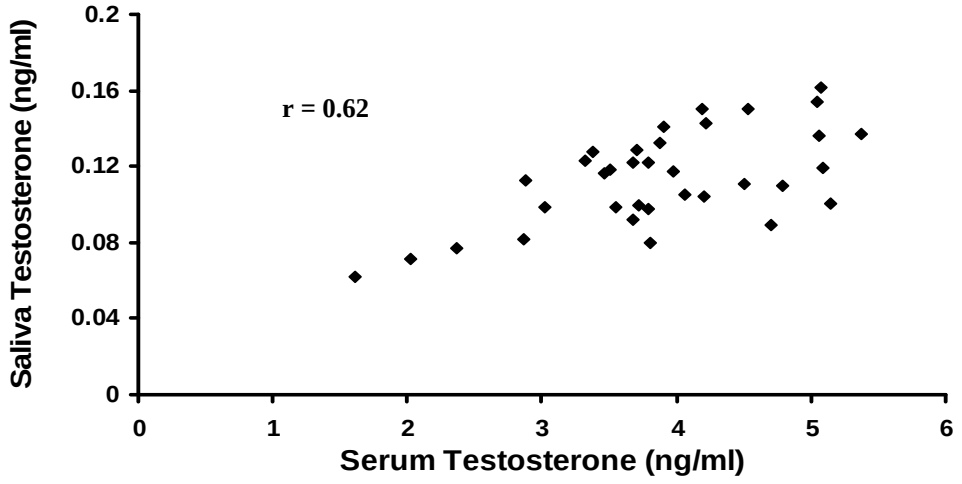

Fig 4.1.5: Correlation between saliva and total serum testosterone concentrations (n=35)

Mean (± SEM) total serum and saliva testosterone to cortisol (T/C) ratio from 09:00 to 17:00 hrs in shown in shown in Figure 4.1.6. A gradual increase throughout the day was observed in serum T/C ratio with a similar pattern shown in saliva T/C ratio. A postprandial decrease (51%) was observed in serum T/C ratio in response to a midday meal and this was mirrored by a similar decrease in saliva T/C ratio (46%).

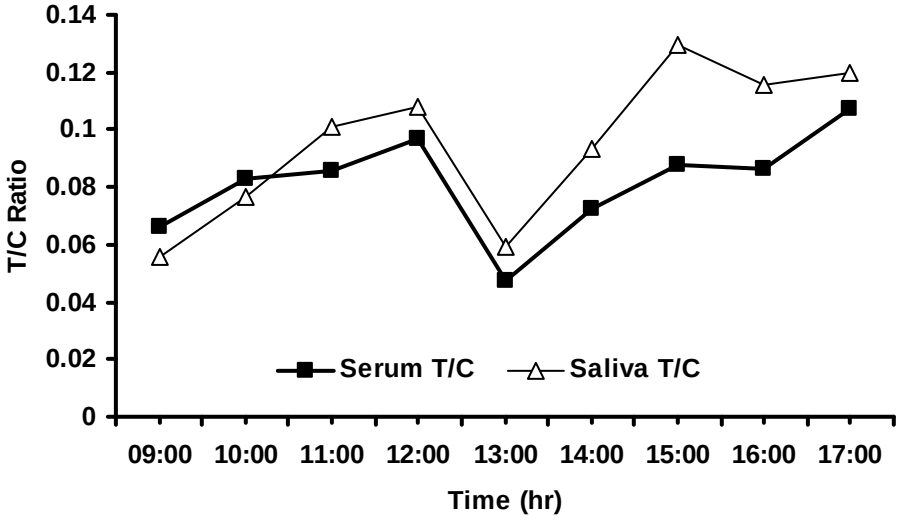

Fig 4.1.6: Mean (± SEM) biological variance of total serum and saliva T/C ratio in 4 male subjects

The correlation between total serum and saliva T/C ratio values from 09:00 to 17:00 hrs is shown in Figure 4.1.7. Total serum T/C ratio showed a strong correlation ($r = 0.87$, $p<0.01$, $n = 35$) with saliva T/C ratio.

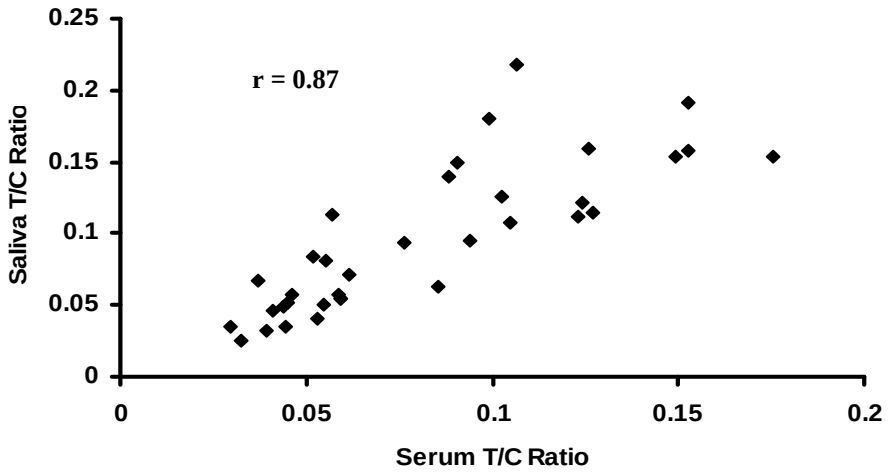

Fig 4.1.7: Correlation between total serum and saliva T/C ratio values (n = 35)

4.1.5 Discussion

The main findings of the current study show a strong correlation between time matched total serum and saliva cortisol ($r = 0.95$, $p<0.01$) samples analysed repeatedly throughout a day (9:00am – 5:00pm) in resting conditions. Conversely, moderate correlations were found in saliva and serum testosterone ($r = 0.62$, $p<0.01$) samples analysed over the same period.

Cortisol

The cortisol values observed in the current study (total serum C: 18.78 – 120.99 ηg/ml and saliva C: 0.52 – 5.73 ηg/ml), are similar to ranges reported in other studies which have explored the influence of circadian rhythm on total serum and/or saliva C. The results confirm the presence of a circadian rhythm in both saliva and total serum C which been observed in previous studies (Nater et al., 2008; Sharma et al., 1989). While these studies have shown similar patterns in total serum or saliva C, to the author's knowledge,

this is the first study to show a parallel rhythm in C within both total serum and saliva. In the previous studies, peak levels occurred at approximately 08:00 hrs, followed by a gradual decrease throughout the day ($p < 0.05$). The results from this present study show a similar pattern. Total serum C peaked at 09:00 hrs with a gradual and statistically significant reduction throughout the day. Similarly, salivary C decreased at a similar rate at each sample point throughout the day, confirming that salivary C reflects the circadian profile of total serum C. However, the gradual reduction in salivary C throughout the day was unable to reach statistical significance due the small sample size ($n = 4$). In the present study a standardized meal (protein: 41.2g, carbohydrate: 79.4g, fat: 61.8g) was consumed by subjects immediately after the 12:00 hr sampling point. A subsequent increase in total serum C (29%) was observed at the following sample point (13:00 hrs), this was mirrored by an increase in salivary C (42%). This postprandial rise in total serum and saliva C has been shown in previous studies (Slag et al., 1981; Gibson et al., 1999). These studies showed that consumption of a moderate to high protein meal results in a significant increase in C concentrations. The standardised meals consumed by subjects in this study resulted in an expected rise in postprandial total serum and saliva C. Cortisol levels in both these biological fluids returned to the normal circadian pattern within 2 hours from the start of the meal as shown in previous studies (Gibson et al., 1999; Slag et al., 1981).

Total serum and saliva C showed a strong ($r = 0.95$, $p<0.01$, $n = 36$) correlation throughout the day. In values above 500 ηmol/l the correlation between serum and saliva has been shown to become non-linear (Obminski and Stupnicki, 1990). However, it is unlikely that total serum C in individuals would reach this level in a resting state. The

current study reconfirms the presence of a circadian variation in both total serum and saliva C (Nater et al., 2008; Dorn et al., 2007). Furthermore a strong correlation between total serum and saliva C was observed, endorsing the use of saliva as a practical means in the monitoring of stress levels in resting humans throughout the day.

Testosterone

The testosterone values observed in the current study (total serum T: 1.61 – 5.37 ηg/ml, saliva T: 0.06 – 0.16 ηg/ml), are similar to ranges observed in other studies investigating the influence of circadian rhythms on either total serum and saliva T (Leymarie et al., 1973; Granger et al., 2004). The results confirm the presence of a circadian rhythm in both total serum and saliva T which been shown in previous studies (Faiman and Winter, 1971; Umehara et al., 1991). While these previous studies have shown similar patterns in either total serum or saliva T, to the author's knowledge, the current study is the first to show a parallel rhythm in both total serum and saliva T. Similar to the findings of past studies, both total serum and saliva T peaked in the morning with levels gradually decreasing throughout the day. Both biological fluids showed a similar circadian pattern with total serum and saliva T peaked at 09:00 hrs, however, a nadir was reached at 15:00 hrs in total serum T and at 13:00 hrs (post meal) in saliva T. This variation in nadir levels shows that T concentrations in both fluids did not mirror each other exactly throughout the day despite similar reduction in both total serum and salivary T concentrations from 09:00 hrs until 17:00 hrs (24% and 20% respectively). T concentrations both biological fluids showed a similar response to the standardised meal consumption. Total serum and saliva T decreased significantly ($p<0.05$) and by a similar magnitude (26% and 20%

respectively) post meal. Previous studies (Meikle et al., 1990; Volek et al., 2001) into the postprandial response of total serum and saliva T have shown that a fat-containing meal reduces serum total and free T by approximately 25%. In the present study, consumption of a standardized meal (fat: 61.8g) resulted in an expected decrease in total serum T. Testosterone values in saliva also mirrored this response.

Total serum T showed a moderate ($r = 0.62$) and significant ($p<0.01$) correlation with saliva T throughout the day. This is a lower correlation than those reported in other studies (Wang et al., 1981: $r = 0.94$; Johnson et al., 1987: $r = 0.92$). The methods used in the collection and analysis of saliva were similar to those recommended by Granger et al. (2004) to ensure the validity of the measurement of saliva T. A possible reason for this weaker correlation could be metabolic transformation of androgens by the salivary gland. A previous study (Rey et al., 1988) concluded that androgen metabolism is the primary reason for the excess or deficit of salivary T concentration when compared to the serum free concentration. These authors suggested that androstenedione conversion into testosterone, together with testosterone conversion into undefined metabolites in the salivary gland should be taken into account when assessing the clinical measurement of salivary T. However, Blom et al. (1993) reported that the rate of metabolism is constant in resting situation and concluded that metabolism of androgens in saliva is probably not an important source of error in the measurement of T in saliva. Another factor could be the transfer of testosterone bound proteins into saliva via blood contamination. Blood and its components can leak into the oral cavity as a result of micro-injury or poor oral health. This is the route via which albumin and SHBG (testosterone bound proteins) enter the saliva (Granger et al., 2004). Analysis of saliva transferrin was used to detect blood

contamination of saliva samples during this study. Any samples that exceeded transferrin levels of 1.2 mg/dl were omitted from testosterone analysis as shown in a previous study by Shirctliff et al. 2002. However a recent study (Schwartz and Granger, 2004) suggested that saliva samples with transferrin levels above 0.5 mg/dl should be omitted when measuring testosterone. Apart from the singular sample already omitted from analysis due to contamination, no other samples contained transferrin levels above 0.5 mg/dl (mean transferrin = 0.247 mg/dl), however, a few samples were very near to this level and may have resulted in testosterone values being slightly distorted. The time it takes for passage of steroid hormones from blood to saliva may also be a factor in the weaker correlation observed. Vining et al. (1983b) has proposed that equilibrium between serum and saliva concentrations occurs between 1 -5 minutes, however, in resting conditions, serum hormone do not fluctuate rapidly and are quite constant. As a result, this probably has a minimal effect on the serum/saliva correlation.

T/C Ratio

The range of T/C ratio values observed in the current study (total serum T/C: 0.01 – 0.17, saliva T/C: 0.02 – 0.21) are similar to those observed in previous studies investigating biological variance of T/C ratio (Obminski and Stupnicki, 1990; Elloumi et al., 2003). The T/C ratio has been suggested to be indicative of a disturbance in the anabolic-catabolic balance (Hoogeveen and Zonderland, 1996). The ratio is considered to reflect states of anabolism and tapering off when it is high, and inversely, states of catabolism when it falls by 30% or more (Maso et al., 2004). The index has therefore been used widely in the sporting field as a marker of training induced fatigue or overtraining

(Hackney, 1991; Maso et al., 2004). The results confirm that biological variance of saliva T/C ratio is in parallel with that of total serum T/C ratio. The strong correlation observed between saliva T/C and total serum T/C ($r = 0.87$, $p<0.01$) reconfirm previous findings on the relationship between serum and saliva T/C ratio (Obminski and Stupnicki, 1990: $r = 0.83$). This strong relationship makes saliva measurement of T/C ratio a practical and stress free method of assessing the anabolic-catabolic balance in individuals at rest.

Limitations

There are several limitations associated with the present study. The sample size of four subjects severely limits the statistical assessment and correlation of both hormones and, as a consequence, the robustness of any conclusion drawn from the results. A larger number of subjects would have provided a better insight into any individual variation in serum/saliva correlations. Secondly, sampling over a period of 24 hrs as opposed to the 8 hr time span used in this study may give a better indication of overall association between analytes in both biological fluids across time.

The current study reconfirms results from previous studies observing the existence of circadian rhythms in total serum and saliva concentrations of C and T; with strong correlations between C and T/C ratio concentrations and moderate correlations between T concentrations in saliva and serum samples. The current study also shows that any observed changes in these hormones throughout the day are mirrored in saliva samples taken at the same time. Present and past data demonstrate that analysis of saliva samples for C, T and T/C ratio offers a practical, stress free and non-invasive method of

monitoring serum hormone levels in an unsupervised setting. However, the timing of sampling with regards to the consumption of meals must be considered due to the postprandial variation of both C and T levels. Regarding testosterone, further studies are required to determine the effect of saliva gland metabolism and minimal blood contamination on the total serum/saliva correlation.

STUDY TWO

4.2 CORTISOL AND TESTOSTERONE RESPONSES TO ACUTE EXERCISE

4.2.1 Introduction

Previous studies have shown that acute exercise elicits a response in serum cortisol (C) and testosterone (T) (Luger et al., 1987; Fahrner & Hackney, 1998), and saliva C and T (McGuigan et al. 2004, Hoffman et al. 2002). Authors in these studies observed that acute exercise such as running, cycling (at above 60% $\dot{V}O_{2max}$) and resistance exercise, in addition to exercise in a sporting environment (rugby, football and hockey) elicit a post exercise increase in C and T in both biological fluids. Exercise has also been shown to elicit a change in plasma volume both during and post exercise (Fellman et al., 1989; van Beaumont et al., 1981).

While many studies have investigated the response of serum C and T and saliva C and T to exercise independently, to the author's knowledge, no studies have investigated both serum C and T and saliva C and T concurrently in response to acute exercise.

The aim of this study was to investigate the correlation between serum and saliva C, and serum and saliva T post-exercise. This study also examined the possible effects of post exercise plasma volume change on the hormonal data obtained.

4.2.2 Methodology

Nine recreationally active males were recruited to take part in this study. Their characteristics are listed below.

Age (years)	28.8 ± 4.5
Height (cm)	178.5 ± 5.7
Weight (kg)	83.06 ± 8.13
$\dot{V}O_{2max}$ ($ml.kg^{-1}.min^{-1}$)	54.2 ± 3.4

Table 4.2.1: Mean (± SD) subject characteristics (n=9)

All subjects were asked to refrain from supplementation of Vitamin C for 7 days prior to and during the study. Subjects entered the laboratory on two separate days during the course of the experiment. On the first visit, the subject's maximum oxygen uptake ($\dot{V}O_{2max}$) was recorded (see section 3.7). A treadmill speed corresponding to 70% $\dot{V}O_{2max}$ was then established. This served as the exercise intensity for the main trial.

On the second visit the subject's were asked to perform a 30 min treadmill run at 70% $\dot{V}O_{2max}$ (see section 3.8). Blood and saliva samples were taken pre-exercise, immediately after and at 10, 20, 30, 60 and 90 minutes post exercise. Subjects were asked to remain in a standing position for the duration of the study to standardize the sampling posture. All samples were subsequently frozen (-80℃) for future analysis.

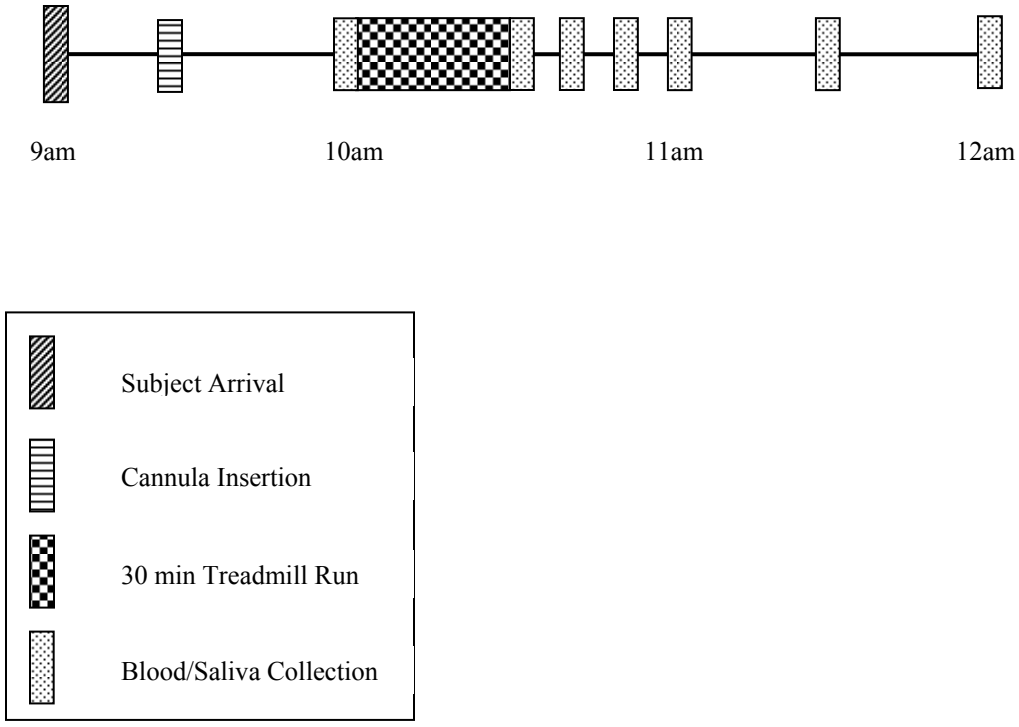

Fig 4.2.1: Experimental Timeline (cannula insertion and blood collection as described in section 3.3; saliva collection as described in section 3.4; 30 min treadmill run as described in section 3.8)

Analysis of saliva cortisol and testosterone is described in sections 3.6.1 and 3.6.2, respectively. Intra and inter-assay coefficients of variance for the above mentioned assays were 7.5% and 3.8% for cortisol and 7% and 3.2% for testosterone.

Analysis of serum cortisol and testosterone is described in section 3.5.1 and 3.5.2, respectively. Intra and inter-assay coefficients of variance for the above mentioned assays were 2.6% and 3.4% for cortisol and 2.5% and 4.6% for testosterone.

Analysis of saliva transferrin is described in section 3.6.3. Intra-assay coefficients of variance was 2.5%.

Analysis of plasma volume change is described in section 3.5.3. Coefficients of variance for the analysis of haemoglobin and haematocrit were 1% and 0.7%, respectively.

4.2.3 Statistical Analysis

Normal distribution of the data were tested using the Kolmogorov-Smirnov test. A repeated measures ANOVA was used to determine if there was a significant difference over time within samples of serum C and saliva T. Fisher LSD post hoc procedures were used to locate significant differences among means following a significant ANOVA ($p<0.05$). Because saliva C and serum T data were not normally distributed, the non-parametric Friedman test was used to determine significant differences over time for samples. Wilcoxon matched pairs test were used post hoc where relevant. The relationship between total serum and saliva cortisol concentrations and between serum and saliva testosterone concentrations was evaluated by means of Spearman rank correlation analysis.

After correction for plasma volume change, serum C and T were again tested for normal distribution. Serum C and T were normally distributed and a repeated measures ANOVA was used to determine significant differences over time between samples. The relationship between C and T concentrations in total serum and saliva was evaluated by means of Spearman rank correlation analysis.

4.2.4 Results

Following analysis of saliva transferrin levels, 7 samples (11%) were omitted from testosterone analysis due to blood contamination (transferrin > 1.2 mg/dl, Shirtcliff et al. 2002).

The mean (n = 9) total serum cortisol and saliva cortisol responses to exercise is shown in Fig 4.2.2. Mean (± SEM) pre-exercise total serum cortisol measured 112.82 ηg/ml ± 11.51. Total serum cortisol increased to 132.45 ηg/ml ± 9.19 at the 10 min post exercise sample point with levels gradually reducing thereafter until 90 min post exercise (84.34 ηg/ml ± 7.07). No sample points were significantly different from pre-exercise serum cortisol values. Mean (± SEM) pre-exercise saliva cortisol measured 3.17ng/ml ± 0.68 and increased to 4.43 ηg/ml ± 0.78 at 20 min post exercise with levels then gradually reducing thereafter. Saliva cortisol was significantly lower (p = 0.03) at 90 min post exercise (1.54 ηg/ml ± 0.24) compared to pre-exercise values.

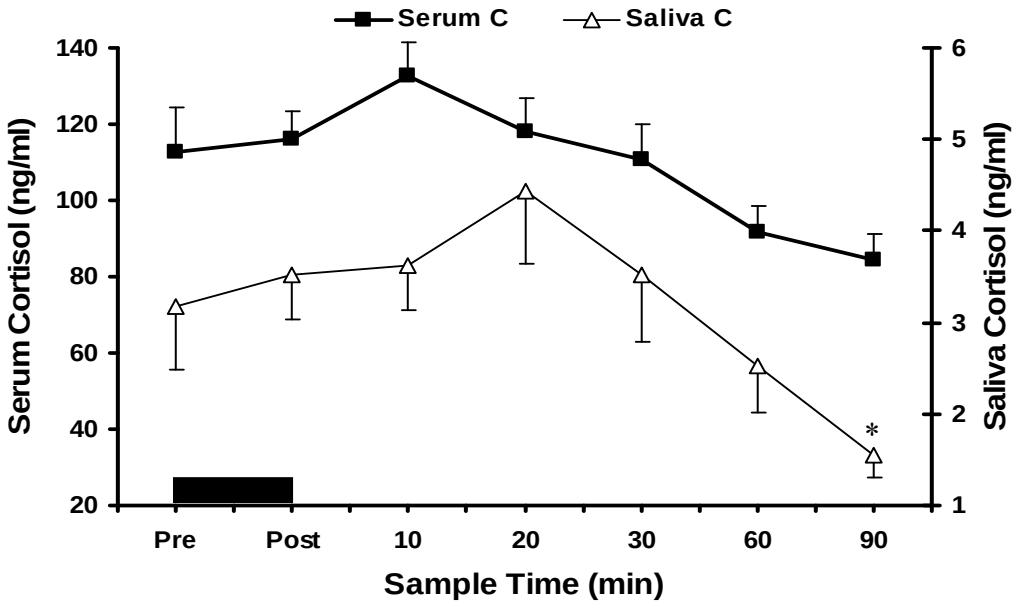

Fig 4.2.2: Mean (± SEM) total serum and saliva cortisol with exercise in 9 subjects
* Significantly different ($p<0.05$) from pre-exercise values

The correlation between total serum C and saliva C samples concentrations taken from pre-exercise to 90min post exercise is shown in Fig 4.2.3. Saliva C levels showed a strong correlation ($r = 0.82$, $p<0.01$, $n = 63$) with total serum C.

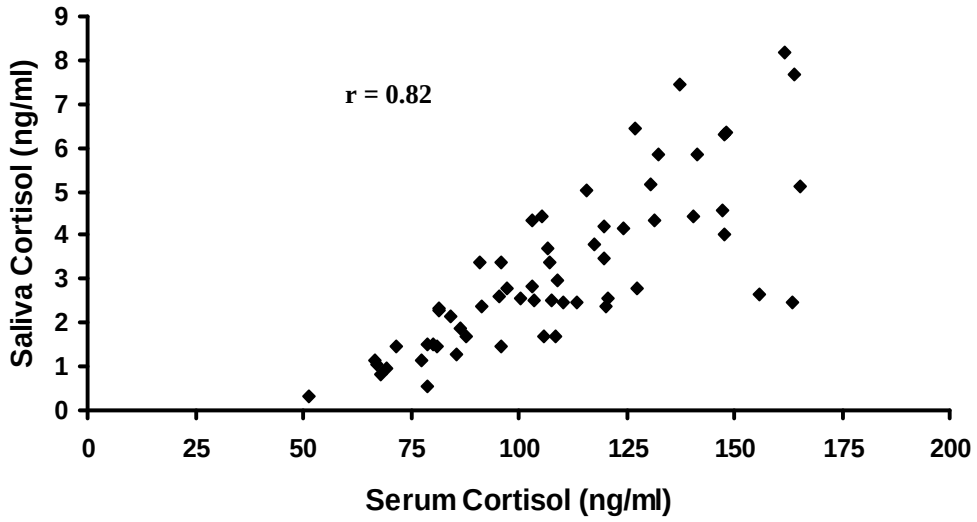

Fig 4.2.3: Correlation between saliva and total serum cortisol with exercise (n=63)

The mean (± SEM) total serum and saliva testosterone response to exercise is shown in Fig 4.2.4. Pre-exercise total serum testosterone measured 3.92 ηg/ml ± 0.59. Exercise resulted in a significant increase ($p<0.05$) in total serum testosterone (+26.5%) immediately post exercise (4.96 ηg/ml ± 0.67) with levels reducing thereafter. Mean pre-exercise saliva testosterone measured 0.27 ηg/ml (± 0.05) with levels increasing to 0.35 ηg/ml (± 0.07) at 20 min post exercise, levels gradually reduced thereafter until 90 min post exercise (0.26 ηg/ml ± 0.09). No post exercise saliva T levels were significantly different from pre-exercise values.

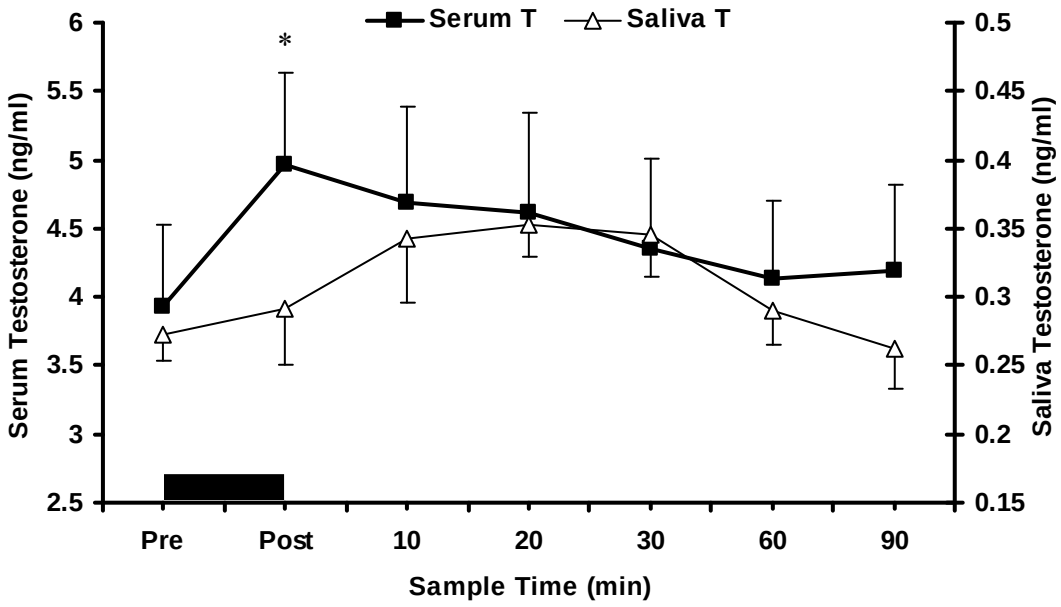

Fig 4.2.4: Mean (± SEM) total serum and saliva testosterone with exercise in 9 subjects
* Significantly different ($p<0.05$) from pre-exercise values

The correlation between total serum T and saliva T sample concentrations taken from pre-exercise to 90 min post exercise is shown in Fig 4.2.5. Saliva T levels showed a low correlation and non-significant ($r = 0.22$, $p>0.05$, $n = 56$) with total serum T.

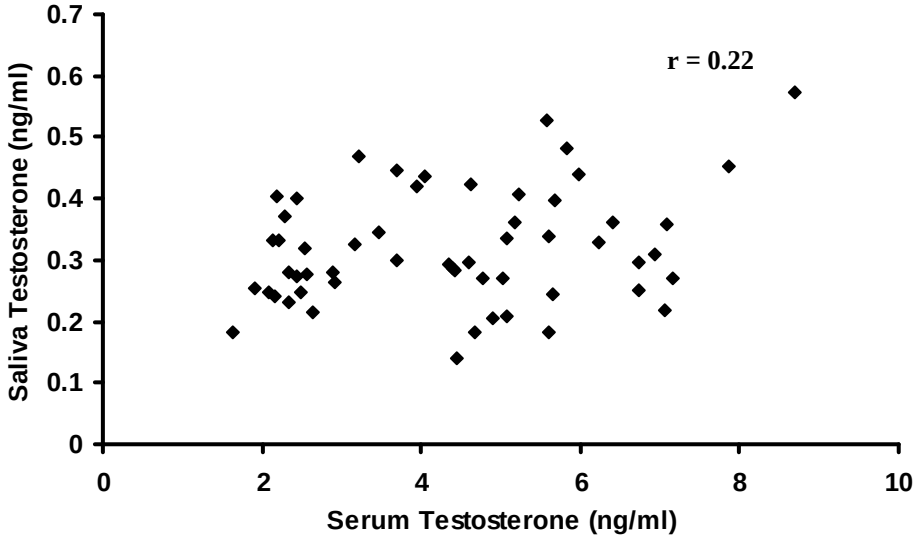

Fig 4.2.5: Correlation between saliva and total serum testosterone with exercise (n=56)

With Plasma Volume Correction

Whole blood was corrected for plasma volume (PV) change using the methodology described in section 3.5.3. Data was then applied to serum C and serum T concentrations to give PV corrected values.

The mean (± SEM) total serum cortisol and saliva cortisol response to exercise (corrected for PV) is shown in Fig 4.2.6. Peak total serum cortisol (128.67 ηg/ml ± 9.01) occurred 10 min post exercise with levels gradually reducing thereafter until 90 min post exercise (79.08 ηg/ml ± 7.56). Exercise resulted in an increase in total serum cortisol with peak levels (+14%) occurring 10 min post exercise. No sample points were significantly different from pre-exercise serum cortisol values. Saliva cortisol was as described earlier.

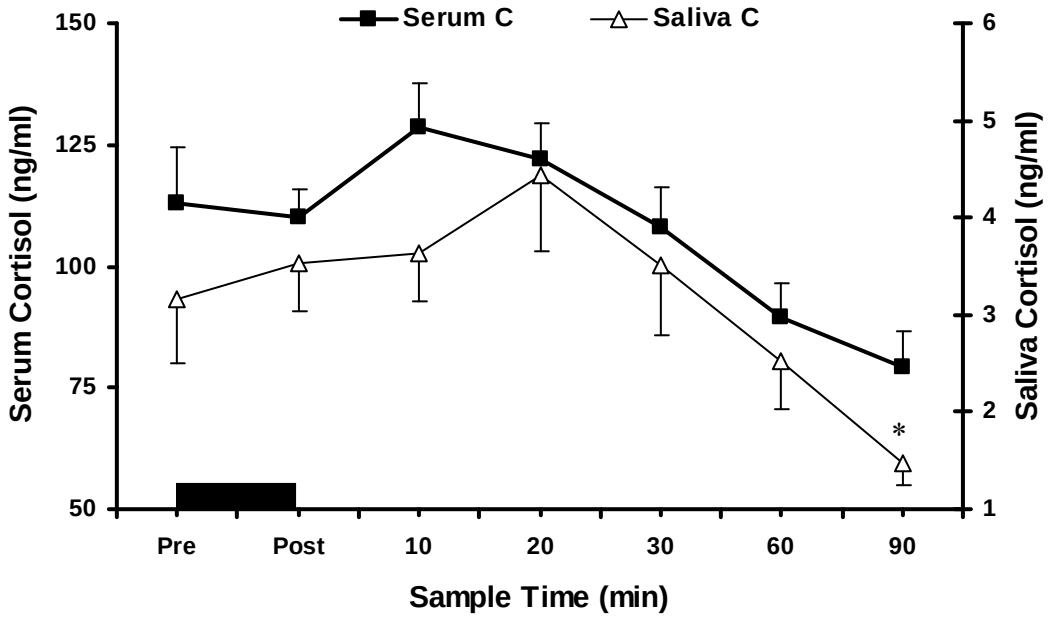

Fig 4.2.6: Mean (± SEM) total serum and saliva cortisol with exercise (corrected for PV)
* Significantly different ($p<0.05$) from pre-exercise values

The correlation between saliva and total serum cortisol sample concentrations taken from pre exercise to 90 min post exercise is shown in Fig 4.2.7. Similar to non-corrected data, saliva C levels showed a strong correlation ($r = 0.8$, $p<0.01$, $n = 63$) with total serum C.

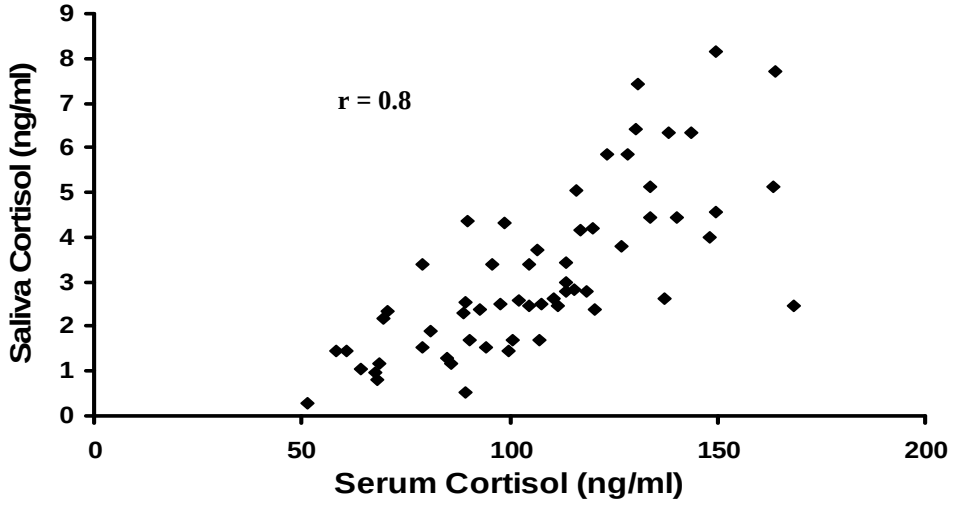

Fig 4.2.7: Correlation between saliva and total serum testosterone with exercise (corrected for PV)

The mean (± SEM) total serum and testosterone response to exercise (corrected for PV) is shown in Fig 4.2.8. Similar to non-corrected data, exercise elicited its greatest response in serum testosterone (+19%) immediately post exercise (4.68 ηg/ml ± 0.64) with levels gradually reducing thereafter until 90 min post exercise (3.82 ηg/ml ± 0.52). Serum T was significantly higher immediately after and 10 min post-exercise ($p < 0.05$) compared to pre-exercise values (3.92 ηg/ml ± 0.59). Saliva testosterone was as described earlier.

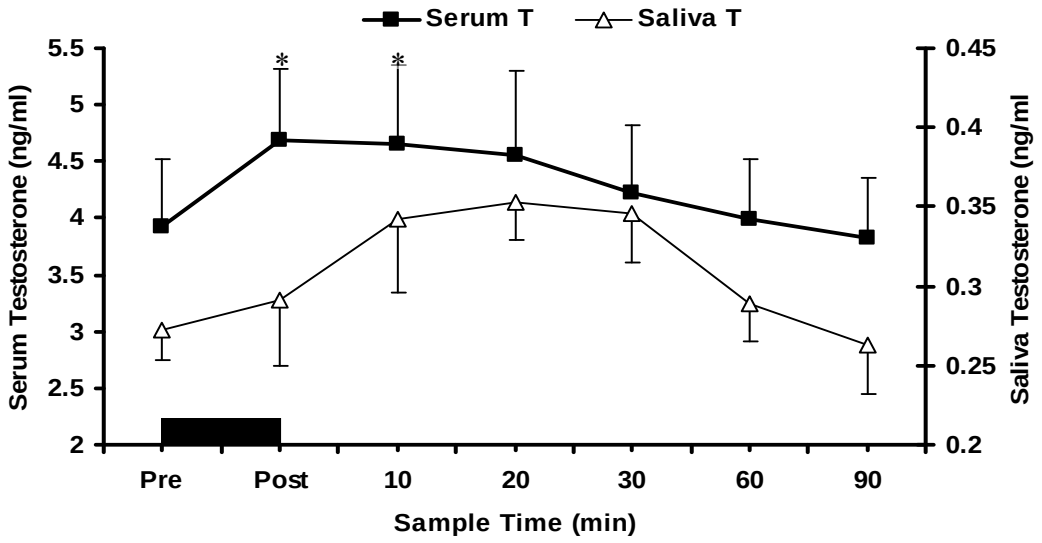

Fig 4.2.8: Mean (± SEM) total serum and saliva testosterone with exercise (corrected for PV)
* Significantly different ($p<0.05$) from pre-exercise values

The correlation between saliva and total serum testosterone sample concentrations taken from pre exercise to 90 min post exercise is shown in Fig 4.2.9. Similar to non-corrected values, saliva T levels showed a low but significant correlation ($r = 0.35$, $p<0.05$, $n = 56$) with total serum T.

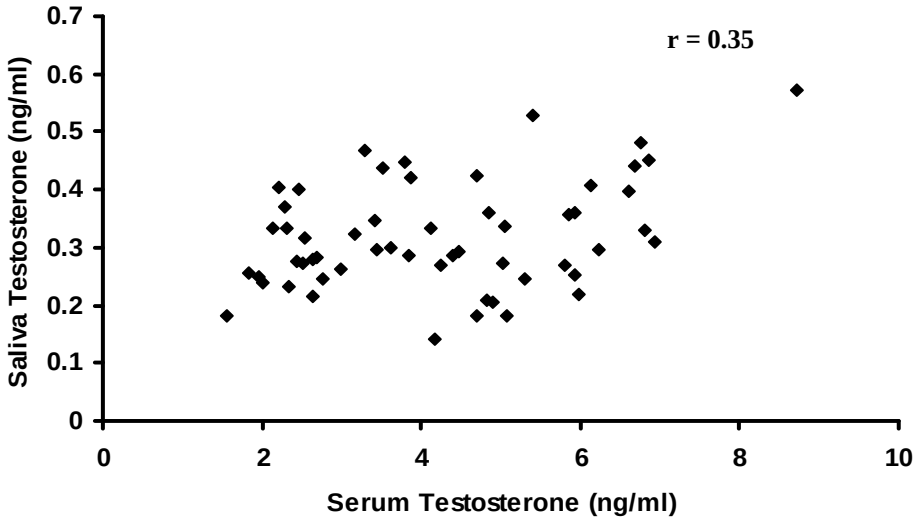

Fig 4.2.9: Correlation between saliva and total serum testosterone with exercise (corrected for PV)

4.2.5 Discussion

The main finding of the current study is the strong correlation observed between total serum and corresponding saliva cortisol (C) values before and after 30 min of exercise (treadmill run at ~ 70% $\dot{V}O_{2max}$). Conversely, no significant correlation was found between total serum and saliva testosterone (T) samples in response to the same exercise protocol.

Cortisol

The range of values observed in the current study (total serum C: 51.29 – 165.12 ηg/ml, saliva C: 0.3 – 8.17 ηg/ml) are similar to ranges reported in other studies which have investigated the response of total serum or saliva C to exercise (Kokalas et al., 2004; Paccotti et al., 2005; Davies and Few, 1973; Luger et al., 1987). The results confirm an acute increase in total serum and saliva C following an exercise protocol of ~ 70% $\dot{V}O_{2max}$ which has been observed in previous studies (Luger et al., 1987; Davies and Few, 1973). However, while these studies have shown similar post exercise response in either total serum or saliva C, few studies the investigated the dynamics of C in both serum and saliva following exercise concurrently (Paccotti et al., 2005; Gozansky et al., 2005). In the current study, mean (± SEM) total serum C increased to a peak (132.49 ηg/ml ± 8.73) at 10 min post exercise with levels gradually reducing thereafter. A similar response in serum C has been observed in previous studies (Davies and Few. 1973, Luger et al. 1987, Paccotti et al. 2005). Authors in these latter studies observed that serum C peaked at 10 – 30 min post exercise when exercising at an intensity of 60 – 90% $\dot{V}O_{2max}$. Similarly, in

the current study, salivary C increased to a peak (4.43 ηg/ml ± 0.78) post exercise, however, this occurred at 20 min post exercise with levels gradually reducing thereafter. The general pattern of a post exercise peak followed by a gradual decrease in C was similar in serum and saliva. However, peak saliva C was observed 10 min after peak total serum. The lag time of saliva C compared to total serum C in the current study has been observed in other studies investigating the response of both serum and saliva C to exercise (Paccotti et al. 2005, Gozansky et al. 2005). In these studies, peak saliva C post exercise occurred between 10 – 20 min following peak total serum concentrations. In the present study, the peak response of saliva C in individuals compared to total serum C was variable. Of the nine subjects involved in the present study, peak saliva and serum levels were found to occur concurrently in five subjects, while peak saliva was reached 10 min after peak serum in the remaining four subjects. The reason for the observed lag time of saliva C compared to serum values is not fully understood. Vining et al. (1983a) states that it takes approximately 1 - 5 minutes for serum C to represent itself in saliva C. This study and those referenced previously collected both fluids at intervals of a minimum 10 min post exercise. Because of the rapid change in C levels in response to exercise, actual time of peak levels of both fluids could have been over or underestimated with this sampling protocol. A protocol of sampling blood and saliva every 5 min may have provided greater clarity on this issue. However, Paccotti et al. (2005) observed lag times of up to 20 min in certain individuals and a reduction in sampling intervals would not account for a lag time of this duration.

Total serum and saliva C showed a strong and significant correlation ($r = 0.82$, $p = 0.01$) throughout the exercise protocol. These results are in agreement with similar studies

observing the correlation between serum and saliva C in response to exercise (Port, 1991; Paccotti et al., 2005; Gozansky et al., 2005). The weaker correlations observed by Paccotti et al. (2005) and Gozansky et al. (2005) [r = 0.6 and 0.61 respectively] were due to the intensity of exercise protocol used. In these studies, exercise elicited a response in serum cortisol which exceeded 500 ηmol/l. Above this level, cortisol binding globulin (CBG) capacity becomes saturated (Obminski and Stupnicki, 1990), thus the levels of free cortisol in the blood increases more rapidly which then appears in saliva cortisol. As a consequence, the correlation between serum and saliva C becomes non-linear beyond serum C levels of 500 ηmol/l. In the current study and in a previous investigation (Port, 1991), serum C levels did not exceed this threshold and a stronger correlation was therefore observed (Port, (1991): r = 0.86).

Testosterone

The range of values observed in the current study (total serum T: 1.64 – 8.7 ηg/ml, saliva T: 0.14 – 0.57 ηg/ml) are similar to ranges reported in previous studies which have investigated the response of total serum and saliva T to exercise (Hackney et al., 1995; Cook et al., 1986; Kuoppasalmi et al., 1980). The results from the current study confirm the acute response of total serum and saliva T to an exercise protocol of ~ 70% $\dot{V}O_{2max}$. However, while these studies have confirmed the post exercise response of either total serum or saliva T, it would seem that this is the first study to investigate both total serum and saliva T concurrently post exercise and over the subsequent recovery period. Mean (± SEM) total serum T increased to a peak immediately post exercise (4.97 ηg/ml ± 0.68) with levels gradually reducing thereafter. This response in total serum T is in agreement

with other studies using a similar exercise protocol (Kuoppasalmi et al., 1980; Hackney et al., 1995). However, mean (± SEM) saliva C increased immediately post exercise up to a maximum (0.35 ηg/ml ± 0.02) at 20 min post exercise. There are very few studies that have investigated the post exercise saliva T via serial sampling which would facilitate a comparison with the present study. Cook et al. (1986) investigated saliva T response to a marathon. Saliva T peaked immediately post marathon, however, the following sample point was 1 hr post marathon (a decrease in saliva T) and saliva T may have fluctuated significantly during the first hour of recovery post marathon. No other studies have investigated the post exercise response of saliva T via serial sampling. In the current study, of the nine subjects, four had one or more samples omitted due to blood contamination and for these subjects it was difficult to interpret where peak saliva T actually occurred. Of the remaining five, peak serum T and saliva T occurred simultaneously in two subjects, while in the remaining three subjects; peak saliva T occurred 20 – 30 min later than peak total serum T. This presents a similar dilemma to serum and saliva cortisol response with exercise and is not fully understood. Previous studies have estimated the time for equilibrium between serum and saliva hormone levels to be in the range of 1 – 5 minutes and this would not account for the length of time lag observed (Vining et al., 1983a). A possible reason for the prolonged lag time could be the effect of high circulating cortisol on the binding of testosterone to plasma proteins. A previous study investigated the effect of administration of synthetic corticotropin on the concentrations of plasma total, free and non-SHBG testosterone in normal women (Swinkels et al., 1991). This study observed that administration of corticotropin elicited an increase in the fraction of free testosterone in plasma. This observation was attributed

to the impairment of albumin and CBG binding to testosterone, due to cortisol occupying the binding sites of these proteins. It is reasonable to suggest that an increase in post exercise serum C, as seen in this current study, may increase the free fraction of testosterone in the blood. In turn, this would then represent itself in saliva testosterone concentrations. Considering the five subjects with no saliva samples omitted due to blood contamination, two of these subjects showed a parallel post exercise peak in serum and saliva T. Interestingly, these were the only subjects that showed no post exercise increase in serum C. In these subjects, there was no increase in post exercise total serum C compared to pre-exercise concentrations. In the remaining three subjects, a post exercise peak in serum C occurred at 10 – 20 min. This was then mirrored by a post exercise peak in saliva T. Therefore, the high cortisol concentrations observed in these subjects post exercise may have had an effect on the levels of saliva T measured; however, further investigation is needed to determine the effect of blood concentrations of C on the binding of plasma T in men.

Total serum and saliva T showed a low and non-significant correlation ($r = 0.22$, $p > 0.05$) throughout the exercise protocol. No other studies have investigated the correlation between total serum and saliva T in response to exercise. However, Cadore et al. (2008) investigated the correlation between serum free and saliva T in response to a resistance exercise protocol. In this study, the response of both serum free and saliva T was measured immediately post resistance exercise. In keeping with to the current study, no correlation was found between both biological fluids ($r = 0.26$). A possible reason for the weaker correlation with exercise compared to resting situations could be the impact of blood contamination of saliva. In the current study, saliva samples with transferrin levels

exceeding 1.2 mg/dl were omitted from saliva T analysis. A previous study though has stated that saliva samples with transferrin levels above 0.5 mg/dl should be omitted from saliva T analysis (Schwartz and Granger, 2004). However, omitting saliva samples above this threshold had no significant effect on the overall testosterone correlation (r = 0.28). In the current study, four out of nine subjects showed strong correlations between serum and saliva T (r = 0.71 – 0.95), while a weak correlation was observed in remaining subjects (r = -0.37 – 0.5). Again, as mentioned previously, the individual variation in correlation results may be due to the effect of serum cortisol concentrations or minimal blood contamination on the validity of saliva T measurements in a post exercise environment.

Correction for PV

The influence of plasma volume (PV) change on the serum/saliva correlation of C and T was also investigated in this study. Kargotich et al. (1998) suggested that the influence of PV on biochemical data obtained during the recovery period from exercise should be considered when investigating hormonal response post exercise. The authors recommended that blood hormonal data should be corrected for PV to avoid misinterpretation of the actual response of hormones. It is reasonable to suggest that PV changes due to exercise may influence the serum/saliva correlation of hormones post exercise. To the author's knowledge, this is the first study to investigate the influence of PV on serum and saliva C and T correlations. After correcting total serum C and T concentrations for PV change, data were again statistically analysed. Similar to original serum C data, PV-corrected data showed an increase in serum C post exercise. Peak

levels occurred at 10 min post exercise with concentrations gradually reducing thereafter with the general pattern mirroring the original serum C data. Correcting for PV had a minimal effect on the correlation between total serum and saliva C (original data: r = 0.82, PVC corrected data: r = 0.8). PV corrected serum T data showed a significant increase in levels immediately post exercise as was observed in original serum T data. Peak levels occurred at this point with concentrations gradually decreasing thereafter thus mirroring original total serum T data. Once more, correcting for PV had a minimal effect on the total serum/saliva T correlation with the strength of the correlation improving slightly (original data: r = 0.22, PVC data: r = 0.35).

Limitations

Limitations of the present study must be considered. Firstly, the protocol of sampling at intervals of 10 min for the first 30 min post-exercise may have hindered the assessment of peak levels of serum and saliva hormone in response to the exercise protocol. Sampling at intervals of 5 min for the 30 min post-exercise may have given a better indication of peak hormone levels in both biological fluids and hence a better insight into the time lag observed for saliva concentrations compared to serum levels. Secondly, measurement of serum free hormones in addition to total serum hormones may have given a better insight into the effect of exercise on the proportion of bound and free hormones in serum and its consequent effect on saliva hormone measurements.

In summary, the current study confirms results from previous studies showing a strong correlation between total serum and saliva C under exercise conditions. However, the

present study suggests that measurement of salivary T does not reflect total serum T concentrations under exercise conditions. Correcting serum C and T data for changes in plasma volume had little effect on the post exercise serum response or the correlation with saliva concentrations in either hormone. With the above in mind, analysis of saliva C offers a practical, non-invasive and stress-free method of monitoring serum concentrations of cortisol in an exercise environment. Regarding salivary T, further investigations are required to establish whether there is any correlation between total serum and saliva values in an exercise environment. In addition, the effect of high plasma cortisol levels on the binding of testosterone to plasma proteins needs further investigation.

STUDY THREE

4.3 PRE-COMPETITION HORMONAL AND PSYCHOLOGICAL LEVELS IN ELITE RUGBY UNION

4.3.1 Introduction

Competition can be a stressful situation which stimulates a physiological and psychological response in participants (Salvador et al., 2003). Cortisol (C) and Testosterone (T) have been shown to alter in anticipation of competition. Studies have shown that pre-competitive anxiety elicits a response in salivary C and T (Salvador et al., 2003; Neave et al., 2003; Carre et al., 2006). Pre-competitive anxiety has also been shown to have an influence on an athlete's psychological state (Doan et al., 2007; Mazur et al., 1992).

While many studies have investigated pre-competitive hormone responses and psychological stress independently (Passelergue and Lac, 1995; Swain and Jones, 1993), few have reported both hormone response and psychological state concurrently. To date, very few investigations have been carried out within elite team sports such as rugby union (Maso et al., 2004). It is feasible that environmental issues such as travel, team selection and spectator numbers could have an influence on psychological and physiological state. Further knowledge on the effect of environmental and psychological factors on performance in elite rugby union players could aid in appropriate tailoring of future pre-competitive programs.

The main aim of this study was to investigate the hormonal and psychological responses of a professional rugby team to pre-competition stress, travel to away fixtures and the stress of away games compared to home games. The Newport Gwent Dragons are a professional regional rugby union team in Wales. They compete in the Heineken cup, Magners league and EDF cup competitions during their playing season. The team was studied over a period of home and away fixtures against opposing teams in the Magners

league competition. Study 1 has shown that at rest, saliva C and T are representative of serum C and T concentrations. Previous studies have also shown that saliva hormone concentrations correlate strongly with serum hormone concentrations in a resting state (Lac et al., 1993; Vining et al., 1983; Johnson et al., 1987). Due to its stress free and non-invasive procedure, saliva sampling is ideal for the monitoring of hormonal concentrations in a sporting environment. It was hoped that this study will give greater insight into pre-competitive stress through separate analysis of the effect of travel and game location on overall stress response.

4.3.2 Methodology

The Newport Gwent Dragons were monitored over a series of home and away matches at the end of the 2007/2008 season.

The itinerary was as follows:

Match 1: Ospreys (Away) 25/04/08: The Liberty Stadium, Swansea K/O19:35

Match 2: Leinster (Away) 03/05/08: Donnybrook, Dublin K/O 18:30

Match 3: Ospreys (Home) 06/05/08: Rodney Parade, Newport K/O 19:35

Match 4: Leinster (Home) 09/05/08: Rodney Parade, Newport K/O 19:10

(All of the above were Magners league fixtures)

At 90 min prior to the start of each match, the match day squad of 22 players (15 starters, 7 replacements) were asked to provide a saliva sample (section 3.4) and complete the CSAI-2R questionnaire provided (Appendix C).

To investigate the possible effects of day long travel the day before Match 2 another sampling point was introduced post flight. Players were asked to provide samples on arrival at the airport following the flight.

A control day was included to measure baseline psychological and hormonal levels. On the control day participants were asked to refrain from exercise for 24 hr prior and to fast for 2 hours prior to sampling. All participants were asked to refrain from supplementation of Vitamin C for 7 days prior to and during the study.

Saliva samples were placed on ice for 24 hr before being frozen at -80℃. This was a standardized procedure for all samples collected.

The sampling itinerary was as follows:

Sample 1: Match 1 25/04/08: Time 18:00

Sample 2: Post Flight 02/05/08: Time 18:10

Sample 3: Match 2 03/05/08: Time 16:30

Sample 4: Match 3 06/05/08: Time 18:00

Sample 5: Match 4 09/05/08: Time 17:40

Sample 6: Control Day 13/05/08: Time 18:00

The characteristics of all male players involved in the study are listed in table 4.3.1

Age (years)	26.15 ± 4.45
Height (cm)	186.19 ± 7.46
Weight (kg)	101.94 ± 13.97

Table 4.3.1: Mean (± SD) subject characteristics (n=31)

Analysis of salivary cortisol and testosterone is described in section 3.6.1 and 3.6.2, respectively. Intra and inter-assay coefficients of variance for the above mentioned assays were 4.64% and 6.1% for cortisol and 3.8% and 3.9% for testosterone.

Analysis of saliva transferrin is described in section 3.6.3. Analysis for saliva osmolality was as described in section 3.6.4.

Dehydration levels of 5% bodyweight or more have been shown to cause a significant increase in blood cortisol (Maresh et al., 2006; Judelson et al., 2008) while saliva osmolalities of 160 $mOsmol \cdot kg^{-1}$ and above has been shown to correspond to this level of dehydration (Walsh et al., 2004). With this in mind, any samples over and above this osmolality level were excluded from cortisol analysis.

Evaluation of CSAI-2R questionnaires is described in section 3.9. Four questions were added to the questionnaire to monitor the behavioural patterns of subjects over the previous 24 hours. The four additional questions enquired about the subject's sleep, relaxation, eating and exercise patterns over the previous 24 hrs compared to a normal day. Subjects answered using a Likert scale rating from 1-5 with the ratings corresponding to the following; 1 – much more, 2 – somewhat more, 3 – normal, 4 – somewhat less, 5 – much less.

Data are presented separately for all of the fifteen players starting the game and the fifteen plus replacements. The data for all players (players starting plus replacement) will be referred to as 'Squad'. Data for starting players will be referred to as 'Starting XV'.

4.3.3 Statistical Analysis

All hormonal data were tested for normality using the Kolmogorov-Smirnov test. A one way ANOVA was used to determine significant differences over time within groups for T/C ratio data. Fisher LSD post hoc procedures were used to locate significant differences among means. A one way ANOVA was used to determine if there were significant differences over time with samples for cortisol difference and testosterone difference data. Fisher LSD post hoc procedures were used to locate significant differences among means following a significant ANOVA. Home and away game data is presented as mean (SEM) but without statistical analysis because the inconsistency among player selection (only two players started in all four games) meant that a repeated measures analysis would have encountered too much missing data to provide a reliable result.

CSAI-2R psychological variables were tested for normal distribution using the Kolmogorov-Smirnov test. Somatic and Cognitive anxiety, and self confidence scores were normally distributed and a one way ANOVA was used to determine significant differences over time within scores. Behavioural pattern data was also evaluated for normality using the Kolmogorov-Smirnov test. A Kruskal-Wallis test was used to determine significant differences over time with Mann-Whitney post hoc procedures used to determine differences among groups.

The relationship between hormonal data and psychological variables was evaluated using Pearson correlation analysis.

4.3.4 Results

Hormonal Data

A total of 31 players were involved during the matches investigated in this study and 28 of these players started in one or more games. Only two players started all games, a total of 151 saliva samples were taken over course of the study. Two (1.3%) samples were omitted from salivary testosterone analysis due to blood contamination (transferrin levels > 1.2 mg/dl, Shirtcliff et al., 2002). Mean (± SEM) saliva osmolality concentrations for all samples were 74.77 mOsmol·kg^{-1} (± 3.1). Only one sample (>160 mOsmol·kg$^{-1)}$ was omitted from cortisol analysis due to dehydration.

Mean (± SEM) saliva cortisol and testosterone concentration for Squad and Starting XV (in brackets) at each sample point are shown in Table 4.3.2. Peak cortisol levels for Squad and Starting XV (2325.2 pg/ml and 2407 pg/ml respectively) occurred prior to match 3 while the lowest levels were observed for post-flight samples (806.6 pg/ml and 662.3 pg/ml). Peak testosterone levels for Squad and Starting XV (119.5 pg/ml and 126 pg/ml respectively) occurred prior to match 3 with lowest levels recorded post flight (84.1 pg/ml and 84.5 pg/ml). Peak T/C ratio was recorded post flight for both Squad and Starting XV (0.13 and 0.14 respectively). T/C ratio was at its lowest for Squad and Starting XV prior to Match 2 (both 0.06). Mean (± SEM) Starting XV T/C ratio was significantly different among sample points ($F = 3.59$, $p = 0.005$). Match 2 T/C ratio was significantly lower ($p < 0.01$) than control day values.

	Cortisol (pg/ml)			Testosterone (pg/ml)			T/C Ratio		
	n	Mean	SEM	N	Mean	SEM	n	Mean	SEM
Match 1	22	1458.2	217.2	21	98.6	8.9	21	0.10	0.01
	(15)	(1242)*	(170.54)	(15)	(98.8)	7.5	(15)	(0.11)	(0.02)
Post Flight	22	806.6	93.5	22	84.1	5.4	22	0.13	0.02
	(15)	(662.3)	47.7	(15)	(84.5)	9.9	(15)	(0.14)	(0.02)
Match 2	22	2325.2	268.3	22	119.5	9.3	22	0.06	0.01
	(15)	(2407)*	237.33	(15)	(126)*	10.9	(15)	(0.06)*	(0.01)
Match 3	22	1521.1	186.1	22	109.8	11.2	22	0.09	0.01
	(15)	(1637)*	173.25	(15)	(120.4)*	10.0	(15)	(0.09)	(0.01)
Match 4	22	1595.2	169.8	21	106.8	9.1	21	0.08	0.01
	(15)	(1633.6)*	140.2	(14)	(113)	5.8	(14)	(0.08)	(0.01)
Control	**33**	**1013.6**	**93.2**	**33**	**102.7**	**6.7**	**33**	**0.11**	**0.01**

Table 4.3.2: Mean (± SEM) cortisol, testosterone and T/C ratio values of all samples at each sample point (starting XV in brackets). * Significantly different ($p < 0.05$) from control day.

Mean (± SEM) cortisol and testosterone concentrations for home matches (match 1 and 2) and away matches (match 3 and 4) are shown in Table 4.3.3. Higher cortisol and testosterone levels were observed for away matches (Squad; 1891.7 pg/ml and 109.3 pg/ml respectively) compared to home matches (1557.4 pg/ml and 108.3 pg/ml respectively). For the Starting XV, cortisol was higher for away matches (1824.5 pg/ml) while testosterone was higher at home matches (116.7 pg/ml). T/C ratio for Squad and Starting XV was similar for home and away matches.

	Cortisol (pg/ml)			Testosterone (pg/ml)			T/C Ratio		
	n	Mean	SEM	N	Mean	SEM	N	Mean	SEM
Home	44	1557.4	124.9	43	108.3	7.2	43	0.08	0.01
	(30)	(1635.3)	(155.3)	(29)	(116.7)	(7.7)	(29)	(0.08)	(0.01)
Away	44	1891.7	182.9	43	109.3	6.6	43	0.08	0.01
	(30)	(1824.5)	(230.1)	(30)	(112.4)	(7.0)	(30)	(0.09)	(0.01)

Table 4.3.3: Mean (± SEM) cortisol, testosterone and T/C ratio values for home and away matches (starting XV in brackets)

To account for natural variation in circadian rhythms, and the large variation of individual cortisol and testosterone concentrations, statistical analysis was performed on hormonal data minus baseline measurements. Control day cortisol and testosterone values were subtracted from each respective sample point cortisol and testosterone concentrations. Data are therefore presented as 'cortisol difference' and 'testosterone difference' as observed in similar previous studies (Doan et al. 2006, Stupnicki and Obminski. 1992).

Figure 4.3.1 shows mean (± SEM) cortisol difference over all sample points. Individual cortisol difference across all sample points ranged from a decrease of 960 pg/ml to an increase of 4390 pg/ml. Match 2 elicited the largest increase in mean cortisol difference (1573.7 pg/ml ± 340.4) with post flight eliciting a decrease of 171.0 pg/ml ± 143.4. Mean cortisol difference was significantly different among sample points ($F = 6.84$, $p < 0.01$). Match 2 was significantly different to all other sample points ($p < 0.02$). Post flight concentrations were significantly different to match 2, 3, and 4 ($p < 0.015$).

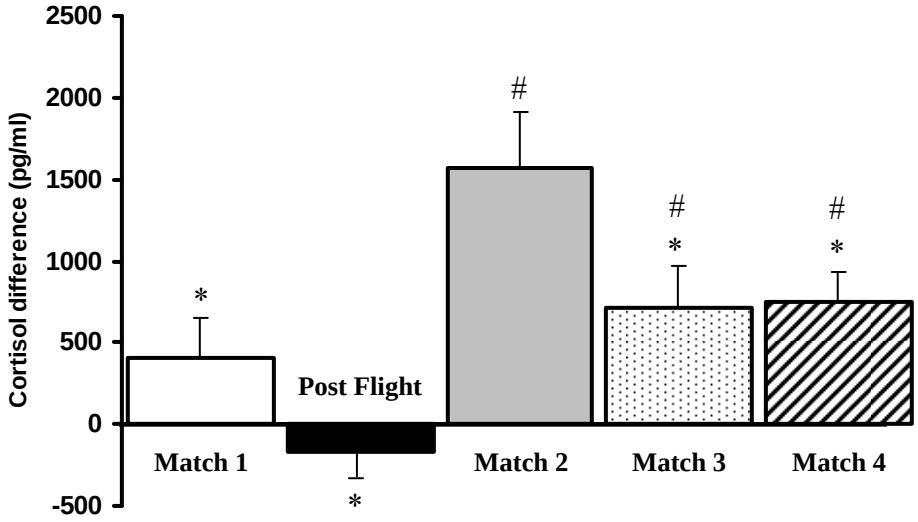

Fig 4.3.1: Mean (± SEM) cortisol difference over all sample points
*Significantly different ($p < 0.02$) from Match 2, #Significantly different ($p < 0.015$) from Post Flight

Mean (± SEM) home and away match cortisol difference is shown in Figure 4.3.2. Away matches elicited the largest cortisol difference (992.5 pg/ml ± 273.4) with home games resulting in a difference of 730.5 pg/ml ± 158.1.

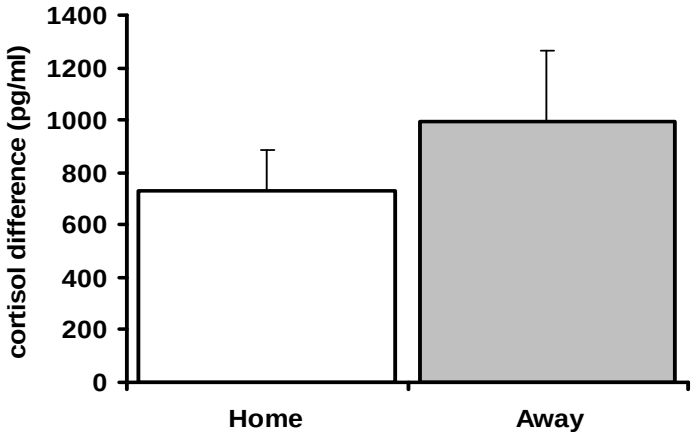

Fig 4.3.2: Mean (± SEM) cortisol difference for home and away matches

Mean (± SEM) testosterone difference over all sample points is shown in Figure 4.3.3. Individual testosterone difference across all sample points ranged from a decrease of 42.5 to an increase of 125.4 pg/ml. Match 2 elicited the largest increase in testosterone difference (38.6 pg/ml ± 10.9) with post flight resulting in a decrease of 3.7 pg/ml ± 9.9. Mean testosterone difference was significantly different among sample points ($F = 3.46$, $p = 0.012$). Match 2 was significantly different to Match 1, Post flight and Match 4 ($p < 0.04$). Post flight concentrations were significantly different to Match 2 and Match 3 ($p < 0.02$).

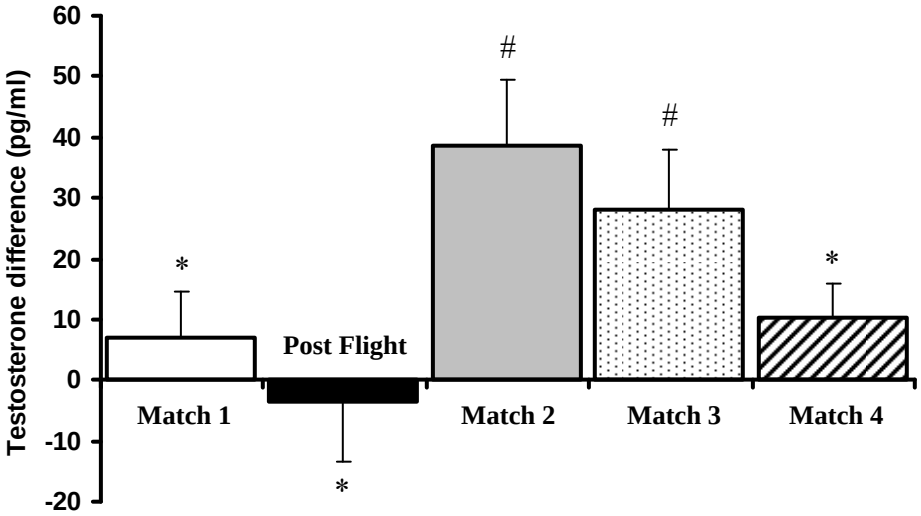

Fig 4.3.3: Mean (± SEM) testosterone difference across all sample points
*Significantly different ($p < 0.04$) from Match 2
#Significantly different ($p < 0.02$) from Post Flight

Mean (± SEM) home and away testosterone difference is shown in Figure 4.3.4. Away matches elicited the largest testosterone difference (22.85 pg/ml ± 7.27) with home games resulting in a difference of 19.69 pg/ml ± 6.11.

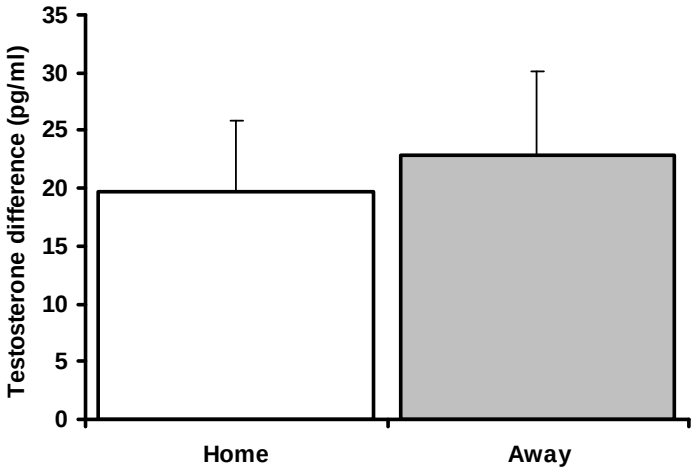

Fig 4.3.4: Mean (± SEM) testosterone difference for home and away matches

Psychological Data

Mean (± SEM) CSAI-2R psychological variables for starting XV over all sample points are shown in Table 4.3.4. Peak mean somatic anxiety occurred prior to Match 4 (18.67 ± 1.79). Peak mean cognitive anxiety occurred prior to Match1 (20.29 ± 1.33). Match 1 also elicited the highest score (34.86 ± 1.10) in self confidence. No psychological variables were significantly different across sample points.

	Somatic Anxiety	**Cognitive Anxiety**	**Self Confidence**
Match 1	17.24 (1.31)	20.29 (1.33)	34.86 (1.10)
Post Flight	13.71 (1.22)	19.73 (1.91)	32 (1.70)
Match 2	16.95 (1.11)	19.6 (1.71)	32.53 (1.73)
Match 3	16.67 (1.96)	18.4 (1.80)	32.93 (1.55)
Match 4	18.67 (1.79)	18.67 (1.83)	32.27 (1.31)

Table 4.3.4: Mean (± SEM) pre-game CSAI- 2R psychological scores for starting XV at each sample point

Mean (± SEM) CSAI-2R psychological variable scores for starting XV at home and away matches are shown in Table 4.3.5. Somatic anxiety was greater for home matches (17.67 ± 1.34). Cognitive anxiety and self confidence was greater for away matches (19.93 and 33.66 respectively).

	Somatic Anxiety	**Cognitive Anxiety**	**Self Confidence**
Home	17.67 (1.34)	18.53 (1.28)	32.6 (1.01)
Away	17.09 (0.84)	19.93 (1.07)	33.66 (1.04)

Table 4.3.5: Mean (± SEM) pre-game CSAI-2R psychological scores for starting XV at different match locations

24 hr Behaviour Pattern Data

Mean (± SEM) behaviour pattern data for staring XV at each sample point is shown in Table 4.3.6. Players reported significantly less sleep in the 24 hrs prior to post flight samples compared to matches 1, 3 and 4. Relaxation patterns were not significantly different over any sample points. Players reported consuming significantly more food than normal before Match 1 and 4 compared to other sample points and also reported significantly less exercise prior to Match 1 compared to all other sample points.

	Sleep	Relaxation	Eating	Exercise
Match 1	2.43 (0.05) [a]	2.93 (0.06)	3.5 (0.04)	4.29 (0.05)
Post Flight	3.33 (0.05)	3.33 (0.03)	2.87 (0.03) [b c]	3.27 (0.04) [d]
Match 2	2.93 (0.06)	2.63 (0.04)	2.75 (0.07) [b c]	3.4 (0.05) [d]
Match 3	2.53 (0.04) [a]	2.8 (0.05)	3.07 (0.04) [c]	3.6 (0.05) [d]
Match 4	2.62 (0.05) [a]	2.75 (0.18)	3.38 (0.03)	3.5 (0.06) [d]

Table 4.3.6: Mean (± SEM) 24hr behaviour pattern scores for starting XV at each sample point.
[a] Significantly different ($p < 0.05$) from Post Flight scores, [b] Significantly different ($p < 0.05$) from Match 1, [c] Significantly different ($p < 0.05$) from Match 4, [d] Significantly different ($p < 0.05$) from Match 1

Correlations among Hormonal and Psychological Data

The correlations between biochemical and psychological variables are shown in Table 4.3.7. No significant correlations were found between CSAI-2R variables and hormonal data at any sample point throughout the study.

Variable	Somatic Anxiety	Cognitive Anxiety	Self Confidence
Testosterone Difference	0.08	0.04	-0.10
Cortisol Difference	0.12	-0.03	0.08
T/C Ratio	-0.17	0.12	-0.17

Table 4.3.7: Correlation between hormonal and psychological variables

4.3.5 Discussion

To the author's knowledge, this is the first investigation to examine both pre-competition hormone responses and psychological state in elite rugby union. The primary finding of the present study suggests that the onset of competition induced a significant change in hormonal and psychological measures in Elite rugby union players. Cortisol, testosterone and somatic and cognitive anxiety increased in individuals in anticipation of competition. In addition, pre-competition cortisol, testosterone and T/C ratio showed high variability between games and may affect subsequent performance.

Hormonal Data

Cortisol

The range of saliva cortisol (C) concentrations (320 – 4205 pg/ml) measured in participants during this study is similar to those reported in studies investigating pre-competitive hormone response (Carre et al., 2006; Salvador et al., 2003). Pre-match saliva C was significantly higher ($p < 0.05$) for all games compared to control day samples showing an anticipatory C response to competition. The existence of an anticipatory C response prior to stressful events of a physical nature has long been

recognized (Mason et al., 1973). Sapolsky, (1994) stated that every anxiety arousing situation is characterised by its being perceived as a threat, by its being only partially controlled and by uncertainty about its outcome and/or its consequences. Sporting situations meet these characteristics and can therefore be considered as anxiety arousing situations. An anticipatory rise in C could also be considered as physiologically advantageous as it facilitates greater energy availability prior to effort. The pre-competition rise observed in C prior to each game in the current study confirm previous investigations on the existence of a C response to this type of stressor (Salvador et al., 2003, Passelergue and Lac, 1999). Given the intense physical nature of rugby union, existence of injures and for player development reasons, only two players started all games analysed in the current study. This made statistical assessment of cortisol data between games difficult. Therefore, the statistical assessment of C response to each match was performed on cortisol data minus baseline measurements. Individual baseline C values were subtracted from each respective sample point concentrations and presented as 'cortisol difference' as used in previous studies (Doan et al., 2006, Stupnicki and Obminski. 1992). It was hoped that this would provide a more accurate indication of the actual C response to each match and account for the high variability of individual cortisol levels.

Mean (± SEM) cortisol difference was higher for away matches (992.5 pg/ml ± 273.4) than for home matches (730.5 pg/ml ± 158.1). A previous investigation (Carre et al., 2006) reported no statistical differences between pre-game cortisol levels for home games compared to away games in elite hockey players. In the present study, C response to each match was variable. The cortisol response to Match 2 (Away) was significantly

higher ($p < 0.02$) than pre-match C concentrations for all other matches. Match 2 was played away from the team's home arena and an increase in pre-match cortisol was expected compared to home games (Match 3 and 4). However, the reason that Match 2 cortisol difference was higher than the other away game (Match 1) is not fully understood. A possible factor may have been the crowd attendance at both matches. The attendance for Match 2 was 17260 compared to Match 1 (9116). Interestingly, Match 2 was also a championship deciding game for the opposition. The larger attendance, in addition to the greater importance of the game, may have resulted in the heightened cortisol response in Match 2. To the authors knowledge there have been no studies investigating the effect of attendance on competitor's hormonal states. Further investigations are needed to determine if crowd size and density affect measures of pre-competition C. Another factor influencing the difference in cortisol response to both away matches could have been the hotel stay prior to Match 2. Match 1 was against a local rival team and each player was able to stay at home prior to the game. An overnight stay in an unfamiliar setting and the consequent disruption to normal routines prior to Match 2 may have had an effect on the subsequent C response. In addition, the effect of flight travel may have also been a factor. Travel to Match 2 involved a 1 hr flight with approximately 1 hr bus travel to and from the airport. A previous study has investigated C response to long haul flight travel across multiple time zones (Bullock et al., 2008) and reported a suppression in C levels post flight. There have been no studies investigating the effect of short haul flights on subsequent C concentrations. Post Flight cortisol was not significantly different to control. While C values did not differ between pre and post flight, it is possible that this in combination with the above factors may have further

disrupted the normal routines of individuals and contributed to the heightened C response to Match 2. Interestingly, the result in Match 2 (Lost: 8 v 41) was considered by coaches to be the worst performance over the series of games (Match 1 – Lost: 3 v 16, Match 3 – Won: 18 v 10, Match 4 – Won: 31 v 26). It is reasonable to suggest that the significantly higher C response prior to Match 2 may have influenced teams' performance, which was perceived by coaches as the worst performance following post match video analysis. Further studies, are needed to assess whether acutely high levels of C prior to competition have an enhancing or debilitating effect on athletic performance.

Analysis of 24 hr behavioural pattern data revealed that players reported significantly less exercise before Match 1 than before all other matches. Interestingly, this accompanied the lowest C difference values for all matches. This reduction in exercise before Match 1 compared to all other matches may therefore have had an effect on pre-game cortisol levels. However, previous studies (Hooper et al., 1995; Vuorimaa et al., 1999) have reported the effect of strenuous exercise on the following recovery day's cortisol concentrations. Authors found no changes in C levels during the following days and it can be assumed that the less exercise reported before Match 1 had little effect on subsequent cortisol levels.

Testosterone

The range of saliva testosterone (T) concentrations (24.82 – 224.85 pg/ml) measured during the course of this study is comparable with previous studies that reported pre-competition saliva T concentrations (Neave and Wolfson, 2003; Carre et al., 2006). Pre-match saliva T was significantly higher ($p < 0.05$) than control concentrations prior to

Match 2 and 3 but no significant difference was observed for Match 1 and 4. The relationship between aggressive and assertive behaviour, territoriality and T has been documented (Archer, 1991; Book et al., 2001). Higher T concentrations prior to competition may facilitate the expression of these psychological characteristics in preparation for the oncoming contest. Higher T levels have also been associated with improved reaction times (Muller, 1994), an increase in the metabolic rate of muscles (Tsai et al., 1996) and may represent an adaptive response in anticipation of competition. The response of T in anticipation of competition has been investigated by several studies (Salvador et al., 2003; Mazur et al., 1992). Authors observed rises in T concentrations prior to both physical and non-physical contests although this was variable amongst individuals. In the current study, a pre-match rise in T compared to control was observed in a high percentage of individuals (74%). However, in Match 1 and 4 this was more variable and resulted in no change in T prior to these matches. As mentioned previously, statistical comparison of the Starting XV T response for each match was performed on 'testosterone difference' data.

Mean (± SEM) testosterone difference was higher for away matches (22.85 pg/ml ± 7.27) than for matches played at the home venue (19.69 pg/ml ± 6.1). These findings are in disagreement with previous studies (Carre et al., 2006; Neave and Wolfson, 2003), who reported that pre-competition T was higher for games played at home venues compared to away games. In the present study, T difference response to each match was variable. Testosterone difference was significantly higher for Match 2 (Away) compared to Match 1 (Away) and 4 (Home) but not from Match 3 (Home). No other matches were different from each other. The reason for the heightened T response in Match 2 is not understood.

Match 2 was played away from the team's home venue and does not agree with previous observations on higher T concentrations for home games (Carre et al., 2006; Neave and Wolfson, 2003). A possible reason could be the significant increase in cortisol response observed in Match 2 compared to other matches. A previous study (Swinkels et al., 1991) investigated total serum and saliva T following administration of synthetic corticotropin. Authors observed a significant increase in both total serum and saliva T following administration of corticotropin suggesting a sympathetic rise in T in relation to increasing levels of cortisol (Swinkels et al., 1991). It is reasonable to suggest that the significant increase in cortisol prior to Match 2 may have influenced pre-match testosterone concentrations. Another factor could be the large attendance that the match attracted. This together with importance of the game (a championship decider for the opposition) may have resulted in a more aggressive behaviour in preparation for the match. Subsequently, this may have increased the T response. While previous studies have indicated that testosterone levels are associated with alertness and competitiveness (Muller, 1994), others have shown that high T levels may be detrimental to performance (Booth et al., 1999; Muller et al., 2005). The statistically higher T response prior to Match 2 may therefore have influenced the poor performance of the team during the game. Further studies are required to determine the effect of high pre-competition levels of T on subsequent performance.

T/C Ratio

The range of T/C ratio levels observed in the current study (0.017 – 0.371) is comparable with those measured in a previous study (Elloumi et al., 2003). T/C ratio was significantly lower ($p < 0.05$) prior to Match 2 compared with control. No other pre-match T/C values were significantly different to control day T/C. A decrease in the T/C ratio is suggested to be indicative of a disturbance in the anabolic-catabolic balance, which may express itself in decreased performance (Hoogeveen and Zonderland, 1996). The ratio is considered to reflect states of anabolism and tapering off when it is high, and inversely, states of catabolism when it falls by 30% or more (Maso et al., 2004). In the current study, Match 2 T/C ratio was significantly lower ($p < 0.05$) than control day values. A decrease of 49% in T/C ratio was measured prior to the match compared to control. Match 2 was also perceived by coaches to be the worst performance by the team (Lost: 8 v 41) over the series of games. A decrease in T/C levels of 30% or more has been suggested as a measure of overreaching or overtraining, which in turn may result in decreased performance (Maso et al., 2004). However, this study considered whether a prolonged disturbance (weeks to months) in the T/C ratio manifested itself in a deterioration of performance. It is reasonable to suggest that an acute decrease of 30% or more in the T/C ratio may have a debilitating effect on subsequent performance as observed for Match 2 in the current study. Further studies are required to assess if acute changes in the T/C ratio, due to pre-competition anticipatory and stress responses, have an effect on subsequent athletic performance.

Psychological Data

Somatic and cognitive anxiety scores using the CSAI-2R questionnaire were significantly ($p < 0.05$) higher prior to each game when compared to control. No change was observed between self confidence scores for games compared to control. The results confirm observations in previous studies that reported an elevation in cognitive and somatic anxiety prior to competition (Mckay et al., 1997; Filaire et al., 2009). Statistical analysis of CSAI-2R showed no significant differences over matches between variables (self confidence, somatic anxiety, cognitive anxiety) indicating no difference in psychological stress irrespective of opposition or venue. Previous literature (Carre et al., 2006) observed higher pre-match somatic and cognitive anxiety at games played in their opponents' venue compared to their home venue. In the current study, there were no significant differences between home and away somatic and cognitive anxiety. The reason for no change in psychological variables between games or between home and away venues is not fully understood. A possible reason could the confidentiality of the players data during the study. Despite assurances, players may have been concerned that their coaches may have had access to the questionnaire results and altered their answers to perceive themselves as more confident or less anxious. Another factor could be the playing experience of the players involved in the study. The majority of players involved have been participating in professional elite rugby union for a number of years, with 20-30 games per year. Having played against the opposing teams and played at their venues on multiple occasions, it is possible that they may have become accustomed to playing in unfamiliar settings and against opposing teams and perceive games to be no more or less stressful than others. Thirdly, it has been hypothesised previously (Neave and Wolfson,

2003) that players may modify their answers in order to feel personally impervious to the playing conditions, thus increasing their mental stability. In summary, the results from the present study question the sensitivity of psychological questionnaires in assessing anxiety in a competitive environment.

Correlations among measures

Pearson correlations were computed to evaluate to relationship between pre-match physiological and psychological measures. No significant correlations were found between CSAI-2R variables and hormonal measures (cortisol, testosterone and T/C ratio). This was not unexpected considering there was no significant change in CSAI-2R variables between games while concurrently there were significant changes in C, T and T/C ratio between games. Previous studies have reported a relationship between pre-competition cortisol and CSAI-2R somatic anxiety (Filaire et al., 2009; Doan et al., 2007), similar to the present study, others have reported no correlation (Carre et al., 2006; Mckay et al., 1997) demonstrating a variable association between physiological and questionnaire psychological measures of anxiety.

Limitations

There are limitations associated with the present study. Physiological and psychological responses were measured over two home and away games. A longitudinal study incorporating more games over the course of a rugby season may give a better indication of the effect of playing venue, attendance and travel itinerary on individual hormonal and competitive anxiety responses. When assessing performance, self reported individual

performance ratings may have yielded a more reliable assessment of performance, as opposed to the coaches' assessment.

The present study supports previous findings of a rise in cortisol and testosterone in anticipation of competition. In addition, high concentrations of C and T and a reduction in T/C ratio may be an indicator of subsequent performance in elite rugby union players. No changes were observed among psychological variables between games, and no relationship was found between hormonal and psychological measures of competitive anxiety. Further research is needed to assess if a change in pre-competition hormone concentrations can affect actual individual performance.

CHAPTER FIVE

5.1 Summary of Findings

The findings of this research suggest that salivary measurements of testosterone, cortisol and T/C ratio can be used as a reference for their respective blood concentrations in resting conditions.

It has also demonstrated that salivary measurement of cortisol is reflective of total serum concentrations in a post exercise environment. However, no such relationship was reported between total serum and saliva testosterone concentrations in response to the same exercise protocol.

Salivary measurement of testosterone and cortisol in a resting state, and cortisol in a post exercise setting provide a reliable, non-invasive and palatable method of monitoring plasma concentrations of these hormones.

The research demonstrates that competition elicits an anticipatory hormone and stress response in participants. A significant increase in testosterone, cortisol and somatic and cognitive anxiety was reported in elite rugby union players prior to matches. Pre-game salivary testosterone and cortisol responses were significantly different between games suggesting that venue and/or opposition may influence levels of both hormones. Depending on age and/or experience of individuals, the effect of venue and opposition may have different influences on hormonal response. Results also suggest that pre-competition levels of testosterone and cortisol may affect subsequent performance.

5.2 Null Hypotheses

Null Hypothesis 1

Ho – Saliva cortisol does not correlate with total serum cortisol in resting conditions.

Hypothesis rejected

Saliva cortisol showed a strong and significant correlation with total serum cortisol ($r = 0.95$, $p<0.05$) in resting conditions.

Null Hypothesis 2

Ho – Saliva testosterone does not correlate with total serum testosterone in resting conditions.

Hypothesis rejected

Saliva testosterone showed a moderate and significant correlation with total serum testosterone ($r = 0.62$, $p<0.05$) in resting conditions.

Null Hypothesis 3

Ho – Saliva cortisol does not correlate with total serum testosterone in response to exercise.

Hypothesis rejected

Saliva cortisol showed a strong and significant correlation with total serum testosterone ($r = 0.82$, $p<0.05$) in response to exercise.

Null Hypothesis 4

Ho – Saliva testosterone does not correlate with total serum testosterone in response to exercise.

Hypothesis accepted

Saliva testosterone showed no correlation with total serum testosterone ($r = 0.22$, $p>0.05$) in response to exercise.

Null Hypothesis 5

Ho – Correction for plasma volume has no effect on the relationship between total serum and saliva concentrations of testosterone and cortisol.

Hypothesis accepted

Correction for plasma volume had little effect on the correlation between total serum and saliva concentrations of testosterone and cortisol.

Null Hypothesis 6

Ho – The anticipation of competition does not have an effect on salivary measures of testosterone and cortisol.

Hypothesis rejected

Salivary testosterone and cortisol were significantly higher ($p<0.05$) prior to all games compared to control.

Null Hypothesis 7

Ho – The anticipation of competition has no effect on CSAI-2R somatic and cognitive anxiety scores.

Hypothesis rejected

Somatic and Cognitive anxiety scores were significantly higher ($p<0.05$) before all games compared to control.

5.3 Directions for Further Research

The research in study one was conducted on a small sample size (n = 4). A larger sample size would have given a more accurate assessment of the relationship between saliva and serum concentrations in both hormones.

Analysis of free, in addition to total serum testosterone and cortisol may aid in the measurement of saliva hormones post exercise. This will help in determining if high serum levels of cortisol have a reciprocal effect on the binding of testosterone to plasma proteins, and subsequently the measurement of testosterone in saliva.

A longer study, incorporating more games throughout the rugby playing season would give a more accurate indication on the effect of venue and opposition on pre-competition hormone values. This would also help in determining if high levels of cortisol or testosterone have a detrimental effect on subsequent performance. Recent data examining the relationship between hormones, cytokines and inflammation with reference to recovery cycles following activity is a critical area for future research.

The analysis of salivary lysozyme, salivary alpha-amylase and plasma catecholamine's (noradrenaline and adrenaline) in addition to cortisol may assist in the assessment of pre-competitive stress. Previous studies have proposed salivary lysozyme as a potential marker of psychological stress (Perera et al., 1997; Yang et al., 2002). Furthermore, salivary alpha-amylase has been suggested to reflect changes in plasma noradrenaline and increased sympathetic activity under a variety of stressful conditions (Chatterton et al., 1996).

CHAPTER SIX

6.0 General References

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APPENDICES

APPENDIX A

SALIVRY TESTOSTERONE AND CORTISOL RESPONSES IN PROFESSIONAL ELITE RUGBY PLAYERS

HEALTH SCREEN FOR STUDY VOLUNTEERS **Name or Number** .....…….

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

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a)	on medication, prescribed or otherwise.....	Yes		No	
(b)	attending your general practitioner.....	Yes		No	
(c)	on a hospital waiting list.....	Yes		No	

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

(a)	consult your GP	Yes		No	
(b)	attend a hospital outpatient department.....	Yes		No	
(c)	be admitted to hospital	Yes		No	

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

(a)	Convulsions/epilepsy	Yes		No	
(b)	Asthma	Yes		No	
(c)	Eczema	Yes		No	
(d)	Diabetes	Yes		No	
(e)	A blood disorder	Yes		No	
(f)	Head injury	Yes		No	
(g)	Digestive problems	Yes		No	
(h)	Heart problems	Yes		No	
(i)	Problems with bones or joints	Yes		No	
(j)	Disturbance of balance/coordination	Yes		No	
(k)	Numbness in hands or feet	Yes		No	
(l)	Disturbance of vision	Yes		No	
(m)	Ear / hearing problems	Yes		No	
(n)	Thyroid problems	Yes		No	
(o)	Kidney or liver problems	Yes		No	

(p) Allergy to nuts ..... Yes ☐ No ☐

(q) Have any metal pins/plates inserted in your body..... Yes ☐ No ☐

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

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

.....

……

……

……

……

Thank you for your cooperation!

APPENDIX B

GLAMORGAN UNIVERSITY
SCHOOL OF SPORT AND EXERCISE SCIENCES

INFORMED CONSENT

SALIVARY TESTOSTERONE AND CORTISOL RESPONSES IN PROFFESIONAL ELITE RUGBY UNION PLAYERS

NAME OF VOLUNTEER:.....……

PRINCIPAL INVESTIGATOR(S): Prof. Bruce Davies,
Dr Julian Baker,
Mr Kevin Morgan

I have read the volunteer information sheet on the above study and have had the opportunity to discuss the details and ask questions. The investigator has explained to me the nature and purpose of the tests to be undertaken. I understand fully what is proposed to be done.

I have agreed to take part in the study as it has been outlined to me, but I understand that I am completely free to withdraw from the study at any time I wish.

I understand that these trials are part of a research project designed to promote scientific knowledge, which has been approved by the Glamorgan University Ethics Committee, and may be of no benefit to me personally.

I hereby fully and freely consent to participate in the study which has been fully explained to me.

SIGNATURE OF VOLUNTEER:.....……

Date: .....

I confirm that I have explained to the volunteer named above, the nature and purpose of the tests to be undertaken.

SIGNATURE OF INVESTIGATOR: .....…….

Date : .....

APPENDIX C

Evaluation of Stress-Induced Hormonal Disturbances in Professional Rugby Union.

Please note: this information is anonymised and player confidentiality will be maintained at all times. There is no need to note player name on this form.

SECTION 1 PLAYER INFORMATION

PLAYER INITIALS ____ AGE ____ CURRENT PLAYING POSITION ____ DATE ____ TIME ____

PLAYING EXPERIENCE (Please Tick √) REGIONAL SEMI-PRO ☐ REGIONAL ☐ INT 'U19' ☐ INT 'U21' ☐ INT 'A' ☐ INT '7's' ☐ INTERNATIONAL ☐

SECTION 2 – YOUR CURRENT FEELINGS/MOOD

Directions: A number of statements that athletes have used to describe their feelings before competition are given below. Read each statement and then ***circle the appropriate number*** to the right of the statement to indicate ***how you feel right now*** – at this moment. There are no right or wrong answers. Do *not* spend too much time on any one statement, but choose the answer which describes *your feelings right now*.

	NOT AT ALL	SOMEWHAT	MODERATELY SO	VERY MUCH SO
(1) I feel jittery	1	2	3	4
(2) I am concerned I may not do as well in this competition as I could	1	2	3	4
(3) I feel self-confident	1	2	3	4
(4) My body feels tense	1	2	3	4
(5) I am concerned about losing	1	2	3	4
(6) I feel tense in my stomach	1	2	3	4
(7) I'm confident I can meet the challenge	1	2	3	4
(8) I am concerned about choking under pressure	1	2	3	4
(9) My heart is racing	1	2	3	4
(10) I'm confident about performing well	1	2	3	4
(11) I'm concerned about performing poorly	1	2	3	4
(12) I feel my stomach sinking	1	2	3	4

SECTION 2 – Continued

	NOT AT ALL	SOMEWHAT	MODERATELY SO	VERY MUCH SO
(13) I'm confident because I mentally picture myself reaching my goals	1	2	3	4
(14) I'm concerned that others will be disappointed with my performance	1	2	3	4
(15) My hands are clammy	1	2	3	4
(16) I'm confident of coming through under pressure	1	2	3	4
(17) My body feels tight	1	2	3	4

SECTION 3 – BEHAVIOURAL PATTERNS OVER LAST 24 HOURS

In comparison to a 'normal' day, how do you rate the following occurrences over the last 24-h. PLEASE CIRCLE APPROPRIATE NUMBER

	Much more	Somewhat more	Normal	Somewhat less	Much Less
Quality/Quantity sleep	1	2	3	4	5
Relaxation	1	2	3	4	5
Eating	1	2	3	4	5
Exercise	1	2	3	4	5

If there are any **QUESTIONS** relating to the above, please contact Mr Brian Cunniffe or Mr Kevin Morgan on +44 7709566375 or bcunniff@glam.ac.uk. **Thank you for your cooperation!**